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Role of endoscopic ultrasound in the field of hepatology: Recent advances and future trends

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Abstract

The role of endoscopic ultrasound (EUS) as a diagnostic and therapeutic modality for the management of various gastrointestinal diseases has been expanding. The imaging or intervention for various liver diseases has primarily been the domain of radiologists. With the advances in EUS, the domain of endosonologists is rapidly expanding in the field of hepatology. The ability to combine endoscopy and sonography in one hybrid device is a unique property of EUS, together with the ability to bring its probe/transducer near the liver, the area of interest. Its excellent spatial resolution and ability to provide real-time images coupled with several enhancement techniques, such as contrast-enhanced (CE) EUS, have facilitated the growth of EUS. The concept of "Endo-hepatology" encompasses the wide range of diagnostic and therapeutic procedures that are now gradually becoming feasible for managing various liver diseases. Diagnostic advancements can enable a wide array of techniques from elastography and liver biopsy for liver parenchymal diseases, to CE-EUS for focal liver lesions to portal pressure measurements for managing various liver conditions. Similarly, therapeutic advancements range from EUS-guided eradication of varices, drainage of bilomas and abscesses to various EUS-guided modalities of liver tumor management. We provide a comprehensive review of all the different diagnostic and therapeutic EUS modalities available for the management of various liver diseases. A synopsis of all the technical details involving each procedure and the available data has been tabulated, and the future trends in this area have been highlighted.

Key Words: Endoscopic ultrasound; Liver disease; Elastography; Varices; Liver tumor; Liver biopsy

Core Tip: The advancements in the field of endoscopic ultrasound (EUS) have enabled endosonologists to rapidly expand their wings in the field of hepatology. "Endo-hepatology" encompasses the wide range of diagnostic and therapeutic endoscopic procedures that can be used for the management of various liver diseases. Diagnostic advancements range from elastography for liver parenchymal diseases, contrast-enhanced EUS for a focal liver lesion to portal pressure measurements. Therapeutic advancements range from EUS-guided eradication of varices to drainage of abscesses to liver tumor ablation. In this comprehensive review, all the various diagnostic and therapeutic EUS modalities available for the management of liver diseases have been detailed.

INTRODUCTION

The armamentarium of endoscopic ultrasound (EUS) has grown considerably in recent years, both as an investigative and a therapeutic modality. The established diagnostic tools for the study of liver diseases include trans-abdominal ultrasound (USG), computed tomography (CT) scan and magnetic resonance imaging (MRI). While in the past, interventions in liver disease have predominantly been performed by the percutaneous or vascular route, EUS is now more and more being used for both diagnostic and therapeutic purposes. The ability to combine endoscopy and sonography in one hybrid device is a unique property of EUS, together with the ability to bring its probe/transducer in close proximity to the liver, the area of interest. In addition, its excellent spatial resolution and ability to provide real-time images, along with additional techniques, such as contrast-enhanced (CE) EUS, have facilitated the growth of EUS.

Furthermore, EUS guided intervention is also used as a rescue modality when the percutaneous approach is not favorable. EUS has opened doors to a variety of other procedures which are being explored, such as portal vein (PV) sampling for cancer cells, delivery of chemotherapy in the PV, measurement of portosystemic pressure gradient, and EUS guided transjugular intrahepatic portosystemic shunt (TIPS) creation. Harnessing its use in various liver-related interventions paves the way for a new zone of specialty, "Endo-hepatology." Herein we provide a comprehensive review on the use of EUS in the field of hepatology, both diagnostic and therapeutic, discussing the various recent advances and future trends (Figure 1).

LITERATURE SEARCH

A search was performed in PubMed and Embase and the search strategy is outlined in Supplementary Doc 1. All studies such as case reports, series, clinical studies, animal models and reviews regarding EUS applications in liver disorders, including portal hypertension (PHTN), were reviewed. Non-English language literature was not included in the review. EUS applications for extrahepatic bile duct obstruction, gallbladder, *etc.*, including their interventions, are beyond the scope of this review and have been excluded.

EUS FOR LIVER PARENCHYMA ASSOCIATED DISEASES

EUS can be used for the diagnosis, assessment and therapeutic management of ascites, liver parenchymal pathologies, space-occupying lesions (SOLs), liver biopsy, drainage of liver abscesses, bilomas and the management of hepatic tumors.
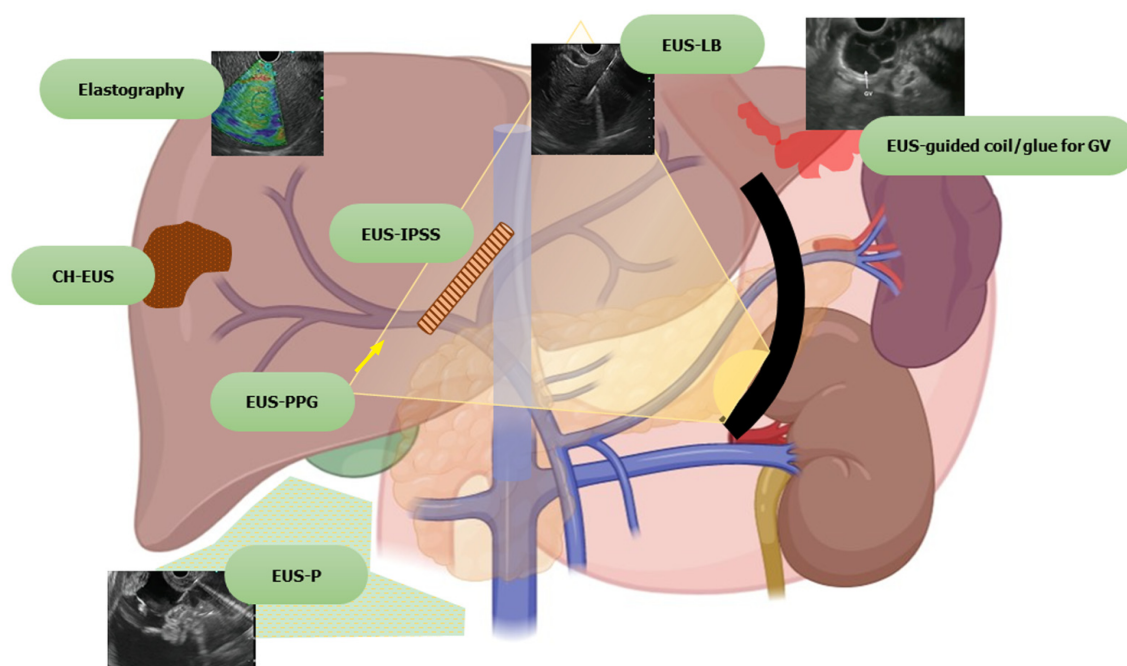
Figure 1 Spectrum of endoscopic ultrasound in hepatology. EUS: Endoscopic ultrasound; CH-EUS: Contrast harmonic endoscopic ultrasound; EUS-IPSS: Endoscopic ultrasound guided intrahepatic portosystemic shunt; EUS-LB: Endoscopic ultrasound guided liver biopsy; EUS-PPG: Endoscopic ultrasound guided portal pressure gradient; EUS-P: Endoscopic ultrasound guided paracentesis; GV: Gastric varices.

Ascites: Assessment and paracentesis

Ascites can be due to benign or malignant diseases. Although the differential diagnosis is broad, around 80%-90% of cases are attributed to underlying cirrhosis and PHTN [1]. Traditionally, routine paracentesis is performed bedside and sometimes with abdominal ultrasound guidance. However, abdominal paracentesis may become difficult in the presence of multiple abdominal scars, previous puncture marks, obesity, dilated bowel loops, dilated/tortuous veins, or the presence of omental or peritoneal nodules[1-3]. EUS guided paracentesis (EUS-P) is more sensitive than CT in detecting ascites[2,4]. The presence of ascites not visualized on imaging (CT/USG) as well as compartmentalization of fluid (such as benign etiologies like tuberculosis or tumor implants in peritoneal carcinomatosis) makes EUS-P a very promising tool in these areas[4,5]. With EUS-P, even small amounts of fluid (as little as 2.7 mL) can be aspirated and provide valuable diagnostic information[6]. In addition, EUS-P can be used as a rescue procedure in the case of previously failed percutaneous paracentesis or part of diagnostic workup during diagnostic EUS (Figure 2).

Additionally, EUS guided fine needle aspiration (EUS-FNA) of suspicious nodules in the omentum/peritoneum can be performed simultaneously while performing paracentesis for targeted cytological diagnosis[7]. Contrast-enhanced EUS (CE-EUS) has also been evaluated to identify enhancement patterns of peritoneal nodules or omental caking and differentiate benign or malignant causes of undiagnosed ascites [8].

The technique of EUS-P: The technique of EUS-P is detailed in Table 1.

Future trends: Since the first report of EUS-FNA of ascites and pleural fluid performed in 1995, various reports of EUS-P with/out FNA of peritoneal deposits have been published subsequently with excellent diagnostic capability and correlation with intraoperative findings[12]. Some cases of development of infectious complications (attributed to traversing the contaminated gastrointestinal wall) such as self-limited fever (3.3%) and bacterial peritonitis (4%) have been reported[5,10]. Recent developments include the deployment of double plastic stents in loculated ascites (benign/malignant), leading to internal drainage causing significant improvement in quality of life[13,14] (Figure 3). A clinical trial is also recruiting patients for EUS guided placement of a plastic prosthesis for refractory malignant ascites[15]. The various studies on EUS-P are summarized in Table 2.

Thus, EUS-P is an excellent tool (sensitivity 94%, specificity 100%) to detect a small quantity of ascites[10] and therapeutic drainage where the percutaneous approach is

Table 1 Technique of endoscopic ultrasound guided paracentesis[1-3,9-11]

Pre-procedure requirements

(1) No recommendations exist for EUS-P, although most studies have been performed under the cover of pre/peri-procedural antibiotics; and (2) Patient is usually fasted for 4-6 h before the procedure

Technical aspects

(1) EUS-P is usually performed using a 22 G/25 G FNA needle. A specialized spring-loaded 22 G FNA needle can also be used for the same; (2) The approach can be transgastric or transduodenal. The tip of the needle is visualized under EUS guidance in the ascites; (3) At the time of puncture, care is taken to avoid a trajectory involving any tumor/vessels to avoid peritoneal seeding or bleeding; (4) For therapeutic paracentesis, a suction tube attached to a vacuum canister can be used; (5) Repositioning of the needle is carried out in case it gets blocked by the tumor or omentum; (6) Two and fro motion is usually not needed; (7) CE-EUS followed by FNA of the peritoneal/omental nodules can also be done for added diagnostic value; and (8) The sample aspirated is sent for routine cytological assessment and for any additional tests that might be needed

Post procedure

The administration of albumin post 5 L of paracentesis and post procedure observation are carried out as per standard recommendations (EASL, AASLD)

EUS: Endoscopic ultrasound; EUS-FNA: Endoscopic ultrasound guided fine needle aspiration; EUS-P: Endoscopic ultrasound guided paracentesis; G: Gauge; CE-EUS: Contrast enhanced endoscopic ultrasound; EASL: European Association for the Study of Liver; AASLD: American Association for the Study of Liver Diseases.

not amenable. Furthermore, FNA of peritoneal/omental nodules is an added advantage that can increase the diagnostic yield.

Assessment of liver parenchyma/SOLs: Anatomy of the liver, its segments and surrounding structures

The requirement for three-dimensional conceptualization of the liver parenchyma makes EUS assessment of the liver and surrounding structures different from the conventional methods of USG/CT/MRI. Depending on the position of the EUS scope, either in the stomach or duodenum, various structures can be identified (Table 3 and Figure 4) such as[17]: (1) From the gastric end: Segments I (caudate lobe), left lobe segments (II, III, IV), right lobe (V, VIII), umbilical part of the left PV and ligamentum teres, ligamentum venosum, inferior vena cava, and hilum; and (2) From the duodenal bulb: Segments VI, VII; the hepatoduodenal ligament structures and PV and hepatic artery branches, the liver hilum and the segmental divisions of the right PV and hepatic artery.

Although transabdominal USG or CT scan is the first-line approach for evaluation of liver parenchyma or focal lesions, EUS has additional features which can add to its diagnostic/therapeutic potential[18,19]: (1) Transducer proximity enables better identification of the structures; (2) Combination of real-time images with elastography enables semi-quantitative measurements of liver parenchymal stiffness; (3) Newer generation EUS machines with color, power and pulsed Doppler systems helps easy assessment of the vasculature; (4) CE-EUS or harmonic EUS increases the diagnostic performance of focal liver lesions; and (5) Simultaneous assessment and interventions such as management of varices and liver biopsy can be performed in a single setting.

Techniques of assessment: Elastography and contrast enhancement techniques

Real-time elastography (RTE) has been developed for the assessment and quantification of liver tissue stiffness. Qualitative RTE uses the degree of deformation by the compression of structures as an indicator of tissue stiffness and is depicted using a color map wherein hard tissue is blue, intermediate stiffness is green and soft tissue is red. Quantitative RTE, on the other hand, uses hue histogram and strain ratio. While the former is a graphical representation of the color distribution in a selected image field, the strain ratio is calculated as the ratio of the target area (A) by reference area (B) (Figure 5)[20].

CE-EUS is a more valuable technique to improve the diagnostic performance of focal liver lesions. It is of 2 types: CE-EUS with the Doppler method (CE-EUS-D) and CE-EUS with harmonic imaging (CE-EUS-H). The former helps distinguish vascular-rich and hypovascular areas of a liver SOL, whereas the latter helps provide a detailed roadmap of the vasculature of the same. Of the contrast agents available, Sonovue and Sonazoid are more commonly used[21].

The concept of CE-EUS depends on the dual blood supply of the liver and has 3 phases: arterial phase (20-45 s), portal venous phase (lasting up to 120 s), and the late phase (contrast agent clearance, around 6 min)[21].

Table 2 Studies on endoscopic ultrasound guided paracentesis

Ref.	Study design	Patient population	Imaging	Age (yr)	Gender (M/F)	Needle	Route (TG/TD)	Amount of fluid aspirated	Diagnosis on EUS	Actual diagnosis	Complications
Chang *et al*[12], 1995	Case report	2 cases	CT (pleural effusion and ascites)	-	-	-	-	-	-	Malignant effusion and ascites	-
Romero-Castro *et al* [14], 2017	Case series	3 cases	DLBCL (1 case), HCC (2 cases)	60/74/55	3/-	19 G FNA (all cases)	TG (3 cases)	Double Pigtail placement (3 cases)	-	Malignant ascites (3 cases)	None
Wardeh *et al*[16], 2011	Retrospective study	101	Ascites not detected in 6/9 cases on CT	68.3	54/47	19 G FNA	NA	10 mL (max) in 90 cases, 2 smears in 11 cases	74 negative	84 malignant	None
Suzuki *et al*[11], 2014	Retrospective study	11 cases	CT (no ascites in 4)	66.4	7/4	22 G (automatedspring-loaded)	NA	14.1 mL (range 0.5-38 mL)	Benign 5; malignant 6	NA	None
Kaushik *et al*[10], 2006	Retrospective study	25	NA	66-70	16/9	22/25 G FNA	Both	6.8 mL (range, 1-20 mL)	64% malignant (benign 9; malignant 16)	Benign 8; malignant 17	1 cases (4%) (bacterial peritonitis)
Lee *et al*[4], 2005	Retrospective study	250 cases	CT in all	60.3	160/90	NA	NA	NA	37% ascites, 28% peritoneal metastasis	All malignant	None
Dewitt *et al*[5], 2007	Retrospective study	60	CT/MRI/USG in all (ascites 31 cases (51%)	67	33/27	22 G	55 (TG), 5 (TD)	8.9 (1-40) mL	Benign 42; malignant/atypical 18	Benign 15; malignant 45	2 cases fever
Köck *et al*[13], 2018	Case report	2 cases	Rectal cancer, ovarian cancer	36, 56	-/2	19 G	Both TG	Pigtail (plastic) placed	-	-	None
Nguyen and Chang [2], 2001	Retrospective study	31 cases (of 85)	CT had ascites in 14/79 (18%)	NA	NA	NA	NA	7.9 (1-40 mL)	Malignant 5; benign 26	NA	None
Varadarajulu and Drelichman[3], 2008	Case report	1	SCC anus	31	-/1	19 G	TG (1)	10 mL (diagnostic); 5 L (therapeutic)	Malignant ascites	NA	None

DLBCL: Diffuse large B cell lymphoma; TG: Transgastric; TD: Transduodenal; M: Male; F: Female; G: Gauge; EUS: Endoscopic ultrasound; CT: Computed tomography; FNA: Fine needle aspiration; SCC: Squamous cell carcinoma; USG: Ultrasound; MRI: Magnetic resonance imaging.

The advantages of CE-EUS over CT and MRI are that: (1) It provides real-time imaging; (2) Contrast is not excreted by the kidneys, and thus can be used in cases with renal insufficiency; (3) Contrast is confined to the vascular space only and so has prolonged enhancement of vascular system; (4) Higher resolution helps in targeted biopsies; and (5) Can characterize lesions less than 1 cm.

EUS imaging in chronic liver diseases
Certain tests such as transient elastography (TE), Fibroscan, and RTE can aid in the diagnosis of the degree of liver fibrosis. However, these tests are fraught with

Table 3 Structures visualized with endoscopic ultrasound in the liver

Structure	Features	Doppler
Portal vein branches	Thick and hyperechoic walls	Positive signal
Hepatic vein branches	Thin, non-reflective walls, straight course	Positive signal
Biliary radical	Hyperechoic walls, irregular course	Negative signal
Ligaments (teres and venosum)	Thick, hyperechoic (no lumen) (between vessels and Glisson's capsule)	Negative signal
Gallbladder	Cystic structure, hyperechoic walls, anechoic content	Negative signal
Falciform ligament	Thick, hyperechoic (no lumen); on the left anterior to segment III, on the right anterior to segment IVa and IVb	Negative signal
Hepatic artery	Thick with reflective walls	Positive signal

Figure 2 Endoscopic ultrasound guided paracentesis. Needle is visualized in the ascitic fluid.

limitations in people with obesity and ascites. EUS can be used similarly with probably better diagnostic sensitivity for the same. Schulman *et al*[22] reported that liver fibrosis index (LFI) correlated with abdominal imaging (LFI in normal, fatty liver and cirrhosis patients were 0.8, 1.4 and 3.2, respectively). Similar findings were replicated in liver fibrosis assessment for chronic hepatitis C cases (LFI of 2.38 had an area under the receiver operating characteristic curve of 0.73) compared with the gold standard of liver biopsy. Histogram acquisition was successful in 82% of patients[23]. A recent study by Tu *et al*[24] in early-stage cirrhosis showed that the accuracy of a combination of EUS, EUS-RTE, acoustic radiation force impulse (AFRI) and aspartate aminotrans-ferase-to-platelet ratio (APRI) had the highest diagnostic rate (sensitivity 87%). Thus, EUS can provide a one-stop diagnostic modality to screen and rule out a host of conditions in patients with liver disease, from the screening of varices, pancre-aticobiliary pathology to hepatic parenchymal/SOL assessment.

EUS imaging in focal liver lesions
The diagnostic accuracy of EUS in detecting focal liver lesions, mostly less than 1 cm, exceeds that of USG, CT, and MRI[25,26]. Singh *et al*[27] addressed the diagnostic yield of EUS *vs* CT for hepatic metastasis (98% *vs* 92%), wherein EUS identified a significantly greater number of metastatic lesions (40 *vs* 19). Diagnostic criteria proposed by Fujii-Lau *et al*[28] can be used to differentiate between benign and malignant metastatic hepatic lesions based on EUS findings with a positive predictive value of 82%. Lesion shape, borders, echogenicity, homogeneity, and size are used to delineate malignant lesions. It is said to be neoplastic if it meets at least three criteria: (1) Lack of isoechoic/slightly hyperechoic center; (2) Post-acoustic enhancement; (3) Adjacent structures distortion; (4) Hypoechogenicity (slightly or distinctly); and (5) At least 10 mm in size.
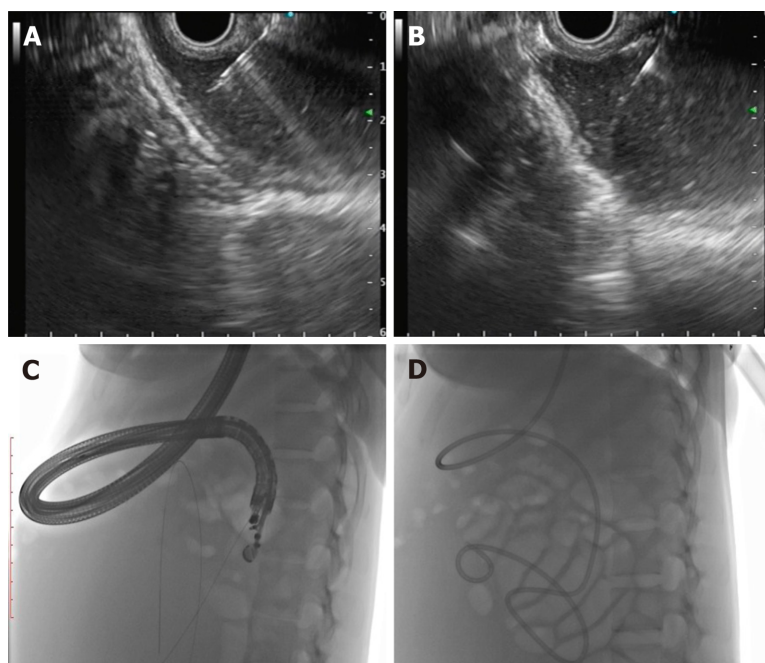
Figure 3 Endoscopic ultrasound-guided internal drainage of loculated ascites. A: Puncture of the loculated ascites with 19-G aspiration needle; B: Guidewire negotiated across as visualized on endoscopic ultrasound; C: Fluoroscopic view of guidewire coiled inside the loculated ascites; D: Naso-cystic drain placed inside the loculated ascites.

With the advent of EUS-RTE, the characterization of liver SOLs and their biopsies have become better (Figure 6). A study reported a hue histogram cutoff of 170 to discriminate between benign and malignant tumors (sensitivity 92.5%, accuracy 88.6%) [29]. In addition, the use of contrast agents in CE-EUS helps in differentiating primary tumors and metastasis[30]. CE-EUS has also been utilized for the assessment of treatment response in hepatocellular carcinoma (HCC) post-trans-arterial catheter embolization[31]. Hence, EUS with RTE, CE-EUS and CE-EUS-H might be a promising tool for diagnosing focal liver lesions and targeted intervention.

EUS-FNA of focal liver lesions

Several studies exist on the use of EUS-FNA/FNB (fine needle biopsy) for solid liver lesions with a complication rate of 0%-6% (Table 4). A recent systematic review by Ichim *et al*[42] showed the diagnostic yield of EUS-FNA to be 80%-100%.

Future trends

Studies have reported additional assessment of KRAS mutation in inconclusive cytological samples, which has resulted in an improved diagnostic yield from 89.3% to 96.4%[43]. Similarly, an animal study has evaluated the art of *in vivo* cytological observation using a high-resolution micro-endoscopy (HRME) system under EUS guidance[44] to decrease the number of needle-passes and subsequent adverse events. Recently, Minaga *et al*[45] have reported the additive role of CE-EUS-H in the detection of left lobe liver metastasis from pancreatic ductal adenocarcinoma. The diagnostic accuracy of CH-EUS was 98.4% compared to 90.6% with CECT.

EUS guided liver biopsy

Despite the advances in various non-invasive testing available to determine the degree of fibrosis, liver biopsy remains the gold standard method for accurate assessment in diagnosis and staging. As first described in 1883 by Dr. Paul Ehlrich, percutaneous liver biopsy (PC-LB) has evolved from a mere percussion method to an "image-guided" technique in the last ten years using ultrasound/CT imaging to accomplish it. However, despite image guidance, the risk of bleeding persists, occurring in up to 0.6% of cases, including other adverse events like pneumothorax and gallbladder puncture and even death in a few cases[46]. The transjugular technique of liver biopsy, introduced in 1973, can help reduce this risk, especially in patients with underlying coagulopathy. However, this method also carried added risks of local site hematoma, intraperitoneal bleeding, arrhythmia and carotid puncture[47].

Table 4 Studies on endoscopic ultrasound guided fine needle aspiration/fine needle biopsy of focal liver lesions

Ref.	Design	Patients	Diagnostic yield (%)	Needle passes (median)	Complications
EUS-FNA					
Nguyen *et al*[32]	Prospective	14	100	2	0
TenBerge *et al*[33]	Retrospective	26	88.6	-	3.8% (fever)
DeWitt *et al*[34]	Retrospective	77	91	3.4 (mean)	0
Hollerbach *et al*[35]	Prospective	33	94	1.4 ± 0.6	6.1% (self-limited bleeding)
McGrath *et al*[36]	Prospective	7	100	2	0
Singh *et al*[26]	Prospective	9	88.9	2	0
Singh *et al*[27]	Prospective	26	96	2.1	0
Crowe *et al*[37]	Retrospective	16	75	3 (minimum)	0
Prachayakul *et al*[38]	Retrospective	14	100		0
Oh *et al*[39]	Prospective	47	90.5	3	0
Ichim *et al*[25]	Prospective	48	98	2	0
EUS-FNB					
Lee *et al*[40]	Prospective	21	90.5	2	0
Chon *et al*[41]	Retrospective	58	89.7	2	1.7% (bleed)

EUS: Endoscopic ultrasound; FNA: Fine needle aspiration; FNB: Fine needle biopsy.

EUS guided liver biopsy (EUS-LB) initiated as early as 2007 is currently emerging as a cost-effective, safe and well-tolerated procedure and helps in more representative sampling. The American Association for the Study of Liver Diseases recommends a tissue length of at least 2-3 cm with ≥ 11 or more complete portal tracts (CPTs) for determining the adequacy of liver biopsy samples[48]. The mean tissue length and CPTs for EUS-LB, PC-LB and TJLB, as shown in various studies is 36.9, 9 and 17.7 mm, and 7.7, 13.5 and 6.8 mm, respectively[49,50]. This can be achieved with a regular 19 G EUS-FNA needle (71). Similarly, a meta-analysis on EUS-LB revealed that pooled successful histological diagnosis was achieved in 93.9% of cases. Adverse event rates with EUS-LB, PC-LC and TJLB were 2.3%, 0.09%-3.1% and 0.56%-6.5%, respectively [48,51,52]. A recent meta-analysis between the three techniques revealed that EUS-LB was comparable to PC-LB in terms of CPT, but tissue length was better with the former with no complication rates[53].

EUS-LB has been used in the setting where patients undergo other endoscopic procedures such as screening of the biliary tree, assessment of surrounding structures and lymph nodes and variceal screening in those not affected with ascites and obesity [50], thereby saving time and resources. Furthermore, EUS-LB is theoretically less painful as it does not require skin puncture, eliminates the need for breath-hold and allows visualization and avoidance of blood vessels even 1 mm in size and is suitable for anxious patients by using adequate sedation (Figure 7). Moreover, bilobar biopsy can be achieved, reducing sampling error and helping in better assessment of disease activity and fibrosis[54].

Technique: The technique of EUS-LB is described in Table 5.

Future trends: In attempts to acquire better quality and quantity of specimens, various studies have been published on different needles and methods of executing a EUS-LB procedure. A recent RCT comparing a 19 G FNB needle (fork-tip) *vs* 19 G standard FNA needle yielded better results with the former (pre-processing length 2.09 cm *vs* 1.47 cm and more CPTs)[55]. In contrast, a recent meta-analysis showed the superiority of FNA needles over core biopsy needles in terms of better tissue acquisition[51]. Thus, 19 G FNA needle may be used for EUS-LB procedures except for the cases where immunohistochemistry and architecture characterization are warranted, in whom core biopsy needle may be used.

Mok *et al*[56] showed that the "wet heparin" suction technique had greater tissue yield compared to "dry suction" (aggregate specimen length 49.2 mm *vs* 23.9 mm;

Table 5 Technique of endoscopic ultrasound guided liver biopsy[50,51]

Pre-biopsy: The following workup is needed in all cases of liver biopsy

(1) Coagulation work up including platelet count, PT/INR and BT/CT; (2) Prior to the biopsy, the medications should be stopped as follows: anti-platelet medications 7 d, warfarin 5 d, heparin and related products discontinued 12-24 h prior to biopsy; and (3) Use of conscious sedation such as midazolam and nalbuphine or propofol as per operator's preference or patient comfort

Procedural details of EUS-LB

(1) A linear array echoendoscope (Olympus GF-UCT180, Center Valley, United States) is generally used for the procedure; (2) Prior to the procedure, Doppler imaging is done to ensure that no vascular structures are present along the expected trajectory of the needle; (3) The EUS-LB can be performed using a 19 G EUS-FNA/FNB needle; (4) The left lobe is identified first, as that liver parenchyma which is a few centimeters below the gastro-esophageal junction with the scope torqued clockwise. The right lobe if needed to be biopsied, is accessed from the duodenal bulb. Two site biopsy can be undertaken at the discretion of the endosonographer; (5) A preferably long vessel free trajectory allowing free passage of the needle to a depth of at least 3 cm or more is usually selected; (6) For wet heparin suction, the stylet is removed and the needle is primed with a heparin flush and the suction syringe is reattached to the needle hub; (7) The needle is then introduced into the echoendoscope channel; (8) Once liver parenchymal penetration is achieved with the needle (around 1-2 cm), full suction is applied with the 20 mL vacuum syringe with fluid column; (9) One pass consists of a total of 4-5 to-and-fro needle motions using the fanning technique under direct EUS guided visualization of the tip of the needle. Two such passes are usually taken (maximum 10 actuations); and (10) The specimen is pushed from the needle directly into the formalin solution using the stylet or saline flush

Post-liver biopsy: The following instructions are to be followed in all cases post liver biopsy

(1) The patient post biopsy, irrespective of the type of procedure, is transferred to the post procedure recovery room and monitored as per the AASLD protocol[69]; (2) The minimum observation period is 2-4 h; (3) Post-procedure pain and need for analgesics to be noted and provided; and (4) Patient is asked to report adverse events at specific time intervals (as per institute policy)

EUS: Endoscopic ultrasound; PT Prothrombin time; INR International normalized ratio; BT: Bleeding time; CT: Clotting time; EUS-LB: Endoscopic ultrasound guided liver biopsy; FNA: Fine needle aspiration; FNB: Fine needle biopsy; AASLD: American Association for the Study of Liver Diseases.
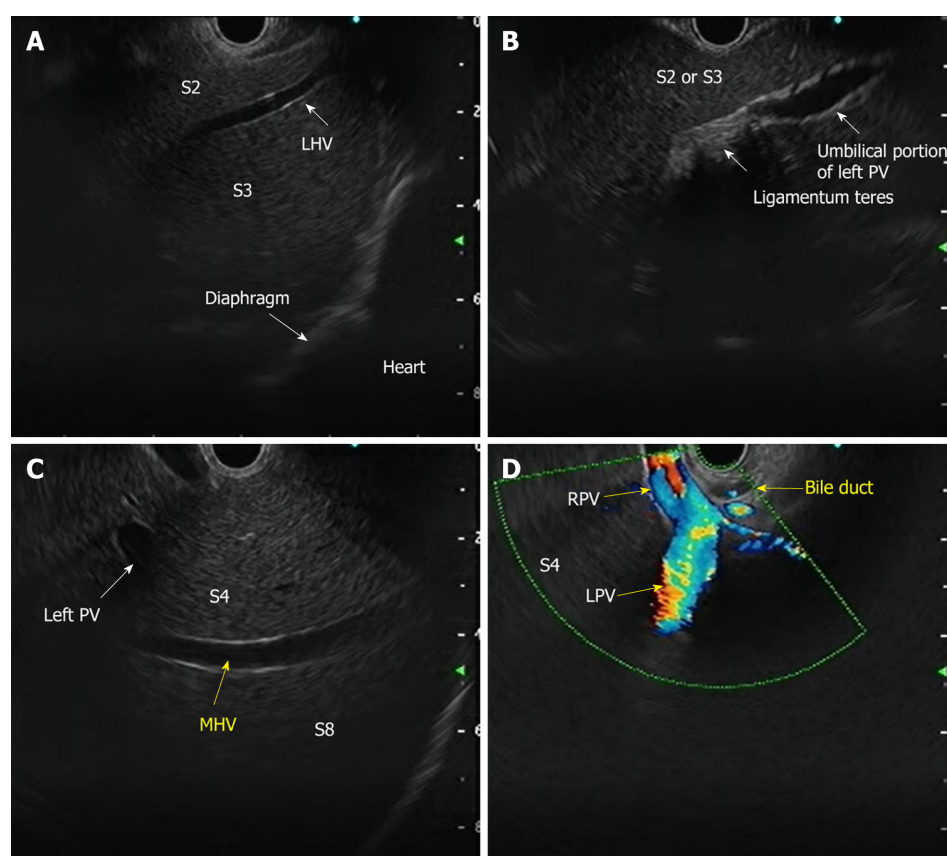
Figure 4 Endoscopic ultrasound anatomy of liver segments. A: Anatomy of the left lobe with S2 and S3 segments; B: Ligamentum teres with umbilical portion of the left portal vein; C: Middle hepatic vein with segments of the liver; D: Anatomy of the bifurcation of portal vein from the duodenal bulb. PV: Portal vein; MHV: Middle hepatic vein; LHV: Left hepatic vein; RPV: Right portal vein; LPV: Left portal vein.

mean CPT count 7 *vs* 4). Thus, the combination of wet-heparinized suction and a 19-G second-generation (FNA/FNB) needle might help achieve better specimens with minimal fragmentation.
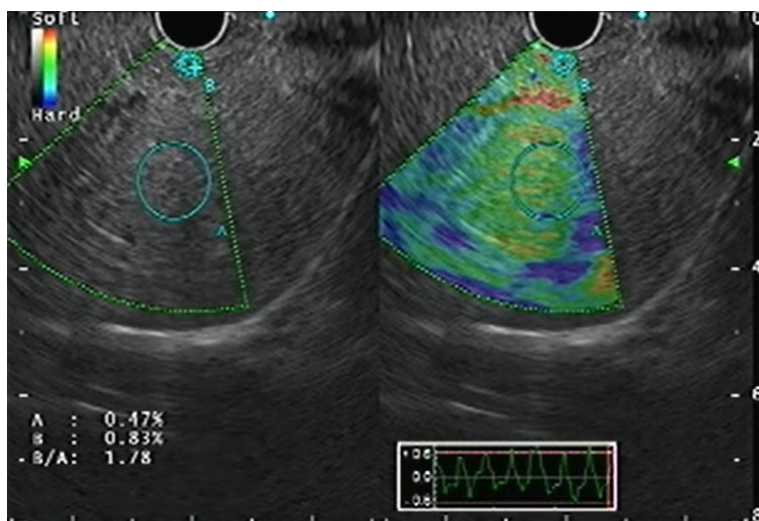
Figure 5 Endoscopic ultrasound elastography of the liver parenchyma.
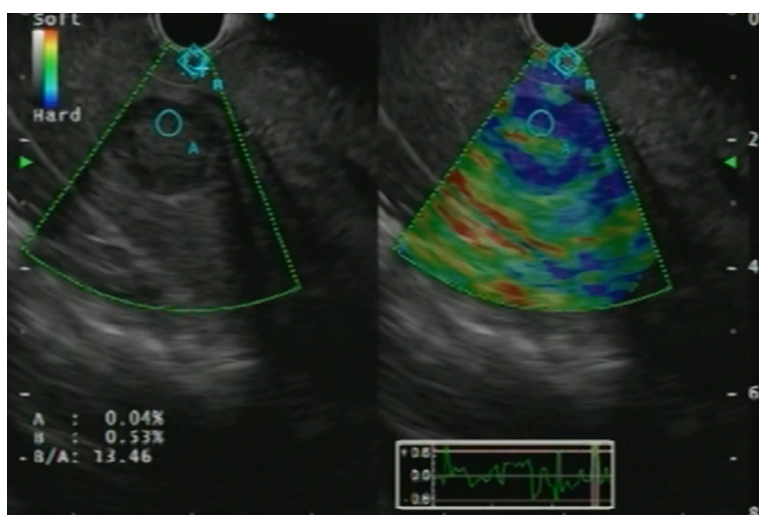
Figure 6 Endoscopic ultrasound elastography of a focal liver lesion with strain ratio calculation.

The various studies using EUS-LB (FNA/FNB) in patients with chronic liver disease are highlighted in Table 6. The average technical success and diagnostic yield for EUS-FNA and EUS-FNB-guided liver biopsy are 100% and 89.8%, respectively, with a complication rate of 3.3%, consisting entirely of minor events[70]. In addition, studies reporting the use of EUS-LB in patients with NAFLD (overall technical success rate 100%, yield 96.8% with 7.7% complication rate) are reported in Supplementary Table 1.

EUS guided therapeutic management of liver cysts, liver abscess and biloma
Symptomatic liver cysts, abscesses and bilomas may require drainage. Traditionally, these were approached through surgical or interventional radiology using percutaneous catheter drainage (PCD). Recently, EUS guidance has been used to drain simple intrahepatic cysts of varied etiologies, liver abscesses and bilomas. EUS guided drainage may be superior to PCD as it enables a one-step approach, leading to internal drainage and thus avoiding the complications of catheter dislodgement, pericatheter leak, multiple interventions and movement restrictions.

EUS guided treatment of hepatic cysts: The most frequent liver cysts encountered for drainage *via* EUS include simple hepatic cysts and intrahepatic pancreatic pseudocysts. Those located in the left lobe of the liver or the caudate lobe can be drained *via* EUS guidance. PCD would be preferred for right lobe cysts as it is difficult to access the right lobe in the duodenal bulb with an unstable scope position. Therapies offered by EUS include fine-needle aspiration, ethanol lavage and

Table 6 Studies on endoscopic ultrasound guided fine needle aspiration guided and endoscopic ultrasound guided fine needle biopsy guided liver biopsy in patients with chronic liver disease

Ref.	Design of the study	Patients	Technical success (%)	Diagnostic yield (%)	Specimen length (median, range) (mm)	CPT (median, range)	Needle used for EUS-LB	Needle passes (median)	Complications, *n* (%)
EUS-FNA guided liver biopsy									
Pineda *et al* [57]	Retrospective	110	100	98	38 (24-81)	14 (9-27)	19 G	-	0
Shuja *et al*[58]	Retrospective	69	100	100	45.8 (mean)	10.84 (mean)	19 G	3	0
Stavropoulos *et al*[50]	Prospective case series	22	100	91	36.9 (2-184.6)	9 (1-73)	19 G	2 (1-3)	0
Diehl *et al*[59]	Prospective non randomized	110	100	98	38 (0-203)	14 (0-68)	19 G	1.5 (1-2)	1 (0.9) (mild bleeding)
Gor *et al*[60]	Retrospective case series	10	100	100	13 (6-23)	8 (6-15)	19 G	-	0
EUS-FNB guided liver biopsy									
Shah *et al*[61]	Retrospective	24	100	96	65.6 (17-167.4)	32.5 (5-85)	19 G (SharkCore)	2 (1-3)	2 (8.3)
Nieto *et al*[62]	Retrospective	165	100	100	60 (43-80)	18 (13-24)	19 G (SharkCore)	1	3 (1.8)
Mathew[63]	Case report	2	100	100	-	-	19 G (QuickCore)	-	0
Ching *et al* [55]	Prospective (RCT)	20; 20	100; 100	100; 100	114 (mean); 153.2 (mean)	16.5 (6-38); 38 (0-81)	19 G (FNA); 19 G (Acquire)	--	8 (40); 7 (35)
Mok *et al*[56]	Prospective (RCT)	40; 40	100; 100	88; 68	-; -	-; -	19 G (FNA); 22 G (SharkCore)	-; -	0; 1 (2.5)
Patel *et al*[64]	Retrospective	30; 50; 28; 27	100; 100; 100; 100	66.7; 46; 82.1; 81.5	1.8 (mean); 4.7 (mean); 1.9 (mean); 8.4 (mean)	6.9 (mean); 3 (mean); 7.3 (mean); 16.9 (mean)	Acquire 22 G; QuickCore 19 G; ProCore 19 G; Expect 19 G	-; -; -; -	-; -; -; -
Gleeson *et al* [65]	Retrospective	9	100	100	13 (8-28)	7 (5-8)	19 G (QuickCore)	2 (1-3)	0
DeWitt *et al* [66]	Prospective case series	21	100	90.5	9 (1-23)	2 (0-10)	19 G (QuickCore)	3 (1-4)	0
Nakai *et al* [67]	Case report	1	100	100	15	8	ProCore 19 G		0
Sey *et al*[68]	Prospective cross sectional study	45; 30	100; 100	73.3; 96.7	9 (0-25); 20 (5-60)	2 (0-15); 5 (0-24)	QuickCore 19 G; ProCore 19 G	3; 2	2 (4.4); 0
Hasan *et al* [69]	Prospective (RCT)	40	100	100	55 (44.5-68)	42 (28.5-53)	Acquire 22 G	-	6 (15)

CPT: Complete portal triad; EUS-LB: Endoscopic ultrasound guided liver biopsy; FNA: Fine needle aspiration; FNB: Fine needle biopsy; RCT: Randomized controlled trial; G: Gauge.

transmural stent placement.

In a retrospective study by Lee *et al*[71], 19 cases of hepatic cysts were treated by PCD and EUS guided ethanol lavage and reported a 97.5% reduction in cyst volume at 11.5 mo of follow-up in the PCD group and a 100% reduction at 15 mo in the EUS arm. The studies on EUS guided treatment of hepatic cysts are outlined in Supplementary Table 2.

EUS guided drainage of liver abscess: Traditionally, pyogenic and amoebic liver abscesses have been drained by PCD with a high technical success rate. However, EUS guided drainage of liver abscesses is a promising new approach, especially for difficult-to-reach locations. Additionally, the advantage of internal drainage with a
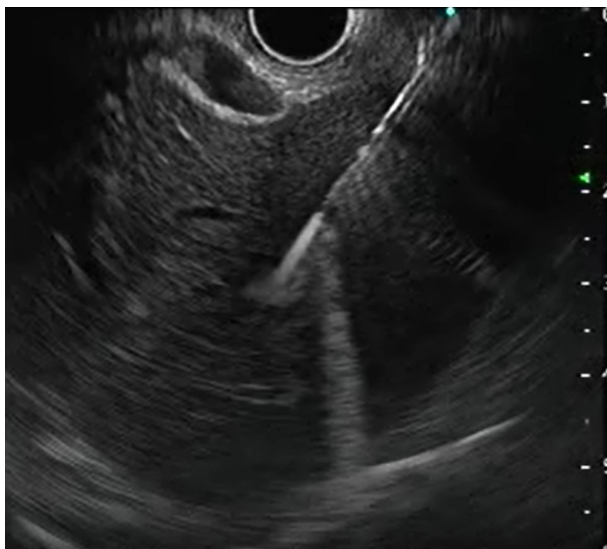
Figure 7 Endoscopic ultrasound-guided liver biopsy.

single-step procedure and easy access from the stomach makes transmural drainage of left and caudate lobe abscess convenient.

The technique was first described by Seewald *et al*[72], who reported complete resolution 4 weeks post-procedure. Literature on EUS guided drainage is limited to retrospective case series only in which the majority have been drained with double pigtail plastic stents[73-75]. Recently, data are emerging on the use of fully covered self-expandable metal stents (SEMS)[76] for the same. Ogura *et al*[77] reported retrospective comparative data on EUS *vs* PCD guided abscess drainage wherein EUS guided abscess drainage (EUS-AD) cases showed greater clinical success (100% *vs* 89%) with shorter hospital stay (21 d *vs* 41 d). Studies on EUS-AD are listed in Supplementary Table 3.

EUS guided drainage of biloma: Biloma is defined as a well-demarcated collection of bile outside the biliary tree, which can be extrahepatic or intrahepatic, encapsulated or without a capsule[78]. It is most frequently caused by iatrogenic biliary tree injury during cholecystectomy. It has been traditionally managed with PCD or surgery. However, large bilomas in opposition to the gastric wall can be taken up for transmural drainage (Figure 8). Similar to EUS-AD, earlier plastic stents were utilized for the same, but now SEMS has been in vogue for biloma drainage with excellent results. Post drainage, such patients should be evaluated to determine the need for endoscopic retrograde cholangiopancreatography, or sphincterotomy with/out biliary stenting or surgery[79]. Studies on EUS guided drainage of bilomas are described in Supplementary Table 4.

Despite it being a point of contention, EUS guided drainage of intrahepatic lesions (cysts, abscesses and bilomas) is an upcoming promising technique and may be considered in conditions where PCD is not amenable or has failed.

EUS guided treatment of liver tumors

A thrilling offshoot of EUS guided therapeutic interventions has been EUS guided local treatment of tumor lesions (both pancreatic and hepatic tumors)[80]. EUS-guided tumor management is a new experimental application that has shown promise in reaching difficult lesions (left lobe, caudate lobe), provided a rescue option in refractory cases, and has potential to improve quality of life by minimizing systemic side effects[81,82]. This procedure has been extensively studied in cases of pancreatic neoplasm, but its role in hepatic tumors (primary or metastatic) is still in its infancy.

Various techniques of EUS guided liver tumor management have been described.

Fine needle injection therapy: Ethanol ablation

Percutaneous injection of ablative injections is most commonly used worldwide to manage HCC, although EUS guided fine needle injection can be performed using acetic acid or ethanol (pure alcohol 95%-99%)[83]. Its advantage is that it enables real-time imaging during delivery of ethanol to the tumorous lesion and thus can help
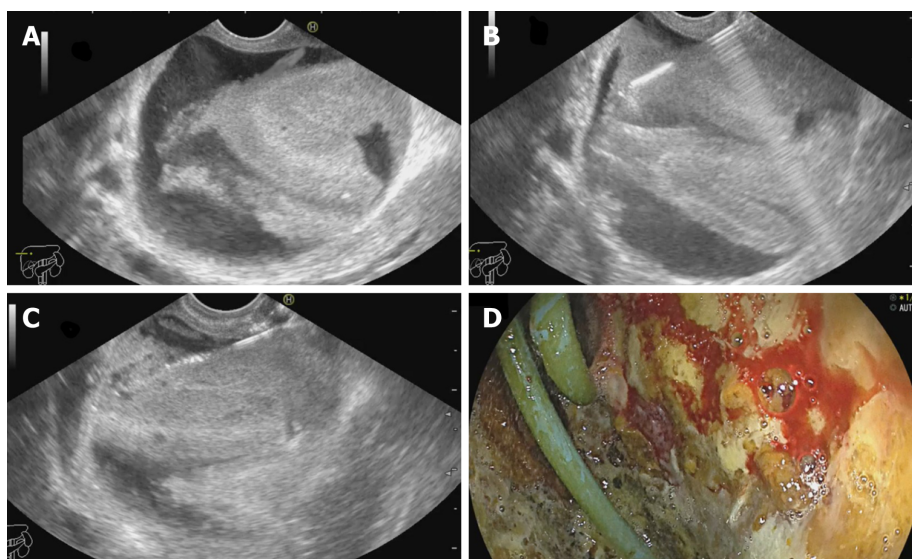
Figure 8 Endoscopic ultrasound-guided drainage of biloma. A: Post-operative biloma noted on endoscopic ultrasound (EUS) with internal echoes; B: EUS-guided puncture of the biloma; C: Guidewire negotiated into the collection followed by placement of naso-cystic drain; D: Endoscopic view of the cavity entered with catheter noted *in situ*.

avoid collateral damage.

Initial case reports using 22 G and 25 G FNA needles have been reported with excellent technical success and complete resolution of HCC[84-87]. For example, Nakaji *et al*[87] reported a high-resolution rate at 31 mo in 12 cases of caudate lobe HCC, whereas Jiang *et al*[88] only showed 30% complete resolution at 12 mo. This technique has also been evaluated for the treatment of hepatic metastasis from pancreatic adenocarcinoma[89].

Thermal ablative therapy

Radiofrequency ablation: Radiofrequency ablation (RFA) uses a high-frequency alternating current (375 kHz to 500 kHz) and is minimally invasive with good tolerability[90]. It can be delivered percutaneously, intraoperatively, *via* an endoluminal approach or endosonographic (transmural) route. Emerging data on the latter have resulted in its application in cases wherein the percutaneous approach fails. Obesity, tumor nodules in the left lobe or caudate lobe, deep-seated and sub-capsular/sub-diaphragmatic lesions that carry an inherent risk of hemothorax or pleural effusion are some of the conditions where it has been applied[81,90]. A specifically designed needle tip electrode for performing EUS-RFA (EUSRA RFA Electrode, STARmed, Koyang, Korea) with a designed internally cooled needle electrode is the most extensively studied. Only a few case reports exist on EUS-RFA using EUSRA in HCC[91-93]. Also, hybrid models combining EUS-RFA with cryoab-lation in the bovine liver have demonstrated better efficacy of the combination treatment[94].

Laser ablation by neodymium:yttrium-aluminum-garnet: Neodymium:yttrium-aluminum-garnet (Nd-YAG) is a type of LITT (laser interstitial thermotherapy) wherein laser waves are introduced through the EUS needle directly into the tumor tissue leading to cell apoptosis and eventual necrosis. Only two human studies have been published so far for the treatment of HCC. Di Matteo *et al*[95] reported complete HCC resolution in 2 mo in a case of previously failed caudate lobe HCC. Similarly, Jiang *et al*[96] reported resolution at 3 mo with an encouraging safety profile.

Cryotherapy ablation: Cryotherapy ablation (CYA) destroys tissue through multiple freezing-thawing cycles leading to osmotic dehydration and injury to the intracellular structures and cell death[90]. No human study exists for its use in liver lesions. However, a single animal study showed the efficacy of a hybrid EUS-RFA and cryoderm device in a porcine model[97].

High-intensity focused ultrasound: This is a non-invasive technique that causes tissue necrosis *via* heat generation and acoustic cavitation by the formation and collapse of bubbles produced by intense USG waves[90]. Its use in EUS has only been tested in

animal models[98,99], showing complete necrosis of the lesions with no immediate side effects.

Brachytherapy

This treatment modality has been used for various cancers with the advantage of less toxicity to surrounding tissues over external beam radiotherapy[81,90]. For example, EUS guided brachytherapy with permanent seed placement of Iodine (I125) or palladium (Pd103) has been performed for head-neck, esophageal, and pancreatic cancer[100-102]. In addition, Jiang *et al*[88] have used EUS guided I125 seed implantation for liver tumors with high efficacy and safety.

Studies on EUS guided liver tumor treatments are outlined in Table 7.

EUS GUIDED VASCULAR INTERVENTIONS

The presence of real-time, high-resolution sonographic imaging with Doppler, along with the relative proximity of the gastrointestinal tract to the major blood vessels in the abdomen and the mediastinum, has led to a growing interest to explore the role of EUS in the field of vascular interventions. EUS may be preferred over the percutaneous route, especially in obesity, ascites and overlying distended bowel[104].

Esophageal and gastric varices: diagnosis and management

EUS guided vascular intervention in patients with PHTN has been well established in managing varices (esophageal, gastric, duodenal, and ectopic).

Management of esophageal varices: Endoscopic variceal band ligation (EVL) has been the standard treatment of esophageal varices (EV) (both primary and secondary prophylaxis). However, re-bleeding rates of 15%-65% have been reported due to the failure to obliterate perforating veins and collaterals feeding the varices[105]. Lahoti *et al*[106] described the first report of EUS guided sclerotherapy in 5 cases, wherein sclerosant (sodium morrhuate) was injected under EUS guidance (2-4 mL per injection site) directed at the perforating vessels as determined by color Doppler with complete eradication of the varices. An RCT comparing EUS *vs* direct sclerotherapy revealed no difference in both arms[107]. Thus, although EUS carries a theoretical advantage for identifying the feeders, more studies are needed to assess its practical clinical benefit.

Management of gastric varices: In patients with PHTN, gastric varices (GV) are present in up to 20% with a 50%-65% re-bleeding rate[108]. Endoscopic injection of CYA glue for GVs has been the treatment of choice since its first description in 1986 but is still prone to a re-bleeding rate of 40%[109]. In the current era of EUS guided vascular interventions, management of GVs by EUS has many conceptual advantages, both diagnostic and therapeutic such as[110,111]: (1) A higher detection rate (6 times) over conventional endoscopy; (2) Greater success in differentiating varices from thick gastric folds; (3) Confirmation of the cessation of blood flow post-treatment; (4) Real-time varix visualization and hence accurate delivery of hemostatic agent to the varix; and (5) Targeted treatment for feeder vessels.

The first description of EUS guided CYA injection in GVs was given by Romero-Castro *et al*[111] and Lee *et al*[112]. To reduce the chances of embolization with CYA, stainless steel coils alone or in combination with CYA glue have been introduced. The advantage is three-pronged: additive hemostasis and varix obliteration, reducing the volume of glue needed and acting as a scaffold to retain the glue within the varix, thereby decreasing embolization. Various studies, including RCTs, have favored coil over glue. Bhat *et al*[113] reported a complete obliteration in 93% with only 3% re-bleeding rates using coils and glue combination. Similarly, two RCTs and a meta-analysis have reported the combination therapy of coil with glue to be superior to either agent alone[114-116]. Newer treatments of utilizing coils with gelatin sponge and sclerotherapy or isolated thrombin injection have been reported in various case series and have shown good results[117-119].

The technical steps of the EUS guided coil and glue placement for the obliteration of GV are outlined in Table 8 and Figure 9.

Use of EUS in the prediction of re-bleeding from EV/GV: EUS with Doppler has a higher sensitivity for detecting esophageal and GV than upper GI endoscopy and can also be used to predict re-bleeding. Certain parameters can help guide us in this direction[120,121]: (1) EUS can help in demonstrating collaterals or feeders, a strong

Table 7 Studies in humans demonstrating the role of endoscopic ultrasound guided therapies for liver lesions

EUS guided treatment	Study design	Patients	Location of the lesion	Technical success (%)	Response to therapy	Complications
Ethanol ablation in HCC						
Nakaji *et al*[84]	Case report	1	Segment 8	100	Complete	0
Lisotti *et al*[85]	Case report	1	Segment 2	100	Complete	0
Nakaji *et al*[86]	Case report	1	Segment 3	100	Complete	0
Nakaji *et al*[87]	Retrospective	12	Caudate lobe	100	Complete	2 (16.7%)
Jiang *et al*[88]	RCT	10	Left lobe	92	Partial (30%)	0
Alcohol ablation in liver metastasis						
Barclay *et al*[89]	Case report	1	Left lobe	100	Complete	Self-limited sub-capsular hematoma
Hu *et al*[103]	Case report	1	Left lobe	100	Complete	Low grade fever
RFA (radiofrequency ablation) in HCC						
Armellini *et al*[91]	Case report	1	Left lobe	100	Complete	None
Attili *et al*[92]	Case report	1	Segment 3	100	Complete	None
de Nucci *et al*[93]	Case report	1	Segment 2-3-4b	100	70% reduction	None
Ablation by Nd-YAG						
Di Matteo *et al*[95]	Case report	1	Caudate lobe	100	Complete	0
Jiang *et al*[96]	Prospective	10	Left lobe	100	Complete	0
Brachytherapy (Iodine-125)						
Jiang *et al*[88]	RCT	13	Left lobe	92	Near complete	0

EUS: Endoscopic ultrasound; HCC: Hepatocellular carcinoma; RCT: Randomized controlled trial; Nd-YAG: Neodymium:yttium-aluminum-garnet; RFA: Radiofrequency ablation.

Table 8 Steps of endoscopic ultrasound guided coil and glue placement for gastric varices obliteration

Pre-procedure requirements

(1) All procedures are done under the cover of pre/peri-procedural antibiotics; (2) Patient is usually fasted for 4-6 h before the procedure; and (3) Adequate resuscitation of the patient, in case of active bleeding is ensured, prior to the procedure

Technical aspects

(1) The echoendoscope is usually positioned either in the distal esophagus or the gastric fundus; (2) Water is filled intra-luminally in the fundus. This enables a good acoustic coupling for better visualization of the gastric varices. Adequate examination of the fundus, the intramural varices and the feeder vessels is carried out; (3) The approach can be trans-esophageal or transgastric, wherein the trans-esophageal route is given preference; (4) EUS-guided coil and glue embolization is usually performed using a 22 G/19 G (gauge) FNA needle. The size of the coil is determined by the short axis of the diameter of the varix; (5) After puncture of the varix, blood is aspirated to confirm the location. This is followed by flushing of the needle with saline; (6) The coils are then deployed into the varix using the stylet as a pusher. Once the coils are deployed, flushing of the needle is done with normal saline; (7) After coil deployment, 1-2 mL of cyanoacrylate glue is injected over 30-45 s followed by rapid flushing with saline; and (8) Once, the varix is obliterated, visualized by absence of flow on color Doppler, the sheath of the needle is advanced beyond the endoscope tip for 2-3 cm before withdrawing the scope. This avoids contact of glue with the endoscope tip. The sample aspirated is sent for routine cytological assessment as well as for any additional tests that might be needed

Post procedure

(1) The patients are kept under observation for 12 h; (2) Repeat EUS can be done after 2 d to look for residual varices; and (3) Follow-up EUS can be performed at 1- and 3-mo intervals

EUS: Endoscopic ultrasound; FNA: Fine needle aspiration; G: Gauge.

indicator to a future occurrence of a re-bleed; (2) Hematocystic spots on EVs identified as saccular aneurysms on EUS is associated with a high risk of variceal rupture; (3) Digital image analysis on EUS can help to determine the cross-sectional area of EVs in the distal esophagus and a cutoff of 0.45 cm^2 has a sensitivity of 83% for future re-
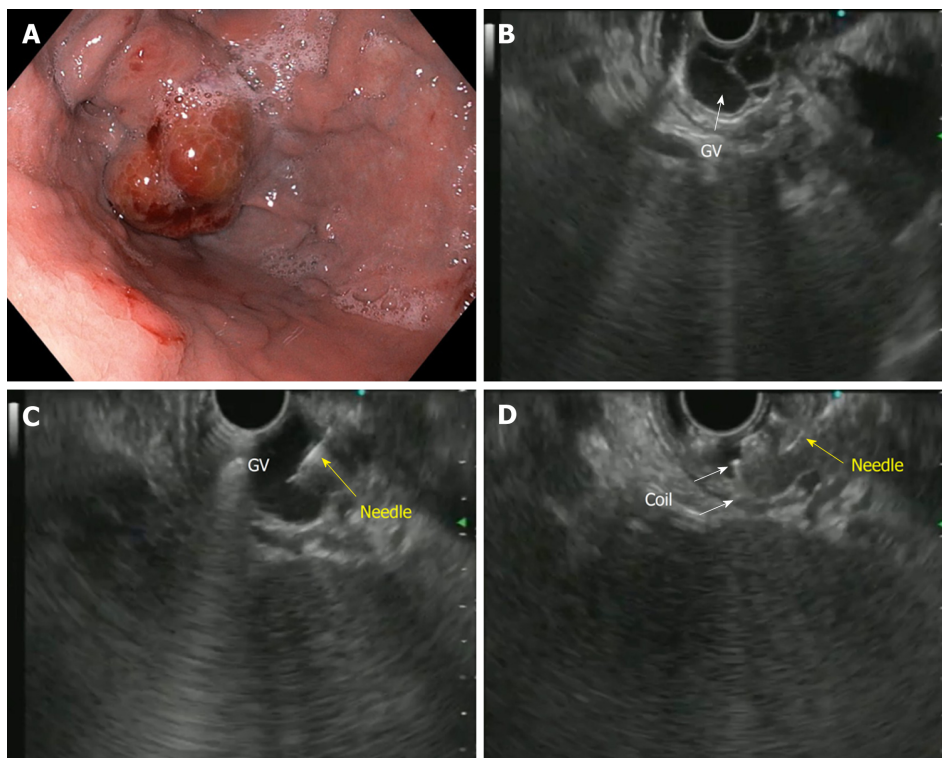
Figure 9 Endoscopic ultrasound-guided coil embolization of fundal varix. A: Endoscopic view of the fundal varix; B: Endoscopic ultrasound (EUS) view of the fundal varix; C: EUS guided puncture of the varix with a 22-G needle; D: Coil deployment inside the varix. GV: Gastric varices.

bleeding; and (4) Para-esophageal diameter after EVL is a better recurrence predictor (cutoff 4 mm has a 70.6% sensitivity).

Thus, there is a huge prospect for using EUS in PHTN, namely in the evaluation of vascular changes of the digestive wall, hemodynamic assessment by measurement of PV pressure gradient, management of variceal bleeding and re-bleeding prediction and currently liquid biopsy *via* PV sampling. Nonetheless, despite the diversity of possible uses, more data on efficacy and safety are warranted.

EUS guided PV access

The PV can be accessed from both the stomach and duodenum and is in very close juxtaposition with the tip of the echoendoscope. The most frequent location to target is the intrahepatic PV through the hepatic parenchyma. The other less commonly used technique is the extrahepatic PV *via* the duodenum[122,123].

Technique of the procedure: After confirming the vascular structure with color Doppler and pulse-wave verification, PV puncture is done *via* the EUS-FNA needle. Studies have shown that 25 G needle causes the least trauma. The trans-gastric, trans-hepatic approach is safer than the trans-duodenal approach. CO_2 is better than using iodine as a contrast (allows better PV visualization and easier intravascular administration through the small-caliber FNA needle). After PV puncture, on withdrawal of the needle, the track is monitored with color Doppler to check for bleeding. In cases of blood flow being identified, the needle is kept in place until the flow has stopped[122, 123].

Animal studies: The first case of PV access was reported in 2004 by Lai *et al*[124], wherein a EUS guided trans-duodenal access to extrahepatic PV was adopted with a 22 G FNA needle in 21 swine models, proving the technical feasibility of the procedure. Thereafter, PV angiography was reported for the first time in 2007 by Magno *et al*[125], wherein autopsies revealed no injuries with a 25 G needle and a hematoma with 19 G needle. Subsequently, Giday *et al*[123,126] reported trans-hepatic access to the PV with a 25 G needle.

EUS guided portal pressure gradient measurement

Measurement of PHTN is useful in determining the stage, progression, prognosis and complications of cirrhosis. Currently, the standard practice of measuring the portal

pressure gradient (PPG) is the percutaneous route. However, both direct PV access and hepatic venous pressure gradient (HVPG) measurement are invasive procedures and have high complication rates. Moreover, HVPG correlated poorly in presinusoidal PHTN cases. Therefore, EUS guided PPG can be performed to overcome these difficulties. Moreover, additional analyses such as assessment of varices and liver biopsy can be carried out in the same sitting. The technique of PPG measurement and the studies (human and animal models) on the same are shown in Supplementary Tables 5 and 6.

EUS guided TIPS

TIPS has an established role in managing PHTN-related complications like variceal bleeding (pre-emptive or rescue) and refractory ascites. EUS-guided TIPS creation in a live porcine model (8 cases) was first described by Buscaglia *et al*[127], wherein the hepatic vein (HV) and PV were sequentially punctured, and a metal stent was inserted with the distal end in the PV and proximal end in the HV. In addition, Binmoeller and Shah[128], and Schulman *et al*[129] have both reported using a lumen apposing metal stent (LAMS) in porcine models for the same purpose.

EUS guided PV sampling

"Liquid biopsy" for hepatobiliary malignancies is gaining momentum in view of the PV harboring circulating tumor cells (CTCs) from the primary tumor. These CTCs are the forerunners of future metastasis of solid organ cancers and help predict the development of liver metastasis[130]. They have been inconsistently found in the peripheral blood due to hepatic sequestration. They reflect tumor signature, help in prognostic stratification, and potentially form organoids for future tumor study.

Catenacci *et al*[131] reported the first human study of PV sampling wherein a 19 G FNA needle was used to sample the PV as four 7.5 mL aliquots of blood. CTCs were detected in 100% cases from the PV *vs* 4 (22.2%) cases from peripheral blood. Liu *et al* [132] reported similar findings in cases of advanced pancreatic cancer (100% detection of CTCs in PV *vs* 54% in peripheral blood). Besides these, further studies are needed to establish the clinical utility of EUS guided liquid biopsies.

EUS guided FNA of PV thrombosis

The presence of malignant PV thrombosis (PVT) usually portends a poor prognosis. Therefore, differentiating bland and malignant thrombus needs FNA confirmation. Various case reports have suggested the use of EUS guided FNA of the PVT by overcoming the complications encountered *via* the percutaneous route[133-135] with excellent results.

EUS guided PV injection chemotherapy

Both systemic palliative chemotherapy and transarterial microbead injection into the hepatic artery for diffuse liver metastasis are fraught with complications. However, Faigel *et al*[136] reported the feasibility of EUS guided PV injection chemotherapy in 24 porcine models using drug-eluting microbeads and nanoparticles. In comparison with systemic injection, systemic levels were halved, but the hepatic concentration of drugs was doubled. Human studies are warranted for the same.

EUS guided PV embolization

Preoperative PV embolization before liver resection in hepatobiliary malignancies induces affected lobe atrophy and ultimately hypertrophy in the functional liver[137]. However, preliminary studies in the animal model by Matthes *et al*[138] and Park *et al* [139] using EUS guided PV embolization using ethylene-vinyl alcohol copolymer and coil with CYA glue embolization, respectively, reported high success rates.

EUS guided PV stent placement

EUS directed PV access has opened up avenues for stent placement *via* this route in PV occlusion or thrombosis. Park *et al*[140] reported 100% technical success (all uncovered stents) in 6 swine models.

FUTURE ADVANCES

Photodynamic therapy

Photodynamic therapy (PDT) is a commonly used modality for treating malignant

biliary obstruction, requiring pretreatment with a photosensitizer followed by exposure to selective tissue wavelength of light-generating singlet oxygen species (tissue necrosis from 6-40 mm depth)[141]. Preliminary animal studies exist on the use of EUS guided PDT on the porcine pancreas[141,142] and pancreaticobiliary malignancies (with lesions in the caudate lobe)[143].

EUS guided fiducial marker placement
Stereotactic body radiation therapy demands high targeting accuracy to minimize toxicity to surrounding organs. Placement of fiducial markers can help localize and track the target and can be placed *via* a percutaneous or surgical approach. EUS guided fiducial marker placement has come into the forefront for targeting even deeper abdominal lesions not amenable *via* standard means[144,145]. However, no studies exist on its use in liver malignancies.

Artificial intelligence
Artificial intelligence (AI) is a prediction technique using mathematical algorithms to create automated learning and recognize patterns in the fed data. Artificial neural network (ANN) and deep learning (DL) are powerful machine-learning-based tools used to provide high yield predictions and are being used more and more in the medical field to aid in diagnosis. Just like its widespread use in the field of endoscopic diagnosis of polyps and other lesions, AI has also found its place in the arena of diagnostic EUS. Studies have used ANN for the interpretation of EUS-elastography and CE-EUS[146]. However, to date, only two studies have used DL for EUS image analysis. With the availability of additional studies, AI can add to the diagnostic armamentarium of EUS and lead to much better accuracy.

CONCLUSION

Hepatologists have always turned to radiologists for imaging and intervention of various liver-related conditions. However, with the expansion of this intersection of endoscopy in EUS and hepatology, the field of "Endo-hepatology" may soon evolve into a sub-specialty with hepatologists trained in interventional EUS. Starting from EUS-guided liver biopsy to PV interventions, the merger of EUS and hepatology seems to show invigorating scope in the future. However, more studies are needed to establish the safety and efficacy of these newer modalities in regular mainstream practice.

REFERENCES

1   **European Association for the Study of the Liver**. EASL Clinical Practice Guidelines for the management of patients with decompensated cirrhosis. *J Hepatol* 2018; **69**: 406-460 [PMID: 29653741 DOI: 10.1016/j.jhep.2018.03.024]
2   **Nguyen PT**, Chang KJ. EUS in the detection of ascites and EUS-guided paracentesis. *Gastrointest Endosc* 2001; **54**: 336-339 [PMID: 11522974 DOI: 10.1067/mge.2001.117544]
3   **Varadarajulu S**, Drelichman ER. EUS-guided therapeutic paracentesis. *Gastrointest Endosc* 2008; **67**: 758-759 [PMID: 18178210 DOI: 10.1016/j.gie.2007.08.025]
4   **Lee YT**, Ng EK, Hung LC, Chung SC, Ching JY, Chan WY, Chu WC, Sung JJ. Accuracy of endoscopic ultrasonography in diagnosing ascites and predicting peritoneal metastases in gastric cancer patients. *Gut* 2005; **54**: 1541-1545 [PMID: 15955787 DOI: 10.1136/gut.2004.055772]
5   **DeWitt J**, LeBlanc J, McHenry L, McGreevy K, Sherman S. Endoscopic ultrasound-guided fine-needle aspiration of ascites. *Clin Gastroenterol Hepatol* 2007; **5**: 609-615 [PMID: 17336593 DOI: 10.1016/j.cgh.2006.11.021]
6   **Chin MA**. EUS-guided paracentesis and ascitic fluid analysis. *Endosc Ultrasound* 2018; **7**: 223-227 [PMID: 30117483 DOI: 10.4103/eus.eus_31_18]
7   **Levy MJ**, Abu Dayyeh BK, Fujii LL, Clayton AC, Reynolds JP, Lopes TL, Rao AS, Clain JE, Gleeson FC, Iyer PG, Kendrick ML, Rajan E, Topazian MD, Wang KK, Wiersema MJ, Chari ST. Detection of peritoneal carcinomatosis by EUS fine-needle aspiration: impact on staging and resectability (with videos). *Gastrointest Endosc* 2015; **81**: 1215-1224 [PMID: 25660979 DOI: 10.1016/j.gie.2014.10.028]
8   **Que Y**, Wang X, Tao C, Zhang Y, Wan W, Chen B. Peritoneal metastases: evaluation with contrast-enhanced ultrasound. *Abdom Imaging* 2011; **36**: 327-332 [PMID: 20959976 DOI: 10.1007/s00261-010-9651-3]
9   **ASGE Standards of Practice Committee**, Khashab MA, Chithadi KV, Acosta RD, Bruining DH,

Chandrasekhara V, Eloubeidi MA, Fanelli RD, Faulx AL, Fonkalsrud L, Lightdale JR, Muthusamy VR, Pasha SF, Saltzman JR, Shaukat A, Wang A, Cash BD. Antibiotic prophylaxis for GI endoscopy. *Gastrointest Endosc* 2015; **81**: 81-89 [PMID: 25442089 DOI: 10.1016/j.gie.2014.08.008]

10   **Kaushik N**, Khalid A, Brody D, McGrath K. EUS-guided paracentesis for the diagnosis of malignant ascites. *Gastrointest Endosc* 2006; **64**: 908-913 [PMID: 17140897 DOI: 10.1016/j.gie.2005.11.058]

11   **Suzuki R**, Irisawa A, Bhutani MS, Hikichi T, Takagi T, Shibukawa G, Sato A, Sato M, Ikeda T, Watanabe K, Nakamura J, Annangi S, Tasaki K, Obara K, Ohira H. An automated spring-loaded needle for endoscopic ultrasound-guided abdominal paracentesis in cancer patients. *World J Gastrointest Endosc* 2014; **6**: 55-59 [PMID: 24567793 DOI: 10.4253/wjge.v6.i2.55]

12   **Chang KJ**, Albers CG, Nguyen P. Endoscopic ultrasound-guided fine needle aspiration of pleural and ascitic fluid. *Am J Gastroenterol* 1995; **90**: 148-150 [PMID: 7801920]

13   **Köck R**, Jürgensen C, Fischer-Lampsatis R. Endosonography-guided drainage of loculated malignant ascites using double-pigtail plastic stents. *Endosc Int Open* 2018; **6**: E902-E906 [PMID: 29978013 DOI: 10.1055/a-0605-3587]

14   **Romero-Castro R**, Jimenez-Garcia VA, Boceta-Osuna J, Castilla-Guerra L, Pellicer-Bautista F, Caunedo-Alvarez A, Herrerias-Gutierrez JM, Romero-Gómez M, Giovannini M. Endoscopic ultrasound-guided placement of plastic pigtail stents for the drainage of refractory malignant ascites. *Endosc Int Open* 2017; **5**: E1096-E1099 [PMID: 29250586 DOI: 10.1055/s-0043-118746]

15   **Romero-Castro R**.   EUS-Guided Drainage of Refractory Malignant Ascites. [accessed 2021 Feb 15]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT03550560 ClinicalTrials.gov Identifier: NCT03550560

16   **Wardeh R**, Lee JG, Gu M. Endoscopic ultrasound-guided paracentesis of ascitic fluid: a morphologic study with ultrasonographic correlation. *Cancer Cytopathol* 2011; **119**: 27-36 [PMID: 21072835 DOI: 10.1002/cncy.20123]

17   **Bhatia V**, Hijioka S, Hara K, Mizuno N, Imaoka H, Yamao K. Endoscopic ultrasound description of liver segmentation and anatomy. *Dig Endosc* 2014; **26**: 482-490 [PMID: 24355092 DOI: 10.1111/den.12216]

18   **Friedberg SR**, Lachter J. Endoscopic ultrasound: Current roles and future directions. *World J Gastrointest Endosc* 2017; **9**: 499-505 [PMID: 29085560 DOI: 10.4253/wjge.v9.i10.499]

19   **Campos S**, Poley JW, van Driel L, Bruno MJ. The role of EUS in diagnosis and treatment of liver disorders. *Endosc Int Open* 2019; **7**: E1262-E1275 [PMID: 31579708 DOI: 10.1055/a-0958-2183]

20   **Iglesias-García J**, Lariño-Noia J, Domínguez-Muñoz JE. New Imaging Techniques: Endoscopic Ultrasound-Guided Elastography. *Gastrointest Endosc Clin N Am* 2017; **27**: 551-567 [PMID: 28918798 DOI: 10.1016/j.giec.2017.06.001]

21   **Alvarez-Sánchez MV**, Napoléon B. Contrast-enhanced harmonic endoscopic ultrasound imaging: basic principles, present situation and future perspectives. *World J Gastroenterol* 2014; **20**: 15549-15563 [PMID: 25400439 DOI: 10.3748/wjg.v20.i42.15549]

22   **Schulman AR**, Lin MV, Rutherford A, Chan WW, Ryou M. A Prospective Blinded Study of Endoscopic Ultrasound Elastography in Liver Disease: Towards a Virtual Biopsy. *Clin Endosc* 2018; **51**: 181-185 [PMID: 29566479 DOI: 10.5946/ce.2017.095]

23   **Marques S**, Carmo J, Túlio MA, Bispo M, Matos L, Chagas C. Diagnostic Performance of Real-Time Elastography in the Assessment of Advanced Fibrosis in Chronic Hepatitis C. *GE Port J Gastroenterol* 2016; **23**: 13-18 [PMID: 28868425 DOI: 10.1016/j.jpge.2015.10.008]

24   **Tu CH**, Li J, Wang CY, Zhou L, Ma Y, Gao M, Wang J, Zeng QM, Lu W. [Diagnostic value of endoscopic ultrasonography, fibroscan, acoustic radiation pulse imaging, serological index, and their combination for early stage liver cirrhosis]. *Zhonghua Gan Zang Bing Zazhi* 2019; **27**: 615-620 [PMID: 31594079 DOI: 10.3760/cma.j.issn.1007-3418.2019.08.006]

25   **Ichim VA**, Chira RI, Mircea PA, Nagy GA, Crisan D, Socaciu MA. Accuracy of endoscopic ultrasound-guided biopsy of focal liver lesions. *Med Ultrason* 2020; **22**: 20-25 [PMID: 32096783 DOI: 10.11152/mu-2078]

26   **Singh P**, Erickson RA, Mukhopadhyay P, Gopal S, Kiss A, Khan A, Ulf Westblom T. EUS for detection of the hepatocellular carcinoma: results of a prospective study. *Gastrointest Endosc* 2007; **66**: 265-273 [PMID: 17543307 DOI: 10.1016/j.gie.2006.10.053]

27   **Singh P**, Mukhopadhyay P, Bhatt B, Patel T, Kiss A, Gupta R, Bhat S, Erickson RA. Endoscopic ultrasound *vs* CT scan for detection of the metastases to the liver: results of a prospective comparative study. *J Clin Gastroenterol* 2009; **43**: 367-373 [PMID: 18981929 DOI: 10.1097/MCG.0b013e318167b8cc]

28   **Fujii-Lau LL**, Abu Dayyeh BK, Bruno MJ, Chang KJ, DeWitt JM, Fockens P, Forcione D, Napoleon B, Palazzo L, Topazian MD, Wiersema MJ, Chak A, Clain JE, Faigel DO, Gleeson FC, Hawes R, Iyer PG, Rajan E, Stevens T, Wallace MB, Wang KK, Levy MJ. EUS-derived criteria for distinguishing benign from malignant metastatic solid hepatic masses. *Gastrointest Endosc* 2015; **81**: 1188-96.e1 [PMID: 25660980 DOI: 10.1016/j.gie.2014.10.035]

29   **Sandulescu L**, Padureanu V, Dumitrescu C, Braia N, Streba CT, Gheonea DI, Cazacu S, Ciurea T, Rogoveanu I, Saftoiu A. A pilot study of real time elastography in the differentiation of focal liver lesions. *Curr Health Sci J* 2012; **38**: 32-35 [PMID: 24778839]

30   **Kitano M**, Kamata K. Contrast-enhanced harmonic endoscopic ultrasound: Future perspectives. *Endosc Ultrasound* 2016; **5**: 351-354 [PMID: 28000625 DOI: 10.4103/2303-9027.195852]

31   **Liu M**, Lin MX, Lu MD, Xu ZF, Zheng KG, Wang W, Kuang M, Zhuang WQ, Xie XY. Comparison of contrast-enhanced ultrasound and contrast-enhanced computed tomography in evaluating the treatment response to transcatheter arterial chemoembolization of hepatocellular carcinoma using modified RECIST. *Eur Radiol* 2015; **25**: 2502-2511 [PMID: 25702094 DOI: 10.1007/s00330-015-3611-9]

32   **Nguyen P**, Feng JC, Chang KJ. Endoscopic ultrasound (EUS) and EUS-guided fine-needle aspiration (FNA) of liver lesions. *Gastrointest Endosc* 1999; **50**: 357-361 [PMID: 10462656 DOI: 10.1053/ge.1999.v50.97208]

33   **TenBerge J**, Hoffman BJ, Hawes RH, Van Enckevort C, Giovannini M, Erickson RA, Catalano MF, Fogel R, Mallery S, Faigel DO, Ferrari AP, Waxman I, Palazzo L, Ben-Menachem T, Jowell PS, McGrath KM, Kowalski TE, Nguyen CC, Wassef WY, Yamao K, Chak A, Greenwald BD, Woodward TA, Vilmann P, Sabbagh L, Wallace MB. EUS-guided fine needle aspiration of the liver: indications, yield, and safety based on an international survey of 167 cases. *Gastrointest Endosc* 2002; **55**: 859-862 [PMID: 12024141 DOI: 10.1067/mge.2002.124557]

34   **DeWitt J**, LeBlanc J, McHenry L, Ciaccia D, Imperiale T, Chappo J, Cramer H, McGreevy K, Chriswell M, Sherman S. Endoscopic ultrasound-guided fine needle aspiration cytology of solid liver lesions: a large single-center experience. *Am J Gastroenterol* 2003; **98**: 1976-1981 [PMID: 14499774 DOI: 10.1111/j.1572-0241.2003.07638.x]

35   **Hollerbach S**, Willert J, Topalidis T, Reiser M, Schmiegel W. Endoscopic ultrasound-guided fine-needle aspiration biopsy of liver lesions: histological and cytological assessment. *Endoscopy* 2003; **35**: 743-749 [PMID: 12929021 DOI: 10.1055/s-2003-41593]

36   **McGrath K**, Brody D, Luketich J, Khalid A. Detection of unsuspected left hepatic lobe metastases during EUS staging of cancer of the esophagus and cardia. *Am J Gastroenterol* 2006; **101**: 1742-1746 [PMID: 16790035 DOI: 10.1111/j.1572-0241.2006.00665.x]

37   **Crowe DR**, Eloubeidi MA, Chhieng DC, Jhala NC, Jhala D, Eltoum IA. Fine-needle aspiration biopsy of hepatic lesions: computerized tomographic-guided *vs* endoscopic ultrasound-guided FNA. *Cancer* 2006; **108**: 180-185 [PMID: 16634071 DOI: 10.1002/cncr.21912]

38   **Prachayakul V**, Aswakul P, Kachintorn U. EUS guided fine needle aspiration cytology of liver nodules suspicious for malignancy: yields, complications and impact on management. *J Med Assoc Thai* 2012; **95** Suppl 2: S56-S60 [PMID: 22574530]

39   **Oh D**, Seo DW, Hong SM, Jun JH, Song TJ, Park DH, Son BK, Lee SS, Lee SK, Kim MH. The usefulness of contrast-enhanced harmonic EUS-guided fine-needle aspiration for evaluation of hepatic lesions (with video). *Gastrointest Endosc* 2018; **88**: 495-501 [PMID: 29859228 DOI: 10.1016/j.gie.2018.05.019]

40   **Lee YN**, Moon JH, Kim HK, Choi HJ, Choi MH, Kim DC, Lee TH, Cha SW, Kim SG, Kim YS. Usefulness of endoscopic ultrasound-guided sampling using core biopsy needle as a percutaneous biopsy rescue for diagnosis of solid liver mass: Combined histological-cytological analysis. *J Gastroenterol Hepatol* 2015; **30**: 1161-1166 [PMID: 25684303 DOI: 10.1111/jgh.12922]

41   **Chon HK**, Yang HC, Choi KH, Kim TH. Endoscopic Ultrasound-Guided Liver Biopsy Using a Core Needle for Hepatic Solid Mass. *Clin Endosc* 2019; **52**: 340-346 [PMID: 31302987 DOI: 10.5946/ce.2018.175]

42   **Ichim VA**, Chira RI, Mircea PA. Diagnostic yield of endoscopic ultrasound-guided biopsy of focal liver lesions. *Med Pharm Rep* 2019; **92**: 15-20 [PMID: 30957081 DOI: 10.15386/cjmed-1066]

43   **Choi HJ**, Moon JH, Kim HK, Lee YN, Lee TH, Cha SW, Cho YD, Park SH. KRAS mutation analysis by next-generation sequencing in endoscopic ultrasound-guided sampling for solid liver masses. *J Gastroenterol Hepatol* 2017; **32**: 154-162 [PMID: 27118240 DOI: 10.1111/jgh.13423]

44   **Suzuki R**, Shin D, Richards-Kortum R, Coghlan L, Bhutani MS. In vivo cytological observation of liver and spleen by using high-resolution microendoscopy system under endoscopic ultrasound guidance: A preliminary study using a swine model. *Endosc Ultrasound* 2016; **5**: 239-242 [PMID: 27503155 DOI: 10.4103/2303-9027.187867]

45   **Minaga K**, Kitano M, Nakai A, Omoto S, Kamata K, Yamao K, Takenaka M, Tsurusaki M, Chikugo T, Matsumoto I, Chiba Y, Watanabe T, Kudo M. Improved detection of liver metastasis using Kupffer-phase imaging in contrast-enhanced harmonic EUS in patients with pancreatic cancer (with video). *Gastrointest Endosc* 2021; **93**: 433-441 [PMID: 32592778 DOI: 10.1016/j.gie.2020.06.051]

46   **Tapper EB**, Lok AS. Use of Liver Imaging and Biopsy in Clinical Practice. *N Engl J Med* 2017; **377**: 756-768 [PMID: 28834467 DOI: 10.1056/NEJMra1610570]

47   **Rösch J**, Hanafee WN, Snow H. Transjugular portal venography and radiologic portacaval shunt: an experimental study. *Radiology* 1969; **92**: 1112-1114 [PMID: 5771827 DOI: 10.1148/92.5.1112]

48   **Rockey DC**, Caldwell SH, Goodman ZD, Nelson RC, Smith AD; American Association for the Study of Liver Diseases. Liver biopsy. *Hepatology* 2009; **49**: 1017-1044 [PMID: 19243014 DOI: 10.1002/hep.22742]

49   **Cholongitas E**, Senzolo M, Standish R, Marelli L, Quaglia A, Patch D, Dhillon AP, Burroughs AK. A systematic review of the quality of liver biopsy specimens. *Am J Clin Pathol* 2006; **125**: 710-721 [PMID: 16707372 DOI: 10.1309/W3XC-NT4H-KFBN-2G0B]

50   **Stavropoulos SN**, Im GY, Jlayer Z, Harris MD, Pitea TC, Turi GK, Malet PF, Friedel DM, Grendell JH. High yield of same-session EUS-guided liver biopsy by 19-gauge FNA needle in patients undergoing EUS to exclude biliary obstruction. *Gastrointest Endosc* 2012; **75**: 310-318 [PMID: 22248599 DOI: 10.1016/j.gie.2011.09.043]

51   **Mohan BP**, Shakhatreh M, Garg R, Ponnada S, Adler DG. Efficacy and safety of EUS-guided liver biopsy: a systematic review and meta-analysis. *Gastrointest Endosc* 2019; **89**: 238-246.e3 [PMID: 30389469 DOI: 10.1016/j.gie.2018.10.018]

52   **Kalambokis G**, Manousou P, Vibhakorn S, Marelli L, Cholongitas E, Senzolo M, Patch D, Burroughs AK. Transjugular liver biopsy--indications, adequacy, quality of specimens, and complications--a systematic review. *J Hepatol* 2007; **47**: 284-294 [PMID: 17561303 DOI: 10.1016/j.jhep.2007.05.001]

53   **Mony S**, Shah I, Vyas N, Sofi A, Das A. Su1375 Meta-analysis of EUS guided liver biopsy in comparison with percutaneous and transjugular liver biopsy for the diagnosis of parenchymal liver disease. *Gastrointest Endosc* 2018; **87**: AB328-AB329

54   **Khurana S**, Butt W, Khara HS, Johal AS, West SF, Chen ZE, Berger AL, Diehl DL. Bi-lobar liver biopsy *via* EUS enhances the assessment of disease severity in patients with non-alcoholic steatohepatitis. *Hepatol Int* 2019; **13**: 323-329 [PMID: 30993598 DOI: 10.1007/s12072-019-09945-4]

55   **Ching-Companioni RA**, Diehl DL, Johal AS, Confer BD, Khara HS. 19 G aspiration needle *vs* 19 G core biopsy needle for endoscopic ultrasound-guided liver biopsy: a prospective randomized trial. *Endoscopy* 2019; **51**: 1059-1065 [PMID: 31342474 DOI: 10.1055/a-0956-6922]

56   **Mok SRS**, Diehl DL, Johal AS, Khara HS, Confer BD, Mudireddy PR, Kirchner HL, Chen ZE. A prospective pilot comparison of wet and dry heparinized suction for EUS-guided liver biopsy (with videos). *Gastrointest Endosc* 2018; **88**: 919-925 [PMID: 30120956 DOI: 10.1016/j.gie.2018.07.036]

57   **Pineda JJ**, Diehl DL, Miao CL, Johal AS, Khara HS, Bhanushali A, Chen EZ. EUS-guided liver biopsy provides diagnostic samples comparable with those *via* the percutaneous or transjugular route. *Gastrointest Endosc* 2016; **83**: 360-365 [PMID: 26301407 DOI: 10.1016/j.gie.2015.08.025]

58   **Shuja A**, Alkhasawneh A, Fialho A, Shukri A, Harris C, Smotherman C, Malespin M, de Melo SW Jr. Comparison of EUS-guided *vs* percutaneous and transjugular approaches for the performance of liver biopsies. *Dig Liver Dis* 2019; **51**: 826-830 [PMID: 30755347 DOI: 10.1016/j.dld.2019.01.006]

59   **Diehl DL**, Johal AS, Khara HS, Stavropoulos SN, Al-Haddad M, Ramesh J, Varadarajulu S, Aslanian H, Gordon SR, Shieh FK, Pineda-Bonilla JJ, Dunkelberger T, Gondim DD, Chen EZ. Endoscopic ultrasound-guided liver biopsy: a multicenter experience. *Endosc Int Open* 2015; **3**: E210-E215 [PMID: 26171433 DOI: 10.1055/s-0034-1391412]

60   **Gor N**, Salem SB, Jakate S, Patel R, Shah N, Patil A. Histological adequacy of EUS-guided liver biopsy when using a 19-gauge non-Tru-Cut FNA needle. *Gastrointest Endosc* 2014; **79**: 170-172 [PMID: 23916397 DOI: 10.1016/j.gie.2013.06.031]

61   **Shah ND**, Sasatomi E, Baron TH. Endoscopic Ultrasound-guided Parenchymal Liver Biopsy: Single Center Experience of a New Dedicated Core Needle. *Clin Gastroenterol Hepatol* 2017; **15**: 784-786 [PMID: 28126424 DOI: 10.1016/j.cgh.2017.01.011]

62   **Nieto J**, Khaleel H, Challita Y, Jimenez M, Baron TH, Walters L, Hathaway K, Patel K, Lankarani A, Herman M, Holloman D, Saab S. EUS-guided fine-needle core liver biopsy sampling using a novel 19-gauge needle with modified 1-pass, 1 actuation wet suction technique. *Gastrointest Endosc* 2018; **87**: 469-475 [PMID: 28551024 DOI: 10.1016/j.gie.2017.05.013]

63   **Mathew A**. EUS-guided routine liver biopsy in selected patients. *Am J Gastroenterol* 2007; **102**: 2354-2355 [PMID: 17897349 DOI: 10.1111/j.1572-0241.2007.01353_7.x]

64   **Patel HK**, Saxena R, Rush N, Patel SK, Dasari CS, Mneimneh W, Quickery A, Rahal MA, Temnykh L, DeWitt J, Al-Haddad M. A Comparative Study of 22G *vs* 19G Needles for EUS-Guided Biopsies for Parenchymal Liver Disease: Are Thinner Needles Better? *Dig Dis Sci* 2021; **66**: 238-246 [PMID: 32128647 DOI: 10.1007/s10620-020-06165-x]

65   **Gleeson FC**, Clayton AC, Zhang L, Clain JE, Gores GJ, Rajan E, Smyrk TC, Topazian MD, Wang KK, Wiersema MJ, Levy MJ. Adequacy of endoscopic ultrasound core needle biopsy specimen of nonmalignant hepatic parenchymal disease. *Clin Gastroenterol Hepatol* 2008; **6**: 1437-1440 [PMID: 19081532 DOI: 10.1016/j.cgh.2008.07.015]

66   **Dewitt J**, McGreevy K, Cummings O, Sherman S, Leblanc JK, McHenry L, Al-Haddad M, Chalasani N. Initial experience with EUS-guided Tru-cut biopsy of benign liver disease. *Gastrointest Endosc* 2009; **69**: 535-542 [PMID: 19231495 DOI: 10.1016/j.gie.2008.09.056]

67   **Nakai Y**, Samarasena JB, Iwashita T, Park DH, Lee JG, Hu KQ, Chang KJ. Autoimmune hepatitis diagnosed by endoscopic ultrasound-guided liver biopsy using a new 19-gauge histology needle. *Endoscopy* 2012; **44** Suppl 2 UCTN: E67-E68 [PMID: 22396285 DOI: 10.1055/s-0031-1291567]

68   **Sey MS**, Al-Haddad M, Imperiale TF, McGreevy K, Lin J, DeWitt JM. EUS-guided liver biopsy for parenchymal disease: a comparison of diagnostic yield between two core biopsy needles. *Gastrointest Endosc* 2016; **83**: 347-352 [PMID: 26278654 DOI: 10.1016/j.gie.2015.08.012]

69   **Hasan MK**, Kadkhodayan K, Idrisov E, Ali S, Rafiq E, Ben-Ami Shor D, Abdel-Jalil A, Navaneethan U, Bang J, Varadarajulu S, Hawes R, Pernicone P. Endoscopic ultrasound-guided liver biopsy using a 22-G fine needle biopsy needle: a prospective study. *Endoscopy* 2019; **51**: 818-824 [PMID: 31365947 DOI: 10.1055/a-0967-3640]

70   **Sbeit W**, Kadah A, Mahamid M, Pellicano R, Mari A, Khoury T. A State-of-the-Art Review on the Evolving Utility of Endoscopic Ultrasound in Liver Diseases Diagnosis. *Diagnostics (Basel)* 2020; **10** [PMID: 32717886 DOI: 10.3390/diagnostics10080512]

71   **Lee S**, Seo DW, Paik WH, Park DH, Lee SS, Lee SK, Kim MH. Ethanol lavage of huge hepatic cysts by using EUS guidance and a percutaneous approach. *Gastrointest Endosc* 2014; **80**: 1014-1021 [PMID: 24890421 DOI: 10.1016/j.gie.2014.03.037]

72   **Seewald S**, Imazu H, Omar S, Groth S, Seitz U, Brand B, Zhong Y, Sikka S, Thonke F, Soehendra N. EUS-guided drainage of hepatic abscess. *Gastrointest Endosc* 2005; **61**: 495-498 [PMID: 15758937 DOI: 10.1016/s0016-5107(04)02848-2]

73   **Noh SH**, Park DH, Kim YR, Chun Y, Lee HC, Lee SO, Lee SS, Seo DW, Lee SK, Kim MH. EUS-guided drainage of hepatic abscesses not accessible to percutaneous drainage (with videos). *Gastrointest Endosc* 2010; **71**: 1314-1319 [PMID: 20400078 DOI: 10.1016/j.gie.2009.12.045]

74   **Itoi T**, Ang TL, Seewald S, Tsuji S, Kurihara T, Tanaka R, Itokawa F. Endoscopic ultrasonography-guided drainage for tuberculous liver abscess drainage. *Dig Endosc* 2011; **23** Suppl 1: 158-161 [PMID: 21535224 DOI: 10.1111/j.1443-1661.2011.01115.x]

75   **Medrado BF**, Carneiro FO, Vilaça TG, Gouveia TS, Frazão MS, de Moura EG, Sakai P, Otoch JP, Artifon EL. Endoscopic ultrasound-guided drainage of giant liver abscess associated with transgastric migration of a self-expandable metallic stent. *Endoscopy* 2013; **45** Suppl 2: E331-E332 [PMID: 24150733 DOI: 10.1055/s-0033-1344128]

76   **Alcaide N**, Vargas-Garcia AL, de la Serna-Higuera C, Sancho Del Val L, Ruiz-Zorrilla R, Perez-Miranda M. EUS-guided drainage of liver abscess by using a lumen-apposing metal stent (with video). *Gastrointest Endosc* 2013; **78**: 941-942 [PMID: 24016354 DOI: 10.1016/j.gie.2013.07.034]

77   **Ogura T**, Masuda D, Saori O, Wataru T, Sano T, Okuda A, Miyano A, Kitano M, Abdel-Aal UM, Takeuchi T, Fukunishi S, Higuchi K. Clinical Outcome of Endoscopic Ultrasound-Guided Liver Abscess Drainage Using Self-Expandable Covered Metallic Stent (with Video). *Dig Dis Sci* 2016; **61**: 303-308 [PMID: 26254774 DOI: 10.1007/s10620-015-3841-3]

78   **Della Valle V**, Eshja E, Bassi EM. Spontaneous biloma: a case report. *J Ultrasound* 2015; **18**: 293-296 [PMID: 26261461 DOI: 10.1007/s40477-013-0053-6]

79   **Sandha GS**, Bourke MJ, Haber GB, Kortan PP. Endoscopic therapy for bile leak based on a new classification: results in 207 patients. *Gastrointest Endosc* 2004; **60**: 567-574 [PMID: 15472680 DOI: 10.1016/s0016-5107(04)01892-9]

80   **Sbeit W**, Kadah A, Mari A, Mahamid M, Khoury T. A Comprehensive Narrative Review on the Evolving Role of Endoscopic Ultrasound in Focal Solid Liver Lesions Diagnosis and Management. *Diagnostics (Basel)* 2020; **10** [PMID: 32932960 DOI: 10.3390/diagnostics10090688]

81   **Lakhtakia S**, Seo DW. Endoscopic ultrasonography-guided tumor ablation. *Dig Endosc* 2017; **29**: 486-494 [PMID: 28171697 DOI: 10.1111/den.12833]

82   **Chua T**, Faigel DO. Endoscopic Ultrasound-Guided Ablation of Liver Tumors. *Gastrointest Endosc Clin N Am* 2019; **29**: 369-379 [PMID: 30846159 DOI: 10.1016/j.giec.2018.11.007]

83   **Schoppmeyer K**, Weis S, Mössner J, Fleig WE. Percutaneous ethanol injection or percutaneous acetic acid injection for early hepatocellular carcinoma. *Cochrane Database Syst Rev* 2009; CD006745 [PMID: 19588401 DOI: 10.1002/14651858.CD006745.pub2]

84   **Nakaji S**, Hirata N, Iwaki K, Shiratori T, Kobayashi M, Inase M. Endoscopic ultrasound (EUS)-guided ethanol injection for hepatocellular carcinoma difficult to treat with percutaneous local treatment. *Endoscopy* 2012; **44** Suppl 2 UCTN: E380 [PMID: 23139031 DOI: 10.1055/s-0032-1309918]

85   **Lisotti A**, Piscaglia F, Fusaroli P. Contrast-enhanced harmonic endoscopic ultrasound-guided ethanol injection for a small hepatocellular carcinoma. *Endoscopy* 2019; **51**: E317-E318 [PMID: 31163490 DOI: 10.1055/a-0915-1385]

86   **Nakaji S**, Hirata N, Kobayashi M, Shiratori T, Sanagawa M. Endoscopic ultrasonography-guided ethanol injection as a treatment for ruptured hepatocellular carcinoma in the left hepatic lobe. *Endoscopy* 2015; **47** Suppl 1: E558-E560 [PMID: 26610083 DOI: 10.1055/s-0034-1393394]

87   **Nakaji S**, Hirata N, Mikata R, Kobayashi M, Shiratori T, Ogasawara S, Ooka Y, Tsuyuguchi T, Yamaguchi T, Yokosuka O. Clinical outcomes of endoscopic ultrasound-guided ethanol injection for hepatocellular carcinoma in the caudate lobe. *Endosc Int Open* 2016; **4**: E1111-E1115 [PMID: 27747288 DOI: 10.1055/s-0042-116146]

88   **Jiang TA**, Deng Z, Tian G, Zhao QY, Wang WL. Efficacy and safety of endoscopic ultrasonography-guided interventional treatment for refractory malignant left-sided liver tumors: a case series of 26 patients. *Sci Rep* 2016; **6**: 36098 [PMID: 27958384 DOI: 10.1038/srep36098]

89   **Barclay RL**, Perez-Miranda M, Giovannini M. EUS-guided treatment of a solid hepatic metastasis. *Gastrointest Endosc* 2002; **55**: 266-270 [PMID: 11818938 DOI: 10.1067/mge.2002.120784]

90   **Nault JC**, Sutter O, Nahon P, Ganne-Carrié N, Séror O. Percutaneous treatment of hepatocellular carcinoma: State of the art and innovations. *J Hepatol* 2018; **68**: 783-797 [PMID: 29031662 DOI: 10.1016/j.jhep.2017.10.004]

91   **Armellini E**, Leutner M, Stradella D, Ballarè M, Occhipinti P. EUS-guided radiofrequency ablation: an option for the extrapancreatic region. *Endosc Ultrasound* 2018; **7**: 282-283 [PMID: 29536950 DOI: 10.4103/eus.eus_103_17]

92   **Attili F**, Boškoski I, Bove V, Familiari P, Costamagna G. EUS-guided radiofrequency ablation of a hepatocellular carcinoma of the liver. *VideoGIE* 2018; **3**: 149-150 [PMID: 29916463 DOI: 10.1016/j.vgie.2018.02.006]

93   **de Nucci G**, Della Corte C, Reati R, Imperatore N, Arena I, Larghi A, Manes G. Endoscopic ultrasound-guided radiofrequency ablation for hepatocellular carcinoma in cirrhosis: a case report test for efficacy and future perspectives. *Endosc Int Open* 2020; **8**: E1713-E1716 [PMID: 33140029 DOI: 10.1055/a-1236-3105]

94   **Hines-Peralta A**, Hollander CY, Solazzo S, Horkan C, Liu ZJ, Goldberg SN. Hybrid radiofrequency and cryoablation device: preliminary results in an animal model. *J Vasc Interv Radiol* 2004; **15**:

1111-1120 [PMID: 15466798 DOI: 10.1097/01.RVI.0000136031.91939.EC]

95   **Di Matteo F**, Grasso R, Pacella CM, Martino M, Pandolfi M, Rea R, Luppi G, Silvestri S, Zardi E, Costamagna G. EUS-guided Nd:YAG laser ablation of a hepatocellular carcinoma in the caudate lobe. *Gastrointest Endosc* 2011; **73**: 632-636 [PMID: 21030019 DOI: 10.1016/j.gie.2010.08.019]

96   **Jiang T**, Tian G, Bao H, Chen F, Deng Z, Li J, Chai W. EUS dating with laser ablation against the caudate lobe or left liver tumors: a win-win proposition? *Cancer Biol Ther* 2018; **19**: 145-152 [PMID: 29303406 DOI: 10.1080/15384047.2017.1414760]

97   **Carrara S**, Arcidiacono PG, Albarello L, Addis A, Enderle MD, Boemo C, Neugebauer A, Campagnol M, Doglioni C, Testoni PA. Endoscopic ultrasound-guided application of a new internally gas-cooled radiofrequency ablation probe in the liver and spleen of an animal model: a preliminary study. *Endoscopy* 2008; **40**: 759-763 [PMID: 18702032 DOI: 10.1055/s-2008-1077520]

98   **Pioche M**, Lafon C, Constanciel E, Vignot A, Birer A, Gincul R, Lépilliez V, Prat F, Roman S, Chapelon JY, Saurin JC, Ponchon T. High-intensity focused ultrasound liver destruction through the gastric wall under endoscopic ultrasound control: first experience in living pigs. *Endoscopy* 2012; **44** Suppl 2 UCTN: E376-E377 [PMID: 23012031 DOI: 10.1055/s-0032-1310061]

99   **Li T**, Khokhlova T, Maloney E, Wang YN, D'Andrea S, Starr F, Farr N, Morrison K, Keilman G, Hwang JH. Endoscopic high-intensity focused US: technical aspects and studies in an *in vivo* porcine model (with video). *Gastrointest Endosc* 2015; **81**: 1243-1250 [PMID: 25759124 DOI: 10.1016/j.gie.2014.12.019]

100   **Lah JJ**, Kuo JV, Chang KJ, Nguyen PT. EUS-guided brachytherapy. *Gastrointest Endosc* 2005; **62**: 805-808 [PMID: 16246706 DOI: 10.1016/j.gie.2005.07.019]

101   **Martínez-Monge R**, Subtil JC, López-Picazo JM. Transoesophageal endoscopic-ultrasonography-guided 125I permanent brachytherapy for unresectable mediastinal lymphadenopathy. *Lancet Oncol* 2006; **7**: 781-783 [PMID: 16945775 DOI: 10.1016/S1470-2045(06)70865-8]

102   **Sun S**, Xu H, Xin J, Liu J, Guo Q, Li S. Endoscopic ultrasound-guided interstitial brachytherapy of unresectable pancreatic cancer: results of a pilot trial. *Endoscopy* 2006; **38**: 399-403 [PMID: 16680642 DOI: 10.1055/s-2006-925253]

103   **Hu YH**, Tuo XP, Jin ZD, Liu Y, Guo Y, Luo L. Endoscopic ultrasound (EUS)-guided ethanol injection in hepatic metastatic carcinoma: a case report. *Endoscopy* 2010; **42** Suppl 2: E256-E257 [PMID: 20931470 DOI: 10.1055/s-0030-1255653]

104   **Samarasena JB**, Chang KJ. Endoscopic Ultrasound-Guided Interventions for the Measurement and Treatment of Portal Hypertension. *Gastrointest Endosc Clin N Am* 2019; **29**: 311-320 [PMID: 30846155 DOI: 10.1016/j.giec.2018.12.004]

105   **Helmy A**, Hayes PC. Review article: current endoscopic therapeutic options in the management of variceal bleeding. *Aliment Pharmacol Ther* 2001; **15**: 575-594 [PMID: 11328251 DOI: 10.1046/j.1365-2036.2001.00950.x]

106   **Lahoti S**, Catalano MF, Alcocer E, Hogan WJ, Geenen JE. Obliteration of esophageal varices using EUS-guided sclerotherapy with color Doppler. *Gastrointest Endosc* 2000; **51**: 331-333 [PMID: 10699783 DOI: 10.1016/s0016-5107(00)70363-4]

107   **de Paulo GA**, Ardengh JC, Nakao FS, Ferrari AP. Treatment of esophageal varices: a randomized controlled trial comparing endoscopic sclerotherapy and EUS-guided sclerotherapy of esophageal collateral veins. *Gastrointest Endosc* 2006; **63**: 396-402; quiz 463 [PMID: 16500386 DOI: 10.1016/j.gie.2005.10.039]

108   **Sarin SK**, Lahoti D, Saxena SP, Murthy NS, Makwana UK. Prevalence, classification and natural history of gastric varices: a long-term follow-up study in 568 portal hypertension patients. *Hepatology* 1992; **16**: 1343-1349 [PMID: 1446890 DOI: 10.1002/hep.1840160607]

109   **Garcia-Tsao G**, Sanyal AJ, Grace ND, Carey W; Practice Guidelines Committee of the American Association for the Study of Liver Diseases; Practice Parameters Committee of the American College of Gastroenterology. Prevention and management of gastroesophageal varices and variceal hemorrhage in cirrhosis. *Hepatology* 2007; **46**: 922-938 [PMID: 17879356 DOI: 10.1002/hep.21907]

110   **Iwase H**, Suga S, Morise K, Kuroiwa A, Yamaguchi T, Horiuchi Y. Color Doppler endoscopic ultrasonography for the evaluation of gastric varices and endoscopic obliteration with cyanoacrylate glue. *Gastrointest Endosc* 1995; **41**: 150-154 [PMID: 7721004 DOI: 10.1016/s0016-5107(05)80599-1]

111   **Romero-Castro R**, Pellicer-Bautista FJ, Jimenez-Saenz M, Marcos-Sanchez F, Caunedo-Alvarez A, Ortiz-Moyano C, Gomez-Parra M, Herrerias-Gutierrez JM. EUS-guided injection of cyanoacrylate in perforating feeding veins in gastric varices: results in 5 cases. *Gastrointest Endosc* 2007; **66**: 402-407 [PMID: 17643723 DOI: 10.1016/j.gie.2007.03.008]

112   **Lee YT**, Chan FK, Ng EK, Leung VK, Law KB, Yung MY, Chung SC, Sung JJ. EUS-guided injection of cyanoacrylate for bleeding gastric varices. *Gastrointest Endosc* 2000; **52**: 168-174 [PMID: 10922086 DOI: 10.1067/mge.2000.107911]

113   **Bhat YM**, Weilert F, Fredrick RT, Kane SD, Shah JN, Hamerski CM, Binmoeller KF. EUS-guided treatment of gastric fundal varices with combined injection of coils and cyanoacrylate glue: a large U.S. experience over 6 years (with video). *Gastrointest Endosc* 2016; **83**: 1164-1172 [PMID: 26452992 DOI: 10.1016/j.gie.2015.09.040]

114   **Robles-Medranda C**, Oleas R, Valero M, Puga-Tejada M, Baquerizo-Burgos J, Ospina J, Pitanga-Lukashok H. Endoscopic ultrasonography-guided deployment of embolization coils and cyanoacrylate injection in gastric varices *vs* coiling alone: a randomized trial. *Endoscopy* 2020; **52**: 268-275 [PMID: 32126576 DOI: 10.1055/a-1123-9054]

115 **Lôbo MRA**, Chaves DM, DE Moura DTH, Ribeiro IB, Ikari E, DE Moura EGH. Safety and efficacy of EUS-guided coil plus cyanoacrylate versus conventional cyanoacrylate technique in the treatment of gastric varices: a randomized controlled trial. *Arq Gastroenterol* 2019; **56**: 99-105 [PMID: 31141079 DOI: 10.1590/S0004-2803.201900000-08]

116 **McCarty TR**, Bazarbashi AN, Hathorn KE, Thompson CC, Ryou M. Combination therapy *versus* monotherapy for EUS-guided management of gastric varices: A systematic review and meta-analysis. *Endosc Ultrasound* 2020; **9**: 6-15 [PMID: 31417066 DOI: 10.4103/eus.eus_37_19]

117 **Irisawa A**, Shibukawa G, Hoshi K, Yamabe A, Sato A, Maki T, Yoshida Y, Yamamoto S, Obara K. Endoscopic ultrasound-guided coil deployment with sclerotherapy for isolated gastric varices: Case series of feasibility, safety, and long-term follow-up. *Dig Endosc* 2020; **32**: 1100-1104 [PMID: 32147871 DOI: 10.1111/den.13666]

118 **Bazarbashi AN**, Wang TJ, Thompson CC, Ryou M. Endoscopic ultrasound-guided treatment of gastric varices with coil embolization and absorbable hemostatic gelatin sponge: a novel alternative to cyanoacrylate. *Endosc Int Open* 2020; **8**: E221-E227 [PMID: 32010757 DOI: 10.1055/a-1027-6708]

119 **Frost JW**, Hebbar S. EUS-guided thrombin injection for management of gastric fundal varices. *Endosc Int Open* 2018; **6**: E664-E668 [PMID: 29868631 DOI: 10.1055/a-0599-0440]

120 **Carneiro FO**, Retes FA, Matuguma SE, Albers DV, Chaves DM, Dos Santos ME, Herman P, Chaib E, Sakai P, Carneiro D'Albuquerque LA, Maluf Filho F. Role of EUS evaluation after endoscopic eradication of esophageal varices with band ligation. *Gastrointest Endosc* 2016; **84**: 400-407 [PMID: 26905936 DOI: 10.1016/j.gie.2016.02.006]

121 **Kuramochi A**, Imazu H, Kakutani H, Uchiyama Y, Hino S, Urashima M. Color Doppler endoscopic ultrasonography in identifying groups at a high-risk of recurrence of esophageal varices after endoscopic treatment. *J Gastroenterol* 2007; **42**: 219-224 [PMID: 17380280 DOI: 10.1007/s00535-006-1992-x]

122 **Hashimoto R**, Chang KJ. Endoscopic ultrasound guided hepatic interventions. *Dig Endosc* 2021; **33**: 54-65 [PMID: 32145117 DOI: 10.1111/den.13661]

123 **Giday SA**, Clarke JO, Buscaglia JM, Shin EJ, Ko CW, Magno P, Kantsevoy SV. EUS-guided portal vein catheterization: a promising novel approach for portal angiography and portal vein pressure measurements. *Gastrointest Endosc* 2008; **67**: 338-342 [PMID: 18226699 DOI: 10.1016/j.gie.2007.08.037]

124 **Lai L**, Poneros J, Santilli J, Brugge W. EUS-guided portal vein catheterization and pressure measurement in an animal model: a pilot study of feasibility. *Gastrointest Endosc* 2004; **59**: 280-283 [PMID: 14745408 DOI: 10.1016/s0016-5107(03)02544-6]

125 **Magno P**, Ko CW, Buscaglia JM, Giday SA, Jagannath SB, Clarke JO, Shin EJ, Kantsevoy SV. EUS-guided angiography: a novel approach to diagnostic and therapeutic interventions in the vascular system. *Gastrointest Endosc* 2007; **66**: 587-591 [PMID: 17725951 DOI: 10.1016/j.gie.2007.01.011]

126 **Giday SA**, Ko CW, Clarke JO, Shin EJ, Magno P, Jagannath SB, Buscaglia JM, Kantsevoy SV. EUS-guided portal vein carbon dioxide angiography: a pilot study in a porcine model. *Gastrointest Endosc* 2007; **66**: 814-819 [PMID: 17905028 DOI: 10.1016/j.gie.2007.05.056]

127 **Buscaglia JM**, Dray X, Shin EJ, Magno P, Chmura KM, Surti VC, Dillon TE, Ducharme RW, Donatelli G, Thuluvath PJ, Giday SA, Kantsevoy SV. A new alternative for a transjugular intrahepatic portosystemic shunt: EUS-guided creation of an intrahepatic portosystemic shunt (with video). *Gastrointest Endosc* 2009; **69**: 941-947 [PMID: 19327481 DOI: 10.1016/j.gie.2008.09.051]

128 **Binmoeller KF**, Shah JN. Sa1428 EUS-guided transgastric intrahepatic portosystemic shunt using the axios stent. *Gastrointest Endosc* 2011; **73**: AB167

129 **Schulman AR**, Ryou M, Aihara H, Abidi W, Chiang A, Jirapinyo P, Sakr A, Ajeje E, Ryan MB, Thompson CC. EUS-guided intrahepatic portosystemic shunt with direct portal pressure measurements: a novel alternative to transjugular intrahepatic portosystemic shunting. *Gastrointest Endosc* 2017; **85**: 243-247 [PMID: 27468858 DOI: 10.1016/j.gie.2016.07.041]

130 **Chapman CG**, Waxman I. Portal-vein blood samples as a new diagnostic entity for pancreatic cancer. *Expert Rev Gastroenterol Hepatol* 2016; **10**: 665-667 [PMID: 27077275 DOI: 10.1080/17474124.2016.1176911]

131 **Catenacci DV**, Chapman CG, Xu P, Koons A, Konda VJ, Siddiqui UD, Waxman I. Acquisition of Portal Venous Circulating Tumor Cells From Patients With Pancreaticobiliary Cancers by Endoscopic Ultrasound. *Gastroenterology* 2015; **149**: 1794-1803.e4 [PMID: 26341722 DOI: 10.1053/j.gastro.2015.08.050]

132 **Liu X**, Li C, Li J, Yu T, Zhou G, Cheng J, Li G, Zhou Y, Lou W, Wang X, Gong G, Liu L, Chen Y. Detection of CTCs in portal vein was associated with intrahepatic metastases and prognosis in patients with advanced pancreatic cancer. *J Cancer* 2018; **9**: 2038-2045 [PMID: 29896289 DOI: 10.7150/jca.23989]

133 **Lai R**, Stephens V, Bardales R. Diagnosis and staging of hepatocellular carcinoma by EUS-FNA of a portal vein thrombus. *Gastrointest Endosc* 2004; **59**: 574-577 [PMID: 15044903 DOI: 10.1016/s0016-5107(04)00007-0]

134 **Storch I**, Gomez C, Contreras F, Schiff E, Ribeiro A. Hepatocellular carcinoma (HCC) with portal vein invasion, masquerading as pancreatic mass, diagnosed by endoscopic ultrasound-guided fine needle aspiration (EUS-FNA). *Dig Dis Sci* 2007; **52**: 789-791 [PMID: 17268833 DOI: 10.1007/s10620-006-9325-8]

135   **Moreno M**, Gimeno-García AZ, Corriente MM, Nicolás-Pérez D, Brito-García A, García-Castro C, Quintero E. EUS-FNA of a portal vein thrombosis in a patient with a hidden hepatocellular carcinoma: confirmation technique after contrast-enhanced ultrasound. *Endoscopy* 2014; **46** Suppl 1 UCTN: E590-E591 [PMID: 25502254 DOI: 10.1055/s-0034-1390734]

136   **Faigel DO**, Lake DF, Landreth TL, Kelman CC, Marler RJ. EUS-guided portal injection chemotherapy for treatment of hepatic metastases: feasibility in the acute porcine model. *Gastrointest Endosc* 2016; **83**: 444-446 [PMID: 26358330 DOI: 10.1016/j.gie.2015.08.064]

137   **Loffroy R**, Favelier S, Chevallier O, Estivalet L, Genson PY, Pottecher P, Gehin S, Krausé D, Cercueil JP. Preoperative portal vein embolization in liver cancer: indications, techniques and outcomes. *Quant Imaging Med Surg* 2015; **5**: 730-739 [PMID: 26682142 DOI: 10.3978/j.issn.2223-4292.2015.10.04]

138   **Matthes K**, Sahani D, Holalkere NS, Mino-Kenudson M, Brugge WR. Feasibility of endoscopic ultrasound-guided portal vein embolization with Enteryx. *Acta Gastroenterol Belg* 2005; **68**: 412-415 [PMID: 16432991]

139   **Park TY**, Seo DW, Kang HJ, Song TJ, Park DH, Lee SS, Lee SK, Kim MH. Feasibility and safety of EUS-guided selective portal vein embolization with a coil and cyanoacrylate in a live porcine model. *Endosc Ultrasound* 2018; **7**: 389-394 [PMID: 30246708 DOI: 10.4103/eus.eus_18_18]

140   **Park TY**, Seo DW, Kang HJ, Cho MK, Song TJ, Park DH, Lee SS, Lee SK, Kim MH. Endoscopic ultrasonography-guided placement of a transhepatic portal vein stent in a live porcine model. *Endosc Ultrasound* 2016; **5**: 315-319 [PMID: 27803904 DOI: 10.4103/2303-9027.191611]

141   **Chan HH**, Nishioka NS, Mino M, Lauwers GY, Puricelli WP, Collier KN, Brugge WR. EUS-guided photodynamic therapy of the pancreas: a pilot study. *Gastrointest Endosc* 2004; **59**: 95-99 [PMID: 14722560 DOI: 10.1016/s0016-5107(03)02361-7]

142   **Yusuf TE**, Matthes K, Brugge WR. EUS-guided photodynamic therapy with verteporfin for ablation of normal pancreatic tissue: a pilot study in a porcine model (with video). *Gastrointest Endosc* 2008; **67**: 957-961 [PMID: 18178203 DOI: 10.1016/j.gie.2007.08.020]

143   **Choi JH**, Oh D, Lee JH, Park JH, Kim KP, Lee SS, Lee YJ, Lim YS, Song TJ, Seo DW, Lee SK, Kim MH, Park DH. Initial human experience of endoscopic ultrasound-guided photodynamic therapy with a novel photosensitizer and a flexible laser-light catheter. *Endoscopy* 2015; **47**: 1035-1038 [PMID: 26070006 DOI: 10.1055/s-0034-1392150]

144   **DiMaio CJ**, Nagula S, Goodman KA, Ho AY, Markowitz AJ, Schattner MA, Gerdes H. EUS-guided fiducial placement for image-guided radiation therapy in GI malignancies by using a 22-gauge needle (with videos). *Gastrointest Endosc* 2010; **71**: 1204-1210 [PMID: 20598247 DOI: 10.1016/j.gie.2010.01.003]

145   **Varadarajulu S**, Trevino JM, Shen S, Jacob R. The use of endoscopic ultrasound-guided gold markers in image-guided radiation therapy of pancreatic cancers: a case series. *Endoscopy* 2010; **42**: 423-425 [PMID: 20232282 DOI: 10.1055/s-0029-1243989]

146   **Kuwahara T**, Hara K, Mizuno N, Haba S, Okuno N, Koda H, Miyano A, Fumihara D. Current status of artificial intelligence analysis for endoscopic ultrasonography. *Dig Endosc* 2021; **33**: 298-305 [PMID: 33098123 DOI: 10.1111/den.13880]

Porta-caval fibrous connections — the lesser-known structure of intrahepatic connective-tissue framework: A unified view of liver extracellular matrix

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Abstract

Knowledge about the connective-tissue framework of the liver is not systematized, the terminology is inconsistent and some perspectives on the construction of the hepatic matrix components are contradictory. In addition, until the last two decades of the 20th century, the connective-tissue sheaths of the portal tracts and the hepatic veins were considered to be independent from each other in the liver and that they do not make contact with each other. The results of the research carried out by Professor Shalva Toidze and his colleagues started in the 1970s in the Department of Operative Surgery and Topographic Anatomy at the Tbilisi State Medical Institute have changed this perception. In particular, Chanukvadze I showed that in some regions where they intersect with each other, the connective tissue sheaths of the large portal complexes and hepatic veins fuse. The areas of such fusion are called porta-caval fibrous connections (PCFCs). This opinion review aims to promote a systematic understanding of the hepatic connective-tissue skeleton and to demonstrate the hitherto underappreciated PCFC as a genuine structure with high biological and clinical significance. The components of the liver connective-tissue framework — the capsules, plates,

sheaths, covers — are described, and their intercommunication is discussed. The analysis of the essence of the PCFC and a description of its various forms are provided. It is also mentioned that analogs of different forms of PCFC are found in different mammals.

Key Words: Hepatic capsule; Hilar plate; Perivascular fibrous sheath; Glissonean pedicle; Portal tract; Caval port

Core Tip: In the places of spatial intersection of the Glissonean pedicles with the main hepatic veins, the fusion of their connective tissue sheaths is described. The sites of the above-mentioned fusion are called porta-caval fibrous connections. Various forms of porta-caval fibrous connections are discussed as well as their clinical and scientific implications.

INTRODUCTION

The extracellular matrix — the connective-tissue framework of the liver — determines the shape of the organ and creates specialized compartments for the liver cell populations and blood and lymph circulations, the synergy of which determines the diverse functioning of the organ. The structure and components of the human liver extracellular matrix were comprehensively analyzed in a series of studies performed in the 1980s and the 90s[1,2].

The last five years saw a new wave of studies on hepatic connective-tissue structures. This "revisiting" is thanks to the introduction of new methods and computer technologies in morphological studies[3] and includes studies not only of the human liver but also of the liver of various animals and birds[4-7].

The emergence of endoscopic anatomic liver resections strengthened the need to specify the anatomy and interrelationship of the connective-tissue structures within the liver[8-11]. Additionally, the prospects for the use of human and animal liver matrices as scaffolds for the creation of bioartificial livers (thanks to the development of stem cells and bioengineering technologies)[11-14] also contribute to the resurgence of interests in the hepatic connective-tissue structures.

However, upon reviewing these studies, we noticed that knowledge on the connective-tissue skeleton of the liver were not systematized, the terminology was inconsistent, and the literature concerning the construction of one or another component of the hepatic matrix were sometimes contradictory[15].

Until the last two decades of the 20th century, the branches of the portal vein and the hepatic veins were considered to be independent from each other in the liver and that their connective-tissue sheaths did not make contact with each other[16-18]. Modern hepatology textbooks usually perpetuate this notion that the Glissonean portal pedicles and the main hepatic veins intersect spatially, but some liver parenchyma always remains between them. Thus, it was believed that they are anatomically independent of each other[19].

The results of the research carried out by Professor Shalva Toidze and his colleagues started in the 1970s in the Department of Operative Surgery, and the Topographic Anatomy of Tbilisi State Medical Institute changed this perception. In particular, Chanukvadze[20] showed that in some regions where they intersect with each other, the connective-tissue sheaths of the main portal complex and a hepatic vein fuse. The regions of such fusion he called porta-caval fibrous connections (PCFCs). Several forms of PCFC have been described. It has also been revealed that PCFC, as an

anatomical formation, develops in the 11th-12th weeks of gestation. Despite numerous publications, these data have not yet received proper acknowledgement in scientific discourse and, as a result, in clinical hepatology. This opinion review aims to promote a systematic understanding of the connective-tissue skeleton of the liver, standardize the definition and the nomenclature of its structural components, and highlight the importance of the hitherto underappreciated PCFC as a genuine structure.

Since the same connective-tissue structure of the liver is often referred to by different names, we have tried to standardize the terms used throughout this article. The following terms will be used in the ensuing discussion: (1) Liver capsule is the same as Laennec's capsule (but not Glisson's capsule); (2) Hilar plate is the same as Walaeus vasculo-biliary sheath (but not Glisson's plate); (3) Perivascular fibrous capsule is the same as Glisson's capsule; (4) Proper hepatic capsule (PHC) is the same as the intrahepatic part of Laennec's capsule covering the liver parenchyma; (5) Portal hilus is the same as portal port; (6) Caval port is the same as hepatic venous port (where the inferior vena cava adjoins to the liver and incorporates the hepatic veins); and (7) Glissonean pedicle is the same as the portal tract surrounded by Glisson's capsule.

DISCUSSION

Liver capsule and its derivatives

Laennec's capsule (liver capsule) covers the entire liver surface, including its bare area (aperitoneal area). In the portal hilus and venous port of the liver, Laennec's capsule around the Glissonean pedicles and the hepatic veins enters the hepatic parenchyma, covers it, and separates it from the portal tracts and hepatic vein tributaries[21].

In the hepatic hilus, the liver capsule directly touches the hilar plate (also known as Walaeus vascular-biliary sheath) covering the portal vein, the hepatic artery, and the bile ducts, while within the liver, the intrahepatic part of the liver capsule — PHC — covering the parenchyma, sets against the perivascular fibrous capsule (Glisson's capsule), which is a direct extension of the Walaeus sheath and envelops the lobar, sectoral, and segmental portal tracts[15,22]. These two fibrous fascial structures — PHC and Glisson's capsule — are separated by a narrow fissure[10] (Figure 1A and C). The individual fibers of the connective tissue (or their bundles) are located in this fissure and connect the outer side of Gleason's capsule with the PHC. On the other hand, soft collagen fibers (type I and III collagen) separate from the internal side of PHC and extend within the liver lobule (Disse's spaces), fusing to the intralobular matrix[3].

In the region of the thinner portal tracts (subsegmental, zonal), Glisson's capsule tapers off, and cross-banded collagen fibers from portal spaces are in continuity with similar fibers in the immediately adjacent lobular interstitium, which in turn are in continuity with those in central spaces; in this manner, collagen type I fibers and bundles form the structural scaffold of the liver lobule[2]. Meanwhile, the portal, extralobular and intralobular matrices of the liver are united by creating a complex labyrinth that represents the circulation area for tissue fluid and prelymph[23,24].

Laennec's capsule covering the liver parenchyma is related to the adventitia of the hepatic veins and their tributaries, represented by type I and type III collagen fibers and single muscle fibers, mainly running along the veins. Thick collagen fibers were found external to thin elastic fibers, which were intimately related to smooth muscle. The above-mentioned features are consistent with the observation that all veins of the infracardiac region in humans are mainly propulsive veins[25]. The increase in collagen content on the adventitial side of the interface may strengthen it and prevent rupture of the vein during extreme liver movements[26].

The PHC is often separated from the adventitia of the hepatic veins and their large tributaries by a narrow slit (similar to that described in relation to Glissonean pedicles), in which the tissue fluid and prelymph circulate[23,24] (Figure 1C). The average distance between the PHC and the Glissonean pedicle is 32 ± 8.7 μm, while that between the PHC and the hepatic veins is 26 ± 6.3 μm[8]. Some authors suggest that Laennec's capsule, Glisson's capsule and the sheath for the hepatic vein tributaries can be characterized by a high content of thin, wavy elastic fibers. The Waleus vasculo-biliary sheath of the thick vessels and ducts does not contain elastic fibers[15]. However, some researchers believe that there is no fibrous sheath around the hepatic veins and that the adventitia of the hepatic veins is in direct contact with the PHC covering the liver parenchyma[27]. With the reduction of the diameter (caliber) of the tributaries of the hepatic veins, the adventitia of these veins thins out, PHC tapers off,
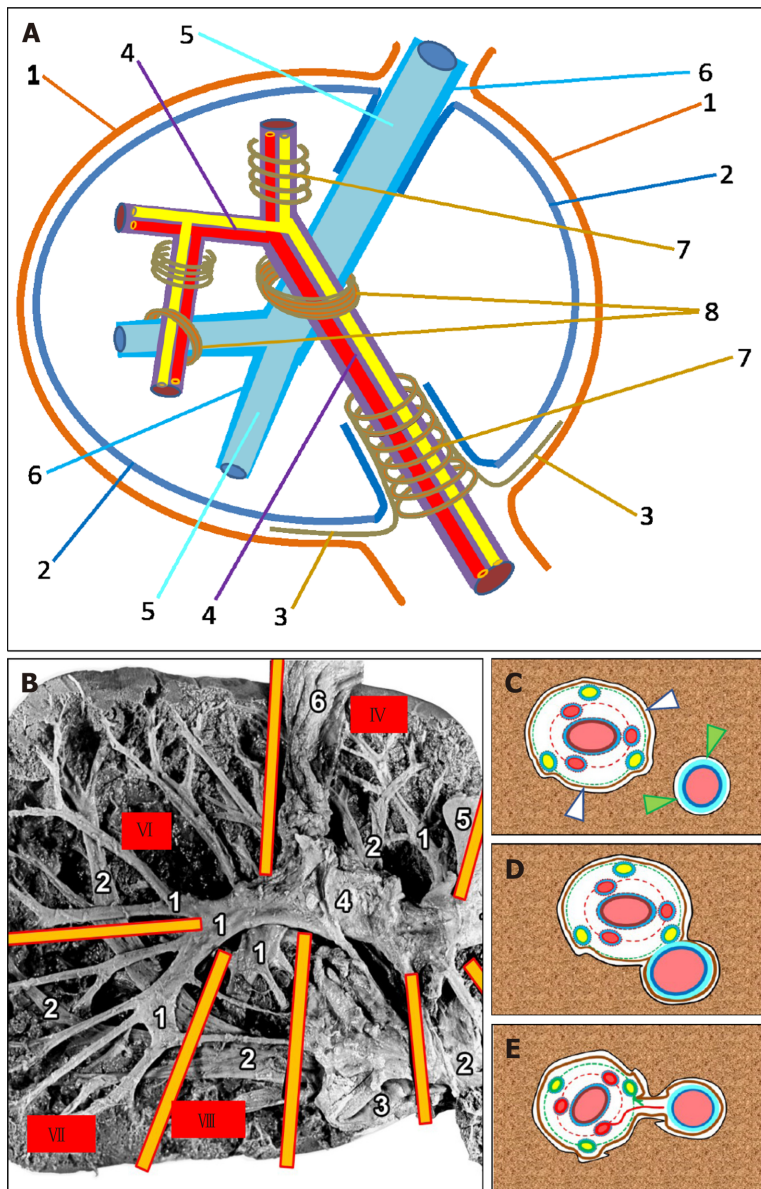
Figure 1 Connective tissue structures and their relationship in the liver. A: 1: Peritoneum; 2: Liver capsule (Laennec's capsule); 3: Hilar plate (Walaeus vasculo-biliary sheath); 4: Portal tract; 5: Hepatic vein and its tributaries; 6: Connective tissue sheath of a hepatic vein; 7: Portal tract surrounded by Glisson's capsule (Glissonean pedicle); 8: Porta-caval fibrous connection (PCFC); Arrowhead: the fissure among the Laennec's capsule (proper hepatic capsule, PHC) and the Glisson's capsule; B: Intrahepatic portal tracts and hepatic veins of the human liver after maceration from the visceral surface (preparation from the private archive of Professor Chanukvadze I); Intersection of portal tracts and the hepatic veins. Yellow lines show the borders among the liver segments enumeration of which is shown in red quadrats. 1: Portal tract; 2: Hepatic veins and their tributaries; 3: Inferior vena cava; 4: Walaeus vasculo-biliary sheath; 5: Round ligament; 6: Gallbladder; C: Section of liver tissue containing the portal tract and hepatic vein (scheme). White arrowhead: the fissure among the Laennec's capsule (PHC) and the Glisson's capsule; Green arrowhead: the fissure among the Laennec's capsule (PHC) and connective-tissue sheath surrounding the hepatic vein; D: Area of complete fusion of the Glisson's capsule and a connective-tissue sheath surrounding the hepatic vein (scheme); E: Plate-shaped PCFC (scheme).

and intralobular connective-tissue fibers connect directly to the connective-tissue fibers of adventitia of the small tributaries of the hepatic veins[2,28]. Such a relationship further reinforces the notion that the merger of the intralobular and extralobular connective-tissue fibers and that of the capsule covering the organ create a complex, yet well-regulated, structure of the extracellular matrix, which is the connective-tissue skeleton of the liver, coordinating the synergy between the cell populations and the neural and circulatory tubular structures. The PHC is mainly composed of reticular fibers (RFs) that cover the hepatic lobules. The ring of hepatocytes abutting the connective tissue of the portal region is called the periportal limiting plate. The RF bordering the hepatocytes constituting the limiting plate forms a capsule. This capsule covers the hepatic lobule from one side and abuts the perivascular fibrous capsule (Glisson's capsule) enveloping the portal tract, from another side[3].

Based on computer software analysis of liver specimens (histotopograms), the same authors distinguish loose fiber construction (and not the fissure described above) between Glisson's capsule and the PHC and called it the private hepatic ligament (PHL). The PHL is a structure in which collagen fibers have invaded from the portal region into the lattice-like or mesh-like RF that originally surrounded the lost hepatocytes[3]. However, it should be noted that the existence of such a formation has to be confirmed by additional studies.

There is a system of connective-tissue plates in the area of the hepatic port, whose origin and structure continue to be the subject of debate. This system includes a cystic plate, a round ligament plate, an Arantial plate, and a hilar plate (Walaeus vasculo-biliary sheath)[27,29].

The names of the plates are determined by their location: the gallbladder bed, round ligament gutter, Arantial ligament (obliterated venous duct) gutter, and hilus of the liver[30,31]. Several researchers have further described the caval plate, the connective-tissue sheath situated between the hepatic parenchyma and the adventitia of the hepatic part of the inferior vena cava[26,32]. Some researchers believe that these plates are derivatives of Laennec's capsule, which is attached to the liver capsule as an additional outer layer in the above-mentioned areas[30]. Other researchers indicate that the plate complexes, especially the hilar plate (which has special functional and clinical significance), is not an embryological derivative of Laennec's capsule and is connected with the fibrous part of the hepatoduodenal ligament and the connective tissues surrounding the blood vessels and bile ducts located in the portal area[27]. However, another group of researchers believes that the hilar plate does not exist at all as an independent entity; it is part of the liver capsule, which thickens in the area of the hepatic port due to a large number of thin-walled bile ducts (so-called "vaginal ductuli"). During surgery and dissection, it should be kept in mind that the hilar plate is likely to be artificially generated when, the surgeon unintentionally bundles collagenous fibers around the vaginal ductuli[15,29,33]. Taken together, the origin of the plates located on the visceral surface of the liver requires additional studies. Furthermore, we can state with confidence that the hilar plate (Walaeus vasculo-biliary sheath) covers the structures entering or exiting the liver at the hepatic port — the branches of the portal vein, hepatic artery, and bile ducts and accompanying lymphatic vessels and nerve cords. In combination with the accompanying connective-tissue fibers, afore-mentioned structures form the portal tracts that branch inside the liver. Large portal tracts, such as lobar, sectoral, segmental, and sometimes subsegmental tracts, are enveloped by a perivascular fibrous capsule (Glisson's capsule), which forms the so-called Glissonean pedicle[30]. Glisson's capsule is an intrahepatic extension of the hilar plate (Walaeus sheath). Thus, the portal tracts at the hepatic port are surrounded by the Walaeus sheath and inside the liver with Glisson's capsule. As mentioned above, Glisson's capsule is prominent around the large-caliber portal tracts but tapers off or completely disappears in thinner tracts[7].

Taking all of the above into consideration, Hu *et al*[8] concluded that the plate system represented a fibrous, thickened part of the Walaeus vasculo-biliary sheath and that Laennec's capsule had no continuity with the Glissonean pedicle. However, Laennec's capsule, which is dissociated from the main Glissonean capsule, extends to the peripheral portal tracts, where the structural integrity loosens and directly continues into the intralobular connective tissue fibers.

Laennec's capsule is the critical structure for understanding the comprehensive surgical anatomy of the liver and standardizing extrahepatic Glissonean pedicle isolation in anatomical liver resection[21]. Its precise understanding may rewrite the descriptions in the hepatology textbooks on the relationship between the hepatic capsule and intrahepatic and extrahepatic portal pedicle sheaths as follows: the connective tissue that constitutes the hepatic capsule wraps around the portal vein, hepatic artery, bile duct, lymphatics, and nerves that enter and exit the liver from the hilar part and then enters the liver where it is distributed as a skeleton in the parenchyma[34].

Portal tracts and their connective-tissue structures

The blood vessels, bile ducts and nerves located in the portal tracts are covered by their own fascial connective tissue. These structures are individually encased by a typical membrane containing laminin, collagen type IV, entactin, and heparan sulfate proteoglycan. The surrounding portal interstitium contains collagen types I, III, V, and VI, fibronectin and tenascin[2]. The fibrous covers are separated from the blood vessel walls by a space called the conceptual paravasal body[35].

In the liver hilus and adjacent proximal part of the hepatoduodenal ligament, the connective tissue cover of the portal vein is well distinguished. It surrounds the blood vessel in the form of a sheath, inside of which there is the aforementioned paravasal fissure, which contains connective tissue fibers running in different directions, connecting the portal vein adventitia with the inner surface of its fibrous cover. Likewise, in the same regions, the hepatic artery is also surrounded by a layer of fibrous connective tissue called the fibrous cover. It is separated from the blood vessel wall by a well-defined fissure containing the bundles of connective-tissue fibers connecting the inner wall of the fibrous cover with the adventitia of the artery[20,32].

The Brisbane Meeting of the International Society of Hepatobiliary-Pancreatic Surgery in 2000 formed a consensus on the uniform anatomical term/terminology classification to remedy the confusion that was present at that time. Their consensus was that first-order divisions of the elements of the portal triad were those that supplied the right and left halves of the liver, second-order divisions were those that supplied the liver sectors, and third-order divisions were those that supplied the segments[36].

The perivascular fibrous capsule abruptly appears in the area of the sectoral portal tract. It is dense and easily separates from the liver tissue, which in turn is covered by the PHC (the intrahepatic part of Laennec's capsule)[3].

The perivascular fibrous capsule is formed by collagen fibers running in various directions (elastic fibers are relatively rare). In addition, the outer layer of the capsule is denser. The relatively loose inner layer is contiguous to the connective tissue that surrounds the covers of individual elements of the portal triad. The thickness of the sectoral perivascular fibrous capsule is 45-110 µm (average 70-75 µm). Gradually, with the decrease in the caliber of the portal tract, the perivascular fibrous capsule also becomes thinner. The perivascular fibrous capsule of the 2-3 mm caliber subsegmental portal tract loses its sheath-like structure and transforms into loose connective tissue located between the individual elements of the portal triad.

The thickness of the proper cover of sectoral and segmental branches of the portal vein ranges from 50 µm to 150 µm (on average 90-100 µm) and it is directly proportional to the caliber of the blood vessel. The portal vein cover, within the subsegmental tract, gradually becomes thinner and looser. In addition, studies have shown that in 15% of cases, the identification of the connective tissue cover of the portal vein is hampered, even around the sectoral and segmental branches[32,37].

The number of bile ducts in sectoral and segmental portal tracts always exceeds three. Bile ducts are enveloped by the fibrous parabiliary sheath. The sheath has circularly oriented internal bundles, while the external bundles form septa oriented in various directions and connect closely to both the adjacent bile duct wall and the perivascular fibrous capsule. Bile ducts are accompanied by the peribiliary glands, which are connected to the ducts mainly along their opposite edges. The glands can be distinguished between intramural and extramural parts. The extramural part of the glands is several times larger in size than the intramural part. It is covered by the fibers of the fibrous parabiliary sheath, extends a considerable distance from the duct wall, is closely related to the connective tissue sheaths of other elements of the portal complex, and sometimes directly attaches to the perivascular fibrous capsule. Occasionally, the fibers covering the peribiliary glands and that of the internal surface of the perivascular capsule are so intertwined that no border can be identified between them[32,38-40].

The number of branches of the hepatic artery with a caliber larger than 1 mm varies from 2 to 5 in each sectoral and/or segmental portal tract. They are located more centrally (closer to the portal vein branch) than the bile ducts. The covers of the hepatic artery are not as distinct in sectoral and segmental tracts as in the hepatic hilus or hepatoduodenal ligament. The paravasal fissure is invisible as the adventitia is virtually contiguous with its own cover. The covers of the arteries at the peripheral edges of the blood vessels extend into the septa, which often interconnect and create the circular layer of para-arterial connective tissue located between the portal vein and the parabiliary fibrous sheath (Figure 1C). The degree of differentiation of the connective tissue covers of the arteries strongly depends on the caliber of the portal tract. In the small (subsegmental and thinner) portal tracts, the arteries have no connective tissue covers at all, and they are surrounded only by loose connective tissue that forms a bed for all elements of the portal triad[32,37]. Therefore, a combination of paravasal and parabiliary connective-tissue formations concentrated around the portal vein makes the skeletons of the hepatic portal tracts. The perivascular fibrous capsule, with adjacent parabiliary tissue with bile ducts and peribiliary glands, is located on the periphery of Glissonean pedicles[32,37].

PCFCs

In the liver, at the site of the spatial intersection of the main portal tracts and the hepatic veins, there is a little-known anatomical formation generated by the fusion of the connective-tissue fibrous sheaths of the portal tracts and the hepatic veins where these two structures come into contact with each other. The perivascular fibrous capsule extends from the portal complex to the wall of the hepatic vein and it becomes an additional element (Figure 1A, B, D and E). An anatomical formation created by the fusion of the sheaths of portal tracts and hepatic veins is called the intrahepatic PCFC [20,32].

Anatomical classification of PCFCs

Various forms of PCFC are distinguished.

Complete fusion: This type of porta-caval connection is characterized by the complete fusion of the surfaces of connective tissue sheaths of the portal tract and hepatic vein directed towards each other (Figure 1D and 2B). This type of connection is mainly found in segments II and III of the liver. The connective tissue sheaths of the hepatic veins are highly developed in the PCFC area, and its thickness reaches 90 μm. It represents a thick network of the collagen fibers running in various directions and the spiral bundles of elastic fibers and separate cellular elements are located between them. At the same time, irrespective of the density of the elements of the portal triad that merge with the hepatic veins in the area of the PCFC, there is always a narrow gap between them, filled with loose connective tissue. Small blood vessels (up to 1.5 mm in diameter), which are separated from the branches of the hepatic artery located in the portal tract, might pass through this place. They extend to the wall of the hepatic vein and supply it with blood.

Touching connection: This type of PCFC occurs when the perivascular fibrous capsule and the sheath of the hepatic vein merge only with the parts of the surface facing each other, while the rest of the space between them is filled with liver tissue. Similar to complete fusion, this form of PCFC also contains small blood vessels, but rarely the nerves or lymphatics. Touching PCFCs are often found within segments II, III, VI, and VII of the liver.

Fan-shaped connection: The fan-shaped connection, a special form of connection, is formed when the 2-5 mm caliber portal tract touches the wall of the inferior vena cava or large hepatic vein and immediately splits into thinner branches. The fan-shaped PCFC is constantly found within segment I (caudal lobe), including the inferior vena cava wall. The branches feeding the wall of the inferior vena cava or large caliber hepatic veins are separated from the arteries of the portal tract within this connection [20,32]. Within the complete fusion, touching and fan-shaped PCFCs, the hepatic vein is most often bordered by the bile ducts and their peribiliary glands. Such direct contacts may facilitate the spread of the inflammatory process from the bile ducts to the liver[32].

Plate and thread-shaped connections: The plate or thread-shaped PCFCs are represented by a fibrous plate or a cone that stretches between the perivascular fibrous capsule and the hepatic venous sheath. The plate may contain small blood and lymphatic vessels[20,32] (Figure 1E and 2A).

It should also be noted that the presence of various forms of PCFC has been confirmed in other mammals (pigs, sheep, dogs, rats). In the histological liver specimens of these animals, the sites of the crossing of different size portal tracts and hepatic vein tributaries with integration (fusion) of their connective-tissue sheaths were described. At the same time, in rat livers, the translocation of biliary structures from the portal tract toward hepatic veins was shown. This translocation causes the appearance of ductular profiles accompanying hepatic veins and their tributaries on histological specimens[32] (Figure 2C and D).

Clinical significance of PCFCs

Today, among the modern methods of surgical treatment of portal hypertension complicated by bleeding from varicose veins, the transjugular method of intrahepatic porta-caval anastomosis, which has a palliative effect, is widely used[41]. However, this method is often accompanied by complications; the most common ones are thrombotic or proliferative occlusion of the endoprosthetic shunt implanted between the branches of portal and hepatic veins, as well as stent migration-transposition[20]. This is exacerbated by the fact that the tubular shunt-prosthesis is often placed between the right branch of the portal vein and the right hepatic vein, which are
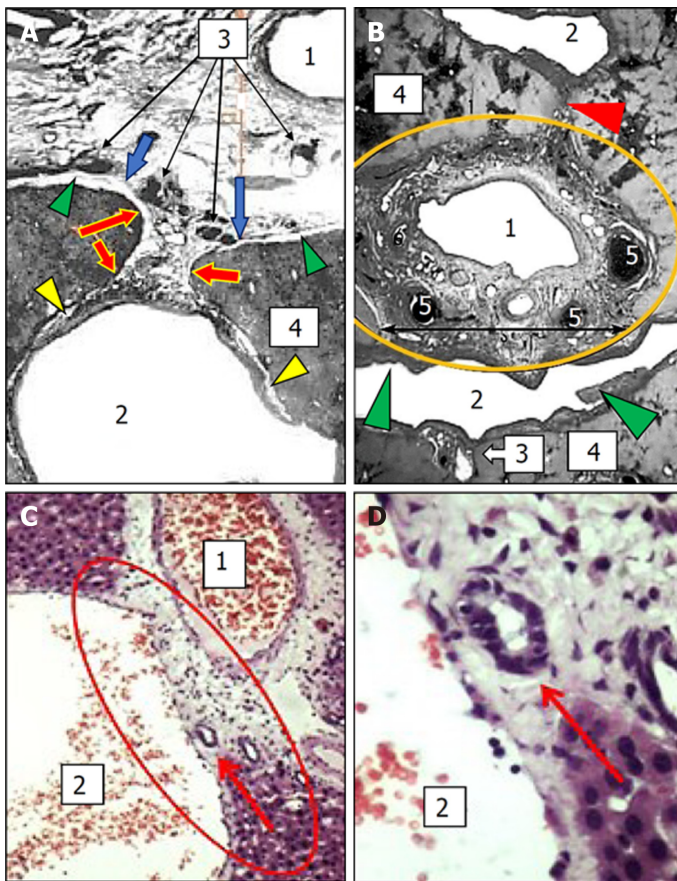
Figure 2 Porta-caval fibrous connections in humans and animals. A: Plate-shaped porta-caval fibrous connection (PCFC) (histotopogram of liver tissue): 1: Lumen of the portal vein; 2: Lumen of hepatic vein tributary; 3: Bile ducts and biliary glands (filled with Indian ink); 4: Liver parenchyma; Rad arrow: Proper hepatic capsule (PHC); Blue arrow: Perivascular fibrous capsule (Glisson's capsule); Green arrowhead: Fissure among the PHC and Glisson's capsule; Yellow arrowhead: Fissure among the PHC and perivenous connective-tissue sheath (preparation from the private archive of Professor Chanukvadze I); B: PCFCs (histotopogram of liver tissue): Large portal tract is surrounded by a yellow ellipse; 1: Lumen of the portal vein; 2: Lumen of hepatic vein tributaries; 3: Small portal tract; 4: Liver parenchyma; 5: Bile ducts (filled with Indian ink); Green arrowhead: The area of complete fusion; Red arrowhead: Thread-shaped PCFC (preparation from the private archive of Professor Ilya Chanukvadze); C: PCFC in rat liver (surrounded by a red ellipse). 1: Lumen of the portal vein; 2: Lumen of a hepatic vein; Red arrow: Bile ductule abutted to hepatic vein connective tissue sheath (preparation from the private archive of Professor Dimitri Kordzaia). Hematoxylin-eosin, Ob × 10, Oc × 10; D: Fragment of Figure C. Hematoxylin-eosin, Ob × 40, Oc × 10. C and D: Citation: Kordzaia D, Jangavadze M. Unknown bile ductuli accompanying hepatic vein tributaries (experimental study). Georgian Med News 2014: 121-129. Copyright ©Georgian Medical News 2014. Published by Georgian Medical News[43].

significantly separated from each other (from 2 cm to 9 cm). The longer the shunting prosthesis is, the higher the likelihood of thrombosis, suppression and/or transposition[41,42].

It is quite probable that the endovascular method may be more successful in developing portocaval anastomoses in the area of PCFCs, where parenchyma-free areas of direct contact between the walls of large branches (5 mm to 20 mm) of the hepatic and portal veins already exist. It is preferable to perform endovascular intervention on liver segments II and III, where the left hepatic vein passes below the main portal complex and is in direct contact with the portal vein branch, as well as between the right hepatic vein and the portal vein branch of segment VII. The various types of branching of the portal and caval veins determine a large variation in the number of PCFCs — from 4 to 20; however, despite this, the above-mentioned PCFCs in segments III and VII are characterized by high stability. In addition, the sites of integration within the connective-tissue sheaths of the large portal tracts and hepatic veins with the standard topography can be visualized by magnetic resonance imaging [20].

CONCLUSION

In the human liver where the portal tracts and hepatic veins spatially intersect (spatial crossing), the fusion of their connective-tissue sheaths develops an anatomical structural element in the form of a nodal fibrous connection — "porta-caval fibrous

connection" — allowing the hepatic vein to interact closely with the elements of the portal complex. The PCFC is a stable structure, whose formation begins at the 11th-12th week of embryogenic development. Based on the above discussion, intrahepatic PCFC can be considered an independent anatomical element of the liver, which deserves to be reflected in international anatomical nomenclature. Knowledge of the existence and features of PCFC enhance our understanding of the liver connective tissue framework and support the development of new surgical approaches for the treatment of various liver pathologies.

REFERENCES

1 **Rojkind M**, Ponce-Noyola P. The extracellular matrix of the liver. *Coll Relat Res* 1982; **2**: 151-175 [PMID: 7049553 DOI: 10.1016/s0174-173x(82)80031-9]

2 **Martinez-Hernandez A**, Amenta PS. The hepatic extracellular matrix. I. Components and distribution in normal liver. *Virchows Arch A Pathol Anat Histopathol* 1993; **423**: 1-11 [PMID: 8212529 DOI: 10.1007/BF01606425]

3 **Ikeda T**, Okano S, Hashimoto N, Kimura K, Kudo K, Tsutsumi R, Sasaki S, Kawasaki J, Miyashita Y, Wada H. Histomorphological investigation of intrahepatic connective tissue for surgical anatomy based on modern computer imaging analysis. *J Hepatobiliary Pancreat Sci* 2021; **28**: 76-85 [PMID: 32697892 DOI: 10.1002/jhbp.753]

4 **Lada E**, Anna M, Patrik M, Zbynek T, Miroslav J, Hynek M, Richard P, Sarah L, Vaclav L. Porcine Liver Anatomy Applied to Biomedicine. *J Surg Res* 2020; **250**: 70-79 [PMID: 32018145 DOI: 10.1016/j.jss.2019.12.038]

5 **Al-Samawy ER**, Waad SK, Hashim WS, Alabbas G. Comparative Histology of Human, Rats and Rabbits Liver. *Indian J Public Heal Res Dev* 2019; **10**: 1441 [DOI: 10.5958/0976-5506.2019.01134.3]

6 **Ota N**, Hirose H, Kato H, Maeda H, Shiojiri N. Immunohistological analysis on distribution of smooth muscle tissues in livers of various vertebrates with attention to different liver architectures. *Ann Anat* 2021; **233**: 151594 [PMID: 32911068 DOI: 10.1016/j.aanat.2020.151594]

7 **Patarashvili L**, Azmaipharashvili E, Jandieri K, Gvidiani S, Tsomaia K, Kikalishvili L, Sareli M, Chanukvadze I, Kordzaia D. Liver extracellular matrix peculiarities in mammals and avians. *Georgian Med News* 2021; 124-133 [PMID: 33658421]

8 **Hu Y**, Shi J, Wang S, Zhang W, Sun X, Sun B, Yu D. Laennec's approach for laparoscopic anatomic hepatectomy based on Laennec's capsule. *BMC Gastroenterol* 2019; **19**: 194 [PMID: 31752706 DOI: 10.1186/s12876-019-1107-9]

9 **Morimoto M**, Tomassini F, Berardi G, Mori Y, Shirata C, Abu Hilal M, Asbun HJ, Cherqui D, Gotohda N, Han HS, Kato Y, Rotellar F, Sugioka A, Yamamoto M, Wakabayashi G; Study group of Precision Anatomy for Minimally Invasive Hepato-Biliary-Pancreatic surgery (PAM-HBP surgery). Glissonean approach for hepatic inflow control in minimally invasive anatomic liver resection: A systematic review. *J Hepatobiliary Pancreat Sci* 2021 [PMID: 33528877 DOI: 10.1002/jhbp.908]

10 **Morimoto M**, Matsuo Y, Ueda G, Kato T, Aoyama Y, Hayashi Y, Omi K, Imafuji H, Saito K, Tsuboi K, Ogawa R. Exploring the Fine-Layer Structure Around a Glissonean Pedicle in Cadaveric Models. *Surg Gastroenterol Oncol* 2020; **25**: 67-72 [DOI: 10.21614/sgo-25-2-67]

11 **Navarro-Tableros V**, Herrera Sanchez MB, Figliolini F, Romagnoli R, Tetta C, Camussi G. Recellularization of rat liver scaffolds by human liver stem cells. *Tissue Eng Part A* 2015; **21**: 1929-1939 [PMID: 25794768 DOI: 10.1089/ten.TEA.2014.0573]

12 **Liu Y**, Yang R, He Z, Gao WQ. Generation of functional organs from stem cells. *Cell Regen* 2013; **2**: 1 [PMID: 25408873 DOI: 10.1186/2045-9769-2-1]

13 **Lee JS**, Shin J, Park HM, Kim YG, Kim BG, Oh JW, Cho SW. Liver extracellular matrix providing dual functions of two-dimensional substrate coating and three-dimensional injectable hydrogel platform for liver tissue engineering. *Biomacromolecules* 2014; **15**: 206-218 [PMID: 24350561 DOI: 10.1021/bm4015039]

14 **Felgendreff P**, Schindler C, Mussbach F, Xie C, Gremse F, Settmacher U, Dahmen U. Identification of tissue sections from decellularized liver scaffolds for repopulation experiments. *Heliyon* 2021; **7**: e06129 [PMID: 33644446 DOI: 10.1016/j.heliyon.2021.e06129]

15 **Yamamoto M**, Katagiri S, Ariizumi S, Kotera Y, Takahashi Y. Glissonean pedicle transection method for liver surgery (with video). *J Hepatobiliary Pancreat Sci* 2012; **19**: 3-8 [PMID: 21938411 DOI: 10.1007/s00534-011-0443-0]

16 **ELIAS H**, PETTY D. Gross anatomy of the blood vessels and ducts within the human liver. *Am J Anat* 1952; **90**: 59-111 [PMID: 14902689 DOI: 10.1002/aja.1000900104]

17 **Ostroverkhov**, G Zabrodskaya V. Surgical anatomy of the liver and biliary tract. In: Maksimenkov A. Surgical anatomy of the abdomen. Leningrad: 1972: 297-380

18 **Fegershanu N**, Ionescu-Bujar K, Aloman D, Albu A. Surgery of the liver and intrahepatic biliary tract. Bucharest: 1976

19 **Dancygier H**. Clinical Hepatology. Berlin, Heidelberg: Springer Berlin Heidelberg, 2010

20 **Chanukvadze I**. Portacaval Fibrous Connections: Little Known Anatomical Structures of Liver. *Rom Med J* 2017; **64**: 43-48

21   **Sugioka A**, Kato Y, Tanahashi Y. Systematic extrahepatic Glissonean pedicle isolation for anatomical liver resection based on Laennec's capsule: proposal of a novel comprehensive surgical anatomy of the liver. *J Hepatobiliary Pancreat Sci* 2017; **24**: 17-23 [PMID: 28156078 DOI: 10.1002/jhbp.410]

22   **Kawarada Y**, Das BC, Taoka H. Anatomy of the hepatic hilar area: the plate system. *J Hepatobiliary Pancreat Surg* 2000; **7**: 580-586 [PMID: 11180890 DOI: 10.1007/s005340070007]

23   **Poonkhum R**, Pisetpaisan K, Wang BJ, Anupunpisit V, Ohtani Y, Ohtani O. Origins and pathways of fluid entering sublobular lymphatic vessels in cat livers. *Arch Histol Cytol* 2003; **66**: 317-326 [PMID: 14692687 DOI: 10.1679/aohc.66.317]

24   **Ohtani O**, Ohtani Y. Lymph circulation in the liver. *Anat Rec (Hoboken)* 2008; **291**: 643-652 [PMID: 18484610 DOI: 10.1002/ar.20681]

25   **Medeiros de Mello J**, Orsi AM, Piffer CR, Torrejais MM. Architecture of the caudal vena cava wall of the dog at the level of the liver caudate lobe. *Ann Anat* 2000; **182**: 525-528 [PMID: 11125802 DOI: 10.1016/S0940-9602(00)80096-5]

26   **Karau PB**, Ogeng'o JA, Hassanali J, Odula PO. The histormorphological organization of the hepato-caval interface in the human. *J Morphol Sci* 2010; **27**: 148-151

27   **Takasaki K**, Yamamoto M.   Surgical Anatomy of the Liver in the Glissonean Pedicle Approach: What We Need to Know. In: Madoff D, Makuuchi M, Nagino M, Vauthey JN. Venous Embolization of the Liver. London: Springer London, 2011: 23-27

28   **Crawford AR**, Lin XZ, Crawford JM. The normal adult human liver biopsy: a quantitative reference standard. *Hepatology* 1998; **28**: 323-331 [PMID: 9695993 DOI: 10.1002/hep.510280206]

29   **Hayashi S**, Murakami G, Ohtsuka A, Itoh M, Nakano T, Fukuzawa Y. Connective tissue configuration in the human liver hilar region with special reference to the liver capsule and vascular sheath. *J Hepatobiliary Pancreat Surg* 2008; **15**: 640-647 [PMID: 18987936 DOI: 10.1007/s00534-008-1336-8]

30   **Couinaud C**.   Surgical anatomy of the liver revisited. Paris: Semantic Scholar, 1989: 29-39

31   **Couinaud C**.   Le foie; études anatomiques et chirurgicales. Masson, 1957

32   **Kordzaia D**, Chanukvadze I, Jangavadze M.   Functional Anatomy of Intrahepatic Biliary System (Clinical and Experimental Data). In: Miguel Ángel Mercado. Bile Duct: Functional Anatomy, Disease and Injury Classification and Surgical Management. Nova Science Publishers Inc, 2014: 1-87

33   **Guglielmi A**, Ruzzenente A, Iacono C.   Surgical Anatomy of the Hepatic Hilus. In: Guglielmi A, Ruzzenente A, Iacono C. Surgical Treatment of Hilar and Intrahepatic Cholangiocarcinoma. Milano: Springer, 2008: 101-111

34   **Kuntz E**, Kuntz H-D. Hepatology Textbook and Atlas. *Hepatol Textb Atlas* 2008 [DOI: 10.1007/978-3-540-76839-5]

35   **Kovanov V**, Anikina M. Surgical anatomy of human paravasal connective tissue structures [in Russian]. *Moscow Med* 1985

36   **Strasberg SM**, Belghiti J, Clavien P-A, Gadzijev E, Garden JO, Lau W-Y, Makuuchi M, Strong RW. The Brisbane 2000 Terminology of Liver Anatomy and Resections. *HPB* 2000; **2**: 333-339 [DOI: 10.1016/s1365-182x(17)30755-4]

37   **Chanukvadze I**. Vasculo-fibrous architecture of the magistral portal complexes. *Rom Med J* 2016; **11**: 214-218

38   **de Jong IE**, van Leeuwen OB, Lisman T, Gouw AS, Porte RJ. Repopulating the biliary tree from the peribiliary glands. *Biochim Biophys Acta Mol Basis Dis* 2018; **1864**: 1524-1531

39   **Nakanuma Y**, Katayanagi K, Terada T, Saito K. Intrahepatic peribiliary glands of humans. I. Anatomy, development and presumed functions. *J Gastroenterol Hepatol* 1994; **9**: 75-79 [PMID: 8155872 DOI: 10.1111/j.1440-1746.1994.tb01220.x]

40   **Nakanuma Y**, Sasaki M, Terada T, Harada K. Intrahepatic peribiliary glands of humans. II. Pathological spectrum. *J Gastroenterol Hepatol* 1994; **9**: 80-86 [PMID: 8155873 DOI: 10.1111/j.1440-1746.1994.tb01221.x]

41   **Nolte W**, Manke H, Schndler C. Dopplerronographsche kurz und landzeitunterrsuchunger der patalen Hamodynamik nach transjugular intrahepatischem portosistemischem Stent-Shunt. *Ztsch fur Gastroenterol* 1998; 491-499

42   **Richter GM**, Roeren T, Brado M, Theilmann L, Sauer P, Kauffmann GW. [Portal hypertension and percutaneous transjugular portasystemic stent shunt]. *Chirurg* 1995; **66**: 555-565 [PMID: 7664584]

43   **Kordzaia D**, Jangavadze M. Unknown bile ductuli accompanying hepatic vein tributaries (experimental study). *Georgian Med News* 2014; 121-129 [PMID: 25341252]

REVIEW

Promising diagnostic biomarkers of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis: From clinical proteomics to microbiome

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Abstract

Fatty liver has been present in the lives of patients and physicians for almost two centuries. Vast knowledge has been generated regarding its etiology and consequences, although a long path seeking novel and innovative diagnostic biomarkers for nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) is still envisioned. On the one hand, proteomics and lipidomics have emerged as potential noninvasive resources for NAFLD diagnosis. In contrast, metabolomics has been able to distinguish between NAFLD and NASH, even detecting degrees of fibrosis. On the other hand, genetic and epigenetic markers have been useful in monitoring disease progression, eventually functioning as target therapies. Other markers involved in immune dysregulation, oxidative stress, and inflammation are involved in the instauration and evolution of the disease. Finally, the fascinating gut microbiome is significantly involved in NAFLD and NASH. This review presents state-of-the-art biomarkers related to NAFLD and NASH and new promises that could eventually be positioned as diagnostic resources for this disease. As is evident, despite great advances in studying these biomarkers, there is still a long path before they translate into clinical benefits.

Key Words: Fatty liver; Biomarkers; Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis

Core Tip: Nonalcoholic fatty liver disease is increasing in prevalence worldwide. Liver biopsy is considered the gold standard for diagnosis, but it has several limitations. Given the burden on the healthcare system caused by liver fibrosis in a population with metabolic syndrome, there is a priority for noninvasive and accurate diagnostic biomarkers that differentiate patients with steatosis from those with nonalcoholic steatohepatitis, stage fibrosis, predict progression, and monitor treatment response. These biomarkers could assist clinicians in early interventions, avoiding complications and improving prognosis. Here, we summarize the current evidence and future directions.

INTRODUCTION

Thomas Addison first described "fatty liver" in 1836 in England; however, it was not until 1885 when Bartholow made an association between obesity and fatty liver. In 1938, Charles Connor demonstrated a link between fatty liver and progression to cirrhosis in diabetic patients. Throughout the 1950s and up to the 1970s, pathologists reported similarities between alcoholic liver disease and hepatic histological changes in obese and diabetic patients. In 1980, Jurgen Ludwig[1] described patients who denied excessive alcohol consumption yet still had chronic liver disease and histological characteristics of alcoholic fatty liver disease. There was no name for the disease, so Ludwig coined the terms nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH)[1].

As reported in the most recent guidelines, NAFLD is defined as the presence of steatosis in > 5% of hepatocytes in the absence of significant ongoing or recent alcohol consumption and other known causes of liver disease. While in 2005 it had a global prevalence of 15%, a rapid increase in sedentarism and excessive calorie intake independent of diet has pushed it to 24%, with the highest rates in South America (31%) and the Middle East (32%), followed by Asia (27%), the United States (24%), and Europe (23%)[2]. In persons with obesity or type 2 diabetes, it increases up to 70%-90% [3]. Although there is a significant difference between ethnicities within these populations, the exact explanation remains unknown[2].

NAFLD is a necessary and opportune diagnosis, given that 59% progress to NASH. From this stage, 41% continue to fibrosis, with 40% ending with cirrhosis, increasing their risk of a liver transplant, cardiovascular disease, and mortality if there are no interventions[4]. In our country, the Mexican population has several risk factors for the disease because there is a high incidence of overweight and obesity[5], making the NAFLD prevalence likely to surpass 50%. Up to 82% of obese patients who have undergone bariatric surgery present NAFLD, alongside 36% of women with obesity[6].

An international panel has now proposed to rename the disease metabolic dysfunction-associated fatty liver disease to represent the hepatic manifestation of a multisystemic disorder. Until now, the diagnosis was reached by the exclusion of other liver diseases; however, as the pathogenesis is better understood, it is now perceived as a distinct disease and requires a positive diagnosis, which is why it is proposed that the criteria be based on histological, imaging, or blood biomarker evidence of fat accumulation in the liver in addition to one of the following three: Overweight/obesity, type 2 diabetes mellitus, or evidence of metabolic dysregulation (at least two metabolic risk abnormalities)[1].

Today, the liver biopsy remains the gold standard for diagnosing and monitoring liver disease, with the disadvantage of being a costly and invasive procedure[7], which is why it is important to look into possible new noninvasive diagnostic tools, such as biomarkers, use of transcriptomics, proteomics, metabolomics, and now "glycomics" [8]. These should aid in predicting liver disease severity, progression, and response to lifestyle changes and pharmacological treatment[9]. The objective of this article is to review concisely and present the potential diagnostic biomarkers for NAFLD and NASH (Figure 1).

PROTEOMICS

The concentrations of several plasma components are determined in routine clinical practice, including electrolytes, molecules, and proteins. Plasma proteins, which constitute the plasma proteome, are released as a result of inflammation, apoptosis, and oxidative stress (OS)[10]. Mass spectrometry-based proteomics[9] and two-dimensional electrophoresis are powerful tools for studying differences[11] in the plasma proteome. There are differences in protein expression among patients with NAFLD and healthy controls. Proteomics technologies have gained relevance as potential non-invasive diagnostic methods for NAFLD.

Plasma proteomics

Plasma proteomics may be secreted by the liver or as a result of the response of the host to steatosis. Hemoglobin is currently the most replicated proteomic biomarker in NAFLD[12]. Studies have found that higher hemoglobin levels are associated with a higher incidence of NAFLD[12]. Circulating aminotransferase [aspartate aminotransferase (AST) and alanine aminotransferase (ALT)] levels are markers of several liver diseases, including NASH. Changes in these enzymes are one of the most commonly observed abnormalities[10].

Fibroblast growth factor 21 is another protein secreted in response to peroxisome proliferator-activated receptor (PPAR)-α activation, and several studies support its potential use as a biomarker for NAFLD[13,14]. The elevation of retinol-binding protein 4 has also been associated with liver fat accumulation[15]. Some glycoproteins like serum fucosylated haptoglobin and Mac-2 binding protein are predictors of hepatocyte ballooning and liver fibrosis[16].

Cytokeratin-18 fragments, such as CK18Asp396, are other proteins that have been extensively studied. These are produced during apoptosis (M30) or cell death (M65). CK18 is the most reviewed biomarker to evaluate liver inflammation[15], but current knowledge does not support its use in clinical practice[17] because of its modest accuracy[8].

Increased cytokeratin-18 levels have good predictive value for NASH *vs* normal livers but do not differentiate NASH *vs* simple steatosis[18,19]. Cytokeratin-18 serum levels decrease parallel with histological improvement, but its predictive value is not better than ALT in identifying histological responders[20].

Circulating concentrations of cytokeratin-18 fragments were proposed as the most reliable predictors of NASH in patients with NAFLD[21].

Circulating extracellular vesicles

Another important plasma component includes circulating extracellular vesicles (EVs), which are small cell-derived membrane-surrounded structures enclosed by a phospholipid bilayer, with a specific cargo of bioactive molecules of cell origin. There are three types according to their size: Exosomes (40-100 nm), microvesicles or microparticles (0.1-1 μm), and apoptotic bodies (1-4 μm)[22].

They can be detected in several body fluids and can serve several functions by delivering a variety of bioactive molecules, including non-coding RNAs, proteins, lipids, and nucleic acids[23]. Recent studies have provided insight on the bioavailability of circulating EVs in various fluids and, as a consequence, on their potential use as biomarkers for various diseases such as cancer[20,24,25], cardiovascular disease [26], renal disease[27], and liver disease[28,29].

Some authors consider them noninvasive "liquid biopsies" for NASH diagnosis, and studies suggest they can assess disease severity[30]. Serum levels of total and hepatocyte-derived EVs correlate with NASH clinical characteristics, and disease severity in experimental models of NASH, liver and blood levels of EVs are increased and correlate positively with changes in liver histology[31].
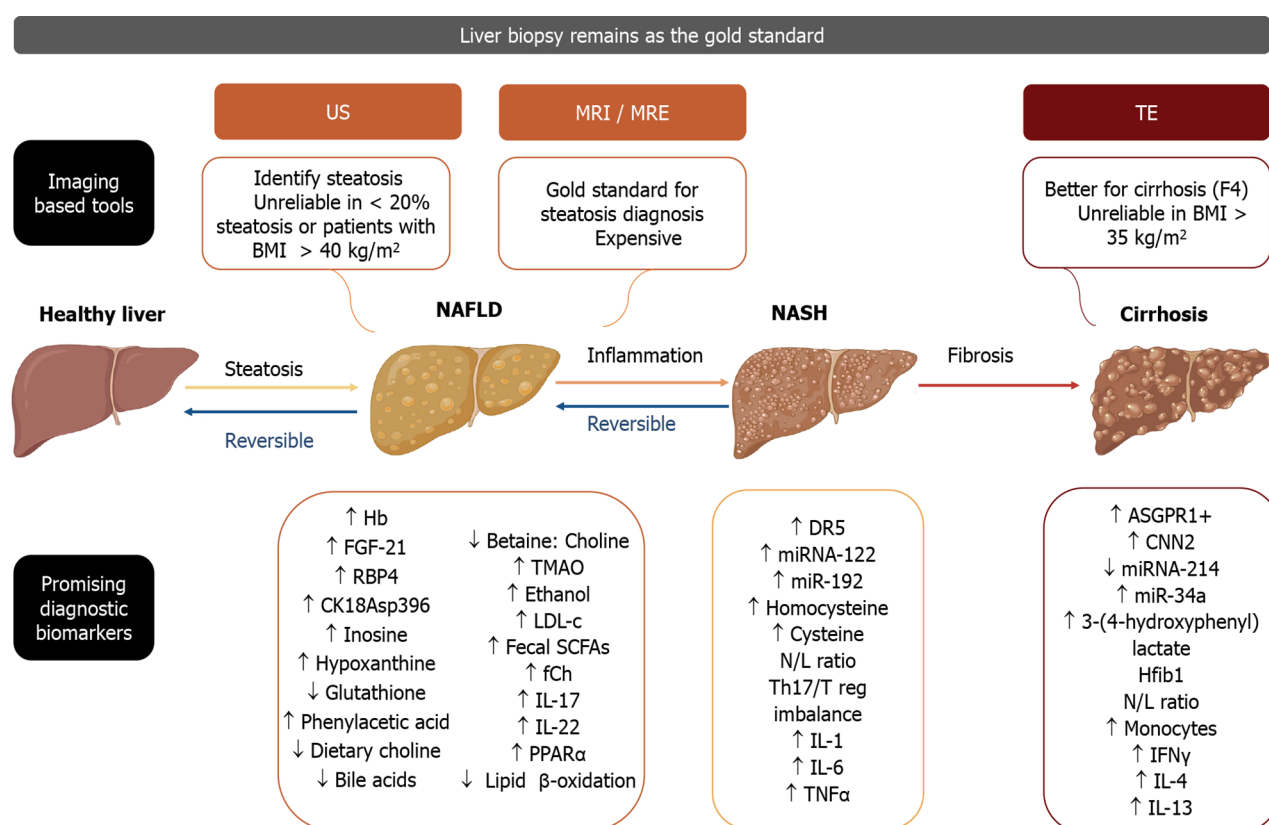
Figure 1 Although liver biopsy remains as the gold standard for the diagnosis of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis, other current imaging studies are shown, along with promising diagnostic and/or monitoring biomarkers that may be present in each of the stages of hepatic pathology, ranging from reversible steatosis and inflammation to irreversible fibrosis and eventually cirrhosis (Figure 1 created with BioRender.com). US: Ultrasound; TE: Transient elastography; BMI: Body mass index; Hb: Hemoglobin; FGF-21: Fibroblast growth factor 21; RBP4: Retinol binding protein 4; CK18Asp396: Caspase cleaved cytokeratin-18 fragment; TMAO: Trimethylamine N-oxide; LDL-c: Low density lipoprotein cholesterol; Fecal SCFAs: Fecal Short chain fatty acids; fCh: Ferrochelatase; IL-17: Interleukin-17; IL-22: Interleukin-22; PPARα: Peroxisome proliferator-activated receptor α; DR5: Death receptor 5; miRNA-122: MicroRNA 122; miR-192: MicroRNA 192; N/L ratio: Neutrophil/lymphocyte ratio; Th17/Treg imbalance: T helper 17/T regulatory cells imbalance; IL-1: Interleukin-1; IL-6: Interleukin-6; TNFα: Tumor necrosis factor alpha; ASGPR1+: Asialoglycoprotein receptor 1; CNN2: Calponin 2; miRNA-214: MicroRNA 214; miR-34a: MicroRNA 34a; Hfib1: Hepatic fibrosis 1; N/L ratio: Neutrophil/lymphocyte ratio; IFNγ: Interferon γ; IL-4: Interleukin-4; IL-13: Interleukin-13.

Povero *et al*[30] performed a study isolating EVs from controls with histologically confirmed NASH without cirrhosis and patients with cirrhotic NASH[30]. After the characterization of EV structural features, they found that differences in the quantity and protein components of circulating EVs could be potentially useful for differentiating patients with NASH from controls and patients with pre-cirrhotic NASH from patients with cirrhotic NASH[30].

Notably, asialoglycoprotein receptor 1-positive hepatocyte-specific EVs may represent a surrogate noninvasive biomarker of portal hypertension in patients with cirrhotic NASH. If confirmed, these findings may support the clinical utility of asialoglycoprotein receptor 1-positive EVs (hepatocyte-specific EVs) as a potential alternative to an invasive hepatic venous pressure gradient[30].

Patients with NAFLD or NASH secrete increased levels of microvesicles derived from macrophages/monocytes [CD14(+)] and natural killer (NK) T cells; these levels correlate with NASH severity based on histology[28]. Hirsova *et al*[32] have demonstrated that lipids that stimulate death receptor 5 on hepatocytes also induce the release of hepatocyte EVs that activate an inflammatory phenotype in macrophages that lead to NASH[32].

However, a major problem in translating this research into clinically useful information is a lack of reproducibility and rigorous criteria for reporting these biomarkers. Proteomics analysis of EVs from patients with advanced NASH is currently limited.

Exosomes

Exosomes are a type of EVs secreted in most cells[22]. These nanovesicles of endocytic origin are present in nearly all-human fluids. Exosomes have several bioactive

molecules, including proteins, lipids, and genetic materials[33]. They are conduits for intracellular transfer, and their signals can induce fibrosis, macrophage activation, cytokine secretion, and remodeling extracellular matrix (ECM) production and inactivate hepatic stellate cells (HSC)[34]. Hepatocytes are exosome-secreting cells that are also regulated by hepatic and extrahepatic exosomes[33].

Koeck *et al*[35] found that exosomes from visceral adipose tissue were involved in the progression of NAFLD by inducing dysregulation of the transforming growth factor-beta (TGF-β) pathway in hepatocytes and HSCs *in vitro*[35]. Another study by Seo *et al*[36] detected that during liver injury, damaged hepatocytes produce exosomes that activate toll-like receptor 3, which exacerbates liver fibrosis by enhancing interleukin-17A (IL-17A) production by γδ T cells[36].

Liver fibrogenic pathways are primarily controlled by HSC, which produces and responds to fibrotic mediators such as connective tissue growth factor (CCN2)[37]. Tadokoro *et al*[29] found that CCN2 upregulation in fibrotic or steatotic livers is associated with the downregulation of microRNA-214 (miRNA-214). miR-214 levels increased in quiescent HSC-secreted exosomes compared with active HSC-released exosomes[29]. On the other hand, exosomal CCN2 may amplify fibrogenic signaling and might be useful for assessing hepatic fibrosis[37].

Chen *et al*[38] found that the miR-214 promoter binds to the basic helix-loop-helix transcription factor (Twist1), which drives miR-214 expression and results in CNN2 suppression. Twist 1 expression was suppressed during HSC activation. The amounts of Twist1, miR-214, or CCN2 in circulating exosomes from fibrotic mice reflected fibrosis-induced changes in the liver[38]. These findings suggest that during liver fibrosis, exosomes contain specific types of biomarkers, which could be helpful in the diagnosis and progression of liver diseases.

miRNA

Circulating microRNAs (miRNA) are RNA molecules that do not encode proteins but regulate gene expression in the body, binding to target mRNAs and interfering with their translation[22]. They are expressed in several liver cell types and may offer a biologically stable blood-based biomarker tool for the detection and stratification of liver disease[29].

Tadokoro *et al*[29] have suggested that serum/plasma miR-122 correlates with liver damage. They have also identified that miR-155 might serve as a liver inflammation biomarker. The one limitation found is that this miRNA cannot differentiate different liver damage etiologies[29].

Another study reported that miRNA-122 and miR-192 levels are dynamic and increase over time, closely correlating with the histopathological severity of NASH[31]. The miR-29 family (miR-29a, miR-29b, miR-29c) mediates the regulation of liver fibrosis through several cellular signaling pathways such as the nuclear transcription factor-kappa B pathway, TGF, and phosphatidylinositol 3-kinase/AKT signaling in HSC with upregulation of ECM genes for the progression of liver fibrosis[39].

Members of the miR-34 family (miR-34a, miR-34b, miR-34c) have pleiotropic roles in the cell cycle and promote the progression of hepatic fibrosis by activation of HSC[39]. miR-34a appears to have an important role in liver fibrosis by regulating the deposition of ECM[40]. miR-30c and miR-193 are also involved in fibrotic remodeling processes that modify the TGF-β-dependent regulation of ECM-related genes in HSCs [41].

The miR-15 family mainly regulates the TGF-β pathway. The activation of HSCs relates to miR15a and miR15b, and they are thought to be essential for apoptosis by targeting Bcl-2 and the caspase signaling pathway[42]. The miR-378 family (specially miR-378a-3p) suppresses the activation of HSCs by directly targeting Gli3[43]. miR-571 closely correlates with the liver cirrhosis stage, and it is upregulated in human hepatocytes and HSC[44]. miR-503 also acts on HSC activation and hepatic fibrosis through the TGF-β/SMAD pathway[45].

The miR-199 family and miR-200 family are responsible for ECM deposition and the release of profibrotic cytokines, which might play profibrotic or anti-fibrotic roles[39]. HSCs also have anti-fibrotic miRNAs, and these include miR-19b, miR-29, miR-30, miR-101, miR-122, miR-133a, miR-144, miR-146a, miR-150-5p, miR-155, miR-195, miR-200a, miR-214, miR-335, miR-370, miR-454, miR-483, *etc*. The latter are responsible for the maintenance of the quiescent phenotype of normal HSCs[46]. Thus, these studies evidence the role of microRNAs as potential biomarkers of liver damage in NAFLD.

METABOLOMICS

Technological advances in metabolomic analyses on feces, serum, plasma, urine, or liver biopsies led to identifying different metabolites in patients with NAFLD or NASH[47]. Recent studies have found that the severity of fibrosis is associated with serum metabolite changes[48-50].

Remarkably, some metabolites come from the host or the diet, but most need the participation of gut microbes. Notably, inosine and hypoxanthine are enriched in serum samples from patients with mild or moderate NAFLD[47]. Another study found that liver steatosis correlates with phenylacetic acid levels in humans[51]. Glutathione plasma concentration is significantly lower in subjects with liver steatosis, while in subjects with NASH, homocysteine and cysteine concentrations in plasma are higher [52].

Gut microbially-derived metabolomics

Choline, betaine, and circulating methylamines: Choline is an essential component of phosphatidylcholine (a precursor of acetylcholine), mostly obtained from the diet[53]. It is known that a reduction in dietary choline is related to an increase in liver fat. Mice fed with a choline deficient diet are identified as a characteristic model of NAFLD[54]. Choline can be oxidized to betaine, and it has been found that patients with increasing severity of NAFLD have a decreased betaine to choline ratio[55]. The gut microbiota metabolizes choline into trimethylamine (TMA), which is further metabolized into trimethylamine-N-oxide (TMAO) in the liver[56]. Studies suggest that NAFLD severity is associated with increased urinary levels of TMA and TMAO, while TMAO seems to be associated with NAFLD severity[47].

TMAO and bile acids: Gut microbiota regulates secondary bile acid metabolism and inhibits the liver synthesis of lipids by alleviating farnesoid X-activated receptor inhibition[57]. TMAO is a gut-dependent metabolite of choline. A decreased level of bile acids could be associated with TMAO production and NAFLD since it induces a decrease in the bile acid pool by inhibiting two key enzymes of bile acid metabolism: Cytochrome P450 (CYP)7A1 and CYP27A1[55]. Some studies have found adverse associations between the circulating TMAO levels and the presence and severity of NAFLD and a favorable betaine-NAFLD relationship in participants[55].

Three-(4-hydroxyphenyl) lactate: Three-(4-hydroxyphenyl) lactate is a derived product of amino acid metabolism. It was consistently associated with increased liver fibrosis severity in a test and validation cohort[48].

Ethanol: Gut microbiota leads to endogenous ethanol production, which might be a liver toxin involved in NAFLD and NASH development[47]. A study showed that *Klebsiella pneumoniae* can produce ethanol from glucose in the absence of alcohol consumption, and it might be associated with NAFLD[58].

LIPIDOMICS AND LIPOTOXICITY

Human serum and plasma are composed of lipids that play important roles in energy storage, metabolic regulation, signaling, *etc.*[10]. Technological advances have made possible the identification of specific alterations in lipids and metabolites in the feces, serum, plasma, urine, and liver of patients with NAFLD[47].

Choline is a dietary component metabolized in the liver, necessary for cell function. Epidemiological studies suggest that increased free choline levels are related to the degree of hepatic steatosis fibrosis[59].

Kalhan *et al*[60] have shown that plasma levels of triglycerides[60] and low-density lipoprotein cholesterol are higher in patients with NAFLD[52]; however, differences in this lipidomic profile are also observed in obesity. Therefore, this lack of specificity remains a limitation for their use. Barr *et al*[61] described a lipidomic signature associated with NAFLD progression to distinguish NASH from steatosis, depending on the body mass index in a large cohort of samples[61].

Gorden *et al*[62] described a panel of 20 lipids that differentiate patients with NASH and liver steatosis[62]. Later, Kimberly *et al*[63] identified the association between anandamide (endocannabinoid derived from arachidonic acid metabolism) and NAFLD severity[63]. Tokushige *et al*[64] reported 28 metabolites associated with liver fibrosis, showing a decrease of dehydroepiandrosterone sulfate and etiocholanolone-S with the progression of fibrosis[64].

Puri *et al*[65] analyzed plasma lipids and eicosanoid metabolites in NAFLD and NASH patients. They reported increased plasma monounsaturated fatty acids and primary palmitoleic and oleic acids and decreased linoleic acid. Plasmalogen levels were significantly decreased in NASH, and 11-HETE (a nonenzymatic product of arachidonic acid) was increased in NASH[65]. Loomba *et al*[66] assessed the lipidomic profile in NAFLD and NASH patients and reported that 11,12-dihydroxy- eicosatrie-noic acid (11,12-diHETrE) was the best biomarker for differentiating NAFLD from NASH[66].

Short-chain fatty acids (SCFAs) are comprised of butyrate, acetate, and propionate. They are produced in the colon through microbial fermentation of dietary fiber and are a substrate that increases liver triglyceride levels[67]. They are also involved in fatty acid synthesis and gluconeogenesis[68]. Human studies have observed an increased fecal concentration of SCFAs in patients with NAFLD and/or NASH[69].

In NAFLD, lipid metabolism is disrupted, and lipotoxicity is a key mechanism for NAFLD progression. Lipidomic profiling might provide a novel biomarker for the noninvasive prediction of NASH.

GENETIC MARKERS

The role of genetic and epigenetic factors in the progression of liver fibrosis is well documented. It is known that key regulatory genes partially control the cell phenotype. Several genes are involved in the pathogenesis and histological stage of liver fibrosis, although the mechanisms underlying gene regulation are highly complex and need additional research[70].

Chromosome 15, designated Hfib1 (hepatic fibrogenic gene 1), affects the stage of liver fibrosis[71]. The core of risk genes that control fibrosis progression has been defined by quantitative trait locus analysis in mouse strains by genome-wide interval mapping, which identified several genomic loci related to fibrosis phenotypes on chromosomes 4, 5, 7, 12, and 17[72].

Bruschi *et al*[73] reported that PLPNA3 quantification correlates with the liver fibrosis stage. Expression of PLPNA3 in biopsies from NASH patients is increased during progression from mild to severe liver fibrosis. Carriers of the I148M single-nucleotide polymorphism (C>G) had higher PLPNA3 and serum liver enzyme (ALT/AST) levels, along with steatosis grade inflammation ballooning and NAFLD activity score, compared with non-polymorphism carriers[73]. On the other hand, Sharma *et al*[74] stated that neurocan is associated with NASH and liver fibrosis in patients of European ancestry. Another study found that patients of Indian descent with neurocan variations had higher ALT levels[74].

EPIGENETIC MARKERS

Epigenetics describes reversible gene expression changes that do not imply changes in the DNA sequence and are entirely cell type-specific. Epigenetic mechanisms initiate and sustain chromatin modifications by facilitating gene transcription, cell phenotype, and consequently, organ function. These mechanisms include DNA methylation, histone modifications, and noncoding RNAs mediating gene silencing[75].

Aberrant DNA methylation is associated with fibrosis. Komatsu *et al*[76] suggested that DNA hypomethylation in fibrogenic genes is crucial for the onset and progression of liver fibrosis[76]. Mann *et al*[77] confirmed this functional association of DNA methylation with liver fibrosis. The transdifferentiation of HSC to profibrogenic myofibroblast phenotype was suppressed *in vitro* by the DNMT inhibitor 5′-aza-deoxycytidine[77]. The development of fibrosis is also related to changes in the expression of enzymes that regulate DNA methylation and hydroxymethylation[78].

Epigenetic modulation on the PPAR-γ gene promoter is involved in HSC differentiation. Aberrant expression of a series of chemokines in HSCs aggravate inflammation and OS[79].

Small non-coding RNAs contribute to various pathologic states of liver disease, but miRNA has been previously reviewed. The detection of genetic and epigenetic markers may be helpful in the recognition and monitoring of disease evolution and can eventually be applied for targeted therapies.

IMMUNE DYSREGULATION

NASH pathology encompasses an intricate network of mechanisms. OS activates Kupffer cells (KC), and KC activation triggers an innate and adaptive immune response, including the release of cytokines and chemokines that activate NK T (NKT) cells and HSCs[80]. Besides, there is augmented infiltration of different immune cells, such as monocytes, T lymphocytes, and neutrophils, in the activation and *in situ* expansion of liver cells, like KC or stellate cells. Activated KC and NKT cells promote additional fat accumulation in the liver. KC, neutrophils, NKT cells, and inflammatory T cells [T helper (Th)1, Th17, CD8+ T cells] enhance liver inflammation and contribute to the development of fibrosis[81].

The neutrophil to lymphocyte ratio (N:L ratio) has been proposed as a novel noninvasive marker to predict NASH and advanced fibrosis in patients with NAFLD [82]. In patients with cirrhosis, these cells are functionally deficient, with impaired chemotaxis, phagocytosis, and intracellular killing. Their function correlates with 90-d survival[83].

On the other hand, monocytes are myeloid-derived cells that migrate to inflammation sites, phagocytose microbes, and secrete cytotoxins. They are spontaneously activated in patients with liver fibrosis. Cirrhotic patients have an increased peripheral frequency of monocytes, impaired phagocytosis, and reduced responses to stimulation [84].

Studies have reported that NK cells are dysregulated in liver diseases. One study found that IL-17- and IL-22- secreting iNKT cells are dominant at the beginning of liver steatosis, and IFNγ/IL-4/IL-13-secreting iNKT cells are prevalent at the most advanced course of the disease[85].

Notably, CD4+ T cells are reduced in patients with liver fibrosis. This finding could explain the increased risk of spontaneous bacterial peritonitis in these patients[86]. CD8+ T cells isolated from mice hepatic cells expressed an increased cytotoxic IL-10 phenotype and CD8+ T cell depletion[87].

Th17 cells and T regulatory cells (Treg) originate from naïve T cell precursors. Th17 cells are important for pathogen clearance and inflammation. Treg cells in patients with liver fibrosis are significant[88]. There is a Th17/Treg imbalance that positively correlates with NASH histological progression[89].

Innate lymphoid cells are lymphocytes that secrete cytokines and chemokines in response to pathogenic tissue damage. They have a role in inflammation and fibrogenesis that progresses with advancing chronic liver disease[90].

OS AND INFLAMMATION

Detoxification is a crucial hepatic activity. It is vulnerable to OS and inflammation. An increase in free fatty acids is critical for the elevation of reactive oxygen species (ROS). A balance between the ROS and antioxidant systems is necessary for adequate cell function[80]. OS causes liver damage by altering DNA molecules, proteins, and lipids and modulating pathways associated with gene transcription, protein expression, cell apoptosis, and HSC activation. Inflammation is manifested as inflammatory cell infiltration in the liver to fight pathogen invasion. When the stimuli are persistent, it can lead to cell injury and lipid accumulation associated with an increased risk of severe liver disease, including steatohepatitis and fibrosis[91].

In NASH, ROS are generated in several ways that can alter signaling pathways, such as cell kinases, phosphatases, and transcription factors, which impact cell proliferation, differentiation, and apoptosis. They can lead to cirrhosis *via* the rebuilding of stellate cells and ECM within the liver. Substantial hepatic ROS is produced by excessive angiotensin II and activated CYP2E1, resulting in impaired beta-oxidation and eventually fatty liver[91].

Lipotoxicity in NAFLD causes OS and induces organelle damage due to decreased antioxidant systems, mitochondrial dysfunction, and an increase in unfolded protein response by endoplasmic reticulum stress[80]. On the other hand, there is an impairment of α-oxidation due to a decrease in PPARα activity, which upturns hepatic lipid levels. Fatty acid overload is the major source of reducing equivalents responsible for increased ROS production. Also, TNF-α and lipid peroxidation products could induce mitochondrial dysfunction. Mitochondrial damage will result in secondary lipid α-oxidation inhibition and a further increase in the degree of steatosis[80].

Furthermore, inflammatory cytokines such as IL-1-β, TNF-α, and IL-17/20/33, chemokines, like monocyte chemoattractant protein-1 and C-X-C chemokine ligand 10,

and the toll-like receptor pathway are intensively involved in the regulation of hepatic fibrogenesis[91]. Macrophage activation and influx in the liver are important for the progression of NAFLD since hepatic macrophages promote NASH development *via* cytokines IL-1, IL-6, and TNF-α[92]. Liver failure causes an increase of TNF-α, IL-6, and angiotensin II[80].

OTHER NOVEL MARKERS

Gut permeability markers

The intestinal barrier is composed of chemical, physical, and immunological barriers. Maintaining a healthy barrier is essential to prevent microbial translocation and keep the liver safe to prevent systemic inflammation[93].

Differences in the taxonomic composition of the intestinal microbiome in NAFLD (an increased proportion of *Firmicutes* and a reduced proportion of *Bacteriodetes)* change metabolic function. The availability of bile acids, endogenous alcohols, and voltaic organic compounds increases. When these changes are combined with reduced SCFAs and choline, the integrity of the intestinal barrier is reduced[93].

Gut barrier disruption is recognized in patients with cirrhosis. The epithelial layers show structural abnormalities related to increased intestinal permeability or bacterial translocation[94]. Permeability can be measured by the urinary excretion of radiolabeled ^{51}chromium-ethylenediamine tetraacetic acid or by measuring volatile organic compounds formed by the fermentation of some dietary polysaccharides[95].

CTC–cardiotonic steroids

Cardiotonic steroids (CTS) are part of a group of specific ligands of Na^+, K^+-ATPase, a ubiquitously expressed enzyme responsible for the maintenance of electrochemical gradients across the cell membrane through active transport[96] that provokes a variety of cell signals[70]. In the last decades, studies have revealed the role of Na^+, K^+-ATPase and its signaling in various diseases, including inflammation and fibrosis[97].

CTS increase cholesterol synthesis in liver HepG2 cells, which augments the activity and expression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase[98]. Disturbed cholesterol balance underlies cardiovascular disease and an increasing number of other diseases, such as neurodegenerative diseases, cancers, and liver disease[99].

Elevated CTS might encourage increased cholesterol levels in the liver and worsen liver fibrosis by activating HSCs[100] and other redox-inflammatory pathways[101]. This increase in cholesterol levels could precipitate hepatocyte injury and macrophage activation that could lead to liver fibrosis progression. However, even CTS seem to have an important role in hepatocyte lipotoxicity and fibrosis; to our knowledge, they have not been studied as biomarkers for liver disease progression.

GUT MICROBIOTA

A large community of viruses, bacteria, archaea, and fungi live in the gastrointestinal tract and composes the gut microbiota[102]. It has critical roles in digestion, immunity, and metabolism[103]. Recently, the characterization of gut microbiota has evolved rapidly due to the advances in sequencing technology, permitting the creation of a gut microbiota gene catalogue[102]. The collective genetic material of the microbiota is often referred to as the "gut microbiome". It encodes pathways that produce small bioactive molecules derived from dietary or metabolic precursors and may alter human health[104].

Thus, knowledge of microbiome characteristics in different metabolic diseases has increased in the past years. There has been great interest in dysbiosis (alterations in the composition and balance of microbiota[104]). Microbiota alterations are being studied as possible diagnostic biomarkers to improve personalized care. Animal studies have demonstrated a potential causal role of gut microbiota in NAFLD development[105]. However, extrapolating mouse model experimental information to humans has several limitations[106]. Consequently, signatures specific to liver alterations would be useful as NAFLD diagnostic biomarkers. However, discrepant microbiome signatures might be linked to the heterogeneity of diet, drugs, infections, environmental exposures, among others[104].

Bacterial microbiome

Alterations in the gut microbiome have been associated with the progression and severity of NAFLD[107]. Proteobacteria are enriched in steatosis[103,108,109]. Patients with NAFLD, compared with healthy individuals, also have significant changes at the phylum (increased Enterobacteriaceae[109] and decreased Rikenellaceae and Ruminococcaceae[109]) and genera level (increased *Escherichia*[109], *Dorea*, and *Peptoniphilus* and decreased *Anaerosporobacter, Coprococcus, Faecalibacterium,* and *Prevotella*)[103].

When comparing people with NASH *vs* healthy controls, some patterns are observed that also overlap with the NAFLD microbiome: Phylum (increased Proteobacteria[50,109-111]), family (increased Enterobacteriaceae[109,110] and decreased Ruminococcaceae[110-113] and Rikenellaceae[110]), and genera (increased *Dorea*[111] and decreased *Faecalibacterium*[110,113,114], *Coprococcus*[110,112,113], and *Anaerosporobacter*[112,114]).

Few projects have studied microbial composition as a function of fibrosis progression. *Bacteroides vulgatus* and *Escherichia coli* are the most abundant species in advanced fibrosis (F3–F4)[50]. Models have been proposed to use the microbiome as a reservoir for diagnostic signatures of NAFLD fibrosis[50], but further confirmation in independent cohorts and across geographical regions is necessary to assess their clinical relevance.

Microbial signatures of liver fibrosis are related to a severe shift in taxa conformation, leading to a growth in pathogenic taxa and a decline in metabolically beneficial taxa[115]. However, the evaluation of gut microbiota contribution to liver disease progression (from steatosis to NASH and NASH cirrhosis) is limited and bacterial markers are frequently identified in a given study yet not confirmed in independent cohorts.

Although some studies consider gut bacterial groups as promising markers of different stages of liver disease, if the microbiota is a causal factor and how it interacts with the complex pathophysiological processes driving disease progression from mild fibrosis to severe fibrosis is still under investigation[50,109].

Virome

Dense and complex populations of intestinal viruses reside in the gut and interact with other microorganisms and the human host[116,117]. Most intestinal viruses are bacteriophages (phages), viruses that can specifically infect bacteria[118]. Phages may serve as important microbiota genetic diversity reservoirs by acting as vehicles for the horizontal transfer of virulence, antibiotic resistance, and metabolic determinants among bacteria[119].

Lang *et al*[120] studied the fecal viromes from NAFLD patients and controls. They found associated histologic markers of NAFLD severity with significant decreases in viral diversity and proportion of bacteriophages[120]. The intestinal virome is specific for every individual, and viral diversity measures were the third and fifth most important variables following a higher AST and higher age. The most important viral species belonged to *Lactococcus* phages, and several *Lactococcus* phages were less present in patients with NAFLD and NASH.

Protozoa and fungi

Fungi and archaea are important components of the human microbiota. Recent findings have revealed that mycobiome (commensal fungi at barrier surfaces) can influence host immunity and the development and progression of human inflammatory diseases[121]. The human gut mycobiome is dominated by *Saccharomyces, Malassezia, Candida*, and *Cladosporium* and are an important modulator for local and peripheral immune responses. Patients with liver fibrosis have decreased fungal diversity and increased *Candida*[122]. Gut mycobiota disturbance might produce metabolites called mycotoxins (trichothecenes, zearalenone, fumonisins, ochratoxins, aflatoxins) that can alter gut health by compromising intestinal epithelia[123,124].

LIMITATION

The increasing burden of NAFLD worldwide has encouraged the search for novel biomarkers to detect liver diseases. Liver biopsy is currently the gold standard for diagnosis and staging, but it has several limitations, including sampling errors, invasiveness, inter-observer variability, and related procedure risks. Researchers have faced the challenge of developing novel biomarkers in past decades, and significant advances have been made. A promising biomarker should be liver-specific, accessible

	Proteomics	Metabolomics	Lipidomics	Genetic markers	Epigenetic markers	Immune disregulation	Oxidative stress and inflammation	Gut microbiota
NAFLD (Steatosis)	↑ Hb ↑ FGF-21 ↑ RBP4 ↑ CK18Asp396	↑ Inosine ↑ Hypoxanthine ↓ Glutathione ↑ Phenylacetic acid ↓ Dietary choline ↓ Betaine: Choline ↑ TMAO ↑ Ethanol ↓ Bile acids	↑ LDL-c ↑ Fecal SCFAs ↑ fCh		PPAR-γ	↑ IL-17 ↑ IL-22	↓ PPARα ↓ Lipid β-oxidation	↑ Proteobacteria ↑ Enterobacteriaceae ↓ Rikenellaceae ↓ Ruminococcaceae ↓ Lactococcus phages
NASH (Inflammation)	↑ Aminotransferases ↑ Fuc-Hpt ↑ Mac2bp ↑ CK18Asp396 ↑ DR5 ↑ miRNA-122 ↑ miR-192	↑ Homocysteine ↑ Cysteine ↑ Ethanol	↑ Fecal SCFAs ↑ fCh ↑ 11-HETE ↑ 11,12-diHETrE	↑PLPNA3		N/L ratio Th17/T reg imbalance	↑ ROS ↑ Angiotensin II ↑ CYP2E1 ↑ IL-1 ↑ IL-6 ↑ TNFα	↑ Proteobacteria ↑ Enterobacteriaceae ↓ Rikenellaceae ↓ Ruminococcaceae ↑ Dorea ↓ Faecalibacterium ↓ Coprococcus ↓ Anaerosporobacter ↓ Lactococcus phages
Cirrhosis (Fibrosis)	↑ ASGPR1+ ↑ CNN2 ↓ miRNA-214 ↑ miR-34a	↑ 3-(4-hydroxyphenyl) lactate	↑ fCh ↓ DHEA-S ↓ Etiocholanolone	↑PLPNA3 Hfib1	Hypomethylation in fibrogenic genes	N/L ratio ↑ Monocytes ↑ IFNγ ↑ IL-4 ↑ IL-13 ↓ CD4+T ↑ T reg ↑ ILCs	↑ ROS ↑ TNFα ↑ IL-6 ↑ Angiotensin II	↑ Bacteroides vulgatus ↑ Escherichia coli ↑ Candida

Figure 2 Potential biomarkers involved in hepatic pathophysiology. Hb: Hemoglobin; FGF-21: Fibroblast growth factor 21; RBP4: Retinol binding protein 4; CK18Asp396: Caspase cleaved cytokeratin-18 fragment (M30); Fuc-Hpt: Fucosylated haptoglobin; Mac2bp: Mac-2-binding protein; DR5: Death receptor 5; miRNA-122: MicroRNA 122; miR-192: MicroRNA 192; ASGPR1+: Asialoglycoprotein receptor 1; CNN2: Calponin 2; miRNA-214: MicroRNA 214; miR-34a: MicroRNA 34a; TMAO: Trimethylamine N-oxide; LDL-c: Low density lipoprotein cholesterol; Fecal SCFAs: Fecal Short chain fatty acids; fCh: Ferrochelatase; 11-HETE: 11-Hydroxyeicosatetraenoic Acid; 11,12-diHETrE: 11,12-dihydroxyicosatrienoic acid; DHEA-S: Dehydroepiandrosterone sulphate; PPAR-γ: Peroxisome proliferator-activated receptor γ; IL-17: Interleukin-17; IL-22: Interleukin-22; N/L ratio: Neutrophil/lymphocyte ratio; Th17/Treg imbalance: T helper 17/T regulatory cells imbalance; IFNγ: Interferon gamma; IL-4: Interleukin-4; IL-13: Interleukin-13; CD4+T: Cluster of differentiation 4, T helper cells; T reg: Regulatory T cells; ILCs: Innate lymphoid cells.

and accurate, replicable, and available in clinical laboratories. As summarized in this article, most studies have focused on proteomics, metabolomics, genome-wide association studies, microbiome, and inflammation markers. Still, some may be more specific for NAFLD while others for NASH, although the challenge for determining the etiology and staging the degree of severity remains a limitation (Figure 2).

The evaluation of future biomarkers for the assessment of liver fibrosis could greatly impact the health system. There is a priority for non-invasive diagnostic tools to fulfil medical needs, differentiate patients with steatosis from those with NASH and fibrosis, predict disease progression, and monitor patients to evaluate the therapeutic response. In the following years, it would be expected that a physician who faces a hepatic patient could suspect hepatic disease, perform imaging studies, and from there have a set of potential biomarkers that they may request to have a concrete and specific diagnosis. Some of these biomarkers have strong diagnostic performance, but current evidence shows a lack of reproducibility. Besides, the analytical, clinical validity of the methodology is lacking. Validity is necessary to translate basic research into real clinical application. Even if we perform this validation, it is unlikely that a single biomarker could fulfil this necessity. A combination of these biomarkers could soon be used to create a diagnostic panel. This panel, combined with the patient´s clinical history and clinical data, could certainly lead to a medical decision that results in an accurate diagnosis and treatment. This result must be the goal in the following years.

CONCLUSION

Through this review, we have shown that despite a wide range of potential biomarkers for the different stages of hepatic steatosis and fibrosis, there is still a long path to the translation of these resources. We provide evidence of the current absence of an efficient, non-invasive, and widely accessible test for NAFLD and NASH detection. Biomarkers are still in early stages. Rigorous, well-designed comprehensive studies are required to determine the actual benefit these may pose for determining the risk, diagnosis, and progression of the hepatic patient. In conclusion, our review compiles significant efforts to find new promising biomarkers for liver disease, still leaving great challenges. There is still a need to define normal reference levels in healthy individuals and the different stages of the disease and to determine the clinical sensitivity and specificity of biomarkers to develop a clinical diagnostic panel.

REFERENCES

1   **Fouad Y**, Waked I, Bollipo S, Gomaa A, Ajlouni Y, Attia D. What's in a name? *Liver Int* 2020; **40**: 1254-1261 [PMID: 32301554 DOI: 10.1111/liv.14478]

2   **Younossi Z**, Anstee QM, Marietti M, Hardy T, Henry L, Eslam M, George J, Bugianesi E. Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. *Nat Rev Gastroenterol Hepatol* 2018; **15**: 11-20 [PMID: 28930295 DOI: 10.1038/nrgastro.2017.109]

3   **Byrne CD**, Patel J, Scorletti E, Targher G. Tests for diagnosing and monitoring non-alcoholic fatty liver disease in adults. *BMJ* 2018; **362**: k2734 [PMID: 30002017 DOI: 10.1136/bmj.k2734]

4   **Younossi ZM**, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* 2016; **64**: 73-84 [PMID: 26707365 DOI: 10.1002/hep.28431]

5   **Méndez-Sánchez N**, Villa AR, Chávez-Tapia NC, Ponciano-Rodriguez G, Almeda-Valdés P, González D, Uribe M. Trends in liver disease prevalence in Mexico from 2005 to 2050 through mortality data. *Ann Hepatol* 2005; **4**: 52-55 [PMID: 15798662]

6   **Bernal-Reyes R**, Castro-Narro G, Malé-Velázquez R, Carmona-Sánchez R, González-Huezo MS, García-Juárez I, Chávez-Tapia N, Aguilar-Salinas C, Aiza-Haddad I, Ballesteros-Amozurrutia MA, Bosques-Padilla F, Castillo-Barradas M, Chávez-Barrera JA, Cisneros-Garza L, Flores-Calderón J, García-Compeán D, Gutiérrez-Grobe Y, Higuera de la Tijera MF, Kershenobich-Stalnikowitz D, Ladrón de Guevara-Cetina L, Lizardi-Cervera J, López-Cossio JA, Martínez-Vázquez S, Márquez-Guillén E, Méndez-Sánchez N, Moreno-Alcantar R, Poo-Ramírez JL, Ramos-Martínez P, Rodríguez-Hernández H, Sánchez-Ávila JF, Stoopen-Rometti M, Torre-Delgadillo A, Torres-Villalobos G, Trejo-Estrada R, Uribe-Esquivel M, Velarde-Ruiz Velasco JA. The Mexican consensus on nonalcoholic fatty liver disease. *Rev Gastroenterol Mex (Engl Ed)* 2019; **84**: 69-99 [PMID: 30711302 DOI: 10.1016/j.rgmx.2018.11.007]

7   **Long MT**, Gandhi S, Loomba R. Advances in non-invasive biomarkers for the diagnosis and monitoring of non-alcoholic fatty liver disease. *Metabolism* 2020; **111S**: 154259 [PMID: 32387227 DOI: 10.1016/j.metabol.2020.154259]

8   **Giraudi PJ**, Stephenson AM, Tiribelli C, Rosso N. Novel high-throughput applications for NAFLD diagnostics and biomarker dis-covery. *Hepatoma Res* 2021; 2021 [DOI: 10.20517/2394-5079.2020.92]

9   **Geyer PE**, Holdt LM, Teupser D, Mann M. Revisiting biomarker discovery by plasma proteomics. *Mol Syst Biol* 2017; **13**: 942 [PMID: 28951502 DOI: 10.15252/msb.20156297]

10 **Iruarrizaga-Lejarreta M**, Bril F, Noureddin M, Ortiz P, Lu SC, Mato JM. Emerging circulating biomarkers for the diagnosis and assess-ment of treatment responses in patients with hepatic fat accumula-tion, nash and liver fibrosis. Translational Research Methods in Diabetes, Obesity, and Nonalcoholic Fatty Liver Disease: A Focus on Early Phase Clinical Drug Development. Gainesville: Springer, 2019: 423–448

11 **Rodriguez-Suarez E**, Mato JM, Elortza F. Proteomics analysis of human nonalcoholic fatty liver. *Methods Mol Biol* 2012; **909**: 241-258 [PMID: 22903720 DOI: 10.1007/978-1-61779-959-4_16]

12 **Yilmaz Y**. Serum proteomics for biomarker discovery in nonalcoholic fatty liver disease. *Clin Chim Acta* 2012; **413**: 1190-1193 [PMID: 22546610 DOI: 10.1016/j.cca.2012.04.019]

13 **Li H**, Dong K, Fang Q, Hou X, Zhou M, Bao Y, Xiang K, Xu A, Jia W. High serum level of fibroblast growth factor 21 is an independent predictor of non-alcoholic fatty liver disease: a 3-year prospective study in China. *J Hepatol* 2013; **58**: 557-563 [PMID: 23142063 DOI: 10.1016/j.jhep.2012.10.029]

14 **Seo JA**, Kim NH, Park SY, Kim HY, Ryu OH, Lee KW, Lee J, Kim DL, Choi KM, Baik SH, Choi DS, Kim SG. Serum retinol-binding protein 4 levels are elevated in non-alcoholic fatty liver disease. *Clin Endocrinol (Oxf)* 2008; **68**: 555-560 [PMID: 17941908 DOI: 10.1111/j.1365-2265.2007.03072.x]

15 **Leoni S**, Tovoli F, Napoli L, Serio I, Ferri S, Bolondi L. Current guidelines for the management of non-alcoholic fatty liver disease: A systematic review with comparative analysis. *World J*

Gastroenterol 2018; **24**: 3361-3373 [PMID: 30122876 DOI: 10.3748/wjg.v24.i30.3361]

16   **Kamada Y**, Akita M, Takeda Y, Yamada S, Fujii H, Sawai Y, Doi Y, Asazawa H, Nakayama K, Mizutani K, Yakushijin T, Miyazaki M, Ezaki H, Hiramatsu N, Yoshida Y, Kiso S, Imai Y, Kawada N, Takehara T, Miyoshi E. Serum Fucosylated Haptoglobin as a Novel Diagnostic Biomarker for Predicting Hepatocyte Ballooning and Nonalcoholic Steatohepatitis. *PLoS One* 2013; **8**: e66328 [PMID: 23805214 DOI: 10.1371/journal.pone.0066328]

17   **European Association for the Study of the Liver (EASL)**; European Association for the Study of Diabetes (EASD); European Association for the Study of Obesity (EASO). EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. *Diabetologia* 2016; **59**: 1121-1140 [PMID: 27053230 DOI: 10.1007/s00125-016-3902-y]

18   **Shen J**, Chan HL, Wong GL, Chan AW, Choi PC, Chan HY, Chim AM, Yeung DK, Yu J, Chu WC, Wong VW. Assessment of non-alcoholic fatty liver disease using serum total cell death and apoptosis markers. *Aliment Pharmacol Ther* 2012; **36**: 1057-1066 [PMID: 23066946 DOI: 10.1111/apt.12091]

19   **Estep M**, Mehta R, Bratthauer G, Alaparthi L, Monge F, Ali S, Abdelatif D, Younoszai Z, Stepanova M, Goodman ZD, Younossi ZM. Hepatic sonic hedgehog protein expression measured by computer assisted morphometry significantly correlates with features of non-alcoholic steatohepatitis. *BMC Gastroenterol* 2019; **19**: 27 [PMID: 30744560 DOI: 10.1186/s12876-019-0951-y]

20   **Skog J**, Würdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, Curry WT Jr, Carter BS, Krichevsky AM, Breakefield XO. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol* 2008; **10**: 1470-1476 [PMID: 19011622 DOI: 10.1038/ncb1800]

21   **Stefan N**, Häring HU, Cusi K. Non-alcoholic fatty liver disease: causes, diagnosis, cardiometabolic consequences, and treatment strategies. *Lancet Diabetes Endocrinol* 2019; **7**: 313-324 [PMID: 30174213 DOI: 10.1016/S2213-8587(18)30154-2]

22   **Shah R**, Patel T, Freedman JE. Circulating Extracellular Vesicles in Human Disease. *N Engl J Med* 2018; **379**: 958-966 [PMID: 30184457 DOI: 10.1056/NEJMra1704286]

23   **Maas SLN**, Breakefield XO, Weaver AM. Extracellular Vesicles: Unique Intercellular Delivery Vehicles. *Trends Cell Biol* 2017; **27**: 172-188 [PMID: 27979573 DOI: 10.1016/j.tcb.2016.11.003]

24   **Juan T**, Fürthauer M. Biogenesis and function of ESCRT-dependent extracellular vesicles. *Semin Cell Dev Biol* 2018; **74**: 66-77 [PMID: 28807885 DOI: 10.1016/j.semcdb.2017.08.022]

25   **Hornick NI**, Huan J, Doron B, Goloviznina NA, Lapidus J, Chang BH, Kurre P. Serum Exosome MicroRNA as a Minimally-Invasive Early Biomarker of AML. *Sci Rep* 2015; **5**: 11295 [PMID: 26067326 DOI: 10.1038/srep11295]

26   **Dickhout A**, Koenen RR. Extracellular Vesicles as Biomarkers in Cardiovascular Disease; Chances and Risks. *Front Cardiovasc Med* 2018; **5**: 113 [PMID: 30186839 DOI: 10.3389/fcvm.2018.00113]

27   **Erdbrügger U**, Le TH. Extracellular Vesicles in Renal Diseases: More than Novel Biomarkers? *J Am Soc Nephrol* 2016; **27**: 12-26 [PMID: 26251351 DOI: 10.1681/ASN.2015010074]

28   **Kornek M**, Lynch M, Mehta SH, Lai M, Exley M, Afdhal NH, Schuppan D. Circulating microparticles as disease-specific biomarkers of severity of inflammation in patients with hepatitis C or nonalcoholic steatohepatitis. *Gastroenterology* 2012; **143**: 448-458 [PMID: 22537612 DOI: 10.1053/j.gastro.2012.04.031]

29   **Tadokoro T**, Morishita A, Masaki T. Diagnosis and Therapeutic Management of Liver Fibrosis by MicroRNA. *Int J Mol Sci* 2021; **22** [PMID: 34360904 DOI: 10.3390/ijms22158139]

30   **Povero D**, Yamashita H, Ren W, Subramanian MG, Myers RP, Eguchi A, Simonetto DA, Goodman ZD, Harrison SA, Sanyal AJ, Bosch J, Feldstein AE. Characterization and Proteome of Circulating Extracellular Vesicles as Potential Biomarkers for NASH. *Hepatol Commun* 2020; **4**: 1263-1278 [PMID: 32923831 DOI: 10.1002/hep4.1556]

31   **Povero D**, Eguchi A, Li H, Johnson CD, Papouchado BG, Wree A, Messer K, Feldstein AE. Circulating extracellular vesicles with specific proteome and liver microRNAs are potential biomarkers for liver injury in experimental fatty liver disease. *PLoS One* 2014; **9**: e113651 [PMID: 25470250 DOI: 10.1371/journal.pone.0113651]

32   **Hirsova P**, Ibrahim SH, Krishnan A, Verma VK, Bronk SF, Werneburg NW, Charlton MR, Shah VH, Malhi H, Gores GJ. Lipid-Induced Signaling Causes Release of Inflammatory Extracellular Vesicles From Hepatocytes. *Gastroenterology* 2016; **150**: 956-967 [PMID: 26764184 DOI: 10.1053/j.gastro.2015.12.037]

33   **Cai S**, Cheng X, Pan X, Li J. Emerging role of exosomes in liver physiology and pathology. *Hepatol Res* 2017; **47**: 194-203 [PMID: 27539153 DOI: 10.1111/hepr.12794]

34   **Chen L**, Brenner DA, Kisseleva T. Combatting Fibrosis: Exosome-Based Therapies in the Regression of Liver Fibrosis. *Hepatol Commun* 2019; **3**: 180-192 [PMID: 30766956 DOI: 10.1002/hep4.1290]

35   **Koeck ES**, Iordanskaia T, Sevilla S, Ferrante SC, Hubal MJ, Freishtat RJ, Nadler EP. Adipocyte exosomes induce transforming growth factor beta pathway dysregulation in hepatocytes: a novel paradigm for obesity-related liver disease. *J Surg Res* 2014; **192**: 268-275 [PMID: 25086727 DOI: 10.1016/j.jss.2014.06.050]

36   **Seo W**, Eun HS, Kim SY, Yi HS, Lee YS, Park SH, Jang MJ, Jo E, Kim SC, Han YM, Park KG, Jeong WI. Exosome-mediated activation of toll-like receptor 3 in stellate cells stimulates interleukin-17 production by γδ T cells in liver fibrosis. *Hepatology* 2016; **64**: 616-631 [PMID: 27178735 DOI:

10.1002/hep.28644]

37   **Charrier A**, Chen R, Chen L, Kemper S, Hattori T, Takigawa M, Brigstock DR. Exosomes mediate intercellular transfer of pro-fibrogenic connective tissue growth factor (CCN2) between hepatic stellate cells, the principal fibrotic cells in the liver. *Surgery* 2014; **156**: 548-555 [PMID: 24882759 DOI: 10.1016/j.surg.2014.04.014]

38   **Chen L**, Chen R, Kemper S, Charrier A, Brigstock DR. Suppression of fibrogenic signaling in hepatic stellate cells by Twist1-dependent microRNA-214 expression: Role of exosomes in horizontal transfer of Twist1. *Am J Physiol Gastrointest Liver Physiol* 2015; **309**: G491-G499 [PMID: 26229009 DOI: 10.1152/ajpgi.00140.2015]

39   **Jiang XP**, Ai WB, Wan LY, Zhang YQ, Wu JF. The roles of microRNA families in hepatic fibrosis. *Cell Biosci* 2017; **7**: 34 [PMID: 28680559 DOI: 10.1186/s13578-017-0161-7]

40   **Yan G**, Li B, Xin X, Xu M, Ji G, Yu H. MicroRNA-34a Promotes Hepatic Stellate Cell Activation *via* Targeting ACSL1. *Med Sci Monit* 2015; **21**: 3008-3015 [PMID: 26437572 DOI: 10.12659/MSM.894000]

41   **Roy S**, Benz F, Vargas Cardenas D, Vucur M, Gautheron J, Schneider A, Hellerbrand C, Pottier N, Alder J, Tacke F, Trautwein C, Roderburg C, Luedde T. miR-30c and miR-193 are a part of the TGF-β-dependent regulatory network controlling extracellular matrix genes in liver fibrosis. *J Dig Dis* 2015; **16**: 513-524 [PMID: 26120970 DOI: 10.1111/1751-2980.12266]

42   **Guo CJ**, Pan Q, Li DG, Sun H, Liu BW. miR-15b and miR-16 are implicated in activation of the rat hepatic stellate cell: An essential role for apoptosis. *J Hepatol* 2009; **50**: 766-778 [PMID: 19232449 DOI: 10.1016/j.jhep.2008.11.025]

43   **Hyun J**, Wang S, Kim J, Rao KM, Park SY, Chung I, Ha CS, Kim SW, Yun YH, Jung Y. MicroRNA-378 limits activation of hepatic stellate cells and liver fibrosis by suppressing Gli3 expression. *Nat Commun* 2016; **7**: 10993 [PMID: 27001906 DOI: 10.1038/ncomms10993]

44   **Roderburg C**, Mollnow T, Bongaerts B, Elfimova N, Vargas Cardenas D, Berger K, Zimmermann H, Koch A, Vucur M, Luedde M, Hellerbrand C, Odenthal M, Trautwein C, Tacke F, Luedde T. Micro-RNA profiling in human serum reveals compartment-specific roles of miR-571 and miR-652 in liver cirrhosis. *PLoS One* 2012; **7**: e32999 [PMID: 22412969 DOI: 10.1371/journal.pone.0032999]

45   **Xie X**, Dou CY, Zhou Y, Zhou Q, Tang HB. MicroRNA-503 Targets Mothers Against Decapentaplegic Homolog 7 Enhancing Hepatic Stellate Cell Activation and Hepatic Fibrosis. *Dig Dis Sci* 2021; **66**: 1928-1939 [PMID: 32648079 DOI: 10.1007/s10620-020-06460-7]

46   **Ezhilarasan D**. MicroRNA interplay between hepatic stellate cell quiescence and activation. *Eur J Pharmacol* 2020; **885**: 173507 [PMID: 32858048 DOI: 10.1016/j.ejphar.2020.173507]

47   **Chu H**, Duan Y, Yang L, Schnabl B. Small metabolites, possible big changes: a microbiota-centered view of non-alcoholic fatty liver disease. *Gut* 2019; **68**: 359-370 [PMID: 30171065 DOI: 10.1136/gutjnl-2018-316307]

48   **Caussy C**, Loomba R. Gut microbiome, microbial metabolites and the development of NAFLD. *Nat Rev Gastroenterol Hepatol* 2018; **15**: 719-720 [PMID: 30158571 DOI: 10.1038/s41575-018-0058-x]

49   **Dong S**, Zhan ZY, Cao HY, Wu C, Bian YQ, Li JY, Cheng GH, Liu P, Sun MY. Urinary metabolomics analysis identifies key biomarkers of different stages of nonalcoholic fatty liver disease. *World J Gastroenterol* 2017; **23**: 2771-2784 [PMID: 28487615 DOI: 10.3748/wjg.v23.i15.2771]

50   **Loomba R**, Seguritan V, Li W, Long T, Klitgord N, Bhatt A, Dulai PS, Caussy C, Bettencourt R, Highlander SK, Jones MB, Sirlin CB, Schnabl B, Brinkac L, Schork N, Chen CH, Brenner DA, Biggs W, Yooseph S, Venter JC, Nelson KE. Gut Microbiome-Based Metagenomic Signature for Non-invasive Detection of Advanced Fibrosis in Human Nonalcoholic Fatty Liver Disease. *Cell Metab* 2017; **25**: 1054-1062.e5 [PMID: 28467925 DOI: 10.1016/j.cmet.2017.04.001]

51   **Hoyles L**, Fernández-Real JM, Federici M, Serino M, Abbott J, Charpentier J, Heymes C, Luque JL, Anthony E, Barton RH, Chilloux J, Myridakis A, Martinez-Gili L, Moreno-Navarrete JM, Benhamed F, Azalbert V, Blasco-Baque V, Puig J, Xifra G, Ricart W, Tomlinson C, Woodbridge M, Cardellini M, Davato F, Cardolini I, Porzio O, Gentileschi P, Lopez F, Foufelle F, Butcher SA, Holmes E, Nicholson JK, Postic C, Burcelin R, Dumas ME. Molecular phenomics and metagenomics of hepatic steatosis in non-diabetic obese women. *Nat Med* 2018; **24**: 1070-1080 [PMID: 29942096 DOI: 10.1038/s41591-018-0061-3]

52   **Engin A**. Non-Alcoholic Fatty Liver Disease. *Adv Exp Med Biol* 2017; **960**: 443-467 [PMID: 28585211 DOI: 10.1007/978-3-319-48382-5_19]

53   **Sherriff JL**, O'Sullivan TA, Properzi C, Oddo JL, Adams LA. Choline, Its Potential Role in Nonalcoholic Fatty Liver Disease, and the Case for Human and Bacterial Genes. *Adv Nutr* 2016; **7**: 5-13 [PMID: 26773011 DOI: 10.3945/an.114.007955]

54   **Sergeeva KV**. [Blood serum and liver phospholipids in rats with chronic choline-protein deficiency]. *Biull Eksp Biol Med* 1975; **79**: 47-49 [PMID: 1222249]

55   **Chen YM**, Liu Y, Zhou RF, Chen XL, Wang C, Tan XY, Wang LJ, Zheng RD, Zhang HW, Ling WH, Zhu HL. Associations of gut-flora-dependent metabolite trimethylamine-N-oxide, betaine and choline with non-alcoholic fatty liver disease in adults. *Sci Rep* 2016; **6**: 19076 [PMID: 26743949 DOI: 10.1038/srep19076]

56   **Zeisel SH**, daCosta KA, Youssef M, Hensey S. Conversion of dietary choline to trimethylamine and dimethylamine in rats: dose-response relationship. *J Nutr* 1989; **119**: 800-804 [PMID: 2723829 DOI: 10.1093/jn/119.5.800]

57   **Sayin SI**, Wahlström A, Felin J, Jäntti S, Marschall HU, Bamberg K, Angelin B, Hyötyläinen T, Orešič M, Bäckhed F. Gut microbiota regulates bile acid metabolism by reducing the levels of tauro-beta-muricholic acid, a naturally occurring FXR antagonist. *Cell Metab* 2013; **17**: 225-235 [PMID: 23395169 DOI: 10.1016/j.cmet.2013.01.003]

58   **Yuan J**, Chen C, Cui J, Lu J, Yan C, Wei X, Zhao X, Li N, Li S, Xue G, Cheng W, Li B, Li H, Lin W, Tian C, Zhao J, Han J, An D, Zhang Q, Wei H, Zheng M, Ma X, Li W, Chen X, Zhang Z, Zeng H, Ying S, Wu J, Yang R, Liu D. Fatty Liver Disease Caused by High-Alcohol-Producing Klebsiella pneumoniae. *Cell Metab* 2019; **30**: 675-688.e7 [PMID: 31543403 DOI: 10.1016/j.cmet.2019.08.018]

59   **Imajo K**, Fujita K, Yoneda M, Shinohara Y, Suzuki K, Mawatari H, Takahashi J, Nozaki Y, Sumida Y, Kirikoshi H, Saito S, Nakamuta M, Matsuhashi N, Wada K, Nakajima A. Plasma free choline is a novel non-invasive biomarker for early-stage non-alcoholic steatohepatitis: A multi-center validation study. *Hepatol Res* 2012; **42**: 757-766 [PMID: 22780848 DOI: 10.1111/j.1872-034X.2012.00976.x]

60   **Kalhan SC**, Guo L, Edmison J, Dasarathy S, McCullough AJ, Hanson RW, Milburn M. Plasma metabolomic profile in nonalcoholic fatty liver disease. *Metabolism* 2011; **60**: 404-413 [PMID: 20423748 DOI: 10.1016/j.metabol.2010.03.006]

61   **Barr J**, Caballería J, Martínez-Arranz I, Domínguez-Díez A, Alonso C, Muntané J, Pérez-Cormenzana M, García-Monzón C, Mayo R, Martín-Duce A, Romero-Gómez M, Lo Iacono O, Tordjman J, Andrade RJ, Pérez-Carreras M, Le Marchand-Brustel Y, Tran A, Fernández-Escalante C, Arévalo E, García-Unzueta M, Clement K, Crespo J, Gual P, Gómez-Fleitas M, Martínez-Chantar ML, Castro A, Lu SC, Vázquez-Chantada M, Mato JM. Obesity-dependent metabolic signatures associated with nonalcoholic fatty liver disease progression. *J Proteome Res* 2012; **11**: 2521-2532 [PMID: 22364559 DOI: 10.1021/pr201223p]

62   **Gorden DL**, Myers DS, Ivanova PT, Fahy E, Maurya MR, Gupta S, Min J, Spann NJ, McDonald JG, Kelly SL, Duan J, Sullards MC, Leiker TJ, Barkley RM, Quehenberger O, Armando AM, Milne SB, Mathews TP, Armstrong MD, Li C, Melvin WV, Clements RH, Washington MK, Mendonsa AM, Witztum JL, Guan Z, Glass CK, Murphy RC, Dennis EA, Merrill AH Jr, Russell DW, Subramaniam S, Brown HA. Biomarkers of NAFLD progression: a lipidomics approach to an epidemic. *J Lipid Res* 2015; **56**: 722-736 [PMID: 25598080 DOI: 10.1194/jlr.P056002]

63   **Kimberly WT**, O'Sullivan JF, Nath AK, Keyes M, Shi X, Larson MG, Yang Q, Long MT, Vasan R, Peterson RT, Wang TJ, Corey KE, Gerszten RE. Metabolite profiling identifies anandamide as a biomarker of nonalcoholic steatohepatitis. *JCI Insight* 2017; **2** [PMID: 28469090 DOI: 10.1172/jci.insight.92989]

64   **Tokushige K**, Hashimoto E, Kodama K, Tobari M, Matsushita N, Kogiso T, Taniai M, Torii N, Shiratori K, Nishizaki Y, Ohga T, Ohashi Y, Sato T. Serum metabolomic profile and potential biomarkers for severity of fibrosis in nonalcoholic fatty liver disease. *J Gastroenterol* 2013; **48**: 1392-1400 [PMID: 23478936 DOI: 10.1007/s00535-013-0766-5]

65   **Puri P**, Wiest MM, Cheung O, Mirshahi F, Sargeant C, Min HK, Contos MJ, Sterling RK, Fuchs M, Zhou H, Watkins SM, Sanyal AJ. The plasma lipidomic signature of nonalcoholic steatohepatitis. *Hepatology* 2009; **50**: 1827-1838 [PMID: 19937697 DOI: 10.1002/hep.23229]

66   **Loomba R**, Quehenberger O, Armando A, Dennis EA. Polyunsaturated fatty acid metabolites as novel lipidomic biomarkers for noninvasive diagnosis of nonalcoholic steatohepatitis. *J Lipid Res* 2015; **56**: 185-192 [PMID: 25404585 DOI: 10.1194/jlr.P055640]

67   **Samuel BS**, Shaito A, Motoike T, Rey FE, Backhed F, Manchester JK, Hammer RE, Williams SC, Crowley J, Yanagisawa M, Gordon JI. Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41. *Proc Natl Acad Sci U S A* 2008; **105**: 16767-16772 [PMID: 18931303 DOI: 10.1073/pnas.0808567105]

68   **den Besten G**, Lange K, Havinga R, van Dijk TH, Gerding A, van Eunen K, Müller M, Groen AK, Hooiveld GJ, Bakker BM, Reijngoud DJ. Gut-derived short-chain fatty acids are vividly assimilated into host carbohydrates and lipids. *Am J Physiol Gastrointest Liver Physiol* 2013; **305**: G900-G910 [PMID: 24136789 DOI: 10.1152/ajpgi.00265.2013]

69   **Rau M**, Rehman A, Dittrich M, Groen AK, Hermanns HM, Seyfried F, Beyersdorf N, Dandekar T, Rosenstiel P, Geier A. Fecal SCFAs and SCFA-producing bacteria in gut microbiome of human NAFLD as a putative link to systemic T-cell activation and advanced disease. *United European Gastroenterol J* 2018; **6**: 1496-1507 [PMID: 30574320 DOI: 10.1177/2050640618804444]

70   **Zehra M**, Curry JC, Pillai SS, Lakhani HV, Edwards CE, Sodhi K. Elucidating Potential Profibrotic Mechanisms of Emerging Biomarkers for Early Prognosis of Hepatic Fibrosis. *Int J Mol Sci* 2020; **21** [PMID: 32635162 DOI: 10.3390/ijms21134737]

71   **Haston CK**, Zhou X, Gumbiner-Russo L, Irani R, Dejournett R, Gu X, Weil M, Amos CI, Travis EL. Universal and radiation-specific loci influence murine susceptibility to radiation-induced pulmonary fibrosis. *Cancer Res* 2002; **62**: 3782-3788 [PMID: 12097289]

72   **Liebe R**, Hall RA, Williams RW, Dooley S, Lammert F. Systems genetics of hepatocellular damage in vivo and in vitro: identification of a critical network on chromosome 11 in mouse. *Physiol Genomics* 2013; **45**: 931-939 [PMID: 23943854 DOI: 10.1152/physiolgenomics.00078.2013]

73   **Bruschi FV**, Tardelli M, Herac M, Claudel T, Trauner M. Metabolic regulation of hepatic PNPLA3 expression and severity of liver fibrosis in patients with NASH. *Liver Int* 2020; **40**: 1098-1110 [PMID: 32043752 DOI: 10.1111/liv.14402]

74   **Sharma M**, Mitnala S, Vishnubhotla RK, Mukherjee R, Reddy DN, Rao PN. The Riddle of Nonalcoholic Fatty Liver Disease: Progression From Nonalcoholic Fatty Liver to Nonalcoholic Steatohepatitis. *J Clin Exp Hepatol* 2015; **5**: 147-158 [PMID: 26155043 DOI:

10.1016/j.jceh.2015.02.002]

75   **Moran-Salvador E**, Mann J. Epigenetics and Liver Fibrosis. *Cell Mol Gastroenterol Hepatol* 2017; **4**: 125-134 [PMID: 28593184 DOI: 10.1016/j.jcmgh.2017.04.007]

76   **Komatsu Y**, Waku T, Iwasaki N, Ono W, Yamaguchi C, Yanagisawa J. Global analysis of DNA methylation in early-stage liver fibrosis. *BMC Med Genomics* 2012; **5**: 5 [PMID: 22281153 DOI: 10.1186/1755-8794-5-5]

77   **Mann J**, Oakley F, Akiboye F, Elsharkawy A, Thorne AW, Mann DA. Regulation of myofibroblast transdifferentiation by DNA methylation and MeCP2: implications for wound healing and fibrogenesis. *Cell Death Differ* 2007; **14**: 275-285 [PMID: 16763620 DOI: 10.1038/sj.cdd.4401979]

78   **You H**, Sun YM. [Cellular mechanism in the fibrogenesis of liver fibrosis]. *Zhonghua Gan Zang Bing Za Zhi* 2012; **20**: 563-564 [PMID: 23227528]

79   **Zhao Q**, Qin CY, Zhao ZH, Fan YC, Wang K. Epigenetic modifications in hepatic stellate cells contribute to liver fibrosis. *Tohoku J Exp Med* 2013; **229**: 35-43 [PMID: 23238615 DOI: 10.1620/tjem.229.35]

80   **Gonzalez A**, Huerta-Salgado C, Orozco-Aguilar J, Aguirre F, Tacchi F, Simon F, Cabello-Verrugio C. Role of Oxidative Stress in Hepatic and Extrahepatic Dysfunctions during Nonalcoholic Fatty Liver Disease (NAFLD). *Oxid Med Cell Longev* 2020; **2020**: 1617805 [PMID: 33149804 DOI: 10.1155/2020/1617805]

81   **Nati M**, Haddad D, Birkenfeld AL, Koch CA, Chavakis T, Chatzigeorgiou A. The role of immune cells in metabolism-related liver inflammation and development of non-alcoholic steatohepatitis (NASH). *Rev Endocr Metab Disord* 2016; **17**: 29-39 [PMID: 26847547 DOI: 10.1007/s11154-016-9339-2]

82   **Alkhouri N**, Morris-Stiff G, Campbell C, Lopez R, Tamimi TA, Yerian L, Zein NN, Feldstein AE. Neutrophil to lymphocyte ratio: a new marker for predicting steatohepatitis and fibrosis in patients with nonalcoholic fatty liver disease. *Liver Int* 2012; **32**: 297-302 [PMID: 22097893 DOI: 10.1111/j.1478-3231.2011.02639.x]

83   **Irvine KM**, Ratnasekera I, Powell EE, Hume DA. Causes and Consequences of Innate Immune Dysfunction in Cirrhosis. *Front Immunol* 2019; **10**: 293 [PMID: 30873165 DOI: 10.3389/fimmu.2019.00293]

84   **Sargenti K**, Johansson Å, Bertilsson S, Mattsby-Baltzer I, Klintman D, Kalaitzakis E. Dysfunction of Circulating Polymorphonuclear Leukocytes and Monocytes in Ambulatory Cirrhotics Predicts Patient Outcome. *Dig Dis Sci* 2016; **61**: 2294-2302 [PMID: 27010544 DOI: 10.1007/s10620-016-4132-3]

85   **Maricic I**, Marrero I, Eguchi A, Nakamura R, Johnson CD, Dasgupta S, Hernandez CD, Nguyen PS, Swafford AD, Knight R, Feldstein AE, Loomba R, Kumar V. Differential Activation of Hepatic Invariant NKT Cell Subsets Plays a Key Role in Progression of Nonalcoholic Steatohepatitis. *J Immunol* 2018; **201**: 3017-3035 [PMID: 30322964 DOI: 10.4049/jimmunol.1800614]

86   **Confer BD**, Lopez R, Zein NN. CD4+ T-Cell function and the risk for developing spontaneous bacterial peritonitis in patients with cirrhosis. *J Clin Gastroenterol* 2013; **47**: 807-813 [PMID: 23507764 DOI: 10.1097/MCG.0b013e3182854969]

87   **Breuer DA**, Pacheco MC, Washington MK, Montgomery SA, Hasty AH, Kennedy AJ. CD8+ T cells regulate liver injury in obesity-related nonalcoholic fatty liver disease. *Am J Physiol Gastrointest Liver Physiol* 2020; **318**: G211-G224 [PMID: 31709830 DOI: 10.1152/ajpgi.00040.2019]

88   **Huang CH**, Jeng WJ, Ho YP, Teng W, Chen WT, Chen YC, Lin SM, Chiu CT, Sheen IS, Lin CY. Increased regulatory T cells in patients with liver cirrhosis correlated with hyperbilirubinemia and predict bacterial complications. *J Gastroenterol Hepatol* 2015; **30**: 775-783 [PMID: 25250558 DOI: 10.1111/jgh.12781]

89   **Chackelevicius CM**, Gambaro SE, Tiribelli C, Rosso N. Th17 involvement in nonalcoholic fatty liver disease progression to non-alcoholic steatohepatitis. *World J Gastroenterol* 2016; **22**: 9096-9103 [PMID: 27895397 DOI: 10.3748/wjg.v22.i41.9096]

90   **Panda SK**, Colonna M. Innate Lymphoid Cells in Mucosal Immunity. *Front Immunol* 2019; **10**: 861 [PMID: 31134050 DOI: 10.3389/fimmu.2019.00861]

91   **Li S**, Hong M, Tan HY, Wang N, Feng Y. Insights into the Role and Interdependence of Oxidative Stress and Inflammation in Liver Diseases. *Oxid Med Cell Longev* 2016; **2016**: 4234061 [PMID: 28070230 DOI: 10.1155/2016/4234061]

92   **Shabangu CS**, Huang JF, Hsiao HH, Yu ML, Chuang WL, Wang SC. Liquid Biopsy for the Diagnosis of Viral Hepatitis, Fatty Liver Steatosis, and Alcoholic Liver Diseases. *Int J Mol Sci* 2020; **21** [PMID: 32466319 DOI: 10.3390/ijms21103732]

93   **Tranah TH**, Edwards LA, Schnabl B, Shawcross DL. Targeting the gut-liver-immune axis to treat cirrhosis. *Gut* 2021; **70**: 982-994 [PMID: 33060124 DOI: 10.1136/gutjnl-2020-320786]

94   **Such J**, Guardiola JV, de Juan J, Casellas JA, Pascual S, Aparicio JR, Solá-Vera J, Pérez-Mateo M. Ultrastructural characteristics of distal duodenum mucosa in patients with cirrhosis. *Eur J Gastroenterol Hepatol* 2002; **14**: 371-376 [PMID: 11943948 DOI: 10.1097/00042737-200204000-00006]

95   **Cho MS**, Kim SY, Suk KT, Kim BY. Modulation of gut microbiome in nonalcoholic fatty liver disease: pro-, pre-, syn-, and antibiotics. *J Microbiol* 2018; **56**: 855-867 [PMID: 30377993 DOI: 10.1007/s12275-018-8346-2]

96   **Orellana AM**, Kinoshita PF, Leite JA, Kawamoto EM, Scavone C. Cardiotonic Steroids as Modulators of Neuroinflammation. *Front Endocrinol (Lausanne)* 2016; **7**: 10 [PMID: 26909067

DOI: 10.3389/fendo.2016.00010]

97   **Xu Y**, Marck P, Huang M, Xie JX, Wang T, Shapiro JI, Cai L, Feng F, Xie Z. Biased Effect of Cardiotonic Steroids on Na/K-ATPase-Mediated Signal Transduction. *Mol Pharmacol* 2021; **99**: 217-225 [PMID: 33495275 DOI: 10.1124/molpharm.120.000101]

98   **Campia I**, Gazzano E, Pescarmona G, Ghigo D, Bosia A, Riganti C. Digoxin and ouabain increase the synthesis of cholesterol in human liver cells. *Cell Mol Life Sci* 2009; **66**: 1580-1594 [PMID: 19288057 DOI: 10.1007/s00018-009-9018-5]

99   **Luo J**, Yang H, Song BL. Mechanisms and regulation of cholesterol homeostasis. *Nat Rev Mol Cell Biol* 2020; **21**: 225-245 [PMID: 31848472 DOI: 10.1038/s41580-019-0190-7]

100 **Arguello G**, Balboa E, Arrese M, Zanlungo S. Recent insights on the role of cholesterol in non-alcoholic fatty liver disease. *Biochim Biophys Acta* 2015; **1852**: 1765-1778 [PMID: 26027904 DOI: 10.1016/j.bbadis.2015.05.015]

101 **Liu J**, Lilly MN, Shapiro JI. Targeting Na/K-ATPase Signaling: A New Approach to Control Oxidative Stress. *Curr Pharm Des* 2018; **24**: 359-364 [PMID: 29318961 DOI: 10.2174/1381612824666180110101052]

102 **Sharpton SR**, Ajmera V, Loomba R. Emerging Role of the Gut Microbiome in Nonalcoholic Fatty Liver Disease: From Composition to Function. *Clin Gastroenterol Hepatol* 2019; **17**: 296-306 [PMID: 30196156 DOI: 10.1016/j.cgh.2018.08.065]

103 **Aron-Wisnewsky J**, Vigliotti C, Witjes J, Le P, Holleboom AG, Verheij J, Nieuwdorp M, Clément K. Gut microbiota and human NAFLD: disentangling microbial signatures from metabolic disorders. *Nat Rev Gastroenterol Hepatol* 2020; **17**: 279-297 [PMID: 32152478 DOI: 10.1038/s41575-020-0269-9]

104 **Lynch SV**, Pedersen O. The Human Intestinal Microbiome in Health and Disease. *N Engl J Med* 2016; **375**: 2369-2379 [PMID: 27974040 DOI: 10.1056/NEJMra1600266]

105 **Henao-Mejia J**, Elinav E, Jin C, Hao L, Mehal WZ, Strowig T, Thaiss CA, Kau AL, Eisenbarth SC, Jurczak MJ, Camporez JP, Shulman GI, Gordon JI, Hoffman HM, Flavell RA. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. *Nature* 2012; **482**: 179-185 [PMID: 22297845 DOI: 10.1038/nature10809]

106 **Van Herck MA**, Vonghia L, Francque SM. Animal Models of Nonalcoholic Fatty Liver Disease-A Starter's Guide. *Nutrients* 2017; **9** [PMID: 28953222 DOI: 10.3390/nu9101072]

107 **Brandl K**, Schnabl B. Intestinal microbiota and nonalcoholic steatohepatitis. *Curr Opin Gastroenterol* 2017; **33**: 128-133 [PMID: 28257306 DOI: 10.1097/MOG.0000000000000349]

108 **Grabherr F**, Grander C, Effenberger M, Adolph TE, Tilg H. Gut Dysfunction and Non-alcoholic Fatty Liver Disease. *Front Endocrinol (Lausanne)* 2019; **10**: 611 [PMID: 31555219 DOI: 10.3389/fendo.2019.00611]

109 **Shen F**, Zheng RD, Sun XQ, Ding WJ, Wang XY, Fan JG. Gut microbiota dysbiosis in patients with non-alcoholic fatty liver disease. *Hepatobiliary Pancreat Dis Int* 2017; **16**: 375-381 [PMID: 28823367 DOI: 10.1016/S1499-3872(17)60019-5]

110 **Safari Z**, Gérard P. The links between the gut microbiome and non-alcoholic fatty liver disease (NAFLD). *Cell Mol Life Sci* 2019; **76**: 1541-1558 [PMID: 30683985 DOI: 10.1007/s00018-019-03011-w]

111 **Raman M**, Ahmed I, Gillevet PM, Probert CS, Ratcliffe NM, Smith S, Greenwood R, Sikaroodi M, Lam V, Crotty P, Bailey J, Myers RP, Rioux KP. Fecal microbiome and volatile organic compound metabolome in obese humans with nonalcoholic fatty liver disease. *Clin Gastroenterol Hepatol* 2013; **11**: 868-75.e1 [PMID: 23454028 DOI: 10.1016/j.cgh.2013.02.015]

112 **Wang B**, Jiang X, Cao M, Ge J, Bao Q, Tang L, Chen Y, Li L. Altered Fecal Microbiota Correlates with Liver Biochemistry in Nonobese Patients with Non-alcoholic Fatty Liver Disease. *Sci Rep* 2016; **6**: 32002 [PMID: 27550547 DOI: 10.1038/srep32002]

113 **de Faria Ghetti F**, Oliveira DG, de Oliveira JM, de Castro Ferreira LEVV, Cesar DE, Moreira APB. Influence of gut microbiota on the development and progression of nonalcoholic steatohepatitis. *Eur J Nutr* 2018; **57**: 861-876 [PMID: 28875318 DOI: 10.1007/s00394-017-1524-x]

114 **Tsai MC**, Liu YY, Lin CC, Wang CC, Wu YJ, Yong CC, Chen KD, Chuah SK, Yao CC, Huang PY, Chen CH, Hu TH, Chen CL. Gut Microbiota Dysbiosis in Patients with Biopsy-Proven Nonalcoholic Fatty Liver Disease: A Cross-Sectional Study in Taiwan. *Nutrients* 2020; **12** [PMID: 32204538 DOI: 10.3390/nu12030820]

115 **Bajaj JS**, Heuman DM, Hylemon PB, Sanyal AJ, White MB, Monteith P, Noble NA, Unser AB, Daita K, Fisher AR, Sikaroodi M, Gillevet PM. Altered profile of human gut microbiome is associated with cirrhosis and its complications. *J Hepatol* 2014; **60**: 940-947 [PMID: 24374295 DOI: 10.1016/j.jhep.2013.12.019]

116 **Lima M**, Andrade A, Oliveira G, Nicoli J, Martins F, Kroon E, Abrahão J. Virus and microbiota relationships in humans and other mammals: An evolutionary view. *Hum Micro J* 2019; **11**: 100050 [DOI: 10.1016/j.humic.2018.11.001]

117 **Carding SR**, Davis N, Hoyles L. Review article: the human intestinal virome in health and disease. *Aliment Pharmacol Ther* 2017; **46**: 800-815 [PMID: 28869283 DOI: 10.1111/apt.14280]

118 **Schmelcher M**, Loessner MJ. Bacteriophage endolysins: applications for food safety. *Curr Opin Biotechnol* 2016; **37**: 76-87 [PMID: 26707470 DOI: 10.1016/j.copbio.2015.10.005]

119 **Canchaya C**, Fournous G, Chibani-Chennoufi S, Dillmann ML, Brüssow H. Phage as agents of lateral gene transfer. *Curr Opin Microbiol* 2003; **6**: 417-424 [PMID: 12941415 DOI: 10.1016/s1369-5274(03)00086-9]

120   **Lang S**, Demir M, Martin A, Jiang L, Zhang X, Duan Y, Gao B, Wisplinghoff H, Kasper P, Roderburg C, Tacke F, Steffen HM, Goeser T, Abraldes JG, Tu XM, Loomba R, Stärkel P, Pride D, Fouts DE, Schnabl B. Intestinal Virome Signature Associated With Severity of Nonalcoholic Fatty Liver Disease. *Gastroenterology* 2020; **159**: 1839-1852 [PMID: 32652145 DOI: 10.1053/j.gastro.2020.07.005]

121   **Li XV**, Leonardi I, Iliev ID. Gut Mycobiota in Immunity and Inflammatory Disease. *Immunity* 2019; **50**: 1365-1379 [PMID: 31216461 DOI: 10.1016/j.immuni.2019.05.023]

122   **Jiang L**, Stärkel P, Fan JG, Fouts DE, Bacher P, Schnabl B. The gut mycobiome: a novel player in chronic liver diseases. *J Gastroenterol* 2021; **56**: 1-11 [PMID: 33151407 DOI: 10.1007/s00535-020-01740-5]

123   **Liew WP**, Mohd-Redzwan S. Mycotoxin: Its Impact on Gut Health and Microbiota. *Front Cell Infect Microbiol* 2018; **8**: 60 [PMID: 29535978 DOI: 10.3389/fcimb.2018.00060]

124   **BioRender**.   Adapted from "Nonalcoholic Fatty Liver Disease (NAFLD) Spec-trum. [cited 10 January 2021]. Available from: https://app.biorender.com/biorender-templates

Fatty acid metabolism and acyl-CoA synthetases in the *liver-gut axis*

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Abstract

Fatty acids are energy substrates and cell components which participate in regulating signal transduction, transcription factor activity and secretion of bioactive lipid mediators. The acyl-CoA synthetases (ACSs) family containing 26 family members exhibits tissue-specific distribution, distinct fatty acid substrate preferences and diverse biological functions. Increasing evidence indicates that dysregulation of fatty acid metabolism in the *liver-gut axis*, designated as the bidirectional relationship between the gut, microbiome and liver, is closely associated with a range of human diseases including metabolic disorders, inflammatory disease and carcinoma in the gastrointestinal tract and liver. In this review, we depict the role of ACSs in fatty acid metabolism, possible molecular mechanisms through which they exert functions, and their involvement in hepatocellular and colorectal carcinoma, with particular attention paid to long-chain fatty acids and small-chain fatty acids. Additionally, the *liver-gut* communication and the liver and gut intersection with the microbiome as well as diseases related to microbiota imbalance in the *liver-gut axis* are addressed. Moreover, the development of potentially therapeutic small molecules, proteins and compounds targeting ACSs in cancer treatment is summarized.

Key Words: Long-chain fatty acids; Short-chain fatty acids; Acyl-CoA synthetases; Microbiota; *Liver-gut axis*

Core Tip: To understand the role of acyl-CoA synthetases (ACSs) in the fatty acid metabolism, it is necessary to explore the biological function, gene interactions/regulations and signal pathways in physiological and pathological conditions. Growing evidence demonstrates that the control of microbial balance plays an important role in maintaining homeostasis and normal functions of the *liver-gut* axis, and the bidirectional communication in turn affects microbial communities. As novel therapeutic targets, miRNAs are receiving more and more attention, together with other compounds targeting ACSs.

INTRODUCTION

Lipids, one of three main nutrients, are mainly composed of fatty acids (FAs), triglycerides (TGs), phospholipid and cholesterol. Lipid metabolites are involved in various biological functions and physiological processes, ranging from energy storage and degradation and structural composition to molecule signaling as well as signal transduction cascade[1].

The *liver-gut axis* plays a critical role in the homeostasis of lipid metabolism in the human body during the feed-fast cycle. Free FAs are absorbed by enterocyte and intestine-derived products released into portal blood which is directed to the liver; in turn, the liver responds by secreting bile acids (BAs) to the intestine *via* the biliary tract. BAs are transported back to the liver *via* enterohepatic circulation. Since the Volta group identified the important role of microorganisms in the *liver-gut axis* for the first time[2], a number of studies have confirmed that gut microbiota, described as an invisible metabolic 'organ', has a tight and coordinated connection with the gut and liver[3,4]. The intestinal mucosal barrier either acts as a physical barrier or lives in symbiosis with microbiota. Once the balance of symbiosis is disrupted, microbiota responds to this imbalance, microbiota metabolites (short-chain fatty acids, SCFAs) are modified and circulated into the liver. Aberrant lipid metabolism in the *liver-gut axis* has been linked with intestinal bowel diseases and diverse liver diseases[5].

Around 95% of dietary lipids absorbed are TGs, mainly composed of long-chain fatty acids (LCFAs)[6]. Fatty acid metabolism takes place mainly in intestinal enterocytes and hepatocytes, further assisted by adipocytes and other cell types. To become further involved in both anabolic and catabolic pathways, FAs must be taken up and activated by thioesterification. This ATP-mediated coupling reaction of FAs with coenzyme A is catalyzed by the enzymes called acyl-CoA synthetases (ACSs). ACSs are classified into five groups according to the fatty acid chain length: short-chain, medium-chain, bubblegum-chain, long-chain and very-long-chain acyl CoA synthetases (ACSVLs)[7]. ACSVLs as membrane channel proteins have been identified as a major enzyme responsible for LCFA uptake and activation[8]. Long-chain acyl-CoA synthetases (ACSLs) are responsible for the catalyzation of intracellular free LCFAs which are transported by other transport proteins, such as fatty acid translocase (CD36) and fatty acid binding proteins (FABPs)[9]. Short-chain acyl-CoA synthetases (ACSSs) are involved in the activation of microbiota-derived SCFAs, such as acetate and propionate[10] (Table 1).

In this review, we will summarize the functional role of ACSs in fatty acid metabolism, focusing on LCFAs and SCFAs, as well as potential therapeutic targets of ACSs. Furthermore, we will explore the influence of dietary diversity on microbiota and the microbial metabolites, and their bidirectional communication in the *liver-gut* axis.

Table 1 miRNA and compounds targeting acyl-CoA synthetases

Type	Name	Target	Mechanism	Ref.
miRNA	miR-205	ACSL4/ACSL1	Inhibition of ACSL4/ACSL1 in hepatocellular carcinoma	[155,171]
	miR-211-5p	ACSL4	Inhibition of ACSL4 in hepatocellular carcinoma	[172]
	miR-19b-1	ACSL1/ACSL4/SCD1	Inhibition of ACSL1/ACSL4/SCD1 axis in colorectal cancer	[173]
	miR-142-3p	ACSL1/ACSL4/SCD1	Inhibition of ACSL1/ACSL4/SCD1 axis in colorectal cancer	[173,174]
	miR-34c	ACSL1	Inhibition of ACSL1 and induction of liver fibrogenesis	[175]
	miR-497-5p	ACSL5	Inhibition of ACSL5 in colon cancer	[170]
Compounds	Triacsin C	ACSL1/ACSL3/ACSL4 and ACSL5[1]	Inhibition of ACSL1/ACSL3/ACSL4 and ACSL5[1]	[177,178]
	Roglitazone Pioglitazone Troglitazone	ACSL4	Inhibition of ACSL4	[179-181]
	Lipofermata	FATP2	Inhibition of FATP2	[191,192]
	Grassofermata	FATP2	Inhibition of FATP2	[191,193, 194]
	Ursodiol chenodiol	FATP5	Inhibition of FATP5 in liver	[195]
	Fenofibrate	PPARα	Indirect activation of FATP in liver predominantly	[196,197]

[1]Triacsin C is also competitive inhibitor of ACSL5 when used in higher concentration.

FATTY ACID METABOLISM MEDIATED BY ACYL-COA SYNTHETASES IN THE *LIVER-GUT AXIS*

Circulation of fatty acids and bile acids in the liver-gut axis

Intestinal absorption of FAs is a multistep process that includes digestion, uptake and absorption and needs to cooperate with large numbers of enzymes secreted by series of organs in the gastrointestinal tract[11]. TGs are first released from a fatty diet after digestion with lingual and gastric lipase in the stomach, and released TGs are further hydrolyzed by pancreatic lipase to produce 2-monoacylglycerides and free FAs[12]. Sequentially those digested FAs mix with BAs and emulsify to form spherical water-soluble droplets, called micelles (MCs). With intestinal peristalsis, MCs are transported to the small intestinal lumen and further translocated into the apical membrane of enterocytes.

In intestinal enterocytes, absorbed LCFAs experience a series of catabolic metabolisms for energy supply for massive biological activities, and anabolic metabolism to reconstitute lipids. Newly synthesized lipids are incorporated into transport vehicles, chylomicrons (CMs), that are later liberated from enterocytes, and then transported to the liver through the hepatic portal vein. The liver is the major processing factory of FAs and regulates and balances lipid homeostasis systemically in the *liver-gut axis*. Fatty acid uptake and metabolism occur in hepatocytes. During feeding, hepatocytes take up the influx of FAs and get rid of FAs *via* β-oxidation to produce energy, and reformed TGs integrated into CMs partition into two pathways: (1) Secreted into bloodstream; and (2) transported and stored in adipose tissue. During fasting or starvation, hepatocytes recycle TGs from lipid droplets and adipose tissue, and initiate *de novo* lipogenesis by using other energy sources in the liver, such as carbohydrates [13,14]. Therefore, the pool of FAs is always in dynamic equilibrium between dietary absorption in the enterocytes, process and lipogenesis in the liver and liver feedback regulation *via* BAs during the feed-fast cycle.

As previously mentioned, BAs are involved not only in facilitating MC formation, but also as signaling molecules and metabolic regulators of lipid/glucose metabolism, energy homeostasis and inflammation in the *liver-gut axis*[15]. It has been demonstrated that a higher level of BAs can be detected in the tissues of the *liver-gut axis* compared to peripheral blood[16]. Primary BAs are synthesized in the hepatocytes and secreted into the small intestine; most of them are reabsorbed in the ileum. A small

number of unabsorbed BAs are taken up by microbiota and metabolized into secondary BAs[17]. In enterocytes BAs are reabsorbed through the apical sodium-dependent BA transporter (ASBT), carried by the intestinal bile acid-binding protein (FABP6) and released into portal blood *via* heterodimeric transporter OSTα/OSTβ. BA activation of the nuclear farnesoid X receptor (FXR) also upregulates FABP6, OSTα /OSTβ and fibroblast growth factor 19 (FGF19), which further inhibits BAs synthesis. In hepatocytes, the transport of BAs is mediated by sodium-taurocholate cotrans-porting polypeptide (NTCP) and organic anion transporters (OATPs). BAs acting as an activator of hepatic FXR regulate the expression of genes involved in bile acid transport and synthesis. This enterohepatic circulation of BAs plays a critical role in maintaining the BAs pool in the *liver-gut axis*[18,19].

Long-chain fatty acid transport to enterocytes and hepatocytes

Free fatty acid uptake is requested across the phospholipid bilayer in the mammalian membrane. It is widely known that LCFAs can be taken up into cells *via* flip-flop diffusion with rate limiting[20,21]. High permeability of LCFA transport is mediated by several membrane-associated transport proteins including FA transport proteins (FATPs), FABPs, CD36 and caveolin (CAV)[9].

FATP1-6 (fatp in mice, also called ACSVL1-6) is a group of enzymatic proteins with double capabilities of transport and activation. FATP can trap and activate a broad range of LCFA and VLCFA to form acyl-CoA[9,22]. Different FATP family members have tissue-specific expression patterns[23]. In the intestine, FATP4 (ACSVL5) is strongly expressed in intestinal villi but not in crypts, which plays an important role in fatty acid absorption[24]. Fapt4-null mice display an embryonic lethality with a defective epidermal barrier. Fapt4 depletion alters the ceramide fatty acid composition significantly, especially in saturated VLCFA substitutes C26:0 and C26:0-OH[25]. FAPT5 (ACSVL6) mainly transports BAs but also LCFAs, is only expressed in the liver and particularly in the basal membrane of hepatocytes[8,26]. Fapt5 knockout mice showed this defective bile acid conjugation, indicating that Fapt5 is essential for fatty acid uptake by hepatocytes and maintenance of the lipid balance which further regulates body weight[27]. With the discovery of the topological structure of murine FAPT1 containing one transmembrane domain and a large cytoplasm domain[28], different mechanisms of FATP1 transporting exogenous FAs into cells have been proposed, one of which is vectorial transport or flipase function[29]. Moreover, BAs acting as a FATP5 antagonist dramatically decrease hepatic fatty acid uptake as well as liver triglyceride synthesis[30].

FABP 1-9 (fabp in mice) are a fatty acid binding protein superfamily that binds to FAs, cholesterol or other non-esterified FAs, facilitate fatty acid uptake and lipid metabolism[31]. FABP appears in two distinct forms depending on localization: one is peripheral membrane protein (FABPpm) and the other is intracellular/cytoplasmic protein (FABPc)[32]. Like FATP, different family members of FABPs exhibit organ-specific expression. FABP2 (Intestinal-FABP, I-FABP) encodes the intestinal form which is only expressed in the small intestine, and FABP-1(Liver-FABP, L-FABP) is only expressed in the liver[33]. I-FABP and L-FABP are all cytoplasmic proteins, but it is reported that they deliver FAs through different mechanisms of L-FABP in diffusion and I-FABP in collision[34]. L-fabp-null mice showed a reduced uptake of LCFAs as well as new biosynthesis for lipid storage or secretion, suggesting the important role of L-fabp in fatty acid esterification at endoplasmic reticulum (ER)[35]. Furthermore, L-FABP depletion suppresses lipid catabolism in mitochondria and downregulates the transcription of oxidative enzymes through inhibition of peroxisome proliferator-activated receptor (PPARα) transcriptor in the nucleus[36,37].

CD36, officially designated as scavenger receptor B2 (SR-B2), is a transmembrane glycoprotein which has a broad range of binding profiles including LCFAs, plasma lipoproteins, phospholipids, collagen[38]. CD36 whole body knockout mice showed significantly decreased fatty acid uptake in the heart and skeletal muscle[39]. In the intestine, CD36 is only detected in the duodenal and jejunal parts and plays a critical role for fatty acid and cholesterol uptake in the small intestine[40]. Although CD36 has a very low expression level in the liver, CD36 liver-specific knockout in the steatosis model indicated that CD36 deletion reduces lipid content and inflammation and improves insulin sensitivity[41].

CAV 1-3 (cav in mice) are intramembrane proteins which are responsible for caveolae formation. CAV1 as a cholesterol-binding protein is implicated in cholesterol trafficking and absorption[42]. However, Cav1 knockout mice did not show a compensatory mechanism to increase other family members, such as Cav2 and Cav3, and cholesterol absorption and sterol excretion were also not changed in the intestine [43]. Additionally, CAV1 also acts as a cytosolic intermediate form involved in

lipogenesis and lipid body formation during liver regeneration[44].

It is widely recognized that several fatty acid transport proteins cooperate synergistically to accomplish the process of fatty acid transport (Figure 1). Due to the tissue-specific expression pattern, FATP4, FABPpm, FABP-I, CD36 are main types in the intestine and FATP5, FABPpm, FABP-L, CD36 are major types in the liver. Partial LCFAs are activated during transport *via* FATP. The rest of the LCFAs are grabbed by FABPpm and presented to CD36. Free cytosolic LCFAs is not only activated by ACSLs for esterification of acyl-CoA but also trapped by FABPc for subcellular function. Generated acyl-CoA as a raw material initiates the subsequent metabolism pathway to produce energy or synthesize diverse complex lipids. In addition, acyl-CoA can be deactivated to free FAs and CoA, and this process is mediated by acyl-CoA thioesterases (ACOTs). ACSLs and ACOTs are two critical enzymes helping to control the dynamic balance between acyl-CoA and free FAs.

Long-chain fatty acid activation in enterocytes and hepatocytes

As mentioned previously, most of the abundant dietary FAs are LCFAs so ACSLs are addressed in more details here. In humans and rodents there are five existing ACSL isoforms namely ACSL1, ACSL3, ACSL4, ACSL5 and ACSL6 (acsl in mice), each one coded by the different gene containing several splice variants[45]. Due to the differences in the 5′UTRs, the first coding exon, alternative coding exons and exchangeable motifs, different variants of each ACSL isoform are available[46]. The ACSL isoforms have two motifs: ATP binding and fatty acid binding[47]. The fatty acid binding tunnel located at the N-terminal domain has been linked to the substrate specificity of each ACSL isoform[48]. Since the N-terminal domain varies between the different ACSL isoforms, it contributes to the substrate preference of each family member and its different subcellular localization which is essential for vectorial acylation[49].

ACSL1 is predominantly located in the liver. Knockout of ACSL1 in the liver demonstrated a reduction in total ACSL activity of up to 50%, together with a decrease in the hepatic amount of acyl-CoA and a decreased level of oleic acid-derived TG[1, 50]. Acsl1 deficient mice showed a 50% reduction in the amount of long-chain acyl-carnitines, leading to the conclusion that the loss of Acsl1 impaired partitioning of its products into TG synthesis and oxidation pathways[1]. Due to its both endoplasmic and mitochondrial localization, ACSL1 directs its metabolites to both the anabolic (TG synthesis) and catabolic (β-oxidation) pathway[1].

ACSL3 Localization is linked to the lipid droplets and ER in the liver and other tissue. The increase in fatty acid uptake causes a transition of ACSL3 from ER to the lipid droplets, suggesting its role in neutral lipid synthesis[1]. Knockdown of ACSL3 reduced the activity of transcription factors including PPARγ, ChREBP, SREBP1C and Liver X receptor and their target genes involved in hepatic lipogenesis[1]. ACSL3 activates FAs incorporated into phospholipids, which are used for very-low density lipoprotein (VLDL) production[50]. As revealed by Yan *et al*, ACSL3 knockdown decreased the level of VLDL in hepatic cells[50]. Besides its role in the activation of FAs, overexpression of ACSL3 was found to be able to induce cellular fatty acid uptake[51]**.**

ACSL4 is mostly expressed in adrenal glands and steroid-producing organs[52,53]**.** The role of ACSL4 is related to the activation of polyunsaturated FAs in steroidogenic tissue. ACSL4 has a preference for the arachidonic acid which is involved in the eicosanoid synthesis.

The nuclear-coded ACSL5 is prominent in both the mitochondria and ER of the intestinal mucosa and liver[50]. Highest expression was detected in the jejunum and ACSL5 was assumed to be involved in dietary fatty acid absorption. However, studies in acsl5 null mice showed no alteration in dietary fatty acid absorption but a significant decrease in total ACSL activity[1]. In the liver, ACSL5 activates LCFAs mostly of C18 carbon atoms, which are further incorporated into TGs, phospholipids and cholesterol esters. According to previous reports, ACSL5 plays a role in the metabolism of dietary FAs, but not in *de novo* synthetized ones[50,54,55]**.** Since ACSL5 is localized on the mitochondrial outer membrane, the activity was initially attributed to β-oxidation. Some studies with ectopic expression of ACSL5 failed to prove this, but the increased synthesis of TGs and diglycerides was observed in the liver[54]. ACSL5 is a dominant activator of dietary LCFAs and displayed an 80% lower activity in total acsl of the jejunum in acsl5 knockout mice[56]. ACSL5 is strongly expressed by enterocytes in an ascending gradient along the *crypt-villus* axis with the highest expression level at the villus tip; however, nuclear β-catenin, a hallmark of Wnt activation, is expressed in a descending gradient along the *crypt-villus* axis[57], suggesting an interplay between ACSL5 and Wnt activity during enterocyte differen-
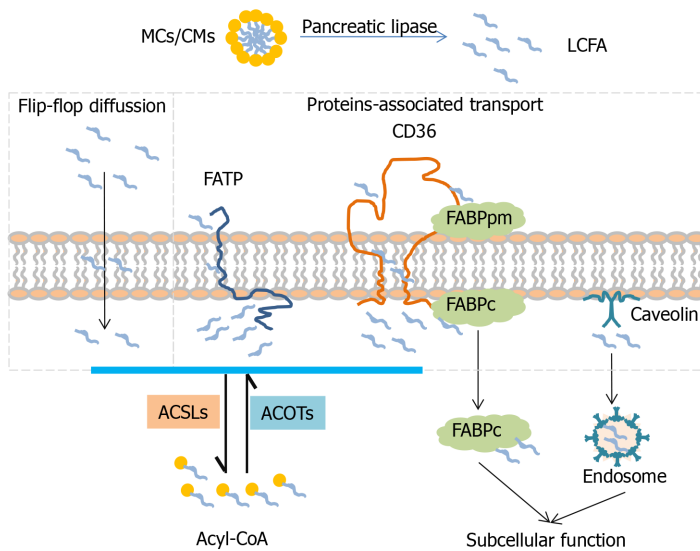
Figure 1 Mechanism of long-chain fatty acid transport across the lipid raft. LCFAs are taken up into cell in two different ways. One is passive transport by a flip-flop with rate limiting. The other is active transport, which is mediated with transport-associated proteins (FATPs, CD36, FABPs and Caveolin). FATPs with tissue-specific distribution integrating both transport and activation functions are responsible for LCFAs uptake. Free FAs trapped by the FABPpm present to CD36 and are transported into cells. Consequently released free FAs bind with FABPc and CAV channel into different organelles and are activated by different subcellular expression of ACSLs into acyl-CoA. In addition, acyl-CoA can be deactivated to free FA and CoA which is mediated by ACOTs. Liver-specific proteins: FATP5, FABP-L, ACSL1; Intestine-specific proteins: FATP4, FABP-I, ACSL5; ACSL: Acyl-CoA synthetase, ACOT: Acyl-CoA thioesterase; MCs: Micelles, CMs: Chylomicrons

tiation and maturation[58].

ACSL6 is highly expressed in the brain where it plays a role in phospholipid synthesis during neurite outgrowth. ACSL expression is controlled by the level of intracellular FAs in physiological conditions[1].

Short-chain fatty acid transport and activation in enterocytes and hepatocytes

Microbiota-derived SCFAs cross the lipid membrane *via* different mechanisms: non-ionized diffusion, Na^+/H^+-dependent gradient exchange[59,60]. Intracellular SCFAs can shuttle between cytosol, nucleus and mitochondria *via* a diffusion mechanism[10,60]. SCFA activation by ACSSs is the first step in utilizing the energy source. ACSS 1-3 (acss in mice) are encoded and designated in humans. ACSS1 and ACSS3 are localized at the mitochondria matrix, while ACSS2 is a nuclear-cytosolic enzyme. ACSS1 and ACSS2 activate acetate to thioester into acetyl-CoA, but ACSS3 favors propionate[10].

In humans, mitochondrial ACSS1 is most highly expressed in the brain, blood, testis and intestine, also to a certain level in the heart, muscle and kidney, but not in the liver or spleen[61]. In mice, ACSS1 is strongly expressed in the heart, kidney, skeletal muscle and brown adipose tissue, which all need high energy expenditure[62]. Acss1 knockout mice showed a remarkably decreased acetate oxidation in the whole body during fasting compared with the wild type, however, no histological changes were detected in multiple tissues including the intestine and liver[63]. ACSS3 displays the character of propionyl-CoA synthetase as well as the highest expression in the liver. Knockdown of ACSS3 in hepG2 significantly decreases the activity of propionyl-CoA synthetase. During fasting, ACSS3 is upregulated, which is probably linked to ketogenesis, and ACSS2 is downregulated[64].

ACSS2 is most highly expressed in the liver and kidney[64,65]. Moffet *et al*[10] introduced the concept that the expression of ACSS2 in different cell types is based on the different physiological conditions to utilize acetate. Therefore, the liver is supposed to be the main organ for processing acetate. With the feature of localization, ACSS2 catalyzes acetate into acetyl-CoA which is correlated with fatty acid biosynthesis in cytosol, and retains acetate released from histone in the nucleus[66]. Acss2-deficient mice with high-fat feeding can lighten fat deposition in the liver by regulating many genes involved in lipid metabolism, suggesting that Acss2 acts as a transcription regulator during lipogenesis[67].

The expression and localization pattern of ACSS 1-3 suggests that ACSS1 and ACSS3 are responsible for energy production by using acetate in the intestine and liver respectively. The majority of acetate is taken up by the liver, ACSS2 in cytoplasm is involved in lipogenesis and is distributed to other organs in ketone bodies through systemic circulation. Acetyl-CoA as a central metabolite can go into either energy

production or lipid biosynthesis. ACSS1-3 plays a key role in regulating the level of acetyl-CoA in the nucleus, mitochondria and cytoplasm (Figure 2).

MICROBIOTA UTILIZATION OF DIET, MICROBIOTA METABOLITES AND THE ROLE OF MICROBIOTA IN THE *LIVER-GUT AXIS*

Dietary structure shapes the composition of microbiota

Gut microbiota, a diverse microbial community with approximately 100 trillion microorganisms, is colonized in the gastrointestinal tract. In human adults, five families microbiota are mainly Firmicutes, Bacteroidetes, Actinobacteria, Proteo-bacteria and Verrucomicrobia, while phylum Firmicutes and Bacteroidetes make up approximately 80% of all species[68].

A high-fiber intake population has higher diversity microbiota and more SCFAs production than a high-calorie diet population, and two populations showed distinct diet favor microbiota[69]. *Bacteroides* and *Prevotella* are two dominant groups which are highly enriched in a high-protein/fat diet population and high-fiber population respectively[70,71]. Moreover, the composition of fecal microbiota varies by age, geography and lifestyle due to the behavior of microbiota dietary preferences[72]. The term microbiota-accessible carbohydrates (MACs) introduced by Sonnenburg *et al* refers to microbiota favorable-carbohydrates that cannot be digested by the host. Mice feeding on a long-term low-MACs diet display a remarkably reduced diversity of microflora containing mostly *Bacteroildales* and *Clostridiales*. Although the microbiota composition cannot be restored after refeeding with a high-MAC diet, it increases again mainly in *Bacteroidales* upon reintroduction of fecal microbiota[73].

SCFAs are metabolic end-products from specialized bacteria utilizing with undigested dietary polysaccharides in human small intestine. The most abundant SCFAs in the intestine are acetate (C2), propionate (C3) and butyrate (C4). The phylum Bacteroidetes, the most abundant gram-negative bacteria with a high flexibility to adapt the environment, are associated with acetate production[74]. Phylum Bacteroidetes and Negativicutes (*Akkermansia muciniphila*, family Veillonellacear and phylum Firmicutes) are dominantly responsible for production of propionate by the succinate pathway, small bacterial genera from phylum Firmicutes have been identified to form propionate through the acrylate pathway, and distant Lachnospiraceae are known to produce propionate by utilizing the propanediol pathway[75]. Several species from families Lachnospiraceae, Ruminococcaceae and Erysipelotrichacear (Phylum Firmicutes) produce butyrate *via* butyrate kinase route and butyryl-CoA:acetate CoA-transferase route[76]. Diverse composition of microbiota has distinct SCFAs profiles, and additionally, SCFAs-metabolic network is a cross-feeding microbial system between different bacterial species[77].

In all, a high intake of MACs is pivotal in shaping the diversity and composition of microbiota. Diverse microbiota-generated SCFAs reversely influence the microbial communities and further act as a mediator is strongly involved in host-microbiota cross-talk.

Utilization of long-chain fatty acid in microbiota

Microbiota can also employ luminal unabsorbed LCFAs directly as energy source once there is a fermentable fiber deficiency[78]. LCFAs cross the cellular envelope in bacteria and yeast, unlike in mammalian cells. In bacteria, FadL transports exogenous LCFAs from outer membrane to periplasm, FadD (role as ACSLs) extracts LCFAs into the cytoplasmic membrane and activates to form acyl-CoA. In yeast, Fat1p and Faa1p/Faa4p are required for LCFAs transport and activation respectively[29]. Moreover, LCFAs can also permeate the bilayers *via* the TolC channel in *E. coli*[79,80].

Subsequently activated acyl-CoA is degraded to acetyl-CoA *via* β-oxidation. Acetyl-CoA is located at the crossroads of central metabolism[81]. During bacterial overgrowth, acetyl-CoA is not only necessary only for energy generation *via* entering citric acid cycle and respiratory chain, but also synthesizes new cell material *via* the glyoxylate cycle. Moreover, the conversion from acetyl-CoA to acetate and ethanol takes place through anaerobic fermentation due to oxidant deficiency[82].

In addition to being a nutrient, LCFAs serve as an environmental factor which guides a series of gram-negative bacteria to colonize and invade intestinal lumen by repressing the expression of the strain-specific pathogenicity island. A pathogenicity island has been reported as a transcriptional activator which is mandatory for tissue invasion, such as *Salmonella* PI1/hilA[80], the *Vibril cholera* AraC/Xyls family ToxT
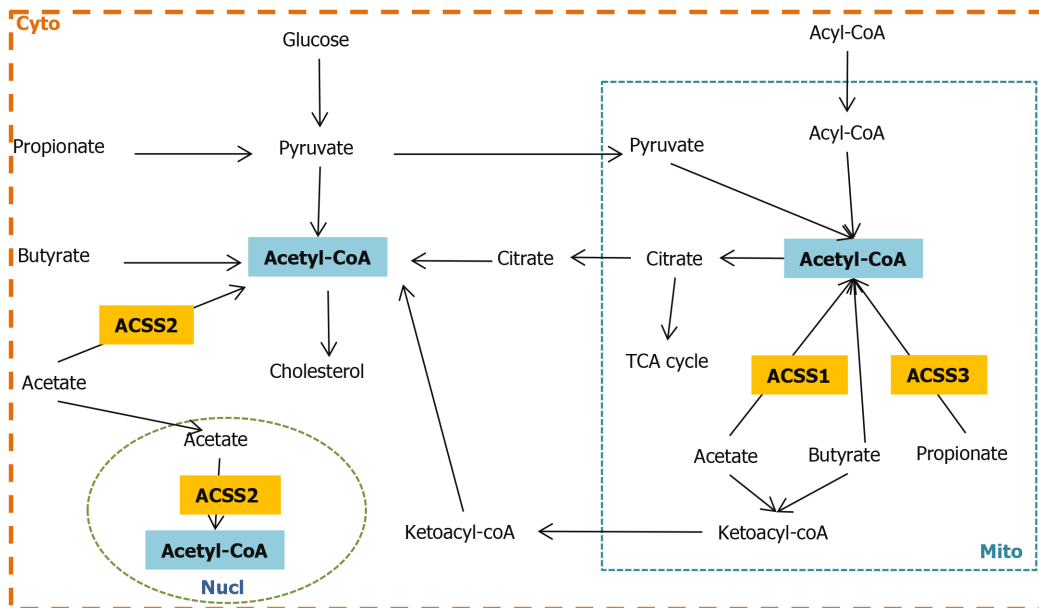
Figure 2 The crosslink between acyl-CoA synthetases and short-chain fatty acids. In mitochondria, acetyl-CoA is generated either from fatty acid β-oxidation and glucose *via* pyruvate or SCFAs through ACSS1 and ACSS3; acetyl-CoA is directed into energy production through the TCA cycle and electron respiration chain, as well as reflux into cytosol *via* citrate and again synthesizes acetyl-CoA. In addition, excessive acetate and butyrate synthesize into ketone bodies and are released into cytosol. In cytosol, acetyl-CoA is produced from pyruvate which is from both glucose and propionate; the source of acetyl-CoA can be converted from butyrate and acetate *via* butyryl-CoA/acetate CoA-transferase and ACSS2 respectively; cytosolic ketone bodies can also either produce acetyl-CoA or enter the blood circulation in the whole body. On the other hand, acetyl-CoA is involved in cholesterol biosynthesis. In the nucleus, acetate synthesizes acetyl-CoA *via* ACSS2 which is responsible for chromosome stability through histone acylation regulation. Cyto: Cytoplasma; Mito: mitochondria; Nucl: Nucleus; TCA: tricarboxylic acid cycle.

[83], *Yersinia enterocolitica* VirF and enterotoxigenic *E. coli* Rns[84].

Microbiota-derived short-chain fatty acids

Microbiota-derived SCFAs make up almost all SCFAs due to the lower level of SCFAs in human blood[85]. SCFAs as the basic substance sources play an important role in regulating lipid metabolism as well as maintaining the host energy homeostasis. In part, SCFAs can be absorbed directly as an energy source by enterocytes or transported to the liver *via* the portal vein; in part, SCFAs are reassigned by the liver and released into bloodstream for the systemic circulation through the whole body[10, 86]. SCFAs are mainly composed of acetate, butyrate and propionate which comprise 60%, 20% and 20% respectively[87]. SCFAs are transported and taken up into cells *via* non-ionized and ionized diffusion. The *liver-gut axis* plays a key role in the absorption, metabolism and systemic circulation of SCFAs[88].

Acetate, which is produced from pyruvate *via* acetyl-CoA and the wood-Ljungdahl pathway in microbiota, is the most abundant SCFA. Acetate is activated by ACSS1-3 to form acetyl-CoA and metabolized for energy production. However, the majority of acetate reaches and is processed in the liver. In cytosol, acetyl-CoA can synthesize cholesterol[89]; in the nucleus, acetate and acetyl-CoA are involved in regulating DNA histone acetylation and deacetylation[90]; in mitochondria, acetyl-CoA can be either for energy supply or ketogenesis in case of glucose deficiency, ketone bodies enter blood circulation for peripheral tissues usages[91]. Moreover, acetate can cross the blood-brain barrier freely and is an energy source for glial cells[92]. Acetate has a direct role in appetite regulation. Acetate is metabolized to generate more adenosine triphosphate, and inhibits adenosine monophosphate-active protein kinase (AMPK), as well as upregulating anorectic neuropeptide POMC and downregulating orexigenic neuropeptide AgRP[93].

Of the SCFAs which are mainly composed of acetate, butyrate and propionate, butyrate is the most widely studied. Butyrate is generated through the butyrate kinase or butyryl-CoA/acetate CoA-transferase route. Butyrate is a major SCFA in the large intestine. In enterocytes, the majority of butyrate is converted into acetyl-CoA that further participates in catabolism for host energy supply[94]; a small amount of butyrate is delivered to the liver and incorporated into ketone bodies (β-hydroxybutyrate) in mitochondrial for ATP production[95]. Butyrate plays a key role in maturing the intestinal barrier function in premature infants[96]. *In vivo* studies

showed that butyrate administration has favorable therapeutic effects on normal colonic health in a safe dose[86]. In a mouse model with globin chain synthesis disorder, the application of a high dose of butyrate resulted in striking neuropathological changes and multiorgan system failure due to harmful systemic concentrations [97]. Therefore, mechanisms underlying the dosage-dependent effects on the intestinal barrier are controversial, but reasonable. A low dose promotes restitution of intestinal epithelial lumen and a high dose impairs the intestinal barrier function with regulation of permeability by inducing apoptosis[98]. The selective paracellular permeability is determined by junction proteins including tight junction, adherence junction and desmosomes[99]. Excessive SCFA accumulation downregulates the expression of junction protein and further impairs the integrity of the membrane, leading to a leaky gut[100]. Moreover, increased intestinal permeability has been linked to inflammatory bowel disease[101].

Propionate is produced *via* the succinate, acrylate and propanediol pathway in microbiota. Propionate is activated by ACSS3 in mitochondria of hepatocytes. The concentration of dietary propionate regulates the balance between lipid and glucose metabolism[102]. Propionate reduces cancer cell proliferation through activation of G-protein-coupled receptors 43 GPR43) in mice liver[103].

In view of the biosynthesis of SCFAs, acetate, butyrate and propionate have crosslinks through acetyl-CoA, pyruvate, oxaloacetate, some of which can be converted between them to meet the physiological need of microbiota[104]. SCFAs as key microbiota metabolites are closely correlated with host health and disease conditions through regulation of diverse physiological processes. Two major signaling pathways related to SCFAs including G-protein-coupled receptors (GPCRs) and histone deacetylases have been characterized[105]. GPCRs, also named free fatty acid receptors (FFAR) are activated by SCFAs. Two SCFA receptors, GPR41 (FFAR3) and GPR43 (FFAR2) have been reported. FFAR2 has preference to acetate and propionate, and FFAR3 has a specificity in butyrate[106]. FFAR2 is expressed along the entire gastrointestinal tract. FFAR2 can be upregulated by propionate during adipocyte differentiation[107]. In addition, FFAR2 activated by SCFAs releases glucagon-like peptide 1(GLP-1) and peptide YY (PYY) in enteroendocrine L cells, GLP-1 and PYY, are involved in gut motility, glucose tolerance and regulation of appetite[108]. Moreover, Butyrate plays a role in anti-inflammation through inhibition of pro-inflammatory mediators/adipokines, adhesion molecules, metalloproteinase production as well as inflammatory signaling pathways (NFκB, MAPKinase, AMPK-α, and PI3K/Akt). However, the anti-inflammatory activity of butyrate was eliminated by FFRA3 knockdown[109]. Supplementation of SCFAs significantly improved hepatic metabolic actiity in FFAR3-dificient mice, but not FFAR-2 deficient mice[110].

SCFAs are also considered a promising supplementary treatment for active intestinal bowel disease[111]. Moreover, SCFAs, as inhibitors of histone deacetylases, show potential anti-inflammatory activity[112,113]. It is demonstrated that three SCFAs alone or in combination protect the intestinal barrier *via* stimulation of tight junction formation and repression of NLRP3 inflammasome and autophagy in the colon cancer cell model[114]. Apart from this, a high-fiber intake, fecal microbiota transplant, prebiotics and probiotics are suggested to have a beneficial effect on colonic health by increasing the level of SCFAs.

Microbiota-imbalance-related diseases in the liver-gut axis

Gut microbiota exert multifunction in maintaining the host homeostasis, including defending against pathogens, affecting immune system, mediating digestion and metabolism, involving in insulin regulation and maintaining the intestinal epithelial cell renewal[115]. Gut microbiota interact with host through producing a serial of metabolites, particularly SCFAs. Imbalance in diversity and composition as well as alterations in the function of gut microbiota is associated with the pathogenesis of diverse gastrointestinal tract diseases, such as small intestinal bacterial overgrowth (SIBO), intestinal bowel disease (IBD), and a serial of liver diseases[116].

SIBO takes place in short bowel syndrome (SBS) and causes variable signs and symptoms resulting in nutrient malabsorption[117]. SIBO is characterized with the small intestinal excessive numbers and types of bacteria overgrowth exceeding 10^5 organisms/mL, which are mainly colonic type with predominantly gram-negative aerobic species (*Streptococcus*, *Escherichia coli, staphylococcus*) and anaerobic species (*Lactobacillus, bacteroides, clostridium and veillonella*)[118]. Enterotoxins expressing in the outer membrane of germ-negative species can damage the intestinal mucosa barrier by stimulation of fluid secretion in enterocytes, and further affect the absorptive function [119]. SIBO is associated with irritable bowel syndrome (IBS), celiac disease (CD) as well as IBD[120], and also involved in the development of nonalcoholic fatty liver

disease[121].

IBD occurs due to the imbalance between the host immune system and gut microbiota in digestive tract and is becoming an increasing health problem. Crohn's disease and ulcerative colitis are the two prevailing types. The worldwide epidemiologic data shows that the higher incidence and prevalence of IBD is associated with industrialization[122]. Differences in dietary habits highly influence the composition of microbiota; a high-fat diet induces microbiota dysbiosis which alters the intestinal permeability[123].

Additionally, the disruption of bacterial colonization with dysbiosis and an exaggerated inflammatory response has been linked with the pathological process of necrotizing enterocolitis (NEC) in preterm infants[124]. In NEC cases, an increased proportion of Proteobacteria and Actinobacteria, a decreased numbers of Bifidobacteria and Bacteroidetes were detected before NEC diagnosis. Moreover, a type of bacteria related to Klebsiella pneumoniae has been strongly correlated with the NEC development later stage[125].

Although the mechanism involved in diverse gastrointestinal tract diseases is still not completely understood, an impaired intestinal mucosal barrier is common feature among them. In addition, Paneth cells located in the crypts of the small intestine are very important for providing a sterile inner mucus layer and maintaining mucosal barrier integrity against microbiota by secreting antibiotic peptides containing α-defensin, angiogenin, lysozyme and lectins[126]. α-defensin 5/6 are the most abundant components. α-defensin 5 can be digested into fragments which exert specific antibiotic activity[127]. However, α-defensin 6 prevents invasion by bacterial pathogens through self-assembly to form fibrils and nanonets[128]. Diminished expression of Paneth cell defensins regulated by the Wnt factor is associated with Crohn's disease (also called Paneth's disease)[129,130]. Paneth-cell-deficient mice showed a dysbiosis in favor of an E. coli expansion and further weakening of the intestinal mucosal barrier with a visceral hypersensitivity[131]. Moreover, active Crohn's disease is accompanied by bile acid malabsorption due to altered expression of the major bile acid transporter[132].

As a consequence of intestinal mucosal barrier disruption, microbial/pathogen-associated molecular patterns (MAMPs/PAMPs) pass through lumen and mucosa to induce the inflammatory signaling nuclear factor kappa B (NFκB) *via* toll-like receptors (TLRs) and nod-like receptors (NLRs). Activation of this signaling induces the release of cytokines and chemokines into portal circulation[133,134].

Both bacterial components and metabolites reach the liver *via* the portal vein to induce hepatocytes damage. Additionally if dysbiosis occurs, secondary BAs including deoxycholic and lithocholic acid, which are toxic for both intestine and liver, are produced more than usual in microbiota[135]. Hepatocytes are damaged due a high level of secondary BAs, bacterial components and metabolites. High lipid peroxides and PAMPs derived from damaged hepatocytes induce liver microphage activation and initiate an immune response through NFκB, p-38/c-Jun-N-terminal kinase, TGF-β 1 and other inflammation cytokines[136]. A macrophage-mediated immune response is a major player in liver fibrogenesis. Chronic liver injury leads to hepatic stellate cells to transition into myofibroblast-like cells which produce an extracellular matrix and further contribute to the progression of fibrosis[137,138]. Moreover, chronic liver inflammation is significantly involved in the pathogenesis of liver fibrosis/cirrhosis and probably contributes to carcinogenesis.

POTENTIAL THERAPEUTIC APPLICATION TARGETING ACYL-COA SYNTHETASES

Long-chain acyl-CoA synthetases and cancer

Alteration in a fatty acid metabolism with a higher fatty acid synthesis and lipid deposition is a major player in the pathogenesis of metabolic disorders and cancer [139]. Deregulation of metabolism is known as a hallmark of cancer[140]. The Warburg effect, one of the hallmarks of cancer, first introduced by Otto Warburg, has been used to describe the deregulated metabolism of cancer cells characterized by increased conversion of glucose into lactate even in the presence of oxygen[141]. Many cancer cells are highly dependent on aerobic glycolysis for their growth and division[142]. Recently, several studies have shown that some cancers, including colon cancer, rather synthetize ATP by oxidative phosphorylation, which has been called the reverse Warburg effect[143-146]. In addition to previously reported abnormalities of glucose and glutamine metabolism in cancers, abnormal lipid metabolism was also found in

different cancer types[143]. Highly proliferative cancer cells are dependent not only on glucose but also on other metabolites including glutamine, serine and FAs[147-151]. It was reported that many cancer cells are characterized by an increased level of *de novo* fatty acid synthesis[152,153]. Upregulation of processes as fatty acid synthesis and FA release from lipid storage on the one hand, and downregulation of β-oxidation of FAs and their reesterification on the other, leads to an increased level of fatty acid in cancer cells. The fatty acid level was reported as a prognostic marker in several types of cancers including colorectal carcinoma (CRC)[7]. A high level of FA is considered a cancer biomarker and is associated with a worse prognosis and survival[7].

There is some evidence from mice with genetic inactivation of the *Muc2* gene that in adenocarcinoma arising in both the small and large intestine, alterations of the glucose metabolism induce expression of genes linked to *de novo* lipogenesis[154]. However, a systematic comparative analysis of adenocarcinomas arising in different locations of the intestinal tract with lipidomics is not available at present. Increased expression of ACSL1 was reported in several cancers, including colon[155,156] and liver[157,158], related to a poor clinical outcome[159]; ACSL4 was also upregulated in multiple cancer types, including colon[155,160] and liver[161-163]. Poorer patient survival in stage II colon cancer was correlated with the expression of ACSL4 and expression of stearoyl CoA desaturase 1 (SCD1)[156]. Concomitant overexpression of ACSL1, ACSL4 and SCD1 was found to induce epithelial-mesenchymal transtion in colorectal cancer [155]. ACSL3 and ACSL4 were upregulated in hepatocellular carcinoma (HCC)[164]. Deregulated expression of both ACSL3 and ACSL4 is associated with disease and especially with cancer[7]. ACSL3 drives tumor growth by increasing both fatty acid β-oxidation[165] and arachidonic acid conversion into prostaglandin[166]. As previously reported, ACSL4 indirectly stabilizes c-Myc by acting on the *ERK/FBW7* axis and driving oncogenesis *via* c-Myc-oncogenic signaling in HCC[167]. ACSL4 expression is highly linked to the cell sensitivity for ferroptosis, known as an iron-mediated non-apoptotic cell death[168]. Reported roles of ACSL4 include metabolic signaling resulting in drug resistance and the activation of intracellular, pro-oncogenic signaling pathways[139]. Impaired expression of ACSL5 is associated with coeliac disease and sporadic colorectal adenocarcinomas[169] and overexpression of ACSL5 induces apoptosis[170] and suppresses proliferation by inhibiting the activation of the Wnt/β-catenin signaling pathway in colon cancer[57].

ACSS1 and ACSS2 are overexpressed in HCC[171]. Both are key players in acetate metabolism which is shown to be highly taken up by several types of cancers, including liver. Gao *et al*[171] reported a role of acetate in epigenetic regulation (Histone acetylation) of a promoter region of FASN. Induction of lipid synthesis driven by increased FASN expression supports tumor cell survival and growth[171].

miRNAs targeting of long-chain acyl-CoA synthetases

Micro RNAs (miRNAs) are non-coding single stranded RNAs which regulate transcription of messenger RNA *via* binding to their 3'-untranslated region[172]. Cancer cells evolved a regulatory mechanism to control the mRNA stability of ACSLs by targeting their 3'-untranslated regions (3'UTR). For example, it was reported that miR-205 was decreased in liver cancer[173]. Negative correlation between miR-205 and ACSL4 expression was reported in human HCC patients[173]. The miR-205 targeting site is reported at the 3'UTR region of ACSL4-mRNA[173]. In addition, it is known that miR-205 binds to the 3'UTR of ACSL1 and induces its degradation[157]. The role of miR-211-5p as a tumor suppressor was reported in HCC[174]. This tumor-suppressive role was accomplished by downregulation of ACSL4 which is highly expressed in HCC[174]. miR-19b-1 showed an inhibitory effect on the *ACSL1/ACSL4/SCD1* axis by downregulating the Wnt/β-catenin pathway[175]. *ACSL/SCD* increases GSK3β phosphorylation, activating Wnt signaling and EMT, therefore, downregulation of β-catenin signaling by miR-19b-1 can be beneficial in colon cancer[175]. miR-142-3p has been reported to target cancer stem cell markers, such as the Wnt target and *LGR5* in colorectal cancer cells[176], in agreement with its action on the *ACSL/SCD* network cancer stem cell feature generation[175,176]. miR-34c was reported to be involved in hepatic fibrogenesis, miR-34c increases lipid droplet formation and hepatic stellate cell activation by downregulating ACSL1 in the liver[177]. miR-497-5p was reported to induce death in colon cancer cells by targeting ACSL5, suggesting its therapeutic potential in colon cancer[172].

Pharmacological targeting of long-chain acyl-CoA synthetases

Triacsin C, a fungal metabolite and a potent competitive inhibitor of ACSs activity[178, 179], competes with FAs for the catalytic domain. It inhibits ACSL1, ACSL3 and ACSL4, and in higher concentration proves effective against ACSL5[179,180]. It is

worth highlighting that triacsin C has a high toxicity (IC50) and consequently normal cells can be damaged[7].

Thiazolidinediones, also known as glitazones, are used for the therapy of diabetes II. Troglitazone and rosiglitazone are PPARγ agonists; interestingly they inhibit ACSL4 *via* PPARγ indirect mechanism[181,182]. Some of these drugs (Troglitazone, Ciglitazone) showed a protective effect against diabetes-promoted cancer[183].

Pharmacological targeting of very-long-chain acyl-CoA synthetases

FATP1 and FATP4 inhibitors were detected using high-throughput screening[184-186]. However, these compounds were not effective as revealed by *in vivo* studies. Screening compounds that specifically target domains involved in fatty acid transport, rather than the ACSL activity domain, might help to discover more effective compounds which could inhibit fatty acid transport. FATP2/ACSVL1, expressed mostly in the liver and intestine, acts as a transport protein and ACS[187]. FATP2 might be considered as an early marker for the development of overweight disorder after a high-fat diet[188]. A high-fat diet significantly upregulated fatp2 expression in the intestine of mice[188,189] It has a role in hepatic long-chain fatty acid uptake[190]. Due to its important role in fatty acid transport, FATP2 can be a promising pharmacological target in diseases which are characterized by an abnormal accumulation of intracellular FAs and lipids which may eventually result in irreversible hepatic cirrhosis[191,192]. Lipofermata and Grassofermata are selected FATP2 inhibitors which show specificity toward attenuating transport of LCFAs and VLCFAs. Lipofermata (5'-bromo-5-phenyl-spiro[3H-1,3,4-thiadiazole-2,3'-indoline]-2'-one) inhibits the function of FATP2 as a transport protein, without compromising its function as an ACS[193,194]. Grassofermata (2-benzyl-3-(4-chlorophenyl)-5-(4-nitrophenyl) pyrazolo[1,5-a] pyrimidin-7(4H)-one) suppresses palmitic acid mediated lipotoxicity[193,195,196]. Both of them reduce intestinal fat absorption of 13C labeled oleate[186]. In addition to its contribution to the development of metabolic liver diseases, FATP2 promotes the growth of cancer cells and induces their resistance to targeted therapies[190]. A study by Veglia *et al*[194] demonstrated that lipofermata abrogated the activity of polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) and substantially delayed tumor progression in colon cancer cell line CT26 tumor-bearing mice. STAT5 signaling induced by granulocyte-macrophage colony stimulating factor (GM-CSF) upregulated the FATP2 in these cells. FATP2 overexpression in these PMN-MDSCs cells induced PGE2 synthesis and its immunosuppressive effect on CD8+ T cell[194]. Interestingly in this study, it was found that lipofermata elevated the therapeutical effect of immune checkpoint inhibitor therapy (anti-PD-1 and anti-CTLA-4) as well as macrophage targeted therapy (anti CSF-1R)[194].

FATP5 can be exclusively found in liver, at the basal plasma membrane of hepatocytes[197]. Both its location and role in long-chain fatty acid uptake make it an attractive target for treatment of metabolic disorders. Interestingly, screening of potential compounds revealed the potential of BAs including the primary BAs produced by the liver and the secondary BA secreted by intestinal bacteria (microbiota) to attenuate specifically FATP5 function without affecting FATP4[197]. The following BAs showed potential for FATP5 inhibition: chenodiol, primary BA, produced by the liver and ursodiol, secondary BA, which is metabolically produced by intestinal bacteria[197].

Experimental *in vivo* studies in rats showed induction of FATP mRNA expression, finding the highest upregulation in the liver. In the intestine, there was an increase in the FATP mRNA level but two times less than in the liver[198], suggesting that fenofibrates show specificity towards liver FATPs. Fibrates are known as PPARα activators, their hypolipidemic effect is accomplished *via* FATP activation, induction of β-oxidation and consequently reduction in triglyceride synthesis[198]. The indirect activation of FATP by the fenofibrate is mediated *via* PPARα[199].

Targeting of short-chain acyl-CoA synthetases

As reported by Bjorson *et al*[200], mitochondrial acetate appears to be the main metabolic energy source under hypoxia in HCC patients. Upregulation of ACSS1 Led to an enhanced level of mitochondrial acetate in HCC, which is associated with several metabolic alterations including decreased fatty acid oxidation, glutamine utilization, gluconeogenesis and increased glycolysis[200] This finding suggests a potential of ACSS1 as a target in cancer treatment. Indeed, the ACSS1 inhibitor showed a growth inhibitory effect on glioma[201].

CONCLUSION

LCFAs and SCFAs are the most abundant energy sources from dietary lipid intake and microbiota-derived fermentation products. Members of ACSs play a critical role in lipid metabolism, participating in fatty acid transport and activation. Abnormal expression of ACSs is closely associated with lipid metabolic disorders and carcinogenesis. Research on ACSs will shed further light on their biological functions and molecular mechanisms in fatty acid metabolism and eventually lead to the development of therapeutic drugs targeting ACSs in the treatment of human metabolic diseases.

REFERENCES

1   **Grevengoed TJ**, Klett EL, Coleman RA. Acyl-CoA metabolism and partitioning. *Annu Rev Nutr* 2014; **34**: 1-30 [PMID: 24819326 DOI: 10.1146/annurev-nutr-071813-105541]

2   **Volta U**, Bonazzi C, Bianchi FB, Baldoni AM, Zoli M, Pisi E. IgA antibodies to dietary antigens in liver cirrhosis. *Ric Clin Lab* 1987; **17**: 235-242 [PMID: 3671996 DOI: 10.1007/BF02912537]

3   **Guinane CM**, Cotter PD. Role of the gut microbiota in health and chronic gastrointestinal disease: understanding a hidden metabolic organ. *Therap Adv Gastroenterol* 2013; **6**: 295-308 [PMID: 23814609 DOI: 10.1177/1756283X13482996]

4   **Konturek PC**, Harsch IA, Konturek K, Schink M, Konturek T, Neurath MF, Zopf Y. Gut‑Liver Axis: How Do Gut Bacteria Influence the Liver? *Med Sci (Basel)* 2018; **6** [PMID: 30227645 DOI: 10.3390/medsci6030079]

5   **Park W**. Gut microbiomes and their metabolites shape human and animal health. *J Microbiol* 2018; **56**: 151-153 [PMID: 29492871 DOI: 10.1007/s12275-018-0577-8]

6   **Niot I**, Poirier H, Tran TT, Besnard P. Intestinal absorption of long-chain fatty acids: evidence and uncertainties. *Prog Lipid Res* 2009; **48**: 101-115 [PMID: 19280719 DOI: 10.1016/j.plipres.2009.01.001]

7   **Rossi Sebastiano M**, Konstantinidou G. Targeting Long Chain Acyl-CoA Synthetases for Cancer Therapy. *Int J Mol Sci* 2019; **20** [PMID: 31344914 DOI: 10.3390/ijms20153624]

8   **Anderson CM**, Stahl A. SLC27 fatty acid transport proteins. *Mol Aspects Med* 2013; **34**: 516-528 [PMID: 23506886 DOI: 10.1016/j.mam.2012.07.010]

9   **Hamilton JA**. New insights into the roles of proteins and lipids in membrane transport of fatty acids. *Prostaglandins Leukot Essent Fatty Acids* 2007; **77**: 355-361 [PMID: 18032007 DOI: 10.1016/j.plefa.2007.10.020]

10 **Moffett JR**, Puthillathu N, Vengilote R, Jaworski DM, Namboodiri AM. Acetate Revisited: A Key Biomolecule at the Nexus of Metabolism, Epigenetics and Oncogenesis-Part 1: Acetyl-CoA, Acetogenesis and Acyl-CoA Short-Chain Synthetases. *Front Physiol* 2020; **11**: 580167 [PMID: 33281616 DOI: 10.3389/fphys.2020.580167]

11 **Wang TY**, Liu M, Portincasa P, Wang DQ. New insights into the molecular mechanism of intestinal fatty acid absorption. *Eur J Clin Invest* 2013; **43**: 1203-1223 [PMID: 24102389 DOI: 10.1111/eci.12161]

12 **Lowe ME**. The triglyceride lipases of the pancreas. *J Lipid Res* 2002; **43**: 2007-2016 [PMID: 12454260 DOI: 10.1194/jlr.r200012-jlr200]

13 **Alves-Bezerra M**, Cohen DE. Triglyceride Metabolism in the Liver. *Compr Physiol* 2017; **8**: 1-8 [PMID: 29357123 DOI: 10.1002/cphy.c170012]

14 **Hardie DG**. Organismal carbohydrate and lipid homeostasis. *Cold Spring Harb Perspect Biol* 2012; **4** [PMID: 22550228 DOI: 10.1101/cshperspect.a006031]

15 **Chiang JY**. Bile acid metabolism and signaling. *Compr Physiol* 2013; **3**: 1191-1212 [PMID: 23897684 DOI: 10.1002/cphy.c120023]

16 **Zhang YK**, Guo GL, Klaassen CD. Diurnal variations of mouse plasma and hepatic bile acid concentrations as well as expression of biosynthetic enzymes and transporters. *PLoS One* 2011; **6**: e16683 [PMID: 21346810 DOI: 10.1371/journal.pone.0016683]

17 **Ramírez-Pérez O**, Cruz-Ramón V, Chinchilla-López P, Méndez-Sánchez N. The Role of the Gut Microbiota in Bile Acid Metabolism. *Ann Hepatol* 2017; **16**: s15-s20 [PMID: 29080339 DOI: 10.5604/01.3001.0010.5494]

18 **de Aguiar Vallim TQ**, Tarling EJ, Edwards PA. Pleiotropic roles of bile acids in metabolism. *Cell Metab* 2013; **17**: 657-669 [PMID: 23602448 DOI: 10.1016/j.cmet.2013.03.013]

19 **Li T**, Chiang JY. Nuclear receptors in bile acid metabolism. *Drug Metab Rev* 2013; **45**: 145-155 [PMID: 23330546 DOI: 10.3109/03602532.2012.740048]

20 **Massey JB**, Bick DH, Pownall HJ. Spontaneous transfer of monoacyl amphiphiles between lipid and protein surfaces. *Biophys J* 1997; **72**: 1732-1743 [PMID: 9083677 DOI: 10.1016/S0006-3495(97)78819-2]

21 **Kamp F**, Hamilton JA. How fatty acids of different chain length enter and leave cells by free diffusion. *Prostaglandins Leukot Essent Fatty Acids* 2006; **75**: 149-159 [PMID: 16829065 DOI: 10.1016/j.plefa.2006.05.003]

22 **Schaffer JE**, Lodish HF. Expression cloning and characterization of a novel adipocyte long chain

fatty acid transport protein. *Cell* 1994; **79**: 427-436 [PMID: 7954810 DOI: 10.1016/0092-8674(94)90252-6]

23 **Pohl J**, Ring A, Hermann T, Stremmel W. Role of FATP in parenchymal cell fatty acid uptake. *Biochim Biophys Acta* 2004; **1686**: 1-6 [PMID: 15522816 DOI: 10.1016/j.bbalip.2004.06.004]

24 **Stahl A**, Hirsch DJ, Gimeno RE, Punreddy S, Ge P, Watson N, Patel S, Kotler M, Raimondi A, Tartaglia LA, Lodish HF. Identification of the major intestinal fatty acid transport protein. *Mol Cell* 1999; **4**: 299-308 [PMID: 10518211 DOI: 10.1016/s1097-2765(00)80332-9]

25 **Herrmann T**, van der Hoeven F, Grone HJ, Stewart AF, Langbein L, Kaiser I, Liebisch G, Gosch I, Buchkremer F, Drobnik W, Schmitz G, Stremmel W. Mice with targeted disruption of the fatty acid transport protein 4 (Fatp 4, Slc27a4) gene show features of lethal restrictive dermopathy. *J Cell Biol* 2003; **161**: 1105-1115 [PMID: 12821645 DOI: 10.1083/jcb.200207080]

26 **Doege H**, Baillie RA, Ortegon AM, Tsang B, Wu Q, Punreddy S, Hirsch D, Watson N, Gimeno RE, Stahl A. Targeted deletion of FATP5 reveals multiple functions in liver metabolism: alterations in hepatic lipid homeostasis. *Gastroenterology* 2006; **130**: 1245-1258 [PMID: 16618416 DOI: 10.1053/j.gastro.2006.02.006]

27 **Hubbard B**, Doege H, Punreddy S, Wu H, Huang X, Kaushik VK, Mozell RL, Byrnes JJ, Stricker-Krongrad A, Chou CJ, Tartaglia LA, Lodish HF, Stahl A, Gimeno RE. Mice deleted for fatty acid transport protein 5 have defective bile acid conjugation and are protected from obesity. *Gastroenterology* 2006; **130**: 1259-1269 [PMID: 16618417 DOI: 10.1053/j.gastro.2006.02.012]

28 **Lewis SE**, Listenberger LL, Ory DS, Schaffer JE. Membrane topology of the murine fatty acid transport protein 1. *J Biol Chem* 2001; **276**: 37042-37050 [PMID: 11470793 DOI: 10.1074/jbc.M105556200]

29 **Black PN**, DiRusso CC. Transmembrane movement of exogenous long-chain fatty acids: proteins, enzymes, and vectorial esterification. *Microbiol Mol Biol Rev* 2003; **67**: 454-472, table of contents [PMID: 12966144 DOI: 10.1128/MMBR.67.3.454-472.2003]

30 **Nie B**, Park HM, Kazantzis M, Lin M, Henkin A, Ng S, Song S, Chen Y, Tran H, Lai R, Her C, Maher JJ, Forman BM, Stahl A. Specific bile acids inhibit hepatic fatty acid uptake in mice. *Hepatology* 2012; **56**: 1300-1310 [PMID: 22531947 DOI: 10.1002/hep.25797]

31 **Chmurzyńska A**. The multigene family of fatty acid-binding proteins (FABPs): function, structure and polymorphism. *J Appl Genet* 2006; **47**: 39-48 [PMID: 16424607 DOI: 10.1007/BF03194597]

32 **Glatz JF**, van der Vusse GJ. Cellular fatty acid-binding proteins: their function and physiological significance. *Prog Lipid Res* 1996; **35**: 243-282 [PMID: 9082452 DOI: 10.1016/s0163-7827(96)00006-9]

33 **Sweetser DA**, Birkenmeier EH, Klisak IJ, Zollman S, Sparkes RS, Mohandas T, Lusis AJ, Gordon JI. The human and rodent intestinal fatty acid binding protein genes. A comparative analysis of their structure, expression, and linkage relationships. *J Biol Chem* 1987; **262**: 16060-16071 [PMID: 2824476]

34 **Mansbach CM**, Abumrad NA. Chapter 60 - Enterocyte Fatty Acid Handling Proteins and Chylomicron Formation. In: Johnson LR, Ghishan FK, Kaunitz JD, Merchant JL, Said HM, Wood JD, editors. Physiology of the Gastrointestinal Tract (Fifth Edition). Boston: Academic Press, 2012: 1625-1641 [DOI: 10.1016/b978-0-12-382026-6.00060-9]

35 **Newberry EP**, Xie Y, Kennedy S, Han X, Buhman KK, Luo J, Gross RW, Davidson NO. Decreased hepatic triglyceride accumulation and altered fatty acid uptake in mice with deletion of the liver fatty acid-binding protein gene. *J Biol Chem* 2003; **278**: 51664-51672 [PMID: 14534295 DOI: 10.1074/jbc.M309377200]

36 **Atshaves BP**, McIntosh AM, Lyuksyutova OI, Zipfel W, Webb WW, Schroeder F. Liver fatty acid-binding protein gene ablation inhibits branched-chain fatty acid metabolism in cultured primary hepatocytes. *J Biol Chem* 2004; **279**: 30954-30965 [PMID: 15155724 DOI: 10.1074/jbc.M313571200]

37 **Huang H**, Starodub O, McIntosh A, Atshaves BP, Woldegiorgis G, Kier AB, Schroeder F. Liver fatty acid-binding protein colocalizes with peroxisome proliferator activated receptor alpha and enhances ligand distribution to nuclei of living cells. *Biochemistry* 2004; **43**: 2484-2500 [PMID: 14992586 DOI: 10.1021/bi0352318]

38 **Glatz JF**, Luiken JJ. From fat to FAT (CD36/SR-B2): Understanding the regulation of cellular fatty acid uptake. *Biochimie* 2017; **136**: 21-26 [PMID: 28013071 DOI: 10.1016/j.biochi.2016.12.007]

39 **Coburn CT**, Knapp FF Jr, Febbraio M, Beets AL, Silverstein RL, Abumrad NA. Defective uptake and utilization of long chain fatty acids in muscle and adipose tissues of CD36 knockout mice. *J Biol Chem* 2000; **275**: 32523-32529 [PMID: 10913136 DOI: 10.1074/jbc.M003826200]

40 **Lobo MV**, Huerta L, Ruiz-Velasco N, Teixeiro E, de la Cueva P, Celdrán A, Martín-Hidalgo A, Vega MA, Bragado R. Localization of the lipid receptors CD36 and CLA-1/SR-BI in the human gastrointestinal tract: towards the identification of receptors mediating the intestinal absorption of dietary lipids. *J Histochem Cytochem* 2001; **49**: 1253-1260 [PMID: 11561009 DOI: 10.1177/002215540104901007]

41 **Wilson CG**, Tran JL, Erion DM, Vera NB, Febbraio M, Weiss EJ. Hepatocyte-Specific Disruption of CD36 Attenuates Fatty Liver and Improves Insulin Sensitivity in HFD-Fed Mice. *Endocrinology* 2016; **157**: 570-585 [PMID: 26650570 DOI: 10.1210/en.2015-1866]

42 **Murata M**, Peränen J, Schreiner R, Wieland F, Kurzchalia TV, Simons K. VIP21/caveolin is a cholesterol-binding protein. *Proc Natl Acad Sci U S A* 1995; **92**: 10339-10343 [PMID: 7479780 DOI: 10.1073/pnas.92.22.10339]

43   **Valasek MA**, Weng J, Shaul PW, Anderson RG, Repa JJ. Caveolin-1 is not required for murine intestinal cholesterol transport. *J Biol Chem* 2005; **280**: 28103-28109 [PMID: 15919660 DOI: 10.1074/jbc.M504609200]

44   **Pol A**, Martin S, Fernandez MA, Ferguson C, Carozzi A, Luetterforst R, Enrich C, Parton RG. Dynamic and regulated association of caveolin with lipid bodies: modulation of lipid body motility and function by a dominant negative mutant. *Mol Biol Cell* 2004; **15**: 99-110 [PMID: 14528016 DOI: 10.1091/mbc.e03-06-0368]

45   **Mashek DG**, Bornfeldt KE, Coleman RA, Berger J, Bernlohr DA, Black P, DiRusso CC, Farber SA, Guo W, Hashimoto N, Khodiyar V, Kuypers FA, Maltais LJ, Nebert DW, Renieri A, Schaffer JE, Stahl A, Watkins PA, Vasiliou V, Yamamoto TT. Revised nomenclature for the mammalian long-chain acyl-CoA synthetase gene family. *J Lipid Res* 2004; **45**: 1958-1961 [PMID: 15292367 DOI: 10.1194/jlr.E400002-JLR200]

46   **Soupene E**, Kuypers FA. Mammalian long-chain acyl-CoA synthetases. *Exp Biol Med (Maywood)* 2008; **233**: 507-521 [PMID: 18375835 DOI: 10.3181/0710-MR-287]

47   **Watkins PA**, Maiguel D, Jia Z, Pevsner J. Evidence for 26 distinct acyl-coenzyme A synthetase genes in the human genome. *J Lipid Res* 2007; **48**: 2736-2750 [PMID: 17762044 DOI: 10.1194/jlr.M700378-JLR200]

48   **Hisanaga Y**, Ago H, Nakagawa N, Hamada K, Ida K, Yamamoto M, Hori T, Arii Y, Sugahara M, Kuramitsu S, Yokoyama S, Miyano M. Structural basis of the substrate-specific two-step catalysis of long chain fatty acyl-CoA synthetase dimer. *J Biol Chem* 2004; **279**: 31717-31726 [PMID: 15145952 DOI: 10.1074/jbc.M400100200]

49   **Li LO**, Klett EL, Coleman RA. Acyl-CoA synthesis, lipid metabolism and lipotoxicity. *Biochim Biophys Acta* 2010; **1801**: 246-251 [PMID: 19818872 DOI: 10.1016/j.bbalip.2009.09.024]

50   **Yan S**, Yang XF, Liu HL, Fu N, Ouyang Y, Qing K. Long-chain acyl-CoA synthetase in fatty acid metabolism involved in liver and other diseases: an update. *World J Gastroenterol* 2015; **21**: 3492-3498 [PMID: 25834313 DOI: 10.3748/wjg.v21.i12.3492]

51   **Poppelreuther M**, Rudolph B, Du C, Großmann R, Becker M, Thiele C, Ehehalt R, Füllekrug J. The N-terminal region of acyl-CoA synthetase 3 is essential for both the localization on lipid droplets and the function in fatty acid uptake. *J Lipid Res* 2012; **53**: 888-900 [PMID: 22357706 DOI: 10.1194/jlr.M024562]

52   **Coleman RA**, Lewin TM, Van Horn CG, Gonzalez-Baró MR. Do long-chain acyl-CoA synthetases regulate fatty acid entry into synthetic *vs* degradative pathways? *J Nutr* 2002; **132**: 2123-2126 [DOI: 10.1093/jn/132.8.2123]

53   **Ellis JM**, Frahm JL, Li LO, Coleman RA. Acyl-coenzyme A synthetases in metabolic control. *Curr Opin Lipidol* 2010; **21**: 212-217 [PMID: 20480548 DOI: 10.1097/mol.0b013e32833884bb]

54   **Mashek DG**, McKenzie MA, Van Horn CG, Coleman RA. Rat long chain acyl-CoA synthetase 5 increases fatty acid uptake and partitioning to cellular triacylglycerol in McArdle-RH7777 cells. *J Biol Chem* 2006; **281**: 945-950 [PMID: 16263710 DOI: 10.1074/jbc.M507646200]

55   **Bu SY**, Mashek MT, Mashek DG. Suppression of long chain acyl-CoA synthetase 3 decreases hepatic de novo fatty acid synthesis through decreased transcriptional activity. *J Biol Chem* 2009; **284**: 30474-30483 [PMID: 19737935 DOI: 10.1074/jbc.M109.036665]

56   **Meller N**, Morgan ME, Wong WP, Altemus JB, Sehayek E. Targeting of Acyl-CoA synthetase 5 decreases jejunal fatty acid activation with no effect on dietary long-chain fatty acid absorption. *Lipids Health Dis* 2013; **12**: 88 [PMID: 23767941 DOI: 10.1186/1476-511X-12-88]

57   **Klaus C**, Kaemmerer E, Reinartz A, Schneider U, Plum P, Jeon MK, Hose J, Hartmann F, Schnölzer M, Wagner N, Kopitz J, Gassler N. TP53 status regulates ACSL5-induced expression of mitochondrial mortalin in enterocytes and colorectal adenocarcinomas. *Cell Tissue Res* 2014; **357**: 267-278 [PMID: 24770931 DOI: 10.1007/s00441-014-1826-8]

58   **Klaus C**, Jeon MK, Kaemmerer E, Gassler N. Intestinal acyl-CoA synthetase 5: activation of long chain fatty acids and behind. *World J Gastroenterol* 2013; **19**: 7369-7373 [PMID: 24259967 DOI: 10.3748/wjg.v19.i42.7369]

59   **Paroder V**, Spencer SR, Paroder M, Arango D, Schwartz S Jr, Mariadason JM, Augenlicht LH, Eskandari S, Carrasco N. Na(+)/monocarboxylate transport (SMCT) protein expression correlates with survival in colon cancer: molecular characterization of SMCT. *Proc Natl Acad Sci U S A* 2006; **103**: 7270-7275 [PMID: 16670197 DOI: 10.1073/pnas.0602365103]

60   **Rowe WA**, Lesho MJ, Montrose MH. Polarized Na+/H+ exchange function is pliable in response to transepithelial gradients of propionate. *Proc Natl Acad Sci U S A* 1994; **91**: 6166-6170 [PMID: 8016132 DOI: 10.1073/pnas.91.13.6166]

61   **Castro LF**, Lopes-Marques M, Wilson JM, Rocha E, Reis-Henriques MA, Santos MM, Cunha I. A novel Acetyl-CoA synthetase short-chain subfamily member 1 (Acss1) gene indicates a dynamic history of paralogue retention and loss in vertebrates. *Gene* 2012; **497**: 249-255 [PMID: 22313524 DOI: 10.1016/j.gene.2012.01.013]

62   **Fujino T**, Kondo J, Ishikawa M, Morikawa K, Yamamoto TT. Acetyl-CoA synthetase 2, a mitochondrial matrix enzyme involved in the oxidation of acetate. *J Biol Chem* 2001; **276**: 11420-11426 [PMID: 11150295 DOI: 10.1074/jbc.M008782200]

63   **Sakakibara I**, Fujino T, Ishii M, Tanaka T, Shimosawa T, Miura S, Zhang W, Tokutake Y, Yamamoto J, Awano M, Iwasaki S, Motoike T, Okamura M, Inagaki T, Kita K, Ezaki O, Naito M, Kuwaki T, Chohnan S, Yamamoto TT, Hammer RE, Kodama T, Yanagisawa M, Sakai J. Fasting-induced hypothermia and reduced energy production in mice lacking acetyl-CoA synthetase 2. *Cell*

Metab 2009; **9**: 191-202 [PMID: 19187775 DOI: 10.1016/j.cmet.2008.12.008]

64   **Yoshimura Y**, Araki A, Maruta H, Takahashi Y, Yamashita H. Molecular cloning of rat acss3 and characterization of mammalian propionyl-CoA synthetase in the liver mitochondrial matrix. *J Biochem* 2017; **161**: 279-289 [PMID: 28003429 DOI: 10.1093/jb/mvw067]

65   **Luong A**, Hannah VC, Brown MS, Goldstein JL. Molecular characterization of human acetyl-CoA synthetase, an enzyme regulated by sterol regulatory element-binding proteins. *J Biol Chem* 2000; **275**: 26458-26466 [PMID: 10843999 DOI: 10.1074/jbc.M004160200]

66   **Bulusu V**, Tumanov S, Michalopoulou E, van den Broek NJ, MacKay G, Nixon C, Dhayade S, Schug ZT, Vande Voorde J, Blyth K, Gottlieb E, Vazquez A, Kamphorst JJ. Acetate Recapturing by Nuclear Acetyl-CoA Synthetase 2 Prevents Loss of Histone Acetylation during Oxygen and Serum Limitation. *Cell Rep* 2017; **18**: 647-658 [PMID: 28099844 DOI: 10.1016/j.celrep.2016.12.055]

67   **Huang Z**, Zhang M, Plec AA, Estill SJ, Cai L, Repa JJ, McKnight SL, Tu BP. ACSS2 promotes systemic fat storage and utilization through selective regulation of genes involved in lipid metabolism. *Proc Natl Acad Sci U S A* 2018; **115**: E9499-E9506 [PMID: 30228117 DOI: 10.1073/pnas.1806635115]

68   **Rios-Covian D**, Salazar N, Gueimonde M, de Los Reyes-Gavilan CG. Shaping the Metabolism of Intestinal *Bacteroides* Population through Diet to Improve Human Health. *Front Microbiol* 2017; **8**: 376 [PMID: 28326076 DOI: 10.3389/fmicb.2017.00376]

69   **De Filippo C**, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, Collini S, Pieraccini G, Lionetti P. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc Natl Acad Sci U S A* 2010; **107**: 14691-14696 [PMID: 20679230 DOI: 10.1073/pnas.1005963107]

70   **Wu GD**, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA, Bewtra M, Knights D, Walters WA, Knight R, Sinha R, Gilroy E, Gupta K, Baldassano R, Nessel L, Li H, Bushman FD, Lewis JD. Linking long-term dietary patterns with gut microbial enterotypes. *Science* 2011; **334**: 105-108 [PMID: 21885731 DOI: 10.1126/science.1208344]

71   **Arumugam M**, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, Fernandes GR, Tap J, Bruls T, Batto JM, Bertalan M, Borruel N, Casellas F, Fernandez L, Gautier L, Hansen T, Hattori M, Hayashi T, Kleerebezem M, Kurokawa K, Leclerc M, Levenez F, Manichanh C, Nielsen HB, Nielsen T, Pons N, Poulain J, Qin J, Sicheritz-Ponten T, Tims S, Torrents D, Ugarte E, Zoetendal EG, Wang J, Guarner F, Pedersen O, de Vos WM, Brunak S, Doré J; MetaHIT Consortium, Antolín M, Artiguenave F, Blottiere HM, Almeida M, Brechot C, Cara C, Chervaux C, Cultrone A, Delorme C, Denariaz G, Dervyn R, Foerstner KU, Friss C, van de Guchte M, Guedon E, Haimet F, Huber W, van Hylckama-Vlieg J, Jamet A, Juste C, Kaci G, Knol J, Lakhdari O, Layec S, Le Roux K, Maguin E, Mérieux A, Melo Minardi R, M'rini C, Muller J, Oozeer R, Parkhill J, Renault P, Rescigno M, Sanchez N, Sunagawa S, Torrejon A, Turner K, Vandemeulebrouck G, Varela E, Winogradsky Y, Zeller G, Weissenbach J, Ehrlich SD, Bork P. Enterotypes of the human gut microbiome. *Nature* 2011; **473**: 174-180 [PMID: 21508958 DOI: 10.1038/nature09944]

72   **Yatsunenko T**, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, Magris M, Hidalgo G, Baldassano RN, Anokhin AP, Heath AC, Warner B, Reeder J, Kuczynski J, Caporaso JG, Lozupone CA, Lauber C, Clemente JC, Knights D, Knight R, Gordon JI. Human gut microbiome viewed across age and geography. *Nature* 2012; **486**: 222-227 [PMID: 22699611 DOI: 10.1038/nature11053]

73   **Sonnenburg ED**, Smits SA, Tikhonov M, Higginbottom SK, Wingreen NS, Sonnenburg JL. Diet-induced extinctions in the gut microbiota compound over generations. *Nature* 2016; **529**: 212-215 [PMID: 26762459 DOI: 10.1038/nature16504]

74   **Salonen A**, Lahti L, Salojärvi J, Holtrop G, Korpela K, Duncan SH, Date P, Farquharson F, Johnstone AM, Lobley GE, Louis P, Flint HJ, de Vos WM. Impact of diet and individual variation on intestinal microbiota composition and fermentation products in obese men. *ISME J* 2014; **8**: 2218-2230 [PMID: 24763370 DOI: 10.1038/ismej.2014.63]

75   **Reichardt N**, Duncan SH, Young P, Belenguer A, McWilliam Leitch C, Scott KP, Flint HJ, Louis P. Phylogenetic distribution of three pathways for propionate production within the human gut microbiota. *ISME J* 2014; **8**: 1323-1335 [PMID: 24553467 DOI: 10.1038/ismej.2014.14]

76   **Louis P**, Flint HJ. Formation of propionate and butyrate by the human colonic microbiota. *Environ Microbiol* 2017; **19**: 29-41 [PMID: 27928878 DOI: 10.1111/1462-2920.13589]

77   **Giri S**, Oña L, Waschina S, Shitut S, Yousif G, Kaleta C, Kost C.   Metabolic dissimilarity determines the establishment of cross-feeding interactions in bacteria. bioRxiv 2020: 2020.2010.2009.333336 [DOI: 10.1101/2020.10.09.333336]

78   **Wall R**, Ross RP, Shanahan F, O'Mahony L, O'Mahony C, Coakley M, Hart O, Lawlor P, Quigley EM, Kiely B, Fitzgerald GF, Stanton C. Metabolic activity of the enteric microbiota influences the fatty acid composition of murine and porcine liver and adipose tissues. *Am J Clin Nutr* 2009; **89**: 1393-1401 [PMID: 19357220 DOI: 10.3945/ajcn.2008.27023]

79   **Lennen RM**, Politz MG, Kruziki MA, Pfleger BF. Identification of transport proteins involved in free fatty acid efflux in Escherichia coli. *J Bacteriol* 2013; **195**: 135-144 [PMID: 23104810 DOI: 10.1128/JB.01477-12]

80   **Golubeva YA**, Ellermeier JR, Cott Chubiz JE, Slauch JM. Intestinal Long-Chain Fatty Acids Act as a Direct Signal To Modulate Expression of the Salmonella Pathogenicity Island 1 Type III Secretion System. *mBio* 2016; **7**: e02170-e02115 [PMID: 26884427 DOI: 10.1128/mBio.02170-15]

81   **Wolfe AJ**. The acetate switch. *Microbiol Mol Biol Rev* 2005; **69**: 12-50 [PMID: 15755952 DOI:

10.1128/MMBR.69.1.12-50.2005]

82   **Clark DP**, Cronan JE. Two-Carbon Compounds and Fatty Acids as Carbon Sources. *EcoSal Plus* 2005; **1** [PMID: 26443509 DOI: 10.1128/ecosalplus.3.4.4]

83   **Childers BM**, Cao X, Weber GG, Demeler B, Hart PJ, Klose KE. N-terminal residues of the Vibrio cholerae virulence regulatory protein ToxT involved in dimerization and modulation by fatty acids. *J Biol Chem* 2011; **286**: 28644-28655 [PMID: 21673111 DOI: 10.1074/jbc.M111.258780]

84   **Cruite JT**, Kovacikova G, Clark KA, Woodbrey AK, Skorupski K, Kull FJ. Structural basis for virulence regulation in *Vibrio cholerae* by unsaturated fatty acid components of bile. *Commun Biol* 2019; **2**: 440 [PMID: 31815195 DOI: 10.1038/s42003-019-0686-x]

85   **Ktsoyan ZA**, Mkrtchyan MS, Zakharyan MK, Mnatsakanyan AA, Arakelova KA, Gevorgyan ZU, Sedrakyan AM, Hovhannisyan AI, Arakelyan AA, Aminov RI. Systemic Concentrations of Short Chain Fatty Acids Are Elevated in Salmonellosis and Exacerbation of Familial Mediterranean Fever. *Front Microbiol* 2016; **7**: 776 [PMID: 27252692 DOI: 10.3389/fmicb.2016.00776]

86   **van der Beek CM**, Bloemen JG, van den Broek MA, Lenaerts K, Venema K, Buurman WA, Dejong CH. Hepatic Uptake of Rectally Administered Butyrate Prevents an Increase in Systemic Butyrate Concentrations in Humans. *J Nutr* 2015; **145**: 2019-2024 [PMID: 26156796 DOI: 10.3945/jn.115.211193]

87   **den Besten G**, van Eunen K, Groen AK, Venema K, Reijngoud DJ, Bakker BM. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J Lipid Res* 2013; **54**: 2325-2340 [PMID: 23821742 DOI: 10.1194/jlr.R036012]

88   **Zambell KL**, Fitch MD, Fleming SE. Acetate and butyrate are the major substrates for de novo lipogenesis in rat colonic epithelial cells. *J Nutr* 2003; **133**: 3509-3515 [PMID: 14608066 DOI: 10.1093/jn/133.11.3509]

89   **Sunami Y**, Rebelo A, Kleeff J. Lipid Metabolism and Lipid Droplets in Pancreatic Cancer and Stellate Cells. *Cancers (Basel)* 2017; **10** [PMID: 29295482 DOI: 10.3390/cancers10010003]

90   **Sidoli S**, Trefely S, Garcia BA, Carrer A. Integrated Analysis of Acetyl-CoA and Histone Modification *via* Mass Spectrometry to Investigate Metabolically Driven Acetylation. *Methods Mol Biol* 2019; **1928**: 125-147 [PMID: 30725455 DOI: 10.1007/978-1-4939-9027-6_9]

91   **Puchalska P**, Crawford PA. Multi-dimensional Roles of Ketone Bodies in Fuel Metabolism, Signaling, and Therapeutics. *Cell Metab* 2017; **25**: 262-284 [PMID: 28178565 DOI: 10.1016/j.cmet.2016.12.022]

92   **Wyss MT**, Magistretti PJ, Buck A, Weber B. Labeled acetate as a marker of astrocytic metabolism. *J Cereb Blood Flow Metab* 2011; **31**: 1668-1674 [PMID: 21654698 DOI: 10.1038/jcbfm.2011.84]

93   **Frost G**, Sleeth ML, Sahuri-Arisoylu M, Lizarbe B, Cerdan S, Brody L, Anastasovska J, Ghourab S, Hankir M, Zhang S, Carling D, Swann JR, Gibson G, Viardot A, Morrison D, Louise Thomas E, Bell JD. The short-chain fatty acid acetate reduces appetite *via* a central homeostatic mechanism. *Nat Commun* 2014; **5**: 3611 [PMID: 24781306 DOI: 10.1038/ncomms4611]

94   **Hamer HM**, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, Brummer RJ. Review article: the role of butyrate on colonic function. *Aliment Pharmacol Ther* 2008; **27**: 104-119 [PMID: 17973645 DOI: 10.1111/j.1365-2036.2007.03562.x]

95   **Liu H**, Wang J, He T, Becker S, Zhang G, Li D, Ma X. Butyrate: A Double-Edged Sword for Health? *Adv Nutr* 2018; **9**: 21-29 [PMID: 29438462 DOI: 10.1093/advances/nmx009]

96   **Rouwet EV**, Heineman E, Buurman WA, ter Riet G, Ramsay G, Blanco CE. Intestinal permeability and carrier-mediated monosaccharide absorption in preterm neonates during the early postnatal period. *Pediatr Res* 2002; **51**: 64-70 [PMID: 11756641 DOI: 10.1203/00006450-200201000-00012]

97   **Blau CA**, Constantoulakis P, Shaw CM, Stamatoyannopoulos G. Fetal hemoglobin induction with butyric acid: efficacy and toxicity. *Blood* 1993; **81**: 529-537 [PMID: 8422469]

98   **Peng L**, He Z, Chen W, Holzman IR, Lin J. Effects of butyrate on intestinal barrier function in a Caco-2 cell monolayer model of intestinal barrier. *Pediatr Res* 2007; **61**: 37-41 [PMID: 17211138 DOI: 10.1203/01.pdr.0000250014.92242.f3]

99   **Suzuki T**. Regulation of intestinal epithelial permeability by tight junctions. *Cell Mol Life Sci* 2013; **70**: 631-659 [PMID: 22782113 DOI: 10.1007/s00018-012-1070-x]

100   **Turner JR**. Intestinal mucosal barrier function in health and disease. *Nat Rev Immunol* 2009; **9**: 799-809 [PMID: 19855405 DOI: 10.1038/nri2653]

101   **Bach Knudsen KE**, Lærke HN, Hedemann MS, Nielsen TS, Ingerslev AK, Gundelund Nielsen DS, Theil PK, Purup S, Hald S, Schioldan AG, Marco ML, Gregersen S, Hermansen K. Impact of Diet-Modulated Butyrate Production on Intestinal Barrier Function and Inflammation. *Nutrients* 2018; **10** [PMID: 30322146 DOI: 10.3390/nu10101499]

102   **Weitkunat K**, Schumann S, Nickel D, Kappo KA, Petzke KJ, Kipp AP, Blaut M, Klaus S. Importance of propionate for the repression of hepatic lipogenesis and improvement of insulin sensitivity in high-fat diet-induced obesity. *Mol Nutr Food Res* 2016; **60**: 2611-2621 [PMID: 27467905 DOI: 10.1002/mnfr.201600305]

103   **Bindels LB**, Porporato P, Dewulf EM, Verrax J, Neyrinck AM, Martin JC, Scott KP, Buc Calderon P, Feron O, Muccioli GG, Sonveaux P, Cani PD, Delzenne NM. Gut microbiota-derived propionate reduces cancer cell proliferation in the liver. *Br J Cancer* 2012; **107**: 1337-1344 [PMID: 22976799 DOI: 10.1038/bjc.2012.409]

104   **Koh A**, De Vadder F, Kovatcheva-Datchary P, Bäckhed F. From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites. *Cell* 2016; **165**: 1332-1345 [PMID: 27259147 DOI: 10.1016/j.cell.2016.05.041]

105   **He J**, Zhang P, Shen L, Niu L, Tan Y, Chen L, Zhao Y, Bai L, Hao X, Li X, Zhang S, Zhu L. Short-Chain Fatty Acids and Their Association with Signalling Pathways in Inflammation, Glucose and Lipid Metabolism. *Int J Mol Sci* 2020; **21** [PMID: 32887215 DOI: 10.3390/ijms21176356]

106   **Milligan G**, Stoddart LA, Smith NJ. Agonism and allosterism: the pharmacology of the free fatty acid receptors FFA2 and FFA3. *Br J Pharmacol* 2009; **158**: 146-153 [PMID: 19719777 DOI: 10.1111/j.1476-5381.2009.00421.x]

107   **Hong YH**, Nishimura Y, Hishikawa D, Tsuzuki H, Miyahara H, Gotoh C, Choi KC, Feng DD, Chen C, Lee HG, Katoh K, Roh SG, Sasaki S. Acetate and propionate short chain fatty acids stimulate adipogenesis *via* GPCR43. *Endocrinology* 2005; **146**: 5092-5099 [PMID: 16123168 DOI: 10.1210/en.2005-0545]

108   **Forbes S**, Stafford S, Coope G, Heffron H, Real K, Newman R, Davenport R, Barnes M, Grosse J, Cox H. Selective FFA2 Agonism Appears to Act *via* Intestinal PYY to Reduce Transit and Food Intake but Does Not Improve Glucose Tolerance in Mouse Models. *Diabetes* 2015; **64**: 3763-3771 [DOI: 10.2337/db15-0481]

109   **Pirozzi C**, Francisco V, Guida FD, Gómez R, Lago F, Pino J, Meli R, Gualillo O. Butyrate Modulates Inflammation in Chondrocytes *via* GPR43 Receptor. *Cell Physiol Biochem* 2018; **51**: 228-243 [PMID: 30448827 DOI: 10.1159/000495203]

110   **Shimizu H**, Masujima Y, Ushiroda C, Mizushima R, Taira S, Ohue-Kitano R, Kimura I. Dietary short-chain fatty acid intake improves the hepatic metabolic condition *via* FFAR3. *Sci Rep* 2019; **9**: 16574 [PMID: 31719611 DOI: 10.1038/s41598-019-53242-x]

111   **Parada Venegas D**, De la Fuente MK, Landskron G, González MJ, Quera R, Dijkstra G, Harmsen HJM, Faber KN, Hermoso MA. Short Chain Fatty Acids (SCFAs)-Mediated Gut Epithelial and Immune Regulation and Its Relevance for Inflammatory Bowel Diseases. *Front Immunol* 2019; **10**: 277 [PMID: 30915065 DOI: 10.3389/fimmu.2019.00277]

112   **Fellows R**, Denizot J, Stellato C, Cuomo A, Jain P, Stoyanova E, Balázsi S, Hajnády Z, Liebert A, Kazakevych J, Blackburn H, Corrêa RO, Fachi JL, Sato FT, Ribeiro WR, Ferreira CM, Perée H, Spagnuolo M, Mattiuz R, Matolcsi C, Guedes J, Clark J, Veldhoen M, Bonaldi T, Vinolo MAR, Varga-Weisz P. Microbiota derived short chain fatty acids promote histone crotonylation in the colon through histone deacetylases. *Nat Commun* 2018; **9**: 105 [PMID: 29317660 DOI: 10.1038/s41467-017-02651-5]

113   **Halili MA**, Andrews MR, Sweet MJ, Fairlie DP. Histone deacetylase inhibitors in inflammatory disease. *Curr Top Med Chem* 2009; **9**: 309-319 [PMID: 19355993 DOI: 10.2174/156802609788085250]

114   **Feng Y**, Huang Y, Wang Y, Wang P, Song H, Wang F. Antibiotics induced intestinal tight junction barrier dysfunction is associated with microbiota dysbiosis, activated NLRP3 inflammasome and autophagy. *PLoS One* 2019; **14**: e0218384 [PMID: 31211803 DOI: 10.1371/journal.pone.0218384]

115   **Hasan N**, Yang H. Factors affecting the composition of the gut microbiota, and its modulation. *PeerJ* 2019; **7**: e7502 [PMID: 31440436 DOI: 10.7717/peerj.7502]

116   **Vrancken G**, Gregory AC, Huys GRB, Faust K, Raes J. Synthetic ecology of the human gut microbiota. *Nat Rev Microbiol* 2019; **17**: 754-763 [PMID: 31578461 DOI: 10.1038/s41579-019-0264-8]

117   **Lakhani SV**, Shah HN, Alexander K, Finelli FC, Kirkpatrick JR, Koch TR. Small intestinal bacterial overgrowth and thiamine deficiency after Roux-en-Y gastric bypass surgery in obese patients. *Nutr Res* 2008; **28**: 293-298 [PMID: 19083422 DOI: 10.1016/j.nutres.2008.03.002]

118   **Bouhnik Y**, Alain S, Attar A, Flourié B, Raskine L, Sanson-Le Pors MJ, Rambaud JC. Bacterial populations contaminating the upper gut in patients with small intestinal bacterial overgrowth syndrome. *Am J Gastroenterol* 1999; **94**: 1327-1331 [PMID: 10235214 DOI: 10.1111/j.1572-0241.1999.01016.x]

119   **Walker WA**. Role of the mucosal barrier in toxin/microbial attachment to the gastrointestinal tract. *Ciba Found Symp* 1985; **112**: 34-56 [PMID: 3891256 DOI: 10.1002/9780470720936.ch3]

120   **Pyleris E**, Giamarellos-Bourboulis EJ, Tzivras D, Koussoulas V, Barbatzas C, Pimentel M. The prevalence of overgrowth by aerobic bacteria in the small intestine by small bowel culture: relationship with irritable bowel syndrome. *Dig Dis Sci* 2012; **57**: 1321-1329 [PMID: 22262197 DOI: 10.1007/s10620-012-2033-7]

121   **Schnabl B**, Brenner DA. Interactions between the intestinal microbiome and liver diseases. *Gastroenterology* 2014; **146**: 1513-1524 [PMID: 24440671 DOI: 10.1053/j.gastro.2014.01.020]

122   **Molodecky NA**, Soon IS, Rabi DM, Ghali WA, Ferris M, Chernoff G, Benchimol EI, Panaccione R, Ghosh S, Barkema HW, Kaplan GG. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology* 2012; **142**: 46-54.e42; quiz e30 [PMID: 22001864 DOI: 10.1053/j.gastro.2011.10.001]

123   **Chelakkot C**, Ghim J, Ryu SH. Mechanisms regulating intestinal barrier integrity and its pathological implications. *Exp Mol Med* 2018; **50**: 1-9 [PMID: 30115904 DOI: 10.1038/s12276-018-0126-x]

124   **Grishin A**, Papillon S, Bell B, Wang J, Ford HR. The role of the intestinal microbiota in the pathogenesis of necrotizing enterocolitis. *Semin Pediatr Surg* 2013; **22**: 69-75 [PMID: 23611609 DOI: 10.1053/j.sempedsurg.2013.01.002]

125   **Torrazza RM**, Ukhanova M, Wang X, Sharma R, Hudak ML, Neu J, Mai V. Intestinal microbial ecology and environmental factors affecting necrotizing enterocolitis. *PLoS One* 2013; **8**: e83304 [PMID: 24386174 DOI: 10.1371/journal.pone.0083304]

126   **Wehkamp J**, Stange EF. An Update Review on the Paneth Cell as Key to Ileal Crohn's Disease. *Front Immunol* 2020; **11**: 646 [PMID: 32351509 DOI: 10.3389/fimmu.2020.00646]

127   **Ehmann D**, Wendler J, Koeninger L, Larsen IS, Klag T, Berger J, Marette A, Schaller M, Stange EF, Malek NP, Jensen BAH, Wehkamp J. Paneth cell α-defensins HD-5 and HD-6 display differential degradation into active antimicrobial fragments. *Proc Natl Acad Sci U S A* 2019; **116**: 3746-3751 [PMID: 30808760 DOI: 10.1073/pnas.1817376116]

128   **Chu H**, Pazgier M, Jung G, Nuccio SP, Castillo PA, de Jong MF, Winter MG, Winter SE, Wehkamp J, Shen B, Salzman NH, Underwood MA, Tsolis RM, Young GM, Lu W, Lehrer RI, Bäumler AJ, Bevins CL. Human α-defensin 6 promotes mucosal innate immunity through self-assembled peptide nanonets. *Science* 2012; **337**: 477-481 [PMID: 22722251 DOI: 10.1126/science.1218831]

129   **Wehkamp J**, Wang G, Kübler I, Nuding S, Gregorieff A, Schnabel A, Kays RJ, Fellermann K, Burk O, Schwab M, Clevers H, Bevins CL, Stange EF. The Paneth cell alpha-defensin deficiency of ileal Crohn's disease is linked to Wnt/Tcf-4. *J Immunol* 2007; **179**: 3109-3118 [PMID: 17709525 DOI: 10.4049/jimmunol.179.5.3109]

130   **Wehkamp J**, Stange EF. Paneth's disease. *J Crohns Colitis* 2010; **4**: 523-531 [PMID: 21122555 DOI: 10.1016/j.crohns.2010.05.010]

131   **Riba A**, Olier M, Lacroix-Lamandé S, Lencina C, Bacquié V, Harkat C, Gillet M, Baron M, Sommer C, Mallet V, Salvador-Cartier C, Laurent F, Théodorou V, Ménard S. Paneth Cell Defects Induce Microbiota Dysbiosis in Mice and Promote Visceral Hypersensitivity. *Gastroenterology* 2017; **153**: 1594-1606.e2 [PMID: 28865734 DOI: 10.1053/j.gastro.2017.08.044]

132   **Jahnel J**, Fickert P, Hauer AC, Högenauer C, Avian A, Trauner M. Inflammatory bowel disease alters intestinal bile acid transporter expression. *Drug Metab Dispos* 2014; **42**: 1423-1431 [PMID: 24965812 DOI: 10.1124/dmd.114.058065]

133   **Yiu JH**, Dorweiler B, Woo CW. Interaction between gut microbiota and toll-like receptor: from immunity to metabolism. *J Mol Med (Berl)* 2017; **95**: 13-20 [PMID: 27639584 DOI: 10.1007/s00109-016-1474-4]

134   **Milosevic I**, Vujovic A, Barac A, Djelic M, Korac M, Radovanovic Spurnic A, Gmizic I, Stevanovic O, Djordjevic V, Lekic N, Russo E, Amedei A. Gut-Liver Axis, Gut Microbiota, and Its Modulation in the Management of Liver Diseases: A Review of the Literature. *Int J Mol Sci* 2019; **20** [PMID: 30658519 DOI: 10.3390/ijms20020395]

135   **Payne CM**, Weber C, Crowley-Skillicorn C, Dvorak K, Bernstein H, Bernstein C, Holubec H, Dvorakova B, Garewal H. Deoxycholate induces mitochondrial oxidative stress and activates NF-kappaB through multiple mechanisms in HCT-116 colon epithelial cells. *Carcinogenesis* 2007; **28**: 215-222 [PMID: 16887864 DOI: 10.1093/carcin/bgl139]

136   **Ohtani N**, Kawada N. Role of the Gut-Liver Axis in Liver Inflammation, Fibrosis, and Cancer: A Special Focus on the Gut Microbiota Relationship. *Hepatol Commun* 2019; **3**: 456-470 [PMID: 30976737 DOI: 10.1002/hep4.1331]

137   **Tsuchida T**, Friedman SL. Mechanisms of hepatic stellate cell activation. *Nat Rev Gastroenterol Hepatol* 2017; **14**: 397-411 [PMID: 28487545 DOI: 10.1038/nrgastro.2017.38]

138   **Wandzioch E**, Kolterud A, Jacobsson M, Friedman SL, Carlsson L. Lhx2-/- mice develop liver fibrosis. *Proc Natl Acad Sci U S A* 2004; **101**: 16549-16554 [PMID: 15536133 DOI: 10.1073/pnas.0404678101]

139   **Tang Y**, Zhou J, Hooi SC, Jiang YM, Lu GD. Fatty acid activation in carcinogenesis and cancer development: Essential roles of long-chain acyl-CoA synthetases. *Oncol Lett* 2018; **16**: 1390-1396 [PMID: 30008815 DOI: 10.3892/ol.2018.8843]

140   **Hanahan D**, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**: 646-674 [PMID: 21376230 DOI: 10.1016/j.cell.2011.02.013]

141   **Warburg O**, Wind F, Negelein E. THE METABOLISM OF TUMORS IN THE BODY. *J Gen Physiol* 1927; **8**: 519-530 [PMID: 19872213 DOI: 10.1085/jgp.8.6.519]

142   **Vander Heiden MG**, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* 2009; **324**: 1029-1033 [PMID: 19460998 DOI: 10.1126/science.1160809]

143   **Pakiet A**, Kobiela J, Stepnowski P, Sledzinski T, Mika A. Changes in lipids composition and metabolism in colorectal cancer: a review. *Lipids Health Dis* 2019; **18**: 29 [PMID: 30684960 DOI: 10.1186/s12944-019-0977-8]

144   **Chekulayev V**, Mado K, Shevchuk I, Koit A, Kaldma A, Klepinin A, Timohhina N, Tepp K, Kandashvili M, Ounpuu L, Heck K, Truu L, Planken A, Valvere V, Kaambre T. Metabolic remodeling in human colorectal cancer and surrounding tissues: alterations in regulation of mitochondrial respiration and metabolic fluxes. *Biochem Biophys Rep* 2015; **4**: 111-125 [PMID: 29124194 DOI: 10.1016/j.bbrep.2015.08.020]

145   **Satoh K**, Yachida S, Sugimoto M, Oshima M, Nakagawa T, Akamoto S, Tabata S, Saitoh K, Kato K, Sato S, Igarashi K, Aizawa Y, Kajino-Sakamoto R, Kojima Y, Fujishita T, Enomoto A, Hirayama A, Ishikawa T, Taketo MM, Kushida Y, Haba R, Okano K, Tomita M, Suzuki Y, Fukuda S, Aoki M, Soga T. Global metabolic reprogramming of colorectal cancer occurs at adenoma stage and is induced by MYC. *Proc Natl Acad Sci U S A* 2017; **114**: E7697-E7706 [PMID: 28847964 DOI: 10.1073/pnas.1710366114]

146   **Fu Y**, Liu S, Yin S, Niu W, Xiong W, Tan M, Li G, Zhou M. The reverse Warburg effect is likely to be an Achilles' heel of cancer that can be exploited for cancer therapy. *Oncotarget* 2017; **8**: 57813-57825 [PMID: 28915713 DOI: 10.18632/oncotarget.18175]

147   **DeBerardinis RJ**, Mancuso A, Daikhin E, Nissim I, Yudkoff M, Wehrli S, Thompson CB. Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. *Proc Natl Acad Sci U S A* 2007; **104**: 19345-19350 [PMID: 18032601 DOI: 10.1073/pnas.0709747104]

148   **Locasale JW**, Grassian AR, Melman T, Lyssiotis CA, Mattaini KR, Bass AJ, Heffron G, Metallo CM, Muranen T, Sharfi H, Sasaki AT, Anastasiou D, Mullarky E, Vokes NI, Sasaki M, Beroukhim R, Stephanopoulos G, Ligon AH, Meyerson M, Richardson AL, Chin L, Wagner G, Asara JM, Brugge JS, Cantley LC, Vander Heiden MG. Phosphoglycerate dehydrogenase diverts glycolytic flux and contributes to oncogenesis. *Nat Genet* 2011; **43**: 869-874 [PMID: 21804546 DOI: 10.1038/ng.890]

149   **Possemato R**, Marks KM, Shaul YD, Pacold ME, Kim D, Birsoy K, Sethumadhavan S, Woo HK, Jang HG, Jha AK, Chen WW, Barrett FG, Stransky N, Tsun ZY, Cowley GS, Barretina J, Kalaany NY, Hsu PP, Ottina K, Chan AM, Yuan B, Garraway LA, Root DE, Mino-Kenudson M, Brachtel EF, Driggers EM, Sabatini DM. Functional genomics reveal that the serine synthesis pathway is essential in breast cancer. *Nature* 2011; **476**: 346-350 [PMID: 21760589 DOI: 10.1038/nature10350]

150   **Le A**, Lane AN, Hamaker M, Bose S, Gouw A, Barbi J, Tsukamoto T, Rojas CJ, Slusher BS, Zhang H, Zimmerman LJ, Liebler DC, Slebos RJ, Lorkiewicz PK, Higashi RM, Fan TW, Dang CV. Glucose-independent glutamine metabolism *via* TCA cycling for proliferation and survival in B cells. *Cell Metab* 2012; **15**: 110-121 [PMID: 22225880 DOI: 10.1016/j.cmet.2011.12.009]

151   **Carracedo A**, Cantley LC, Pandolfi PP. Cancer metabolism: fatty acid oxidation in the limelight. *Nat Rev Cancer* 2013; **13**: 227-232 [PMID: 23446547 DOI: 10.1038/nrc3483]

152   **Counihan JL**, Grossman EA, Nomura DK. Cancer Metabolism: Current Understanding and Therapies. *Chem Rev* 2018; **118**: 6893-6923 [PMID: 29939018 DOI: 10.1021/acs.chemrev.7b00775]

153   **Ookhtens M**, Kannan R, Lyon I, Baker N. Liver and adipose tissue contributions to newly formed fatty acids in an ascites tumor. *Am J Physiol* 1984; **247**: R146-R153 [PMID: 6742224 DOI: 10.1152/ajpregu.1984.247.1.R146]

154   **Tadesse S**, Corner G, Dhima E, Houston M, Guha C, Augenlicht L, Velcich A. MUC2 mucin deficiency alters inflammatory and metabolic pathways in the mouse intestinal mucosa. *Oncotarget* 2017; **8**: 71456-71470 [PMID: 29069719 DOI: 10.18632/oncotarget.16886]

155   **Sánchez-Martínez R**, Cruz-Gil S, García-Álvarez MS, Reglero G, Ramírez de Molina A. Complementary ACSL isoforms contribute to a non-Warburg advantageous energetic status characterizing invasive colon cancer cells. *Sci Rep* 2017; **7**: 11143 [PMID: 28894242 DOI: 10.1038/s41598-017-11612-3]

156   **Sánchez-Martínez R**, Cruz-Gil S, Gómez de Cedrón M, Álvarez-Fernández M, Vargas T, Molina S, García B, Herranz J, Moreno-Rubio J, Reglero G, Pérez-Moreno M, Feliu J, Malumbres M, Ramírez de Molina A. A link between lipid metabolism and epithelial-mesenchymal transition provides a target for colon cancer therapy. *Oncotarget* 2015; **6**: 38719-38736 [PMID: 26451612 DOI: 10.18632/oncotarget.5340]

157   **Cui M**, Wang Y, Sun B, Xiao Z, Ye L, Zhang X. MiR-205 modulates abnormal lipid metabolism of hepatoma cells *via* targeting acyl-CoA synthetase long-chain family member 1 (ACSL1) mRNA. *Biochem Biophys Res Commun* 2014; **444**: 270-275 [PMID: 24462768 DOI: 10.1016/j.bbrc.2014.01.051]

158   **Cui M**, Xiao Z, Wang Y, Zheng M, Song T, Cai X, Sun B, Ye L, Zhang X. Long noncoding RNA HULC modulates abnormal lipid metabolism in hepatoma cells through an miR-9-mediated RXRA signaling pathway. *Cancer Res* 2015; **75**: 846-857 [PMID: 25592151 DOI: 10.1158/0008-5472.CAN-14-1192]

159   **Vargas T**, Moreno-Rubio J, Herranz J, Cejas P, Molina S, Mendiola M, Burgos E, Custodio AB, De Miguel M, Martín-Hernández R, Reglero G, Feliu J, Ramírez de Molina A. 3'UTR Polymorphism in ACSL1 Gene Correlates with Expression Levels and Poor Clinical Outcome in Colon Cancer Patients. *PLoS One* 2016; **11**: e0168423 [PMID: 27992526 DOI: 10.1371/journal.pone.0168423]

160   **Cao Y**, Dave KB, Doan TP, Prescott SM. Fatty acid CoA ligase 4 is up-regulated in colon adenocarcinoma. *Cancer Res* 2001; **61**: 8429-8434 [PMID: 11731423]

161   **Xia H**, Lee KW, Chen J, Kong SN, Sekar K, Deivasigamani A, Seshachalam VP, Goh BKP, Ooi LL, Hui KM. Simultaneous silencing of ACSL4 and induction of GADD45B in hepatocellular carcinoma cells amplifies the synergistic therapeutic effect of aspirin and sorafenib. *Cell Death Discov* 2017; **3**: 17058 [PMID: 28900541 DOI: 10.1038/cddiscovery.2017.58]

162   **Sung YK**, Hwang SY, Park MK, Bae HI, Kim WH, Kim JC, Kim M. Fatty acid-CoA ligase 4 is overexpressed in human hepatocellular carcinoma. *Cancer Sci* 2003; **94**: 421-424 [PMID: 12824887 DOI: 10.1111/j.1349-7006.2003.tb01458.x]

163   **Hu C**, Chen L, Jiang Y, Li Y, Wang S. The effect of fatty acid-CoA ligase 4 on the growth of hepatic cancer cells. *Cancer Biol Ther* 2008; **7**: 131-134 [PMID: 18059177 DOI: 10.4161/cbt.7.1.5198]

164   **Ndiaye H**, Liu JY, Hall A, Minogue S, Morgan MY, Waugh MG. Immunohistochemical staining reveals differential expression of ACSL3 and ACSL4 in hepatocellular carcinoma and hepatic gastrointestinal metastases. *Biosci Rep* 2020; **40** [PMID: 32286604 DOI: 10.1042/BSR20200219]

165   **Padanad MS**, Konstantinidou G, Venkateswaran N, Melegari M, Rindhe S, Mitsche M, Yang C, Batten K, Huffman KE, Liu J, Tang X, Rodriguez-Canales J, Kalhor N, Shay JW, Minna JD,

McDonald J, Wistuba II, DeBerardinis RJ, Scaglioni PP. Fatty Acid Oxidation Mediated by Acyl-CoA Synthetase Long Chain 3 Is Required for Mutant KRAS Lung Tumorigenesis. *Cell Rep* 2016; **16**: 1614-1628 [PMID: 27477280 DOI: 10.1016/j.celrep.2016.07.009]

166 **Saliakoura M**, Reynoso-Moreno I, Pozzato C, Rossi Sebastiano M, Galié M, Gertsch J, Konstantinidou G. The ACSL3-LPIAT1 signaling drives prostaglandin synthesis in non-small cell lung cancer. *Oncogene* 2020; **39**: 2948-2960 [PMID: 32034305 DOI: 10.1038/s41388-020-1196-5]

167 **Chen J**, Ding C, Chen Y, Hu W, Lu Y, Wu W, Zhang Y, Yang B, Wu H, Peng C, Xie H, Zhou L, Wu J, Zheng S. ACSL4 promotes hepatocellular carcinoma progression *via* c-Myc stability mediated by ERK/FBW7/c-Myc axis. *Oncogenesis* 2020; **9**: 42 [PMID: 32350243 DOI: 10.1038/s41389-020-0226-z]

168 **Doll S**, Proneth B, Tyurina YY, Panzilius E, Kobayashi S, Ingold I, Irmler M, Beckers J, Aichler M, Walch A, Prokisch H, Trümbach D, Mao G, Qu F, Bayir H, Füllekrug J, Scheel CH, Wurst W, Schick JA, Kagan VE, Angeli JP, Conrad M. ACSL4 dictates ferroptosis sensitivity by shaping cellular lipid composition. *Nat Chem Biol* 2017; **13**: 91-98 [PMID: 27842070 DOI: 10.1038/nchembio.2239]

169 **Gassler N**, Schneider A, Kopitz J, Schnölzer M, Obermüller N, Kartenbeck J, Otto HF, Autschbach F. Impaired expression of acyl-CoA-synthetase 5 in epithelial tumors of the small intestine. *Hum Pathol* 2003; **34**: 1048-1052 [PMID: 14608540 DOI: 10.1053/s0046-8177(03)00431-3]

170 **Gassler N**, Roth W, Funke B, Schneider A, Herzog F, Tischendorf JJ, Grund K, Penzel R, Bravo IG, Mariadason J, Ehemann V, Sykora J, Haas TL, Walczak H, Ganten T, Zentgraf H, Erb P, Alonso A, Autschbach F, Schirmacher P, Knüchel R, Kopitz J. Regulation of enterocyte apoptosis by acyl-CoA synthetase 5 splicing. *Gastroenterology* 2007; **133**: 587-598 [PMID: 17681178 DOI: 10.1053/j.gastro.2007.06.005]

171 **Gao X**, Lin SH, Ren F, Li JT, Chen JJ, Yao CB, Yang HB, Jiang SX, Yan GQ, Wang D, Wang Y, Liu Y, Cai Z, Xu YY, Chen J, Yu W, Yang PY, Lei QY. Acetate functions as an epigenetic metabolite to promote lipid synthesis under hypoxia. *Nat Commun* 2016; **7**: 11960 [PMID: 27357947 DOI: 10.1038/ncomms11960]

172 **Gharib E**, Nasri Nasrabadi P, Reza Zali M. miR-497-5p mediates starvation-induced death in colon cancer cells by targeting acyl-CoA synthetase-5 and modulation of lipid metabolism. *J Cell Physiol* 2020; **235**: 5570-5589 [PMID: 32012265 DOI: 10.1002/jcp.29488]

173 **Cui M**, Xiao Z, Sun B, Wang Y, Zheng M, Ye L, Zhang X. Involvement of cholesterol in hepatitis B virus X protein-induced abnormal lipid metabolism of hepatoma cells *via* up-regulating miR-205-targeted ACSL4. *Biochem Biophys Res Commun* 2014; **445**: 651-655 [PMID: 24576478 DOI: 10.1016/j.bbrc.2014.02.068]

174 **Qin X**, Zhang J, Lin Y, Sun XM, Zhang JN, Cheng ZQ. Identification of MiR-211-5p as a tumor suppressor by targeting ACSL4 in Hepatocellular Carcinoma. *J Transl Med* 2020; **18**: 326 [PMID: 32859232 DOI: 10.1186/s12967-020-02494-7]

175 **Cruz-Gil S**, Sanchez-Martinez R, Gomez de Cedron M, Martin-Hernandez R, Vargas T, Molina S, Herranz J, Davalos A, Reglero G, Ramirez de Molina A. Targeting the lipid metabolic axis *ACSL/SCD* in colorectal cancer progression by therapeutic miRNAs: miR-19b-1 role. *J Lipid Res* 2018; **59**: 14-24 [PMID: 29074607 DOI: 10.1194/jlr.M076752]

176 **Shen WW**, Zeng Z, Zhu WX, Fu GH. MiR-142-3p functions as a tumor suppressor by targeting CD133, ABCG2, and Lgr5 in colon cancer cells. *J Mol Med (Berl)* 2013; **91**: 989-1000 [PMID: 23619912 DOI: 10.1007/s00109-013-1037-x]

177 **Li B**, Liu J, Xin X, Zhang L, Zhou J, Xia C, Zhu W, Yu H. MiR-34c promotes hepatic stellate cell activation and Liver Fibrogenesis by suppressing ACSL1 expression. *Int J Med Sci* 2021; **18**: 615-625 [PMID: 33437196 DOI: 10.7150/ijms.51589]

178 **Tomoda H**, Igarashi K, Omura S. Inhibition of acyl-CoA synthetase by triacsins. *Biochim Biophys Acta* 1987; **921**: 595-598 [PMID: 3117118]

179 **Kaemmerer E**, Peuscher A, Reinartz A, Liedtke C, Weiskirchen R, Kopitz J, Gassler N. Human intestinal acyl-CoA synthetase 5 is sensitive to the inhibitor triacsin C. *World J Gastroenterol* 2011; **17**: 4883-4889 [PMID: 22171129 DOI: 10.3748/wjg.v17.i44.4883]

180 **Van Horn CG**, Caviglia JM, Li LO, Wang S, Granger DA, Coleman RA. Characterization of recombinant long-chain rat acyl-CoA synthetase isoforms 3 and 6: identification of a novel variant of isoform 6. *Biochemistry* 2005; **44**: 1635-1642 [PMID: 15683247 DOI: 10.1021/bi0477211]

181 **Kim JH**, Lewin TM, Coleman RA. Expression and characterization of recombinant rat Acyl-CoA synthetases 1, 4, and 5. Selective inhibition by triacsin C and thiazolidinediones. *J Biol Chem* 2001; **276**: 24667-24673 [PMID: 11319222 DOI: 10.1074/jbc.M010793200]

182 **Askari B**, Kanter JE, Sherrid AM, Golej DL, Bender AT, Liu J, Hsueh WA, Beavo JA, Coleman RA, Bornfeldt KE. Rosiglitazone inhibits acyl-CoA synthetase activity and fatty acid partitioning to diacylglycerol and triacylglycerol *via* a peroxisome proliferator-activated receptor-gamma-independent mechanism in human arterial smooth muscle cells and macrophages. *Diabetes* 2007; **56**: 1143-1152 [PMID: 17259370 DOI: 10.2337/db06-0267]

183 **Weng JR**, Chen CY, Pinzone JJ, Ringel MD, Chen CS. Beyond peroxisome proliferator-activated receptor gamma signaling: the multi-facets of the antitumor effect of thiazolidinediones. *Endocr Relat Cancer* 2006; **13**: 401-413 [PMID: 16728570 DOI: 10.1677/erc.1.01182]

184 **Matsufuji T**, Ikeda M, Naito A, Hirouchi M, Kanda S, Izumi M, Harada J, Shinozuka T. Arylpiperazines as fatty acid transport protein 1 (FATP1) inhibitors with improved potency and pharmacokinetic properties. *Bioorg Med Chem Lett* 2013; **23**: 2560-2565 [PMID: 23528296 DOI:

10.1016/j.bmcl.2013.02.116]

185   **Blackburn C**, Guan B, Brown J, Cullis C, Condon SM, Jenkins TJ, Peluso S, Ye Y, Gimeno RE, Punreddy S, Sun Y, Wu H, Hubbard B, Kaushik V, Tummino P, Sanchetti P, Yu Sun D, Daniels T, Tozzo E, Balani SK, Raman P. Identification and characterization of 4-aryl-3,4-dihydropyrimidin-2(1H)-ones as inhibitors of the fatty acid transporter FATP4. *Bioorg Med Chem Lett* 2006; **16**: 3504-3509 [PMID: 16644217 DOI: 10.1016/j.bmcl.2006.03.102]

186   **Black PN**, Ahowesso C, Montefusco D, Saini N, DiRusso CC. Fatty Acid Transport Proteins: Targeting FATP2 as a Gatekeeper Involved in the Transport of Exogenous Fatty Acids. *Medchemcomm* 2016; **7**: 612-622 [PMID: 27446528 DOI: 10.1039/C6MD00043F]

187   **Uchiyama A**, Aoyama T, Kamijo K, Uchida Y, Kondo N, Orii T, Hashimoto T. Molecular cloning of cDNA encoding rat very long-chain acyl-CoA synthetase. *J Biol Chem* 1996; **271**: 30360-30365 [PMID: 8939997 DOI: 10.1074/jbc.271.48.30360]

188   **Wiśniewski JR**, Friedrich A, Keller T, Mann M, Koepsell H. The impact of high-fat diet on metabolism and immune defense in small intestine mucosa. *J Proteome Res* 2015; **14**: 353-365 [PMID: 25285821 DOI: 10.1021/pr500833v]

189   **van den Bosch HM**, Bünger M, de Groot PJ, van der Meijde J, Hooiveld GJ, Müller M. Gene expression of transporters and phase I/II metabolic enzymes in murine small intestine during fasting. *BMC Genomics* 2007; **8**: 267 [PMID: 17683626 DOI: 10.1186/1471-2164-8-267]

190   **Qiu P**, Wang H, Zhang M, Peng R, Zhao Q, Liu J. FATP2-targeted therapies - A role beyond fatty liver disease. *Pharmacol Res* 2020; **161**: 105228 [PMID: 33027714 DOI: 10.1016/j.phrs.2020.105228]

191   **Leamy AK**, Egnatchik RA, Young JD. Molecular mechanisms and the role of saturated fatty acids in the progression of non-alcoholic fatty liver disease. *Prog Lipid Res* 2013; **52**: 165-174 [PMID: 23178552 DOI: 10.1016/j.plipres.2012.10.004]

192   **Arias-Barrau E**, Dirusso CC, Black PN. Methods to monitor Fatty Acid transport proceeding through vectorial acylation. *Methods Mol Biol* 2009; **580**: 233-249 [PMID: 19784603 DOI: 10.1007/978-1-60761-325-1_13]

193   **Sandoval A**, Chokshi A, Jesch ED, Black PN, Dirusso CC. Identification and characterization of small compound inhibitors of human FATP2. *Biochem Pharmacol* 2010; **79**: 990-999 [PMID: 19913517 DOI: 10.1016/j.bcp.2009.11.008]

194   **Veglia F**, Tyurin VA, Blasi M, De Leo A, Kossenkov AV, Donthireddy L, To TKJ, Schug Z, Basu S, Wang F, Ricciotti E, DiRusso C, Murphy ME, Vonderheide RH, Lieberman PM, Mulligan C, Nam B, Hockstein N, Masters G, Guarino M, Lin C, Nefedova Y, Black P, Kagan VE, Gabrilovich DI. Fatty acid transport protein 2 reprograms neutrophils in cancer. *Nature* 2019; **569**: 73-78 [PMID: 30996346 DOI: 10.1038/s41586-019-1118-2]

195   **Calder PC**. Polyunsaturated fatty acids and inflammation. *Biochem Soc Trans* 2005; **33**: 423-427 [PMID: 15787620 DOI: 10.1042/BST0330423]

196   **Saini N**, Black PN, Montefusco D, DiRusso CC. Fatty acid transport protein-2 inhibitor Grassofermata/CB5 protects cells against lipid accumulation and toxicity. *Biochem Biophys Res Commun* 2015; **465**: 534-541 [PMID: 26284975 DOI: 10.1016/j.bbrc.2015.08.055]

197   **Zhou W**, Madrid P, Fluitt A, Stahl A, Xie XS. Development and validation of a high-throughput screening assay for human long-chain fatty acid transport proteins 4 and 5. *J Biomol Screen* 2010; **15**: 488-497 [PMID: 20448275 DOI: 10.1177/1087057110369700]

198   **Martin G**, Schoonjans K, Lefebvre AM, Staels B, Auwerx J. Coordinate regulation of the expression of the fatty acid transport protein and acyl-CoA synthetase genes by PPARalpha and PPARgamma activators. *J Biol Chem* 1997; **272**: 28210-28217 [PMID: 9353271 DOI: 10.1074/jbc.272.45.28210]

199   **Schoonjans K**, Watanabe M, Suzuki H, Mahfoudi A, Krey G, Wahli W, Grimaldi P, Staels B, Yamamoto T, Auwerx J. Induction of the acyl-coenzyme A synthetase gene by fibrates and fatty acids is mediated by a peroxisome proliferator response element in the C promoter. *J Biol Chem* 1995; **270**: 19269-19276 [PMID: 7642600 DOI: 10.1074/jbc.270.33.19269]

200   **Björnson E**, Mukhopadhyay B, Asplund A, Pristovsek N, Cinar R, Romeo S, Uhlen M, Kunos G, Nielsen J, Mardinoglu A. Stratification of Hepatocellular Carcinoma Patients Based on Acetate Utilization. *Cell Rep* 2015; **13**: 2014-2026 [PMID: 26655911 DOI: 10.1016/j.celrep.2015.10.045]

201   **Min W**, Li Y, Zhang Y, Dai D, Cao Y, Yue Z, Liu J. Role of the anti-glioma drug AT13148 in the inhibition of Notch signaling pathway. *Gene* 2015; **573**: 153-159 [PMID: 26187072 DOI: 10.1016/j.gene.2015.07.033]

REVIEW

Liver involvement in inflammatory bowel disease: What should the clinician know?

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Abstract

Inflammatory bowel disease (IBD) may show a wide range of extraintestinal manifestations. In this context, liver involvement is a focal point for both an adequate management of the disease and its prognosis, due to possible serious comorbidity. The association between IBD and primary sclerosing cholangitis is the most known example. This association is relevant because it implies an increased risk of both colorectal cancer and cholangiocarcinoma. Additionally, drugs such as thiopurines or biologic agents can cause drug-induced liver damage; therefore, this event should be considered when planning IBD treatment. Additionally, particular consideration should be given to the evidence that IBD patients may have concomitant chronic viral hepatitis, such as hepatitis B and hepatitis C. Chronic immunosuppressive regimens may cause a hepatitis flare or reactivation of a healthy carrier state, therefore careful monitoring of these patients is necessary. Finally, the spread of obesity has involved even IBD patients, thus increasing the risk of non-alcoholic fatty liver disease, which has already proven to be more common in IBD patients than in the non-IBD population. This phenomenon is considered an emerging issue, as it will become the leading cause of liver cirrhosis.

Key Words: Inflammatory bowel disease; Liver; Primary sclerosing cholangitis; Viral hepatitis; Immunosuppression; Non-alcoholic fatty liver disease

Core Tip: In the present article, several aspects of liver involvement of inflammatory bowel disease (IBD) have been highlighted. Co-occurrence of primary sclerosing

cholangitis is one of the most well-known comorbidities and deserves more attention by the clinician. Liver damage due to drugs used to cure IBD is also a relevant issue. Finally, some emerging topics such as the spread of liver steatosis or the implications of chronic viral hepatitis have been analyzed.

INTRODUCTION

Inflammatory bowel disease (IBD) consists of two separate disease entities, ulcerative colitis (UC) and Crohn's disease (CD), affecting the gastrointestinal tract[1]. However, IBD does not exclusively affect the gut. The gut-liver axis refers to the bidirectional relationship between the gut and its microbiota, and the liver, resulting from the integration of signals generated by dietary, genetic and environmental factors[2]. Therefore, a perturbation of this axis may mirror pathologic conditions both in the gut and the liver. Based on this consideration, the relationships between IBD and liver disorders are noteworthy and should always be considered by the clinician. The association between IBD and primary sclerosing cholangitis (PSC) is the most known and studied model, as it has several implications, the most important ones are the increased risk of both colorectal cancer and cholangiocarcinoma. Additionally, hepato-toxicity due to drugs such as thiopurines or biologic drugs is a relevant issue that should also be taken into account when planning IBD treatment[3]. It should not be forgotten that IBD patients may have concomitant chronic viral hepatitis, such as hepatitis B (HBV) and hepatitis C (HCV)[3]. Chronic immunosuppressive regimens may cause a hepatitis flare or reactivation of a healthy carrier state; therefore, careful monitoring of these patients is necessary. Finally, the obesity epidemic has involved even IBD patients, thus increasing the risk of non-alcoholic fatty liver disease (NAFLD), which has already proven to be higher than the control population in IBD patients[3]. This phenomenon is considered an emerging issue, as it will become the leading cause of liver cirrhosis.

Therefore, we aimed to perform a narrative review describing the main interactions between IBD and corresponding liver involvement, with a particular focus on PSC and other autoimmune liver disorders, drug-induced hepatitis, HBV, HCV and NAFLD (Table 1).

IBD AND PRIMARY SCLEROSING CHOLANGITIS

IBD and PSC are two pathologic entities that can occur alone or in combination. In this case they create a phenotypically different disease known as PSC-IBD. PSC-IBD prevalence is uncertain and differs in several studies, but it is agreed that it is very low (0.024%-0.041%)[4-6]. PSC and IBD may occur simultaneously or sequentially. Indeed, PSC patients develop IBD in 20%-70% of cases, with a stronger association with UC (80%) than with CD (10%) and indeterminate colitis (IC) (10%)[7]. Conversely only 5% of patients with UC show concomitant PSC.

Primary Sclerosing Cholangitis and Ulcerative Colitis

UC represents the underlying IBD in most cases of PSC-IBD. In patients with PSC and UC (PSC-UC), UC characteristically tends to be mild, quiescent and may even appear endoscopically normal (in this case, the diagnosis is based simply on histological analysis)[8]. Therefore, random biopsies during the first colonoscopy should always be performed to reveal an underlying UC in patients with PSC. Similarly, PSC may be underdiagnosed in patients with UC, as it can be asymptomatic. Thus, liver function tests, including cholestatic and hepatocellular damage markers, should always be recommended in the follow-up of UC. If a patient with UC is found to have hepato-cellular injury or a cholestatic pattern, magnetic resonance cholangiopancreatography

Table 1 Main liver comorbidities associated with inflammatory bowel disease		
Associated diseases	Prevalence in IBD (%)	Notes
PSC	0.024-0.041	Higher risk of cholangiocarcinoma and colorectal cancer; IBD shows less severe lesions than IBD alone
NAFLD	20-30	Associated with the use of corticosteroids, long disease duration, severe disease course; Associated with metabolic syndrome
Viral hepatitis	1-9	More common in the elderly; Association with advanced liver fibrosis; Need for anti-viral treatment before starting immunosuppressive drugs; HBV vaccine recommended

HBV: Hepatitis B virus; NAFLD: Non-alcoholic fatty liver disease; PSC: Primary sclerosing cholangitis; IBD: Inflammatory bowel disease.

(MRCP) should be performed to confirm the diagnosis[9]. The onset of the two disorders may vary. Typically, UC occurs first, with a median time interval of 10 years [10]. Nevertheless, in a minority of cases, UC may appear some years after the diagnosis of PSC, even after orthotopic liver transplantation[11]. The degree and the extension of colorectal inflammation in PSC-UC differ from UC alone. Indeed, the incidence of pancolitis appears increased in PSC-UC patients when compared with UC-only patients, as shown by Boonstra *et al*[12] In their series, PSC-UC patients were affected by pancolitis in 94% of cases, while pancolitis was demonstrated only in 62% of patients affected by UC alone. Patients with PSC-UC usually have a greater prevalence of backwash ileitis and rectal sparing (51% and 52%, respectively) than controls with UC alone (7% and 6%, respectively)[13]. However, the mild degree of colitis and the low rate of endoscopically visible inflammation may overestimate rectal sparing, when random biopsies are not performed[12,14]. Even though, the extension of colitis tends to be more diffuse, and in PSC-UC the severity of the mucosal inflammation seems less pronounced. Patients with PSC-UC have less significant bowel symptoms, a lower need for steroids and undergo fewer hospitalizations than patients with UC alone[15].

Primary Sclerosing Cholangitis and Crohn's Disease
Similar to patients affected by PSC-UC, patients with PSC and CD (PSC-CD) have a phenotypical and clinical pattern that sharply differs from patients with CD alone. Indeed, isolated ileal involvement, which occurs in about 30% of patients affected with CD, is rare in patients with PSC-CD (2%-5%)[12,16]. As shown with PSC-UC, the degree of endoscopically visible inflammation is milder in patient with PSC-CD than in those affected by UC. Likewise, the incidence of CD complications seems low in PSC-CD[12,16,17].

Main characteristics of PSC in IBD
While IBD in PSC-IBD has specific phenotypical patterns as listed above, PSC does not show significant differences in terms of histologic findings such as periductal fibrosis, inflammation and portal edema or fibrosis[18]. From a clinical point of view, according to Yanai *et al*[19] PSC outcomes, including cirrhosis incidence and transplant-free survival, did not differ in PSC-IBD compared with PSC alone patients. Conversely, Fevery *et al*[20] reported higher rates of liver-related death and malignancies in patients with PSC-UC when compared to patients with PSC-CD. Interestingly, Nordenvall *et al*[21] found that patients with PSC-UC who underwent colectomy, seemed to have a lower risk of mortality, morbidity and the need for liver transplantation.

Risk of colorectal cancer (CRC) and hepatobiliary carcinomas in PSC-IBD
Although both PSC and IBD patients do not have a general higher risk of malignancies than the general population, patients with PSC-IBD show a significantly more marked risk of developing colorectal carcinoma (CRC) and cholangiocarcinoma (CCA), and hepatocellular carcinoma (HCC). In a meta-analysis, Zengh *et al*[22] found that patients with PSC-IBD have a strikingly higher risk for the development of CRC than patients with IBD alone. In detail, the stratification by IBD type showed a three-fold increased risk for the development of CRC and colorectal dysplasia in patients with PSC-UC compared to those with UC alone. A non-significant increase in the risk of neoplasia was shown in patients with PSC-CD, in contrast to that found in patients with CD alone. For these reasons, patients with PSC-IBD (especially those with PSC-

UC) require close colorectal neoplasia endoscopic surveillance. Major American and European Societies recommend that annual CRC screening should be started at the time of PSC-IBD diagnosis. In PSC-IBD patients an increased risk of hepatobiliary malignancies such as CCA, gallbladder carcinoma (GBC), and HCC has been demonstrated. Gulamhusein *et al*[23] demonstrated that prolonged duration of IBD is associated with an increased risk of CCA in patients with PSC-IBD. They also observed that the risk of CCA was not modified after colectomy, thus suggesting that colonic resection itself does not reduce the risk of CCA. European and American Societies recommend that CA 19-9 and biliary imaging should be completed every year for these patients[24,25]. IBD could be an additional risk factor that further increases the hazard of CCA in PSC. In particular, a long duration of IBD is associated with CCA with a hazard ratio of 1.37[23].

There are no studies demonstrating an increased risk of GBC in PSC-IBD patients, even if that risk is demonstrated in PSC-alone patients[26]. Said *et al*[27] found in their cohort of patients affected with PSC, that 6% had gallbladder masses, of which 56% were malignant. The American Association for the Study of Liver Disease (AASLD) guidelines support cholecystectomy for polyps of any size in these patients, given the high likelihood of malignancy[28]. HCC seems to be a rare malignancy in PSC-IBD. Zanouzi *et al*[29] analyzed a cohort of PSC-cirrhosis patients and found no cases of HCC. However, in the same cohort of patients, IBD was found in 65%.

As both CCA and CRC are likely to occur in PSC-IBD patients, a chemopreventive strategy could be proposed. A meta-analysis[30] showed that low dose ursodeoxycholic acid may have a protective effect on both CRC and colonic dysplastic lesions, with an odds ratio of 0.19. However, the studies were performed on small populations in tertiary centers, and were often retrospective, therefore the strength of evidence is not high[31]. Even mesalazine has demonstrated, *in vitro* and in animal models, an anti-proliferative effect as well as the ability to inhibit the Wnt/β-Catenin pathway and epithelial growth factor receptor activation; therefore, it may be a promising agent for CRC prevention, despite the chemopreventive effect of mesalazine only being documented for patients with UC alone so far[32]. Unfortunately, no effective approach for CCA chemoprevention has emerged, therefore surveillance remains the mainstay for early CCA detection in PSC patients.

Therapeutic perspectives

The pathogenetic mechanisms underlying PSC-IBD remain unknown, even though many hypotheses have been proposed. Understanding the basis of the disease could lead to the identification of a new targeted therapy. One of the most interesting assumptions suggests that intestinal mucosal lymphocytes may migrate to the liver following activation in the bowel of IBD patients, thus promoting liver inflammation [33]. It has been shown that adhesion molecules and chemokine receptors normally expressed only in the gut can be aberrantly expressed within the liver to promote the homing of gut-associated lymphocytes. One of these adhesion molecules is α4β7 integrin. A monoclonal antibody directed against α4β7, vedolizumab, has been approved for the treatment of IBD. It was hypothesized that vedolizumab could provide hepatic anti-inflammatory benefits. Nevertheless, Christensen *et al* found that, after treatment with vedolizumab, symptoms and intestinal clinical activity were significantly decreased, but the Mayo PSC Risk Score and liver damage biomarkers were only slightly improved[34].

Aberrant microbiota epitope recognition and gut dysbiosis seem to have a role in the pathogenesis of PSC-IBD, while genetics, gut mucosal permeability and autoimmune mechanisms have a controversial role[35]. Further studies are needed to improve our knowledge on the pathogenesis of PSC-IBD in order to provide new and efficient therapeutic strategies.

When PSC causes end-stage liver disease, liver transplantation is the only curative treatment. Regarding this point, some studies found that IBD does not worsen survival in patients who undergo liver transplantation for PSC. Only exposure to azathioprine seems to increase post-transplant mortality, while IBD per se increases the risk of cytomegalovirus infection[36].

PRIMARY BILIARY CHOLANGITIS AND AUTOIMMUNE HEPATITIS IN IBD

PBC is an autoimmune liver disease characterized by inflammatory cell infiltration of intralobular biliary ducts, with consequent biliary duct damage, which can progress towards fibrosis. Currently, there is no solid link between IBD and PBC, as only a few

case reports have been published. The most consistent case series involving six PBC patients in a cohort of IBD subjects during the period 2006-2016 (3 CD and 3 UC), who were diagnosed with PBC by liver biopsy responded to ursodeoxycholic acid therapy [37]. In a genetic association study, it was found that TNFSF15 and ICOSLG-CXCR5 might be a shared pathogenic pathway in the development of PBC and CD[38].

Similarly, only some case reports on the association between IBD and autoimmune hepatitis (AIH) have been published. A systematic review found approximately 109 cases, which were mostly overlap syndrome with PBC. The authors reported that jaundice was the most common onset sign and that response to steroids was good, with a low mortality rate[39]. Interestingly, a case report of AIH onset after starting adalimumab has been described, which underlines the possibility that an immunogenic drug may alter an equilibrium in the immune system[40].

HEPATIC STEATOSIS IN IBD

Hepatic steatosis is defined as intrahepatic fat accumulation of at least 5% of liver weight. Prolonged hepatic lipid storage may lead to liver metabolic dysfunction, inflammation, and advanced forms of NAFLD. Non-alcoholic hepatic steatosis is associated with obesity, type 2 diabetes and dyslipidemia. Several mechanisms are involved in the accumulation of intrahepatic fat, including increased flux of fatty acids to the liver, increased *de novo* lipogenesis, and/or reduced clearance through β-oxidation or very-low-density lipoprotein secretion[41,42] in the absence of secondary causes of lipid overload such as significant alcohol intake.

A link between hepatic steatosis and IBD has been studied since 1873, when Thomas [43] described for the first time the association between "ulceration of the colon" and a "much enlarged fatty liver". In recent years, due to the spread of obesity in the context of IBD[44], fatty liver disease has been increasingly recognized in IBD. The intestinal inflammatory state and gut barrier perturbation secondary to IBD might increase toxin and bacterial constituents translocation from the gut to the portal vein; this event has been recognized as a possible pathophysiologic mechanism underlying NAFLD[45]. Moreover, diets poor in high fiber foods, such as fruits and vegetables, frequently consumed by IBD subjects to avoid intestinal symptoms, could lead to a great prevalence of NAFLD[46]. Moreover, food components and alimentary habits with high proteins and fats, excessive sugar intake and less vegetables and fiber can influence the composition of the intestinal microbiome, and play a role in driving IBD pathogenesis and fat metabolism leading tog NAFLD onset[47].

A recent meta-analysis showed that the overall pooled prevalence of NAFLD in IBD patients was 27.5%[48]. NAFLD, in particular, was more common among patients with features of severe IBD, such as longer disease duration or a history of abdominal surgery.

Another study by Bessisow *et al*[49] showed a frequency of NAFLD in IBD of 33.6% and demonstrated that disease activity, duration of IBD and prior surgery were predictors of NAFLD development.

Conversely, in a Japanese study[50], the ultrasonographic prevalence of NAFLD in CD was 21.8% and this was the only study in which NAFLD was identified as an independent predictor of a negative C-reactive protein level and higher rate of remission, so NAFLD might offer a protective effect in patients with CD.

Nevertheless, most studies did not include non-IBD patients as a control group.

Glassner *et al*[51] examined 3 groups of patients: IBD + NAFLD, IBD alone, and NAFLD alone. A total of 168 patients were evaluated, 56 patients in each group. They found an overall NAFLD prevalence of 13.3% in IBD patients. IBD patients with NAFLD had longer IBD disease duration and developed NAFLD even in the absence of metabolic risk factors when compared to patients with NAFLD alone.

A study performed in 2018 by Principi *et al*[52] included 465 IBD patients and 223 non-IBD patients. The prevalence of NAFLD was higher in IBD than in non-IBD patients (28.0% *vs* 20.1% respectively, *P* = 0.04); furthermore, younger age was observed in NAFLD-IBD than in non-IBD individuals, whereas no other differences were found between these two subgroups. Regarding risk factors, diabetes and fasting blood glucose were associated with development of NAFLD in IBD, without any difference in the populations without IBD, with only a higher waist circumference in IBD compared to non-IBD patients. No IBD-related variable was associated with NAFLD.

There are no studies on the progression of NASH in IBD. However, since IBD may induce gut barrier perturbation and an increase in toxin and bacterial translocation, it

is possible that in patients with NAFLD, the coexistence of IBD can trigger the progression from simple steatosis to NASH. A single study, on the other hand, has shown that progression of fibrosis, estimated by the NAFLD fibrosis score, is quite rare in IBD[53].

In conclusion, NAFLD is common in patients with IBD. Screening, prevention, and early treatment of NAFLD might be recommended in IBD patients. However, a better understanding of the underlying mechanism of the coexistence of IBD and NAFLD is necessary to improve management. The treatment of NAFLD in IBD does not differ from other cases. In particular, so far only diet and physical exercise have been proved to be effective[54].

CHRONIC VIRAL HEPATITIS IN IBD

Chronic viral hepatitis, in particular HBV and HCV-related, is a very common infection and a worldwide health issue. It is estimated that over 350 million people in the world have chronic HBV infection and over 250 million people have chronic HCV infection, with a mean prevalence of 5% and 2% for HBV and HCV, respectively[55, 56].

With regard to the prevalence of chronic hepatitis B (CHB) and chronic hepatitis C (CHC) in IBD, recent evidence[57-61] shows that it was comparable to a control population, ranging from 1% to 9%. A recent Italian study by Losurdo *et al*[62] on 807 IBD patients and 189 controls, found a prevalence of 3.4% for CHC and 0.9% for CHB, a result which agrees with recent literature reports[57,58,61]. This analysis demonstrated that advanced age was independently associated with increased risk of CHB/CHC. It is possible that surgery performed before the diffusion of presurgical hepatitis screening could explain this result, also taking into account that CHC was more common in patients operated before 1990. Indeed, the introduction of the HBV vaccine and HCV routine detection led to an improvement in the prevention measures against viral hepatitis transmission during surgery or blood donation, thus reducing the risk of infection in young generations[62].

As the treatment of IBD is based in selected cases on immunosuppressive agents (thiopurines and biologic drugs such as monoclonal antibodies), an accurate clinical and laboratory assessment is preliminarily required to look for chronic infections that may have a severe flare under biologic drugs[57,63]. Among these, chronic viral hepatitis and in particular CHB and CHC, are advised to be investigated by the guidelines before starting immunosuppressive treatment[64].

According to the guidelines, all IBD patients should be tested for HBV (HBsAg, anti-HBs, anti-HBc) at diagnosis of IBD to determine HBV status. In patients with positive HBsAg, viremia (HBV-DNA) should also be quantified. Moreover, HBV vaccination is recommended in all HBV anti-HBc seronegative patients with IBD. All HBsAg positive subjects should start anti-viral agents before undergoing biologic treatment to prevent potentially serious hepatitis B flares[64,65]. A number of case series and study cohorts suggest that nucleotide/nucleoside analogues are safe and effective in IBD patients on immunomodulator treatment[66]. Entecavir and tenofovir are preferred for IBD patients due to their rapid onset of action, high anti-viral potency and low incidence of resistance. On the other hand, patients with HBsAg positive (chronic HBV infection) should receive anti-viral agents before, during and for at least 12 mo after immunomodulator treatment has ceased[64]. Additionally, HBV vaccination is strongly advised by the guidelines, possibly before starting any immunosuppressive treatment and preferably at the moment of diagnosis, if anti-HBs level is not protective. This approach should be followed in any region, irrespective of HBV prevalence.

With regard to CHC, present knowledge shows in some cases mild liver dysfunction and an amplified detrimental effect by the simultaneous presence of other viruses (HBV/HIV) in relation to immunomodulator assumption[67,68]; therefore, HCV antibody testing and HCV-RNA should be investigated. Immunomodulators are not contraindicated but should be used with caution. The decision depends on the severity of IBD and the stage of liver disease. In the past years, an interferon-based treatment for HCV infection in CD has generally not been recommended, as it could worsened the intestinal disorder; however, this aspect remains controversial[69]. Conversely, in UC, interferon therapy did not appear to have an adverse effect[70]. In addition, the administration of ribavirin plus interferon or triple anti-viral therapy (interferon, ribavirin and protease inhibitors) could have increased the toxicity of drugs used for IBD maintenance (for example azathioprine, methotrexate)[64]. Therefore, the risk that anti-viral therapy or drug interactions with IBD therapy might

exacerbate IBD should assessed cautiously when considering the need for HCV treatment[64]. However, over the last years, concomitant IBD and HCV infection management has completely changed due to the recent introduction of direct-acting anti-virals (DAAs). Recently published data on DAAs are very encouraging also in IBD patients[71]. There are three possible timing strategies for administration in patients requiring biological therapies: (1) Sequential strategy, meaning the choice of treating firstly the active IBD with biologics and then, once the acute phase has been controlled, treating the HCV infection; (2) Concomitant strategy, that is the contemporaneous initiation of DAAs and biologic drug administration; and (3) Inverted sequential strategy, *i.e.,* the administration of anti-viral therapy before biologics. The timing strategy could depend on several factors, including IBD activity and patient comorbidity. This means that a case-by-case decision could be the best choice[72]. The opportunity to eradicate HCV should always be taken into account, as it has demonstrated that a sustained viral response may reduce liver stiffness in these patients[73].

IBD AND DRUG-INDUCED LIVER INJURY

In the last decade, treatment options for IBD have included new molecules acting at different target levels. Usually, as new drugs are introduced, their side effects should also be considered, and liver toxicity is one of the most meaningful among these.

Drug-induced liver injury (DILI) caused by these drugs can be classified into three forms: hepatocellular, cholestatic or a mixed pattern. Moreover, some forms of drug-induced AIH should also be considered. This issue leads to a schedule of specific screening before starting therapy for IBD, and a follow-up to monitor liver enzymes is necessary[74,75].

In Table 2, we summarize the main knowledge on DILI in IBD patients.

Thiopurines

Thiopurines, in particular azathioprine (AZA) and 6-mercaptopurine (6-MP) are used for induction and maintenance of remission in IBD. Studies have shown that AZA/6-MP as add-on to infliximab can reduce the development of antibodies against infliximab. Thiopurines act as DNA synthesis inhibitors by incorporating purine analogues into DNA with cytotoxic and immunosuppressive effects. AZA is metabolized in the liver to 6-MP, which is metabolized by three enzymes, including thiopurine S-methyltransferase (TMPT) to 6-methylmercaptopurine (6-MMP). AZA and 6-MP are prodrugs of 6-thioguanine (6-TGN), the real effective metabolite. Some studies have suggested that some TMPT polymorphisms could cause a rise in 6-MMP level, thereby amplifying hepatotoxicity. In a cohort study of 270 patients treated with 6-MP, 47 patients showed evidence of altered liver function tests (LFT) in the first 20 weeks of treatment and > 80% of these patients had elevated levels of 6-MMP in the first week[76]. Another study proved that patients with high concentrations of 6-MMP had not only a strong risk of side effects but also a reduction in therapeutic response [77]. Conversely, Dong *et al*[78] found that the presence of TMPT polymorphisms increased bone marrow toxicity but not hepatotoxicity. A recent meta-analysis of 10 studies (recruiting 1875 patients) proved that TMPT polymorphisms were not linked with liver injury. The physiopathology of liver injury due to thiopurine is still unclear.

The prevalence of thiopurine-induced liver toxicity can vary between 0% and 17%. In a systematic review of 34 studies with 3485 patients, the prevalence of hepatotoxicity induced by AZA/6-MP was 3.4% with no differences between the two drugs [79]. Additionally, Chaparro *et al*[80] in a study of 3931 patients with IBD treated with thiopurine reported that hepatotoxicity was one of the most common side effects, with a prevalence of 4%. CD, smoking and preexisting NAFLD seemed to be risk factors, while the prevalence was lower in females. In a study by Shroder, who analyzed 259 patients undergoing immunosuppressive treatment with AZA, 6MP and MTX, liver steatosis was found in 28.2% of them, and patients with steatosis also had a higher risk of having elevated alanine transaminase (ALT) blood levels[81].

On the other hand, dose independent, idiosyncratic liver reactions have been described for thiopurines. Acute dose-independent toxicity is caused by an idiosyncratic cholestatic reaction accompanied by fever, rash, lymphadenopathy and hepatomegaly with increased alkaline phosphatase level. The median onset time of hepatotoxicity is 110 days, and in most cases is self-limiting with a good prognosis.

Another atypical, long-term liver injury caused by thiopurines is characterized by vascular endothelial lesions. Nodular regenerative hyperplasia (NRH), is the most

Table 2 Main features of drug-induced liver injury in inflammatory bowel disease

Drug	Characteristics of drug induced liver injury
Aminosalicylates	Increases in LFT; Cholestatic pattern; Rarely eosinophilia
Thiopurines	Influenced by TMPT polymorphisms > increase in 6-MMP, the hepatotoxic molecule; Increases in LFT; Idiosyncratic cholestatic reaction; Fever, rash, lymphadenopathy and hepatomegaly; Nodular regenerative hyperplasia
Anti-TNF	Idiosyncratic reaction > dose-dependent mechanism; Hepatocellular injury > cholestasis; Autoimmune phenomena
Anti-integrins	Rare; Asymptomatic LFT increase
Anti IL12/23	Mild LFT increase

LFT: Liver function test; TMPT: Thiopurine S-methyltransferase; TNF: Tumor necrosis factor.

frequent of these lesions, while peliosis hepatis and sinusoidal obstruction syndrome (SOS) are less common. NRH is frequently asymptomatic. The mechanism underlying NRH is still unknown, it is possible that hepatocyte atrophy and portal venules destruction could be involved; risk factors seem to be male sex, CD with stricturing behavior and previous small bowel resection. In a large French study, NRH was found in 37 cases, with a cumulative risk of 0.5% at five years and a median onset time of 48 mo[82]. A recent study observed a similar prevalence of NRH between patients treated with thiopurines and patients thiopurine-naïve[83]. On the other hand, it was found that thiopurines are associated with NRH when the dose is high (tioguanine > 40 mg/day) or in male patients with small bowel resection > 50 cm[84,85]. The evolution of NRH after stopping thiopurine therapy is still unclear.

There is no agreement on thiopurine toxicity management. In a large study with a long-term follow-up only 3.6% of patients needed to discontinue therapy[86]. In another study, 90% of patients had normalization of LFT by reducing thiopurine doses [87]. It is unclear whether the frequency of hepatotoxicity is the same for AZA and 6-MP treatment: a study of 135 patients reported that 6-MP was well tolerated in 71% patients who had shown liver toxicity with AZA[88]. Coadministration of allopurinol (a xanthine-oxidase inhibitor) seems to reduce 6-MMP levels as it leads to a higher concentration of 6-MP converted to 6-TGN. However, since allopurinol is a xanthine-oxidase inhibitor, the AZA dose should be reduced. A retrospective cohort study of 105 patients reported that coadministration of allopurinol allowed long-lasting therapy and transaminase normalization[89]. Also, in another study by Krejineof, among 211 patients with liver toxicity, 86% experienced an improvement by lowering the dose of thiopurines in association with allopurinol[90]. A larger study by Vasuvedan analyzed 767 patients on thiopurine therapy and demonstrated that allopurinol should be started to reduce side effects, as 94% of patients who had hepatotoxicity achieved resolution by changing to co-therapy[91]. As TMPT polymorphisms are likely to be involved in hepatotoxicity, some authors have proposed that these polymorphisms should be identified before starting therapy, but a review by the American Gastroenterological Association Institute stated that the benefits of these tests were low[92]. On the contrary, a consensus guideline by the British Society of Gastroenterology focused on TMPT activity and recommended the administration of a half-dose of thiopurines to patients with low TMPT activity[93].

LFT should be monitored routinely, but there is no agreement on their timing. Mottet *et al*[93] recommended LTF every wk for the first mo, then twice a mo during the second mo and then once every 3 mo.

Sulfasalazine and mesalamine

Sulfasalazine is used for mild UC. It has been associated with acute hepatitis, cholestatic hepatitis, granulomatous hepatitis and rarely with acute liver failure[94]. The incidence of hepatotoxicity is low: A review by Ransford *et al* who analyzed 4.7 million prescriptions in the period from 1991 and 1998, reported only 9 cases of hepatitis caused by sulfasalazine[95].

Mesalamine (oral and rectal) is approved for mild UC. Authors in the last three years have demonstrated that the prevalence of liver toxicity caused by mesalamine is low, between 0% and 4%. The use of mesalamine may be associated with asymptomatic elevations in LFT, hepatitis and cholestatic hepatitis[96]. A recent review reported that LTF should be monitored every year and therapy should be stopped in the case of abnormal increases, while treatment with corticosteroids should be considered if fever, rash, or eosinophilia are observed. The same review

demonstrated that most cases of hepatotoxicity quickly reversed with drug withdrawal[97].

Methotrexate

Low doses of methotrexate (MTX) are used for mild CD, and it is widely used for rheumatologic disease; therefore, in this field its hepatotoxicity has been more extensively studied. The underlying mechanism is still not clear; several polymorphisms of enzymes involved in folic acid metabolism are thought to be involved. Two systematic reviews on this topic reported opposite results: the first review found an association between MTX hepatotoxicity and C677T polymorphism of methylenetetrahydrofolate reductase (MTHFR) gene, while the second review did not confirm this result[98,99]. MTX can cause different histological liver findings according to the Roenigk's classification including: (1) Normal; (2) Mild fatty infiltration, nuclear alterations or portal inflammation; (3) Moderate to severe fatty infiltration, nuclear alterations, or portal infiltration and mild fibrosis; (4) Moderate to severe fibrosis; and (5) cirrhosis[100].

Some studies reported that the prevalence of abnormal LTF in these patients ranged from 15 to 50%, while most recent evidence demonstrated a lower prevalence. A meta-analysis of patients with IBD treated with MTX reported a rate of abnormal LTF (defined as ALT higher than normal values but less than x2 upper normal limit (ULN)) of 1.4 per 100 person-month and a rate of hepatotoxicity (defined as ALT higher than two times normal values) of 0.9 per 100 person-month[101]. It should be noted that, in CD, methotrexate is given *i.m.,* with a dose of 25 mg/wk at induction and 15 mg/wk for maintaining remission. Considering that this dose is higher than in rheumatologic patients, this could explain the more frequent liver adverse events.

Before starting MTX treatment, patients should be screened for preexisting medical conditions, such as alcohol intake, viral hepatitis, steatosis and family history of liver disease. Rheumatological consensus guidelines recommend monitoring LFT every two wk for the first 2 mo, then every 2 or 3 mo[102]. Liver biopsy should be considered in some cases, such as when liver laboratory tests remain abnormal despite dose reduction or when there are high blood levels of drug in patients with known risk factors for hepatotoxicity. Treatment should be stopped in the case of severe fibrosis or cirrhosis and daily doses should be reduced in the case of LFT elevation. Co-administration with folic acid or folinic acid seems to reduce the frequency of serum transaminase elevation[103]. Elastography (Fibroscan) and laboratory tests are emerging tools to diagnose fibrosis as reported by *Labadie et al*[104]. Furthermore, in a case control study of 518 patients treated with MTX, 8.5% showed Fibroscan and FibroTest abnormalities, *i.e.,* severe fibrosis[105]. A multivariate analysis reported that elastography should be used mainly in patients with an alcohol habit or obesity, or affected by NAFLD. Similar results were reported in a study by Herfath *et al*[106].

Tumor necrosis factor alpha inhibiting agents

Currently several molecules belonging to this class have been approved to treat IBD: infliximab (IFX), adalimumab (ADA), golimumab and certolizumab pegol. Few data are available on the hepatotoxicity of golimumab and certolizumab, while most of the literature reports DILI by IFX and ADA.

The Food and Drug Administration (FDA) in 2004 after 130 cases of liver injury in patients treated with IFX and etanercept (which has no indication in IBD), issued an alarm statement of severe hepatic adverse reactions, including acute liver failure, autoimmune hepatitis (AIH) and cholestatic hepatitis during IFX therapy[107]. In an Icelandic study by Bjornsson that included patients with IBD, rheumatological and dermatological disorders, the occurrence of DILI in patients treated with IFX or ADA was 1:120 and 1:270, respectively[108]. Shelton *et al*[109] in a retrospective study analyzed 1753 patients under anti-TNF therapy (1170 IFX, 575 ADA, 8 certolizumab), and found that 102 patients had high blood levels of ALT, but in 54 of these patients, additional risk factors for liver injury were found and, of the remaining 48 patients (45 IFX, 3 ADA), only 4 were considered to be affected by anti-TNF induced liver injury. Koller *et al*[110] in a recent observational study of 251 patients with IBD, monitored liver injury in 163 receiving IFX. Twenty-six patients (16%) showed a grade 1 liver injury (ALT < x3 ULN), 4 patients (2.5%) a grade 2 (ALT > x3 ULN); grade 1 alkaline phosphatase elevation was seen in 11 patients (6.7%) and grade 2 alkaline phosphatase elevation (> x2.5 ULN) in none. Liver injury in these patients was associated with high BMI, hepatic steatosis and longer duration of IBD[110]. In an Australian retrospective cohort study of adult patients with IBD treated with IFX (IDLE STUDY), out of 175 patients (149 with CD and 26 with UC), 57 showed abnormal liver laboratory tests. In this study, the authors used the Roussel Uclaf Causality Assessment Method

(RUCAM) score to predict the risk of hepatic injury caused by drugs. A score of 0 rules out DILI, 1-2 means unlikely DILI, 3-5 possible DILI, 6-8 probable DILI, and > 8 highly probable DILI. Eleven patients had a RUCAM score > 3, but just one patient had a score > 8. Usually, liver injury due to IFX occurs after multiple infusions and a mean latency of 14-18 wk from induction. In this context, the RUCAM score is not a diagnostic test, but it is useful to predict DILI relying on LFT, timing of drug initiation and cessation, and on liver biopsy, when performed[111].

Although IFX, ADA and etanercept are anti-TNF drugs, they are structurally different. This explains the different responses to these agents and the different capacity to induce liver injury. Some authors have described how patients tolerate successful treatment with another molecule after a prior DILI episode induced by an anti-TNF agent. This suggests a lack of cross-toxicity within this class of drugs.

The pathogenetic mechanism underlying anti-TNF hepatotoxicity is still unknown. As liver injury can occur after a singular infusion it seems more an idiosyncratic injury rather than a dose-dependent one[107]. A genetic predisposition may be considered. Another hypothesis is that anti-TNF agents may trigger a pre-existing autoimmune disorder or generate autoantibodies: the binding of IFX to the transmembrane TNF-alpha can lead to apoptosis of monocytes and T-lymphocytes with exposure of nucleosomal autoantigens and the production of autoantibodies[112,114]. Another possibility is that anti-TNF drugs inhibit T-lymphocytes activity, thus suppressing auto-reactive B cells; this may lead to increased humoral autoimmunity[114]. However, there are several cases without evidence of autoimmunity, in which direct liver injury is involved.

DILI caused by anti-TNF agents can show different patterns: Hepatocellular injury in 75% cases, but also a mixed pattern, most rarely with cholestasis, while few cases of acute liver failure have been described. Colina *et al*[115] reported histological necroinflammation caused by IFX, with bridging and massive necrosis in the most severe cases and some features of autoimmune injury with piecemeal necrosis in the periportal interface and prominent plasma cells infiltration. Liver injury caused by anti-TNF drugs is associated with the presence of autoimmunity markers in some patients: anti-nucleus, anti-DsDNA and anti-smooth muscle actin positivity and/or histologic features of AIH are described for IFX, ADA and etanercept. In a study analyzing 34 patients undergoing anti-TNF treatment with DILI, 22 were positive for such antibodies and showed higher levels of ALT than seronegative patients. Fifteen out of 22 subjects underwent liver biopsy that revealed clear features of autoimmunity [116]. Indeed, it is difficult to distinguish between AIH and drug-induced AIH, since these conditions may have similar clinical, biochemical, serological and histological features. Actually, IFX-induced AIH is rare in IBD patients and is described more often in rheumatology patients. In several studies, autoimmunity features were treated with corticosteroids, achieving in some cases a reduction or disappearance of autoantibodies titer; this suggests an immune-mediated DILI rather than an anti-TNF induced AIH. Ierardi *et al*[117] reported a case of acute liver injury after a single IFX administration. Analogously, Adar *et al*[118] described the first case of AIH caused by ADA that resolved after treatment cessation and corticosteroid therapy.

There is still a lack of consensus on the management of DILI induced by anti-TNF agents. The prognosis is usually favorable with normalization of LFT without cessation of anti-TNF therapy. Liver enzymes should be monitored before starting treatment and then monitored periodically, especially during the first 3 mo. If ALT remains < x3 ULN, anti-TNF can be continued until resolution; if ALT is persistently elevated > x3 ULN or in the case of jaundice, corticosteroids and liver biopsy should be considered. If a DILI is documented, anti-TNF withdrawal is still controversial. Also, the necessity to obtain an autoimmune panel before starting anti-TNF treatment is debated: several studies demonstrated that this practice does not predict the risk of developing drug-induced AIH and that anti-TNF therapy could be continued in the presence of asymptomatic anti-nucleus positivity[102].

Anti-Integrins

Natalizumab and vedolizumab were approved some years ago for the treatment of IBD. Both drugs have shown a good safety profile, but in the post-marketing phase, 6 cases of significant DILI associated with natalizumab were reported to the FDA[119].

Liver injury caused by natalizumab is rare with a 5% rate of asymptomatic liver enzymes elevation and it can manifest with both the hepatocellular and cholestatic pattern and can be associated with jaundice. Some cases with autoimmune features (autoantibodies positive) have also been described[120]. The guidelines recommend monitoring LFT before starting the treatment and then every 3 or 6 mo[121]. Nevertheless, the use of natalizumab is quite rare in IBD due to possible severe

neurologic complications such as progressive multifocal leukoencephalopathy[122].

Similar to natalizumab, liver injury associated with vedolizumab is rare, less than 2% in clinical trials, with both the hepatocellular or cholestatic pattern[123]. Similar to natalizumab, the guidelines recommend monitoring liver enzymes every 3-6 mo.

Anti IL12/23

Ustekimumab was approved for CD treatment in 2016 and UC treatment in 2019. Most of the data regarding hepatotoxicity induced by ustekimumab comes from dermatologic studies. In PHOENIX 1 and 2, both studies evaluated the efficacy and safety of ustekimumab in patients with psoriasis, and the rate of liver enzymes abnormalities was low (between 0.5% and 2%) and similar between the case and control group[124,125]. A small retrospective study including 44 patients with psoriasis treated with ustekimumab described cases of mild elevation of liver enzymes and no cases of severe DILI[126]. Some case reports described spontaneous regression of liver injury after ustekinumab withdrawal[127].

Small molecules

Tofacitinib was approved for UC treatment in 2018. Liver enzymes elevation with a hepatocellular pattern has been rarely described[128]. One case of possible AIH was reported, but liver injury due to other drugs could not be excluded[129]. Monitoring liver enzymes periodically during tofacitinib treatment is recommended.

Ozanimod is a new molecule introduced for IBD treatment. Aspartate transaminase increases 32 wk after drug exposure were described in 2% and 1% of patients treated with 0.5 mg and 1 mg of ozanimod, respectively. Preliminary data suggest a low rate of hepatotoxicity associated with these new therapeutic approaches[102].

PORTAL VEIN THROMBOSIS

Portal vein thrombosis (PVT) is a common event in IBD. Indeed, IBD patients have a high risk of thromboembolism due to systemic inflammation and alterations in the concentrations of some coagulation factors, such as high factor V and VIII or low antithrombin III[130].

In a retrospective study, the incidence of thromboembolic events in patients with IBD rose from 5.65% in 2000 to 7.17% by 2009[131]. In particular, the prevalence of PVT in IBD has been estimated to be about 0.17%[132]. There are several causes of PVT, including inflammation, immobilization, major extent of colon disease, disease severity, surgery, use of corticosteroids and smoking. For that reason, the guidelines recommend starting heparin when facing an acute flare of UC, for PVT prophylaxis [133].

After the onset of PVT, complications such as portal hypertension, bleeding or even death are not common, but early anticoagulation is safe and associated with a better outcome, and the use of novel direct oral anticoagulants was associated with particularly favorable outcomes in this setting[134].

CONCLUSIONS

In conclusion, the scenario of liver involvement of IBD patients is quite extensive. The relationship between IBD and PSC is the most studied. PSC is a disease that currently has no effective medical therapy; therefore, research on drugs that may be effective for both hepatic and intestinal disorders is required. Moreover, the strategies for early neoplasia screening (both CCA and CCR) in these patients are not sufficiently efficient at present, and this is a pitfall that needs to be resolved.

NAFLD in IBD is another focal issue, as this novel comorbidity may complicate the management of IBD patients due to its multifaceted aspects.

As viral hepatitis may soon become a thing of the past, due to the advent of drugs with very high success rates, some patients will still require careful monitoring, especially when immunosuppression for IBD is required.

Among the drugs currently in use to treat IBD, thiopurines, mesalazine derivatives and methotrexate are the most studied, and periodic assessment of LFT is still required. However, the field of DILI is expected to expand quickly, as several novel molecules for the treatment of IBD (tyrosine kinase inhibitors, small molecules and others) have been developed, and their possible hepatotoxicity will be a matter of

debate.

REFERENCES

1   **Jairath V**, Feagan BG. Global burden of inflammatory bowel disease. *Lancet Gastroenterol Hepatol* 2020; **5**: 2-3 [PMID: 31648974 DOI: 10.1016/S2468-1253(19)30358-9]

2   **Bibbò S**, Ianiro G, Dore MP, Simonelli C, Newton EE, Cammarota G. Gut Microbiota as a Driver of Inflammation in Nonalcoholic Fatty Liver Disease. *Mediators Inflamm* 2018; **2018**: 9321643 [PMID: 29563854 DOI: 10.1155/2018/9321643]

3   **Fousekis FS**, Theopistos VI, Katsanos KH, Tsianos EV, Christodoulou DK. Hepatobiliary Manifestations and Complications in Inflammatory Bowel Disease: A Review. *Gastroenterology Res* 2018; **11**: 83-94 [PMID: 29707074 DOI: 10.14740/gr990w]

4   **Lunder AK**, Hov JR, Borthne A, Gleditsch J, Johannesen G, Tveit K, Viktil E, Henriksen M, Hovde Ø, Huppertz-Hauss G, Høie O, Høivik ML, Monstad I, Solberg IC, Jahnsen J, Karlsen TH, Moum B, Vatn M, Negård A. Prevalence of Sclerosing Cholangitis Detected by Magnetic Resonance Cholangiography in Patients With Long-term Inflammatory Bowel Disease. *Gastroenterology* 2016; **151**: 660-669.e4 [PMID: 27342213 DOI: 10.1053/j.gastro.2016.06.021]

5   **Lakatos L**, Kiss LS, David G, Pandur T, Erdelyi Z, Mester G, Balogh M, Szipocs I, Molnar C, Komaromi E, Lakatos PL. Incidence, disease phenotype at diagnosis, and early disease course in inflammatory bowel diseases in Western Hungary, 2002-2006. *Inflamm Bowel Dis* 2011; **17**: 2558-2565 [PMID: 22072315 DOI: 10.1002/ibd.21607]

6   **Burisch J**, Jess T, Martinato M, Lakatos PL; ECCO -EpiCom. The burden of inflammatory bowel disease in Europe. *J Crohns Colitis* 2013; **7**: 322-337 [PMID: 23395397 DOI: 10.1016/j.crohns.2013.01.010]

7   **Loftus EV Jr**, Harewood GC, Loftus CG, Tremaine WJ, Harmsen WS, Zinsmeister AR, Jewell DA, Sandborn WJ. PSC-IBD: a unique form of inflammatory bowel disease associated with primary sclerosing cholangitis. *Gut* 2005; **54**: 91-96 [PMID: 15591511 DOI: 10.1136/gut.2004.046615]

8   **de Vries AB**, Janse M, Blokzijl H, Weersma RK. Distinctive inflammatory bowel disease phenotype in primary sclerosing cholangitis. *World J Gastroenterol* 2015; **21**: 1956-1971 [PMID: 25684965 DOI: 10.3748/wjg.v21.i6.1956]

9   **Dave M**, Elmunzer BJ, Dwamena BA, Higgins PD. Primary sclerosing cholangitis: meta-analysis of diagnostic performance of MR cholangiopancreatography. *Radiology* 2010; **256**: 387-396 [PMID: 20656832 DOI: 10.1148/radiol.10091953]

10   **Sørensen JØ**, Nielsen OH, Andersson M, Ainsworth MA, Ytting H, Bélard E, Jess T. Inflammatory bowel disease with primary sclerosing cholangitis: A Danish population-based cohort study 1977-2011. *Liver Int* 2018; **38**: 532-541 [PMID: 28796371 DOI: 10.1111/liv.13548]

11   **Riley TR**, Schoen RE, Lee RG, Rakela J. A case series of transplant recipients who despite immunosuppression developed inflammatory bowel disease. *Am J Gastroenterol* 1997; **92**: 279-282 [PMID: 9040206]

12   **Boonstra K**, van Erpecum KJ, van Nieuwkerk KM, Drenth JP, Poen AC, Witteman BJ, Tuynman HA, Beuers U, Ponsioen CY. Primary sclerosing cholangitis is associated with a distinct phenotype of inflammatory bowel disease. *Inflamm Bowel Dis* 2012; **18**: 2270-2276 [PMID: 22407885 DOI: 10.1002/ibd.22938]

13   **Fousekis FS**, Theopistos VI, Mitselos IV, Skamnelos A, Kavvadias A, Katsanos KH, Christodoulou DK. Specific Features of Patients With Inflammatory Bowel Disease and Primary Sclerosing Cholangitis. *J Clin Med Res* 2019; **11**: 81-88 [PMID: 30700999 DOI: 10.14740/jocmr3680]

14   **Ricciuto A**, Fish J, Carman N, Walters TD, Church PC, Hansen BE, Crowley E, Siddiqui I, Nguyen GC, Kamath BM, Griffiths AM. Symptoms Do Not Correlate With Findings From Colonoscopy in Children With Inflammatory Bowel Disease and Primary Sclerosing Cholangitis. *Clin Gastroenterol Hepatol* 2018; **16**: 1098-1105.e1 [PMID: 29378308 DOI: 10.1016/j.cgh.2018.01.020]

15   **Sano H**, Nakazawa T, Ando T, Hayashi K, Naitoh I, Okumura F, Miyabe K, Yoshida M, Takahashi S, Ohara H, Joh T. Clinical characteristics of inflammatory bowel disease associated with primary sclerosing cholangitis. *J Hepatobiliary Pancreat Sci* 2011; **18**: 154-161 [PMID: 20740366 DOI: 10.1007/s00534-010-0319-8]

16   **Halliday JS**, Djordjevic J, Lust M, Culver EL, Braden B, Travis SP, Chapman RW. A unique clinical phenotype of primary sclerosing cholangitis associated with Crohn's disease. *J Crohns Colitis* 2012; **6**: 174-181 [PMID: 22325171 DOI: 10.1016/j.crohns.2011.07.015]

17   **Lindström L**, Lapidus A, Ost A, Bergquist A. Increased risk of colorectal cancer and dysplasia in patients with Crohn's colitis and primary sclerosing cholangitis. *Dis Colon Rectum* 2011; **54**: 1392-1397 [PMID: 21979184 DOI: 10.1097/DCR.0b013e31822bbcc1]

18   **Rabinovitz M**, Gavaler JS, Schade RR, Dindzans VJ, Chien MC, Van Thiel DH. Does primary sclerosing cholangitis occurring in association with inflammatory bowel disease differ from that occurring in the absence of inflammatory bowel disease? *Hepatology* 1990; **11**: 7-11 [PMID: 2295474 DOI: 10.1002/hep.1840110103]

19   **Yanai H**, Matalon S, Rosenblatt A, Awadie H, Berdichevski T, Snir Y, Kopylov U, Katz L, Stein A, Mlynarsky L, Tulchinsky H, Konikoff FM, Horin SB, Braun M, Ben-Ari Z, Chowers Y, Baruch Y, Shibolet O, Dotan I. Prognosis of primary sclerosing cholangitis in israel is independent of

coexisting inflammatory bowel Disease. *J Crohns Colitis* 2015; **9**: 177-184 [PMID: 25518055 DOI: 10.1093/ecco-jcc/jju013]

20   **Fevery J**, Van Steenbergen W, Van Pelt J, Laleman W, Hoffman I, Geboes K, Vermeire S, Nevens F. Patients with large-duct primary sclerosing cholangitis and Crohn's disease have a better outcome than those with ulcerative colitis, or without IBD. *Aliment Pharmacol Ther* 2016; **43**: 612-620 [PMID: 26748470 DOI: 10.1111/apt.13516]

21   **Nordenvall C**, Olén O, Nilsson PJ, von Seth E, Ekbom A, Bottai M, Myrelid P, Bergquist A. Colectomy prior to diagnosis of primary sclerosing cholangitis is associated with improved prognosis in a nationwide cohort study of 2594 PSC-IBD patients. *Aliment Pharmacol Ther* 2018; **47**: 238-245 [PMID: 29064110 DOI: 10.1111/apt.14393]

22   **Zheng HH**, Jiang XL. Increased risk of colorectal neoplasia in patients with primary sclerosing cholangitis and inflammatory bowel disease: a meta-analysis of 16 observational studies. *Eur J Gastroenterol Hepatol* 2016; **28**: 383-390 [PMID: 26938805 DOI: 10.1097/MEG.0000000000000576]

23   **Gulamhusein AF**, Eaton JE, Tabibian JH, Atkinson EJ, Juran BD, Lazaridis KN. Duration of Inflammatory Bowel Disease Is Associated With Increased Risk of Cholangiocarcinoma in Patients With Primary Sclerosing Cholangitis and IBD. *Am J Gastroenterol* 2016; **111**: 705-711 [PMID: 27002801 DOI: 10.1038/ajg.2016.55]

24   **Lindor KD**, Kowdley KV, Harrison ME; American College of Gastroenterology. ACG Clinical Guideline: Primary Sclerosing Cholangitis. *Am J Gastroenterol* 2015; **110**: 646-59; quiz 660 [PMID: 25869391 DOI: 10.1038/ajg.2015.112]

25   **Harbord M**, Annese V, Vavricka SR, Allez M, Barreiro-de Acosta M, Boberg KM, Burisch J, De Vos M, De Vries AM, Dick AD, Juillerat P, Karlsen TH, Koutroubakis I, Lakatos PL, Orchard T, Papay P, Raine T, Reinshagen M, Thaci D, Tilg H, Carbonnel F; European Crohn's and Colitis Organisation. The First European Evidence-based Consensus on Extra-intestinal Manifestations in Inflammatory Bowel Disease. *J Crohns Colitis* 2016; **10**: 239-254 [PMID: 26614685 DOI: 10.1093/ecco-jcc/jjv213]

26   **Lewis JT**, Talwalkar JA, Rosen CB, Smyrk TC, Abraham SC. Prevalence and risk factors for gallbladder neoplasia in patients with primary sclerosing cholangitis: evidence for a metaplasia-dysplasia-carcinoma sequence. *Am J Surg Pathol* 2007; **31**: 907-913 [PMID: 17527079 DOI: 10.1097/01.pas.0000213435.99492.8a]

27   **Said K**, Glaumann H, Bergquist A. Gallbladder disease in patients with primary sclerosing cholangitis. *J Hepatol* 2008; **48**: 598-605 [PMID: 18222013 DOI: 10.1016/j.jhep.2007.11.019]

28   **Chapman R**, Fevery J, Kalloo A, Nagorney DM, Boberg KM, Shneider B, Gores GJ; American Association for the Study of Liver Diseases. Diagnosis and management of primary sclerosing cholangitis. *Hepatology* 2010; **51**: 660-678 [PMID: 20101749 DOI: 10.1002/hep.23294]

29   **Zenouzi R**, Weismüller TJ, Hübener P, Schulze K, Bubenheim M, Pannicke N, Weiler-Normann C, Lenzen H, Manns MP, Lohse AW, Schramm C. Low risk of hepatocellular carcinoma in patients with primary sclerosing cholangitis with cirrhosis. *Clin Gastroenterol Hepatol* 2014; **12**: 1733-1738 [PMID: 24530461 DOI: 10.1016/j.cgh.2014.02.008]

30   **Singh S**, Khanna S, Pardi DS, Loftus EV Jr, Talwalkar JA. Effect of ursodeoxycholic acid use on the risk of colorectal neoplasia in patients with primary sclerosing cholangitis and inflammatory bowel disease: a systematic review and meta-analysis. *Inflamm Bowel Dis* 2013; **19**: 1631-1638 [PMID: 23665966 DOI: 10.1097/MIB.0b013e318286fa61]

31   **Carey EJ**, Lindor KD. Chemoprevention of colorectal cancer with ursodeoxycholic acid: cons. *Clin Res Hepatol Gastroenterol* 2012; **36** Suppl 1: S61-S64 [PMID: 23141896 DOI: 10.1016/S2210-7401(12)70023-2]

32   **Stolfi C**, Pallone F, Monteleone G. Colorectal cancer chemoprevention by mesalazine and its derivatives. *J Biomed Biotechnol* 2012; **2012**: 980458 [PMID: 22701310 DOI: 10.1155/2012/980458]

33   **Tsaitas C**, Semertzidou A, Sinakos E. Update on inflammatory bowel disease in patients with primary sclerosing cholangitis. *World J Hepatol* 2014; **6**: 178-187 [PMID: 24799986 DOI: 10.4254/wjh.v6.i4.178]

34   **Christensen B**, Micic D, Gibson PR, Yarur A, Bellaguarda E, Corsello P, Gaetano JN, Kinnucan J, Rao VL, Reddy S, Singh S, Pekow J, Rubin DT. Vedolizumab in patients with concurrent primary sclerosing cholangitis and inflammatory bowel disease does not improve liver biochemistry but is safe and effective for the bowel disease. *Aliment Pharmacol Ther* 2018; **47**: 753-762 [DOI: 10.1111/apt.14525]

35   **Shah A**, Macdonald GA, Morrison M, Holtmann G. Targeting the Gut Microbiome as a Treatment for Primary Sclerosing Cholangitis: A Conceptional Framework. *Am J Gastroenterol* 2020; **115**: 814-822 [PMID: 32250997 DOI: 10.14309/ajg.0000000000000604]

36   **Irlès-Depé M**, Roullet S, Neau-Cransac M, Dumortier J, Dharancy S, Houssel-Debry P, Boillot O, Chiche L, Laurent C, Laharie D, De Lédinghen V. Impact of Preexisting Inflammatory Bowel Disease on the Outcome of Liver Transplantation for Primary Sclerosing Cholangitis. *Liver Transpl* 2020; **26**: 1477-1491 [PMID: 32603007 DOI: 10.1002/lt.25838]

37   **Liberal R**, Gaspar R, Lopes S, Macedo G. Primary biliary cholangitis in patients with inflammatory bowel disease. *Clin Res Hepatol Gastroenterol* 2020; **44**: e5-e9 [PMID: 31171469 DOI: 10.1016/j.clinre.2019.05.002]

38   **Aiba Y**, Yamazaki K, Nishida N, Kawashima M, Hitomi Y, Nakamura H, Komori A, Fuyuno Y,

Takahashi A, Kawaguchi T, Takazoe M, Suzuki Y, Motoya S, Matsui T, Esaki M, Matsumoto T, Kubo M, Tokunaga K, Nakamura M. Disease susceptibility genes shared by primary biliary cirrhosis and Crohn's disease in the Japanese population. *J Hum Genet* 2015; **60**: 525-531 [PMID: 26084578 DOI: 10.1038/jhg.2015.59]

39   **Ballotin VR**, Bigarella LG, Riva F, Onzi G, Balbinot RA, Balbinot SS, Soldera J. Primary sclerosing cholangitis and autoimmune hepatitis overlap syndrome associated with inflammatory bowel disease: A case report and systematic review. *World J Clin Cases* 2020; **8**: 4075-4093 [PMID: 33024765 DOI: 10.12998/wjcc.v8.i18.4075]

40   **Miranda-Bautista J**, Menchén L. Adalimumab-induced autoimmune hepatitis in a patient with Crohn's disease. *Gastroenterol Hepatol* 2019; **42**: 306-307 [PMID: 30029925 DOI: 10.1016/j.gastrohep.2018.06.006]

41   **Nassir F**, Rector RS, Hammoud GM, Ibdah JA. Pathogenesis and Prevention of Hepatic Steatosis. *Gastroenterol Hepatol (N Y)* 2015; **11**: 167-175 [PMID: 27099587]

42   **Sunny NE**, Parks EJ, Browning JD, Burgess SC. Excessive hepatic mitochondrial TCA cycle and gluconeogenesis in humans with nonalcoholic fatty liver disease. *Cell Metab* 2011; **14**: 804-810 [PMID: 22152305 DOI: 10.1016/j.cmet.2011.11.004]

43   C. Ulceration of the colon with a much enlarged fatty liver. *Trans Pathol Soc Philadelphia* 1873; **4**: 87-88 [DOI: 10.1056/nejm187304100881506]

44   **Losurdo G**, La Fortezza RF, Iannone A, Contaldo A, Barone M, Ierardi E, Di Leo A, Principi M. Prevalence and associated factors of obesity in inflammatory bowel disease: A case-control study. *World J Gastroenterol* 2020; **26**: 7528-7537 [PMID: 33384552 DOI: 10.3748/wjg.v26.i47.7528]

45   **Miele L**, Valenza V, La Torre G, Montalto M, Cammarota G, Ricci R, Mascianà R, Forgione A, Gabrieli ML, Perotti G, Vecchio FM, Rapaccini G, Gasbarrini G, Day CP, Grieco A. Increased intestinal permeability and tight junction alterations in nonalcoholic fatty liver disease. *Hepatology* 2009; **49**: 1877-1887 [PMID: 19291785 DOI: 10.1002/hep.22848]

46   **Principi M**, Losurdo G, Iannone A, Contaldo A, Deflorio V, Ranaldo N, Pisani A, Ierardi E, Di Leo A, Barone M. Differences in dietary habits between patients with inflammatory bowel disease in clinical remission and a healthy population. *Ann Gastroenterol* 2018; **31**: 469-473 [PMID: 29991892 DOI: 10.20524/aog.2018.0273]

47   **Ruemmele FM**. Role of Diet in Inflammatory Bowel Disease. *Ann Nutr Metab* 2016; **68** Suppl 1: 33-41 [PMID: 27355913 DOI: 10.1159/000445392]

48   **Zou ZY**, Shen B, Fan JG. Systematic Review With Meta-analysis: Epidemiology of Nonalcoholic Fatty Liver Disease in Patients With Inflammatory Bowel Disease. *Inflamm Bowel Dis* 2019; **25**: 1764-1772 [PMID: 30918952 DOI: 10.1093/ibd/izz043]

49   **Bessissow T**, Le NH, Rollet K, Afif W, Bitton A, Sebastiani G. Incidence and Predictors of Nonalcoholic Fatty Liver Disease by Serum Biomarkers in Patients with Inflammatory Bowel Disease. *Inflamm Bowel Dis* 2016; **22**: 1937-1944 [PMID: 27379445 DOI: 10.1097/MIB.0000000000000832]

50   **Sagami S**, Ueno Y, Tanaka S, Fujita A, Hayashi R, Oka S, Hyogo H, Chayama K. Significance of non-alcoholic fatty liver disease in Crohn's disease: A retrospective cohort study. *Hepatol Res* 2017; **47**: 872-881 [PMID: 27737498 DOI: 10.1111/hepr.12828]

51   **Glassner K**, Malaty HM, Abraham BP. Epidemiology and Risk Factors of Nonalcoholic Fatty Liver Disease Among Patients with Inflammatory Bowel Disease. *Inflamm Bowel Dis* 2017; **23**: 998-1003 [PMID: 28511199 DOI: 10.1097/MIB.0000000000001085]

52   **Principi M**, Iannone A, Losurdo G, Mangia M, Shahini E, Albano F, Rizzi SF, La Fortezza RF, Lovero R, Contaldo A, Barone M, Leandro G, Ierardi E, Di Leo A. Nonalcoholic Fatty Liver Disease in Inflammatory Bowel Disease: Prevalence and Risk Factors. *Inflamm Bowel Dis* 2018; **24**: 1589-1596 [PMID: 29688336 DOI: 10.1093/ibd/izy051]

53   **Ritaccio G**, Stoleru G, Abutaleb A, Cross RK, Shetty K, Sakiani S, Wong U. Nonalcoholic Fatty Liver Disease Is Common in IBD Patients However Progression to Hepatic Fibrosis by Noninvasive Markers Is Rare. *Dig Dis Sci* 2020 [DOI: 10.1007/s10620-020-06588-6]

54   **Franco I**, Bianco A, Mirizzi A, Campanella A, Bonfiglio C, Sorino P, Notarnicola M, Tutino V, Cozzolongo R, Giannuzzi V, Aballay LR, Buongiorno C, Bruno I, Osella AR. Physical Activity and Low Glycemic Index Mediterranean Diet: Main and Modification Effects on NAFLD Score. Results from a Randomized Clinical Trial. *Nutrients* 2020; **13** [PMID: 33379253 DOI: 10.3390/nu13010066]

55   **Hou JK**, Velayos F, Terrault N, Mahadevan U. Viral hepatitis and inflammatory bowel disease. *Inflamm Bowel Dis* 2010; **16**: 925-932 [PMID: 20480515 DOI: 10.1002/ibd.21284]

56   **Basnayake SK**, Easterbrook PJ. Wide variation in estimates of global prevalence and burden of chronic hepatitis B and C infection cited in published literature. *J Viral Hepat* 2016; **23**: 545-559 [PMID: 27028545 DOI: 10.1111/jvh.12519]

57   **Sansone S**, Guarino M, Castiglione F, Rispo A, Auriemma F, Loperto I, Rea M, Caporaso N, Morisco F. Hepatitis B and C virus reactivation in immunosuppressed patients with inflammatory bowel disease. *World J Gastroenterol* 2014; **20**: 3516-3524 [PMID: 24707134 DOI: 10.3748/wjg.v20.i13.3516]

58   **Loras C**, Saro C, Gonzalez-Huix F, Mínguez M, Merino O, Gisbert JP, Barrio J, Bernal A, Gutiérrez A, Piqueras M, Calvet X, Andreu M, Abad A, Ginard D, Bujanda L, Panés J, Torres M, Fernández-Bañares F, Viver JM, Esteve M; GETECCu (Grupo Español de Enfermedades de Crohn y Colitis Ulcerosa). Prevalence and factors related to hepatitis B and C in inflammatory bowel disease patients

in Spain: a nationwide, multicenter study. *Am J Gastroenterol* 2009; **104**: 57-63 [PMID: 19098850 DOI: 10.1038/ajg.2008.4]

59   **Chevaux JB**, Bigard MA, Bensenane M, Oussalah A, Jarlot S, Belle A, Nani A, Bronowicki JP, Peyrin-Biroulet L. Inflammatory bowel disease and hepatitis B and C. *Gastroenterol Clin Biol* 2009; **33**: 1082-1093 [PMID: 19896313 DOI: 10.1016/j.gcb.2009.03.021]

60   **Morisco F**, Castiglione F, Rispo A, Stroffolini T, Sansone S, Vitale R, Guarino M, Biancone L, Caruso A, D'Inca R, Marmo R, Orlando A, Riegler G, Donnarumma L, Camera S, Zorzi F, Renna S, Bove V, Tontini G, Vecchi M, Caporaso N. Effect of immunosuppressive therapy on patients with inflammatory bowel diseases and hepatitis B or C virus infection. *J Viral Hepat* 2013; **20**: 200-208 [PMID: 23383659 DOI: 10.1111/j.1365-2893.2012.01643.x]

61   **Papa A**, Felice C, Marzo M, Andrisani G, Armuzzi A, Covino M, Mocci G, Pugliese D, De Vitis I, Gasbarrini A, Rapaccini GL, Guidi L. Prevalence and natural history of hepatitis B and C infections in a large population of IBD patients treated with anti-tumor necrosis factor-α agents. *J Crohns Colitis* 2013; **7**: 113-119 [PMID: 22464811 DOI: 10.1016/j.crohns.2012.03.001]

62   **Losurdo G**, Iannone A, Contaldo A, Barone M, Ierardi E, Di Leo A, Principi M. Chronic Viral Hepatitis in a Cohort of Inflammatory Bowel Disease Patients from Southern Italy: A Case-Control Study. *Pathogens* 2020; **9** [PMID: 33113974 DOI: 10.3390/pathogens9110870]

63   **Borman ZA**, Côté-Daigneault J, Colombel JF. The risk for opportunistic infections in inflammatory bowel disease with biologics: an update. *Expert Rev Gastroenterol Hepatol* 2018; **12**: 1101-1108 [PMID: 30277409 DOI: 10.1080/17474124.2018.1530983]

64   **Rahier JF**, Magro F, Abreu C, Armuzzi A, Ben-Horin S, Chowers Y, Cottone M, de Ridder L, Doherty G, Ehehalt R, Esteve M, Katsanos K, Lees CW, Macmahon E, Moreels T, Reinisch W, Tilg H, Tremblay L, Veereman-Wauters G, Viget N, Yazdanpanah Y, Eliakim R, Colombel JF; European Crohn's and Colitis Organisation (ECCO). Second European evidence-based consensus on the prevention, diagnosis and management of opportunistic infections in inflammatory bowel disease. *J Crohns Colitis* 2014; **8**: 443-468 [PMID: 24613021 DOI: 10.1016/j.crohns.2013.12.013]

65   **Lok AS**, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; **45**: 507-539 [PMID: 17256718 DOI: 10.1002/hep.21513]

66   **Katsanos KH**, Tsianos VE, Zois CD, Zioga H, Vagias I, Zervou E, Christodoulou DK, Tsianos EV; Northwest Greece IBD Study Group. Inflammatory bowel disease and hepatitis B and C in Western Balkans: a referral centre study and review of the literature. *J Crohns Colitis* 2010; **4**: 450-465 [PMID: 21122543 DOI: 10.1016/j.crohns.2010.03.001]

67   **Magro F**, Gionchetti P, Eliakim R, Ardizzone S, Armuzzi A, Barreiro-de Acosta M, Burisch J, Gecse KB, Hart AL, Hindryckx P, Langner C, Limdi JK, Pellino G, Zagórowicz E, Raine T, Harbord M, Rieder F; European Crohn's and Colitis Organisation [ECCO]. Third European Evidence-based Consensus on Diagnosis and Management of Ulcerative Colitis. Part 1: Definitions, Diagnosis, Extra-intestinal Manifestations, Pregnancy, Cancer Surveillance, Surgery, and Ileo-anal Pouch Disorders. *J Crohns Colitis* 2017; **11**: 649-670 [PMID: 28158501 DOI: 10.1093/ecco-jcc/jjx008]

68   **Brunasso AM**, Puntoni M, Gulia A, Massone C. Safety of anti-tumour necrosis factor agents in patients with chronic hepatitis C infection: a systematic review. *Rheumatology (Oxford)* 2011; **50**: 1700-1711 [PMID: 21690185 DOI: 10.1093/rheumatology/ker190]

69   **Scherzer TM**, Staufer K, Novacek G, Steindl-Munda P, Schumacher S, Hofer H, Ferenci P, Vogelsang H. Efficacy and safety of antiviral therapy in patients with Crohn's disease and chronic hepatitis C. *Aliment Pharmacol Ther* 2008; **28**: 742-748 [PMID: 19145730 DOI: 10.1111/j.1365-2036.2008.03779.x]

70   **Tilg H**, Vogelsang H, Ludwiczek O, Lochs H, Kaser A, Colombel JF, Ulmer H, Rutgeerts P, Krüger S, Cortot A, D'Haens G, Harrer M, Gasche C, Wrba F, Kuhn I, Reinisch W. A randomised placebo controlled trial of pegylated interferon alpha in active ulcerative colitis. *Gut* 2003; **52**: 1728-1733 [PMID: 14633951 DOI: 10.1136/gut.52.12.1728]

71   **Koff RS**. Review article: the efficacy and safety of sofosbuvir, a novel, oral nucleotide NS5B polymerase inhibitor, in the treatment of chronic hepatitis C virus infection. *Aliment Pharmacol Ther* 2014; **39**: 478-487 [PMID: 24387618 DOI: 10.1111/apt.12601]

72   **Imperatore N**, Castiglione F, Rispo A, Sessa A, Caporaso N, Morisco F. Timing Strategies of Direct-Acting Antivirals and Biologics Administration in HCV-Infected Subjects with Inflammatory Bowel Diseases. *Front Pharmacol* 2017; **8**: 867 [PMID: 29209223 DOI: 10.3389/fphar.2017.00867]

73   **Losurdo G**, Iannone A, Contaldo A, Barone M, Ierardi E, Di Leo A, Principi M. Trends of Liver Stiffness in Inflammatory Bowel Disease with Chronic Hepatitis C. *Diagnostics (Basel)* 2020; **10** [PMID: 33276638 DOI: 10.3390/diagnostics10121037]

74   **Andrade RJ**, Chalasani N, Björnsson ES, Suzuki A, Kullak-Ublick GA, Watkins PB, Devarbhavi H, Merz M, Lucena MI, Kaplowitz N, Aithal GP. Drug-induced liver injury. *Nat Rev Dis Primers* 2019; **5**: 58 [PMID: 31439850 DOI: 10.1038/s41572-019-0105-0]

75   **Yaccob A**, Mari A. Practical clinical approach to the evaluation of hepatobiliary disorders in inflammatory bowel disease. *Frontline Gastroenterol* 2019; **10**: 309-315 [PMID: 31281626 DOI: 10.1136/flgastro-2018-101037]

76   **Wong DR**, Coenen MJ, Derijks LJ, Vermeulen SH, van Marrewijk CJ, Klungel OH, Scheffer H, Franke B, Guchelaar HJ, de Jong DJ, Engels LG, Verbeek AL, Hooymans PM; TOPIC Recruitment Team. Early prediction of thiopurine-induced hepatotoxicity in inflammatory bowel disease. *Aliment*

Pharmacol Ther 2017; **45**: 391-402 [PMID: 27943397 DOI: 10.1111/apt.13879]

77   **Marinaki AM**, Arenas-Hernandez M. Reducing risk in thiopurine therapy. *Xenobiotica* 2020; **50**: 101-109 [PMID: 31682552 DOI: 10.1080/00498254.2019.1688424]

78   **Dong X**, Zheng Q, Zhu M, Tong JL, Ran ZH. Thiopurine Smethyltransferase polymorphisms and thiopurine toxicity in treatment of inflammatory bowel disease. *World J Gastroenterol* 2010; **16**: 3187-3195 [DOI: 10.3748/wjg.v16.i25.3187]

79   **Gisbert JP**, González-Lama Y, Maté J. Thiopurine-induced liver injury in patients with inflammatory bowel disease: a systematic review. *Am J Gastroenterol* 2007; **102**: 1518-1527 [PMID: 17391318 DOI: 10.1111/j.1572-0241.2007.01187.x]

80   **Chaparro M**, Ordás I, Cabré E, Garcia-Sanchez V, Bastida G, Peñalva M, Gomollón F, García-Planella E, Merino O, Gutiérrez A, Esteve M, Márquez L, Garcia-Sepulcre M, Hinojosa J, Vera I, Muñoz F, Mendoza JL, Cabriada JL, Montoro MA, Barreiro-de Acosta M, Ceña G, Saro C, Aldeguer X, Barrio J, Maté J, Gisbert JP. Safety of thiopurine therapy in inflammatory bowel disease: long-term follow-up study of 3931 patients. *Inflamm Bowel Dis* 2013; **19**: 1404-1410 [PMID: 23665964 DOI: 10.1097/MIB.0b013e318281f28f]

81   **Schröder T**, Schmidt KJ, Olsen V, Möller S, Mackenroth T, Sina C, Lehnert H, Fellermann K, Büning J. Liver steatosis is a risk factor for hepatotoxicity in patients with inflammatory bowel disease under immunosuppressive treatment. *Eur J Gastroenterol Hepatol* 2015; **27**: 698-704 [PMID: 25923946 DOI: 10.1097/MEG.0000000000000350]

82   **Romagnuolo J**, Sadowski DC, Lalor E, Jewell L, Thomson AB. Cholestatic hepatocellular injury with azathioprine: a case report and review of the mechanisms of hepatotoxicity. *Can J Gastroenterol* 1998; **12**: 479-483 [PMID: 9812167 DOI: 10.1155/1998/294752]

83   **Seinen ML**, van Asseldonk DP, de Boer NK, Bouma G, van Nieuwkerk CM, Mulder CJ, Bloemena E, van Bodegraven AA. Nodular Regenerative Hyperplasia of the Liver in Patients with IBD Treated with Allopurinol-Thiopurine Combination Therapy. *Inflamm Bowel Dis* 2017; **23**: 448-452 [PMID: 28151736 DOI: 10.1097/MIB.0000000000001036]

84   **Musumba CO**. Review article: the association between nodular regenerative hyperplasia, inflammatory bowel disease and thiopurine therapy. *Aliment Pharmacol Ther* 2013; **38**: 1025-1037 [PMID: 24099468 DOI: 10.1111/apt.12490]

85   **Seksik P**, Mary JY, Beaugerie L, Lémann M, Colombel JF, Vernier-Massouille G, Cosnes J. Incidence of nodular regenerative hyperplasia in inflammatory bowel disease patients treated with azathioprine. *Inflamm Bowel Dis* 2011; **17**: 565-572 [PMID: 20848502 DOI: 10.1002/ibd.21330]

86   **Gisbert JP**, Luna M, González-Lama Y, Pousa ID, Velasco M, Moreno-Otero R, Maté J. Liver injury in inflammatory bowel disease: long-term follow-up study of 786 patients. *Inflamm Bowel Dis* 2007; **13**: 1106-1114 [PMID: 17455203 DOI: 10.1002/ibd.20160]

87   **Shaye OA**, Yadegari M, Abreu MT, Poordad F, Simon K, Martin P, Papadakis KA, Ippoliti A, Vasiliauskas E, Tran TT. Hepatotoxicity of 6-mercaptopurine (6-MP) and Azathioprine (AZA) in adult IBD patients. *Am J Gastroenterol* 2007; **102**: 2488-2494 [PMID: 17764490 DOI: 10.1111/j.1572-0241.2007.01515.x]

88   **Hindorf U**, Johansson M, Eriksson A, Kvifors E, Almer SH. Mercaptopurine treatment should be considered in azathioprine intolerant patients with inflammatory bowel disease. *Aliment Pharmacol Ther* 2009; **29**: 654-661 [PMID: 19183142 DOI: 10.1111/j.1365-2036.2008.03925.x]

89   **Meijer B**, Seinen ML, van Egmond R, Bouma G, Mulder CJJ, van Bodegraven AA, de Boer NKH. Optimizing Thiopurine Therapy in Inflammatory Bowel Disease Among 2 Real-life Intercept Cohorts: Effect of Allopurinol Comedication? *Inflamm Bowel Dis* 2017; **23**: 2011-2017 [PMID: 28617756 DOI: 10.1097/MIB.0000000000001168]

90   **Kreijne JE**, de Veer RC, de Boer NK, Dijkstra G, West R, Moorsel SAW, de Jong DJ, van der Woude CJ, de Vries AC; of the Dutch Initiative on Crohn, Colitis (ICC). Real-life study of safety of thiopurine-allopurinol combination therapy in inflammatory bowel disease: myelotoxicity and hepatotoxicity rarely affect maintenance treatment. *Aliment Pharmacol Ther* 2019; **50**: 407-415 [PMID: 31359480 DOI: 10.1111/apt.15402]

91   **Vasudevan A**, Beswick L, Friedman AB, Moltzen A, Haridy J, Raghunath A, Sparrow M, van Langenberg D. Low-dose thiopurine with allopurinol co-therapy overcomes thiopurine intolerance and allows thiopurine continuation in inflammatory bowel disease. *Dig Liver Dis* 2018; **50**: 682-688 [PMID: 29525182 DOI: 10.1016/j.dld.2018.02.001]

92   **Vande Casteele N**, Herfarth H, Katz J, Falck-Ytter Y, Singh S. American Gastroenterological Association Institute Technical Review on the Role of Therapeutic Drug Monitoring in the Management of Inflammatory Bowel Diseases. *Gastroenterology* 2017; **153**: 835-857.e6 [PMID: 28774547 DOI: 10.1053/j.gastro.2017.07.031]

93   **Mottet C**, Schoepfer AM, Juillerat P, Cosnes J, Froehlich F, Kessler-Brondolo V, Seibold F, Rogler G, Vavricka SR, Michetti P. Experts Opinion on the Practical Use of Azathioprine and 6-Mercaptopurine in Inflammatory Bowel Disease. *Inflamm Bowel Dis* 2016; **22**: 2733-2747 [PMID: 27760078 DOI: 10.1097/MIB.0000000000000923]

94   **Khokhar OS**, Lewis JH. Hepatotoxicity of agents used in the management of inflammatory bowel disease. *Dig Dis* 2010; **28**: 508-518 [PMID: 20926880 DOI: 10.1159/000320410]

95   **Ransford RA**, Langman MJ. Sulphasalazine and mesalazine: serious adverse reactions re-evaluated on the basis of suspected adverse reaction reports to the Committee on Safety of Medicines. *Gut* 2002; **51**: 536-539 [PMID: 12235076 DOI: 10.1136/gut.51.4.536]

96   **Sehgal P**, Colombel JF, Aboubakr A, Narula N. Systematic review: safety of mesalazine in

ulcerative colitis. *Aliment Pharmacol Ther* 2018; **47**: 1597-1609 [PMID: 29722441 DOI: 10.1111/apt.14688]

97   **Food and Drug Administration**.   Highlights of prescribing information: Delzicol. 2015. [cited 20 February 2021]. Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2015204412s006lbl.pdf

98   **Fisher MC**, Cronstein BN. Metaanalysis of methylenetetrahydrofolate reductase (MTHFR) polymorphisms affecting methotrexate toxicity. *J Rheumatol* 2009; **36**: 539-545 [PMID: 19208607 DOI: 10.3899/jrheum.080576]

99   **Cáliz R**, del Amo J, Balsa A, Blanco F, Silva L, Sanmarti R, Martínez FG, Collado MD, Ramirez Mdel C, Tejedor D, Artieda M, Pascual-Salcedo D, Oreiro N, Andreu JL, Graell E, Simon L, Martínez A, Mulero J. The C677T polymorphism in the MTHFR gene is associated with the toxicity of methotrexate in a Spanish rheumatoid arthritis population. *Scand J Rheumatol* 2012; **41**: 10-14 [PMID: 22044028 DOI: 10.3109/03009742.2011.617312]

100   **Berends MA**, van Oijen MG, Snoek J, van de Kerkhof PC, Drenth JP, Han van Krieken J, de Jong EM. Reliability of the Roenigk classification of liver damage after methotrexate treatment for psoriasis: a clinicopathologic study of 160 liver biopsy specimens. *Arch Dermatol* 2007; **143**: 1515-1519 [PMID: 18087000 DOI: 10.1001/archderm.143.12.1515]

101   **Khan N**, Abbas AM, Whang N, Balart LA, Bazzano LA, Kelly TN. Incidence of liver toxicity in inflammatory bowel disease patients treated with methotrexate: a meta-analysis of clinical trials. *Inflamm Bowel Dis* 2012; **18**: 359-367 [PMID: 21751301 DOI: 10.1002/ibd.21820]

102   **Tran-Minh ML**, Sousa P, Maillet M, Allez M, Gornet JM. Hepatic complications induced by immunosuppressants and biologics in inflammatory bowel disease. *World J Hepatol* 2017; **9**: 613-626 [PMID: 28539989 DOI: 10.4254/wjh.v9.i13.613]

103   **Saibeni S**, Bollani S, Losco A, Michielan A, Sostegni R, Devani M, Lupinacci G, Pirola L, Cucino C, Meucci G, Basilisco G, D'Incà R, Bruno S. The use of methotrexate for treatment of inflammatory bowel disease in clinical practice. *Dig Liver Dis* 2012; **44**: 123-127 [PMID: 22051323 DOI: 10.1016/j.dld.2011.09.015]

104   **Labadie JG**, Jain M. Noninvasive Tests to Monitor Methotrexate-Induced Liver Injury. *Clin Liver Dis (Hoboken)* 2019; **13**: 67-71 [PMID: 30988939 DOI: 10.1002/cld.765]

105   **Laharie D**, Seneschal J, Schaeverbeke T, Doutre MS, Longy-Boursier M, Pellegrin JL, Chabrun E, Villars S, Zerbib F, de Lédinghen V. Assessment of liver fibrosis with transient elastography and FibroTest in patients treated with methotrexate for chronic inflammatory diseases: a case-control study. *J Hepatol* 2010; **53**: 1035-1040 [PMID: 20801541 DOI: 10.1016/j.jhep.2010.04.043]

106   **Herfarth HH**, Kappelman MD, Long MD, Isaacs KL. Use of Methotrexate in the Treatment of Inflammatory Bowel Diseases. *Inflamm Bowel Dis* 2016; **22**: 224-233 [PMID: 26457382 DOI: 10.1097/MIB.0000000000000589]

107   **Food and Drug Administration**.   Approved Drug Products. [cited 20 February 2021]. Available from: http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm?fuseaction=Search.Label_ApprovalHistory#apphist

108   **Björnsson ES**, Gunnarsson BI, Gröndal G, Jonasson JG, Einarsdottir R, Ludviksson BR, Gudbjörnsson B, Olafsson S. Risk of drug-induced liver injury from tumor necrosis factor antagonists. *Clin Gastroenterol Hepatol* 2015; **13**: 602-608 [PMID: 25131534 DOI: 10.1016/j.cgh.2014.07.062]

109   **Shelton E**, Chaudrey K, Sauk J, Khalili H, Masia R, Nguyen DD, Yajnik V, Ananthakrishnan AN. New onset idiosyncratic liver enzyme elevations with biological therapy in inflammatory bowel disease. *Aliment Pharmacol Ther* 2015; **41**: 972-979 [PMID: 25756190 DOI: 10.1111/apt.13159]

110   **Koller T**, Galambosova M, Filakovska S, Kubincova M, Hlavaty T, Toth J, Krajcovicova A, Payer J. Drug-induced liver injury in inflammatory bowel disease: 1-year prospective observational study. *World J Gastroenterol* 2017; **23**: 4102-4111 [PMID: 28652663 DOI: 10.3748/wjg.v23.i22.4102]

111   **Worland T**, Chin KL, van Langenberg D, Garg M, Nicoll A. Retrospective study of idiosyncratic drug-induced liver injury from infliximab in an inflammatory bowel disease cohort: the IDLE study. *Ann Gastroenterol* 2020; **33**: 162-169 [PMID: 32127737 DOI: 10.20524/aog.2020.0453]

112   **Van den Brande JM**, Braat H, van den Brink GR, Versteeg HH, Bauer CA, Hoedemaeker I, van Montfrans C, Hommes DW, Peppelenbosch MP, van Deventer SJ. Infliximab but not etanercept induces apoptosis in lamina propria T-lymphocytes from patients with Crohn's disease. *Gastroenterology* 2003; **124**: 1774-1785 [PMID: 12806611 DOI: 10.1016/s0016-5085(03)00382-2]

113   **Cantaert T**, De Rycke L, Mavragani CP, Wijbrandts CA, Niewold TB, Niers T, Vandooren B, Veys EM, Richel D, Tak PP, Crow MK, Baeten D. Exposure to nuclear antigens contributes to the induction of humoral autoimmunity during tumour necrosis factor alpha blockade. *Ann Rheum Dis* 2009; **68**: 1022-1029 [PMID: 18625621 DOI: 10.1136/ard.2008.093724]

114   **Gershov D**, Kim S, Brot N, Elkon KB. C-Reactive protein binds to apoptotic cells, protects the cells from assembly of the terminal complement components, and sustains an antiinflammatory innate immune response: implications for systemic autoimmunity. *J Exp Med* 2000; **192**: 1353-1364 [PMID: 11067883 DOI: 10.1084/jem.192.9.1353]

115   **Colina F**, Molero A, Casís B, Martínez-Montiel P. Infliximab-related hepatitis: a case study and literature review. *Dig Dis Sci* 2013; **58**: 3362-3367 [PMID: 23645381 DOI: 10.1007/s10620-013-2698-6]

116   **Ghabril M**, Bonkovsky HL, Kum C, Davern T, Hayashi PH, Kleiner DE, Serrano J, Rochon J, Fontana RJ, Bonacini M; US Drug-Induced Liver Injury Network. Liver injury from tumor necrosis

factor-α antagonists: analysis of thirty-four cases. *Clin Gastroenterol Hepatol* 2013; **11**: 558-564.e3 [PMID: 23333219 DOI: 10.1016/j.cgh.2012.12.025]

117 **Ierardi E**, Della Valle N, Nacchiero MC, De Francesco V, Stoppino G, Panella C. Infliximab single administration followed by acute liver injury. *Inflamm Bowel Dis* 2006; **12**: 1089-1091 [PMID: 17075352 DOI: 10.1097/01.mib.0000246783.75753.9f]

118 **Adar T**, Mizrahi M, Pappo O, Scheiman-Elazary A, Shibolet O. Adalimumab-induced autoimmune hepatitis. *J Clin Gastroenterol* 2010; **44**: e20-e22 [PMID: 19593165 DOI: 10.1097/MCG.0b013e3181a745e7]

119 **Bezabeh S**, Flowers CM, Kortepeter C, Avigan M. Clinically significant liver injury in patients treated with natalizumab. *Aliment Pharmacol Ther* 2010; **31**: 1028-1035 [PMID: 20163378 DOI: 10.1111/j.1365-2036.2010.04262.x]

120 **Lisotti A**, Azzaroli F, Brillanti S, Mazzella G. Severe acute autoimmune hepatitis after natalizumab treatment. *Dig Liver Dis* 2012; **44**: 356-357 [PMID: 22154948 DOI: 10.1016/j.dld.2011.11.003]

121 **Lamb CA**, Kennedy NA, Raine T, Hendy PA, Smith PJ, Limdi JK, Hayee B, Lomer MCE, Parkes GC, Selinger C, Barrett KJ, Davies RJ, Bennett C, Gittens S, Dunlop MG, Faiz O, Fraser A, Garrick V, Johnston PD, Parkes M, Sanderson J, Terry H; IBD guidelines eDelphi consensus group, Gaya DR, Iqbal TH, Taylor SA, Smith M, Brookes M, Hansen R, Hawthorne AB. British Society of Gastroenterology consensus guidelines on the management of inflammatory bowel disease in adults. *Gut* 2019; **68**: s1-s106 [PMID: 31562236 DOI: 10.1136/gutjnl-2019-318484]

122 **Bellizzi A**, Barucca V, Fioriti D, Colosimo MT, Mischitelli M, Anzivino E, Chiarini F, Pietropaolo V. Early years of biological agents therapy in Crohn's disease and risk of the human polyomavirus JC reactivation. *J Cell Physiol* 2010; **224**: 316-326 [PMID: 20432445 DOI: 10.1002/jcp.22146]

123 **Food and Drug Administration**. Highlights of prescribing information: Entyvio (Vedolizumab). 2014. [cited 20 February 2021]. Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2014/125476s000lbl.pdf

124 **Leonardi CL**, Kimball AB, Papp KA, Yeilding N, Guzzo C, Wang Y, Li S, Dooley LT, Gordon KB; PHOENIX 1 study investigators. Efficacy and safety of ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with psoriasis: 76-wk results from a randomised, double-blind, placebo-controlled trial (PHOENIX 1). *Lancet* 2008; **371**: 1665-1674 [PMID: 18486739 DOI: 10.1016/S0140-6736(08)60725-4]

125 **Papp KA**, Langley RG, Lebwohl M, Krueger GG, Szapary P, Yeilding N, Guzzo C, Hsu MC, Wang Y, Li S, Dooley LT, Reich K; PHOENIX 2 study investigators. Efficacy and safety of ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with psoriasis: 52-wk results from a randomised, double-blind, placebo-controlled trial (PHOENIX 2). *Lancet* 2008; **371**: 1675-1684 [PMID: 18486740 DOI: 10.1016/S0140-6736(08)60726-6]

126 **Llamas-Velasco M**, Concha-Garzón MJ, García-Diez A, Daudén E. Liver Injury in Psoriasis Patients Receiving Ustekinumab: A Retrospective Study of 44 Patients Treated in the Clinical Practice Setting. *Actas Dermosifiliogr* 2015; **106**: 470-476 [PMID: 25912374 DOI: 10.1016/j.ad.2015.02.002]

127 **Lovero R**, Losurdo G, Mastromauro M, Castellaneta NM, Mongelli A, Gentile A, Di Leo A, Principi M. A Case of Severe Transaminase Elevation Following a Single Ustekinumab Dose with Remission After Drug Withdrawal. *Curr Drug Saf* 2018; **13**: 221-223 [PMID: 30027852 DOI: 10.2174/1574886313666180719165212]

128 **Valenzuela F**, Korman NJ, Bissonnette R, Bakos N, Tsai TF, Harper MK, Ports WC, Tan H, Tallman A, Valdez H, Gardner AC. Tofacitinib in patients with moderate-to-severe chronic plaque psoriasis: long-term safety and efficacy in an open-label extension study. *Br J Dermatol* 2018; **179**: 853-862 [PMID: 29782642 DOI: 10.1111/bjd.16798]

129 **Wollenhaupt J**, Silverfield J, Lee EB, Curtis JR, Wood SP, Soma K, Nduaka CI, Benda B, Gruben D, Nakamura H, Komuro Y, Zwillich SH, Wang L, Riese RJ. Safety and efficacy of tofacitinib, an oral janus kinase inhibitor, for the treatment of rheumatoid arthritis in open-label, longterm extension studies. *J Rheumatol* 2014; **41**: 837-852 [PMID: 24692527 DOI: 10.3899/jrheum.130683]

130 **Rojas-Feria M**, Castro M, Suárez E, Ampuero J, Romero-Gómez M. Hepatobiliary manifestations in inflammatory bowel disease: the gut, the drugs and the liver. *World J Gastroenterol* 2013; **19**: 7327-7340 [PMID: 24259964 DOI: 10.3748/wjg.v19.i42.7327]

131 **Kuy S**, Dua A, Chappidi R, Seabrook G, Brown KR, Lewis B, Rossi PJ, Lee CJ. The increasing incidence of thromboembolic events among hospitalized patients with inflammatory bowel disease. *Vascular* 2015; **23**: 260-264 [PMID: 24986868 DOI: 10.1177/1708538114541799]

132 **Maconi G**, Bolzacchini E, Dell'Era A, Russo U, Ardizzone S, de Franchis R. Portal vein thrombosis in inflammatory bowel diseases: a single-center case series. *J Crohns Colitis* 2012; **6**: 362-367 [PMID: 22405175 DOI: 10.1016/j.crohns.2011.10.003]

133 **Zezos P**, Kouklakis G, Saibil F. Inflammatory bowel disease and thromboembolism. *World J Gastroenterol* 2014; **20**: 13863-13878 [PMID: 25320522 DOI: 10.3748/wjg.v20.i38.13863]

134 **Naymagon L**, Tremblay D, Zubizarreta N, Moshier E, Naymagon S, Mascarenhas J, Schiano T. The Natural History, Treatments, and Outcomes of Portal Vein Thrombosis in Patients With Inflammatory Bowel Disease. *Inflamm Bowel Dis* 2021; **27**: 215-223 [PMID: 32185400 DOI: 10.1093/ibd/izaa053]

REVIEW

Chelation therapy in liver diseases of childhood: Current status and response

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Abstract

Chelation is the mainstay of therapy in certain pediatric liver diseases. Copper and iron related disorders require chelation. Wilson's disease (WD), one of the common causes of cirrhosis in children is treated primarily with copper chelating agents like D-penicillamine and trientine. D-Penicillamine though widely used due its high efficacy in hepatic WD is fraught with frequent adverse effects resulting discontinuation. Trientine, an alternative drug has comparable efficacy in hepatic WD but has lower frequency of adverse effects. The role of ammonium tetra-thiomolybdate is presently experimental in hepatic WD. Indian childhood cirrhosis is related to excessive copper ingestion, rarely seen in present era. D-Penicillamine is effective in the early part of this disease with reversal of clinical status. Iron chelators are commonly used in secondary hemochromatosis of liver in hemolytic anemias. There are strict chelation protocols during bone marrow transplant. The role of iron chelation in neonatal hemochromatosis is presently not in vogue due to its poor efficacy and availability of other modalities of therapy. Hereditary hemochromatosis is rare in children and the use of iron chelators in this condition is limited.

Key Words: Wilson's disease; D-Penicillamine; Trientine; Indian childhood cirrhosis; Deferoxamine; Deferasirox; Hemochromatosis

Core Tip: Chelation forms the most important part of management of certain liver diseases in children. In Wilson's disease and secondary hemochromatosis related to transfusion, chelation is well established treatment modality with proven efficacy. In other diseases like copper associated childhood cirrhosis and neonatal hemochromatosis the role of chelation is doubtful. In hereditary hemochromatosis, chelation is

recommended as alternative therapy. The selection of chelating agents for treatment depends on the efficacy, feasibility and risk of adverse effects known from literature. The review discusses the concepts of chelation and reviews the literature to assess the role of chelation in treatment of various pediatric liver diseases.

INTRODUCTION

Chelation is a process in which a synthetic compound is administered to remove an excess mineral or heavy metal from the body. There are various liver diseases that are caused by excess deposition of various heavy metals such as copper, iron and arsenic. Some of these are genetic-metabolic, others are due to environmental exposure. In the landmarks of chelation therapy in hepatology, Walshe documented cupriuresis after administering dimethyl cysteine (penicillamine) in Wilson's disease (WD) in 1956[1]. Chelation was thereafter used in non-Wilsonian liver diseases. In the subsequent years newer chelators such as trientine and ammonium tetra thiomolybdate were identified for WD. From the 1970s, transfusion-related liver siderosis of hemolytic anemias was revolutionized by the use of deferoxamine[2]. The use of iron chelators was attempted in gestational alloimmune liver disease and hereditary hemochromatosis. This review explores the rationale and outcome of chelation therapy in various pediatric liver diseases.

MECHANISM OF CHELATION

Metal ion (M) complexes with cheating agent (L) through an equilibrium reaction to form metal-ligand complex (ML) or chelate. The concentration of the chelate in the solution is directly proportional to the concentration of metal ion [M] and the ligand [L].
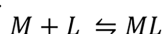
$$M + L \leftrightharpoons ML$$

$$[M][L] \propto [ML]$$

$$[M][L]k = [ML]$$

Where k is the effective stability constant. Value k denotes the affinity of the chelating agent. High k values suggest high affinity of the chelating agent. The value of k depends on the nature of the chelating agent, temperature, pH of the solution[3]. The *in-vivo* milieu is not similar to the *in-vitro* chemical reaction. The presence of weak acids in the body fluids like glutamate, sulfate, citrate, amino acids, albumin, macroglobulin *etc*. affect the chelation. These are called biological ligands. Chelating agent binds to the biological ligands and the effective concentration in the body fluid is lowered. Hence the equation becomes.

$$[Mt][Lt]k = [ML]$$

Where Mt, Lt is the total concentration of the metal ion and chelating agent respectively which is very difficult to assess in the clinical setting[4].

Effective chelation occurs when concentration of M and/or L is high, when affinity of the chelator (k) is high or when the concentration of the chelate [ML] is low. The metal ion concentration [M] in the body depends on the severity of the disease. For example, in a WD presenting as acute liver failure, serum copper (Cu) levels are usually very high. The concentration of chelating agent [L] is increased by increasing the dosing and/or frequency as tolerated by the patient. For the chelation to progress, urinary excretion of chelate [ML] is very important as it effectively reduces the concentration[3]. Ideal chelating agents must have good oral absorption, acceptable bioavailability, high affinity to metal ions, low toxicity at appropriate plasma concentration, undergo rapid elimination or detoxification after combining with metal ions and more

importantly should be available in affordable price[5].

CHELATION IN WD

WD is an autosomal recessive disorder caused by mutation of ATP7B gene that encodes for a protein P-type ATPase which transports copper into trans Golgi network and for biliary excretion of copper. In lysosomes, copper is incorporated into ceruloplasmin. In WD, due to defect in ATPase transport protein, ceruloplasmin formation is defective and biliary excretion of copper is impaired[6,7]. This causes excess accumulation of intracellular copper subsequently increasing the levels in blood causing accumulation in extra-hepatic organs (Figure 1).

Chelating drugs

D-Penicillamine (3, 3-dimethylcysteine) is the most commonly used medication for WD worldwide. The L-isomer of this drug is not advised for treatment due to its neurotoxicity. The chelation property of DPA is due to the presence of thiol (-SH), which is responsible for its high affinity towards divalent metal ions such as copper. The mechanism of action of D-Penicillamine (DPA) is by inducing cuprieuresis, inducing hepatic metallothionine synthesis, reducing fibrosis (by preventing collagen formation). DPA also has an anti-inflammatory property[8]. It is rapidly absorbed in proximal intestine but only 40%-70% are absorbed[9]. The peak plasma concentration occurs after 1-3 h after ingestion. It circulates in the plasma predominantly by binding to albumin (80%), while the rest of the compound is present as free or disulphide forms. DPA is metabolized in the liver by conjugation with sulfide or by methylation (phase II reaction) and excreted in urine with almost 80% being eliminated within 10 h of ingestion. After discontinuation of therapy, the drug is eliminated in about 3-6 d [10]. Food, antacids, iron and zinc preparations reduce the bioavailability by almost 50%. Plasma concentration reduces significantly when the drug is taken with food[11]. It is recommended to give the drug either 1- hour before or 2- h after food. The drug is given in the dose of 20 mg/kg per day (up to 1500 mg) rounded to nearest 250 mg in 2-4 divided doses and can be maintained at 1000 mg/d once the disease is in remission [12]. As DPA causes pyridoxine deficiency, pyridoxine should be supplemented at 25-50 mg/d. In case of neurological WD, to prevent paradoxical neurological worsening, the drug is started at low dose (125-250 mg) and slowly increased (125-250 mg every week) to reach the desired dose by 4-6 wk[13].

Trientine (triethylenetetramine) is an alternative chelating agent in WD. It is a derivative of spermine and putrescine and binds to copper in the ratio 1:1 to form a stable complex, which is eliminated in the urine. Trientine dihydrochloride is the oral ingestible form requiring storage at 2-8 degree Celsius to maintain stability. 10% of the trientine is absorbed in the proximal small intestine and achieves its peak concentration 1.5-4 h after ingestion. Trientine is extensively metabolized in tissues by acetylation but the enzyme responsible for it is not identified. 1% of ingested trientine and 8% trientine metabolite acetyltrien, appears in the urine. Plasma concentration of the trientine significantly reduces when given with food due to its affinity to dietary copper in the lumen thereby compromising the removal of tissue copper and the other reason could be due to the physiological polyamines secreted during food intake inhibits effective trientine absorption[14]. Trientine is not to be given with iron as it forms toxic complexes. The dose recommended is 20 mg/kg per day with the maximum of 1500 mg/d rounded to nearest 250 mg (300 mg capsules in North America) and maintenance dose of 1000 mg/d. Similar to DPA, trientine also should be ingested 1 h before or 2 h after food intake[12,15]. The decoppering efficacy of any chelating agent is evident from the effective stability constant (k) which denotes copper affinity. The comparison of k-value of DPA (2.38×10^{-16}) and trientine (1.74×10^{-16}) suggests the decoppering efficacy of DPA is much higher than trientine[16].

Efficacy of chelation

Improvement in symptoms and biochemical parameters in WD takes around 2-6 mo in hepatic forms whereas in isolated neurological forms it may take up to 12-24 mo[12]. DPA in WD children shows an efficacy of almost 70%-90%[17-20]. The response depends on whether it is hepatic or neurological form and severity of the disease at presentation. Long term of follow up of WD (median duration- 15.1 years) studied by Bruha *et al*[19] showed the response to DPA to hepatic forms is 82% compared to 69% for neurological forms. One of the largest series of WD patients (*n* = 327) from Euro Wilson consortium, showed hepatic forms had 91% response compared to only 68% in
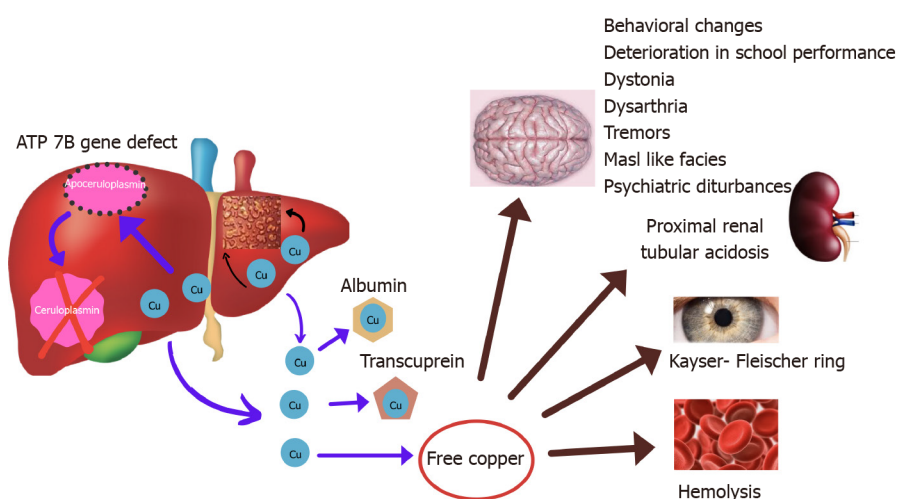
Figure 1 Pathophysiology of Wilson's disease. Due to mutation in *ATP 7B* gene, P type ATPase is defective and copper is not incorporated in ceruloplasmin. Free copper increases in blood and is deposited in liver and extrahepatic sites (brain, kidneys, bones, cornea, RBC).

neurological forms after a median follow up duration of 13.3 years[20]. In most series, trientine is used as a second line either due to poor response or due to toxicity to DPA. Hence, there are no head-to-head randomized trials comparing the efficacy of DPA and trientine. Overall efficacy of trientine is reported to be 80%-92%[21,22]. Retrospective analysis of efficacy of the two drugs by Hölscher *et al*[23] showed response in hepatic forms with DPA was 92% compared to 84% response with trientine after a median follow up duration of 13.3 years. In neurological forms, DPA fares significantly better (68%) than trientine (48%, $P = 0.008$)[23]. In Euro Wilson consortium, the response of both the DPA and trientine were comparable when used as a first line in both hepatic (90.7% *vs* 92.6%, $P = 0.98$) and neurological forms (67.5% *vs* 55%, $P = 0.76$). However when used as a second line therapy, trientine *vs* DPA showed similar response in hepatic form (75% *vs* 68.9%, $P = 0.76$) but better response in neurological form (51% *vs* 23.1%, $P = 0.01$)[20].

Adverse effects of copper chelators

Adverse effects of DPA are always a major concern with up to 30% of the patients develop one or more adverse effects (Table 1)[20,24,25]. Adverse effect can be early onset (less than 3 wk of therapy) or late (more than 3 wk to up to 2-3 years of initiation of therapy). Early adverse effects like fever, rash, arthralgia, lymphadenopathy, pancytopenia are predominantly immune mediated[26]. Nephropathy, the most common late adverse effect of DPA is seen in 5%-30%. Presentations include proteinuria, glomerulonephritis, nephrotic syndrome less commonly as Good Pasture's syndrome[27-29]. More than 90% of the nephropathy occurs within 12 mo of therapy. High doses of DPA, decompensated liver disease, intrinsic renal diseases or presence of HLA-B8/DR3 are probable risk factors of nephropathy[30]. Eighty percent are membranous glomerulonephritis on renal biopsy. In a study by Hall *et al*[27] of 33 patients with DPA nephropathy, one-third each showed resolution at 6, 12 and 18 mo respectively, after drug discontinuation. There are no clear recommendations as to whether the drug can be rechallenged after resolution of nephropathy. However, in such situations, it is prudent to continue the patient on an alternative drug such as trientine or zinc. DPA related myelotoxicity occur in up to 7% patients undergoing chelation with DPA[31-33]. Two types of myelotoxicity are known to occur, idiosyncratic (usually with in 1 year of therapy) or dose dependent (more than after 1 year therapy)[34]. Though, there are no definite guidelines for monitoring and treatment of myelotoxicity, European society of Pediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) suggests weekly blood counts initially, 1-3 mo till remission and 3-6 monthly thereafter[35]. If two or more values of total leukocyte count less than 3.5×10^3 per cubic mm, drug is to be discontinued. Bone marrow examination and reticulocyte counts differentiates this condition if concomitant hypersplenism is present[36,37]. Blood products, colony stimulating factor and anti-thymocyte globulin may improve the counts. Usual time of spontaneous recovery is 4-12 wk. Rarely hematopoietic stem cell transplantation may be required in refractory and prolonged cases. Once bone marrow toxicity has ensued, the drug should not be re-challenged. Adverse effects of DPA related to skin may be due to either acute hypersensitivity

Table 1 Adverse effects of copper chelating drugs

Name of the drug	Side effects
D-Penicillamine	Early (1-3 wk): Fever, rash, arthralgia, cytopenia, proteinuriaLate: (1) Skin: degenerative dermatoses elastosis perforans serpingosa, cutis laxa, pseudoxanthoma elasticum, bullous dermatoses, psoriasiform dermatoses, lichen planus, seborrheic dermatitis alopecia, aphthous ulcerations, hair loss; (2) Connective tissue disorders: Lupus like syndrome, arthralgia, Rheumatoid arthritis, polymyositis; (3) Renal: proteinuria, hematuria, glomerulonephritis, nephrotic syndrome, renal vasculitis, Goodpasture's syndrome; (4) Nervous system: paradoxical neurological worsening, neuropathies, myasthenia, hearing abnormalities, serous retinitis; (5) Gastrointestinal: Nausea, vomiting, diarrhea, elevated transaminases, cholestasis, hepatic siderosis; (6) Respiratory: pneumonitis, pulmonary fibrosis, pleural effusion; (7) Hematological: cytopenia, agranulocytosis, aplastic anemia, hemolytic anemia; and (8) Others: Immunoglobulin deficiency, breast enlargement, pyridoxine deficiency
Trientine	Paradoxical neurological worsening (10%-50%), sideroblastic anemia, bone marrow suppression, gastritis, skin rash, arthralgia, myalgia, hirsutism
Ammonium tetra thiomolybdate	Neurological dysfunction (rare), hepatotoxicity, bone marrow suppression

reaction presenting as morbilliform rash, urticaria, degenerative dermatoses (cutis laxa or elastosis perforans serpingosa) or an autoimmune phenomenon (pemphigus, scleroderma or lichen planus[38]. Rare musclar adverse effects of DPA include myasthenia (1%-2%) and ptosis. Anti- nicotinic acetyl choline receptor or Anti- MuSK (Anti- Muscle Specific tyrosine Kinase) is present in up to 70%[39]. Systemic lupus erythematosus can occur within 6-12 mo after the onset of DPA therapy presenting as pleurisy, arthritis, rash with or without presence of anti-nuclear antibody[40]. Deutscher *et al*[41] noted 3 out of 50 WD children with elevated transaminases within 6 wk of DPA therapy who resolved subsequently following discontinuation. Trientine also present with similar adverse effects as DPA like nausea, vomiting, arthralgia, myalgia, leukopenia, elevation in anti-nuclear antibody (ANA), nephropathy but adverse effects requiring discontinuation of trientine is significantly lower compared to DPA[20].

In hepatic WD, paradoxical neurological worsening occurs commonly within 6 mo of therapy, in patients with an underlying overt or occult neuropsychiatric feature. Paradoxical neurological worsening occurs even when dosing and compliance is good [42]. It occurs due to the sudden release of Cu from the liver following chelation therapy causing oxidative brain injury. Overall incidence of paradoxical neurological worsening ranges from 7%-26%. Those with previous known neurological WD, the incidence of worsening is up to 75%[19,24,25]. Both DPA and TA have shown to cause neurological worsening. In series from Euro Wilson consortium, paradoxical neurological worsening occurred significantly more with TA compared to DPA[20]. Litwin *et al*[13] studied natural history of 143 WD (70 Neuro/Neurohepatic WD and 73 hepatic WD), of whom 23% neurological cohort and none of the hepatic cohort developed early neurological worsening on chelation. In this series, median time of onset of neurological worsening was 2.3 mo. Fifty-three percent were completely reversible and 13% were partially reversible on drug discontinuation with median time of reversibility of 9.2 mo[13]. Prior neurological involvement, lesions in brain stem or thalamus and concomitant anti-dopaminergic drugs had higher chances of neurological worsening. Treatment consists of drug discontinuation and addition of zinc for a transition period. Chelators can be restarted in lower doses with gradual increment once the symptoms improve[13].

Assessment of adequacy of chelation: Clinical parameters
Currently there is no fool-proof, gold standard yardstick to assess chelation adequacy. All have fallacies in assessment and hence multiple parameters are considered. Chelation adequacy can be assessed firstly by assessing compliance to drug intake. Compliance is assessed by having a pill count, self-reporting by patients themselves or by checking empty blister packs during follow up outpatient visits[43]. There are various scales being developed assessing medication adherence (MAQ: Medication adherence questionnaire, MARS: Medication adherence Rating scale) but none have been validated in children[44]. More objective way of assessing compliance is by measuring drug levels but it is not routinely available under clinical setting. Secondly, follow up of clinical parameters assess the adequacy of chelation like improvement in jaundice, ascites, encephalopathy which usually take 2-6 mo post therapy. Resolution of neurological symptoms may take longer than 2-3 years[12]. The resolution of Kayser-Fleischer ring on de-coppering therapy has considerable controversies to the

same. Studies have heterogeneity in their assessment and reports. It appears to be independent on type of presentation (neurologic *vs* hepatic), stage of disease (pre-symptomatic *vs* symptomatic) and choice of chelator and compliance. Initial reports showed, Kayser-Fleischer (KF) ring disappearance in 81% of the patients (completely in 41% and incompletely in 59%), more in pre-symptomatic stage (60%) than those in symptomatic phase with ongoing therapy (2%) over 22 years of follow-up on DPA (90%) and zinc or trientine (10%). Conversely one-third of asymptomatic patients the rings did not reabsorb even after therapy of > 10 years. In this study, the fading of KF rings seemed to be independent of the stage of the disease and effectiveness of the decopperizing treatment[45]. In a study by Fenu *et al*[46] where 66% were hepatic and 31% were neuro-hepatic (90% on DPA ± zinc therapy), partial or total KF ring resolution was observed in 28%, deterioration in 6% and static in the rest of the cohort over 1-3 years of therapy. Other smaller cohorts report reduction of KF ring in neuropsychiatric manifestation or disappearance over 10 years on maintenance zinc and molybdate therapy in pediatric hepatic WD[47,48]. KF rings may reappear with non-compliance, and occasionally even with successful maintenance therapy[49].

Liver status can be appropriately assessed by Pediatric end-stage liver disease or Child-Turcotte-Pugh score. Biochemical parameters like serum albumin, total bilirubin and prothrombin time normalizes by 6 mo but liver enzymes might take longer[12]. In the author's experience it takes 9-12 mo for complete normalization of Liver function tests in majority of the cases[50]. In patients who have additional neurological involvement, neurological response is monitored by indices such as Global assessment scale (GAS)[51]. Even with neurological WD with significant MRI changes, 50% show improvement with long term chelation[52].

Assessment of adequacy of chelation: Biochemical parameters

Presently the most widely acceptable way to assess adequacy of chelation is by 24-h urine copper and non-ceruloplasmin copper. Twenty-four hours urine copper (UCu) increases immediately following chelation and takes around 12-18 mo to reach a stable level[53]. European Association for the Study of the Liver (EASL) and American Association for the Study of Liver diseases (AALSD) recommends targeting 24-h urine copper between 200-500 mg/d for adequate chelation[12,15]. Values > 500 mg/d suggest under chelation as lot of unchelated copper is remaining in the body. Values < 200 mg/d may be either due to over chelation or poor compliance (Table 2). This can be differentiated by non-ceruloplasmin copper (NCC) levels calculated by the formula (serum copper (mg/L) - 0.3 x serum ceruloplasmin(mg/L)[54]. NCC has a few fallacies. Firstly, almost 20% of NCC are negative values, seen mostly when immunoassay method was used to measure ceruloplasmin as it measures both holoceruloplasmin and apoceruloplasmin. NCC calculation becomes inappropriate when inactive apoceruloplasmin is included. Secondly, there are variabilities in reference ranges in ceruloplasmin values between various laboratories across the world creating disparities in NCC cut-offs[55]. According to EASL guidelines, NCC > 15 mg/dL suggest poor compliance and < 5 mg/dL suggest over chelation. Additionally, 24-h urine copper after 48-h cessation of therapy has been recommended by EASL. Values > 100 mg/d is suggestive of under chelation or poor compliance while values < 100 mg/d suggest adequate treatment[15].

A novel and upcoming modality to assess chelation is the use of exchangeable copper. Exchangeable copper is the fraction of copper bound to albumin, peptide and amino acids which are easily chelated by chelating agents. It denotes a direct estimation of non-ceruloplasmin copper (NCC)[56]. On WD with chelation for long time, exchangeable copper values tend to reduce comparable to non-Wilson children. In a pilot study by the authors, the role of exchangeable copper was assessed in a cohort of 96 children with hepatic WD. Exchangeable copper was significantly higher in newly diagnosed WD compared to WD on chelation for more than 1 year (3 ± 7 µmol/L *vs* 0.9 ± 0.6 µmol/L, P = 0.03). Exchangeable copper values were lower in stable liver disease compared to unstable liver disease (0.86 ± 0.5mmol/L *vs* 1.3 ± 0.6 mmol/L, P = 0.01). Exchangeable copper values showed excellent correlation with non-ceruloplasmin copper (r = 0.92, P < 0.001). Predictive model incorporating exchangeable copper into standard monitoring tools improved the yield of disease control assessment by 21%[57].

Comparison of single vs dual chelation: Which is better in hepatic WD?

Strictly zinc is not considered as a systemic chelator. Oral zinc (Zn) induces metallo-thionine in enterocyte. Metallothionine is an endogenous chelator that has high affinity to copper. Hence induced metallothionine combines with luminal Cu, preventing its entry into circulation. This Cu is removed through feces when enterocyte is shed. Zn

Table 2 Twenty-four hours urine copper and non-ceruloplasmin copper in various stages of Wilson's disease treatment	
Early stages of treatment (< 1 yr)	UCu > 500 µg/dNCC > 25 µg/dL
Good control (treatment > 1 yr)	UCu 200-500 µg/dNCC < 15 µg/dL
Poor compliance/uncontrolled disease	UCu > 500 µg/dNCC > 15 µg/dL
Inadequate dose	UCu < 200 µg/dNCC > 15 µg/dL
Over-treatment	UCu < 200 µg/dNCC < 5 µg/dL

UCu: Twenty-four hours urinary copper; NCC: Non ceruloplasmin copper.

also induces hepatic metallothionine[58]. Hence, Zn is used in pre-symptomatic WD, stable well chelated WD on maintenance therapy, severe neurological WD. It is also used as a last resort in those with DPA or trientine intolerance. In severe hepatic disease, many centers consider giving a trial of dual chelation DPA and zinc for rapid chelation and quick stabilization. In a study conducted by the authors, 65 children with > 9 mo chelation were followed up for long term outcome. Majority had advanced disease at presentation. 83% of children were treated with DPA monotherapy and 17% treated with DPA and zinc combination. Trientine was started in 4 children due to DPA toxicity. 77% of children responded to DPA monotherapy even when the disease is severe at presentation and 50% responded when DPA and zinc combination was started. The overall response to oral chelation is 71%[50]. Hence, DPA should be the first line of therapy for any hepatic WD and zinc is added in those who failed to show optimal response with DPA in desperate circumstances with the hope of rapid synergistic chelation and quicker liver recuperation[50]. Though there are no comparative trials of dual or single chelation therapy, there are limited case series that have used DPA or trientine with zinc for WD presenting with ascites, coagulopathy and encephalopathy[59-61]. Though the efficacy of dual therapy in these studies were 91%-100%, sample sizes were small. Systematic review of 17 studies that assessed the efficacy of dual therapy (DPA/ Trientine with zinc) showed pooled efficacy rate (60.4%, 95%CI: 55.8-65.0) compared to DPA (73.7%, 95%CI: 65.1-85.4) and trientine monotherapy (82.6%, 95%CI: 75.4-89.5). Adverse effects following monotherapy is also lesser with either DPA or trientine compared to combination therapy[62]. Another retrospective study assessed 30 of 313 patients on dual chelator therapy, showed long term discontinuation and non-adherence was higher as compared to monotherapy (P = 0.006). Combination therapy, may fare better in neurological WD compared to exclusive hepatic forms[63]. Compliance and adequate spacing with chelating agent need careful consideration in the treatment schedule. If consumed together, chelator can combine with zinc in the lumen and effective absorption of both the medication gets reduced. Animal studies have shown that hepatic zinc stores is also significantly reduced during decoppering[64]. Hence, when chelator is combined with zinc, a proportion of chelator is used up in removing the body zinc thereby compromising the efficacy.

Efficacy of ammonium tetra thiomolybdate
Ammonium tetra thiomolybdate is a strong decoppering agent used in limited trials. It prevents intestinal absorption of copper if given with meals but also reduces serum copper when given in between meals. Ammonium tetra thiomolybdate (ATM) is predominantly advised for neurological forms due to it low risk of neurological worsening[65]. In the comparative study of ATM with trientine in neurological WD, paradoxical neurological worsening is significantly lower with ATM (4%) compared to trientine (26.1%, P = 0.01)[66]. At larger doses, ATM can form toxic insoluble complex that gets deposited in liver causing hepatotoxicity[67]. Hence the role of ATM in hepatic WD is precarious. Up to 10% of patients receiving ATM might develop bone marrow toxicity also[68]. Bis-choline tetra thiomolybdate (WTX101) is an investigational derivative of ATM being studied recently in neurological WD with better stability and lower toxicity[69]. Twenty-four weeks treatment of the drug caused improvement in 71% of neurological WD. Seven percent developed leukopenia and almost 39% developed elevated liver enzymes post therapy[69]. Robust experience in exclusive hepatic WD is not yet available.

CHELATION IN INDIAN CHILDHOOD CIRRHOSIS

Indian childhood cirrhosis is commonly seen in children between 6 mo and 5 years of age in Indian subcontinent with its peak incidence seen during 1970-1990[70]. Presently this entity seems to be waning in the Indian subcontinent. Predominant etiology advocated was excessive copper ingestion with use of copper utensils[71]. There was also a possibility of genetic predisposition affecting copper metabolism[70]. Clinical features consist of nonspecific symptoms to start with like fever, lethargy, easy fatiguability, palpable liver with leafy edges in stage I, splenomegaly and ascites in stage II and jaundice, coagulopathy and encephalopathy in stage III. Histopathological examination of liver shows diffuse hepatocyte necrosis, presence of Mallory bodies and granular orcein staining. Treatment monitoring is by liver function tests (LFT), serum copper and in many studies, by repeat hepatic copper and liver histology, while on treatment. Mortality is almost 60% in stage II but reaching almost 90% in stage III [72]. In the study by Bavdekar *et al*[73] 65 children with Indian childhood cirrhosis (ICC) on treatment with DPA were followed up for the mean duration of 3.5 years, showed response in 60% of the children in pre-icteric phase compared to only 6% response (P < 0.01) in icteric phase (Table 3). Another study in ICC children who received DPA or DPA with steroids showed 50% survival as compared to10% in placebo group (P = 0.002)[74].In a pediatric study, DPA therapy has showed better response compared to DPA with intravenous immunoglobulin (P = 0.018)[75]. Chelation may improve symptoms if given early as prognosis is poor in advanced disease despite treatment[75].

CHELATION IN NON-WILSONIAN COPPER RELATED DISORDERS

Non-Wilsonian copper related diseases termed by Baker *et al*[76] as copper associated childhood cirrhosis includes ICC from India and ICC-like illness from western countries. This ICC like illnesses is otherwise called idiopathic copper toxicosis. Type I copper associated childhood cirrhosis (CACC) resembles ICC, with an early onset of disease and related to increased copper intake. Type II CACC has onset later than 4 years of age and possibly has an autosomal recessive inheritance without an obvious increase in copper intake[77]. Although there are few case reports of ICC- like illnesses, meagre number of reports use chelation therapy probably due to its conflicting results. One child from Bangladeshi origin, presented with jaundice, anorexia, weight loss at 7 years, with normal serum ceruloplasmin, and elevated hepatic copper 2319 mg/g. Improvement in symptoms and decrease in liver copper (35 mg/g) was noted after 19 mo of DPA therapy (Table 3)[77]. In contrast, a 10 year old Italian child with ascites and hepatomegaly, normal ceruloplasmin levels and liver copper of 1970 mg/g did not show any improvement clinically and biochemically even after 2 years of DPA[78]. Largest cohort of endemic Tyrolean infantile cirrhosis studied by Muller *et al*[79] showed both genetics and copper contamination were responsible for the disease. However there is paucity of chelation therapy experience in this condition.

IRON CHELATION IN GESTATIONAL ALLOIMMUNE LIVER DISEASES

In Gestational alloimmune liver disease alloimmunization of fetal liver antigen occurs in maternal blood resulting in IgG fetal liver antibody causing complement activation in fetal liver and significant impairment in hepcidin production (Figure 2)[80]. This causes iron storage in various organs like liver, heart, gonads, pancreas *etc.* Gestational alloimmune liver disease (GALD) causes liver failure as a result of hemochromatosis in newborn period and has high mortality if not intervened earlier. The liver injury causes reduced production of hepcidin resulting in uncontrolled iron absorption through placenta. This excess iron might further aggravate liver injury and also result in extra-hepatic iron deposition[81,82]. There have been few studies of GALD being treated with iron chelators (intravenous deferoxamine) and antioxidants with no clear-cut benefit. In the series by Flynn *et al*[83] five infants with neonatal hemochromatosis received intravenous deferoxamine but only one survived without liver transplantation. In the study by Rodrigues *et al*[84] 10 infants received iron chelation but only one survived without transplantation. In another series by Sigurdsson *et al*[85] six infants with neonatal hemochromatosis received supportive measures whereas eight infants received combination of deferoxamine and antioxidants. Two out of six who

Table 3 Pediatric studies of chelation in liver diseases

Ref.	Disease	Drug	Follow up duration	Response	Adverse effects
Dhawan *et al* [60]	WD	DPA (*n* = 32)	Median:11.78 (1.45-34.2) yr	20/32 (62.5%)	Minor- 6.3%; Major-21.9%
Wang *et al* [106]	WD	DPA/TA (*n* = 9)	Mean: 5.1 4.1 yr	All responded	Not mentioned
Das *et al*[50]	WD	DPA (*n* = 65), TA(*n* = 4)	Median: 3.6 (0.8-12) yr	DPA (42/65) 64.6%, TA (3/4) 75%	DPA 10.8%
Arnon *et al* [107]	WD	TA (*n* = 10)	Treatment duration: 18 mo. Follow up:12-60 mo	All responded	1/10 (10%) reported hepatotoxicity
Taylor *et al* [108]	WD	TA (*n* = 16)	6.4 (0.78-18.6) yr	14/16 (87.5%)	1 had allergic reaction
Santos Silva *et al*[59]	WDAll decompensated liver disease	DPA (*n* = 1)TA (*n* = 4)	18-60 mo	All responded one still had raised transaminase	3/4 (75%) on DPA developed cytopenia
Bavdekar *et al* [73]	ICC	DPA (*n* = 68)	3.5 (1-7) yr	29/68 (42.6%) alive after follow up	5 children had proteinuria
Tomar *et al* [75]	ICC	DPA (*n* = 60)	12 mo duration	13/17 (76.5%) of grade III survived	11.8% drug rash, 5.9% fever
Tanner *et al* [74]	ICC (15 children treated with DPA in both trials together)	DPA (*n* = 15)	6 yr	Trial I: 1/15 (6.7%) survived in 6 yr, Trial II: 5/10 (50%) survived in 6 yr	Not mentioned
Horselen *et al* [77]	Case report CACC (age 7 yr)	DPA	19 mo	Hepatic copper normalized	none
Maggiore *et al* [78]	Case report CACC (age 10 yr)	DPA	24 mo	No improvement	Not mentioned
Rodeck *et al* [109]	CACC (age 6 and 10 mo)	DPA	18 mo, other child deteriorated immediately following DPA initiation	One child improved and other developed acute liver failure requiring liver transplantation	None
Flynn *et al*[83] 2002	NH	DFO (*n* = 5) with antioxidant	Follow up at 48 mo	2/5 (40%) survived without transplantation	Not mentioned
Rodrigues *et al* [84] 2005	NH	DFO with antioxidant (*n* = 9)	Follow up 3-9.8 yr	1/9 (11.1%) survived without transplantation	Not mentioned
Sigurrdson *et al*[85] 1998	NH	DFO with antioxidant (*n* = 8)	Not mentioned	None survived without transplantation	Not mentioned
Masera *et al* [110] 2013	HJV hemochromatosis Case report (7/F)	DFX	12 mo of treatment	Iron indices improved on 12 mo treatment	Not mentioned

DPA: D-Penicillamine; TA: Trientine; WD: Wilson's disease; ICC: Indian childhood cirrhosis; NH: Neonatal Hemochromatosis; DFO: Deferoxamine; DFX: Deferasirox; CACC: Copper associated childhood cirrhosis.

received supportive measures survived compared to only one who received chelation. It is not clear if the small proportion of response to chelation is due to efficacy of the drug in already advanced disease or due to natural history. In the recent years, it now clear that intravenous immunoglobulin has a superior role than chelation therapy in GALD.

IRON CHELATION IN HEREDITARY HEMOCHROMATOSIS

Hemochromatosis is due to iron accumulation in various organs with secondary causes being commoner in children than hereditary hemochromatosis. Secondary causes of hemochromatosis are commonly related to repeated transfusions in hemolytic anemia especially thalassemia major. In normal individuals, increased plasma iron induces the genes like HFE, TFR2 and HJV. This causes release in hepcidin, binding with ferroportin in enterocytes and macrophages, reducing iron absorption. Hereditary hemochromatosis (HH), most commonly due to mutation in
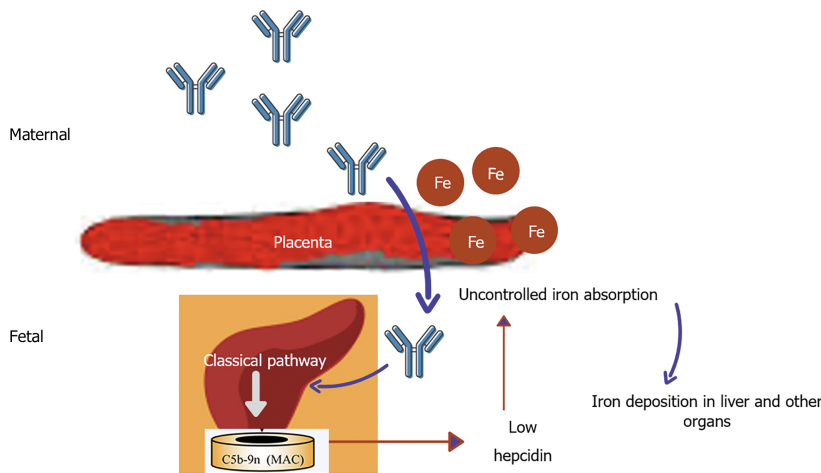
Figure 2 Pathogenesis of gestational alloimmune liver disease. Alloimmunization of fetal liver antigen by maternal blood produces IgG antibody passively transferred through the placenta to cause fetal liver injury by complement activation. Liver injury reduces the hepatic synthesis of hepcidin resulting in uncontrolled placental iron absorption. Excess iron is deposited in liver, pancreas, heart, gonads, *etc*.

HFE, cause impaired production of hepcidin making checkpoint for iron absorption defective[86]. Animal studies showed excessive fat intake causes impaired hepcidin production and increased transferrin receptor 1 and divalent metal transporter 1 Levels by altering mRNA expression. Hence, increased iron absorption and iron related liver injury may be responsible for development of non-alcoholic steatohepatitis[87]. Hereditary hemochromatosis (HH) is extremely rare in children. Excess iron in the serum causes liver cirrhosis, skin pigmentation, pancreatic insufficiency, cardiac dysfunction and hypothyroidism[88]. Iron chelation forms the mainstay of therapy in transfusion related siderosis in various hemolytic anemias in children. In a few studies, iron chelators have been implicated in treatment of HH also. Deferoxamine is parenteral iron chelator, given either as subcutaneous or intravenous infusion (20-50 mg/kg per day) over 8-24 h. Adverse effects seen are local reaction in injection site, hearing abnormalities, bone abnormalities *etc.* Deferasirox is an oral chelator with a similar efficacy as deferoxamine in removing hepatic iron but prone for its gastrointestinal side effects. Deferiprone, also an oral chelator is prone for gastrointestinal side effects and agranulocytosis and is highly effective in removing cardiac iron compared to other chelators (Table 4)[89]. Phatak *et al*[90] from Italy studied multiple doses of deferoxamine in HH, showed 10 mg/kg is the dose with optimal response and lower side effects. Nagler *et al*[91] analyzed 2 patients treated for 6 mo and 10 mo respectively who showed significant reduction in serum ferritin in the follow up. EASL and AASLD guidelines on HH recommend phlebotomy as the treatment of choice in HH[92,93]. Chelation may be considered in HH when phlebotomy is not tolerated due to severe congestive cardiac failure, anemia and in case of difficult venous access.

IRON CHELATION IN SECONDARY HEMOCHROMATOSIS

In children, secondary hemochromatosis is more common than HH and is usually caused by transfusion related iron overload seen in chronic hemolytic anemia especially beta thalassemia[94]. Each milliliter of packed RBC adds 1mg of iron to the body stores. Iron is usually bound to transferrin in plasma. However when the iron load increases, transferrin sites saturate and excess iron spills as labile plasma iron causing free radical injury to heart, liver and endocrine organs[95]. Multiple transfusion causes liver injury by various mechanisms such as siderosis causing hepatitis eventually progressing to fibrosis and cirrhosis. Hepatic foci of hemopoiesis and transfusion related hepatitis B and C infection are also seen[96].

Iron overload related liver injury can be assessed by various modalities. Serum ferritin is easily available and an inexpensive method to assess iron overload but its utility is limited in the presence of infection and inflammation. Liver iron concentration > 15 mg/g dry weight of liver is associated with significant mortality and morbidity[97]. The superconducting quantum interface device (SQUID) measures liver iron stores non-invasively but the SQUID scanners are not available in many centers

Table 4 Properties of iron-chelators

Properties	Deferoxamine (DFO)	Deferasirox (DFX)	Deferiprone (DFP)
Chelator: Iron ratio	1:1	2:1	3:1
Plasma $t_{1/2}$	30 min	12-16 h	2-3 h
Usual dose	20-50 mg/kg per day over 8-24 h	20-40 mg/kg per day once daily	75-100 mg/kg per day in 3 divided doses
Route of administration	Subcutaneous, intravenous	Oral	Oral
Clearance	Renal, hepatic	Hepatic	Renal
Efficacy in removing liver iron stores	Good	Good	Moderate
Efficacy in removing cardiac iron	Moderate	Moderate	Good
Advantages	Long safety data available, strongest chelator on molar basis	Oral once daily dose is sufficient	Oral, effective in removing cardiac iron
Adverse effects	Local reactions	Gastric intolerance	Nausea
	Sensorineural hearing loss	Rash	Vomiting
	Bone abnormalities	Diarrhea	Diarrhea
	Retinopathy	Elevation in creatinine	Arthralgia
	Pulmonary disease	Elevation in transaminases	Elevated liver enzymes
	Allergic reaction	Peptic ulcer	Agranulocytosis
	Bacterial infections (*e.g.*, *Listeria, Klebsiella*)	Renal dysfunction	
		Hepatic dysfunction	

worldwide[98]. Magnetic resonance imaging estimates liver iron by R2 and R2∗ techniques and it correlates well with liver iron concentration attained from biopsy. Magnetic resonance imaging (MRI) has now become the primary monitoring tool for both liver and cardiac iron[99].

Liver injury due to iron overload was common in children in pre-chelation era. Liver biopsies obtained in 80 children with beta thalassemia during splenectomy showed cirrhosis in 40% of children > 11 years with risk of cirrhosis increasing with age. 60% of the children showed hypoalbuminemia and 70% showed elevated transaminases[96]. Iron-chelators are well established treatment modality to prevent iron overload related liver injury. In a retrospective study by Maira *et al*[100] deferasirox for a duration of 4 ± 1.5 years showed significant improvement in liver stiffness measurement by transient elastography (7.4 ± 3.2 kPa *vs* 6.6 ± 3.2 kPa, *P* = 0.017) and liver iron concentration (LIC) (4.81 ± 3.82 mg/g *vs* 3.65 ± 3.45 mg/g, *P* = 0.001). Thus, iron chelation not only prevents progression of liver injury but also reverses inflammation and fibrosis. In the multicentric cross-sectional study from Italy, 924 beta-thalassemia patients were evaluated for iron overload assessment and management. The study showed serum ferritin had an excellent correlation with liver iron concentration. Deferasirox (38.3%) was most preferred chelator, especially in children because of its safety and easy administration[101]. Deferiprone was less commonly used when transaminases were elevated due to its concern of hepatic fibrosis[97]. Combination of two chelators were used whenever serum ferritin > 2500 ng/mL or MRI R2∗ values < 20 ms. Guidelines suggest that LIC assessment should be done at 1-2 yearly intervals [102]. Iron over load needs to be monitored and treated pre- and post-alloimmune hematopoietic stem cell transplantation (HSCT) for hemolytic anemia. Pre-transplant serum ferritin > 1000 ng/mL is associated with increased risk of post-transplant complications such as chronic liver disease, graft *vs* host disease (GVHD), sinusoidal obstruction syndrome and infection[103,104]. Hence it is mandatory to rapidly reduce ferritin levels before HSCT. Gruppo Italiano Trapianto di Midollo Osseo (GITMO) study group recommends switching to intravenous deferoxamine for rapid lowering of serum ferritin pre-transplant. From 6 mo post-transplant, iron overload is to be assessed by serum ferritin and MRI R2∗. If LIC in MRI > 7 mg/g phlebotomy is preferred, but when LIC > 15 mg/g phlebotomy along with iron chelators are required to prevent complications[105].

CONCLUSION

Copper chelation by D-penicillamine and trientine forms the mainstay of treatment in childhood WD. Appropriate dosing, compliance to medications and scheduled monitoring with liver function tests, 24-h urine copper and non- ceruloplasmin copper are required for better control of the disease. D-penicillamine is a promising treatment for Indian childhood cirrhosis especially in early stages. The role in other non-Wilsonian copper diseases is doubtful. The use of iron chelator in Gestational alloimmune liver disease is waning due to its poor efficacy. Iron chelator may be considered as an alternative therapy in hereditary hemochromatosis when the primary treatment fails or not feasible but in case of secondary hemochromatosis chelation forms the main treatment.

REFERENCES

1 **Walshe JM**. Penicillamine, a new oral therapy for Wilson's disease. *Am J Med* 1956; **21**: 487-495 [PMID: 13362281 DOI: 10.1016/0002-9343(56)90066-3]

2 **Mettananda S**. Management of thalassaemia. *Sri Lanka J Child Heal* 2018; **47**: 159-165 [DOI: 10.4038/sljch.v47i2.8484]

3 **Aaseth J**, Skaug MA, Cao Y, Andersen O. Chelation in metal intoxication--Principles and paradigms. *J Trace Elem Med Biol* 2015; **31**: 260-266 [PMID: 25457281 DOI: 10.1016/j.jtemb.2014.10.001]

4 Al-Karadaghi S, Franco R, Hansson M, Shelnutt JA, Isaya G, Ferreira GC. Chelatases: distort to select? *Trends Biochem Sci.* 2006; 31(3):135-42 [DOI: 10.1016/j.tibs.2006.01.001.]

5 **Flora SJ**, Pachauri V. Chelation in metal intoxication. *Int J Environ Res Public Health* 2010; **7**: 2745-2788 [PMID: 20717537 DOI: 10.3390/ijerph7072745]

6 **de Bie P**, Muller P, Wijmenga C, Klomp LW. Molecular pathogenesis of Wilson and Menkes disease: correlation of mutations with molecular defects and disease phenotypes. *J Med Genet* 2007; **44**: 673-688 [PMID: 17717039 DOI: 10.1136/jmg.2007.052746]

7 **Lalioti V**, Sandoval I, Cassio D, Duclos-Vallée JC. Molecular pathology of Wilson's disease: a brief. *J Hepatol* 2010; **53**: 1151-1153 [PMID: 20832891 DOI: 10.1016/j.jhep.2010.07.008]

8 **Peisach J**, Blumberg WE. A mechanism for the action of penicillamine in the treatment of Wilson's disease. *Mol Pharmacol* 1969; **5**: 200-209 [PMID: 4306792]

9 **Kukovetz WR**, Beubler E, Kreuzig F, Moritz AJ, Nirnberger G, Werner-Breitenecker L. Bioavailability and pharmacokinetics of D-penicillamine. *J Rheumatol* 1983; **10**: 90-94 [PMID: 6842492]

10 **Netter P**, Bannwarth B, Péré P, Nicolas A. Clinical pharmacokinetics of D-penicillamine. *Clin Pharmacokinet* 1987; **13**: 317-333 [PMID: 3319347 DOI: 10.2165/00003088-198713050-00003]

11 **Langlois DK**, Lehner AF, Buchweitz JP, Ross DE, Johnson MB, Kruger JM, Bailie MB, Hauptman JG, Schall WD. Pharmacokinetics and relative bioavailability of D-penicillamine in fasted and nonfasted dogs. *J Vet Intern Med* 2013; **27**: 1071-1076 [PMID: 23875792 DOI: 10.1111/jvim.12147]

12 **Roberts EA**, Schilsky ML; American Association for Study of Liver Diseases (AASLD). Diagnosis and treatment of Wilson disease: an update. *Hepatology* 2008; **47**: 2089-2111 [PMID: 18506894 DOI: 10.1002/hep.22261]

13 **Litwin T**, Dzieżyc K, Karliński M, Chabik G, Czepiel W, Członkowska A. Early neurological worsening in patients with Wilson's disease. *J Neurol Sci* 2015; **355**: 162-167 [PMID: 26071888 DOI: 10.1016/j.jns.2015.06.010]

14 **Lu J**. Triethylenetetramine pharmacology and its clinical applications. *Mol Cancer Ther* 2010; **9**: 2458-2467 [PMID: 20660601 DOI: 10.1158/1535-7163.MCT-10-0523]

15 **European Association for Study of Liver**. EASL Clinical Practice Guidelines: Wilson's disease. *J Hepatol* 2012; **56**: 671-685 [PMID: 22340672 DOI: 10.1016/j.jhep.2011.11.007]

16 **Smirnova J**, Kabin E, Järving I, Bragina O, Tõugu V, Plitz T, Palumaa P. Copper(I)-binding properties of de-coppering drugs for the treatment of Wilson disease. α-Lipoic acid as a potential anti-copper agent. *Sci Rep* 2018; **8**: 1463 [PMID: 29362485 DOI: 10.1038/s41598-018-19873-2]

17 **Lau JY**, Lai CL, Wu PC, Pan HY, Lin HJ, Todd D. Wilson's disease: 35 years' experience. *Q J Med* 1990; **75**: 597-605 [PMID: 2217665]

18 **Svetel M**, Pekmezović T, Petrović I, Tomić A, Kresojević N, Jesić R, Kazić S, Raicević R, Stefanović D, Delibasić N, Zivanović D, Dordević M, Kostić VS. Long-term outcome in Serbian patients with Wilson disease. *Eur J Neurol* 2009; **16**: 852-857 [PMID: 19473354 DOI: 10.1111/j.1468-1331.2009.02607.x]

19 **Bruha R**, Marecek Z, Pospisilova L, Nevsimalova S, Vitek L, Martasek P, Nevoral J, Petrtyl J, Urbanek P, Jiraskova A, Ferenci P. Long-term follow-up of Wilson disease: natural history, treatment, mutations analysis and phenotypic correlation. *Liver Int* 2011; **31**: 83-91 [PMID: 20958917 DOI: 10.1111/j.1478-3231.2010.02354.x]

20 **Weiss KH**, Thurik F, Gotthardt DN, Schäfer M, Teufel U, Wiegand F, Merle U, Ferenci-Foerster D,

Maieron A, Stauber R, Zoller H, Schmidt HH, Reuner U, Hefter H, Trocello JM, Houwen RH, Ferenci P, Stremmel W; EUROWILSON Consortium. Efficacy and safety of oral chelators in treatment of patients with Wilson disease. *Clin Gastroenterol Hepatol* 2013; **11**: 1028-35.e1 [PMID: 23542331 DOI: 10.1016/j.cgh.2013.03.012]

21   **Walshe JM**. Treatment of Wilson's disease with trientine (triethylene tetramine) dihydrochloride. *Lancet* 1982; **1**: 643-647 [PMID: 6121964 DOI: 10.1016/s0140-6736(82)92201-2]

22   **Scheinberg IH**, Jaffe ME, Sternlieb I. The use of trientine in preventing the effects of interrupting penicillamine therapy in Wilson's disease. *N Engl J Med* 1987; **317**: 209-213 [PMID: 3600712 DOI: 10.1056/NEJM198707233170405]

23   **Hölscher S**, Leinweber B, Hefter H, Reuner U, Günther P, Weiss KH, Oertel WH, Möller JC. Evaluation of the symptomatic treatment of residual neurological symptoms in Wilson disease. *Eur Neurol* 2010; **64**: 83-87 [PMID: 20606453 DOI: 10.1159/000316066]

24   **Medici V**, Trevisan CP, D'Incà R, Barollo M, Zancan L, Fagiuoli S, Martines D, Irato P, Sturniolo GC. Diagnosis and management of Wilson's disease: results of a single center experience. *J Clin Gastroenterol* 2006; **40**: 936-941 [PMID: 17063115 DOI: 10.1097/01.mcg.0000225670.91722.59]

25   **Czlonkowska A**, Gajda J, Rodo M. Effects of long-term treatment in Wilson's disease with D-penicillamine and zinc sulphate. *J Neurol* 1996; **243**: 269-273 [PMID: 8936358 DOI: 10.1007/BF00868525]

26   LiverTox: Clinical and Research Information on Drug-Induced Liver Injury [Internet]. Bethesda (MD): National Institute of Diabetes and Digestive and Kidney Diseases; 2012 [PMID: 31643176]

27   **Hall CL**, Jawad S, Harrison PR, MacKenzie JC, Bacon PA, Klouda PT, MacIver AG. Natural course of penicillamine nephropathy: a long term study of 33 patients. *Br Med J (Clin Res Ed)* 1988; **296**: 1083-1086 [PMID: 3132218 DOI: 10.1136/bmj.296.6629.1083]

28   **Bienaimé F**, Clerbaux G, Plaisier E, Mougenot B, Ronco P, Rougier JP. D-Penicillamine-induced ANCA-associated crescentic glomerulonephritis in Wilson disease. *Am J Kidney Dis* 2007; **50**: 821-825 [PMID: 17954295 DOI: 10.1053/j.ajkd.2007.05.026]

29   **Derk CT**, Jimenez SA. Goodpasture-like syndrome induced by D-penicillamine in a patient with systemic sclerosis: report and review of the literature. *J Rheumatol* 2003; **30**: 1616-1620 [PMID: 12858467]

30   **Billingsley LM**, Stevens MB. The relationship between D-penicillamine--induced proteinuria and prior gold nephropathy. *Johns Hopkins Med J* 1981; **148**: 64-67 [PMID: 7206401]

31   **Steen VD**, Blair S, Medsger TA Jr. The toxicity of D-penicillamine in systemic sclerosis. *Ann Intern Med* 1986; **104**: 699-705 [PMID: 2938530 DOI: 10.7326/0003-4819-104-5-699]

32   Toxicity of longterm low dose D-penicillamine therapy in rheumatoid arthritis. Cooperative Systematic Studies of Rheumatic Disease Group. *J Rheumatol* 1987; **14**: 67-73 [PMID: 2952797]

33   **Kay AG**. Myelotoxicity of D-penicillamine. *Ann Rheum Dis* 1979; **38**: 232-236 [PMID: 485580 DOI: 10.1136/ard.38.3.232]

34   **Jaffe IA**. Adverse effects profile of sulfhydryl compounds in man. *Am J Med* 1986; **80**: 471-476 [PMID: 2937293 DOI: 10.1016/0002-9343(86)90722-9]

35   **Gupta P**, Choksi M, Goel A, Zachariah U, Sajith KG, Ramachandran J, Chandy G, Kurian G, Rebekah G, Eapen CE. Maintenance zinc therapy after initial penicillamine chelation to treat symptomatic hepatic Wilson's disease in resource constrained setting. *Indian J Gastroenterol* 2018; **37**: 31-38 [PMID: 29457214 DOI: 10.1007/s12664-018-0829-x]

36   **Pitman SK**, Huynh T, Bjarnason TA, An J, Malkhasyan KA. A case report and focused literature review of d-penicillamine and severe neutropenia: A serious toxicity from a seldom-used drug. *Clin Case Rep* 2019; **7**: 990-994 [PMID: 31110732 DOI: 10.1002/ccr3.2125]

37   **Petrides PE**, Gerhartz HH. D-penicillamine-induced agranulocytosis: hematological remission upon treatment with recombinant GM-CSF. *Z Rheumatol* 1991; **50**: 328-329 [PMID: 1776370]

38   **Ishak R**, Abbas O. Penicillamine revisited: historic overview and review of the clinical uses and cutaneous adverse effects. *Am J Clin Dermatol* 2013; **14**: 223-233 [PMID: 23605177 DOI: 10.1007/s40257-013-0022-z]

39   **Nishida H**, Sahashi K. [Penicillamine-induced myasthenia gravis]. *Ryoikibetsu Shokogun Shirizu* 2001; 351-353 [PMID: 11596408]

40   **Lee Y**, Lee ST, Cho H. D-penicillamine-induced ANA (+) ANCA (+) vasculitis in pediatric patients with Wilson's disease. *Clin Nephrol* 2016; **85**: 296-300 [PMID: 26784915 DOI: 10.5414/CN108763]

41   **Deutscher J**, Kiess W, Scheerschmidt G, Willgerodt H. Potential hepatotoxicity of penicillamine treatment in three patients with Wilson's disease. *J Pediatr Gastroenterol Nutr* 1999; **29**: 628 [PMID: 10554138 DOI: 10.1097/00005176-199911000-00031]

42   **Kalita J**, Kumar V, Ranjan A, Misra UK. Role of Oxidative Stress in the Worsening of Neurologic Wilson Disease Following Chelating Therapy. *Neuromolecular Med* 2015; **17**: 364-372 [PMID: 26224517 DOI: 10.1007/s12017-015-8364-8]

43   **Cramer JA**, Roy A, Burrell A, Fairchild CJ, Fuldeore MJ, Ollendorf DA, Wong PK. Medication compliance and persistence: terminology and definitions. *Value Health* 2008; **11**: 44-47 [PMID: 18237359 DOI: 10.1111/j.1524-4733.2007.00213.x]

44   **Maselbas W**, Członkowska A, Litwin T, Niewada M. Persistence with treatment for Wilson disease: a retrospective study. *BMC Neurol* 2019; **19**: 278 [PMID: 31718567 DOI: 10.1186/s12883-019-1502-4]

45   **Lössner A**, Lössner J, Bachmann H, Zotter J. The Kayser-Fleischer ring during long-term treatment in Wilson's disease (hepatolenticular degeneration). A follow-up study. *Graefes Arch Clin Exp*

Ophthalmol 1986; **224**: 152-155 [PMID: 3949188 DOI: 10.1007/BF02141489]

46   **Fenu M**, Liggi M, Demelia E, Sorbello O, Civolani A, Demelia L. Kayser-Fleischer ring in Wilson's disease: a cohort study. *Eur J Intern Med* 2012; **23**: e150-e156 [PMID: 22863441 DOI: 10.1016/j.ejim.2012.04.005]

47   **Esmaeli B**, Burnstine MA, Martonyi CL, Sugar A, Johnson V, Brewer GJ. Regression of Kayser-Fleischer rings during oral zinc therapy: correlation with systemic manifestations of Wilson's disease. *Cornea* 1996; **15**: 582-588 [PMID: 8899270]

48   **Marcellini M**, Di Ciommo V, Callea F, Devito R, Comparcola D, Sartorelli MR, Carelli G, Nobili V. Treatment of Wilson's disease with zinc from the time of diagnosis in pediatric patients: a single-hospital, 10-year follow-up study. *J Lab Clin Med* 2005; **145**: 139-143 [PMID: 15871305 DOI: 10.1016/j.lab.2005.01.007]

49   **Suvarna JC**. Kayser-Fleischer ring. *J Postgrad Med* 2008; **54**: 238-240 [PMID: 18626182 DOI: 10.4103/0022-3859.41816]

50   **Das MC**, Sen Sarma M, Srivastava A, Yachha SK, Poddar U. Effect of chelation therapy in pediatric Wilson's disease: Liver and endoscopic outcome. *J Hepatobiliary Pancreat Sci* 2021; **28**: 336-345 [PMID: 32745371 DOI: 10.1002/jhbp.812]

51   **Aggarwal A**, Aggarwal N, Nagral A, Jankharia G, Bhatt M. A novel Global Assessment Scale for Wilson's Disease (GAS for WD). *Mov Disord* 2009; **24**: 509-518 [PMID: 19115420 DOI: 10.1002/mds.22231]

52   **Prashanth LK**, Taly AB, Sinha S, Ravishankar S, Arunodaya GR, Vasudev MK, Swamy HS. Prognostic factors in patients presenting with severe neurological forms of Wilson's disease. *QJM* 2005; **98**: 557-563 [PMID: 16006499 DOI: 10.1093/qjmed/hci095]

53   **Walshe JM**. The pattern of urinary copper excretion and its response to treatment in patients with Wilson's disease. *QJM* 2011; **104**: 775-778 [PMID: 21622540 DOI: 10.1093/qjmed/hcr073]

54   **Twomey PJ**, Viljoen A, Reynolds TM, Wierzbicki AS. Non-ceruloplasmin-bound copper in routine clinical practice in different laboratories. *J Trace Elem Med Biol* 2008; **22**: 50-53 [PMID: 18319140 DOI: 10.1016/j.jtemb.2007.11.001]

55   **Duncan A**, Yacoubian C, Beetham R, Catchpole A, Bullock D. The role of calculated non-caeruloplasmin-bound copper in Wilson's disease. *Ann Clin Biochem* 2017; **54**: 649-654 [PMID: 27742851 DOI: 10.1177/0004563216676843]

56   **Schmitt F**, Podevin G, Poupon J, Roux J, Legras P, Trocello JM, Woimant F, Laprévote O, Nguyen TH, El Balkhi S. Evolution of exchangeable copper and relative exchangeable copper through the course of Wilson's disease in the Long Evans Cinnamon rat. *PLoS One* 2013; **8**: e82323 [PMID: 24358170 DOI: 10.1371/journal.pone.0082323]

57   UEG Week 2020 Poster Presentations. *United European Gastroenterol J* 2020; **8**: 144-887 [PMID: 33043826 DOI: 10.1177/2050640620927345]

58   **Cousins RJ**. Absorption, transport, and hepatic metabolism of copper and zinc: special reference to metallothionein and ceruloplasmin. *Physiol Rev* 1985; **65**: 238-309 [PMID: 3885271 DOI: 10.1152/physrev.1985.65.2.238]

59   **Santos Silva EE**, Sarles J, Buts JP, Sokal EM. Successful medical treatment of severely decompensated Wilson disease. *J Pediatr* 1996; **128**: 285-287 [PMID: 8636833 DOI: 10.1016/s0022-3476(96)70412-2]

60   **Dhawan A**, Taylor RM, Cheeseman P, De Silva P, Katsiyiannakis L, Mieli-Vergani G. Wilson's disease in children: 37-year experience and revised King's score for liver transplantation. *Liver Transpl* 2005; **11**: 441-448 [PMID: 15776453 DOI: 10.1002/Lt.20352]

61   **Askari FK**, Greenson J, Dick RD, Johnson VD, Brewer GJ. Treatment of Wilson's disease with zinc. XVIII. Initial treatment of the hepatic decompensation presentation with trientine and zinc. *J Lab Clin Med* 2003; **142**: 385-390 [PMID: 14713890 DOI: 10.1016/S0022-2143(03)00157-4]

62   **Chen JC**, Chuang CH, Wang JD, Wang CW. Combination Therapy Using Chelating Agent and Zinc for Wilson's Disease. *J Med Biol Eng* 2015; **35**: 697-708 [PMID: 26692828 DOI: 10.1007/s40846-015-0087-7]

63   **Weiss KH**, Gotthardt DN, Klemm D, Merle U, Ferenci-Foerster D, Schaefer M, Ferenci P, Stremmel W. Zinc monotherapy is not as effective as chelating agents in treatment of Wilson disease. *Gastroenterology* 2011; **140**: 1189-1198.e1 [PMID: 21185835 DOI: 10.1053/j.gastro.2010.12.034]

64   **Fieten H**, Dirksen K, van den Ingh TS, Winter EA, Watson AL, Leegwater PA, Rothuizen J. D-penicillamine treatment of copper-associated hepatitis in Labrador retrievers. *Vet J* 2013; **196**: 522-527 [PMID: 23375251 DOI: 10.1016/j.tvjl.2012.12.013]

65   **Brewer GJ**, Hedera P, Kluin KJ, Carlson M, Askari F, Dick RB, Sitterly J, Fink JK. Treatment of Wilson disease with ammonium tetrathiomolybdate: III. Initial therapy in a total of 55 neurologically affected patients and follow-up with zinc therapy. *Arch Neurol* 2003; **60**: 379-385 [PMID: 12633149 DOI: 10.1001/archneur.60.3.379]

66   **Brewer GJ**, Askari F, Lorincz MT, Carlson M, Schilsky M, Kluin KJ, Hedera P, Moretti P, Fink JK, Tankanow R, Dick RB, Sitterly J. Treatment of Wilson disease with ammonium tetrathiomolybdate: IV. Comparison of tetrathiomolybdate and trientine in a double-blind study of treatment of the neurologic presentation of Wilson disease. *Arch Neurol* 2006; **63**: 521-527 [PMID: 16606763 DOI: 10.1001/archneur.63.4.521]

67   **Medici V**, Trevisan CP, Bigotto MA, D'Incà R, Martines D, Dal Pont E, Sturniolo GC. Adverse reaction after tetrathiomolybdate treatment for Wilson's disease: a case report. *Mov Disord* 2006; **21**:

2030-2032 [PMID: 16991142 DOI: 10.1002/mds.21109]

68   **Karunajeewa H**, Wall A, Metz J, Grigg A. Cytopenias secondary to copper depletion complicating ammonium tetrathiomolybdate therapy for Wilson's disease. *Aust N Z J Med* 1998; **28**: 215-216 [PMID: 9612534 DOI: 10.1111/j.1445-5994.1998.tb02975.x]

69   **Weiss KH**, Askari FK, Czlonkowska A, Ferenci P, Bronstein JM, Bega D, Ala A, Nicholl D, Flint S, Olsson L, Plitz T, Bjartmar C, Schilsky ML. Bis-choline tetrathiomolybdate in patients with Wilson's disease: an open-label, multicentre, phase 2 study. *Lancet Gastroenterol Hepatol* 2017; **2**: 869-876 [PMID: 28988934 DOI: 10.1016/S2468-1253(17)30293-5]

70   **Pandit A**, Bhave S. Present interpretation of the role of copper in Indian childhood cirrhosis. *Am J Clin Nutr* 1996; **63**: 830S-835S [PMID: 8615370 DOI: 10.1093/ajcn/63.5.830]

71   **Chawla V**, Chandra RK, Verma IC, Ghai OP. An epidemiologic approach to Indian childhood cirrhosis. *Indian Pediatr* 1973; **10**: 73-79 [PMID: 4719650]

72   **Nayak NC**, Visalakshi S, Singh M, Chawla V, Chandra RK, Ramalingaswami V. Indian childhood cirrhosis--a re-evaluation of its pathomorphologic features and their significance in the light of clinical data and natural history of the disease. *Indian J Med Res* 1972; **60**: 246-259 [PMID: 5064312]

73   **Bavdekar AR**, Bhave SA, Pradhan AM, Pandit AN, Tanner MS. Long term survival in Indian childhood cirrhosis treated with D-penicillamine. *Arch Dis Child* 1996; **74**: 32-35 [PMID: 8660042 DOI: 10.1136/adc.74.1.32]

74   **Tanner MS**, Bhave SA, Pradhan AM, Pandit AN. Clinical trials of penicillamine in Indian childhood cirrhosis. *Arch Dis Child* 1987; **62**: 1118-1124 [PMID: 3318711 DOI: 10.1136/adc.62.11.1118]

75   **Tomar BS**, Saxena S, Prakash P, Tomar S, Verma C. D-penicillamine in the treatment of Indian childhood cirrhosis--a preliminary report. *Indian J Pediatr* 1983; **50**: 613-618 [PMID: 6680110 DOI: 10.1007/BF02957727]

76   **Baker A**, Gormally S, Saxena R, Baldwin D, Drumm B, Bonham J, Portmann B, Mowat AP. Copper-associated liver disease in childhood. *J Hepatol* 1995; **23**: 538-543 [PMID: 8583141 DOI: 10.1016/0168-8278(95)80059-x]

77   **Horslen SP**, Tanner MS, Lyon TD, Fell GS, Lowry MF. Copper associated childhood cirrhosis. *Gut* 1994; **35**: 1497-1500 [PMID: 7959213 DOI: 10.1136/gut.35.10.1497]

78   **Maggiore G**, De Giacomo C, Sessa F, Burgio GR. Idiopathic hepatic copper toxicosis in a child. *J Pediatr Gastroenterol Nutr* 1987; **6**: 980-983 [PMID: 3681585 DOI: 10.1097/00005176-198711000-00028]

79   **Muller T**, Feichtinger H, Berger H, Muller W. Endemic Tyrolean infantile cirrhosis: an ecogenetic disorder. *Lancet* 1996; **347**: 877-880 [PMID: 8622397 DOI: 10.1016/s0140-6736(96)91351-3]

80   **Kelly AL**, Lunt PW, Rodrigues F, Berry PJ, Flynn DM, McKiernan PJ, Kelly DA, Mieli-Vergani G, Cox TM. Classification and genetic features of neonatal haemochromatosis: a study of 27 affected pedigrees and molecular analysis of genes implicated in iron metabolism. *J Med Genet* 2001; **38**: 599-610 [PMID: 11546828 DOI: 10.1136/jmg.38.9.599]

81   **Feldman AG**, Whitington PF. Neonatal hemochromatosis. *J Clin Exp Hepatol* 2013; **3**: 313-320 [PMID: 25755519 DOI: 10.1016/j.jceh.2013.10.004]

82   **Lopriore E**, Mearin ML, Oepkes D, Devlieger R, Whitington PF. Neonatal hemochromatosis: management, outcome, and prevention. *Prenat Diagn* 2013; **33**: 1221-1225 [PMID: 24030714 DOI: 10.1002/pd.4232]

83   **Flynn DM**, Mohan N, McKiernan P, Beath S, Buckels J, Mayer D, Kelly DA. Progress in treatment and outcome for children with neonatal haemochromatosis. *Arch Dis Child Fetal Neonatal Ed* 2003; **88**: F124-F127 [PMID: 12598501 DOI: 10.1136/fn.88.2.f124]

84   **Rodrigues F**, Kallas M, Nash R, Cheeseman P, D'Antiga L, Rela M, Heaton ND, Mieli-Vergani G. Neonatal hemochromatosis--medical treatment vs. transplantation: the king's experience. *Liver Transpl* 2005; **11**: 1417-1424 [PMID: 16237701 DOI: 10.1002/Lt.20497]

85   **Sigurdsson L**, Reyes J, Kocoshis SA, Hansen TW, Rosh J, Knisely AS. Neonatal hemochromatosis: outcomes of pharmacologic and surgical therapies. *J Pediatr Gastroenterol Nutr* 1998; **26**: 85-89 [PMID: 9443126 DOI: 10.1097/00005176-199801000-00015]

86   **Pietrangelo A**. Hereditary hemochromatosis: pathogenesis, diagnosis, and treatment. *Gastroenterology* 2010; **139**: 393-408, 408.e1 [PMID: 20542038 DOI: 10.1053/j.gastro.2010.06.013]

87   **Higuchi T**, Moriyama M, Fukushima A, Matsumura H, Matsuoka S, Kanda T, Sugitani M, Tsunemi A, Ueno T, Fukuda N. Association of mRNA expression of iron metabolism-associated genes and progression of non-alcoholic steatohepatitis in rats. *Oncotarget* 2018; **9**: 26183-26194 [PMID: 29899851 DOI: 10.18632/oncotarget.25488]

88   **Salgia RJ**, Brown K. Diagnosis and management of hereditary hemochromatosis. *Clin Liver Dis* 2015; **19**: 187-198 [PMID: 25454304 DOI: 10.1016/j.cld.2014.09.011]

89   **Mobarra N**, Shanaki M, Ehteram H, Nasiri H, Sahmani M, Saeidi M, Goudarzi M, Pourkarim H, Azad M. A Review on Iron Chelators in Treatment of Iron Overload Syndromes. *Int J Hematol Oncol Stem Cell Res* 2016; **10**: 239-247 [PMID: 27928480]

90   **Phatak P**, Brissot P, Wurster M, Adams PC, Bonkovsky HL, Gross J, Malfertheiner P, McLaren GD, Niederau C, Piperno A, Powell LW, Russo MW, Stoelzel U, Stremmel W, Griffel L, Lynch N, Zhang Y, Pietrangelo A. A phase 1/2, dose-escalation trial of deferasirox for the treatment of iron overload in HFE-related hereditary hemochromatosis. *Hepatology* 2010; **52**: 1671-1779 [PMID:

20814896 DOI: 10.1002/hep.23879]

91   **Nagler M**, Gregor M, Wuillemin WA. Iron chelation with deferasirox in two patients with HFE hemochromatosis and chronic anemia. *Acta Haematol* 2011; **126**: 119-121 [PMID: 21659727 DOI: 10.1159/000328039]

92   **Bacon BR**, Adams PC, Kowdley KV, Powell LW, Tavill AS; American Association for the Study of Liver Diseases. Diagnosis and management of hemochromatosis: 2011 practice guideline by the American Association for the Study of Liver Diseases. *Hepatology* 2011; **54**: 328-343 [PMID: 21452290 DOI: 10.1002/hep.24330]

93   **European Association for the Study of the Liver**. EASL clinical practice guidelines for HFE hemochromatosis. *J Hepatol* 2010; **53**: 3-22 [PMID: 20471131 DOI: 10.1016/j.jhep.2010.03.001]

94   **Ware HM**, Kwiatkowski JL. Evaluation and treatment of transfusional iron overload in children. *Pediatr Clin North Am* 2013; **60**: 1393-1406 [PMID: 24237978 DOI: 10.1016/j.pcl.2013.09.003]

95   **Piga A**, Longo F, Duca L, Roggero S, Vinciguerra T, Calabrese R, Hershko C, Cappellini MD. High nontransferrin bound iron levels and heart disease in thalassemia major. *Am J Hematol* 2009; **84**: 29-33 [PMID: 19006228 DOI: 10.1002/ajh.21317]

96   **Jean G**, Terzoli S, Mauri R, Borghetti L, Di Palma A, Piga A, Magliano M, Melevendi M, Cattaneo M. Cirrhosis associated with multiple transfusions in thalassaemia. *Arch Dis Child* 1984; **59**: 67-70 [PMID: 6696498 DOI: 10.1136/adc.59.1.67]

97   **Olivieri NF**, Nathan DG, MacMillan JH, Wayne AS, Liu PP, McGee A, Martin M, Koren G, Cohen AR. Survival in medically treated patients with homozygous beta-thalassemia. *N Engl J Med* 1994; **331**: 574-578 [PMID: 8047081 DOI: 10.1056/NEJM199409013310903]

98   **Brittenham GM**, Farrell DE, Harris JW, Feldman ES, Danish EH, Muir WA, Tripp JH, Bellon EM. Magnetic-susceptibility measurement of human iron stores. *N Engl J Med* 1982; **307**: 1671-1675 [PMID: 7144866 DOI: 10.1056/NEJM198212303072703]

99   **Wood JC**, Enriquez C, Ghugre N, Tyzka JM, Carson S, Nelson MD, Coates TD. MRI R2 and R2* mapping accurately estimates hepatic iron concentration in transfusion-dependent thalassemia and sickle cell disease patients. *Blood* 2005; **106**: 1460-1465 [PMID: 15860670 DOI: 10.1182/blood-2004-10-3982]

100   **Maira D**, Cassinerio E, Marcon A, Mancarella M, Fraquelli M, Pedrotti P, Cappellini MD. Progression of liver fibrosis can be controlled by adequate chelation in transfusion-dependent thalassemia (TDT). *Ann Hematol* 2017; **96**: 1931-1936 [PMID: 28875336 DOI: 10.1007/s00277-017-3120-9]

101   **Piga A**, Longo F, Musallam KM, Cappellini MD, Forni GL, Quarta G, Chiavilli F, Commendatore F, Mulas S, Caruso V, Galanello R. Assessment and management of iron overload in β-thalassaemia major patients during the 21st century: a real-life experience from the Italian WEBTHAL project. *Br J Haematol* 2013; **161**: 872-883 [PMID: 23600689 DOI: 10.1111/bjh.12340]

102   **Angelucci E**, Barosi G, Camaschella C, Cappellini MD, Cazzola M, Galanello R, Marchetti M, Piga A, Tura S. Italian Society of Hematology practice guidelines for the management of iron overload in thalassemia major and related disorders. *Haematologica* 2008; **93**: 741-752 [PMID: 18413891 DOI: 10.3324/haematol.12413]

103   **Kamble RT**, Selby GB, Mims M, Kharfan-Dabaja MA, Ozer H, George JN. Iron overload manifesting as apparent exacerbation of hepatic graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* 2006; **12**: 506-510 [PMID: 16635785 DOI: 10.1016/j.bbmt.2006.01.004]

104   **Majhail NS**, Lazarus HM, Burns LJ. Iron overload in hematopoietic cell transplantation. *Bone Marrow Transplant* 2008; **41**: 997-1003 [PMID: 18438425 DOI: 10.1038/bmt.2008.99]

105   **Sivgin S**, Eser B. The management of iron overload in allogeneic hematopoietic stem cell transplant (alloHSCT) recipients: where do we stand? *Ann Hematol* 2013; **92**: 577-586 [PMID: 23430087 DOI: 10.1007/s00277-013-1682-8]

106   **Wang LC**, Wang JD, Tsai CR, Cheng SB, Lin CC. Clinical features and therapeutic response in Taiwanese children with Wilson's disease: 12 years of experience in a single center. *Pediatr Neonatol* 2010; **51**: 124-129 [PMID: 20417464 DOI: 10.1016/S1875-9572(10)60022-8]

107   **Arnon R**, Calderon JF, Schilsky M, Emre S, Shneider BL. Wilson disease in children: serum aminotransferases and urinary copper on triethylene tetramine dihydrochloride (trientine) treatment. *J Pediatr Gastroenterol Nutr* 2007; **44**: 596-602 [PMID: 17460493 DOI: 10.1097/MPG.0b013e3180467715]

108   **Taylor RM**, Chen Y, Dhawan A; EuroWilson Consortium. Triethylene tetramine dihydrochloride (trientine) in children with Wilson disease: experience at King's College Hospital and review of the literature. *Eur J Pediatr* 2009; **168**: 1061-1068 [PMID: 19066958 DOI: 10.1007/s00431-008-0886-8]

109   **Rodeck B**, Kardoff R, Melter M. Treatment of copper associated liver disease in childhood. *Eur J Med Res* 1999; **4**: 253-256 [PMID: 10383883]

110   **Masera N**, Cattoni A, Decimi V, D'Apolito Valeria, Arosio C, Mariani R, Piperno A. Efficacy of deferasirox for the treatment of iron overload in a child affected by Juvenile Hemochromatosis. *Case Rep Clin Med* 2013; **2**: 126-128 [DOI: 10.4236/crcm.2013.22033]

Hepatocellular carcinoma: Understanding molecular mechanisms for defining potential clinical modalities

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Abstract

Liver cancer is the sixth most commonly occurring cancer and costs millions of lives per year. The diagnosis of hepatocellular carcinoma (HCC) has relied on scanning techniques and serum-based markers such as α-fetoprotein. These measures have limitations due to their detection limits and asymptomatic conditions during the early stages, resulting in late-stage cancer diagnosis where targeted chemotherapy or systemic treatment with sorafenib is offered. However, the aid of conventional therapy for patients in the advanced stage of HCC has limited outcomes. Thus, it is essential to seek a new treatment strategy and improve the diagnostic techniques to manage the disease. Researchers have used the omics profile of HCC patients for sub-classification of tissues into different groups, which has helped us with prognosis. Despite these efforts, a promising target for treatment has not been identified. The hurdle in this situation is genetic and epigenetic variations in the tumor, leading to disparities in response to treatment. Understanding reversible epigenetic changes along with clinical traits help to define new markers for patient categorization and design personalized therapy. Many clinical trials of inhibitors of epigenetic modifiers (also known as epi-drugs) are in progress. Epi-drugs like azacytidine or belinostat are already approved for other cancer treatments. Furthermore, epigenetic changes have also been observed in drug-resistant HCC tumors. In such cases, combinatorial treatment of epi-drugs with systemic therapy or trans-arterial chemoembolization might re-sensitize resistant cells.

Key Words: Hepatocellular carcinoma; Diagnosis; Treatment; Epigenetics; Epi-drugs; Drug resistance

Core Tip: This review article focuses on the limitations of diagnosis and treatment of hepatocellular carcinoma (HCC). Furthermore, the use of omics technology with clinical attributes for categorizing HCC patients in order that personalized treatment can be designed to prolong survival is discussed. Finally, the potential of epi-drugs in targeting epigenetic changes in the disease and resistance has been proposed.

INTRODUCTION

Liver cancer ranks sixth in cancer incidence globally and accounts for 8.2% of total cancer deaths. The different categories of primary liver cancer are intrahepatic cholangiocarcinoma, hepatocellular carcinoma (HCC), fibrolamellar carcinoma, and hepatoblastoma. These categories have distinct changes in their molecular, histological, and pathological features. HCC alone accounts for 85%-90% of liver cancer cases[1]. Almost 2/3 of the population affected by HCC is found in east Asian and south-east Asian countries, making this disease endemic to the region[2]. Globally, 5-year median survival is below 20% for HCC[3]. Major risk factors for HCC include chronic infection with hepatitis B virus and hepatitis C virus, excessive consumption of alcohol, exposure to aflatoxin, physiological state such as non-alcoholic fatty liver disease, and diabetes[4]. According to the Barcelona Clinic Cancer Liver Classification (BCLC) algorithm, curative care for HCC involves tumor resection, ablation, and liver transplantation[5]. However, this mode of treatment is offered to patients diagnosed in an early stage of the disease. Current research suggests that only 20% of patients are diagnosed in the early stage[6]. The lacunae in diagnosis are the unavailability of promising liquid-based biomarkers and detection limits of scanning techniques. Palliative care involving chemo/radiation-based treatment is given to patients with intermediate and advanced stage disease. Following this, 70% of patients come back with a relapse of disease and suffer treatment side effects[7,8].

A new approach should be considered to identify diagnostic markers and achieve better therapy response to overcome disease management challenges. Recent advances in the omics field shed light on the pathogenesis and molecular classification of HCC [9-11]. The omics approach can help to investigate new markers to improve the therapeutic outcome. Liver carcinogenesis involves both genetic and epigenetic changes. It is impossible to target all genetic variations due to tumor heterogeneity, but gene signature can be manipulated as epigenetic changes are reversible[12]. Therefore, epi-drug-based treatment may act as an alternate treatment strategy instead of targeting a single protein or molecular pathway. Epi-drugs can be beneficial not only for the treatment of HCC but also for dealing with cancer resistance[13,14].

This article focuses on the existing approach for diagnosis and treatment in the management of HCC. We also review transcriptomic-based signatures of HCC for patient sub-categorization and their potential implications for diagnosis and therapy. Finally, we propose an epi-drug based treatment strategy based on the epigenetic landscape of HCC.

DIAGNOSIS OF LIVER CANCER

Five standard WHO-approved guidelines include the European Association for the Study of Liver Disease (EASL)[15], American Association for the Study of Liver Diseases (AASLD)[16], Asia-Pacific Association Study of the Liver[17], EASL-EORTC Clinical Practice Guidelines[18], and the updated AASLD guidelines are used for diagnosis of liver cancer. The diagnosis is primarily based on imaging techniques such

as ultrasound, computed tomography (CT) scan, and conventional magnetic resonance imaging (MRI)[19]. Invasive biopsies are not helpful for the diagnosis of liver tumors. The myriad risk factors involved in biopsy are the local spread of HCC along the needle track and different complications observed in individual patients[20]. The early-stage diagnosis of HCC continues to be crucial due to reduced sensitivity and specificity of the diagnostic methods, due to which an ample number of tumors are undetected. The complete list of diagnostic methods with detection limits is shown in Table 1. The various factors responsible for undetectable tumors involve a lack of specific markers and asymptomatic condition during the early stages of HCC[21]. Thus, the diagnosis of tumor occurs when it has spread and has reached an advanced stage.

The diagnostic marker used most frequently is serum α-fetoprotein (AFP)[22]. AFP level increases beyond 20 ng/mL in more than 70% of patients with HCC. However, AFP elevations are not explicitly associated with HCC as AFP levels from 10-500 ng/mL and even occasionally to 1000 ng/mL may be seen in patients with a high degree of necro-inflammatory activity such as chronic viral hepatitis[23]. Chan *et al*[24] in 2008 have shown that AFP could be better used as a prognostic marker to evaluate response to treatment and detection of recurrence instead of diagnosis[25]. Studies have shown that multiple combinations of markers provide more appropriate results in diagnosis than a single marker. A recent study investigated the use of HSP90α (heat shock protein 90) combined with AFP and thymidine kinase 1 to diagnose HCC with more efficiency[26]. A study from Beijing YouAn Hospital found that for early diagnosis of HBV-related HCC, a combination of AFP, GPC3, and GP73 had the highest diagnostic value[27]. Ghosh *et al*[28] have shown that the exosome encapsulated microRNAs could be used as a circulating diagnostic marker for HCC with low AFP levels.

Another marker, α-L-fucosidase (AFU), is expressed in liver cirrhosis patients[29]. However, limited research is available regarding the utility of AFU in the diagnosis of HCC. In the liver and gallbladder, cell membrane protein 5′-nucleotidase (5′-NT) is released into the blood during hepatic injury or obstruction[30]. It has been observed that 5′-NT levels also increase with age and during pregnancy[31]. Other markers such as AFP-L3, glypican-3, and des-γ-carboxy prothrombin also show inconsistent data due to low sensitivity and specificity. Hence, the discovery of putative liquid biomarkers is required, which can associate with tumor progression, recurrence, and effectiveness of therapeutic programs.

TREATMENT REGIME AND LIMITATIONS OF CHEMOTHERAPY IN LIVER CANCER

Treatment of HCC is decided based on different stages of tumor detection[32]. The BCLC algorithm is widely used for treatment as it considers tumor stage, liver function, performance status, and treatment impact (Figure 1). Early-stage cases are treated with surgery, ablation, or liver transplantation. The patients undergoing surgery showed 70% recurrence within five years[33]. The currently used methods for tumor ablation in HCC are percutaneous ethanol injection (PEI) and radiofrequency ablation (RFA). PEI consists of the direct injection of absolute ethanol into HCC nodules[34]. RFA is responsive in tumors > 4 cm in size. It involves necrosis of the tumor using a needle tip electrode that reaches temperatures up to 100°C[35]. Microwave ablation and irreversible electroporation have shown more promising results than tumor removal with PEI[36].

Patients with an intermediate stage having a tumor size greater than 5 cm or multinodular HCC with no vascular invasion are treated with trans-arterial chemoembolization (TACE). TACE is used to obstruct the nutrient supply to the tumor using the occlusion of arterial blood vessels[37]. Chemotherapeutic drugs such as doxorubicin or cisplatin are given during embolization, allowing prolonged exposure of the drug to tumor cells, resulting in tumor reduction. Yeo *et al*[38] showed that the overall response rate for doxorubicin-treated patients was 10.5%. Moreover, doxorubicin alone and combined with PIAF had no significant difference in response rate but showed treatment-associated toxicity in patients. Another study showed that combinatorial treatment of fluorouracil, leucovorin, and oxaliplatin failed to improve survival compared to doxorubicin[39]. In a multicohort study involving patients with unresectable tumors treated with TACE, overall survival (OS) was approximately 26-40 mo, with only 52% of patients achieving treatment benefits[40,41]. In some cases, selective internal radiation therapy is used in patients with intermediate-stage HCC.

Table 1 Utility and detection limits of existing diagnostic measures of hepatocellular carcinoma

Diagnostic methods	Definition/concept	Diagnostic limit/range	Ref.
Contrast-enhanced ultrasound	Inexpensive, non-invasive, first choice for screening HCC; Real time dynamic of blood supply.	Small HCC less than 1 cm	[101]
Multi phasic enhanced computed tomography	3 dimensional reconstructions, high sensitivity	1-2 cm HCC lesion	[102]
Magnetic resonance imaging	High resolution anatomic details, pre-contrast and multi-phasic enhanced 3D; Diffusion weighted imaging-functional imaging	2-3 cm HCC lesion	[103]
Positron emission tomography	Hepatocyte-specific PET tracer, 2-[18F] fluoro-2-deoxy-D-galactose, is used which accumulates in the liver compared with other tissues	Detection of small intrahepatic; HCC lesions	[104]
AFP	Elevated in HCC, non-specific	Range: > 500 ng/mL	[23]
α-L-fucosidase	Expressed in liver cirrhosis	Cut-off: 870 nmol/L	[105]
Des-γ-carboxy prothrombin	Sensitive; Not expressed in other liver disease	Cut-off: 40 mAU/mL	[105, 106]
HSP90α + AFP +TKI	Combination of markers have improved diagnostic value	HSP90- (76.65-144.00); AFP- (5.33-2000.00); TK1- (0.57-2.30)	[26]
AFP, GPC3, and GP73	Useful markers for early diagnosis and prognosis	Upregulated	[27, 107]
microRNA: miR-21, miR-199, and miR-122, miR-23a	Specific for diagnosis of HCC; Extremely sensitive	Cut-off value of ≥ 210	[108, 109]

HCC: Hepatocellular carcinoma; AFP: α-fetoprotein.
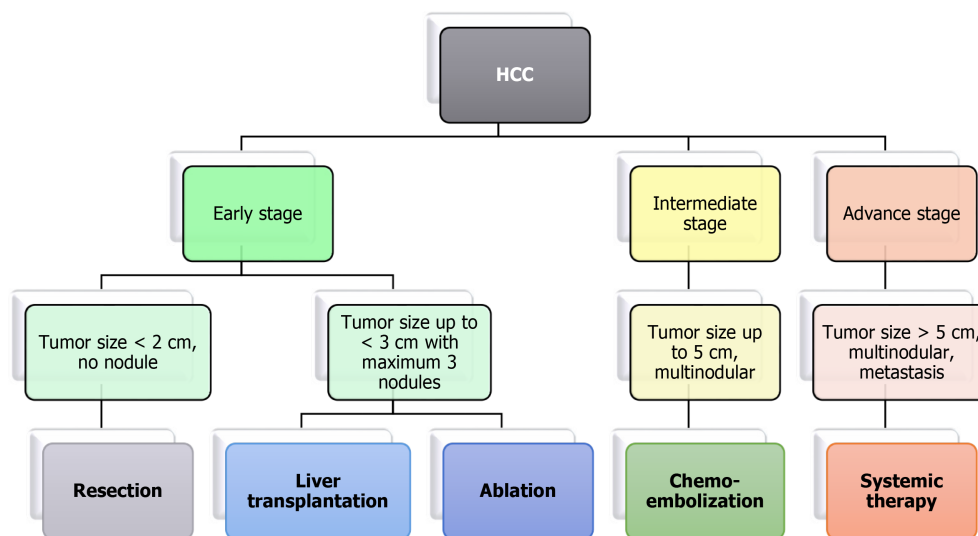
Figure 1 Treatment modalities for hepatocellular carcinoma based on tumor-node-metastasis staging. HCC: Hepatocellular carcinoma.

Intraarterial infusion of radioisotope labeled microspheres is carried out in this modality. Another radiation-based technique known as stereotactic body radiation is used for patients with > 3 cm of the tumor.

Systemic chemotherapy is given for advanced stages of HCC. NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines) have recommended sorafenib and lenvatinib as first-line systemic therapy for patients with unresectable HCC[42]. Brivanib, sunitinib, erlotinib, and regorafenib are other preferred drugs for late-stage HCC treatment. Kudo *et al*[43] observed that treatment with lenvatinib results in significantly higher OS than sorafenib and improvement in all secondary efficacy endpoints. This trial further results in FDA approval of lenvatinib as the first line of therapy for HCC[43]. Sorafenib and sunitinib are protein kinase inhibitors targeting VEGFR, PDGFR, and the Raf kinase pathway. However, a study suggested that sunitinib had an adverse effect in these patients and had no advantage over sorafenib [44]. Moreover, sorafenib has been extensively explored in the systemic treatment of

advanced stage HCC and combination with TACE, but it provided contradictory results[45,46]. Brivanib is an inhibitor of FGF1 and VEGFR2. Phase II clinical trials of brivanib showed the ineffectiveness of the drug compared to sorafenib for improving OS[47,48]. The EGFR inhibitor erlotinib or cetuximab was administered in phase II clinical trials of advanced stages of HCC. However, the trial results did not show the anti-tumor effect of cetuximab in HCC patients[49]. Interestingly, erlotinib showed a positive response in treatment by increasing OS to 13 mo and a response rate of 59% [50].

As discussed earlier, ablation treatment is possible in less than 40% of patients due to late diagnosis, and only 20% are treated with TACE. For the patients with advanced stages of HCC, treatment modalities are limited to systemic therapy, and response rates are also significantly less due to resistance towards available chemotherapy. Multimodal treatment involving more than one therapeutic drug has also failed in different combinations due to cytotoxicity and poor trial outcomes. Despite the significant research in targeted therapy of HCC management, a promising drug is yet to be identified. Thus, the hunt for combinatorial treatment with different therapeutic agents continues (Figure 2).

MOLECULAR LANDSCAPE OF LIVER TUMOR TISSUE FOR PATIENT STRATIFICATION AND IDENTIFICATION OF ALTERNATE TARGETS

Over the past years, HCC classification has mainly focused on histological analysis of tumor tissues. However, the molecular profile and clinical attributes have a significant impact on the prognosis of the disease, thereby redefining HCC into several subgroups. Boyault *et al*[51] published molecular classification systems for HCC composed of 6 groups. The groups were based on mutation profile, disease prognosis, and transcription landscape. The first group included patients with hepatitis B infection and low viral load, increased AFP levels, and high IGF2 expression, whereas the second group included patients with a high viral titer and associated microvascular invasion (MVI) and satellitosis. However, the difference in groups 3 and 4 was based on histological parameters. The third group consisted of poorly differentiated tumors with the worst prognosis; on the other hand, group 4 had well-differentiated tumors. Group 5 and 6 had a low proliferation rate and activated Wnt-signaling pathway. Moreover, pathways are differentially activated in different groups. Another group classified HCC into three groups based on histology and expression analysis of the tumor[52]. In this study, the first group showed the presence of satellitosis and MVI. Group 2 had high AFP expression, and the third group consisted of well-differentiated tumors with a low proliferation rate.

Tumor morphology-based classification has been proposed by Murakata *et al*[53]. The nodal status of the tumor was correlated with survival and recurrence of the disease. Moreover, the miRNA profile of HCC patients has been used to classify sorafenib responders[54]. *c-myc* signaling and EB-1 protein were functionally linked with HCC[55]. Similar findings were observed by Lee *et al*[56] in progenitor-like HCC, which correlated with poor prognosis. In another study, HCC progenitor-like signature consisting of CK-19, Ep-CAM, and CD133 was seen by Woo *et al*[57]. Morofuji *et al*[58] identified the gene signature of early recurrent HCC, including ERK1, PKG, Apaf1, and Bcl-X. Furthermore, ERK1 and Bcl-X were identified as genes associated with the poor prognosis of HCC[58]. However, these studies did not consider the survival status of an individual while proposing subtypes.

Jiang *et al*[59] showed that heterogeneity exists in proteomic profiling of paired early-stage HCC patients. The tumors were segregated into three subtypes: S-I, S-II, and S-III. S-I tumors had increased expression of liver-associated functional proteins. In contrast, S-II and S-III had a more proliferative nature due to overexpression of cell-cycle-related proteins. Furthermore, S-III were more aggressive and had a high expression of KRT19 and MMP9, associated with poor prognosis. Gao *et al*[60] sub-grouped 159 HBV infected patients based on survival, tumor thrombus, and multi-omics profile. These sub-groups were classified based on metabolic rewiring, alterations in the microenvironment, and cellular proliferation. Moreover, the study proposed two prognostic markers PYCR2 and ADH1A.

In the past decade, data generated under the TCGA consortium can be used to understand the gene expression profile of patients and obtain correlations with clinical attributes[9]. Machine learning algorithms are necessary to analyze such multivariate data. The molecular alterations obtained from the cancer genome atlas liver hepatocellular carcinoma (TCGA-LIHC) cohort (423 patients) can be explored to predict new
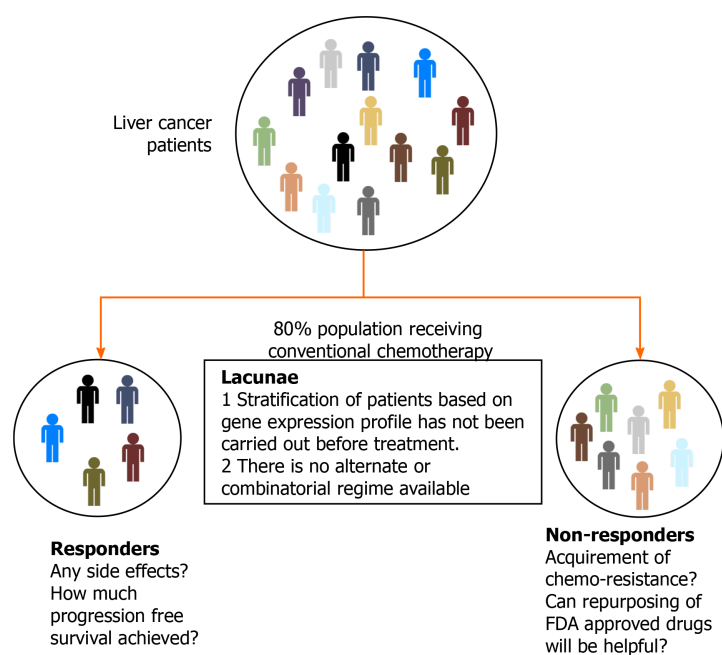
Figure 2 Challenges in the treatment of hepatocellular carcinoma.

targets and rationalize the combinatorial therapy. Transcriptome data generated from TCGA-LIHC identified over 13000 differentially expressed genes compared to cut-margin samples, and around 3330 genes correlated with poor survival (P value < 0.05). Furthermore, 1730 genes overlapped between the DE gene list and genes correlated with patient survival. The majority of overlapped genes showed more than 30% alteration compared to adjacent normal in this cohort and had a significant association with OS. Patients were categorized into different groups using clustering analysis of gene expression. It was observed that these genes belong to metabolism-related pathways and the cellular proliferation-related family (Figure 3). Deep learning computational framework on the TCGA-LIHC dataset suggested that aggressive subtype has TP53 inactivation with high expression of KRT10, EPCAM, and active AKT, WNT signaling[61]. Furthermore, drugs and small molecular compounds are available to target these genes. Schulze *et al*[62] reported that potential gene targets have FDA-approved drugs in 28% of liver tumors. Therefore, these genes can be used for prognosis of the disease, and targeting them may improve patient survival.

Gene expression analysis of liver cancer samples can also be utilized to identify new markers for diagnostic purposes. For example, SPP2 is downregulated at the transcript level in HCC. This gene is deregulated in multiple HCC cohorts. Moreover, a stage-wise decrease at the transcript level was observed in HCC TCGA data. Also, the downregulation of SPP2 leads to a significant decrease in patient survival (Figure 4). This observation indicates that SPP2 level is associated with normal liver function, and a change in levels can be a measure of liver carcinogenesis.

EPI-DRUG BASED TREATMENT FOR IMPROVEMENT OF THERAPEUTIC OUTCOME

The lack of success in disease management can be explained by the multifactorial nature of carcinogenesis involving multiple mutations and global level epigenome alterations[63-65]. Epigenetic changes being reversible can be useful to understand the relationship between tumor biology and help in redefining therapeutic response[12]. Epigenetics deals with changes in gene expression without change in the DNA sequences[66]. Despite all cells having the same DNA sequence, the epigenome decides cell fate regarding differentiation, cell proliferation, and cell death[67,68]. The widely studied epigenetic marks are DNA methylation, histone post-translational modifications, and non-coding RNAs. DNA methylation is the most characterized heritable epigenetic mark. This is where a methyl group is transferred onto the cytosine of the CpG di-nucleotide-rich region in DNA by DNMT enzymes[69]. DNA methylation plays a vital role in gene inactivation, genomic imprinting, attaining
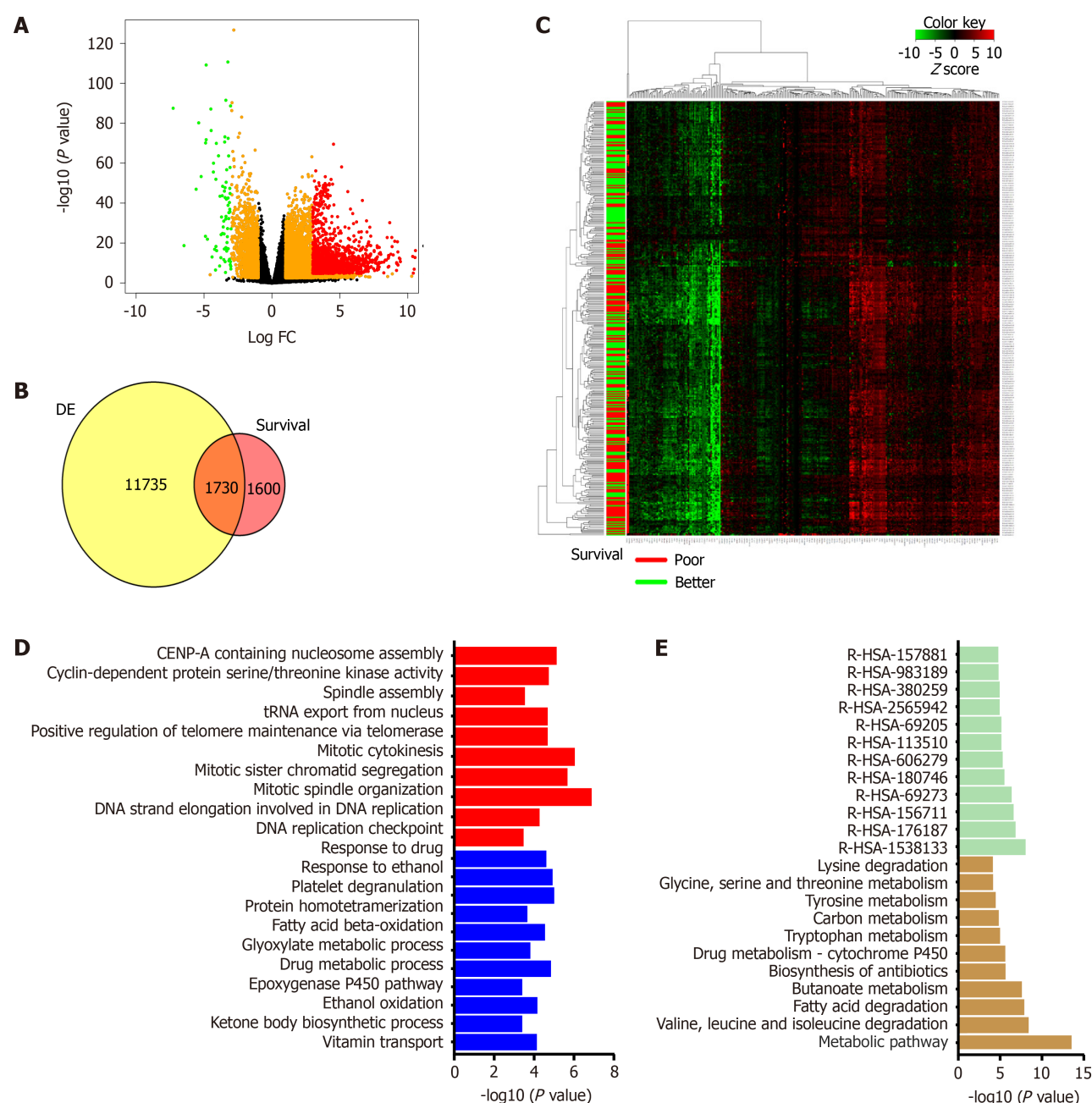
Figure 3 The cancer genome atlas liver hepatocellular carcinoma data analysis. A: Volcano plot representing differential gene expression between 373 tumor samples and 50 normal samples. Genes colored with red or green are most significantly altered; B: Venn diagram showing overlap between differentially expressed gene list and genes affecting survival of patients upon alteration (survival); C: Normalized expression of top 300 genes associated with overall survival represented using heatmap. Patients with overall survival below the median are marked with a red bar while those above the median are marked with a green bar; D: Altered biological process from overlap gene. Upregulated processes highlighted with red and downregulated processes are depicted as blue; E: Pathways analysis for overlap genes. Deregulated KEGG pathways shown by yellow bars and reactome pathways displayed using green bars. DE: Differentially expressed gene list.

tissue-specific gene expression, and X chromosome inactivation[69].

Similar to DNA modification, histone proteins also undergo post-translational modifications carried out by chromatin modifiers, namely writers, readers, and erasers [70]. The well-studied modifications include methylation, acetylation, phosphorylation, and ubiquitination. Histone methylation involves the addition of a methyl group at the lysine or arginine residue on the protruding histone tails. Histone methylation marks can result in repression of transcription or gene activation[71]. A typical example of gene suppression is trimethylation at H3K9, and H3K27 whereas methylation at H3K4, H3K36, and H3K79 enhance transcriptional activity[71]. Histone acetylation is the transfer of an acetyl group from acetyl CoA. This reaction leads to a change in electrostatic interaction between DNA and histones, resulting in the unwinding of chromatin and enhances gene transcription[72]. Histone phosphorylation has an essential role in DNA damage repair, gene transcription, and
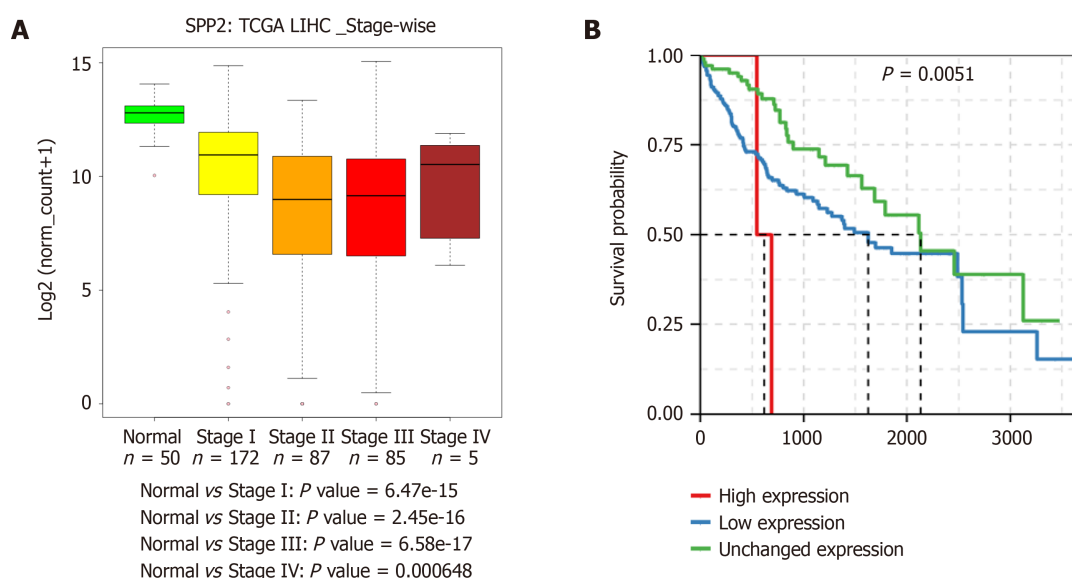
Figure 4 Expression of SPP2 in the cancer genome atlas liver cancer cohort. A: Stage-wise expression of SPP2; B: Patient survival associated with SPP2 expression. TCGA: The cancer genome atlas; LIHC: Liver hepatocellular carcinoma.

chromatin condensation during mitosis[72]. An illustration of chromatin-associated modifications and the role of epigenetic modifiers is shown in Figure 5. Non-coding RNAs are the transcribed intragenic regions of the DNA that are not translated into proteins. These entities govern gene silencing *via* RISC and RNA-induced transcriptional silencing complex formation[73].

Different research groups have extensively studied the epigenetic landscape of liver carcinogenesis. Moreover, in the past few years, researchers are investigating the epigenetic basis of chemoresistance in HCC. Lie *et al*[74] showed that lysine-specific demethylase 1 (LSD1) is upregulated in LGR5+ cells contributing to stemness and chemoresistance properties. Mechanistically, LSD1 removes the H3K4 methylation mark from the promoter of genes which inhibit Wnt-signaling. Thus, promoting pathway activation, which is essential for stemness and chemoresistance[74]. EpCAM+ liver cancer cells have high expression of chromodomain helicase DNA binding protein (CHD4), a DNA damage response protein. The abundance of CHD4 in liver cancer cells leads to epirubicin resistance[75]. Zinc-fingers and homeoboxes 2 (ZHX2) is one of the signature proteins which is downregulated in liver CSCs and is associated with tumor progression. It has been found that low expression of ZHX2 is correlated with epigenetic regulation of OCT4, SOX4, and NANOG by H3K36 methylation[76]. Oriana Lo Re *et al*[77] observed that low expression of MacroH2A1 leads to paracrine mediated chemoresistance and imparts CSCs properties to the tumor cells. Another study showed that the regulator of chromosome condensation 2 promotes metastasis and cisplatin resistance in HCC[78]. Ling *et al*[79] discovered that USP22 helps to attain chemoresistance by hypoxia-driven p53 mutant tumors. Hypoxia-induced expression of carbonyl reductase 1 leading to chemoresistance in HCC was observed by Tak *et al*[80]. H19 long non-coding (lnc)RNA has been shown to sensitize sorafenib or doxorubicin-resistant liver cancer cells[81]. The lncRNA CRNDE has been shown to interact with histone methyltransferase to enhance their effect on the inhibition of tumor suppressors and induce resistance in tumor cells[82].

Epigenetic alterations can be targeted by the class of small-molecule inhibitors that specifically inhibit or reverse the changes[83]. This class of inhibitors are referred to as epi-drugs. Different research groups have synthesized epi-drugs for all three prominent families of epigenetic modifiers- readers, writers, and erasers. Many epi-drugs have cleared pre-clinical trials, and initial phase trials have shown promising results. Few epi-drugs are clinically approved for the treatment of hematological malignancies. In some studies, treatment of solid tumors with an epi-drug helps in sensitizing tumor cells to chemotherapy[84,85]. These findings have promoted the research on inhibitors of HDAC, HAT, and DNMTs in combination with chemotherapeutic drugs. In HCC and gastric cancer, the inactive or suppressed state of tumor suppressor genes (TSGs) is mainly attributed to the overexpression of DNMTs and HDACs, leading to heterochromatinization. Reversion of the chromatin state using epi-drugs further leads to activation of TSGs and prevents tumor growth[86]. Ongoing
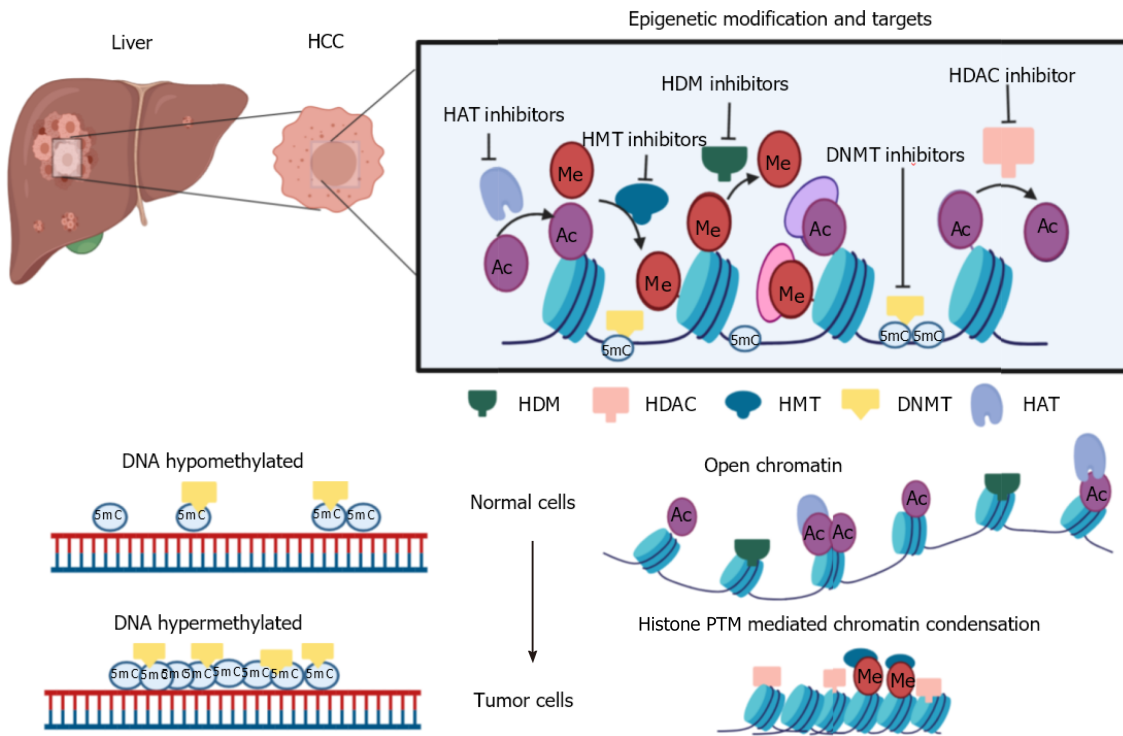
Figure 5 Schematic illustration of epigenetic modifications observed in hepatocellular carcinoma and chromatin modifiers targeted by epi-drugs. The figure represents general epigenetic alterations observed in hepatocellular carcinoma and different epigenetic modifiers that can be targeted *via* small molecule inhibitors. Moreover, DNA and chromatin mediated alterations observed in tumors are highlighted. Changes in DNA methylation and histone post-translational modifications levels inside normal cells lead to tumor formation. HCC: Hepatocellular carcinoma; HDM: Histone demethylase; HDAC: Histone deacetylase; HMT: Histone methyltransferase; DNMT: DNA methyltransferase; HAT: Histone acetyltransferase.

pre-clinical trials have been carried out with HDAC and DNMT inhibitors in combination or in comparison with each other to study the anti-tumor effects of the drugs. Guadecitabine (SGI-110), a DNMT inhibitor with sorafenib and oxaliplatin, is in phase II clinical trials for HCC (NCT01752933). Multicenter phase I/II clinical trials using belinostat (HDAC inhibitor) in patients with unresectable HCC showed a tumor stabilization effect[87]. One study showed that the combination of panobinostat and sorafenib significantly decreased tumor volume by inducing apoptosis in the tumor [88]. A group of researchers observed that the DNMT inhibitor 5'-aza-2' deoxycytidine and HDAC inhibitor SAHA down-regulated DNMT1, DNMT3a, DNMT3b, and HDAC1 and upregulated GSTP1 and SOCS1 gene expression, which further resulted in inhibition of cell viability and induced apoptosis[89]. A detailed list of potential epi-drugs is given in Table 2. These findings indicate the ability of epi-drugs, which can restructure the treatment strategy for HCC.

Future perspectives

The most effective way of controlling HCC is preventing the disease by spreading knowledge of etiological agents and hepatitis B vaccination. An increase in surveillance is one of the strategies to achieve better survival. This practice helps in the early diagnosis of HCC, monitors progression-free survival, and improves quality of life. Diagnosis of HCC at an early stage is crucial in order to start treatment at the right time and improve patient survival. Due to the reduced sensitivity of current diagnostic techniques, ultrasound scanning of high-risk individuals should be carried out every three months. Although ultrasound is cost-effective compared to MRI and CT scans, there is scope for developing more advanced MRI or CT versions to detect small lesions in the liver. Similarly, there is a need for an appropriate combination of liquid biomarkers used for the investigation of liver carcinogenesis. From a treatment perspective, upon early diagnosis, liver transplantation is preferred over surgical removal or ablation as it is has less than 15% chance of recurrence[90].

The primary cause of treatment failure in cancer is resistance to available chemotherapy, which results in relapse. From heterogeneous tumors, cells respond to treatment differently, and a rare small percentage of cells found in the quiescent G0 state of the cell cycle can escape treatment. These cells are inherently resistant to

Table 2 List of Food and Drug Administration approved/under trial epi-drugs

Drugs	Classification	Approved year	Indicated disease	Reference/ clinical trial number
Azacytidine	DNMT inhibitor	2004	MDS	NCT01186939
		2009	AML	NCT00887068
Decitabine	DNMT inhibitor	2006	MDS	NCT01751867
		2011	AML	NCT00260832
Vorinostat	HDAC inhibitor	2006	CTCL	NCT00773747
Romidepsin	HDAC inhibitor	2009	TCL	NCT02296398
Belinostat	HDAC inhibitor	2015	PTCL	NCT01839097
Panobinostat	HDAC inhibitor	2015	MM	NCT01023308
		2016	CML	NCT00451035
		2017	TCL	NCT00490776

MDS: Myelodysplastic syndrome; AML: Acute myeloid leukemia; CTCL: Cutaneous T cell lymphoma; TCL: T-cell lymphoma; PTCL: Peripheral T cell lymphoma; MM: Multiple myeloma; CML: Chronic myeloid leukemia; HDAC: Histone deacetylase; DNMT: DNA methyltransferase.

chemotherapy and involved in relapse. Studies have shown that tumor cells maintain the drug-tolerant state *via* chromatin-mediated changes after drug treatment[13]. The drug-tolerant persister (DTP) stage is reversible; however, prolonged exposure to chemotherapeutic drugs results in stable drug resistance properties[91-93]. DTP cells have non-random differential gene expressions, implicating chromatin-mediated changes leading to hetero-chromatinization of the transposable elements such as LINE1[94]. Recent findings suggest that ablation of the DTP cell population with FDA-approved epi-drugs impedes the development of resistance and relapse[13,94]. Hangauer *et al*[95] have shown DTP cells dependence on mesenchymal state and GPX4 (lipid hydroperoxide) for survival. Furthermore, inhibition of GPX4 triggers cell death of DTP cells *via* the ferroptosis pathway, indicating ferroptosis is required for the survival of DTP cells[95]. Thus, targeting inherently resistant residual cells could be helpful in reducing relapse in patients. However, more research on the identification and characterization of DTP cells is required to choose the appropriate drug combination for treatment purposes.

Targeted drug delivery is the critical factor in improving treatment outcomes and reducing the drug's side effects. Currently, researchers are investigating nanoparticle-mediated drug delivery. In addition, modified liposomal formulation showed a successful therapeutic response in HCC due to tumor-directed delivery and low drug load in the system[96]. Albumin is also a suitable drug-carrier molecule. An albumin-tagged drug has more potent effects compared to the drug alone[97]. Other materials such as dendrimers, micelles, polysaccharides, and silica are also used as carrier molecules[98-100]. Still, the hunt for an effective delivery system continues for targeted delivery.

CONCLUSION

Existing diagnostic methods are inadequate for the early detection of HCC. Similarly, implemented treatment modalities are unsuccessful in improving the survival of patients and result in cytotoxicity in normal cells. The use of credible biomarkers in the prognosis of HCC is essential to reduce mortality due to the disease. In the future, clinicians should focus on patient stratification based on molecular signatures and decide the treatment strategy to achieve maximum therapy outcome. The development of a combinatorial regime consisting of epi-drugs is urgently needed to treat the tumor mass.

ACKNOWLEDGEMENTS

REFERENCES

1   **Sia D**, Villanueva A, Friedman SL, Llovet JM. Liver Cancer Cell of Origin, Molecular Class, and Effects on Patient Prognosis. *Gastroenterology* 2017; **152**: 745-761 [PMID: 28043904 DOI: 10.1053/j.gastro.2016.11.048]

2   **Bray F**, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; **68**: 394-424 [PMID: 30207593 DOI: 10.3322/caac.21492]

3   **Siegel RL**, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin* 2018; **68**: 7-30 [PMID: 29313949 DOI: 10.3322/caac.21442]

4   **Villanueva A**. Hepatocellular Carcinoma. *N Engl J Med* 2019; **380**: 1450-1462 [PMID: 30970190 DOI: 10.1056/NEJMra1713263]

5   **Llovet JM**, Fuster J, Bruix J; Barcelona-Clínic Liver Cancer Group. The Barcelona approach: diagnosis, staging, and treatment of hepatocellular carcinoma. *Liver Transpl* 2004; **10**: S115-S120 [PMID: 14762851 DOI: 10.1002/Lt.20034]

6   **Farinati F**, Sergio A, Baldan A, Giacomin A, Di Nolfo MA, Del Poggio P, Benvegnu L, Rapaccini G, Zoli M, Borzio F, Giannini EG, Caturelli E, Trevisani F. Early and very early hepatocellular carcinoma: when and how much do staging and choice of treatment really matter? *BMC Cancer* 2009; **9**: 33 [PMID: 19171074 DOI: 10.1186/1471-2407-9-33]

7   **Bruix J**, Sala M, Llovet JM. Chemoembolization for hepatocellular carcinoma. *Gastroenterology* 2004; **127**: S179-S188 [PMID: 15508083 DOI: 10.1053/j.gastro.2004.09.032]

8   **Launay-Vacher V**, Deray G. Hypertension and proteinuria: a class-effect of antiangiogenic therapies. *Anticancer Drugs* 2009; **20**: 81-82 [PMID: 19343005 DOI: 10.1097/CAD.0b013e3283161012]

9   **Cancer Genome Atlas Research Network**. Cancer Genome Atlas Research Network. Comprehensive and Integrative Genomic Characterization of Hepatocellular Carcinoma. *Cell* 2017; **169**: 1327-1341.e23 [PMID: 28622513 DOI: 10.1016/j.cell.2017.05.046]

10   **Walther Z**, Jain D. Molecular pathology of hepatic neoplasms: classification and clinical significance. *Patholog Res Int* 2011; **2011**: 403929 [PMID: 21559202 DOI: 10.4061/2011/403929]

11   **Marquardt JU**, Galle PR, Teufel A. Molecular diagnosis and therapy of hepatocellular carcinoma (HCC): an emerging field for advanced technologies. *J Hepatol* 2012; **56**: 267-275 [PMID: 21782758 DOI: 10.1016/j.jhep.2011.07.007]

12   **Brien GL**, Valerio DG, Armstrong SA. Exploiting the Epigenome to Control Cancer-Promoting Gene-Expression Programs. *Cancer Cell* 2016; **29**: 464-476 [PMID: 27070701 DOI: 10.1016/j.ccell.2016.03.007]

13   **Sharma SV**, Lee DY, Li B, Quinlan MP, Takahashi F, Maheswaran S, McDermott U, Azizian N, Zou L, Fischbach MA, Wong KK, Brandstetter K, Wittner B, Ramaswamy S, Classon M, Settleman J. A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. *Cell* 2010; **141**: 69-80 [PMID: 20371346 DOI: 10.1016/j.cell.2010.02.027]

14   **Dalvi MP**, Wang L, Zhong R, Kollipara RK, Park H, Bayo J, Yenerall P, Zhou Y, Timmons BC, Rodriguez-Canales J, Behrens C, Mino B, Villalobos P, Parra ER, Suraokar M, Pataer A, Swisher SG, Kalhor N, Bhanu NV, Garcia BA, Heymach JV, Coombes K, Xie Y, Girard L, Gazdar AF, Kittler R, Wistuba II, Minna JD, Martinez ED. Taxane-Platin-Resistant Lung Cancers Co-develop Hypersensitivity to JumonjiC Demethylase Inhibitors. *Cell Rep* 2017; **19**: 1669-1684 [PMID: 28538184 DOI: 10.1016/j.celrep.2017.04.077]

15   **European Association for the Study of the Liver**. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. *J Hepatol* 2018; **69**: 182-236 [PMID: 29628281 DOI: 10.1016/j.jhep.2018.03.019]

16   **Merriman RB**, Tran TT. AASLD practice guidelines: The past, the present, and the future. *Hepatology* 2016; **63**: 31-34 [PMID: 26565582 DOI: 10.1002/hep.28345]

17   **Omata M**, Cheng AL, Kokudo N, Kudo M, Lee JM, Jia J, Tateishi R, Han KH, Chawla YK, Shiina S, Jafri W, Payawal DA, Ohki T, Ogasawara S, Chen PJ, Lesmana CRA, Lesmana LA, Gani RA, Obi S, Dokmeci AK, Sarin SK. Asia-Pacific clinical practice guidelines on the management of hepatocellular carcinoma: a 2017 update. *Hepatol Int* 2017; **11**: 317-370 [PMID: 28620797 DOI: 10.1007/s12072-017-9799-9]

18   **European Association for the Study of the Liver**; European Organisation for Research and Treatment of Cancer. EASL-EORTC clinical practice guidelines: management of hepatocellular carcinoma. *J Hepatol* 2012; **56**: 908-943 [PMID: 22424438 DOI: 10.1016/j.jhep.2011.12.001]

19   **Bargellini I**, Battaglia V, Bozzi E, Lauretti DL, Lorenzoni G, Bartolozzi C. Radiological diagnosis of hepatocellular carcinoma. *J Hepatocell Carcinoma* 2014; **1**: 137-148 [PMID: 27508183 DOI: 10.2147/JHC.S44379]

20   **Di Tommaso L**, Spadaccini M, Donadon M, Personeni N, Elamin A, Aghemo A, Lleo A. Role of liver biopsy in hepatocellular carcinoma. *World J Gastroenterol* 2019; **25**: 6041-6052 [PMID: 31686761 DOI: 10.3748/wjg.v25.i40.6041]

21   **Dimitroulis D**, Damaskos C, Valsami S, Davakis S, Garmpis N, Spartalis E, Athanasiou A, Moris

D, Sakellariou S, Kykalos S, Tsourouflis G, Garmpi A, Delladetsima I, Kontzoglou K, Kouraklis G. From diagnosis to treatment of hepatocellular carcinoma: An epidemic problem for both developed and developing world. *World J Gastroenterol* 2017; **23**: 5282-5294 [PMID: 28839428 DOI: 10.3748/wjg.v23.i29.5282]

22   **Galle PR**, Foerster F, Kudo M, Chan SL, Llovet JM, Qin S, Schelman WR, Chintharlapalli S, Abada PB, Sherman M, Zhu AX. Biology and significance of alpha-fetoprotein in hepatocellular carcinoma. *Liver Int* 2019; **39**: 2214-2229 [PMID: 31436873 DOI: 10.1111/liv.14223]

23   **Johnson PJ**. The role of serum alpha-fetoprotein estimation in the diagnosis and management of hepatocellular carcinoma. *Clin Liver Dis* 2001; **5**: 145-159 [PMID: 11218912 DOI: 10.1016/s1089-3261(05)70158-6]

24   **Chan SL**, Mo FK, Johnson PJ, Hui EP, Ma BB, Ho WM, Lam KC, Chan AT, Mok TS, Yeo W. New utility of an old marker: serial alpha-fetoprotein measurement in predicting radiologic response and survival of patients with hepatocellular carcinoma undergoing systemic chemotherapy. *J Clin Oncol* 2009; **27**: 446-452 [PMID: 19064965 DOI: 10.1200/JCO.2008.18.8151]

25   **Merani S**, Majno P, Kneteman NM, Berney T, Morel P, Mentha G, Toso C. The impact of waiting list alpha-fetoprotein changes on the outcome of liver transplant for hepatocellular carcinoma. *J Hepatol* 2011; **55**: 814-819 [PMID: 21334400 DOI: 10.1016/j.jhep.2010.12.040]

26   **Tang Y**, Li K, Cai Z, Xie Y, Tan X, Su C, Li J. HSP90α combined with AFP and TK1 improved the diagnostic value for hepatocellular carcinoma. *Biomark Med* 2020; **14**: 869-878 [PMID: 32490681 DOI: 10.2217/bmm-2019-0484]

27   **Wu M**, Liu Z, Li X, Zhang A, Li N. Dynamic Changes in Serum Markers and Their Utility in the Early Diagnosis of All Stages of Hepatitis B-Associated Hepatocellular Carcinoma. *Onco Targets Ther* 2020; **13**: 827-840 [PMID: 32095079 DOI: 10.2147/OTT.S229835]

28   **Ghosh S**, Bhowmik S, Majumdar S, Goswami A, Chakraborty J, Gupta S, Aggarwal S, Ray S, Chatterjee R, Bhattacharyya S, Dutta M, Datta S, Chowdhury A, Dhali GK, Banerjee S. The exosome encapsulated microRNAs as circulating diagnostic marker for hepatocellular carcinoma with low alpha-fetoprotein. *Int J Cancer* 2020; **147**: 2934-2947 [PMID: 32441313 DOI: 10.1002/ijc.33111]

29   **Fawzy Montaser M**, Amin Sakr M, Omar Khalifa M. Alpha-L-fucosidase as a tumour marker of hepatocellular carcinoma. *Arab J Gastroenterol* 2012; **13**: 9-13 [PMID: 22560818 DOI: 10.1016/j.ajg.2012.03.006]

30   **Hyder MA**, Hasan M, Mohieldein A. Comparative Study of 5'-Nucleotidase Test in Various Liver Diseases. *J Clin Diagn Res* 2016; **10**: BC01-BC03 [PMID: 27042442 DOI: 10.7860/JCDR/2016/12754.7163]

31   **Mikolasevic I**, Filipec-Kanizaj T, Jakopcic I, Majurec I, Brncic-Fischer A, Sobocan N, Hrstic I, Stimac T, Stimac D, Milic S. Liver Disease During Pregnancy: A Challenging Clinical Issue. *Med Sci Monit* 2018; **24**: 4080-4090 [PMID: 29905165 DOI: 10.12659/MSM.907723]

32   **Bruix J**, Sherman M; Practice Guidelines Committee, American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma. *Hepatology* 2005; **42**: 1208-1236 [PMID: 16250051 DOI: 10.1002/hep.20933]

33   **Ishizawa T**, Hasegawa K, Aoki T, Takahashi M, Inoue Y, Sano K, Imamura H, Sugawara Y, Kokudo N, Makuuchi M. Neither multiple tumors nor portal hypertension are surgical contraindications for hepatocellular carcinoma. *Gastroenterology* 2008; **134**: 1908-1916 [PMID: 18549877 DOI: 10.1053/j.gastro.2008.02.091]

34   **Zuo CJ**, Wang PJ, Shao CW, Wang MJ, Tian JM, Xiao Y, Ren FY, Hao XY, Yuan M. CT-guided percutaneous ethanol injection with disposable curved needle for treatment of malignant liver neoplasms and their metastases in retroperitoneal lymph nodes. *World J Gastroenterol* 2004; **10**: 58-61 [PMID: 14695769 DOI: 10.3748/wjg.v10.i1.58]

35   **Sharma A**, Moore WH, Lanuti M, Shepard JA. How I do it: radiofrequency ablation and cryoablation of lung tumors. *J Thorac Imaging* 2011; **26**: 162-174 [PMID: 21508737 DOI: 10.1097/RTI.0b013e3182171b75]

36   **Yu H**, Burke CT. Comparison of percutaneous ablation technologies in the treatment of malignant liver tumors. *Semin Intervent Radiol* 2014; **31**: 129-137 [PMID: 25071303 DOI: 10.1055/s-0034-1373788]

37   **Lencioni R**, Petruzzi P, Crocetti L. Chemoembolization of hepatocellular carcinoma. *Semin Intervent Radiol* 2013; **30**: 3-11 [PMID: 24436512 DOI: 10.1055/s-0033-1333648]

38   **Yeo W**, Mok TS, Zee B, Leung TW, Lai PB, Lau WY, Koh J, Mo FK, Yu SC, Chan AT, Hui P, Ma B, Lam KC, Ho WM, Wong HT, Tang A, Johnson PJ. A randomized phase III study of doxorubicin *vs* cisplatin/interferon alpha-2b/doxorubicin/fluorouracil (PIAF) combination chemotherapy for unresectable hepatocellular carcinoma. *J Natl Cancer Inst* 2005; **97**: 1532-1538 [PMID: 16234567 DOI: 10.1093/jnci/dji315]

39   **Qin S**, Bai Y, Lim HY, Thongprasert S, Chao Y, Fan J, Yang TS, Bhudhisawasdi V, Kang WK, Zhou Y, Lee JH, Sun Y. Randomized, multicenter, open-label study of oxaliplatin plus fluorouracil/Leucovorin *vs* doxorubicin as palliative chemotherapy in patients with advanced hepatocellular carcinoma from Asia. *J Clin Oncol* 2013; **31**: 3501-3508 [PMID: 23980077 DOI: 10.1200/JCO.2012.44.5643]

40   **Lencioni R**, de Baere T, Soulen MC, Rilling WS, Geschwind JF. Lipiodol transarterial chemoembolization for hepatocellular carcinoma: A systematic review of efficacy and safety data. *Hepatology* 2016; **64**: 106-116 [PMID: 26765068 DOI: 10.1002/hep.28453]

41   **Kudo M**, Han G, Finn RS, Poon RT, Blanc JF, Yan L, Yang J, Lu L, Tak WY, Yu X, Lee JH, Lin SM, Wu C, Tanwandee T, Shao G, Walters IB, Dela Cruz C, Poulart V, Wang JH. Brivanib as adjuvant therapy to transarterial chemoembolization in patients with hepatocellular carcinoma: A randomized phase III trial. *Hepatology* 2014; **60**: 1697-1707 [PMID: 24996197 DOI: 10.1002/hep.27290]

42   **Benson AB**, D'Angelica MI, Abbott DE, Abrams TA, Alberts SR, Anaya DA, Anders R, Are C, Brown D, Chang DT, Cloyd J, Covey AM, Hawkins W, Iyer R, Jacob R, Karachristos A, Kelley RK, Kim R, Palta M, Park JO, Sahai V, Schefter T, Sicklick JK, Singh G, Sohal D, Stein S, Tian GG, Vauthey JN, Venook AP, Hammond LJ, Darlow SD. Guidelines Insights: Hepatobiliary Cancers, Version 2.2019. *J Natl Compr Canc Netw* 2019; **17**: 302-310 [PMID: 30959462 DOI: 10.6004/jnccn.2019.0019]

43   **Kudo M**, Finn RS, Qin S, Han KH, Ikeda K, Piscaglia F, Baron A, Park JW, Han G, Jassem J, Blanc JF, Vogel A, Komov D, Evans TRJ, Lopez C, Dutcus C, Guo M, Saito K, Kraljevic S, Tamai T, Ren M, Cheng AL. Lenvatinib *vs* sorafenib in first-line treatment of patients with unresectable hepatocellular carcinoma: a randomised phase 3 non-inferiority trial. *Lancet* 2018; **391**: 1163-1173 [PMID: 29433850 DOI: 10.1016/S0140-6736(18)30207-1]

44   **Cheng AL**, Kang YK, Lin DY, Park JW, Kudo M, Qin S, Chung HC, Song X, Xu J, Poggi G, Omata M, Pitman Lowenthal S, Lanzalone S, Yang L, Lechuga MJ, Raymond E. Sunitinib *vs* sorafenib in advanced hepatocellular cancer: results of a randomized phase III trial. *J Clin Oncol* 2013; **31**: 4067-4075 [PMID: 24081937 DOI: 10.1200/JCO.2012.45.8372]

45   **Park JW**, Kim YJ, Kim DY, Bae SH, Paik SW, Lee YJ, Kim HY, Lee HC, Han SY, Cheong JY, Kwon OS, Yeon JE, Kim BH, Hwang J. Sorafenib with or without concurrent transarterial chemoembolization in patients with advanced hepatocellular carcinoma: The phase III STAH trial. *J Hepatol* 2019; **70**: 684-691 [PMID: 30529387 DOI: 10.1016/j.jhep.2018.11.029]

46   **Zhao Y**, Wang WJ, Guan S, Li HL, Xu RC, Wu JB, Liu JS, Li HP, Bai W, Yin ZX, Fan DM, Zhang ZL, Han GH. Sorafenib combined with transarterial chemoembolization for the treatment of advanced hepatocellular carcinoma: a large-scale multicenter study of 222 patients. *Ann Oncol* 2013; **24**: 1786-1792 [PMID: 23508822 DOI: 10.1093/annonc/mdt072]

47   **Llovet JM**, Decaens T, Raoul JL, Boucher E, Kudo M, Chang C, Kang YK, Assenat E, Lim HY, Boige V, Mathurin P, Fartoux L, Lin DY, Bruix J, Poon RT, Sherman M, Blanc JF, Finn RS, Tak WY, Chao Y, Ezzeddine R, Liu D, Walters I, Park JW. Brivanib in patients with advanced hepatocellular carcinoma who were intolerant to sorafenib or for whom sorafenib failed: results from the randomized phase III BRISK-PS study. *J Clin Oncol* 2013; **31**: 3509-3516 [PMID: 23980090 DOI: 10.1200/JCO.2012.47.3009]

48   **Johnson PJ**, Qin S, Park JW, Poon RT, Raoul JL, Philip PA, Hsu CH, Hu TH, Heo J, Xu J, Lu L, Chao Y, Boucher E, Han KH, Paik SW, Robles-Aviña J, Kudo M, Yan L, Sobhonslidsuk A, Komov D, Decaens T, Tak WY, Jeng LB, Liu D, Ezzeddine R, Walters I, Cheng AL. Brivanib *vs* sorafenib as first-line therapy in patients with unresectable, advanced hepatocellular carcinoma: results from the randomized phase III BRISK-FL study. *J Clin Oncol* 2013; **31**: 3517-3524 [PMID: 23980084 DOI: 10.1200/JCO.2012.48.4410]

49   **Zhu AX**, Stuart K, Blaszkowsky LS, Muzikansky A, Reitberg DP, Clark JW, Enzinger PC, Bhargava P, Meyerhardt JA, Horgan K, Fuchs CS, Ryan DP. Phase 2 study of cetuximab in patients with advanced hepatocellular carcinoma. *Cancer* 2007; **110**: 581-589 [PMID: 17583545 DOI: 10.1002/cncr.22829]

50   **Philip PA**, Mahoney MR, Allmer C, Thomas J, Pitot HC, Kim G, Donehower RC, Fitch T, Picus J, Erlichman C. Phase II study of Erlotinib (OSI-774) in patients with advanced hepatocellular cancer. *J Clin Oncol* 2005; **23**: 6657-6663 [PMID: 16170173 DOI: 10.1200/JCO.2005.14.696]

51   **Boyault S**, Rickman DS, de Reyniès A, Balabaud C, Rebouissou S, Jeannot E, Hérault A, Saric J, Belghiti J, Franco D, Bioulac-Sage P, Laurent-Puig P, Zucman-Rossi J. Transcriptome classification of HCC is related to gene alterations and to new therapeutic targets. *Hepatology* 2007; **45**: 42-52 [PMID: 17187432 DOI: 10.1002/hep.21467]

52   **Hoshida Y**, Nijman SM, Kobayashi M, Chan JA, Brunet JP, Chiang DY, Villanueva A, Newell P, Ikeda K, Hashimoto M, Watanabe G, Gabriel S, Friedman SL, Kumada H, Llovet JM, Golub TR. Integrative transcriptome analysis reveals common molecular subclasses of human hepatocellular carcinoma. *Cancer Res* 2009; **69**: 7385-7392 [PMID: 19723656 DOI: 10.1158/0008-5472.CAN-09-1089]

53   **Murakata A**, Tanaka S, Mogushi K, Yasen M, Noguchi N, Irie T, Kudo A, Nakamura N, Tanaka H, Arii S. Gene expression signature of the gross morphology in hepatocellular carcinoma. *Ann Surg* 2011; **253**: 94-100 [PMID: 21233610 DOI: 10.1097/SLA.0b013e3181f9bc00]

54   **Nishida N**, Arizumi T, Hagiwara S, Ida H, Sakurai T, Kudo M. MicroRNAs for the Prediction of Early Response to Sorafenib Treatment in Human Hepatocellular Carcinoma. *Liver Cancer* 2017; **6**: 113-125 [PMID: 28275578 DOI: 10.1159/000449475]

55   **Orimo T**, Ojima H, Hiraoka N, Saito S, Kosuge T, Kakisaka T, Yokoo H, Nakanishi K, Kamiyama T, Todo S, Hirohashi S, Kondo T. Proteomic profiling reveals the prognostic value of adenomatous polyposis coli-end-binding protein 1 in hepatocellular carcinoma. *Hepatology* 2008; **48**: 1851-1863 [PMID: 18937283 DOI: 10.1002/hep.22552]

56   **Lee JS**, Heo J, Libbrecht L, Chu IS, Kaposi-Novak P, Calvisi DF, Mikaelyan A, Roberts LR, Demetris AJ, Sun Z, Nevens F, Roskams T, Thorgeirsson SS. A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells. *Nat Med* 2006; **12**: 410-416 [PMID:

16532004 DOI: 10.1038/nm1377]

57   **Woo HG**, Lee JH, Yoon JH, Kim CY, Lee HS, Jang JJ, Yi NJ, Suh KS, Lee KU, Park ES, Thorgeirsson SS, Kim YJ. Identification of a cholangiocarcinoma-like gene expression trait in hepatocellular carcinoma. *Cancer Res* 2010; **70**: 3034-3041 [PMID: 20395200 DOI: 10.1158/0008-5472.CAN-09-2823]

58   **Morofuji N**, Ojima H, Hiraoka N, Okusaka T, Esaki M, Nara S, Shimada K, Kishi Y, Kondo T. Antibody-based proteomics to identify an apoptosis signature for early recurrence of hepatocellular carcinoma. *Clin Proteomics* 2016; **13**: 28 [PMID: 27799868 DOI: 10.1186/s12014-016-9130-0]

59   **Jiang Y**, Sun A, Zhao Y, Ying W, Sun H, Yang X, Xing B, Sun W, Ren L, Hu B, Li C, Zhang L, Qin G, Zhang M, Chen N, Huang Y, Zhou J, Liu M, Zhu X, Qiu Y, Sun Y, Huang C, Yan M, Wang M, Liu W, Tian F, Xu H, Wu Z, Shi T, Zhu W, Qin J, Xie L, Fan J, Qian X, He F; Chinese Human Proteome Project (CNHPP) Consortium. Proteomics identifies new therapeutic targets of early-stage hepatocellular carcinoma. *Nature* 2019; **567**: 257-261 [PMID: 30814741 DOI: 10.1038/s41586-019-0987-8]

60   **Gao Q**, Zhu H, Dong L, Shi W, Chen R, Song Z, Huang C, Li J, Dong X, Zhou Y, Liu Q, Ma L, Wang X, Zhou J, Liu Y, Boja E, Robles AI, Ma W, Wang P, Li Y, Ding L, Wen B, Zhang B, Rodriguez H, Gao D, Zhou H, Fan J. Integrated Proteogenomic Characterization of HBV-Related Hepatocellular Carcinoma. *Cell* 2019; **179**: 561-577.e22 [PMID: 31585088 DOI: 10.1016/j.cell.2019.08.052]

61   **Chaudhary K**, Poirion OB, Lu L, Garmire LX. Deep Learning-Based Multi-Omics Integration Robustly Predicts Survival in Liver Cancer. *Clin Cancer Res* 2018; **24**: 1248-1259 [PMID: 28982688 DOI: 10.1158/1078-0432.CCR-17-0853]

62   **Schulze K**, Imbeaud S, Letouzé E, Alexandrov LB, Calderaro J, Rebouissou S, Couchy G, Meiller C, Shinde J, Soysouvanh F, Calatayud AL, Pinyol R, Pelletier L, Balabaud C, Laurent A, Blanc JF, Mazzaferro V, Calvo F, Villanueva A, Nault JC, Bioulac-Sage P, Stratton MR, Llovet JM, Zucman-Rossi J. Exome sequencing of hepatocellular carcinomas identifies new mutational signatures and potential therapeutic targets. *Nat Genet* 2015; **47**: 505-511 [PMID: 25822088 DOI: 10.1038/ng.3252]

63   **Sharma S**, Kelly TK, Jones PA. Epigenetics in cancer. *Carcinogenesis* 2010; **31**: 27-36 [PMID: 19752007 DOI: 10.1093/carcin/bgp220]

64   **Herceg Z**, Hainaut P. Genetic and epigenetic alterations as biomarkers for cancer detection, diagnosis and prognosis. *Mol Oncol* 2007; **1**: 26-41 [PMID: 19383285 DOI: 10.1016/j.molonc.2007.01.004]

65   **Shah MA**, Denton EL, Arrowsmith CH, Lupien M, Schapira M. A global assessment of cancer genomic alterations in epigenetic mechanisms. *Epigenetics Chromatin* 2014; **7**: 29 [PMID: 25484917 DOI: 10.1186/1756-8935-7-29]

66   **Weinhold B**. Epigenetics: the science of change. *Environ Health Perspect* 2006; **114**: A160-A167 [PMID: 16507447 DOI: 10.1289/ehp.114-a160]

67   **Kanherkar RR**, Bhatia-Dey N, Csoka AB. Epigenetics across the human lifespan. *Front Cell Dev Biol* 2014; **2**: 49 [PMID: 25364756 DOI: 10.3389/fcell.2014.00049]

68   **Srinageshwar B**, Maiti P, Dunbar GL, Rossignol J. Role of Epigenetics in Stem Cell Proliferation and Differentiation: Implications for Treating Neurodegenerative Diseases. *Int J Mol Sci* 2016; **17** [PMID: 26848657 DOI: 10.3390/ijms17020199]

69   **Moore LD**, Le T, Fan G. DNA methylation and its basic function. *Neuropsychopharmacology* 2013; **38**: 23-38 [PMID: 22781841 DOI: 10.1038/npp.2012.112]

70   **Gillette TG**, Hill JA. Readers, writers, and erasers: chromatin as the whiteboard of heart disease. *Circ Res* 2015; **116**: 1245-1253 [PMID: 25814685 DOI: 10.1161/CIRCRESAHA.116.303630]

71   **Greer EL**, Shi Y. Histone methylation: a dynamic mark in health, disease and inheritance. *Nat Rev Genet* 2012; **13**: 343-357 [PMID: 22473383 DOI: 10.1038/nrg3173]

72   **Bannister AJ**, Kouzarides T. Regulation of chromatin by histone modifications. *Cell Res* 2011; **21**: 381-395 [PMID: 21321607 DOI: 10.1038/cr.2011.22]

73   **Shimada Y**, Mohn F, Bühler M. The RNA-induced transcriptional silencing complex targets chromatin exclusively *via* interacting with nascent transcripts. *Genes Dev* 2016; **30**: 2571-2580 [PMID: 27941123 DOI: 10.1101/gad.292599.116]

74   **Lei ZJ**, Wang J, Xiao HL, Guo Y, Wang T, Li Q, Liu L, Luo X, Fan LL, Lin L, Mao CY, Wang SN, Wei YL, Lan CH, Jiang J, Yang XJ, Liu PD, Chen DF, Wang B. Lysine-specific demethylase 1 promotes the stemness and chemoresistance of Lgr5+ liver cancer initiating cells by suppressing negative regulators of β-catenin signaling. *Oncogene* 2015; **34**: 3214 [PMID: 26062983 DOI: 10.1038/onc.2015.182]

75   **Nio K**, Yamashita T, Okada H, Kondo M, Hayashi T, Hara Y, Nomura Y, Zeng SS, Yoshida M, Sunagozaka H, Oishi N, Honda M, Kaneko S. Defeating EpCAM(+) liver cancer stem cells by targeting chromatin remodeling enzyme CHD4 in human hepatocellular carcinoma. *J Hepatol* 2015; **63**: 1164-1172 [PMID: 26095183 DOI: 10.1016/j.jhep.2015.06.009]

76   **Lin Q**, Wu Z, Yue X, Yu X, Wang Z, Song X, Xu L, He Y, Ge Y, Tan S, Wang T, Song H, Yuan D, Gong Y, Gao L, Liang X, Ma C. ZHX2 restricts hepatocellular carcinoma by suppressing stem cell-like traits through KDM2A-mediated H3K36 demethylation. *EBioMedicine* 2020; **53**: 102676 [PMID: 32114388 DOI: 10.1016/j.ebiom.2020.102676]

77   **Lo Re O**, Mazza T, Giallongo S, Sanna P, Rappa F, Vinh Luong T, Li Volti G, Drovakova A, Roskams T, Van Haele M, Tsochatzis E, Vinciguerra M. Loss of histone macroH2A1 in

hepatocellular carcinoma cells promotes paracrine-mediated chemoresistance and CD4+CD25+FoxP3+ regulatory T cells activation. *Theranostics* 2020; **10**: 910-924 [PMID: 31903159 DOI: 10.7150/thno.35045]

78 **Chen Q**, Jiang P, Jia B, Liu Y, Zhang Z. RCC2 contributes to tumor invasion and chemoresistance to cisplatin in hepatocellular carcinoma. *Hum Cell* 2020; **33**: 709-720 [PMID: 32239438 DOI: 10.1007/s13577-020-00353-7]

79 **Ling S**, Shan Q, Zhan Q, Ye Q, Liu P, Xu S, He X, Ma J, Xiang J, Jiang G, Wen X, Feng Z, Wu Y, Feng T, Xu L, Chen K, Zhang X, Wei R, Zhang C, Cen B, Xie H, Song P, Liu J, Zheng S, Xu X. USP22 promotes hypoxia-induced hepatocellular carcinoma stemness by a HIF1α/USP22 positive feedback loop upon TP53 inactivation. *Gut* 2020; **69**: 1322-1334 [PMID: 31776228 DOI: 10.1136/gutjnl-2019-319616]

80 **Tak E**, Lee S, Lee J, Rashid MA, Kim YW, Park JH, Park WS, Shokat KM, Ha J, Kim SS. Human carbonyl reductase 1 upregulated by hypoxia renders resistance to apoptosis in hepatocellular carcinoma cells. *J Hepatol* 2011; **54**: 328-339 [PMID: 21056497 DOI: 10.1016/j.jhep.2010.06.045]

81 **Schultheiss CS**, Laggai S, Czepukojc B, Hussein UK, List M, Barghash A, Tierling S, Hosseini K, Golob-Schwarzl N, Pokorny J, Hachenthal N, Schulz M, Helms V, Walter J, Zimmer V, Lammert F, Bohle RM, Dandolo L, Haybaeck J, Kiemer AK, Kessler SM. The long non-coding RNA *H19* suppresses carcinogenesis and chemoresistance in hepatocellular carcinoma. *Cell Stress* 2017; **1**: 37-54 [PMID: 31225433 DOI: 10.15698/cst2017.10.105]

82 **Xie SC**, Zhang JQ, Jiang XL, Hua YY, Xie SW, Qin YA, Yang YJ. LncRNA CRNDE facilitates epigenetic suppression of CELF2 and LATS2 to promote proliferation, migration and chemoresistance in hepatocellular carcinoma. *Cell Death Dis* 2020; **11**: 676 [PMID: 32826865 DOI: 10.1038/s41419-020-02853-8]

83 **Berdasco M**, Esteller M. Clinical epigenetics: seizing opportunities for translation. *Nat Rev Genet* 2019; **20**: 109-127 [PMID: 30479381 DOI: 10.1038/s41576-018-0074-2]

84 **Italiano A**, Soria JC, Toulmonde M, Michot JM, Lucchesi C, Varga A, Coindre JM, Blakemore SJ, Clawson A, Suttle B, McDonald AA, Woodruff M, Ribich S, Hedrick E, Keilhack H, Thomson B, Owa T, Copeland RA, Ho PTC, Ribrag V. Tazemetostat, an EZH2 inhibitor, in relapsed or refractory B-cell non-Hodgkin lymphoma and advanced solid tumours: a first-in-human, open-label, phase 1 study. *Lancet Oncol* 2018; **19**: 649-659 [PMID: 29650362 DOI: 10.1016/S1470-2045(18)30145-1]

85 **Li J**, Hao D, Wang L, Wang H, Wang Y, Zhao Z, Li P, Deng C, Di LJ. Epigenetic targeting drugs potentiate chemotherapeutic effects in solid tumor therapy. *Sci Rep* 2017; **7**: 4035 [PMID: 28642588 DOI: 10.1038/s41598-017-04406-0]

86 **Amnekar RV**, Khan SA, Rashid M, Khade B, Thorat R, Gera P, Shrikhande SV, Smoot DT, Ashktorab H, Gupta S. Histone deacetylase inhibitor pre-treatment enhances the efficacy of DNA-interacting chemotherapeutic drugs in gastric cancer. *World J Gastroenterol* 2020; **26**: 598-613 [PMID: 32103870 DOI: 10.3748/wjg.v26.i6.598]

87 **Yeo W**, Chung HC, Chan SL, Wang LZ, Lim R, Picus J, Boyer M, Mo FK, Koh J, Rha SY, Hui EP, Jeung HC, Roh JK, Yu SC, To KF, Tao Q, Ma BB, Chan AW, Tong JH, Erlichman C, Chan AT, Goh BC. Epigenetic therapy using belinostat for patients with unresectable hepatocellular carcinoma: a multicenter phase I/II study with biomarker and pharmacokinetic analysis of tumors from patients in the Mayo Phase II Consortium and the Cancer Therapeutics Research Group. *J Clin Oncol* 2012; **30**: 3361-3367 [PMID: 22915658 DOI: 10.1200/JCO.2011.41.2395]

88 **Lachenmayer A**, Toffanin S, Cabellos L, Alsinet C, Hoshida Y, Villanueva A, Minguez B, Tsai HW, Ward SC, Thung S, Friedman SL, Llovet JM. Combination therapy for hepatocellular carcinoma: additive preclinical efficacy of the HDAC inhibitor panobinostat with sorafenib. *J Hepatol* 2012; **56**: 1343-1350 [PMID: 22322234 DOI: 10.1016/j.jhep.2012.01.009]

89 **Sanaei M**, Kavoosi F, Esmi Z. The Effect of 5-Aza-2'-Deoxycytidine in Combination to and in Comparison with Vorinostat on DNA Methyltransferases, Histone Deacetylase 1, Glutathione S-Transferase 1 and Suppressor of Cytokine Signaling 1 Genes Expression, Cell Growth Inhibition and Apoptotic Induction in Hepatocellular LCL-PI 11 Cell Line. *Int J Hematol Oncol Stem Cell Res* 2020; **14**: 45-55 [PMID: 32337014]

90 **Mehta N**, Heimbach J, Harnois DM, Sapisochin G, Dodge JL, Lee D, Burns JM, Sanchez W, Greig PD, Grant DR, Roberts JP, Yao FY. Validation of a Risk Estimation of Tumor Recurrence After Transplant (RETREAT) Score for Hepatocellular Carcinoma Recurrence After Liver Transplant. *JAMA Oncol* 2017; **3**: 493-500 [PMID: 27838698 DOI: 10.1001/jamaoncol.2016.5116]

91 **Ravindran Menon D**, Das S, Krepler C, Vultur A, Rinner B, Schauer S, Kashofer K, Wagner K, Zhang G, Bonyadi Rad E, Haass NK, Soyer HP, Gabrielli B, Somasundaram R, Hoefler G, Herlyn M, Schaider H. A stress-induced early innate response causes multidrug tolerance in melanoma. *Oncogene* 2015; **34**: 4448-4459 [PMID: 25417704 DOI: 10.1038/onc.2014.372]

92 **Hata AN**, Niederst MJ, Archibald HL, Gomez-Caraballo M, Siddiqui FM, Mulvey HE, Maruvka YE, Ji F, Bhang HE, Krishnamurthy Radhakrishna V, Siravegna G, Hu H, Raoof S, Lockerman E, Kalsy A, Lee D, Keating CL, Ruddy DA, Damon LJ, Crystal AS, Costa C, Piotrowska Z, Bardelli A, Iafrate AJ, Sadreyev RI, Stegmeier F, Getz G, Sequist LV, Faber AC, Engelman JA. Tumor cells can follow distinct evolutionary paths to become resistant to epidermal growth factor receptor inhibition. *Nat Med* 2016; **22**: 262-269 [PMID: 26828195 DOI: 10.1038/nm.4040]

93 **Ramirez M**, Rajaram S, Steininger RJ, Osipchuk D, Roth MA, Morinishi LS, Evans L, Ji W, Hsu CH, Thurley K, Wei S, Zhou A, Koduru PR, Posner BA, Wu LF, Altschuler SJ. Diverse drug-resistance mechanisms can emerge from drug-tolerant cancer persister cells. *Nat Commun* 2016; **7**:

10690 [PMID: 26891683 DOI: 10.1038/ncomms10690]

94   **Guler GD**, Tindell CA, Pitti R, Wilson C, Nichols K, KaiWai Cheung T, Kim HJ, Wongchenko M, Yan Y, Haley B, Cuellar T, Webster J, Alag N, Hegde G, Jackson E, Nance TL, Giresi PG, Chen KB, Liu J, Jhunjhunwala S, Settleman J, Stephan JP, Arnott D, Classon M. Repression of Stress-Induced LINE-1 Expression Protects Cancer Cell Subpopulations from Lethal Drug Exposure. *Cancer Cell* 2017; **32**: 221-237.e13 [PMID: 28781121 DOI: 10.1016/j.ccell.2017.07.002]

95   **Hangauer MJ**, Viswanathan VS, Ryan MJ, Bole D, Eaton JK, Matov A, Galeas J, Dhruv HD, Berens ME, Schreiber SL, McCormick F, McManus MT. Drug-tolerant persister cancer cells are vulnerable to GPX4 inhibition. *Nature* 2017; **551**: 247-250 [PMID: 29088702 DOI: 10.1038/nature24297]

96   **Shah SM**, Goel PN, Jain AS, Pathak PO, Padhye SG, Govindarajan S, Ghosh SS, Chaudhari PR, Gude RP, Gopal V, Nagarsenker MS. Liposomes for targeting hepatocellular carcinoma: use of conjugated arabinogalactan as targeting ligand. *Int J Pharm* 2014; **477**: 128-139 [PMID: 25311181 DOI: 10.1016/j.ijpharm.2014.10.014]

97   **Zhou Q**, Ching AK, Leung WK, Szeto CY, Ho SM, Chan PK, Yuan YF, Lai PB, Yeo W, Wong N. Novel therapeutic potential in targeting microtubules by nanoparticle albumin-bound paclitaxel in hepatocellular carcinoma. *Int J Oncol* 2011; **38**: 721-731 [PMID: 21240457 DOI: 10.3892/ijo.2011.902]

98   **Madaan K**, Kumar S, Poonia N, Lather V, Pandita D. Dendrimers in drug delivery and targeting: Drug-dendrimer interactions and toxicity issues. *J Pharm Bioallied Sci* 2014; **6**: 139-150 [PMID: 25035633 DOI: 10.4103/0975-7406.130965]

99   **Zhang N**, Wardwell PR, Bader RA. Polysaccharide-based micelles for drug delivery. *Pharmaceutics* 2013; **5**: 329-352 [PMID: 24300453 DOI: 10.3390/pharmaceutics5020329]

100   **Bharti C**, Nagaich U, Pal AK, Gulati N. Mesoporous silica nanoparticles in target drug delivery system: A review. *Int J Pharm Investig* 2015; **5**: 124-133 [PMID: 26258053 DOI: 10.4103/2230-973X.160844]

101   **Maida M**, Orlando E, Cammà C, Cabibbo G. Staging systems of hepatocellular carcinoma: a review of literature. *World J Gastroenterol* 2014; **20**: 4141-4150 [PMID: 24764652 DOI: 10.3748/wjg.v20.i15.4141]

102   **Bruix J**, Sherman M; American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma: an update. *Hepatology* 2011; **53**: 1020-1022 [PMID: 21374666 DOI: 10.1002/hep.24199]

103   **Chou R**, Cuevas C, Fu R, Devine B, Wasson N, Ginsburg A, Zakher B, Pappas M, Graham E, Sullivan SD. Imaging Techniques for the Diagnosis of Hepatocellular Carcinoma: A Systematic Review and Meta-analysis. *Ann Intern Med* 2015; **162**: 697-711 [PMID: 25984845 DOI: 10.7326/M14-2509]

104   **Sørensen M**, Frisch K, Bender D, Keiding S. The potential use of 2-[¹⁸F]fluoro-2-deoxy-D-galactose as a PET/CT tracer for detection of hepatocellular carcinoma. *Eur J Nucl Med Mol Imaging* 2011; **38**: 1723-1731 [PMID: 21553087 DOI: 10.1007/s00259-011-1831-z]

105   **Bialecki ES**, Di Bisceglie AM. Diagnosis of hepatocellular carcinoma. *HPB (Oxford)* 2005; **7**: 26-34 [PMID: 18333158 DOI: 10.1080/13651820410024049]

106   **Lok AS**, Sterling RK, Everhart JE, Wright EC, Hoefs JC, Di Bisceglie AM, Morgan TR, Kim HY, Lee WM, Bonkovsky HL, Dienstag JL; HALT-C Trial Group. Des-gamma-carboxy prothrombin and alpha-fetoprotein as biomarkers for the early detection of hepatocellular carcinoma. *Gastroenterology* 2010; **138**: 493-502 [PMID: 19852963 DOI: 10.1053/j.gastro.2009.10.031]

107   **El-Saadany S**, El-Demerdash T, Helmy A, Mayah WW, El-Sayed Hussein B, Hassanien M, Elmashad N, Fouad MA, Basha EA.   Diagnostic Value of Glypican-3 for Hepatocellular Carcinomas Asian Pac J Cancer Prev 2018; 19: 811-817 [PMID: 29582639 DOI: 10.22034/APJCP.2018.19.3.811]

108   **Elhendawy M**, Abdul-Baki EA, Abd-Elsalam S, Hagras MM, Zidan AA, Abdel-Naby AY, Watny M, Elkabash IA, Salem ML, Elshanshoury M, Soliman S, Abdou S. MicroRNA signature in hepatocellular carcinoma patients: identification of potential markers. *Mol Biol Rep* 2020; **47**: 4945-4953 [PMID: 32430845 DOI: 10.1007/s11033-020-05521-4]

109   **Abdalla MA**, Haj-Ahmad Y. Promising Candidate Urinary MicroRNA Biomarkers for the Early Detection of Hepatocellular Carcinoma among High-Risk Hepatitis C Virus Egyptian Patients. *J Cancer* 2012; **3**: 19-31 [PMID: 22211142 DOI: 10.7150/jca.3.19]

REVIEW

Heterogeneity of non-alcoholic fatty liver disease: Implications for clinical practice and research activity

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is a heterogeneous condition with a wide spectrum of clinical presentations and natural history and disease severity. There is also substantial inter-individual variation and variable response to a different therapy. This heterogeneity of NAFLD is in turn influenced by various factors primarily demographic/dietary factors, metabolic status, gut microbiome, genetic predisposition together with epigenetic factors. The differential impact of these factors over a variable period of time influences the clinical phenotype and natural history. Failure to address heterogeneity partly explains the sub-optimal response to current and emerging therapies for fatty liver disease. Consequently, leading experts across the globe have recently suggested a change in nomenclature of NAFLD to metabolic-associated fatty liver disease (MAFLD) which can better reflect current knowledge of heterogeneity and does not exclude concomitant factors for fatty liver disease (*e.g.* alcohol, viral hepatitis, *etc.*). Precise identification of disease phenotypes is likely to facilitate clinical trial recruitment and expedite translational research for the development of novel and effective therapies for NAFLD/MAFLD.

Key Words: Non-alcoholic fatty liver disease; Metabolic-associated fatty liver disease; Heterogeneity; Phenotypes; nomenclature; Clinical trial; Effective therapies

Core Tip: It is being increasingly recognized that non-alcoholic fatty liver disease (NAFLD) is a heterogenous condition with wide variability in clinical presentation and natural history. This heterogeneity is driven by genetic predisposition, metabolic factors, gut microbiota, diet and demographic factors. The suboptimal response to current pharmacotherapy in NAFLD highlights the failure to recognize this heterogeneity. Experts believe that updating NAFLD nomenclature is the first step towards this. Identification of disease subtypes can help development of preclinical model evaluating novel targets. This would in turn help clinical trial design by comparing and pooling results and thus improve disease outcomes.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is increasing in both developed and developing countries, in parallel with the global obesity epidemic. Nevertheless, much is still unknown on the NAFLD phenotype. Moreover, since the term NAFLD was coined by Ludwig *et al*[1] in 1980, the nomenclature and diagnostic criteria have not been revisited. With a deeper understanding of the natural history of NAFLD, it has become gradually more obvious that this term is inherently complicated, chiefly due to the heterogeneity of NAFLD and principal driving factors between individuals. This heterogeneity in clinical presentation and the course of NAFLD is probably influenced by several factors which include age, gender, ethnicity, diet, alcohol consumption, genetic predisposition, microbiota, and metabolic milieu[2].The combined effect of the dynamic and complex systems-level interactions of these drivers is probably reflected in the phenotypic manifestations of NAFLD. Therefore, comprehensive phenotyping will translate into individual-level risk prediction and preventive strategies, and improvements in the design of clinical trials[2]. The heterogeneity of NAFLD and the presence of multiple pathophysiological pathways intrinsic to its progression suggest that the nomenclature should be revised and NAFLD may be classified in a way that takes into account the various underlying processes[3]. However, a change of name of any disease has considerable implications for both clinical practice as well as public health policy. Based on these evolving paradigms, this review will explore the factors contributing to NAFLD heterogeneity and its clinical and therapeutic implications. Besides, proposed changes in the current nomenclature and definition of NAFLD are discussed along with future perspectives.

HETEROGENEITY OF NAFLD: NEED FOR A NEW TERMINOLOGY

NAFLD represents an umbrella term with considerable heterogeneity among its subtypes. This is evidenced by variable disease severity and progression (disease phenotype) among patients with NAFLD[4]. The disease phenotype in NAFLD is in turn influenced by primary drivers of the disease and dynamic interaction between various disease modifiers (age, sex, ethnicity, co-existing disease, diet, alcohol consumption, smoking, hormonal status, genetic and epigenetic factors, gut microbiota, and metabolic risk factors)[2]. Although steatosis is highly prevalent, progression to steatohepatitis or other liver-related complications like cirrhosis and hepatocellular carcinoma (HCC) is highly unpredictable. The rate of fibrosis progression can also vary widely among patients. Moreover, there is growing evidence that HCC can develop in NAFLD without cirrhosis[5].

The suboptimal response rates of current investigational therapies (20%-40%) reflect a lack of consideration of heterogeneity of NAFLD[2,6]. Hence, a structured dissection of the key pathogenetic pathway and precise disease sub-typing based on genetic background, metabolic profile and anthropometric parameters shall help predict individualized risk and provide effective treatment[2]. The term NAFLD was coined in

1980 by Ludwig *et al*[1] and it was used to describe fatty liver disease without a history of significant alcohol intake. Although the prevalence of NAFLD has grown to epidemic proportions involving one-fourth of the population, the nomenclature and the diagnostic criteria have not been reevaluated[2]. The term NAFLD does not consider the heterogeneity of the disease and hence does not reflect current knowledge.

Based on recent epidemiological studies, it has been increasingly recognized that there is no cut-off for safe drinking in so-called NAFLD as there is frequent co-existence of at-risk drinking and dysmetabolism[7]. Moreover, accurate assessment of alcohol intake is often challenging especially in subpopulations like children and women due to cultural interdiction[8]. To further confuse the issue, there is evidence that an altered gut microbiome can lead to excess production of endogenous alcohol in non-drinkers[9]. Hence, the dichotomy between alcoholic liver disease and NAFLD should be abandoned. Until now, diagnosis of NAFLD was based on the exclusion of excess alcohol intake, concomitant viral hepatitis/other liver diseases, and secondary cause of fatty liver (*e.g.* drug-induced). With the increasing prevalence of NAFLD and the high prevalence of other liver diseases such as viral hepatitis particularly in countries like Middle East and north Africa, dual causes of liver disease should be considered[8]. The current definition of metabolic-associated fatty liver disease (MAFLD) does not require the exclusion of the above, considering the co-existence of different pathology for fatty liver disease (Figure 1). However, it requires the presence of overweight/obesity, type 2 diabetes mellitus (T2DM), or 2 metabolic risk factors. The term "non" in "nonalcoholic fatty liver disease" trivializes a disease that has major hepatic, cardiovascular (CV), and oncological sequelae[2,10]. Due to the "non"-rubric, it could be misinterpreted as something not serious and even encourage alcohol consumption. The term "alcohol" makes the nomenclature derogatory and thus stigmatizing the condition blaming the patient for their condition[2]. This has profound implications on recognition of the disease as a major public health problem and resource allocation by regulatory authorities to intercept this potentially deadly disease.

Due to the aforementioned reasons, the term MAFLD was proposed by Lonardo and Carulli 16 years back[11]. However, NAFLD nomenclature remained unchanged until now. For the same reasons, Polyzos and Mantzoros[12] have proposed the term dysmetabolism associated fatty liver disease (DAFLD). Recently two consensus guidelines have proposed a change in the nomenclature of NAFLD to MAFLD and have redefined the condition based on the presence of hepatic steatosis and metabolic risk factors[2,13] (Figure 2). The impact of such change was reflected in the identi-fication of patients with hepatic steatosis with a higher risk of disease progression in a cross-sectional study of more than 13000 patients based on data from the third National Health and Nutrition Examination Surveys of the United States[14]. Another study from Hong Kong has shown that MAFLD definition reduces the incidence of fatty liver disease by 25% [more so in patients with low body mass index (BMI)], while the prevalence remains unchanged. Patients with a fatty liver disease not fulfilling the criteria of MAFLD were unlikely to have significant liver disease.

However, the future implications of change in the nomenclature are still unknown. Hence, Younossi *et al*[15], on behalf of the American Association for the Study of Liver Disease[15] have cautioned about the impact of premature change in terminology to MAFLD. While there are still existing challenges in widespread disease awareness, identification of treatment endpoints, and biomarkers for risk stratification, changing terminology may negatively impact the field[15]. Moreover, international consensus involving all scientific societies, regulatory bodies, pharmacological industry, and patient organizations is required before a change in terminology. No matter what is the terminology for fatty liver disease, it is clear that it is a heterogeneous disease with varying manifestations.

NAFLD AND CARDIOVASCULAR RISK

Patients with NAFLD are more likely to have morbidity and mortality from cardiovascular disease (CVD). Currently proposed term MAFLD is closely linked to DM, dyslipidemia, hypertension, systemic inflammation which are known to increase CVD risk. A higher risk of CVD and CVD associated events have been noted in epidemiological and observational studies in NAFLD[16,17]. NAFLD not only damages the coronary arteries (atherosclerosis and ischemic heart disease), but also the other cardiac structures like myocardium (heart failure), cardiac valves (aortic stenosis,
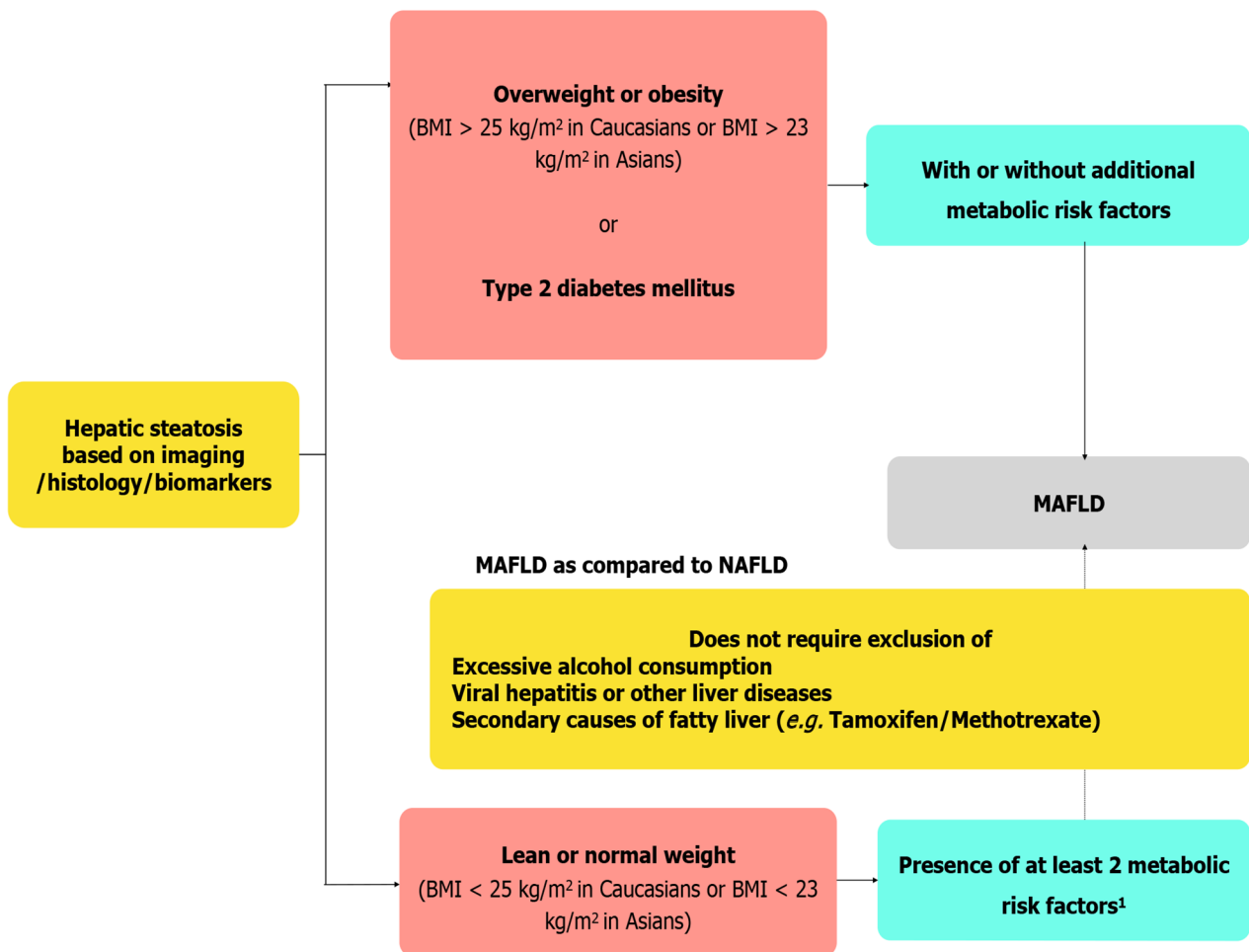
Figure 1 Proposed diagnostic criteria of metabolic associated fatty liver disease and key differences with non-alcoholic fatty liver disease definition. [1]Metabolic risk factors include (1) Waist circumference ≥ 102/88 cm in Caucasian men and women (≥ 90/80 cm for Asian men and women); (2) Blood pressure ≥ 130/85 mmHg or on drug treatment; (3) Triglyceride levels ≥ 150 mg/dL (≥ 1.70 mmol/L) or on drug treatment; (4) Plasma high density lipoprotein [HDL < 40 mg/dL (< 1.0 mmol/L) for men and < 50 mg/dL (< 1.3 mmol/L)] for women or on drug treatment; (5) Pre-diabetes [*i.e.*, fasting glucose levels 100 to 125 mg/dL (5.6 to 6.9 mmol/L), or 2-h post-load glucose levels 140 to 199 mg/dL (7.8 to 11.0 mmoL) or HbA1c 5.7% to 6.4% (39 to 47 mmol/moL)]; (6) Homeostasis model assessment of insulin resistance score ≥ 2.5; and (7) Plasma high-sensitivity C-reactive protein level > 2 mg/L. BMI: Body mass index; MAFLD: Metabolic-associated fatty liver disease; NAFLD: Non-alcoholic fatty liver disease.

mitral annular calcification), and conduction system (atrial fibrillation, conduction defects)[18]. CV disease in NAFLD can be subclinical (coronary and courted athero-sclerosis) or clinical (myocardial infarction, stroke). Pathophysiological factors include dyslipidemia, oxidative stress, systemic inflammation, endothelial dysfunction, and a pro-thrombotic state leading to structural and functional cardiac changes including arterial stiffness, atherogenic plaque formation, and coronary calcification[19]. Among genetic factors related to NAFLD, MBOAT7 may promote venous thromboembolism whereas Transmembrane 6 superfamily 2 (TM6SF2) appears to be protective and PNPLA3 seems not to be associated with the risk of CVD. Other pathogenetic mechanisms of NAFLD such as environmental factors (diet, obesity, *etc.*), gut micro-biota (through the gut liver axis and altered intestinal permeability), and epigenetic alterations also influence the CV risk[16].

Lifestyle modification and weight loss help in primary and secondary prevention of CVD in NAFLD. Aspirin and statins may be considered for primary and secondary prevention in individuals with NAFLD who are at high risk of CVD. Newer anti-diabetic medications such as SGLT2 inhibitors and GLP-1 receptor agonists are known to reduce CV events in T2DM and may be useful in this regard. Additional data are required on CV risk modification by farnesoid X receptor (FXR) agonists such as obeticholic acid. Future studies will likely address the predictive factors responsible for elevated CVD risk in NAFLD as there is a lack of targeted pharmacological therapy. Hence, CV endpoints should be included in clinical trials in NAFLD/MAFLD [16,19].
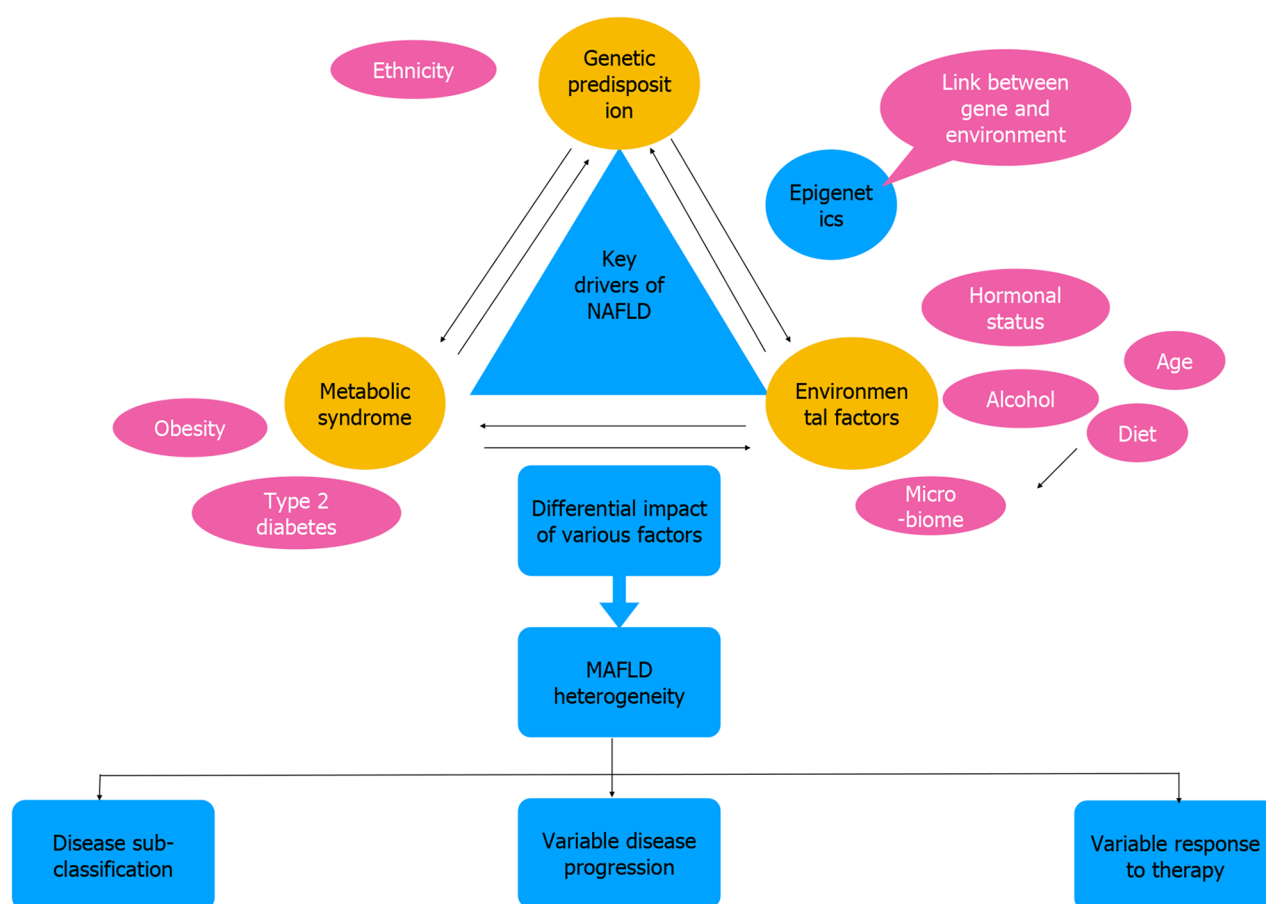
Figure 2 Key drivers of metabolic-associated fatty liver disease, resulting in disease heterogeneity and its clinical implications. Genetic predisposition, metabolic health, and environmental factors influence molecular and phenotypical heterogeneity of metabolic-associated fatty liver disease leading to various disease subtypes, variable disease progression, and response to therapy. MAFLD: Metabolic-associated fatty liver disease; NAFLD: Non-alcoholic fatty liver disease.

FACTORS FOR HETEROGENEITY

Age

The prevalence, risk of hepatic/extra-hepatic complications, and all-cause mortality of NAFLD increase with age. This is due to multiple factors like reduction in hepatic blood flow/volume, decrease in bile acid synthesis, altered cholesterol metabolism, increase in oxidative respiration due to decrease in mitochondria numbers, cellular aging, increased exposure to disease drivers over a prolonged period, and progressive increase in insulin resistance (IR) due to change in body composition (sarcopenia, abdominal and visceral adiposity with ectopic fat deposition)[20-23].

Gender and menopause effect

The prevalence of NAFLD and degree of hepatic fibrosis are lower in pre-menopausal women compared to men and postmenopausal women with better overall survival rates in the former[24]. Changes in body fat distribution (abdominal obesity after menopause), differences in metabolic risk factors, sexual dimorphism of key metabolic pathways (lipid metabolism, insulin signaling, and inflammation), and differences in hepatic gene expression of various metabolic pathways (*e.g.* FXR, liver X receptor) are likely mechanisms for the difference[25-27]. The prevalence of NAFLD and fibrosis risk is lower in postmenopausal women on hormone replacement therapy (HRT) compared to those who are not on HRT[28]. The extent of hepatic fibrosis increases with the prolonged duration of estrogen deficiency in postmenopausal women[29]. Hence, risk stratification in NAFLD should be based on gender and menopausal status.

Ethnicity

The prevalence of NAFLD and risk of nonalcoholic steatohepatitis (NASH) are seen in

decreasing order of frequency in Hispanics, non-Hispanic whites, and African Americans[30]. It is important to note that the risk of fibrosis did not vary based on ethnicity. The plausible explanations for such racial disparity are differences in genetic predisposition, metabolic traits (IR and body fat distribution), environmental factors (dietary habits like increased carbohydrate consumption, physical inactivity, and cultural factors). For example, the frequency of risk alleles of Patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) gene in Hispanics, non-Hispanic whites, and African-Americans are 49%, 23%, and 17% respectively[31]. Importantly, Asian individuals tend to accumulate liver fat at lower BMI, have a higher degree of inflammation, and have a possibly higher risk of fibrosis compared to other ethnicities[32, 33]. PNPLA3 rs738409 risk allele frequency is more common in East Asians compared to Caucasians[34].

Diet and gut microbiota

It is well known that a Western diet with high fat and fruit content leads to a higher incidence of NAFLD. On the other hand, the adoption of the Mediterranean diet is associated with decreased liver fat content and CV risk[35]. Gut microbial composition changes rapidly according to changing dietary patterns. The effect of diet in fatty liver disease is difficult to differentiate from those due to diet-induced change in gut microbial composition[36]. Gut microbiome composition can identify individuals with a higher risk of NAFLD progression[37]. The gut microbiome and its metabolites influence bile acid metabolism, which in turn influences lipid, choline, and glucose metabolism. Alteration in gut microbial composition and intestinal permeability in NAFLD leads to the circulation of bacterial metabolites such as lipopolysaccharide which is in turn sensed by hepatic Toll-like receptors which induce activation of hepatic pro-inflammatory cells and stellate cells leading to inflammation and fibrosis progression[38,39]. Apart from dietary factors, genetic makeup and ethnicity influence gut microbiome composition[40,41].

Metabolic health

Obese *vs* lean NASH: Although intra-hepatic fat content is closely influenced by obesity, 45% of the obese are said to be metabolically healthy as they don't have any components of metabolic syndrome (MetS)[42]. It is not clear whether these individuals have a lower risk of CV complications compared to normal-weight, metabolically healthy individuals[43]. On the other hand, 30% of normal-weight individuals have MetS and higher cardiometabolic risk. This is because the distribution and nature of fat are more important than the amount of fat in predicting metabolic risk[2]. Visceral fat is associated with higher metabolic risk compared to peripheral and subcutaneous fat. Fat distribution is influenced by ethnicity (higher visceral adiposity in Asians) and genetic makeup[44]. 5%-45% of NAFLD (20% among Europeans) are also lean NAFLD as defined by the presence of hepatic steatosis with normal BMI in the absence of significant alcohol intake[45]. Lean NAFLD has distinct genetic predisposition, metabolic and microbial profiles. Increased prevalence of TM6SF2 risk allele, increased bile acids/Farnesoid receptor activity due to intact metabolic adaptation, and gut microbial profile which facilitates liver fat generation have been seen in lean NAFLD. Individuals with lean NALFD have a better metabolic profile compared to their obese counterparts[46]. The data on the natural history of disease progression in lean NAFLD have shown variable outcomes. Distinct pathways of liver fat accumulation are being recognized. In type 1/metabolic NAFLD, calorie excess due to dietary intake and physical inactivity leads to increased hepatic fatty acid supply by peripheral lipolysis and hepatic lipogenesis[4]. This is associated with IR and other components of MetS thus leading to increased cardiometabolic risk. The accumulated liver fat is composed of monounsaturated triacylglycerols and free fatty acids enriched with ceramides. In type 2/PLNPLA3 NAFLD (with rs738409 risk allele), there is increased intra-hepatic lipogenesis and impaired lipolysis leading to steatosis[47]. The fat composition is predominantly polyunsaturated triacylglycerols. This is not associated with IR and adverse cardiometabolic outcomes although the risk of NASH and HCC is increased. Increasingly various metabolomic signatures leading to hepatic steatosis are being recognized based on RNA-sequencing analysis study [48]. Identification of the key pathway for hepatic steatosis by genetic and molecular profiling may thus help in predicting the risk of progression, cardio-metabolic, and treatment outcomes.

Genetics and epigenetics

Among the multiple variant genes associated with NAFLD identified on genome-wide

association studies, few common variants (PNPLA3, TM6SF2, GCKR, MBOAT7, HSD17B13) are worth mentioning which have divergent metabolic effects[49]. PNPLA3 and TM6SF2 variants increase the risk of NAFLD and advanced fibrosis[50, 51]. PLPLA3, TM6SF2, and GCKR variants are associated with T2DM[52]. MBOAT7 and HSD17B13 variants do not affect serum lipid or glucose levels and do not increase cardiometabolic risk[53,54]. These variants explain only a minority of NAFLD. That is why it is important to consider the effect of other variants, gene-environment interactions (described with the *PNPLA3* gene), and epigenetics. Epigenetic alterations of key regulators of metabolic, inflammatory, and fibrotic pathways represent a bridge between variant genes and the environment in NAFLD. Micro-RNAs such as miRNA-122, miRNA-192, and miRNA-34a are unregulated in NAFLD[55]. miRNA-34A also correlates with disease activity. The role of long non-coding RNAs (lncRNAs) in NAFLD is limited requiring further elucidation[56]. Reversible alteration of methylation signatures of key regulatory pathways is seen in NAFLD which reverses following weight reduction surgery[57]. Methylation signatures can help identify patients with advanced fibrosis [*e.g.* hyper-methylation of peroxisome proliferator-activated receptor gamma (PPARγ)][58]. Epigenetic alterations can alter the expression of PNPLA3 explaining the gene-environment link[59]. There is increasing evidence that maternal high fat diet leads to epigenetic alterations in fetal liver and increasing the possibility of NAFLD in adolescence in the offspring[60,61]. Higher maternal BMI is associated with hypermethylation of the PPARγ coactivator 1(*PGC1*) gene which regulates energy metabolism in the newborn[62].

Familial risk

Twin studies, prospective and retrospective family studies have shown heritable factors in hepatic steatosis and fibrosis. In a prospective study, the risk of advanced fibrosis in first-degree relatives of patients with NAFLD-cirrhosis was 18% which is significantly higher than the general population risk[63,64]. Hence family history also should be considered while doing risk stratification of NAFLD patients.

Alcohol intake

The effect of alcohol use in fatty liver disease has a dose-dependent response which synergistically increases in the presence of metabolic risk factors[65]. This is contrary to the earlier belief that alcohol consumption has a "J" shaped effect on fatty liver disease progression with a beneficial effect on light to moderate use and deleterious effect on excessive use[66]. Hence, it is being increasingly revealed that there is no safe cutoff of alcohol consumption in fatty liver disease.

CLINICAL IMPLICATIONS OF NAFLD HETEROGENEITY

NAFLD sub-classification

The heterogeneity in NAFLD due to its multifactorial etiology, pathophysiological diversity, genetic polymorphisms, and on the other side, the ultimate unifying fate of steatosis and its progression, made NAFLD more like an umbrella disease with multiple subtypes. The proposed change of nomenclature as MAFLD, will not truly represent the full spectrum of the disease pathophysiology and thus this over-generalized new nomenclature has been criticized. Singh *et al*[3] had proposed the 'MEGA-D' classification representing the 'Mega-diversity' of the NAFLD. They had proposed five sub-types of the disease, each representing a major pathophysiological hypothesis behind each subtype. The subtypes are as follows: M-Metabolic syndrome, E-Environmental stressor, G-Genetic Factor, A-Bile Acid dysregulation, and D-Gut dysbiosis related NAFLD. Moreover, it is also suggested to consider fatty liver disease as an umbrella term to include the whole spectrum of cryptogenic to classic to alcohol-associated fatty liver disease. Till any consensus-driven widely accepted terminology and sub-classification of NAFLD comes into place, it is prudent to consider fatty liver disease as common outcome pathology with different etiological triggers.

Alteration of lipid metabolism is one of the major pathophysiological factors behind the development and progression of NAFLD. Lipidomics based sub-classification of patients with NAFLD had been proposed which depends upon the signature patterns of alteration in the fatty acid homeostasis pathway[67]. 'M-subtype' is characterized by increased hepatic fatty acid uptake and reduced hepatic glutathione and S-adenosine methionine (SAM) content. On the other hand, the 'non-M subtype' occurs due to increased de novo hepatic lipogenesis and is characterized by normal hepatic SAM levels. Gut microbiota composition-based sub-classification of NAFLD had also been

proposed. However, till now no studies had been able to reveal any signature gut microbiota profile suitable for phenotypical classification of NAFLD patients.

Automated algorithm-driven cluster sub-classification, based on demographic factors (age, gender, ethnicity), clinical and laboratory findings[68], had been evaluated in a cohort of 13290 NAFLD patients in the United States. The whole cohort had been divided into 5 subtypes and evaluated for disease outcomes including survival rates. In subtype 1, there were mostly female Hispanics with mild metabolic comorbidities with minimal fibrosis, but on the other hand subtype 2 had mostly patients with MetS with signs of developing liver dysfunction. Subtype 3 was a mostly young and healthy population with mild disease and minimal abnormalities. Subtype 4 patients were predominantly elderly male Caucasians who had more severe disease at baseline with features of fibrosis and also showed features of progression to cirrhosis stage. Subtype 5 patients were the oldest with more severe cirrhosis and associated with significant co-morbidities. Among the disease outcome, subtype 5 was at the highest risk mortality and subtype 4 had the highest risk of cirrhosis and HCC. Although this type of cluster-based subtyping of the disease needs to be validated clinically it can help to identify relevant disease subtypes in future studies.

In a gene expression study by Hoang *et al*[48], the disease progression score of individual genes had been evaluated and it showed a strong correlation with histological manifestations of disease severity. In this study, the authors proposed NAS (gene-level NAFLD activity score) and gene-level fibrosis stage (gFib) scores. These score-based subtypes of NAFLD not only can assess the risk of disease progression but also can predict the response to therapy. This molecular-based cluster classification either can be the forerunner of different clinical subtypes of NAFLD or can represent different phases of a dynamic spectrum of the disease.

Though genetic, clinical cluster, and pathophysiological based sub-classification of NAFLD had been proposed as discussed above, none of them are universally accepted. Moreover, detailed literature is mainly limited to disease phenotypes depending upon demographic factors, obesity, and clinical outcomes.

Inter-individual variation

Demography (Asian *vs* Western countries): The prevalence of NAFLD is now showing an increasing trend in Asian countries. A meta-analysis done in 2016[69] showed a higher prevalence in Asia (27.4%) than North America (24%) or European Union (23.7%). In a recent meta-analysis[70], the prevalence in Asia was found to have increased further (29.62%) and a secular trend of the rising prevalence in the last few decades had been reported. The increase in prevalence in Asia is likely due to an increase in obesity, sedentary lifestyle, changing westernized eating habits, and various socio-economic factors[71]. The prevalence in the rural area was significantly lower than in the urban areas, suggesting the detrimental effect of urbanization on obesity and the consequent NAFLD[72]. In both Asian and western countries, the prevalence increases with age. Prevalence is higher in males as well as among elderly women indicating protective effects of estrogen in females in the reproductive age group. Apart from the increased prevalence of metabolically unhealthy obesity and excessive visceral obesity, alteration of gut microbiota and bile acid profiles has also been postulated as possible contributing factors behind the development of steatosis [40]. Among the genetic factors, PNPLA3 polymorphism (rs738409) had been strongly associated with hepatic steatosis in both western and eastern studies[31]. However, a higher prevalence of PNPLA3 risk allele had been reported in Asia than in African or European countries[73,74]. Genetic polymorphisms of other genes like *TM6SF2*, *AGTR1*, *HSD17B13*, and *GCKR* genes had also been linked with increased suscept-ibility of NAFLD in Asian subjects[54,75-77]. Sarcopenia and hypovitaminosis D also was associated with NAFLD development[78,79]. One of the major differences in Asian countries from their western counterpart is the increased prevalence of lean NAFLD (discussed later) in the former. Though the overall prevalence of NAFLD is almost similar in eastern and western countries, however, the rate of complications is still lesser in Asian countries. In a retrospective study from Japan with a median follow-up of 5.8 years, only 0.25% of patients developed HCC with an annual incidence of 0.043%[80]. In contrast to western countries, NAFLD still contributes only to a minor proportion of liver-related complications requiring liver transplantation in Asia. In a Japanese nationwide survey, only 2.1% of patients with cirrhosis had NASH and almost two-thirds of the patients had viral hepatitis[81]. The indolent course of NAFLD in Asian countries is likely due to relatively short disease duration in the majority of the patients in this part of the world. As there is a considerable lag in economic growth and consequent obesity epidemic in Asian countries, the rise in NAFLD and its complications are likely to follow the western trend in the coming

years. Moreover, the relatively higher chance of co-existence of viral hepatitis and NAFLD in Asian countries increases the risk of hepatic complications further[82].

Ethnicity: Irrespective of ethnic variability, a trend of overall increased prevalence of NAFLD had been seen globally. In the world, Middle East had the highest prevalence of NAFLD, and in Africa; it is the lowest[69]. Studies from the United States reported that Hispanics had shown the highest risk of NAFLD and on the other hand, the risk is much less in the Alaskan Native. Among Asian ethnicity, the prevalence is highest among Indonesian and lowest in Japanese[70]. Interestingly, people of South Asian origin who are living in the United Kingdom, also showed higher risk[83]. In a recent meta-analysis, which evaluated ethnic heterogeneity of NAFLD in the United States, both higher overall prevalence of NAFLD and risk of progression to NASH had been reported in Hispanics and the risks were lowest among Blacks[30]. Although there was no significant difference in patients with fibrosis among different ethnicities. The reasons behind the ethnic variation are multifactorial. A significantly high risk of NAFLD among American Japanese than the native Japanese suggests the impact of socio-economic development and differences in lifestyles in the pathogenesis[70]. Specific western dietary patterns in different ethnicities, like consumption of red meat and hydrogenated fat, had also been associated with an increased risk of fibrosis[84]. Intake of saturated fatty acids increases and on the other hand, consumption of omega 3 fatty acid-rich food reduces the risk of steatosis. Genetic factors can explain the heterogeneity of NAFLD across different ethnicities. Among genetic variants of the *PNPLA3* gene, rs738409 increases the risk of NAFLD in Hispanics and Southeast Asians[85]. On the other hand, the increased prevalence of protective polymorphism of the same *PNPLA3* gene (rs6006460) can explain the reduced risk of NAFLD among African Americans[31]. The rs738409 variant had been also associated with an increased risk of progression to NASH and hepatic fibrosis[86,87]. However, in a study from Malaysia, though the frequency of *PNPLA3* risk allele was higher among Chinese individuals but the prevalence of NAFLD was much less in them in comparison to Malay and Indian participants[87]. This paradox can be explained by the involvement of multiple candidate genes in disease pathophysiology among different ethnicities. With the advent of Genome Wise Association studies, the role of predisposing polymorphisms of other candidate genes like *TM6SF2* and *GCKR* gene had been explored further. The rs58542926 variants of the *TM6SF2* gene were significantly associated with intra-hepatic fat (triglyceride) accumulation in White and African-American but not among Hispanic individuals[88]. Different polymorphisms in the *AGTR1* gene were protective among Indians but not in Chinese and Malay subjects [75]. Recently, polygenic gene scores had been developed to evaluate the cumulative effects of multiple candidate genes in the development and progression of NAFLD [89]. Further studies are needed in the future to explore the complex interaction of different genetic polymorphisms which can explain disease heterogeneity across different ethnic populations.

Age (Children and adolescents): With the increasing prevalence of pediatric obesity, the prevalence of NAFLD in children and adolescents is ever rising. The pooled prevalence of pediatric NAFLD in general population and obesity clinic were 7.6% (95%CI: 5.5%-10.3%) and 34.2% (95%CI: 27.8%-41.2%) respectively[90]. The factors which can influence the intrauterine metabolic milieu of the developing fetus, like maternal obesity and diabetes, had been postulated to increase the future risk of NAFLD[91,92]. Increased consumption of fructose-rich beverages, processed food, saturated fat along with decreased intake of dietary fibers (westernized dietary habits) had been strongly associated with the development of NAFLD in children[93]. On the other hand, breastfeeding was protective against the development of NAFLD[94]. The genes which had been shown to increase the risk of pediatric NAFLD are similar to the adults. Genetic variants of*PNPLA3* (rs738409), *TM6SF2* (rs58542926), and *GCKR* gene had been shown to increase the susceptibility of development of NAFLD in pediatric patients[31,88]. Though histological diagnosis of NAFLD remains ideal, diagnosis by imaging (ultrasound/MRI) is the most practical one in the pediatric population. As the prevalence of obesity in children is ever-increasing, the chance of co-existence of other secondary causes of hepatic steatosis should also be carefully evaluated before confirming the diagnosis of NAFLD. Histological pattern in pediatric NAFLD (peri-portal distribution-Type 2 NASH) differs from that of their adult counter-part (peri-central distribution-Type 1 NASH)[95]. Both fibrosis and steatosis are mainly present in the periportal region in type 2 NASH and are seen more in younger children. Moreover, the classical 'ballooning' change is also seen less frequently in children. On the other hand, type 1 NASH of the adult pattern can be seen in the older adolescent

age group[96]. There is a paucity of longitudinal studies evaluating the natural history of pediatric NAFLD. Around 10%-25% of patients had advanced fibrosis and almost half of the patients had NASH at the time of diagnosis[97]. Though the incidence of HCC in the pediatric age group is extremely rare, a large number of pediatric patients with NAFLD are at increased risk of developing HCC in early adulthood. Weight loss and lifestyle changes were effective in the reversal of steatosis in pediatric patients[98].

BMI (lean/non-obese NAFLD): Lean and non-obese NAFLD is defined as NAFLD in a person with BMI < 25 kg/m² (< 23 for Asian subjects) and < 30 kg/m² (< 25 for Asian subjects) respectively. In a meta-analysis that included 93 studies from 24 countries, the prevalence of lean and non-obese NAFLD in the general population was reported as 5.1% and 12.1% respectively[99]. Globally, the prevalence of non-obese NAFLD among the whole NAFLD group was 40% and in countries like India, it is as high as 47%, indicating that a large proportion of fatty liver disease is now developing in the non-obese population. Though non-obese NAFLD initially was more common in Asian countries, now almost similar prevalence of NAFLD is being reported from the western part of the world (United States 43.2%). Globally the prevalence of lean/non-obese NAFLD is showing an increasing trend over the last 3 decades[100]. Though Shi *et al*[101] had reported a lower prevalence of hypertension, hyperuricemia, and fasting blood glucose in lean/non-obese NAFLD patients compared to obese NAFLD, these lean patients are not necessarily metabolically healthy. Rather lean NAFLD patients are more likely to have visceral obesity, metabolic syndrome, dyslipidemia, hypertension, and DM as co-morbidities than the lean controls[101]. The pathophysiological basis of the development of NAFLD in lean/non-obese individuals is complex and multi-factorial. Increased prevalence of the PNPLA3 G allele had been found in lean NAFLD patients[102]. Other genetic factors like TM6SF2 (T)[46], cholesteryl ester transfer protein, and interferon lambda 3 (IFNL3)/IFNL4(C) had also been found to increase the risk of lean/non-obese NAFLD[103,104]. On the other hand, possible roles of distinct gut microbiota, bile acid profile[46,105], increased lysine, tyrosine, lysophosphatidylcholines, and phosphatidylcholines, had also been implicated in the development of NAFLD among lean individuals[106]. The progression of NAFLD in the lean population can be conceptualized as a state of gradual attenuation of metabolic adaptation. Pathophysiologically, this can be divided into 3 stages- stage of susceptibility, stage of adaptation, and stage of failure[107]. Studies evaluating the true natural history of lean NAFLD are sparse in the literature. In the largest meta-analysis Ye *et al*[99] reported that among lean/non-obese NAFLD patients, NASH and fibrosis (> stage 2) were present in 39% and 29% of patients respectively, which was lesser than the prevalence among obese NAFLD population. However, liver-related mortality was reported as almost twice in lean/non-obese NAFLD patients than in the obese NAFLD group. In another study with a mean longitudinal follow-up of almost 20 years, lean NAFLD patients did not show any significantly increased risk of overall mortality but the risk of progression to severe hepatic diseases was significantly higher (HR 2.69) than the obese NAFLD population [108]. Like obese NAFLD, lifestyle modification in the form of dietary modifications and increased physical activity remains the main therapeutic approach in lean NAFLD patients[109].

Variable natural history
Classic and dynamic model: Previously, the natural history of NAFLD had been conceptualized as a disease spectrum that follows a linear model of disease progression. This classic model hypothesized that there is a gradual progression of the disease from NAFL to NASH to cirrhosis and HCC. However, this progressive worsening of the disease does not occur in all of the patients of NAFLD and significant heterogeneity in the natural history of NAFLD had been observed. Recent literature had identified that not all the patients with NAFLD follow this 'classic linear model' of natural history. A study by Pais *et al*[110], which systemically evaluated serial liver biopsy in NAFLD patients, had shown that 60% of NAFL patients had progressed to NASH and around 25% of patients of NAFL had directly progressed to the fibrotic stage. Various factors like DM, obesity, old age, and a higher degree of baseline abnormality were identified as possible risk factors for disease progression. In another longitudinal follow-up study by McPherson *et al*[111], no significant difference in the rate of fibrosis progression between NAFL and NASH patients was found. In an excellent systematic review by Singh *et al*[112], serial liver biopsy data of 411 biopsy-proven NAFLD from 11 cohort studies were analyzed. They had also re-emphasized that both NAFL and NASH can progress to the fibrotic stage. However, it takes much

longer (14 years) time to progress one fibrosis stage in NAFL than in NASH (7 years). The annual fibrosis progression rate was slower in NAFL (0.07 stage) than in NASH (0.14 stage). Moreover, NAFL and NASH had a comparable rate of CV mortality (OR 0.9) though all-cause and liver-related mortality are higher in NASH[113]. To summarize, NAFL can progress both to the NASH and fibrosis stage directly and on the other hand, NASH can also regress to NAFL or progress to the fibrotic stage. Thus, in the 'dynamic model' of NAFLD, it has been conceptualized that in early NAFLD, there is dynamic cycling between NAFL and NASH[114] (Figure 3).

Slow and rapid progressor: In the same meta-analysis discussed above, Singh *et al* [112] also had identified significant heterogeneity among disease progression in NAFLD. They reported 2 subtypes of NAFLD patients according to fibrosis progression rate- rapid and slow progressor. The rapid progressors were around 20% of the NAFLD group who progressed rapidly from baseline (stage 0 fibrosis) to advanced (stage 3 or 4 fibrosis). On the other hand, the majority of NAFLD patients are slow progressors who only progressed 1 or 2 stage fibrosis in a similar time frame. Older age, low ASL: Alanine aminotransferase (ALT) ratio, co-morbidities like diabetes mellitus or hypertension, and genetic polymorphisms are probable risk factors for rapid progressors[103,115] (Figure 3).

HCC: With the progressive increase in the prevalence of NAFLD worldwide, the risk of HCC and liver-related mortality are likely to rise as a consequence. Viral hepatitis-related HCC usually occurs in the background of the advanced stage of cirrhosis. Though classically HCC usually occurs in the advanced stage of cirrhosis in the NAFLD spectrum, this is not true for all the cases of NAFLD-related HCC[116]. Rather one of the most common causes of chronic liver disease-related HCC without evidence of cirrhosis is NAFLD[5]. Leung *et al*[117] had reported 15% percent of NAFLD-related HCC as non-cirrhotic and they usually had larger hepatic tumor diameter at diagnosis. In a retrospective analysis, Mohamad *et al*[118] also reported that HCC in NAFLD patients without cirrhosis are likely to present in the older age group with a larger tumor size with a high recurrence rate in comparison to those with cirrhosis (Figure 3).

THERAPEUTIC AND RESEARCH IMPLICATIONS

NAFLD progression and prognostication

Many factors may influence the progression of NAFLD to the more advanced stage but are not routinely or easily assessed in day-to-day practice (*e.g.*, genotype, gut microbiome, mitochondrial function, immunological response)[119]. Consequently, we need to consider the natural history studies to help provide clinical, biochemical, and histological variables that can be utilized to decipher which patients will develop severe disease with worse outcomes. With regard to clinical features, a paired biopsy study by McPherson *et al*[111] underscores the impact of IR with 80% of patients with NAFL and progression of fibrosis developing diabetes by the time of follow-up biopsy compared with 25% of nonprogressors. Other studies have also shown that weight gain and worsening IR are associated with fibrosis progression in NAFLD[110]. Data for biochemical predictors are somewhat deficient. However, a study found that in patients with biopsy-proven NASH and compensated cirrhosis; lower levels of serum cholesterol, ALT, and platelets are independently associated with hepatic complications and higher aspartate aminotransferase (AST)/ALT ratio with overall mortality [120]. In NAFLD, baseline histology can provide a good prognostic value. According to a systemic review and meta-analysis of paired-biopsy studies, a third of individuals with NAFLD will have progression of fibrosis with a mean progression rate of 0.14 stages *per* annum for NASH, corresponding to one stage of fibrosis progression over a median of 7.1 years[112]. Nevertheless, many epidemiological studies have de-emphasized the presence of NASH and confirmed the presence and degree of fibrosis as the most important histologic predictor of liver-related morbidity and mortality [121,122].

It is now widely accepted that the severity of fibrosis is the only significant predictor of outcomes in NAFLD. The histological differentiation between NAFL and NASH is unlikely to predict fibrosis progression and carries very little prognostic value. Thus, it is better to consider the diagnosis of patients with advanced fibrosis (F3 and F4) because this stage is a predictor for hepatic and extrahepatic morbidity and mortality [123]. This strategy identifies those with liver disease sufficient to call for specific interventions to prevent complications of cirrhosis and the development of HCC. People with NAFL or NASH with early F0–F2 don't need to be considered as having
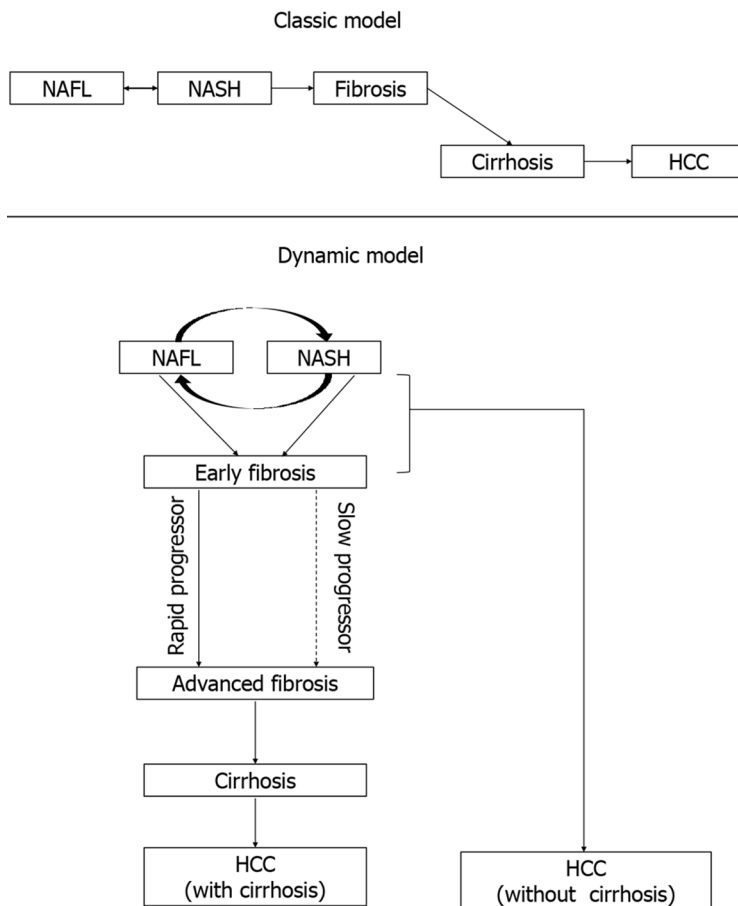
Figure 3 Natural history of non-alcoholic fatty liver disease (classic and dynamic model). HCC: Hepatocellular carcinoma; NAFL: Nonalcoholic fatty liver; NASH: Nonalcoholic steatohepatitis.

liver disease necessitating intervention owing to the low risk of liver-related complications. In these persons, metabolic risk factors like diabetes should be addressed to optimize CV outcomes, with likely benefits on liver disease[123]. As progressive fibrosis indicates a poor prognosis with unfavorable CV and adverse hepatic outcomes, the approach should now focus on the risk stratification of patients and identify those needing liver-specific intervention.

Non-invasive tests of hepatic fibrosis

As the severity of fibrosis is the major driver for the long-term prognosis of NAFLD patients, it is, therefore, critical to identify patients at higher risk of advanced fibrosis to optimize their management[124]. Although required to detect patients with NASH and early fibrosis, liver biopsy is an invasive procedure. Patient acceptability is low, and it is not desirable to perform liver biopsy repetitively to assess disease progression and response to treatment. Moreover, as only a small proportion of the patients would develop liver-related complications, performing non-invasive tests (NITs) as the primary assessment is preferable[125]. This section focuses on the confounding factors that can affect the performance and accuracy of NITs of liver fibrosis in patients with NAFLD.

Impact of confounding factors

Non-invasive fibrosis scores are usually used to detect or exclude advanced fibrosis in individuals with NAFLD. A few studies purposely looked at reasons for imprecise prediction by these scores. In a multicentric European study in subjects with biopsy-proven NAFLD, the AST-to-ALT ratio, NAFLD fibrosis score (NFS) and Fibrosis-4 (FIB-4) index performed poorly for the detection of significant fibrosis in persons aged 35 years or below[126]. The specificity of the FIB-4 index and NFS reduced to unacceptable levels in those aged 65 years and older in the same study. This reason is that age is a component of both the fibrosis scores. The performance of NITs and the used transient elastography (TE) liver stiffness cutoffs in different ethnic populations

and special subpopulations such as individuals with diabetes and obesity also need to be taken into account. For example, depending on the ethnicity, the diagnostic accuracy of the NITs may be altered. Compared to Western populations, South Asians develop more metabolic complications at lower body mass indices. The accuracy of the NFS, AST-to-platelet ratio index, FIB-4, AST/ALT ratio, and BARD score is found to be lower in the South Asian population in comparison with the Caucasian population [127]. In addition, the NFS has a lower sensitivity in individuals of South Asian descent, as the majority had a lower BMI and were younger than Caucasian counterparts with a comparable disease stage, and therefore had a lower score[125]. Serum markers of liver fibrosis and possible confounding factors are summarized in Table 1.

With regards to imaging modalities that estimate liver stiffness as a potential surrogate of hepatic fibrosis, vibration-controlled transient elastography (VCTE) has been widely validated against liver histology[128] and shows correlation with clinical outcomes in longitudinal studies[129]. However, there are a number of factors to be considered while using this modality. Pathologies that increase liver stiffness can lead to a false-positive diagnosis of advanced fibrosis. Besides, high BMI and severe hepatic steatosis have been reported to increase the false positive rate of VCTE[130]. A recent study suggests that when using the XL probe in obese patients, steatosis does not augment liver stiffness independent of fibrosis[128]. Magnetic resonance elastography (MRE) can surmount many of these barriers, except for iron overload and acute inflammation; nonetheless, restricted availability at most centers and cost are the limiting factors. MRE has higher applicability and accuracy than VCTE when compared head-to-head[131].

While it is expected that blood-based parameters or imaging modalities will replace liver biopsy for the diagnosis in people who would benefit from treatment, equally it indicates that validation of any future marker should be done in more specifically defined cohorts. A recent International Consensus Panel suggested that the factors that shape the NAFLD heterogeneity should be taken into account when devising risk-stratification scores and algorithms[2]. Caution should be exercised by clinicians during the interpretation of test results when the tests are applied in patients with potential confounding factors.

Considerations for best practice

Early detection of advanced fibrosis is essential in the efforts to halt the NASH progression. Therefore, screening is vital to ensure that patients, mainly those with advanced F3–F4, are identified and linked to care before they develop end-stage liver disease. With the development of reliable NITs to identify patients with advanced fibrosis, there is now potential to put management strategies earlier in place[132]. Clinicians need to be more proactive in detecting patients with advanced fibrosis due to NASH. Figure 4 shows a diagnostic algorithm that targets screening of patients with characteristics of MetS who are at risk of progressive fibrosis. This is in accordance with guideline recommendations to screen this high-risk group[133]. This pathway includes sequential use of NITs (preferably a serum biomarker and an imaging technique) and can decrease secondary and tertiary referral rates and achieve larger cost savings.

In the Asia–Pacific region, quite a few studies have assessed the cross-sectional accuracy of non-invasive surrogates of liver biopsy among NAFLD patients[134,135]. It has been suggested that the serum tests and physical tools when used in combinations can yield more reliable data than that provided by either method alone[136]. Nevertheless, concerns are there regarding the definition of threshold values in Asian patients and Asia-Pacific Working Party stated that "at the present time, the clinical use of such tools to avoid liver biopsy remains undefined"[137].

Newsome *et al*[138] recently published the FibroScan-AST (FAST) score for the non-invasive identification of patients with significant fibrosis (≥ F2) and a NAFLD activity score (NAS) of ≥ 4 to detect those at increased risk of disease progression. This could reduce unnecessary liver biopsies in patients unlikely to have significant disease. The incorporation of VCTE values in the score enhanced the diagnostic performance. This prospective study was validated in multiple global cohorts from North America, Europe, and Asia. Discrimination was considerably higher for the FAST score when compared with FIB-4 and NFS. Now, further research on the performance of the FAST score is required to transition the use of such predictive models to clinical practice. The diagnostic accuracy of the sequential combination of FIB-4 and VCTE had been evaluated recently in an individual participant data meta-analysis that included 5735 patients. Depending upon the different cut-offs used, this combined algorithm can diagnose cirrhosis with a specificity of 95%-98%, obviating the need for liver biopsy

Table 1 Non-invasive tests of hepatic fibrosis and potential confounding factors

Biomarker panel	Parameters	Validation	Prognostic ability	Confounding factors/limitations
APRI	AST, platelet	Good	Fair	Large number of individuals fall in the indeterminate range
Fibrosis-4 index	Age, AST, ALT, platelet	Very good	Very good	Poor performance in patients aged ≤ 35 yr
				Low specificity in patients aged ≥ 65 yr
				Less sensitive in South Asian Population
NAFLD fibrosis score	Age, BMI, IFG or diabetes, AST, ALT, platelet, albumin	Very good	Good	Different cutoff values needed for younger or older participants
				Albumin may decrease in chronic illnesses, malnutrition, nephrotic syndrome and protein-losing enteropathy
				Less sensitive in South Asian Population
Enhanced liver fibrosis panel	PIIINP, HA, TIMP1	Good	Very good	PIIINP is increased in other fibrotic diseases or bone fracture
				TIMP1 is increased in cancer and inflammation
				Not as widely available as non-patented scores and more expensive
FibroMeter NAFLD	Age, weight, prothrombin index, ALT, AST, ferritin, fasting glucose	Fair	NA	Prothrombin index affected by anti-coagulants
				Ferritin is an acute phase protein
				Glucose is affected by anti-diabetic treatment
				More validation needed
NIS4	miR-34a-5p, α2-M, YKL-40, and glycated hemoglobin	Fair	NA	Not as widely available as non-patented scores and more expensive
				More validation is needed

ALT: Alanine aminotransferase; APRI: AST-to platelet ratio index; AST: Aspartate aminotransferase; BMI: Body mass index; HA: Hyaluronic acid; IFG: Impaired fasting glucose; α2-M: α2 macroglobulin; NA: Not applicable; NAFLD: Non-alcoholic fatty liver disease; PIIINP: Procollagen type III N-terminal peptide; PTI: Prothrombin index; TIMP-1: Tissue inhibitor of matrix metalloproteinase 1.

[139].

Identification of novel therapeutic targets

As the burden of NAFLD has become increasingly evident, so also have hurdles to developing effective therapeutic points of action. The development of progressive steatohepatitis is connected to excess metabolic substrate delivery to the liver that, in turn, induces cell stress, which can activate inflammatory and apoptotic signaling. Eventually, inflammation triggers a fibrogenic response that can lead to cirrhosis in the end[140]. This simplified model facilitates the evaluation of precise mechanisms underlying each of these factors and targeting them for treatment. Table 2 summarizes proposed 'druggable' pathophysiologic targets in NAFLD[141-153].

Quite a few of the recently carried out phase 2 and 3 studies failed to reproduce the encouraging antifibrotic or NASH-resolving effects observed in animal models. Reasons for this discrepancy between preclinical models and clinical settings are likely diverse. Most importantly, no model can ever assess compounds in the actual physiological settings of heterogeneous human populations. This aspect may become further relevant if mechanisms are not entirely translatable between two different species[154]. Additionally, none of the available NASH models used for preclinical trials adequately represents all the human disease aspects from the macroscopic to the molecular level. Moreover, only a few models reflect linked extrahepatic diseases (such as atherosclerosis, obesity, or IR). Finally, a higher heterogeneity in humans in relation to genetics, the gut microbiota, gender, and existing comorbidities leads to even more complications. It is, therefore, critical to recognize the drawbacks of preclinical models to improve clinical trial outcomes in drug development.

There is significant interindividual variability in the NAFLD susceptibility and for progression to liver-related complications[49]. It is becoming more and more apparent that there is substantial heterogeneity in the molecular and cellular processes

Table 2 Liver-targeted therapies in development for the treatment of nonalcoholic fatty liver disease

Treatment targets	Mechanism of action	Agent (oral/injectable)	Current status
Metabolism	FXR agonism	Obeticholic acid	Interim analysis of a phase 3 RCT (REGENERATE) showed significant histological improvement[141]
		Tropifexor (LJN452)	A phase 2 study recently completed (NCT02855164)
		Cilofexor	A phase 2 study in patients with NASH showed a decrease in hepatic fat[142]
	PPAR agonism	Elafibranor	Interim analysis a phase 3 trial (RESOLVE-IT) failed to show any treatment effect
		Lanifibranor (IVA337)	A phase 2 study in patients with T2DM and NAFLD is actively recruiting (NCT03459079)
		Saroglitazar	A phase 2 RCT (EVIDENCES IV) in participants with NAFLD/NASH has shown significant improvement in ALT, LFC, and IR[143]
	Acetyl-CoA Carboxylase inhibition	PF-05221304	Improved liver chemistry and liver fat in an RCT[144]
	GLP-1 agonism	Liraglutide	Only data from small studies have been published and the relative contribution of weight loss and improvement in glycemic control to the observed benefits in NASH are yet to be determined[145-147]
		Semaglutide	In a phase 2 trial, the primary endpoint (resolution of NASH with no worsening in fibrosis), was met[148]
	FGF21 agonism	Pegbelfermin (BMS-986036)	A series of phase 2b trials of pegbelfermin are underway
	MCP2 antagonism	MSDC-0602 K	The EMMINENCE phase 2b trial didn't meet the primary end point[149]
	THRβ agonism	Resmetirom (MGL-3196)	A phase 3 study is actively recruiting (NCT03900429)
Cell stress and apoptosis	Antioxidant	Vitamin E	Resolution of NASH in some studies, but not all; no impact on fibrosis[150]
	Pan-caspase inhibition	Emricasan	Phase 2b clinical trials for NASH failed to meet their primary efficacy end points[151]
	ASK1 inhibition	Selonsertib	Phase 3 STELLAR trials discontinued due to lack of efficacy
Inflammation	CCR2/CCR5 inhibition	Cenicriviroc	Phase 3 trial AURORA terminated due to lack of efficacy
	Inflammasome inhibition	SGM-1019	A phase 2 study is terminated due to a safety event (NCT03676231)
Fibrosis	LOXL2 inhibition	Simtuzumab	No benefit on histological analysis or on clinical outcomes[152]
Gut–liver signaling axis	FGF19 agonism	Aldafermin (NGM282)	In a phase 2 trial of patients with NASH, aldafermin reduced liver fat and produced a trend toward fibrosis improvement[153]

ACC: Acetyl-CoA carboxylase; ALT: Alanine aminotransferase; ASK1: Apoptosis signal-regulating kinase; CCR: C–C motif chemokine receptor; FGF: Fibroblast growth factor; FXR: Farnesoid X receptor; GLP1: Glucagon-like peptide 1; IR: Insulin resistance; LFC: Liver fat content; LOXL2: Lysyl oxidase homolog 2; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; PPAR: Peroxisome proliferator-activated receptor; THRβ: Thyroid hormone receptor β.

propelling the disease from one patient to the next. This understanding raises the possibility of matching specific therapeutic strategies to the particular disease drivers in a given patient. The development of such personalized approaches and the detection of subpopulations with distinctive disease drivers will need a combination of phenotypic, genetic, and molecular data[140]. Furthermore, genetic insights present a powerful approach to deduce and prioritize candidate drugs. Such selection can avoid numerous drawbacks while defining likely benefits[155]. However, drug discovery based on genetics is still in its infancy, and this area will present its challenges. NAFLD is associated with several metabolic disturbances. As many circadian clock-controlled genes are fundamental in the metabolic processes of the body, it is not unexpected that some of these genes can be potential therapeutic targets[156]. Thus, by considering the circadian cycling of their targets, new drugs for NAFLD can be administered in a way that optimizes the benefits and minimizes the side effects.
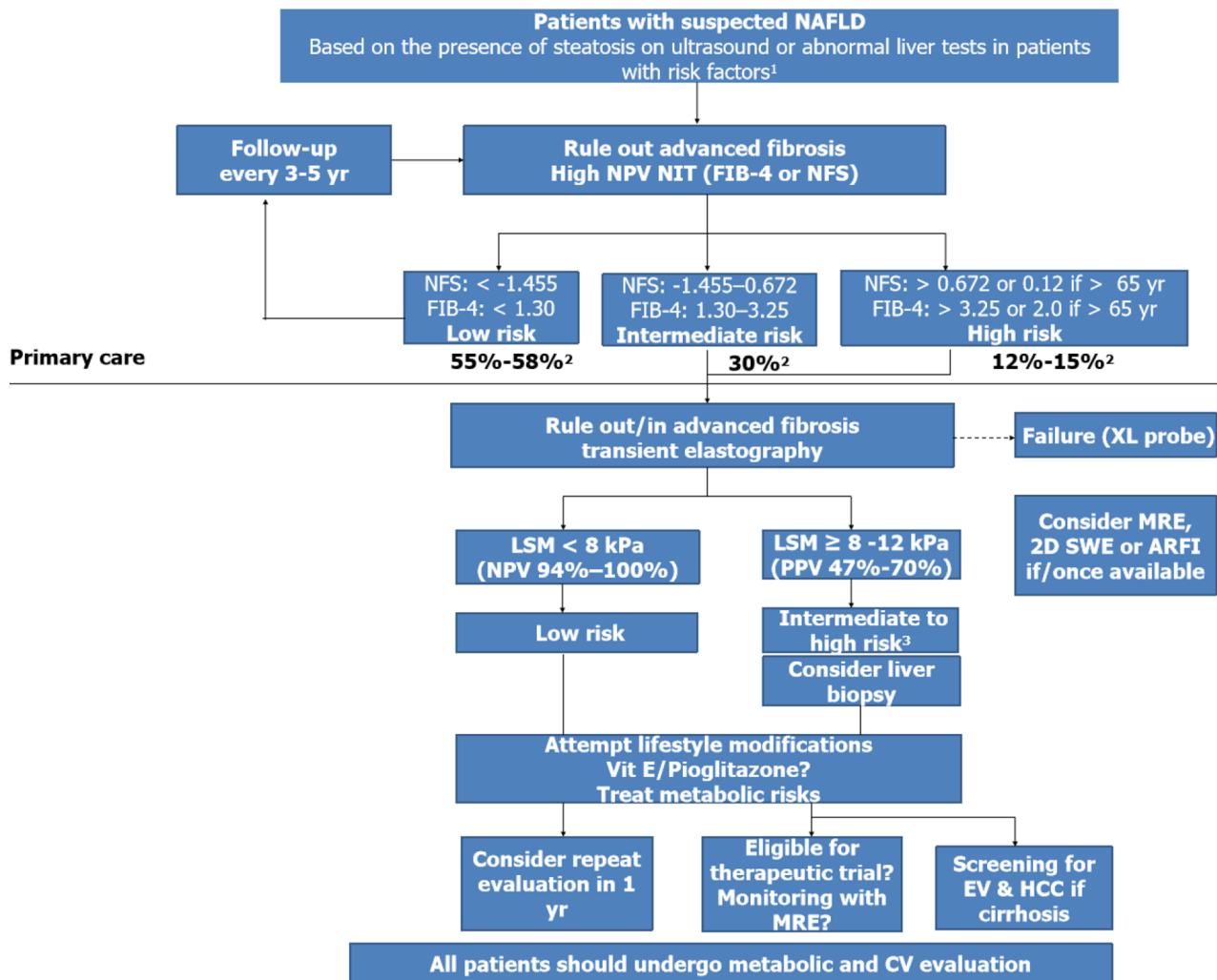
Figure 4 A suggested algorithm for the use of non-invasive tests for risk stratification of patients with suspected non-alcoholic fatty liver disease in clinical practice. [1]Obesity, type 2 diabetes, or metabolic syndrome; [2]Estimated prevalence for low, intermediate, and high risks groups; [3]Patented serum biomarkers (FibroTest, Fibrometer, or ELF) could be considered in patients with intermediate-risk. ARFI: Acoustic radiation force imaging; LSM: Liver stiffness measurement; MRE: Magnetic resonance elastography; NPV: Negative predictive value; PPV: Positive predictive value; SWE: Shear wave elastography.

Impact on clinical trials and endpoints

Given the rising disease burden associated with NAFLD, the development of outcome measures to assess the at-risk population and validate clinically relevant study endpoints is vital. Nevertheless, the natural history of NAFLD is highly variable, often nonlinear in progression. In addition, NAFLD itself is a heterogeneous disease that is shaped by the dynamic interaction between genetic predisposition, environmental factors, and several modifiable risk factors[157]. This pathogenetic background provides numerous potential targets for therapeutic intervention, however, this same complexity limits defining clear, measurable, and objective clinical endpoints[158]. Considering these factors, surrogate endpoints, which can be used to predict outcomes on clinically relevant endpoints, are expected to be beneficial in most patients. Furthermore, NAFLD is a slowly progressive disease, with a gap of many years between onset and development of "hard" clinical outcomes, such as liver-related and all-cause mortality. As stated earlier, the fibrosis stage is the most important predictor of liver-related outcomes. Unfortunately, the progression of fibrosis itself is also slow, with a median of 7.1 years in subjects with NASH[112]. Thus, selecting meaningful clinical endpoints has been a major challenge in drug development and validation. At present, before enrolling patients into NASH clinical trials, identifying which patients with NAFLD have NASH, particularly those with advanced fibrosis, is one of the major stumbling blocks. Once these at-risk patients have been selected, monitoring for fibrosis regression in individuals with advanced fibrosis appears to be the optimal endpoint in clinical trials and should supplant NASH-based endpoints[158]. Surrogate measures of liver-related outcomes also seem reliable. Although important, to assess for all-cause mortality (primarily CV death) and liver-related mortality will require

longer-term follow-up.

Liver biopsy is essentially prone to sampling error and interobserver variability; its invasive nature also makes it a barrier for large clinical trials. Given these limitations, the development of accurate, robust, and reproducible noninvasive surrogate endpoints which may ultimately replace biopsy in trials are eagerly sought in NAFLD research[159]. Algorithms such as NFS and FIB-4 may be useful tools for prescreening, in order to enrich the patient group with an appropriate spectrum of NASH and fibrosis for enrollment. Noninvasive imaging methods such as VCTE and MRE are likely to play a future role but presently lack the ability to differentiate between closely related fibrosis stages[160].

To summarize, a combination of the slow nature of disease progression in NAFLD, heterogeneity of therapeutic targets, and inherent limitations of serial liver biopsy to evaluate effects of intervention have considerably hampered clinical trial design as well as the development of new and effective therapies[158]. Thus, the standard trial design that does not consider the disease heterogeneity may not be the best approach for learning this complex disease. Future clinical trials need to target patients with specific characteristics (gender, hormonal status, genetic susceptibility, metabolic and microbiota signatures, and the presence or absence of comorbidities) once the connections between these characteristics and the therapeutic targets are clearly understood[2].

FUTURE PERSPECTIVES

With increasing recognition of heterogeneous molecular and genetic drivers of NAFLD, there is a possibility of precision medicine based on the identification of specific drivers of the disease. An integrated model of NAFLD development based on genetic, molecular, histology, "omics" based data (transcriptome, metabolite, proteome, microbiome), and disease phenotype to identify disease subpopulations is required for such personalized approaches[140]. Critical data on molecular heterogeneity and its relation to clinical outcomes of NAFLD to going to explore new horizons in the management of this global pandemic[161]. A better understanding of bidirectional and dynamic disease progression and regression (*e.g.* fibrosis), the influence of behavioral factors, and establishing a correlation with end-organ damage is warranted. Prospective follow-up data on the evolution of pediatric NAFLD into adulthood shall shed light on pediatric disease evolution[162]. Identification and validation of non-invasive methods of disease assessment and biomarkers will accelerate the development of pharmacotherapy and testing of combination therapies. Seamless phase II-IV trial designs, virtual placebo cohort analysis, master clinical trials testing multiple agents and multiple disease types, use of effectiveness trials in real-world settings, and patient-reported outcomes would revolutionize clinical trials for NAFLD. Precise terminology, characterization of disease heterogeneity (both molecular and clinical), novel translational models to identify new therapeutic target, and thus better designed clinical trials would help reduce the burden of the disease[2].

CONCLUSION

The impact of the upsurge in NAFLD patients and a rising proportion with advanced disease will be reflected in higher rates of hepatic and extrahepatic morbidity and mortality, which will continue to burden the health care system heavily. On the other hand, a lack of enough consideration of heterogeneity in risk profiles and responsiveness to treatment posing impediments that hampers progress to effective treatments. It is anticipated that a more robust understanding of pathophysiology will result in better characterization and subphenotyping of the disease and its drivers. In turn, this understanding of disease variability may help the introduction of appropriate noninvasive biomarkers for each subtype, thus promoting more individualized interventions. In this regard, any discussions on the update of nomenclature or more appropriate terminology are in the right direction. However, the proposed redefining of the disease should increase the prioritization of research activity on NAFLD to fill current knowledge gaps and find new tools to overcome the challenges. It appears to be important to place NAFLD/MAFLD/DAFLD under the same umbrella with significant comorbidities and approach NAFLD/MAFLD/DAFLD holistically rather than facing NAFLD as a separate entity. Future studies are likely to provide us the necessary prerequisites for designing more appropriate clinical trials to

identify finely tailored diagnostic and treatment strategies for our patients.

REFERENCES

1 **Ludwig J**, Viggiano TR, McGill DB, Oh BJ. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clin Proc* 1980; **55**: 434-438 [PMID: 7382552]

2 **Eslam M**, Sanyal AJ, George J; International Consensus Panel. MAFLD: A Consensus-Driven Proposed Nomenclature for Metabolic Associated Fatty Liver Disease. *Gastroenterology* 2020; **158**: 1999-2014.e1 [PMID: 32044314 DOI: 10.1053/j.gastro.2019.11.312]

3 **Singh SP**, Anirvan P, Reddy KR, Conjeevaram HS, Marchesini G, Rinella ME, Madan K, Petroni ML, Al-Mahtab M, Caldwell SH, Aithal GP, Hamid SS, Farrell GC, Satapathy SK, Duseja A, Acharya SK, Dassanayake AS, Goh KL. Non-alcoholic fatty liver disease: Not time for an obituary just yet! *J Hepatol* 2021; **74**: 972-974 [PMID: 33340575 DOI: 10.1016/j.jhep.2020.10.015]

4 **Yki-Järvinen H**, Luukkonen PK. Heterogeneity of non-alcoholic fatty liver disease. *Liver Int* 2015; **35**: 2498-2500 [PMID: 26386359 DOI: 10.1111/liv.12970]

5 **Mittal S**, El-Serag HB, Sada YH, Kanwal F, Duan Z, Temple S, May SB, Kramer JR, Richardson PA, Davila JA. Hepatocellular Carcinoma in the Absence of Cirrhosis in United States Veterans is Associated With Nonalcoholic Fatty Liver Disease. *Clin Gastroenterol Hepatol* 2016; **14**: 124-31.e1 [PMID: 26196445 DOI: 10.1016/j.cgh.2015.07.019]

6 **Friedman SL**, Neuschwander-Tetri BA, Rinella M, Sanyal AJ. Mechanisms of NAFLD development and therapeutic strategies. *Nat Med* 2018; **24**: 908-922 [PMID: 29967350 DOI: 10.1038/s41591-018-0104-9]

7 **Åberg F**, Helenius-Hietala J, Puukka P, Färkkilä M, Jula A. Interaction between alcohol consumption and metabolic syndrome in predicting severe liver disease in the general population. *Hepatology* 2018; **67**: 2141-2149 [PMID: 29164643 DOI: 10.1002/hep.29631]

8 **Shiha G**, Alswat K, Al Khatry M, Sharara AI, Örmeci N, Waked I, Benazzouz M, Al-Ali F, Hamed AE, Hamoudi W, Attia D, Derbala M, Sharaf-Eldin M, Al-Busafi SA, Zaky S, Bamakhrama K, Ibrahim N, Ajlouni Y, Sabbah M, Salama M, Anushiravani A, Afredj N, Barakat S, Hashim A, Fouad Y, Soliman R. Nomenclature and definition of metabolic-associated fatty liver disease: a consensus from the Middle East and north Africa. *Lancet Gastroenterol Hepatol* 2021; **6**: 57-64 [PMID: 33181119 DOI: 10.1016/S2468-1253(20)30213-2]

9 **Yuan J**, Chen C, Cui J, Lu J, Yan C, Wei X, Zhao X, Li N, Li S, Xue G, Cheng W, Li B, Li H, Lin W, Tian C, Zhao J, Han J, An D, Zhang Q, Wei H, Zheng M, Ma X, Li W, Chen X, Zhang Z, Zeng H, Ying S, Wu J, Yang R, Liu D. Fatty Liver Disease Caused by High-Alcohol-Producing Klebsiella pneumoniae. *Cell Metab* 2019; **30**: 675-688.e7 [PMID: 31543403 DOI: 10.1016/j.cmet.2019.08.018]

10 **Jepsen P**, Vilstrup H, Mellemkjaer L, Thulstrup AM, Olsen JH, Baron JA, Sørensen HT. Prognosis of patients with a diagnosis of fatty liver--a registry-based cohort study. *Hepatogastroenterology* 2003; **50**: 2101-2104 [PMID: 14696473]

11 **Loria P**, Lonardo A, Carulli N. Should nonalcoholic fatty liver disease be renamed? *Dig Dis* 2005; **23**: 72-82 [PMID: 15920328 DOI: 10.1159/000084728]

12 **Polyzos SA**, Mantzoros CS. Making progress in nonalcoholic fatty liver disease (NAFLD) as we are transitioning from the era of NAFLD to dys-metabolism associated fatty liver disease (DAFLD). *Metabolism* 2020; **111S**: 154318 [PMID: 32707055 DOI: 10.1016/j.metabol.2020.154318]

13 **Eslam M**, Newsome PN, Sarin SK, Anstee QM, Targher G, Romero-Gomez M, Zelber-Sagi S, Wai-Sun Wong V, Dufour JF, Schattenberg JM, Kawaguchi T, Arrese M, Valenti L, Shiha G, Tiribelli C, Yki-Järvinen H, Fan JG, Grønbæk H, Yilmaz Y, Cortez-Pinto H, Oliveira CP, Bedossa P, Adams LA, Zheng MH, Fouad Y, Chan WK, Mendez-Sanchez N, Ahn SH, Castera L, Bugianesi E, Ratziu V, George J. A new definition for metabolic dysfunction-associated fatty liver disease: An international expert consensus statement. *J Hepatol* 2020; **73**: 202-209 [PMID: 32278004 DOI: 10.1016/j.jhep.2020.03.039]

14 **Lin S**, Huang J, Wang M, Kumar R, Liu Y, Liu S, Wu Y, Wang X, Zhu Y. Comparison of MAFLD and NAFLD diagnostic criteria in real world. *Liver Int* 2020; **40**: 2082-2089 [PMID: 32478487 DOI: 10.1111/liv.14548]

15 **Younossi ZM**, Rinella ME, Sanyal AJ, Harrison SA, Brunt EM, Goodman Z, Cohen DE, Loomba R. From NAFLD to MAFLD: Implications of a Premature Change in Terminology. *Hepatology* 2021; **73**: 1194-1198 [PMID: 32544255 DOI: 10.1002/hep.31420]

16 **Dongiovanni P**, Paolini E, Corsini A, Sirtori CR, Ruscica M. Nonalcoholic fatty liver disease or metabolic dysfunction-associated fatty liver disease diagnoses and cardiovascular diseases: From epidemiology to drug approaches. *Eur J Clin Invest* 2021; **51**: e13519 [PMID: 33583033 DOI: 10.1111/eci.13519]

17 **Cai J**, Zhang XJ, Ji YX, Zhang P, She ZG, Li H. Nonalcoholic Fatty Liver Disease Pandemic Fuels the Upsurge in Cardiovascular Diseases. *Circ Res* 2020; **126**: 679-704 [PMID: 32105577 DOI: 10.1161/CIRCRESAHA.119.316337]

18 **Targher G**, Corey KE, Byrne CD. NAFLD, and cardiovascular and cardiac diseases: Factors influencing risk, prediction and treatment. *Diabetes Metab* 2021; **47**: 101215 [PMID: 33296704 DOI: 10.1016/j.diabet.2020.101215]

19 **Shroff H**, VanWagner LB. Cardiovascular Disease in Nonalcoholic Steatohepatitis: Screening and

Management. *Curr Hepatol Rep* 2020; **19**: 315-326 [PMID: 33585157 DOI: 10.1007/s11901-020-00530-0]

20   **Frith J**, Day CP, Henderson E, Burt AD, Newton JL. Non-alcoholic fatty liver disease in older people. *Gerontology* 2009; **55**: 607-613 [PMID: 19690397 DOI: 10.1159/000235677]

21   **Frith J**, Jones D, Newton JL. Chronic liver disease in an ageing population. *Age Ageing* 2009; **38**: 11-18 [PMID: 19029099 DOI: 10.1093/ageing/afn242]

22   **Donati B**, Valenti L. Telomeres, NAFLD and Chronic Liver Disease. *Int J Mol Sci* 2016; **17**: 383 [PMID: 26999107 DOI: 10.3390/ijms17030383]

23   **Kuk JL**, Saunders TJ, Davidson LE, Ross R. Age-related changes in total and regional fat distribution. *Ageing Res Rev* 2009; **8**: 339-348 [PMID: 19576300 DOI: 10.1016/j.arr.2009.06.001]

24   **Lonardo A**, Nascimbeni F, Ballestri S, Fairweather D, Win S, Than TA, Abdelmalek MF, Suzuki A. Sex Differences in Nonalcoholic Fatty Liver Disease: State of the Art and Identification of Research Gaps. *Hepatology* 2019; **70**: 1457-1469 [PMID: 30924946 DOI: 10.1002/hep.30626]

25   **Lovejoy JC**, Champagne CM, de Jonge L, Xie H, Smith SR. Increased visceral fat and decreased energy expenditure during the menopausal transition. *Int J Obes (Lond)* 2008; **32**: 949-958 [PMID: 18332882 DOI: 10.1038/ijo.2008.25]

26   **Kurt Z**, Barrere-Cain R, LaGuardia J, Mehrabian M, Pan C, Hui ST, Norheim F, Zhou Z, Hasin Y, Lusis AJ, Yang X. Tissue-specific pathways and networks underlying sexual dimorphism in non-alcoholic fatty liver disease. *Biol Sex Differ* 2018; **9**: 46 [PMID: 30343673 DOI: 10.1186/s13293-018-0205-7]

27   **Cvitanović Tomaš T**, Urlep Ž, Moškon M, Mraz M, Rozman D. *LiverSex* Computational Model: Sexual Aspects in Hepatic Metabolism and Abnormalities. *Front Physiol* 2018; **9**: 360 [PMID: 29706895 DOI: 10.3389/fphys.2018.00360]

28   **Yang JD**, Abdelmalek MF, Pang H, Guy CD, Smith AD, Diehl AM, Suzuki A. Gender and menopause impact severity of fibrosis among patients with nonalcoholic steatohepatitis. *Hepatology* 2014; **59**: 1406-1414 [PMID: 24123276 DOI: 10.1002/hep.26761]

29   **Klair JS**, Yang JD, Abdelmalek MF, Guy CD, Gill RM, Yates K, Unalp-Arida A, Lavine JE, Clark JM, Diehl AM, Suzuki A; Nonalcoholic Steatohepatitis Clinical Research Network. A longer duration of estrogen deficiency increases fibrosis risk among postmenopausal women with nonalcoholic fatty liver disease. *Hepatology* 2016; **64**: 85-91 [PMID: 26919573 DOI: 10.1002/hep.28514]

30   **Rich NE**, Oji S, Mufti AR, Browning JD, Parikh ND, Odewole M, Mayo H, Singal AG. Racial and Ethnic Disparities in Nonalcoholic Fatty Liver Disease Prevalence, Severity, and Outcomes in the United States: A Systematic Review and Meta-analysis. *Clin Gastroenterol Hepatol* 2018; **16**: 198-210.e2 [PMID: 28970148 DOI: 10.1016/j.cgh.2017.09.041]

31   **Romeo S**, Kozlitina J, Xing C, Pertsemlidis A, Cox D, Pennacchio LA, Boerwinkle E, Cohen JC, Hobbs HH. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. *Nat Genet* 2008; **40**: 1461-1465 [PMID: 18820647 DOI: 10.1038/ng.257]

32   **Mohanty SR**, Troy TN, Huo D, O'Brien BL, Jensen DM, Hart J. Influence of ethnicity on histological differences in non-alcoholic fatty liver disease. *J Hepatol* 2009; **50**: 797-804 [PMID: 19231016 DOI: 10.1016/j.jhep.2008.11.017]

33   **Tabibian JH**, Lazo M, Durazo FA, Yeh HC, Tong MJ, Clark JM. Nonalcoholic fatty liver disease across ethno-racial groups: do Asian-American adults represent a new at-risk population? *J Gastroenterol Hepatol* 2011; **26**: 501-509 [PMID: 21332546 DOI: 10.1111/j.1440-1746.2010.06443.x]

34   **Shen J**, Wong GL, Chan HL, Chan HY, Yeung DK, Chan RS, Chim AM, Chan AW, Choi PC, Woo J, Chu WC, Wong VW. PNPLA3 gene polymorphism accounts for fatty liver in community subjects without metabolic syndrome. *Aliment Pharmacol Ther* 2014; **39**: 532-539 [PMID: 24417250 DOI: 10.1111/apt.12609]

35   **Trovato FM**, Catalano D, Martines GF, Pace P, Trovato GM. Mediterranean diet and non-alcoholic fatty liver disease: the need of extended and comprehensive interventions. *Clin Nutr* 2015; **34**: 86-88 [PMID: 24529325 DOI: 10.1016/j.clnu.2014.01.018]

36   **David LA**, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Ling AV, Devlin AS, Varma Y, Fischbach MA, Biddinger SB, Dutton RJ, Turnbaugh PJ. Diet rapidly and reproducibly alters the human gut microbiome. *Nature* 2014; **505**: 559-563 [PMID: 24336217 DOI: 10.1038/nature12820]

37   **Loomba R**, Seguritan V, Li W, Long T, Klitgord N, Bhatt A, Dulai PS, Caussy C, Bettencourt R, Highlander SK, Jones MB, Sirlin CB, Schnabl B, Brinkac L, Schork N, Chen CH, Brenner DA, Biggs W, Yooseph S, Venter JC, Nelson KE. Gut Microbiome-Based Metagenomic Signature for Non-invasive Detection of Advanced Fibrosis in Human Nonalcoholic Fatty Liver Disease. *Cell Metab* 2017; **25**: 1054-1062.e5 [PMID: 28467925 DOI: 10.1016/j.cmet.2017.04.001]

38   **Ilan Y**. Leaky gut and the liver: a role for bacterial translocation in nonalcoholic steatohepatitis. *World J Gastroenterol* 2012; **18**: 2609-2618 [PMID: 22690069 DOI: 10.3748/wjg.v18.i21.2609]

39   **Jialal I**, Kaur H, Devaraj S. Toll-like receptor status in obesity and metabolic syndrome: a translational perspective. *J Clin Endocrinol Metab* 2014; **99**: 39-48 [PMID: 24187406 DOI: 10.1210/jc.2013-3092]

40   **Hall AB**, Tolonen AC, Xavier RJ. Human genetic variation and the gut microbiome in disease. *Nat Rev Genet* 2017; **18**: 690-699 [PMID: 28824167 DOI: 10.1038/nrg.2017.63]

41   **Brooks AW**, Priya S, Blekhman R, Bordenstein SR. Gut microbiota diversity across ethnicities in

the United States. *PLoS Biol* 2018; **16**: e2006842 [PMID: 30513082 DOI: 10.1371/journal.pbio.2006842]

42 **Loos RJF**, Kilpeläinen TO. Genes that make you fat, but keep you healthy. *J Intern Med* 2018; **284**: 450-463 [PMID: 30144199 DOI: 10.1111/joim.12827]

43 **Eckel N**, Li Y, Kuxhaus O, Stefan N, Hu FB, Schulze MB. Transition from metabolic healthy to unhealthy phenotypes and association with cardiovascular disease risk across BMI categories in 90 257 women (the Nurses' Health Study): 30 year follow-up from a prospective cohort study. *Lancet Diabetes Endocrinol* 2018; **6**: 714-724 [PMID: 29859908 DOI: 10.1016/S2213-8587(18)30137-2]

44 **Agbim U**, Carr RM, Pickett-Blakely O, Dagogo-Jack S. Ethnic Disparities in Adiposity: Focus on Non-alcoholic Fatty Liver Disease, Visceral, and Generalized Obesity. *Curr Obes Rep* 2019; **8**: 243-254 [PMID: 31144261 DOI: 10.1007/s13679-019-00349-x]

45 **Ding C**, Chan Z, Magkos F. Lean, but not healthy: the 'metabolically obese, normal-weight' phenotype. *Curr Opin Clin Nutr Metab Care* 2016; **19**: 408-417 [PMID: 27552473 DOI: 10.1097/MCO.0000000000000317]

46 **Chen F**, Esmaili S, Rogers GB, Bugianesi E, Petta S, Marchesini G, Bayoumi A, Metwally M, Azardaryany MK, Coulter S, Choo JM, Younes R, Rosso C, Liddle C, Adams LA, Craxì A, George J, Eslam M. Lean NAFLD: A Distinct Entity Shaped by Differential Metabolic Adaptation. *Hepatology* 2020; **71**: 1213-1227 [PMID: 31442319 DOI: 10.1002/hep.30908]

47 **Park JH**, Cho B, Kwon H, Prilutsky D, Yun JM, Choi HC, Hwang KB, Lee IH, Kim JI, Kong SW. I148M variant in PNPLA3 reduces central adiposity and metabolic disease risks while increasing nonalcoholic fatty liver disease. *Liver Int* 2015; **35**: 2537-2546 [PMID: 26148225 DOI: 10.1111/liv.12909]

48 **Hoang SA**, Oseini A, Feaver RE, Cole BK, Asgharpour A, Vincent R, Siddiqui M, Lawson MJ, Day NC, Taylor JM, Wamhoff BR, Mirshahi F, Contos MJ, Idowu M, Sanyal AJ. Gene Expression Predicts Histological Severity and Reveals Distinct Molecular Profiles of Nonalcoholic Fatty Liver Disease. *Sci Rep* 2019; **9**: 12541 [PMID: 31467298 DOI: 10.1038/s41598-019-48746-5]

49 **Eslam M**, Valenti L, Romeo S. Genetics and epigenetics of NAFLD and NASH: Clinical impact. *J Hepatol* 2018; **68**: 268-279 [PMID: 29122391 DOI: 10.1016/j.jhep.2017.09.003]

50 **Liu YL**, Reeves HL, Burt AD, Tiniakos D, McPherson S, Leathart JB, Allison ME, Alexander GJ, Piguet AC, Anty R, Donaldson P, Aithal GP, Francque S, Van Gaal L, Clement K, Ratziu V, Dufour JF, Day CP, Daly AK, Anstee QM. TM6SF2 rs58542926 influences hepatic fibrosis progression in patients with non-alcoholic fatty liver disease. *Nat Commun* 2014; **5**: 4309 [PMID: 24978903 DOI: 10.1038/ncomms5309]

51 **Diogo D**, Tian C, Franklin CS, Alanne-Kinnunen M, March M, Spencer CCA, Vangjeli C, Weale ME, Mattsson H, Kilpeläinen E, Sleiman PMA, Reilly DF, McElwee J, Maranville JC, Chatterjee AK, Bhandari A, Nguyen KH, Estrada K, Reeve MP, Hutz J, Bing N, John S, MacArthur DG, Salomaa V, Ripatti S, Hakonarson H, Daly MJ, Palotie A, Hinds DA, Donnelly P, Fox CS, Day-Williams AG, Plenge RM, Runz H. Phenome-wide association studies across large population cohorts support drug target validation. *Nat Commun* 2018; **9**: 4285 [PMID: 30327483 DOI: 10.1038/s41467-018-06540-3]

52 **Mahajan A**, Wessel J, Willems SM, Zhao W, Robertson NR, Chu AY, Gan W, Kitajima H, Taliun D, Rayner NW, Guo X, Lu Y, Li M, Jensen RA, Hu Y, Huo S, Lohman KK, Zhang W, Cook JP, Prins BP, Flannick J, Grarup N, Trubetskoy VV, Kravic J, Kim YJ, Rybin DV, Yaghootkar H, Müller-Nurasyid M, Meidtner K, Li-Gao R, Varga TV, Marten J, Li J, Smith AV, An P, Ligthart S, Gustafsson S, Malerba G, Demirkan A, Tajes JF, Steinthorsdottir V, Wuttke M, Lecoeur C, Preuss M, Bielak LF, Graff M, Highland HM, Justice AE, Liu DJ, Marouli E, Peloso GM, Warren HR; ExomeBP Consortium; MAGIC Consortium; GIANT Consortium, Afaq S, Afzal S, Ahlqvist E, Almgren P, Amin N, Bang LB, Bertoni AG, Bombieri C, Bork-Jensen J, Brandslund I, Brody JA, Burtt NP, Canouil M, Chen YI, Cho YS, Christensen C, Eastwood SV, Eckardt KU, Fischer K, Gambaro G, Giedraitis V, Grove ML, de Haan HG, Hackinger S, Hai Y, Han S, Tybjærg-Hansen A, Hivert MF, Isomaa B, Jäger S, Jørgensen ME, Jørgensen T, Käräjämäki A, Kim BJ, Kim SS, Koistinen HA, Kovacs P, Kriebel J, Kronenberg F, Läll K, Lange LA, Lee JJ, Lehne B, Li H, Lin KH, Linneberg A, Liu CT, Liu J, Loh M, Mägi R, Mamakou V, McKean-Cowdin R, Nadkarni G, Neville M, Nielsen SF, Ntalla I, Peyser PA, Rathmann W, Rice K, Rich SS, Rode L, Rolandsson O, Schönherr S, Selvin E, Small KS, Stančáková A, Surendran P, Taylor KD, Teslovich TM, Thorand B, Thorleifsson G, Tin A, Tönjes A, Varbo A, Witte DR, Wood AR, Yajnik P, Yao J, Yengo L, Young R, Amouyel P, Boeing H, Boerwinkle E, Bottinger EP, Chowdhury R, Collins FS, Dedoussis G, Dehghan A, Deloukas P, Ferrario MM, Ferrières J, Florez JC, Frossard P, Gudnason V, Harris TB, Heckbert SR, Howson JMM, Ingelsson M, Kathiresan S, Kee F, Kuusisto J, Langenberg C, Launer LJ, Lindgren CM, Männistö S, Meitinger T, Melander O, Mohlke KL, Moitry M, Morris AD, Murray AD, de Mutsert R, Orho-Melander M, Owen KR, Perola M, Peters A, Province MA, Rasheed A, Ridker PM, Rivadineira F, Rosendaal FR, Rosengren AH, Salomaa V, Sheu WH, Sladek R, Smith BH, Strauch K, Uitterlinden AG, Varma R, Willer CJ, Blüher M, Butterworth AS, Chambers JC, Chasman DI, Danesh J, van Duijn C, Dupuis J, Franco OH, Franks PW, Froguel P, Grallert H, Groop L, Han BG, Hansen T, Hattersley AT, Hayward C, Ingelsson E, Kardia SLR, Karpe F, Kooner JS, Köttgen A, Kuulasmaa K, Laakso M, Lin X, Lind L, Liu Y, Loos RJF, Marchini J, Metspalu A, Mook-Kanamori D, Nordestgaard BG, Palmer CNA, Pankow JS, Pedersen O, Psaty BM, Rauramaa R, Sattar N, Schulze MB, Soranzo N, Spector TD, Stefansson K, Stumvoll M, Thorsteinsdottir U, Tuomi T, Tuomilehto J, Wareham NJ, Wilson JG, Zeggini E, Scott RA,

Barroso I, Frayling TM, Goodarzi MO, Meigs JB, Boehnke M, Saleheen D, Morris AP, Rotter JI, McCarthy MI. Refining the accuracy of validated target identification through coding variant fine-mapping in type 2 diabetes. *Nat Genet* 2018; **50**: 559-571 [PMID: 29632382 DOI: 10.1038/s41588-018-0084-1]

53   **Simons N**, Isaacs A, Koek GH, Kuč S, Schaper NC, Brouwers MCGJ. PNPLA3, TM6SF2, and MBOAT7 Genotypes and Coronary Artery Disease. *Gastroenterology* 2017; **152**: 912-913 [PMID: 28157516 DOI: 10.1053/j.gastro.2016.12.020]

54   **Abul-Husn NS**, Cheng X, Li AH, Xin Y, Schurmann C, Stevis P, Liu Y, Kozlitina J, Stender S, Wood GC, Stepanchick AN, Still MD, McCarthy S, O'Dushlaine C, Packer JS, Balasubramanian S, Gosalia N, Esopi D, Kim SY, Mukherjee S, Lopez AE, Fuller ED, Penn J, Chu X, Luo JZ, Mirshahi UL, Carey DJ, Still CD, Feldman MD, Small A, Damrauer SM, Rader DJ, Zambrowicz B, Olson W, Murphy AJ, Borecki IB, Shuldiner AR, Reid JG, Overton JD, Yancopoulos GD, Hobbs HH, Cohen JC, Gottesman O, Teslovich TM, Baras A, Mirshahi T, Gromada J, Dewey FE. A Protein-Truncating HSD17B13 Variant and Protection from Chronic Liver Disease. *N Engl J Med* 2018; **378**: 1096-1106 [PMID: 29562163 DOI: 10.1056/NEJMoa1712191]

55   **Pirola CJ**, Fernández Gianotti T, Castaño GO, Mallardi P, San Martino J, Mora Gonzalez Lopez Ledesma M, Flichman D, Mirshahi F, Sanyal AJ, Sookoian S. Circulating microRNA signature in non-alcoholic fatty liver disease: from serum non-coding RNAs to liver histology and disease pathogenesis. *Gut* 2015; **64**: 800-812 [PMID: 24973316 DOI: 10.1136/gutjnl-2014-306996]

56   **Sookoian S**, Rohr C, Salatino A, Dopazo H, Fernandez Gianotti T, Castaño GO, Pirola CJ. Genetic variation in long noncoding RNAs and the risk of nonalcoholic fatty liver disease. *Oncotarget* 2017; **8**: 22917-22926 [PMID: 28206970 DOI: 10.18632/oncotarget.15286]

57   **Ahrens M**, Ammerpohl O, von Schönfels W, Kolarova J, Bens S, Itzel T, Teufel A, Herrmann A, Brosch M, Hinrichsen H, Erhart W, Egberts J, Sipos B, Schreiber S, Häsler R, Stickel F, Becker T, Krawczak M, Röcken C, Siebert R, Schafmayer C, Hampe J. DNA methylation analysis in nonalcoholic fatty liver disease suggests distinct disease-specific and remodeling signatures after bariatric surgery. *Cell Metab* 2013; **18**: 296-302 [PMID: 23931760 DOI: 10.1016/j.cmet.2013.07.004]

58   **Hardy T**, Zeybel M, Day CP, Dipper C, Masson S, McPherson S, Henderson E, Tiniakos D, White S, French J, Mann DA, Anstee QM, Mann J. Plasma DNA methylation: a potential biomarker for stratification of liver fibrosis in non-alcoholic fatty liver disease. *Gut* 2017; **66**: 1321-1328 [PMID: 27002005 DOI: 10.1136/gutjnl-2016-311526]

59   **Kitamoto T**, Kitamoto A, Ogawa Y, Honda Y, Imajo K, Saito S, Yoneda M, Nakamura T, Nakajima A, Hotta K. Targeted-bisulfite sequence analysis of the methylation of CpG islands in genes encoding PNPLA3, SAMM50, and PARVB of patients with non-alcoholic fatty liver disease. *J Hepatol* 2015; **63**: 494-502 [PMID: 25776890 DOI: 10.1016/j.jhep.2015.02.049]

60   **Baker PR 2nd**, Friedman JE. Mitochondrial role in the neonatal predisposition to developing nonalcoholic fatty liver disease. *J Clin Invest* 2018; **128**: 3692-3703 [PMID: 30168806 DOI: 10.1172/JCI120846]

61   **Suter MA**, Ma J, Vuguin PM, Hartil K, Fiallo A, Harris RA, Charron MJ, Aagaard KM. In utero exposure to a maternal high-fat diet alters the epigenetic histone code in a murine model. *Am J Obstet Gynecol* 2014; **210**: 463.e1-463.e11 [PMID: 24793723 DOI: 10.1016/j.ajog.2014.01.045]

62   **Gemma C**, Sookoian S, Alvariñas J, García SI, Quintana L, Kanevsky D, González CD, Pirola CJ. Maternal pregestational BMI is associated with methylation of the PPARGC1A promoter in newborns. *Obesity (Silver Spring)* 2009; **17**: 1032-1039 [PMID: 19148128 DOI: 10.1038/oby.2008.605]

63   **Struben VM**, Hespenheide EE, Caldwell SH. Nonalcoholic steatohepatitis and cryptogenic cirrhosis within kindreds. *Am J Med* 2000; **108**: 9-13 [PMID: 11059435 DOI: 10.1016/s0002-9343(99)00315-0]

64   **Caussy C**, Soni M, Cui J, Bettencourt R, Schork N, Chen CH, Ikhwan MA, Bassirian S, Cepin S, Gonzalez MP, Mendler M, Kono Y, Vodkin I, Mekeel K, Haldorson J, Hemming A, Andrews B, Salotti J, Richards L, Brenner DA, Sirlin CB, Loomba R; Familial NAFLD Cirrhosis Research Consortium. Nonalcoholic fatty liver disease with cirrhosis increases familial risk for advanced fibrosis. *J Clin Invest* 2017; **127**: 2697-2704 [PMID: 28628033 DOI: 10.1172/JCI93465]

65   **Boyle M**, Masson S, Anstee QM. The bidirectional impacts of alcohol consumption and the metabolic syndrome: Cofactors for progressive fatty liver disease. *J Hepatol* 2018; **68**: 251-267 [PMID: 29113910 DOI: 10.1016/j.jhep.2017.11.006]

66   **Rehm J**, Taylor B, Mohapatra S, Irving H, Baliunas D, Patra J, Roerecke M. Alcohol as a risk factor for liver cirrhosis: a systematic review and meta-analysis. *Drug Alcohol Rev* 2010; **29**: 437-445 [PMID: 20636661 DOI: 10.1111/j.1465-3362.2009.00153.x]

67   **Mato JM**, Alonso C, Noureddin M, Lu SC. Biomarkers and subtypes of deranged lipid metabolism in non-alcoholic fatty liver disease. *World J Gastroenterol* 2019; **25**: 3009-3020 [PMID: 31293337 DOI: 10.3748/wjg.v25.i24.3009]

68   **Vandromme M**, Jun T, Perumalswami P, Dudley JT, Branch A, Li L. Automated phenotyping of patients with non-alcoholic fatty liver disease reveals clinically relevant disease subtypes. *Pac Symp Biocomput* 2020; **25**: 91-102 [PMID: 31797589]

69   **Younossi ZM**, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* 2016; **64**: 73-84 [PMID: 26707365 DOI: 10.1002/hep.28431]

70   **Li J**, Zou B, Yeo YH, Feng Y, Xie X, Lee DH, Fujii H, Wu Y, Kam LY, Ji F, Li X, Chien N, Wei M, Ogawa E, Zhao C, Wu X, Stave CD, Henry L, Barnett S, Takahashi H, Furusyo N, Eguchi Y, Hsu YC, Lee TY, Ren W, Qin C, Jun DW, Toyoda H, Wong VW, Cheung R, Zhu Q, Nguyen MH. Prevalence, incidence, and outcome of non-alcoholic fatty liver disease in Asia, 1999-2019: a systematic review and meta-analysis. *Lancet Gastroenterol Hepatol* 2019; **4**: 389-398 [PMID: 30902670 DOI: 10.1016/S2468-1253(19)30039-1]

71   **Fan JG**, Kim SU, Wong VW. New trends on obesity and NAFLD in Asia. *J Hepatol* 2017; **67**: 862-873 [PMID: 28642059 DOI: 10.1016/j.jhep.2017.06.003]

72   **Mohan V**, Farooq S, Deepa M, Ravikumar R, Pitchumoni CS. Prevalence of non-alcoholic fatty liver disease in urban south Indians in relation to different grades of glucose intolerance and metabolic syndrome. *Diabetes Res Clin Pract* 2009; **84**: 84-91 [PMID: 19168251 DOI: 10.1016/j.diabres.2008.11.039]

73   **Lin YC**, Chang PF, Hu FC, Yang WS, Chang MH, Ni YH. A common variant in the PNPLA3 gene is a risk factor for non-alcoholic fatty liver disease in obese Taiwanese children. *J Pediatr* 2011; **158**: 740-744 [PMID: 21168155 DOI: 10.1016/j.jpeds.2010.11.016]

74   **Bhatt SP**, Nigam P, Misra A, Guleria R, Pandey RM, Pasha MA. Genetic variation in the patatin-like phospholipase domain-containing protein-3 (PNPLA-3) gene in Asian Indians with nonalcoholic fatty liver disease. *Metab Syndr Relat Disord* 2013; **11**: 329-335 [PMID: 23734760 DOI: 10.1089/met.2012.0064]

75   **Zain SM**, Mohamed Z, Mahadeva S, Rampal S, Basu RC, Cheah PL, Salim A, Mohamed R. Susceptibility and gene interaction study of the angiotensin II type 1 receptor (AGTR1) gene polymorphisms with non-alcoholic fatty liver disease in a multi-ethnic population. *PLoS One* 2013; **8**: e58538 [PMID: 23484035 DOI: 10.1371/journal.pone.0058538]

76   **Yang Z**, Wen J, Tao X, Lu B, Du Y, Wang M, Wang X, Zhang W, Gong W, Ling C, Wu S, Hu R. Genetic variation in the GCKR gene is associated with non-alcoholic fatty liver disease in Chinese people. *Mol Biol Rep* 2011; **38**: 1145-1150 [PMID: 20625834 DOI: 10.1007/s11033-010-0212-1]

77   **Cai W**, Weng DH, Yan P, Lin YT, Dong ZH, Mailamuguli, Yao H. Genetic polymorphisms associated with nonalcoholic fatty liver disease in Uyghur population: a case-control study and meta-analysis. *Lipids Health Dis* 2019; **18**: 14 [PMID: 30646922 DOI: 10.1186/s12944-018-0877-3]

78   **Zhai HL**, Wang NJ, Han B, Li Q, Chen Y, Zhu CF, Chen YC, Xia FZ, Cang Z, Zhu CX, Lu M, Lu YL. Low vitamin D levels and non-alcoholic fatty liver disease, evidence for their independent association in men in East China: a cross-sectional study (Survey on Prevalence in East China for Metabolic Diseases and Risk Factors (SPECT-China)). *Br J Nutr* 2016; **115**: 1352-1359 [PMID: 26888280 DOI: 10.1017/S0007114516000386]

79   **Lim S**, Kim JH, Yoon JW, Kang SM, Choi SH, Park YJ, Kim KW, Lim JY, Park KS, Jang HC. Sarcopenic obesity: prevalence and association with metabolic syndrome in the Korean Longitudinal Study on Health and Aging (KLoSHA). *Diabetes Care* 2010; **33**: 1652-1654 [PMID: 20460442 DOI: 10.2337/dc10-0107]

80   **Kawamura Y**, Arase Y, Ikeda K, Seko Y, Imai N, Hosaka T, Kobayashi M, Saitoh S, Sezaki H, Akuta N, Suzuki F, Suzuki Y, Ohmoto Y, Amakawa K, Tsuji H, Kumada H. Large-scale long-term follow-up study of Japanese patients with non-alcoholic Fatty liver disease for the onset of hepatocellular carcinoma. *Am J Gastroenterol* 2012; **107**: 253-261 [PMID: 22008893 DOI: 10.1038/ajg.2011.327]

81   **Michitaka K**, Nishiguchi S, Aoyagi Y, Hiasa Y, Tokumoto Y, Onji M; Japan Etiology of Liver Cirrhosis Study Group. Etiology of liver cirrhosis in Japan: a nationwide survey. *J Gastroenterol* 2010; **45**: 86-94 [PMID: 19789837 DOI: 10.1007/s00535-009-0128-5]

82   **Wong SW**, Chan WK. Epidemiology of non-alcoholic fatty liver disease in Asia. *Indian J Gastroenterol* 2020; **39**: 1-8 [PMID: 32152903 DOI: 10.1007/s12664-020-01018-x]

83   **Alazawi W**, Mathur R, Abeysekera K, Hull S, Boomla K, Robson J, Foster GR. Ethnicity and the diagnosis gap in liver disease: a population-based study. *Br J Gen Pract* 2014; **64**: e694-e702 [PMID: 25348993 DOI: 10.3399/bjgp14X682273]

84   **Soleimani D**, Ranjbar G, Rezvani R, Goshayeshi L, Razmpour F, Nematy M. Dietary patterns in relation to hepatic fibrosis among patients with nonalcoholic fatty liver disease. *Diabetes Metab Syndr Obes* 2019; **12**: 315-324 [PMID: 30881075 DOI: 10.2147/DMSO.S198744]

85   **Huang Y**, Cohen JC, Hobbs HH. Expression and characterization of a PNPLA3 protein isoform (I148M) associated with nonalcoholic fatty liver disease. *J Biol Chem* 2011; **286**: 37085-37093 [PMID: 21878620 DOI: 10.1074/jbc.M111.290114]

86   **Kawaguchi T**, Sumida Y, Umemura A, Matsuo K, Takahashi M, Takamura T, Yasui K, Saibara T, Hashimoto E, Kawanaka M, Watanabe S, Kawata S, Imai Y, Kokubo M, Shima T, Park H, Tanaka H, Tajima K, Yamada R, Matsuda F, Okanoue T; Japan Study Group of Nonalcoholic Fatty Liver Disease. Genetic polymorphisms of the human PNPLA3 gene are strongly associated with severity of non-alcoholic fatty liver disease in Japanese. *PLoS One* 2012; **7**: e38322 [PMID: 22719876 DOI: 10.1371/journal.pone.0038322]

87   **Zain SM**, Mohamed R, Mahadeva S, Cheah PL, Rampal S, Basu RC, Mohamed Z. A multi-ethnic study of a PNPLA3 gene variant and its association with disease severity in non-alcoholic fatty liver disease. *Hum Genet* 2012; **131**: 1145-1152 [PMID: 22258181 DOI: 10.1007/s00439-012-1141-y]

88   **Kozlitina J**, Smagris E, Stender S, Nordestgaard BG, Zhou HH, Tybjærg-Hansen A, Vogt TF, Hobbs HH, Cohen JC. Exome-wide association study identifies a TM6SF2 variant that confers susceptibility to nonalcoholic fatty liver disease. *Nat Genet* 2014; **46**: 352-356 [PMID: 24531328

DOI: 10.1038/ng.2901]

89   **Goffredo M**, Caprio S, Feldstein AE, D'Adamo E, Shaw MM, Pierpont B, Savoye M, Zhao H, Bale AE, Santoro N. Role of TM6SF2 rs58542926 in the pathogenesis of nonalcoholic pediatric fatty liver disease: A multiethnic study. *Hepatology* 2016; **63**: 117-125 [PMID: 26457389 DOI: 10.1002/hep.28283]

90   **Anderson EL**, Howe LD, Jones HE, Higgins JP, Lawlor DA, Fraser A. The Prevalence of Non-Alcoholic Fatty Liver Disease in Children and Adolescents: A Systematic Review and Meta-Analysis. *PLoS One* 2015; **10**: e0140908 [PMID: 26512983 DOI: 10.1371/journal.pone.0140908]

91   **Brumbaugh DE**, Tearse P, Cree-Green M, Fenton LZ, Brown M, Scherzinger A, Reynolds R, Alston M, Hoffman C, Pan Z, Friedman JE, Barbour LA. Intrahepatic fat is increased in the neonatal offspring of obese women with gestational diabetes. *J Pediatr* 2013; **162**: 930-6.e1 [PMID: 23260099 DOI: 10.1016/j.jpeds.2012.11.017]

92   **Modi N**, Murgasova D, Ruager-Martin R, Thomas EL, Hyde MJ, Gale C, Santhakumaran S, Doré CJ, Alavi A, Bell JD. The influence of maternal body mass index on infant adiposity and hepatic lipid content. *Pediatr Res* 2011; **70**: 287-291 [PMID: 21629154 DOI: 10.1203/PDR.0b013e318225f9b1]

93   **Oddy WH**, Herbison CE, Jacoby P, Ambrosini GL, O'Sullivan TA, Ayonrinde OT, Olynyk JK, Black LJ, Beilin LJ, Mori TA, Hands BP, Adams LA. The Western dietary pattern is prospectively associated with nonalcoholic fatty liver disease in adolescence. *Am J Gastroenterol* 2013; **108**: 778-785 [PMID: 23545714 DOI: 10.1038/ajg.2013.95]

94   **Ayonrinde OT**, Oddy WH, Adams LA, Mori TA, Beilin LJ, de Klerk N, Olynyk JK. Infant nutrition and maternal obesity influence the risk of non-alcoholic fatty liver disease in adolescents. *J Hepatol* 2017; **67**: 568-576 [PMID: 28619255 DOI: 10.1016/j.jhep.2017.03.029]

95   **Molleston JP**, Schwimmer JB, Yates KP, Murray KF, Cummings OW, Lavine JE, Brunt EM, Scheimann AO, Unalp-Arida A; NASH Clinical Research Network. Histological abnormalities in children with nonalcoholic fatty liver disease and normal or mildly elevated alanine aminotransferase levels. *J Pediatr* 2014; **164**: 707-713. e3 [PMID: 24360992 DOI: 10.1016/j.jpeds.2013.10.071]

96   **Schwimmer JB**, Behling C, Newbury R, Deutsch R, Nievergelt C, Schork NJ, Lavine JE. Histopathology of pediatric nonalcoholic fatty liver disease. *Hepatology* 2005; **42**: 641-649 [PMID: 16116629 DOI: 10.1002/hep.20842]

97   **Goyal NP**, Schwimmer JB. The Progression and Natural History of Pediatric Nonalcoholic Fatty Liver Disease. *Clin Liver Dis* 2016; **20**: 325-338 [PMID: 27063272 DOI: 10.1016/j.cld.2015.10.003]

98   **Nobili V**, Marcellini M, Devito R, Ciampalini P, Piemonte F, Comparcola D, Sartorelli MR, Angulo P. NAFLD in children: a prospective clinical-pathological study and effect of lifestyle advice. *Hepatology* 2006; **44**: 458-465 [PMID: 16871574 DOI: 10.1002/hep.21262]

99   **Ye Q**, Zou B, Yeo YH, Li J, Huang DQ, Wu Y, Yang H, Liu C, Kam LY, Tan XXE, Chien N, Trinh S, Henry L, Stave CD, Hosaka T, Cheung RC, Nguyen MH. Global prevalence, incidence, and outcomes of non-obese or lean non-alcoholic fatty liver disease: a systematic review and meta-analysis. *Lancet Gastroenterol Hepatol* 2020; **5**: 739-752 [PMID: 32413340 DOI: 10.1016/S2468-1253(20)30077-7]

100   **Lu FB**, Zheng KI, Rios RS, Targher G, Byrne CD, Zheng MH. Global epidemiology of lean non-alcoholic fatty liver disease: A systematic review and meta-analysis. *J Gastroenterol Hepatol* 2020; **35**: 2041-2050 [PMID: 32573017 DOI: 10.1111/jgh.15156]

101   **Shi Y**, Wang Q, Sun Y, Zhao X, Kong Y, Ou X, Jia J, Wu S, You H. The Prevalence of Lean/Nonobese Nonalcoholic Fatty Liver Disease: A Systematic Review and Meta-Analysis. *J Clin Gastroenterol* 2020; **54**: 378-387 [PMID: 31651571 DOI: 10.1097/MCG.0000000000001270]

102   **Honda Y**, Yoneda M, Kessoku T, Ogawa Y, Tomeno W, Imajo K, Mawatari H, Fujita K, Hyogo H, Ueno T, Chayama K, Saito S, Nakajima A, Hotta K. Characteristics of non-obese non-alcoholic fatty liver disease: Effect of genetic and environmental factors. *Hepatol Res* 2016; **46**: 1011-1018 [PMID: 26763865 DOI: 10.1111/hepr.12648]

103   **Adams LA**, Marsh JA, Ayonrinde OT, Olynyk JK, Ang WQ, Beilin LJ, Mori T, Palmer LJ, Oddy WW, Lye SJ, Pennell CE. Cholesteryl ester transfer protein gene polymorphisms increase the risk of fatty liver in females independent of adiposity. *J Gastroenterol Hepatol* 2012; **27**: 1520-1527 [PMID: 22414273 DOI: 10.1111/j.1440-1746.2012.07120.x]

104   **Eslam M**, Hashem AM, Leung R, Romero-Gomez M, Berg T, Dore GJ, Chan HL, Irving WL, Sheridan D, Abate ML, Adams LA, Mangia A, Weltman M, Bugianesi E, Spengler U, Shaker O, Fischer J, Mollison L, Cheng W, Powell E, Nattermann J, Riordan S, McLeod D, Armstrong NJ, Douglas MW, Liddle C, Booth DR, George J, Ahlenstiel G; International Hepatitis C Genetics Consortium (IHCGC). Interferon-λ rs12979860 genotype and liver fibrosis in viral and non-viral chronic liver disease. *Nat Commun* 2015; **6**: 6422 [PMID: 25740255 DOI: 10.1038/ncomms7422]

105   **Yun Y**, Kim HN, Lee EJ, Ryu S, Chang Y, Shin H, Kim HL, Kim TH, Yoo K, Kim HY. Fecal and blood microbiota profiles and presence of nonalcoholic fatty liver disease in obese vs lean subjects. *PLoS One* 2019; **14**: e0213692 [DOI: 10.1371/journal.pone.0213692]

106   **Feldman A**, Eder SK, Felder TK, Kedenko L, Paulweber B, Stadlmayr A, Huber-Schönauer U, Niederseer D, Stickel F, Auer S, Haschke-Becher E, Patsch W, Datz C, Aigner E. Clinical and Metabolic Characterization of Lean Caucasian Subjects With Non-alcoholic Fatty Liver. *Am J Gastroenterol* 2017; **112**: 102-110 [PMID: 27527746 DOI: 10.1038/ajg.2016.318]

107   **Eslam M**, Chen F, George J. NAFLD in Lean Asians. *Clin Liver Dis (Hoboken)* 2020; **16**: 240-243 [PMID: 33489095 DOI: 10.1002/cld.930]

108   **Hagström H**, Nasr P, Ekstedt M, Hammar U, Stål P, Hultcrantz R, Kechagias S. Risk for development of severe liver disease in lean patients with nonalcoholic fatty liver disease: A long-term follow-up study. *Hepatol Commun* 2018; **2**: 48-57 [PMID: 29404512 DOI: 10.1002/hep4.1124]

109   **Wang AY**, Dhaliwal J, Mouzaki M. Lean non-alcoholic fatty liver disease. *Clin Nutr* 2019; **38**: 975-981 [PMID: 30466956 DOI: 10.1016/j.clnu.2018.08.008]

110   **Pais R**, Charlotte F, Fedchuk L, Bedossa P, Lebray P, Poynard T, Ratziu V; LIDO Study Group. A systematic review of follow-up biopsies reveals disease progression in patients with non-alcoholic fatty liver. *J Hepatol* 2013; **59**: 550-556 [PMID: 23665288 DOI: 10.1016/j.jhep.2013.04.027]

111   **McPherson S**, Hardy T, Henderson E, Burt AD, Day CP, Anstee QM. Evidence of NAFLD progression from steatosis to fibrosing-steatohepatitis using paired biopsies: implications for prognosis and clinical management. *J Hepatol* 2015; **62**: 1148-1155 [PMID: 25477264 DOI: 10.1016/j.jhep.2014.11.034]

112   **Singh S**, Allen AM, Wang Z, Prokop LJ, Murad MH, Loomba R. Fibrosis progression in nonalcoholic fatty liver vs nonalcoholic steatohepatitis: a systematic review and meta-analysis of paired-biopsy studies. *Clin Gastroenterol Hepatol* 2015; **13**: 643-54.e1 [PMID: 24768810 DOI: 10.1016/j.cgh.2014.04.014]

113   **Musso G**, Gambino R, Cassader M, Pagano G. Meta-analysis: natural history of non-alcoholic fatty liver disease (NAFLD) and diagnostic accuracy of non-invasive tests for liver disease severity. *Ann Med* 2011; **43**: 617-649 [PMID: 21039302 DOI: 10.3109/07853890.2010.518623]

114   **De A**, Duseja A. Natural History of Simple Steatosis or Nonalcoholic Fatty Liver. *J Clin Exp Hepatol* 2020; **10**: 255-262 [PMID: 32405182 DOI: 10.1016/j.jceh.2019.09.005]

115   **Kechagias S**, Nasr P, Blomdahl J, Ekstedt M. Established and emerging factors affecting the progression of nonalcoholic fatty liver disease. *Metabolism* 2020; **111S**: 154183 [PMID: 32061907 DOI: 10.1016/j.metabol.2020.154183]

116   **Anastasopoulos NT**, Lianos GD, Tatsi V, Karampa A, Goussia A, Glantzounis GK. Clinical heterogeneity in patients with non-alcoholic fatty liver disease-associated hepatocellular carcinoma. *Expert Rev Gastroenterol Hepatol* 2020; **14**: 1025-1033 [PMID: 32746645 DOI: 10.1080/17474124.2020.1802244]

117   **Leung C**, Yeoh SW, Patrick D, Ket S, Marion K, Gow P, Angus PW. Characteristics of hepatocellular carcinoma in cirrhotic and non-cirrhotic non-alcoholic fatty liver disease. *World J Gastroenterol* 2015; **21**: 1189-1196 [PMID: 25632192 DOI: 10.3748/wjg.v21.i4.1189]

118   **Mohamad B**, Shah V, Onyshchenko M, Elshamy M, Aucejo F, Lopez R, Hanouneh IA, Alhaddad R, Alkhouri N. Characterization of hepatocellular carcinoma (HCC) in non-alcoholic fatty liver disease (NAFLD) patients without cirrhosis. *Hepatol Int* 2016; **10**: 632-639 [PMID: 26558795 DOI: 10.1007/s12072-015-9679-0]

119   **Marjot T**, Moolla A, Cobbold JF, Hodson L, Tomlinson JW. Nonalcoholic Fatty Liver Disease in Adults: Current Concepts in Etiology, Outcomes, and Management. *Endocr Rev* 2020; **41** [PMID: 31629366 DOI: 10.1210/endrev/bnz009]

120   **Bhala N**, Angulo P, van der Poorten D, Lee E, Hui JM, Saracco G, Adams LA, Charatcharoenwitthaya P, Topping JH, Bugianesi E, Day CP, George J. The natural history of nonalcoholic fatty liver disease with advanced fibrosis or cirrhosis: an international collaborative study. *Hepatology* 2011; **54**: 1208-1216 [PMID: 21688282 DOI: 10.1002/hep.24491]

121   **Ekstedt M**, Hagström H, Nasr P, Fredrikson M, Stål P, Kechagias S, Hultcrantz R. Fibrosis stage is the strongest predictor for disease-specific mortality in NAFLD after up to 33 years of follow-up. *Hepatology* 2015; **61**: 1547-1554 [PMID: 25125077 DOI: 10.1002/hep.27368]

122   **Hagström H**, Nasr P, Ekstedt M, Hammar U, Stål P, Hultcrantz R, Kechagias S. Fibrosis stage but not NASH predicts mortality and time to development of severe liver disease in biopsy-proven NAFLD. *J Hepatol* 2017; **67**: 1265-1273 [PMID: 28803953 DOI: 10.1016/j.jhep.2017.07.027]

123   **Rowe IA**, Parker R. The diagnosis of nonalcoholic fatty liver disease should carry important prognostic information. *Nat Rev Gastroenterol Hepatol* 2019; **16**: 449-450 [PMID: 31197261 DOI: 10.1038/s41575-019-0168-0]

124   **Vilar-Gomez E**, Chalasani N. Non-invasive assessment of non-alcoholic fatty liver disease: Clinical prediction rules and blood-based biomarkers. *J Hepatol* 2018; **68**: 305-315 [PMID: 29154965 DOI: 10.1016/j.jhep.2017.11.013]

125   **Wai JW**, Fu C, Wong VW. Confounding factors of non-invasive tests for nonalcoholic fatty liver disease. *J Gastroenterol* 2020; **55**: 731-741 [PMID: 32451628 DOI: 10.1007/s00535-020-01686-8]

126   **McPherson S**, Hardy T, Dufour JF, Petta S, Romero-Gomez M, Allison M, Oliveira CP, Francque S, Van Gaal L, Schattenberg JM, Tiniakos D, Burt A, Bugianesi E, Ratziu V, Day CP, Anstee QM. Age as a Confounding Factor for the Accurate Non-Invasive Diagnosis of Advanced NAFLD Fibrosis. *Am J Gastroenterol* 2017; **112**: 740-751 [PMID: 27725647 DOI: 10.1038/ajg.2016.453]

127   **De Silva S**, Li W, Kemos P, Brindley JH, Mecci J, Samsuddin S, Chin-Aleong J, Feakins RM, Foster GR, Syn WK, Alazawi W. Non-invasive markers of liver fibrosis in fatty liver disease are unreliable in people of South Asian descent. *Frontline Gastroenterol* 2018; **9**: 115-121 [PMID: 29588839 DOI: 10.1136/flgastro-2017-100865]

128   **Xia B**, Wang F, Friedrich-Rust M, Zhou F, Zhu J, Yang H, Ruan W, Zeng Z. Feasibility and Efficacy of Transient Elastography using the XL probe to diagnose liver fibrosis and cirrhosis: A meta-analysis. *Medicine (Baltimore)* 2018; **97**: e11816 [PMID: 30278481 DOI: 10.1097/MD.0000000000011816]

129   **Shili-Masmoudi S**, Wong GL, Hiriart JB, Liu K, Chermak F, Shu SS, Foucher J, Tse YK, Bernard

PH, Yip TC, Merrouche W, Chan HL, Wong VW, de Lédinghen V. Liver stiffness measurement predicts long-term survival and complications in non-alcoholic fatty liver disease. *Liver Int* 2020; **40**: 581-589 [PMID: 31749300 DOI: 10.1111/liv.14301]

130   **Petta S**, Maida M, Macaluso FS, Di Marco V, Cammà C, Cabibi D, Craxì A. The severity of steatosis influences liver stiffness measurement in patients with nonalcoholic fatty liver disease. *Hepatology* 2015; **62**: 1101-1110 [PMID: 25991038 DOI: 10.1002/hep.27844]

131   **Park CC**, Nguyen P, Hernandez C, Bettencourt R, Ramirez K, Fortney L, Hooker J, Sy E, Savides MT, Alquiraish MH, Valasek MA, Rizo E, Richards L, Brenner D, Sirlin CB, Loomba R. Magnetic Resonance Elastography vs Transient Elastography in Detection of Fibrosis and Noninvasive Measurement of Steatosis in Patients With Biopsy-Proven Nonalcoholic Fatty Liver Disease. *Gastroenterology* 2017; **152**: 598-607.e2 [PMID: 27911262 DOI: 10.1053/j.gastro.2016.10.026]

132   **Augustin S**, Ahmed A, Alkhouri N, Canbay A, Corless L, Nakajima A, Okanoue T, Petta S, Ratziu V, Tsochatzis EA, Wong VW, Romero-Gómez M. Identification of Patients with Advanced Fibrosis Due to Nonalcoholic Fatty Liver Disease: Considerations for Best Practice. *J Gastrointestin Liver Dis* 2020; **29**: 235-245 [PMID: 32530991 DOI: 10.15403/jgld-775]

133   Association for the Study of the Liver (EASL).; European Association for the Study of Diabetes (EASD); European Association for the Study of Obesity (EASO). EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. *J Hepatol* 2016; **64**: 1388-1402 [DOI: 10.1159/000443344]

134   **Sumida Y**, Yoneda M, Hyogo H, Itoh Y, Ono M, Fujii H, Eguchi Y, Suzuki Y, Aoki N, Kanemasa K, Fujita K, Chayama K, Saibara T, Kawada N, Fujimoto K, Kohgo Y, Yoshikawa T, Okanoue T; Japan Study Group of Nonalcoholic Fatty Liver Disease (JSG-NAFLD). Validation of the FIB4 index in a Japanese nonalcoholic fatty liver disease population. *BMC Gastroenterol* 2012; **12**: 2 [PMID: 22221544 DOI: 10.1186/1471-230X-12-2]

135   **Yu SJ**, Kim DH, Lee JH, Chung GE, Yim JY, Park MJ, Kim YJ, Yoon JH, Jang JJ, Lee HS. Validation of P2/MS and other noninvasive fibrosis scoring systems in the Korean population with nonalcoholic fatty liver disease. *Korean J Gastroenterol* 2011; **57**: 19-27 [PMID: 21258197 DOI: 10.4166/kjg.2011.57.1.19]

136   **Wong GL**, Chan HL. Two are better than one: noninvasive assessment of liver fibrosis in nonalcoholic fatty liver disease. *Hepatol Int* 2015; **9**: 481-483 [PMID: 25788206 DOI: 10.1007/s12072-015-9625-1]

137   **Wong VW**, Chan WK, Chitturi S, Chawla Y, Dan YY, Duseja A, Fan J, Goh KL, Hamaguchi M, Hashimoto E, Kim SU, Lesmana LA, Lin YC, Liu CJ, Ni YH, Sollano J, Wong SK, Wong GL, Chan HL, Farrell G. Asia-Pacific Working Party on Non-alcoholic Fatty Liver Disease guidelines 2017-Part 1: Definition, risk factors and assessment. *J Gastroenterol Hepatol* 2018; **33**: 70-85 [PMID: 28670712 DOI: 10.1111/jgh.13857]

138   **Newsome PN**, Sasso M, Deeks JJ, Paredes A, Boursier J, Chan WK, Yilmaz Y, Czernichow S, Zheng MH, Wong VW, Allison M, Tsochatzis E, Anstee QM, Sheridan DA, Eddowes PJ, Guha IN, Cobbold JF, Paradis V, Bedossa P, Miette V, Fournier-Poizat C, Sandrin L, Harrison SA. FibroScan-AST (FAST) score for the non-invasive identification of patients with non-alcoholic steatohepatitis with significant activity and fibrosis: a prospective derivation and global validation study. *Lancet Gastroenterol Hepatol* 2020; **5**: 362-373 [PMID: 32027858 DOI: 10.1016/S2468-1253(19)30383-8]

139   **Mózes FE**, Lee JA, Selvaraj EA, Jayaswal ANA, Trauner M, Boursier J, Fournier C, Staufer K, Stauber RE, Bugianesi E, Younes R, Gaia S, Lupșor-Platon M, Petta S, Shima T, Okanoue T, Mahadeva S, Chan WK, Eddowes PJ, Hirschfield GM, Newsome PN, Wong VW, de Ledinghen V, Fan J, Shen F, Cobbold JF, Sumida Y, Okajima A, Schattenberg JM, Labenz C, Kim W, Lee MS, Wiegand J, Karlas T, Yılmaz Y, Aithal GP, Palaniyappan N, Cassinotto C, Aggarwal S, Garg H, Ooi GJ, Nakajima A, Yoneda M, Ziol M, Barget N, Geier A, Tuthill T, Brosnan MJ, Anstee QM, Neubauer S, Harrison SA, Bossuyt PM, Pavlides M; LITMUS Investigators. Diagnostic accuracy of non-invasive tests for advanced fibrosis in patients with NAFLD: an individual patient data meta-analysis. *Gut* 2021 [PMID: 34001645 DOI: 10.1136/gutjnl-2021-324243]

140   **Sanyal AJ**. Past, present and future perspectives in nonalcoholic fatty liver disease. *Nat Rev Gastroenterol Hepatol* 2019; **16**: 377-386 [PMID: 31024089 DOI: 10.1038/s41575-019-0144-8]

141   **Younossi ZM**, Ratziu V, Loomba R, Rinella M, Anstee QM, Goodman Z, Bedossa P, Geier A, Beckebaum S, Newsome PN, Sheridan D, Sheikh MY, Trotter J, Knapple W, Lawitz E, Abdelmalek MF, Kowdley KV, Montano-Loza AJ, Boursier J, Mathurin P, Bugianesi E, Mazzella G, Olveira A, Cortez-Pinto H, Graupera I, Orr D, Gluud LL, Dufour JF, Shapiro D, Campagna J, Zaru L, MacConell L, Shringarpure R, Harrison S, Sanyal AJ; REGENERATE Study Investigators. Obeticholic acid for the treatment of non-alcoholic steatohepatitis: interim analysis from a multicentre, randomised, placebo-controlled phase 3 trial. *Lancet* 2019; **394**: 2184-2196 [PMID: 31813633 DOI: 10.1016/S0140-6736(19)33041-7]

142   **Patel K**, Harrison SA, Elkhashab M, Trotter JF, Herring R, Rojter SE, Kayali Z, Wong VW, Greenbloom S, Jayakumar S, Shiffman ML, Freilich B, Lawitz EJ, Gane EJ, Harting E, Xu J, Billin AN, Chung C, Djedjos CS, Subramanian GM, Myers RP, Middleton MS, Rinella M, Noureddin M. Cilofexor, a Nonsteroidal FXR Agonist, in Patients With Noncirrhotic NASH: A Phase 2 Randomized Controlled Trial. *Hepatology* 2020; **72**: 58-71 [PMID: 32115759 DOI: 10.1002/hep.31205]

143   **Gawrieh S**, Noureddin M, Loo N, Mohseni R, Awasty V, Cusi K, Kowdley KV, Lai M, Schiff E, Parmar D, Patel P, Chalasani N. Saroglitazar, a PPAR-α/γ Agonist, for Treatment of NAFLD: A

Randomized Controlled Double-Blind Phase 2 Trial. *Hepatology* 2021 [DOI: 10.1002/hep.31843]

144   **Amin N**, Carvajal-Gonzalez S, Aggarwal N, Tuthill T, Inglot M, Bergman A, Esler W. Abstract 31: PF-05221304 (PF'1304), a liver-targeted acetyl-CoA carboxylase inhibitor (ACCI), in adults with nonalcoholic fatty liver disease (NAFLD) demonstrates robust reductions in liver fat and ALT: phase 2a, dose-ranging study. *Hepatology* 2019; **70**: 21A-22A [DOI: 10.1016/s0168-8278(20)31392-1]

145   **Armstrong MJ**, Gaunt P, Aithal GP, Barton D, Hull D, Parker R, Hazlehurst JM, Guo K; LEAN trial team, Abouda G, Aldersley MA, Stocken D, Gough SC, Tomlinson JW, Brown RM, Hübscher SG, Newsome PN. Liraglutide safety and efficacy in patients with non-alcoholic steatohepatitis (LEAN): a multicentre, double-blind, randomised, placebo-controlled phase 2 study. *Lancet* 2016; **387**: 679-690 [PMID: 26608256 DOI: 10.1016/S0140-6736(15)00803-X]

146   **Petit JM**, Cercueil JP, Loffroy R, Denimal D, Bouillet B, Fourmont C, Chevallier O, Duvillard L, Vergès B. Effect of Liraglutide Therapy on Liver Fat Content in Patients With Inadequately Controlled Type 2 Diabetes: The Lira-NAFLD Study. *J Clin Endocrinol Metab* 2017; **102**: 407-415 [PMID: 27732328 DOI: 10.1210/jc.2016-2775]

147   **Eguchi Y**, Kitajima Y, Hyogo H, Takahashi H, Kojima M, Ono M, Araki N, Tanaka K, Yamaguchi M, Matsuda Y, Ide Y, Otsuka T, Ozaki I, Ono N, Eguchi T, Anzai K; Japan Study Group for NAFLD (JSG-NAFLD). Pilot study of liraglutide effects in non-alcoholic steatohepatitis and non-alcoholic fatty liver disease with glucose intolerance in Japanese patients (LEAN-J). *Hepatol Res* 2015; **45**: 269-278 [PMID: 24796231 DOI: 10.1111/hepr.12351]

148   **Newsome PN**, Buchholtz K, Cusi K, Linder M, Okanoue T, Ratziu V, Sanyal AJ, Sejling AS, Harrison SA; NN9931-4296 Investigators. A Placebo-Controlled Trial of Subcutaneous Semaglutide in Nonalcoholic Steatohepatitis. *N Engl J Med* 2021; **384**: 1113-1124 [PMID: 33185364 DOI: 10.1056/NEJMoa2028395]

149   **Harrison SA**, Alkhouri N, Davison BA, Sanyal A, Edwards C, Colca JR, Lee BH, Loomba R, Cusi K, Kolterman O, Cotter G, Dittrich HC. Insulin sensitizer MSDC-0602K in non-alcoholic steatohepatitis: A randomized, double-blind, placebo-controlled phase IIb study. *J Hepatol* 2020; **72**: 613-626 [PMID: 31697972 DOI: 10.1016/j.jhep.2019.10.023]

150   **Sanyal AJ**, Chalasani N, Kowdley KV, McCullough A, Diehl AM, Bass NM, Neuschwander-Tetri BA, Lavine JE, Tonascia J, Unalp A, Van Natta M, Clark J, Brunt EM, Kleiner DE, Hoofnagle JH, Robuck PR; NASH CRN. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *N Engl J Med* 2010; **362**: 1675-1685 [PMID: 20427778 DOI: 10.1056/NEJMoa0907929]

151   **Harrison SA**, Goodman Z, Jabbar A, Vemulapalli R, Younes ZH, Freilich B, Sheikh MY, Schattenberg JM, Kayali Z, Zivony A, Sheikh A, Garcia-Samaniego J, Satapathy SK, Therapondos G, Mena E, Schuppan D, Robinson J, Chan JL, Hagerty DT, Sanyal AJ. A randomized, placebo-controlled trial of emricasan in patients with NASH and F1-F3 fibrosis. *J Hepatol* 2020; **72**: 816-827 [PMID: 31887369 DOI: 10.1016/j.jhep.2019.11.024]

152   **Harrison SA**, Abdelmalek MF, Caldwell S, Shiffman ML, Diehl AM, Ghalib R, Lawitz EJ, Rockey DC, Schall RA, Jia C, McColgan BJ, McHutchison JG, Subramanian GM, Myers RP, Younossi Z, Ratziu V, Muir AJ, Afdhal NH, Goodman Z, Bosch J, Sanyal AJ; GS-US-321-0105 and GS-US-321-0106 Investigators. Simtuzumab Is Ineffective for Patients With Bridging Fibrosis or Compensated Cirrhosis Caused by Nonalcoholic Steatohepatitis. *Gastroenterology* 2018; **155**: 1140-1153 [PMID: 29990488 DOI: 10.1053/j.gastro.2018.07.006]

153   **Harrison SA**, Neff G, Guy CD, Bashir MR, Paredes AH, Frias JP, Younes Z, Trotter JF, Gunn NT, Moussa SE, Kohli A, Nelson K, Gottwald M, Chang WCG, Yan AZ, DePaoli AM, Ling L, Lieu HD. Efficacy and Safety of Aldafermin, an Engineered FGF19 Analog, in a Randomized, Double-Blind, Placebo-Controlled Trial of Patients With Nonalcoholic Steatohepatitis. *Gastroenterology* 2021; **160**: 219-231.e1 [PMID: 32781086 DOI: 10.1053/j.gastro.2020.08.004]

154   **Reimer KC**, Wree A, Roderburg C, Tacke F. New drugs for NAFLD: lessons from basic models to the clinic. *Hepatol Int* 2020; **14**: 8-23 [PMID: 31802390 DOI: 10.1007/s12072-019-10001-4]

155   **Eslam M**, George J. Genetic Insights for Drug Development in NAFLD. *Trends Pharmacol Sci* 2019; **40**: 506-516 [PMID: 31160124 DOI: 10.1016/j.tips.2019.05.002]

156   **Saran AR**, Dave S, Zarrinpar A. Circadian Rhythms in the Pathogenesis and Treatment of Fatty Liver Disease. *Gastroenterology* 2020; **158**: 1948-1966. e1 [PMID: 32061597 DOI: 10.1053/j.gastro.2020.01.050]

157   **Hardy T**, Oakley F, Anstee QM, Day CP. Nonalcoholic Fatty Liver Disease: Pathogenesis and Disease Spectrum. *Annu Rev Pathol* 2016; **11**: 451-496 [PMID: 26980160 DOI: 10.1146/annurev-pathol-012615-044224]

158   **Hannah WN Jr**, Torres DM, Harrison SA. Nonalcoholic Steatohepatitis and Endpoints in Clinical Trials. *Gastroenterol Hepatol (N Y)* 2016; **12**: 756-763 [PMID: 28035202]

159   **Thiagarajan P**, Aithal GP. Drug Development for Nonalcoholic Fatty Liver Disease: Landscape and Challenges. *J Clin Exp Hepatol* 2019; **9**: 515-521 [PMID: 31516268 DOI: 10.1016/j.jceh.2019.03.002]

160   **Bawden SJ**, Scott RA, Aithal GP. Current and Future Magnetic Resonance Technologies for Assessing Liver Disease in Clinical and Experimental Medicine. *Dig Dis* 2017; **35**: 314-322 [PMID: 28467990 DOI: 10.1159/000456582]

161   **Cerami E**, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, Antipin Y, Reva B, Goldberg AP, Sander C, Schultz N. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov* 2012; **2**: 401-404 [PMID: 22588877 DOI: 10.1158/2159-8290.CD-12-0095]

162   **Africa JA**, Behling CA, Brunt EM, Zhang N, Luo Y, Wells A, Hou J, Belt PH, Kohil R, Lavine JE, Molleston JP, Newton KP, Whitington PF, Schwimmer JB; Nonalcoholic Steatohepatitis Clinical Research Network. In Children With Nonalcoholic Fatty Liver Disease, Zone 1 Steatosis Is Associated With Advanced Fibrosis. *Clin Gastroenterol Hepatol* 2018; **16**: 438-446.e1 [PMID: 28286193 DOI: 10.1016/j.cgh.2017.02.030]

Newly discovered endocrine functions of the liver

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Abstract

The liver, the largest solid visceral organ of the body, has numerous endocrine functions, such as direct hormone and hepatokine production, hormone metabolism, synthesis of binding proteins, and processing and redistribution of metabolic fuels. In the last 10 years, many new endocrine functions of the liver have been discovered. Advances in the classical endocrine functions include delineation of mechanisms of liver production of endocrine hormones [including 25-hydroxyvitamin D, insulin-like growth factor 1 (IGF-1), and angiotensinogen], hepatic metabolism of hormones (including thyroid hormones, glucagon-like peptide-1, and steroid hormones), and actions of specific binding proteins to glucocorticoids, sex steroids, and thyroid hormones. These studies have furthered insight into cirrhosis-associated endocrinopathies, such as hypogonadism, osteoporosis, IGF-1 deficiency, vitamin D deficiency, alterations in glucose and lipid homeostasis, and controversially relative adrenal insufficiency. Several novel endocrine functions of the liver have also been unraveled, elucidating the liver's key negative feedback regulatory role in the pancreatic α cell-liver axis, which regulates pancreatic α cell mass, glucagon secretion, and circulating amino acid levels. Betatrophin and other hepatokines, such as fetuin-A and fibroblast growth factor 21, have also been discovered to play important endocrine roles in modulating insulin sensitivity, lipid metabolism, and body weight. It is expected that more endocrine functions of the liver will be revealed in the near future.

Key Words: Liver; Endocrine function; Hormone; Amino acids; Hepatokine; Fibroblast growth factor 21

Core Tip: The liver has many newly discovered endocrine functions, most of which are in regulating metabolism, underscoring the functioning of the liver as a major metabolic organ. Convincing evidence has shown that the liver regulates endocrine

functions in mineral and fuel metabolism, especially in the metabolism of glucose and lipids *via* hepatokines and amino acids *via* negative feedback on pancreatic α cells. As research into the endocrine function of the liver is a rapidly evolving field, controversial findings often exist; caution needs to be taken when interpreting novel findings to avoid over-simplification of complex metabolic processes and premature allocation of research resources.

INTRODUCTION

The liver is a dynamic endocrine organ and mediates critical metabolic pathways *via* roles in direct hormone and hepatokine production, hormone metabolism, synthesis of binding proteins, detoxification, and processing and redistribution of metabolic fuels [1-4]. It participates in multiple signaling pathways with other endocrine organs, including the pituitary, pancreas, gut, thyroid, adrenal glands, and bone, with hormones in turn modulating the liver's metabolic and synthetic functions[1,5]. Diseases that affect the liver lead to a variety of endocrine manifestations, including hypogonadism, osteoporosis, effects on glucose metabolism and growth hormone (GH), and controversial effects on cortisol[1,5].

The liver, with its vascularity, is well-positioned to provide and receive endocrine signals, including those from pancreatic and gut hormones[6]. It also receives exposure to antigen-rich blood systemically and from the gastrointestinal system as a lymphoid organ[7] and serves as a principal organ in drug metabolism and clearance[8]. Despite only representing 2.5% of the body weight, the liver receives up to 25% of the total cardiac output at rest[9]. It also receives a unique double afferent blood flow from the hepatic artery and partially deoxygenated portal vein, with around 75% of the blood flow from the latter[9]. The portal vein, in turn, receives blood from the stomach, small and large intestines, pancreas, spleen, and gallbladder[9], with direct physiological implications on the regulation of metabolism by endocrine liver functions[6]. Great progress has been made in the understanding of the endocrine functions of the liver in the last 10 years.

ADVANCES IN CLASSIC ENDOCRINE FUNCTIONS OF THE LIVER

We will first briefly summarize the advances in the understanding of the liver classic endocrine functions (Table 1).

Direct hormone production

The liver directly synthesizes multiple hormones, including 25-hydroxyvitamin D, insulin-like growth factor 1 (IGF-1), and angiotensinogen. Given roles in direct hormone production, the liver also has permissive roles of normal hormone function, in particular with effects on bone health, the GH-IGF-1 axis, and renin-angiotensin-aldosterone (RAA) pathway.

Vitamin D: The liver is the primary site of 25-hydroxylation of vitamin D to 25-hydroxyvitamin D (calcidiol), the main storage form of vitamin D[10]. Vitamin D is a secosteroid hormone well known for its role in calcium and bone homeostasis, with pleiotropic effects on cellular proliferation, differentiation, and immunomodulation [11-13]. 25-hydroxyvitamin D (calcidiol) then undergoes 1-alpha-hydroxylation in the kidney to the activated form 1,25-dihydroxyvitamin D (calcitriol)[10], which provides the active hormonal effects of vitamin D. The hydroxylation of vitamin D to produce calcidiol is mainly carried out in the liver by multiple cytochrome P450 mixed-function oxidases (CYPs) located in the mitochondria, endoplasmic reticulum (ER), and microsomes, though studies also show presence of these CYPs in extrahepatic tissues [10,11].

Table 1 Classic endocrine functions of the liver

Hormone	Liver function	Target organ	Action on target organ	Alteration in liver diseases
25-hydroxyvitamin D	Direct production	Gut	Prohormone of calcitriol which stimulates gut calcium absorption	Decreased production resulting in low bone density
Insulin-like growth factor 1	Direct production	Ubiquitous	Promoting growth and differentiation and regulating nutrients metabolism	Decreased production resulting in dysmetabolism
Angiotensinogen	Direct production	Cardiovascular system	Precursor of angiotensin II which regulates aldosterone level. Both regulate vascular tone, sodium retention, and cardiac remodeling	Near-normal function
Thyroid hormone	Activation through T4 to T3 conversion; inactivation through degradation; TBG production	Ubiquitous	Increasing metabolism and energy expenditure	Low T3 syndrome
Glucagon-like peptide 1 (GLP-1)	Metabolism of GLP-1 *via* dipeptidyl peptidase-4(DPPIV)	Pancreas, gut, and brain	Stimulating insulin production, decreasing gut motility, and suppressing appetite	Increased DPPIV expression resulting in higher risk of diabetes
Sex hormones	Hormone metabolism and SHBG production	Ubiquitous	Numerous (details beyond this review)	Hypogonadism
Glucocorticoids	Hormone metabolism and CBG production	Ubiquitous	Numerous (details beyond this review)	Relative adrenal insufficiency
Mineralocorticoids	Hormone metabolism	Cardiovascular system	Maintaining electrolyte balance and blood pressure	Largely intact

TBG: Thyroxine binding globulin; CBG: Cortisol binding globulin; SHBG: Sex hormone binding globulin.

IGF-1: The liver is the primary source of IGF-1, a 70-amino acid polypeptide hormone with endocrine, paracrine, and autocrine effects[14]. IGF-1 affects almost every tissue and organ[15], and its receptors are ubiquitously expressed[16]. Besides mediating the actions of GH, more recently, non-growth-related actions of IGF-1 are found. IGF-1 binds to the insulin receptor and the hybrid IGF-1/insulin receptors, with implications on the metabolic effects of IGF-1[14]. IGF-1, GH, and insulin are hypothesized to constitute a regulated axis to inform cells about nutritional status, helping direct cells grow and differentiate *vs* induce a state of quiescence, senescence or apoptosis[14]. The IGF-1 receptor also participates in a crosstalk with the thyrotropin receptor by forming heterodimers[17], with implications on cellular growth and pathological implications in Graves' eye disease.

Angiotensinogen: The liver is the primary source of angiotensinogen, which is involved in the RAA system[18]. The RAA system is vital for maintaining blood pressure homeostasis, *via* effects on sodium balance, intra- and extra-vascular volume, and systemic vascular tone[19]. Angiotensinogen, an alpha-globulin, is the only known substrate for renin and the main precursor molecule for angiotensin II (AngII), the major biologically active peptide in the RAA pathway[19]. Despite local tissue production of AngII, liver angiotensinogen is the primary source of renal AngII[18]. Hepatocytes tonically secrete angiotensinogen and primarily determine plasma angiotensinogen levels, with small increases in angiotensinogen levels increasing blood pressure and AngII levels[20].

Hormone metabolism

The liver is involved in the metabolism of multiple endocrine hormones, including thyroid hormones, glucagon-like peptide-1, and steroid hormones, with roles in both activation and inactivation of the hormones.

Thyroid hormone: Hepatic metabolism has roles in both activation and inactivation of thyroid hormones. The biologic activity of thyroid hormone is mainly mediated through the active thyroid hormone T3. The thyroid only secretes 20% of the daily T3 requirement, with the remainder 80% converted from T4 by peripheral selenium-containing deiodinase enzymes (DIO), of which three primary deiodinases (type 1, 2, and 3) have been identified[21]. The liver expresses DIO1, along with the kidney and thyroid, which converts T4 to T3, though with less kinetic efficiency compared to

DIO2, which is expressed by brown adipose tissue and the pituitary. Subsequently, the thyroid hormone is metabolized by conjugation with sulfate or glucuronic acid, which occurs prominently in the liver[22].

Glucagon-like peptide 1: With the discovery of glucagon-like peptide 1 (GLP-1), increasing research has been studying the gut-pancreas-liver axis, and the liver has been shown to play a key role in the hormone's metabolism[23]. GLP-1 is an incretin hormone produced by the intestinal L-cells in response to ingestion of nutrients, including carbohydrates, fatty acids, and fiber[24]. It stimulates insulin secretion in a glucose-dependent manner, with associated inhibition of hepatic gluconeogenesis, and promotes insulin gene transcription and growth and proliferation of islet cells[24]. GLP-1 is inactivated by dipeptidyl peptidase-4 (DPPIV), also known as CD26, a ubiquitous membrane-associated peptidase[25]. DPPIV has pleiotropic effects and widespread tissue distribution in all organs, with expression in capillary endothelial cells and high expression in the liver[25].

Steroid hormone metabolism: The liver participates in most steps of steroid hormone regulation, starting from being the primary site of cholesterol biosynthesis[26,27]. At the liver, steroid hormones undergo phase I metabolism by cytochrome P450 enzymes (CYPs), *via* multiple pathways including hydroxylation or reduction, and phase II metabolism, also *via* various processes including glucuronidation, sulfation, or methylation[27], ultimately leading to excretion of their conjugates in urine or bile.

Steroid hormone metabolism: Sex hormones: The liver is the main site for metabolic conversion of estrogens, progesterone, and androgens to their metabolites *via* CYPs, which are abundantly expressed in the liver[28]. In particular, as part of the first phase of metabolism, estrogens undergo hydroxylation by numerous CYPs, including 2-hydroxylation to 2-hydroxyestradiol and 4-hydroxylation to 4-hydroxestradiol, which represent 80% and 20% of biotransformation of estradiol in the liver, respectively. 2-hydroxylation is mainly catalyzed by CYP1A2 and CYP3A4, which are expressed in the liver, and CYP1A1 in extrahepatic tissues[28]. 4-hydroxestradiol, unlike 2-hydroxestradiol, is associated with free radical generation and cellular damage, with associated increased risk of carcinogenesis in the breast and endometrium. Subsequent phase II metabolism of sex hormones, *via* O-methylation by catechol O-methyltransferase (COMT), glucuronidation, or sulfation, occurs at high levels at the liver, with subsequent elimination in the urine or stool[28-30].

Steroid hormone metabolism: Glucocorticoids and mineralocorticoids: The liver is also the primary site of glucocorticoid and mineralocorticoid metabolism[27]. Cortisol is converted to and from its inactive metabolite cortisone by two isozymes of 11-beta hydroxysteroid dehydrogenase (11-beta-HSD)[31]. 11-beta-HSD type 1 (11-beta-HSD1) is widely distributed, though most abundantly located in the liver and adipose tissue, and is responsible for converting cortisone back to cortisol[31], with *in vitro* activity being greater in omental than subcutaneous adipose tissue[32]. In healthy individuals, local splanchnic cortisol production, including from the liver, can equal or even exceed that produced by extra-splanchnic tissues, including the adrenal gland[32]. In obese, non-diabetic individuals, the liver has been shown to account for virtually all splanchnic cortisol production[32]. Though primarily secreted from the adrenal glands under the regulation of the RAA axis, animal studies suggest possibility of local hepatic aldosterone production during liver injury, which may contribute to fibrogenesis[33]. Glucocorticoids and mineralocorticoids, like other steroid hormones, undergo phase I and phase II metabolism in the liver, with excretion of their conjugates in urine or bile[27].

Binding protein production

Lipophilic hormones, including steroid hormones, are not water soluble and need to be carried in the blood stream by binding proteins[2,34]. The liver is the primary source of binding proteins for many hormones. The liver produces specific binding proteins to multiple lipophilic hormones, including glucocorticoids, mineralocorticoids, sex steroids, thyroid hormones (T3 and T4), and vitamin D metabolites[2,34]. Binding globulins for these lipophilic hormones include cortisol binding globulin (CBG, which binds cortisol, aldosterone, and progesterone), sex hormone binding globulin (SHBG, which binds estradiol, testosterone, and other sex hormones), thyroxine binding globulin (TBG, which binds T3 and T4), and vitamin D binding globulin (DBG, which binds vitamin D metabolites)[2,34]. Binding proteins that are produced by the liver also include transthyretin (which binds thyroid hormone and

retinol), IGF-1 binding proteins (IGFBP, which binds IGF, including IGF-1), and non-specific binding proteins including albumin and lipoproteins. Binding proteins serve as a circulating reservoir for hormones, potentially regulating tissue distribution and target destination in a manner that can be highly selective and targeted[2,35]. Binding protein expression and production, which occur primarily at the liver, is complex and under the regulation and influence of multiple factors[2]. Most binding protein expression increase in response to estrogens, including physiologically with pregnancy or with oral contraceptives[2,34]. Hepatic failure and protein-losing nephropathies lead to decrease of binding proteins in general[2,34].

Endocrine dysregulation in liver disease

The liver mediates the effects of numerous hormonal pathways, whether directly or indirectly; thus, not surprisingly, derangements affecting the liver lead to disruptions of various hormonal pathways. Patients with cirrhosis are characterized by various endocrinopathies, including relative increase in estrogen compared to androgens, hypogonadism, osteoporosis, IGF-1 deficiency, vitamin D deficiency, alterations in glucose and lipid homeostasis, and perhaps more controversially a relative adrenal insufficiency.

Sex hormones: Cirrhosis is characterized by symptoms of estrogen-androgen imbalance, with relatively higher estradiol and lower testosterone concentrations[36]. The etiology of estrogen-testosterone imbalance is at least in part due to conversion of androgens to estrogens in cirrhosis, which in large part occurs peripherally[36]. The pathophysiology of hypogonadism is complex, including potential contribution from hypothalamic-pituitary suppression from a relatively increased estrogen circulation. SHBG is elevated in compensated cirrhotic patients, with subsequent decreases with decompensated cirrhosis, leading to concern for potential underestimation of hypogonadism in cirrhosis[34].

Cortisol: Patients with cirrhosis have relatively lower cortisol levels, also in the setting of lower production of cortisol binding globulin[37]. Some studies suggest the presence of a relative adrenal insufficiency in cirrhosis, also termed critical illness-associated corticosteroid insufficiency[38]. These studies suggest a potential hepatoadrenal syndrome in advanced liver disease, with associated inadequate cortisol production during stress response[38]. The decrease in cortisol binding globulin makes the diagnosis more difficult, though some studies suggest that free cortisol levels are decreased in relative adrenal insufficiency[37]. Hepatoadrenal syndrome and associated low free cortisol are attributed to decreased formation of HDL precursors and formation of proinflammatory cytokines and endotoxins[38].

RAA system: In liver disease, the systemic RAA pathway is upregulated due to systemic and splanchnic arterial vasodilation and associated hypoperfusion of the renal system[39]. Notably, the cirrhotic liver is able to produce angiotensinogen to near-normal plasma levels until the end stages[40].

DPPIV and GLP-1: DPPIV may play a role in linking type 2 diabetes with chronic liver disease. Type 2 diabetes has been associated with a greater than 2-fold increased risk of liver disease[41], and *in vitro* studies have suggested that elevated glucose can induce DPPIV expression in liver cells[42]. The increased DPPIV activity, which degrades the incretin hormone GLP-1, may contribute towards development of IGT, insulin resistance, lipogenesis, and hepatic injury in liver disease[25,43]. Serum DPPIV levels are notably increased in cirrhosis[25], and increased DPPIV expression in the liver has been observed in hepatitis C, NAFLD, experimental liver regeneration, and cirrhosis[25,43]. Cirrhotic nodules show diffuse and uniform staining of DPPIV, with loss of usual zonal expression of DPPIV[43], and degree of hepatic expression of DPPIV has also been shown to correlate with NAFLD grading[25]. Increased DPPIV expression has also been seen in various malignant tumors, including hepatocellular carcinoma, with DPPIV noted to promote resistance to anticancer agents[25].

Thyroid hormone: Given the liver's role in thyroid hormone metabolism, including local conversion of T4 to T3 by DIO1[21], patients with cirrhosis may present with abnormalities in thyroid hormone levels[44]. Though a variety of patterns are seen, the most common pattern is a low total T3 (TT3), low free T3 (FT3), elevated reverse T3 (rT3), low total T4 (TT4), variable literature on elevated *vs* low free T4 (FT4) levels, and possible elevations in TSH[44,45]. The low total hormone levels are attributable to low TBG[44]. The pattern is consistent with low T3 syndrome, which occurs in systemic illnesses, and represents non-thyroidal illness syndrome, previously known as

euthyroid sick syndrome[44].

IGF-1: Systemic IGF-1 deficiency in cirrhosis has been associated with an altered metabolic profile, including diabetes, deregulated lipid profile, and cardiovascular disease[14]. Lack of liver-derived IGF-1, in particular, has been associated with resultant insulin insensitivity in the liver, skeletal muscle, and adipose tissue, and corresponding hyperinsulinemia[46]. In NAFLD, the severity of steatosis has been correlated with a decrease in IGF-1 levels, with statistically significant differences in IGF-1 levels between mild-moderate *vs* severe steatosis[14,47].

Bone health and vitamin D: Chronic liver disease, including cirrhosis regardless of etiology, is associated with osteomalacia, osteopenia, and osteoporosis, and up to 40% of patients with chronic liver disease may develop an osteoporotic fracture[48]. The etiology of hepatic osteodystrophy is not well understood, though potential contributing factors include hypogonadism, and decreased hepatic production of IGF-1 and fibronectin[48]. There is a shift in cytokine production with changes in the receptor activator of nuclear factor kappa-B ligand (RANKL)/osteoprotegerin (OPG) system and an up-regulation of IL-6, which stimulates osteoclasts[48]. Decreased vitamin D synthesis, which is more marked in severely compromised liver function or in cholestatic liver disease, can further contribute to increased osteoporotic risk[49]. History of steroid treatment in chronic liver disease may be a risk factor for osteoporosis as well[48,49]. Different etiologies of liver disease may differ in their pathogenesis of osteoporosis, and in particular, diseases such as hemochromatosis and Wilson's may also directly impact bone health[48].

NOVEL ENDOCRINE FUNCTIONS OF THE LIVER

Besides the advances in the understanding of classic endocrine functions of the liver, novel liver endocrine functions have been unraveled in the last several years (Table 2), including endocrine regulation of pancreatic α cells, adipose tissue, and insulin sensitivity.

Feedback regulation of pancreatic α cells and glucagon

A major novel endocrine function of the liver is its critical role in a pancreatic α cell-liver axis that regulates pancreatic α cell proliferation and circulating glucagon and amino acid levels[50,51]. The pancreatic α cells, unlike the insulin-secreting β cells, have been considered a mysterious cell type until recently[52,53]. The α cells appear first during embryogenesis[54]. The main known function of the α cells is to produce and secrete the hormone glucagon[55]. Glucagon raises circulating glucose levels directly by stimulating gluconeogenesis and glycogenolysis, and indirectly by inhibiting insulin secretion[55,56].

Recently, a new α cell-liver axis has been discovered, endowing the liver with new endocrine functions[50,51]. The first clue of the α cell-liver axis came from glucagon receptor (GCGR) knockout mice[57,58]. The GCGR knockout mice harbor diffusely enlarged pancreas and exhibit extremely high glucagon levels[57-59]. Histologically, the pancreas of GCGR knockout mice contain numerous islets at various sizes, which are composed of mostly α cells as demonstrated by immunochemistry[57-59]. Normally the number of islets is quite small, and the islets are mostly composed of β cells. Mahvash disease, a human autosomal recessive hereditary disease discovered by our group, is caused by biallelic inactivating GCGR mutations, and its universal features are also α cell hyperplasia and hyperglucagonemia[60-62]. GCGR inactivation in zebra fish and non-human primates also result in α cell hyperplasia and hyperglucagonemia[63-66]. Thus, preservation of glucagon function is conserved throughout evolution.

Although a physiological compensation of hyperglucagonemia in animals and humans with inactive GCGR is quite intuitive, the specific mechanism of the compensation was initially not clear[67]. The liver-specific GCGR knockout mice interestingly have similar α cell hyperplasia and hyperglucagonemia, as those in global GCGR knockout mice[57,58,68], suggesting that the liver is the only target organ of glucagon that sends feedback signals to α cells, and that loss of the usual negative feedback mechanism stimulates α cell hyperplasia and glucagon secretion. This theory is also supported by the liver-specific stimulatory G protein α subunit (Gsα) knockout mice, which also exhibit α cell hyperplasia and hyperglucagonemia[69]. As glucagon antagonists were a promising anti-diabetes medication, both academia and pharmaco-

Table 2 Novel endocrine functions of the liver

Liver hormone	Target organ	Action on target organ	Alteration in liver diseases
Amino acids	Pancreatic α cells	Stimulate cell proliferation and glucagon secretion	Not studied yet
Betatrophin	Pancreatic β cells (?)	Stimulate cell proliferation (?)	Increased in cirrhosis
Fetuin	Skeletal muscle; Adipose tissue	Decrease insulin sensitivity; Reduce adiponectin expression	Elevated in nonalcoholic fatty liver disease
FGF21	Adipose tissue; Brain	Increase insulin sensitivity; Reduce food intake	Elevated in nonalcoholic fatty liver disease
Activin E	Adipose tissue	Increase fat oxidation	Increased in nonalcoholic fatty liver disease
Tsukushi	Adipose tissue	Increase thermogenesis	Increased in nonalcoholic fatty liver disease
GPNMB	Adipose tissue	Increase lipogenesis	Increased in nonalcoholic fatty liver disease

FGF21: Fibroblast growth factor 21; GPNMB: Glycoprotein nonmetastatic melanoma protein B.

logical companies became interested in the α cell-liver axis due to potential applications in diabetes drug development[70,71]. Some of the key original large-scale experiments leading to the discovery of the role of amino acids in regulating α cells were performed by pharmaceutical companies[72-74].

The liver may regulate α cells *via* neural or humoral mechanisms[67,68]. Islet transplantation experiments demonstrate that the liver uses a humoral mechanism[68]. Wild-type islets transplanted into the kidney of GCGR knockout mice undergo α cell hyperplasia, while GCGR knockout islets transplanted into wild-type kidney undergo reduced α cell proliferation. Thus, it is assumed that the liver sends a humoral factor (hormone) to stimulate pancreatic α cells, a phenomenon that is pronounced in diseases where the usual negative feedback mechanism is affected.

Initially, it was hoped that a single liver hormone would be isolated from differential liver gene expression patterns of wild-type and GCGR knockout mice[67]. Several groups, including ours, performed liver mRNA arrays of GCGR knockout mice and in wild-type mice treated with inhibitory GCGR antibodies, using wild-type mice as control[67,68,72]. Not surprisingly, many genes are overexpressed (potential stimulatory hormones) or underexpressed (potential inhibitory hormones) in the GCGR knockout liver[67,68,72]. Genes involved in gluconeogenesis are downregulated in the GCGR knockout liver[67,68,72]. On the other hand, genes involved in amino acid synthesis (*e.g.*, asparagine synthetase, Asns) are upregulated, and genes involved in amino acid catabolism (*e.g.*, glutaminase 2, Gls2) are downregulated[67,68,72]. Genes regulating lipid metabolism are also differentially expressed[67,68,72]. Most of the genes with significant differential expression were not bona fide hormone candidates because they were not secreted proteins[67,68,72]. InhbA and DefB1 were the only 2 overexpressed secreted proteins by both the GCGR knockout liver and wild-type liver treated with inhibitory GCGR antibodies; however, these two proteins were are also upregulated by glucagon in primary hepatocytes and thus unlikely the pursued liver hormone[67,68,75].

Another possibility was that the liver hormone may not be a direct gene product such as a protein or polypeptide; rather, the hormone may be a small molecule or metabolite[67]. Metabolomes of the GCGR knockout and wild-type mice were compared[72]. Many differences exist but most notable differences were in glucose, amino acid, nucleotide, and bile acid levels[72]. The GCGR knockout mice have lower glucose levels (70% of wild-type value) and higher levels of most amino acids (up to 15-fold for alanine, glutamine, glycine, lysine, and threonine) and 2 bile acids (cholic acid and glycocholic acid, both about 200-fold) [72]. In humans with Mahvash disease, glucose levels are generally normal, but the levels of amino acids, especially alanine and glutamine, are clearly elevated[62,76-78].

Pinpointing the identity of the novel liver hormone requires tremendous amount of work. Parabiosis of GCGR knockout and wild-type mice was considered, but no such models were published[67]. A more practical *in vitro* islet culture assay was adopted by most groups to screen for the liver hormone that stimulates α cell hyperplasia and hyperglucagonemia[73-75]. With the islet culture assay, it is shown that a < 10 kDa

fraction of serum from GCGR knockout mice sufficiently stimulates α cell proliferation [75]. This fraction contains small proteins or peptides, lipids, amino acids, and metabolites[75]. We have discussed earlier that most proteins or peptides are unlikely the liver hormone. Eliminating lipids from the fraction does not change the activity of the fraction in stimulating α cell proliferation[75]. Finally, as amino acids levels are much higher in GCGR knockout serum, cocktails that mimic the amino acids levels in GCGR knockout mice serum have been tested for their ability to stimulate α cell proliferation, and indeed they do[73-75].

Individual amino acids were further tested to see if a particular amino acid is sufficient to stimulate α cell proliferation[73-75,79]. So far, the data on individual amino acids are still somewhat controversial. Most individual amino acid do not stimulate α cell proliferation or glucagon secretion[73-75,79]. Glutamine alone stimulated α cell proliferation in 2 studies, but it did not stimulate glucagon secretion in another, which is intriguing as α cell hyperplasia and hyperglucagonemia coexist in all models of GCGR inhibition[74,75,79]. Alanine alone stimulated α cell proliferation in one study, but not in another, albeit acutely stimulating glucagon release[75,79]. Experimental conditions may explain some of the different results. It is also possible that α cell proliferation and acute glucagon release may be separate processes.

The α cell receptor for amino acids is under active research. In GCCR knockout mice and in wild-type type mice treated with inhibitory GCGR antibodies, the most upregulated α cell gene is the amino acid transporter Slc38a5 (20-80-fold increase)[74, 75]. Slc38a5 preferentially transports glutamine and several other amino acids, which is concordant with the stimulatory effect of glutamine on α cell proliferation[74,75]. Slc38a5 knockout mice treated with inhibitory glucagon antibodies and Slc38a5 and GCGR double knockout mice exhibited less prominent α cell hyperplasia (approximately 50% less) but similar hyperglucagonemia[74]; this data suggested that Slc38a5 is at least partially responsible for amino acid-stimulated α cell hyperplasia and that α cell hyperplasia and hyperglucagonemia may be regulated separately. Slc38a5, however, is not expressed in human α cells[74]. Another amino acid transporter Slc38a4 is enriched in human α cells when mice with human islet implants are treated with inhibitory GCGR antibodies[80]. In humans with Mahvash disease, Slc38a4 is expressed in the α cells[80], supporting a role of the amino acid transporter in mediating amino acid-stimulated α cell hyperplasia in humans as well. The mTOR pathway in α cells is activated by amino acids as well, contributing to α cell hyperplasia[73-75].

As a result of these studies, the α cell-liver axis has largely been clarified (Figure 1). The α cells secrete glucagon, which signals the liver to increase hepatic amino acid breakdown and reduce amino acid synthesis, consequently leading to desirable amino acid levels in the circulation. After glucagon signaling is inhibited, the liver decreases amino acid breakdown and increases amino acid synthesis, thus raising circulating amino acid levels. The amino acid levels, in turn, act on the α cell amino acid transporters to stimulate α cell proliferation. The evolutionarily conserved α cell-liver axis suggests that glucagon's primary role may be regulating amino acid levels.

Betatrophin

Betatrophin (also known as angiopoietin-like protein 8, ANGPTL8) is a 22-kD protein produced and secreted by the liver and adipose tissue[81,82]. Several years ago, betatrophin was touted as the long sought-after liver hormone that stimulates pancreatic β cell proliferation and insulin production in conditions with insulin resistance[83,84]. An insulin resistance mouse model based on insulin receptor antagonist (S961) infusion exhibits remarkable hyperinsulinemia and beta cell hyperproliferation[83]. As S961 does not directly stimulate β cell proliferation, it was hypothesized that a humoral factor mediates the stimulation of β cell proliferation in this mouse model[83]. Screening of liver genes that were differentially expressed as a result of S961 infusion suggested that betatrophin, a secreted protein that is upregulated by S961 infusion, could be the humoral factor[83]. Betatrophin expression correlated well with β cell proliferation rates. The original report found that liver overexpression of betatrophin stimulated β cell proliferation[83].

The potential of betatrophin as the Holy Grail for diabetes treatment attracted much attention, but later experiments strongly argue against this function of betatrophin[85-87]. Betatrophin knockout mice exhibited normal glucose metabolism and similar hyperinsulinemia and β cell hyperproliferation in response to S961 infusion[85,86]. Detailed analysis of pancreas morphometry by several laboratories definitively showed that betatrophin overexpression does not stimulate β cell proliferation[88]. The only exception was that direct delivery of betatrophin to pancreas does stimulate β cell proliferation in rats[89]. In some mouse models of diabetes, betatrophin lowered
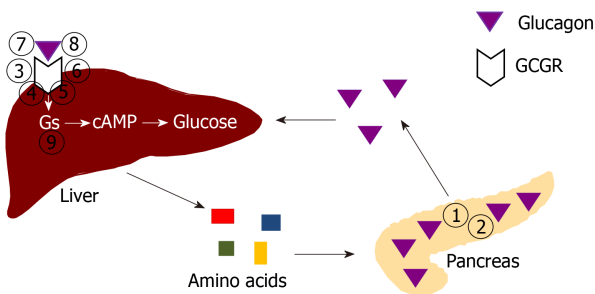
Figure 1 Schematic drawing of regulation of pancreatic α cell number and glucagon secretion by amino acid levels controlled by the liver. The numbers indicate specific ways to disrupt glucagon signaling. (1) Glucagon deletion; (2) Prohormone convertase 2 deletion (with no mature glucagon secretion); (3) Glucagon receptor (GCGR) global deletion; (4) GCGR liver-specific deletion; (5) GCGR inactivating mutation; (6) GCGR antisense RNA; (7) GCGR antagonists; (8) GCGR antibodies; and (9) Gsα liver-specific deletion. See text for details. Citation: Yu R, Zheng Y, Lucas MB, Tong YG. Elusive liver factor that causes pancreatic α cell hyperplasia: A review of literature. *World J Gastrointest Pathophysiol* 2015; 6(4): 131-139. Copyright ©The Author(s) 2015. Published by Baishideng Publishing Group Inc[67]. GCGR: Glucagon receptor.

glucose levels without effects on β cell proliferation[90]. Overall, betatrophin, despite the name, does not appear to stimulate β cell proliferation.

Betatrophin, however, could be a circulating marker of insulin resistance[82]. Early studies of betatrophin levels in various forms of human insulin resistance were quite conflictory, partly due to the differences in measurement methods[82]. Later studies using more standardized methods for measuring betatrophin were summarized by several meta-analyses on the correlation of circulating betatrophin levels and type 2 diabetes, gestational diabetes, polycystic ovary syndrome (PCOS), and obesity — all conditions with insulin resistance[91-95]. Xu *et al*[91] analyzed 25 such studies and showed a positive and significant correlation between circulating betatrophin levels and insulin resistance. Yue *et al*[92] analyzed 11 studies on betatrophin in type 2 diabetes and found that betatrophin is significantly elevated in type 2 diabetes. Kong *et al*[93] analyzed 8 studies on betatrophin in gestational diabetes and concluded that betatrophin is significantly elevated in gestational diabetes. Varikasuvu *et al*[94] analyzed 11 studies on betatrophin in PCOS and concluded that betatrophin is significantly elevated in PCOS. Similarly, Ye *et al*[95] analyzed 6 studies on betatrophin in obesity and concluded that betatrophin is significantly elevated in obesity. Thus, overall, circulating betatrophin is likely a marker of insulin resistance in humans. The high betatrophin liver expression in mice treated with S961, in retrospect, could simply be a sign of insulin resistance caused by S961[83]. It is, however, not clear how insulin resistance upregulates betatrophin. In humans, hyperinsulinemia, often associated with insulin resistance, and metformin, an insulin sensitizer, both decrease betatrophin levels, suggesting that insulin resistance per se upregulates betatrophin levels[96]. Betatrophin overexpression could further worsen hepatocyte sensitivity to insulin, the significance of which needs to be further explored[97].

Betatrophin also has a role in lipids regulation[98]. Betatrophin knockout mice exhibit much reduced triglyceride levels due to reduction in liver VLDL secretion[86]; betatrophin also forms a complex with ANGPTL3, which inhibits lipoprotein lipase (LPL) activity[86]. The increased production of VLDL and decreased LPL activity both contribute to hypertriglyceridemia. Betatrophin overexpression doubles triglyceride levels in mice[86]. In humans, circulating betatrophin levels are positively correlated with triglyceride levels in the general population[99]. In people with dyslipidemia, however, betatrophin levels were lower than in controls[100]. Betatrophin may potentially be a target in dyslipidemia treatment[101].

Hepatokines

Hepatokines are metabolism-regulating proteins produced and secreted by the liver [102,103]. Several hepatokines have been reported and studied. Five of the most studied hepatokines are discussed in this review: Fetuin-A, fibroblast growth factor 21 (FGF21), activin E, Tsukushi, and glycoprotein nonmetastatic melanoma protein B (GPNMB).

Fetuin-A: Fetuin-A, also known as α2-Heremans-Schmid glycoprotein in humans, is one of the first discovered hepatokines[104]. A 52-kD glycoprotein, fetuin-A has diverse metabolic functions[104]. Under physiological conditions, fetuin-A mostly functions as a carrier protein and regulates osteogenesis and inhibits extra-skeletal

calcification[105]. Fetuin-A's role in regulating insulin sensitivity has also been studied in detail[106,107]. Fetuin-A knockout mice exhibit higher insulin sensitivity and have less tendency to develop obesity[106]. At the molecular level, fetuin-A inhibits insulin receptor phosphorylation in myocytes and adipocytes and adiponectin expression in adipocytes[107]. Fetuin-A levels are elevated in patients with insulin resistance or type 2 diabetes, likely mediated by high free fatty acid levels, and high fetuin-A levels are a risk factor for type 2 diabetes[108,109]. The thiazolidinedione-type diabetes medication pioglitazone directly inhibits hepatic production of fetuin-A, partly contributing to its action in improving insulin sensitivity[110].

FGF21: FGF21 is a hepatokine that was first discovered in 2000, but its metabolic regulation functions were not characterized until recently[111,112]. Although FGF21 is also expressed in adipose tissue and the pancreas, circulating FGF21 is predominantly derived from the liver[113]. Hepatic FGF21 expression is regulated by a number of physiological conditions and factors[114]. Prolonged starvation (> 7 d) and overnutrition both upregulate FGF21 expression[115,116]. Glucagon and the thyroid hormone triiodothyronine (T3) both stimulate FGF21 expression, while insulin may inhibit FGF21 expression in liver[117,118]. High-carbohydrate, high-fat diet, and low protein diets stimulate FGF21 expression as well[119,120]. The microRNAs miR-577 and miR-212 target FGF21 mRNA for degradation, thus suppressing FGF21 expression[121,122]. FGF21 is also upregulated by ER stress[123]. At the molecular level, at least some of the above actions are mediated by the nuclear hormone receptor peroxisome proliferation-activated receptor α (PPARα), which binds to regions of the FGF21 promoter and simulates FGF21 expression[124-126].

The human pre-FGF21 (precursor of mature FGF21) includes a 28-amino-acid signaling peptide and a 181-amino-acid FGF21 proper as the circulating form[127]. FGF21 signals through its transmembrane tyrosine kinase receptors, FGFR1c and FGFR3c, and its transmembrane co-receptor, Klotho-β (KLB)[128]. FGF21 downstream signaling is tissue-specific but generally leads to metabolic benefits such as increased insulin sensitivity and weight loss[129]. In the adipose tissue, FGF21 stimulates the Ras/Raf/MAPK pathway, with phosphorylation of ERK1 and ERK2, and the mTOR pathway, contributing to higher insulin sensitivity[130-132]. Other FGF21 metabolic benefits such as weight loss is mediated by non-adipose tissue such as the brain[133]. FGF21 has been a major interest of metabolic drug development. As the native FGF21 is not stable in the usual formulation, re-engineered FGF21 analogues and PEGylated FGF21 have been developed to be more stable[134]. Activating monoclonal antibodies targeting FGFR1–β-klotho have also been developed[135]. Preclinical and clinical studies have demonstrated clear metabolic benefits of the FGF21 analogs and activating antibodies, such as appetite suppression, weight loss, improved glycemia, and favorable lipid profile[134,135].

Activin E: Activin E belongs to the family of transforming growth factor-β (TGF-β) proteins[136]. Activin E is a secreted homodimer of inhibin-βE, which is mainly expressed in the liver[137]. Each mature inhibin-βE monomer has 113 amino acids [137]. In both mice and humans, inhibin-βE is upregulated by obesity and insulin resistance[138]. In mice, hepatic overexpression of inhibin-βE prevents excess weight gain and improves insulin sensitivity by promoting energy expenditure *via* increased fat oxidation[139,140]. Inhibin-βE ablation in mice gives conflictory results[138,139]. In one study using the transcriptional activator-like effector nucleases (TALENs) to remove liver specific inhibin-βE expression, inhibin-βE-deficient mice exhibited normal weight but had impaired thermogenesis during cold exposure[139]. In another study, however, use of small interfering RNA (siRNA) to silence Inhibin-βE expression in the liver reduced weight gain in obese mice[138]. Thus, the roles of Activin E in metabolic regulation are still controversial.

Tsukushi: Tsukushi belongs to the family of small leucine-rich proteoglycan (SLRP) extracellular matrix proteins[141]. The secreted human Tsukushi protein has 337 amino acids. Besides its role in regulating embryonic development, Tsukushi is found to be a hepatokine, potentially regulating adipose tissue, weight, and energy expenditure[142]. In both mice and humans, Tsukushi is upregulated by thyroid hormone[142,143]; in mice, Tsukushi is induced by obesity and cold exposure[142]. Tsukushi deficiency in mice protects them from diet-induced obesity by increasing adipose tissue thermogenesis and energy expenditure[142]. Using mice from a different genetic background, another group could not reproduce the metabolic benefits of Tsukushi deficiency[144]. Furthermore, studies have also failed to show deleterious metabolic effects from Tsukushi overexpression[144]. The roles of
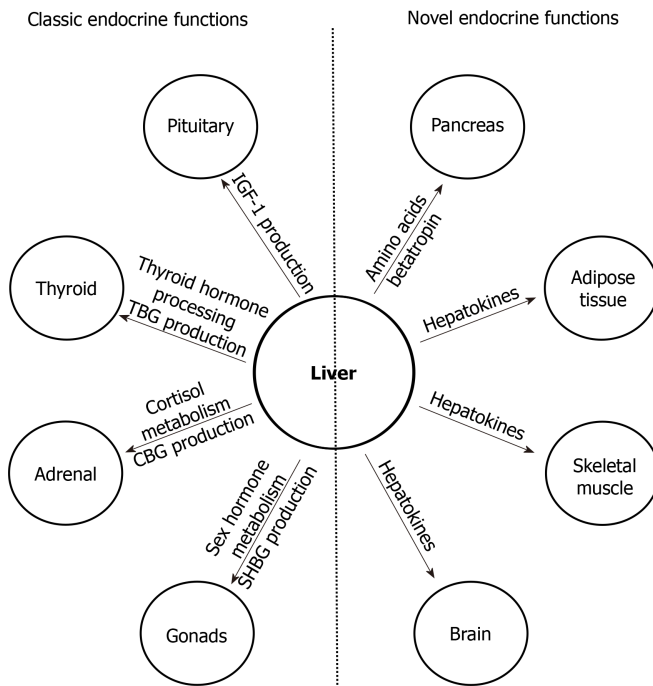
Figure 2 Major classic and novel endocrine functions of the liver. Left, major classic endocrine functions of the liver; right, novel endocrine functions of the liver. See text for details. IGF-1: Insulin-like growth factor 1; TBG: Thyroxine binding globulin; CBG: Cortisol binding globulin; SHBG: Sex hormone binding globulin.

Tsukushi in metabolic regulation thus also remain controversial.

GPNMB: GPNMB is a transmembrane glycoprotein expressed in the liver and other organs[145]. The cleaved extracellular domain of GPNMB (a glycosylated 480-amino-acid protein) is a hepatokine targeting adipose tissue[146,147]. In 2 obese mouse models, GPNMB expression was upregulated in the liver and secreted GPNMB levels were higher as well. Secreted GPNMB stimulates lipogenesis *in vitro* and *in vivo*[147]. A neutralizing antibody targeting GPNMB reduces obesity and improves insulin sensitivity[147]. In both mice and humans, GPNMB levels are positively correlated with obesity and insulin resistance[147]. GPNMB is thus a promising therapeutic target for treatments of obesity and diabetes.

CONCLUSION

The liver has numerous endocrine functions such as direct hormone and hepatokine production, hormone metabolism, synthesis of binding proteins, and processing and redistribution of metabolic fuels. In the last 10 years, many new endocrine functions of the liver have been discovered (Figure 2). Several novel endocrine functions of the liver have been unraveled. The liver plays a key negative feedback regulatory role in the pancreatic α cell-liver axis which regulates pancreatic α cell mass, glucagon secretion, and circulating amino acid levels. Betatrophin and other hepatokines such as fetuin-A and FGF21 play important endocrine roles in modulating insulin sensitivity, lipid metabolism, and body fat weight. It is expected that more endocrine functions of the liver will be discovered in the near future. As endocrine function of the liver is a rapidly evolving field, controversial findings often exist; caution needs to be taken when interpreting novel findings to avoid over-simplification of complex metabolic processes and premature allocation of research resources.

REFERENCES

1   **Watt MJ**, Miotto PM, De Nardo W, Montgomery MK. The Liver as an Endocrine Organ-Linking NAFLD and Insulin Resistance. *Endocr Rev* 2019; **40**: 1367-1393 [PMID: 31098621 DOI: 10.1210/er.2019-00034]

2   **Bikle DD**. The Free Hormone Hypothesis: When, Why, and How to Measure the Free Hormone Levels to Assess Vitamin D, Thyroid, Sex Hormone, and Cortisol Status. *JBMR Plus* 2021; **5**: e10418 [PMID: 33553985 DOI: 10.1002/jbm4.10418]

3   **Grant DM**. Detoxification pathways in the liver. *J Inherit Metab Dis* 1991; **14**: 421-430 [PMID: 1749210 DOI: 10.1007/BF01797915]

4   **Rui L**. Energy metabolism in the liver. *Compr Physiol* 2014; **4**: 177-197 [PMID: 24692138 DOI: 10.1002/cphy.c130024]

5   **Malespin M**, Nassri A. Endocrine Diseases and the Liver: An Update. *Clin Liver Dis* 2019; **23**: 233-246 [PMID: 30947874 DOI: 10.1016/j.cld.2018.12.006]

6   **Svegliati-Baroni G**, Patrício B, Lioci G, Macedo MP, Gastaldelli A. Gut-Pancreas-Liver Axis as a Target for Treatment of NAFLD/NASH. *Int J Mol Sci* 2020; **21** [PMID: 32823659 DOI: 10.3390/ijms21165820]

7   **Crispe IN**. The liver as a lymphoid organ. *Annu Rev Immunol* 2009; **27**: 147-163 [PMID: 19302037 DOI: 10.1146/annurev.immunol.021908.132629]

8   **Corsini A**, Bortolini M. Drug-induced liver injury: the role of drug metabolism and transport. *J Clin Pharmacol* 2013; **53**: 463-474 [PMID: 23436293 DOI: 10.1002/jcph.23]

9   **Eipel C**, Abshagen K, Vollmar B. Regulation of hepatic blood flow: the hepatic arterial buffer response revisited. *World J Gastroenterol* 2010; **16**: 6046-6057 [PMID: 21182219 DOI: 10.3748/wjg.v16.i48.6046]

10   **Bikle DD**. Vitamin D metabolism, mechanism of action, and clinical applications. *Chem Biol* 2014; **21**: 319-329 [PMID: 24529992 DOI: 10.1016/j.chembiol.2013.12.016]

11   **Elangovan H**, Chahal S, Gunton JE. Vitamin D in liver disease: Current evidence and potential directions. *Biochim Biophys Acta Mol Basis Dis* 2017; **1863**: 907-916 [PMID: 28064017 DOI: 10.1016/j.bbadis.2017.01.001]

12   **Christakos S**, Dhawan P, Verstuyf A, Verlinden L, Carmeliet G. Vitamin D: Metabolism, Molecular Mechanism of Action, and Pleiotropic Effects. *Physiol Rev* 2016; **96**: 365-408 [PMID: 26681795 DOI: 10.1152/physrev.00014.2015]

13   **Konstantakis C**, Tselekouni P, Kalafateli M, Triantos C. Vitamin D deficiency in patients with liver cirrhosis. *Ann Gastroenterol* 2016; **29**: 297-306 [PMID: 27366029 DOI: 10.20524/aog.2016.0037]

14   **Kineman RD**, Del Rio-Moreno M, Sarmento-Cabral A. 40 YEARS of IGF1: Understanding the tissue-specific roles of IGF1/IGF1R in regulating metabolism using the Cre/LoxP system. *J Mol Endocrinol* 2018; **61**: T187-T198 [PMID: 29743295 DOI: 10.1530/JME-18-0076]

15   **Lara-Diaz VJ**, Castilla-Cortazar I, Martín-Estal I, García-Magariño M, Aguirre GA, Puche JE, de la Garza RG, Morales LA, Muñoz U. IGF-1 modulates gene expression of proteins involved in inflammation, cytoskeleton, and liver architecture. *J Physiol Biochem* 2017; **73**: 245-258 [PMID: 28124277 DOI: 10.1007/s13105-016-0545-x]

16   **Riedemann J**, Macaulay VM. IGF1R signalling and its inhibition. *Endocr Relat Cancer* 2006; **13** Suppl 1: S33-S43 [PMID: 17259557 DOI: 10.1677/erc.1.01280]

17   **Krieger CC**, Place RF, Bevilacqua C, Marcus-Samuels B, Abel BS, Skarulis MC, Kahaly GJ, Neumann S, Gershengorn MC. TSH/IGF-1 Receptor Cross Talk in Graves' Ophthalmopathy Pathogenesis. *J Clin Endocrinol Metab* 2016; **101**: 2340-2347 [PMID: 27043163 DOI: 10.1210/jc.2016-1315]

18   **Matsusaka T**, Niimura F, Shimizu A, Pastan I, Saito A, Kobori H, Nishiyama A, Ichikawa I. Liver angiotensinogen is the primary source of renal angiotensin II. *J Am Soc Nephrol* 2012; **23**: 1181-1189 [PMID: 22518004 DOI: 10.1681/ASN.2011121159]

19   **Patel S**, Rauf A, Khan H, Abu-Izneid T. Renin-angiotensin-aldosterone (RAAS): The ubiquitous system for homeostasis and pathologies. *Biomed Pharmacother* 2017; **94**: 317-325 [PMID: 28772209 DOI: 10.1016/j.biopha.2017.07.091]

20   **Caulfield M**, Lavender P, Newell-Price J, Kamdar S, Farrall M, Clark AJ. Angiotensinogen in human essential hypertension. *Hypertension* 1996; **28**: 1123-1125 [PMID: 8952609 DOI: 10.1161/01.hyp.28.6.1123]

21   **Luongo C**, Dentice M, Salvatore D. Deiodinases and their intricate role in thyroid hormone homeostasis. *Nat Rev Endocrinol* 2019; **15**: 479-488 [PMID: 31160732 DOI: 10.1038/s41574-019-0218-2]

22   **Findlay KA**, Kaptein E, Visser TJ, Burchell B. Characterization of the uridine diphosphate-glucuronosyltransferase-catalyzing thyroid hormone glucuronidation in man. *J Clin Endocrinol Metab* 2000; **85**: 2879-2883 [PMID: 10946897 DOI: 10.1210/jcem.85.8.6715]

23   **Jin T**, Weng J. Hepatic functions of GLP-1 and its based drugs: current disputes and perspectives. *Am J Physiol Endocrinol Metab* 2016; **311**: E620-E627 [PMID: 27507553 DOI: 10.1152/ajpendo.00069.2016]

24   **MacDonald PE**, El-Kholy W, Riedel MJ, Salapatek AM, Light PE, Wheeler MB. The multiple actions of GLP-1 on the process of glucose-stimulated insulin secretion. *Diabetes* 2002; **51** Suppl 3: S434-S442 [PMID: 12475787 DOI: 10.2337/diabetes.51.2007.s434]

25   **Itou M**, Kawaguchi T, Taniguchi E, Sata M. Dipeptidyl peptidase-4: a key player in chronic liver disease. *World J Gastroenterol* 2013; **19**: 2298-2306 [PMID: 23613622 DOI: 10.3748/wjg.v19.i15.2298]

26   **Trapani L**, Segatto M, Pallottini V. Regulation and deregulation of cholesterol homeostasis: The liver as a metabolic "power station". *World J Hepatol* 2012; **4**: 184-190 [PMID: 22761969 DOI: 10.4254/wjh.v4.i6.184]

27   **Schiffer L**, Barnard L, Baranowski ES, Gilligan LC, Taylor AE, Arlt W, Shackleton CHL, Storbeck KH. Human steroid biosynthesis, metabolism and excretion are differentially reflected by serum and urine steroid metabolomes: A comprehensive review. *J Steroid Biochem Mol Biol* 2019; **194**: 105439 [PMID: 31362062 DOI: 10.1016/j.jsbmb.2019.105439]

28   **Tsuchiya Y**, Nakajima M, Yokoi T. Cytochrome P450-mediated metabolism of estrogens and its regulation in human. *Cancer Lett* 2005; **227**: 115-124 [PMID: 16112414 DOI: 10.1016/j.canlet.2004.10.007]

29   **Raftogianis R**, Creveling C, Weinshilboum R, Weisz J. Estrogen metabolism by conjugation. *J Natl Cancer Inst Monogr* 2000; 113-124 [PMID: 10963623 DOI: 10.1093/oxfordjournals.jncimonographs.a024234]

30   **Wilson VS**, LeBlanc GA. The contribution of hepatic inactivation of testosterone to the lowering of serum testosterone levels by ketoconazole. *Toxicol Sci* 2000; **54**: 128-137 [PMID: 10746940 DOI: 10.1093/toxsci/54.1.128]

31   **Quinkler M**, Stewart PM. Hypertension and the cortisol-cortisone shuttle. *J Clin Endocrinol Metab* 2003; **88**: 2384-2392 [PMID: 12788832 DOI: 10.1210/jc.2003-030138]

32   **Basu R**, Basu A, Grudzien M, Jung P, Jacobson P, Johnson M, Singh R, Sarr M, Rizza RA. Liver is the site of splanchnic cortisol production in obese nondiabetic humans. *Diabetes* 2009; **58**: 39-45 [PMID: 18852327 DOI: 10.2337/db08-1079]

33   **Fujisawa G**, Muto S, Okada K, Kusano E, Ishibashi S. Mineralocorticoid receptor antagonist spironolactone prevents pig serum-induced hepatic fibrosis in rats. *Transl Res* 2006; **148**: 149-156 [PMID: 16938653 DOI: 10.1016/j.trsl.2006.03.007]

34   **Hammond GL**. Plasma steroid-binding proteins: primary gatekeepers of steroid hormone action. *J Endocrinol* 2016; **230**: R13-R25 [PMID: 27113851 DOI: 10.1530/JOE-16-0070]

35   **Hammond GL**. Potential functions of plasma steroid-binding proteins. *Trends Endocrinol Metab* 1995; **6**: 298-304 [PMID: 18406714 DOI: 10.1016/1043-2760(95)00162-x]

36   **Sinclair M**, Grossmann M, Gow PJ, Angus PW. Testosterone in men with advanced liver disease: abnormalities and implications. *J Gastroenterol Hepatol* 2015; **30**: 244-251 [PMID: 25087838 DOI: 10.1111/jgh.12695]

37   **Fede G**, Spadaro L, Tomaselli T, Privitera G, Scicali R, Vasianopoulou P, Thalassinos E, Martin N, Thomas M, Purrello F, Burroughs AK. Comparison of total cortisol, free cortisol, and surrogate markers of free cortisol in diagnosis of adrenal insufficiency in patients with stable cirrhosis. *Clin Gastroenterol Hepatol* 2014; **12**: 504-12.e8; quiz e23 [PMID: 23978347 DOI: 10.1016/j.cgh.2013.08.028]

38   **Rakici H**. Adrenal Insufficiency in Cirrhosis Patients: Evaluation of 108 Case Series. *Euroasian J Hepatogastroenterol* 2017; **7**: 150-153 [PMID: 29201798 DOI: 10.5005/jp-journals-10018-1237]

39   **Di Pascoli M**, La Mura V. Renin-angiotensin-aldosterone system in cirrhosis: There's room to try! *Dig Liver Dis* 2019; **51**: 297-298 [PMID: 30220630 DOI: 10.1016/j.dld.2018.07.038]

40   **Sansoè G**, Aragno M, Wong F. Pathways of hepatic and renal damage through non-classical activation of the renin-angiotensin system in chronic liver disease. *Liver Int* 2020; **40**: 18-31 [PMID: 31580514 DOI: 10.1111/liv.14272]

41   **Jarvis H**, Craig D, Barker R, Spiers G, Stow D, Anstee QM, Hanratty B. Metabolic risk factors and incident advanced liver disease in non-alcoholic fatty liver disease (NAFLD): A systematic review and meta-analysis of population-based observational studies. *PLoS Med* 2020; **17**: e1003100 [PMID: 32353039 DOI: 10.1371/journal.pmed.1003100]

42   **Miyazaki M**, Kato M, Tanaka K, Tanaka M, Kohjima M, Nakamura K, Enjoji M, Nakamuta M, Kotoh K, Takayanagi R. Increased hepatic expression of dipeptidyl peptidase-4 in non-alcoholic fatty liver disease and its association with insulin resistance and glucose metabolism. *Mol Med Rep* 2012; **5**: 729-733 [PMID: 22179204 DOI: 10.3892/mmr.2011.707]

43   **Matsumoto Y**, Bishop GA, McCaughan GW. Altered zonal expression of the CD26 antigen (dipeptidyl peptidase IV) in human cirrhotic liver. *Hepatology* 1992; **15**: 1048-1053 [PMID: 1350563 DOI: 10.1002/hep.1840150613]

44   **Moura Neto A**, Zantut-Wittmann DE. Abnormalities of Thyroid Hormone Metabolism during Systemic Illness: The Low T3 Syndrome in Different Clinical Settings. *Int J Endocrinol* 2016; **2016**: 2157583 [PMID: 27803712 DOI: 10.1155/2016/2157583]

45   **Green JR**. Thyroid function in chronic liver disease. *Z Gastroenterol* 1979; **17**: 447-451 [PMID: 112792]

46   **Ohlsson C**, Mohan S, Sjögren K, Tivesten A, Isgaard J, Isaksson O, Jansson JO, Svensson J. The role of liver-derived insulin-like growth factor-I. *Endocr Rev* 2009; **30**: 494-535 [PMID: 19589948 DOI: 10.1210/er.2009-0010]

47   **Mallea-Gil MS**, Ballarino MC, Spiraquis A, Iriarte M, Kura M, Gimenez S, Oneto A, Guitelman M, Machado R, Miguel CM. IGF-1 Levels in different stages of liver steatosis and its association with metabolic syndrome. *Acta Gastroenterol Latinoam* 2012; **42**: 20-26 [PMID: 22616493]

48   **Handzlik-Orlik G**, Holecki M, Wilczyński K, Duława J. Osteoporosis in liver disease: pathogenesis and management. *Ther Adv Endocrinol Metab* 2016; **7**: 128-135 [PMID: 27293541 DOI: 10.1177/2042018816641351]

49   **Nair S**. Vitamin d deficiency and liver disease. *Gastroenterol Hepatol (N Y)* 2010; **6**: 491-493 [PMID: 20978551]

50   **Wewer Albrechtsen NJ**, Pedersen J, Galsgaard KD, Winther-Sørensen M, Suppli MP, Janah L, Gromada J, Vilstrup H, Knop FK, Holst JJ. The Liver-α-Cell Axis and Type 2 Diabetes. *Endocr Rev*

2019; **40**: 1353-1366 [PMID: 30920583 DOI: 10.1210/er.2018-00251]

51   **Dean ED**. A Primary Role for α-Cells as Amino Acid Sensors. *Diabetes* 2020; **69**: 542-549 [PMID: 31653720 DOI: 10.2337/dbi19-0021]

52   **Gromada J**, Franklin I, Wollheim CB. Alpha-cells of the endocrine pancreas: 35 years of research but the enigma remains. *Endocr Rev* 2007; **28**: 84-116 [PMID: 17261637 DOI: 10.1210/er.2006-0007]

53   **Habener JF**, Stanojevic V. Alpha cells come of age. *Trends Endocrinol Metab* 2013; **24**: 153-163 [PMID: 23260869 DOI: 10.1016/j.tem.2012.10.009]

54   **Habener JF**, Stanojevic V. α-cell role in β-cell generation and regeneration. *Islets* 2012; **4**: 188-198 [PMID: 22847495 DOI: 10.4161/isl.20500]

55   **Quesada I**, Tudurí E, Ripoll C, Nadal A. Physiology of the pancreatic alpha-cell and glucagon secretion: role in glucose homeostasis and diabetes. *J Endocrinol* 2008; **199**: 5-19 [PMID: 18669612 DOI: 10.1677/JOE-08-0290]

56   **Habegger KM**, Heppner KM, Geary N, Bartness TJ, DiMarchi R, Tschöp MH. The metabolic actions of glucagon revisited. *Nat Rev Endocrinol* 2010; **6**: 689-697 [PMID: 20957001 DOI: 10.1038/nrendo.2010.187]

57   **Parker JC**, Andrews KM, Allen MR, Stock JL, McNeish JD. Glycemic control in mice with targeted disruption of the glucagon receptor gene. *Biochem Biophys Res Commun* 2002; **290**: 839-843 [PMID: 11785978 DOI: 10.1006/bbrc.2001.6265]

58   **Gelling RW**, Du XQ, Dichmann DS, Romer J, Huang H, Cui L, Obici S, Tang B, Holst JJ, Fledelius C, Johansen PB, Rossetti L, Jelicks LA, Serup P, Nishimura E, Charron MJ. Lower blood glucose, hyperglucagonemia, and pancreatic alpha cell hyperplasia in glucagon receptor knockout mice. *Proc Natl Acad Sci U S A* 2003; **100**: 1438-1443 [PMID: 12552113 DOI: 10.1073/pnas.0237106100]

59   **Yu R**, Dhall D, Nissen NN, Zhou C, Ren SG. Pancreatic neuroendocrine tumors in glucagon receptor-deficient mice. *PLoS One* 2011; **6**: e23397 [PMID: 21853126 DOI: 10.1371/journal.pone.0023397]

60   **Yu R**, Nissen NN, Dhall D, Heaney AP. Nesidioblastosis and hyperplasia of alpha cells, microglucagonoma, and nonfunctioning islet cell tumor of the pancreas: review of the literature. *Pancreas* 2008; **36**: 428-431 [PMID: 18437091 DOI: 10.1097/MPA.0b013e31815ceb23]

61   **Zhou C**, Dhall D, Nissen NN, Chen CR, Yu R. Homozygous P86S mutation of the human glucagon receptor is associated with hyperglucagonemia, alpha cell hyperplasia, and islet cell tumor. *Pancreas* 2009; **38**: 941-946 [PMID: 19657311 DOI: 10.1097/MPA.0b013e3181b2bb03]

62   **Yu R**. Mahvash Disease: 10 Years After Discovery. *Pancreas* 2018; **47**: 511-515 [PMID: 29702528 DOI: 10.1097/MPA.0000000000001044]

63   **Yu R**. Pancreatic α-cell hyperplasia: facts and myths. *J Clin Endocrinol Metab* 2014; **99**: 748-756 [PMID: 24285676 DOI: 10.1210/jc.2013-2952]

64   **Li M**, Dean ED, Zhao L, Nicholson WE, Powers AC, Chen W. Glucagon receptor inactivation leads to α-cell hyperplasia in zebrafish. *J Endocrinol* 2015; **227**: 93-103 [PMID: 26446275 DOI: 10.1530/JOE-15-0284]

65   **Bai X**, Jia J, Kang Q, Fu Y, Zhou Y, Zhong Y, Zhang C, Li M. Integrated Metabolomics and Lipidomics Analysis Reveal Remodeling of Lipid Metabolism and Amino Acid Metabolism in Glucagon Receptor-Deficient Zebrafish. *Front Cell Dev Biol* 2020; **8**: 605979 [PMID: 33520988 DOI: 10.3389/fcell.2020.605979]

66   **Yan H**, Gu W, Yang J, Bi V, Shen Y, Lee E, Winters KA, Komorowski R, Zhang C, Patel JJ, Caughey D, Elliott GS, Lau YY, Wang J, Li YS, Boone T, Lindberg RA, Hu S, Véniant MM. Fully human monoclonal antibodies antagonizing the glucagon receptor improve glucose homeostasis in mice and monkeys. *J Pharmacol Exp Ther* 2009; **329**: 102-111 [PMID: 19129372 DOI: 10.1124/jpet.108.147009]

67   **Yu R**, Zheng Y, Lucas MB, Tong YG. Elusive liver factor that causes pancreatic α cell hyperplasia: A review of literature. *World J Gastrointest Pathophysiol* 2015; **6**: 131-139 [PMID: 26600971 DOI: 10.4291/wjgp.v6.i4.131]

68   **Longuet C**, Robledo AM, Dean ED, Dai C, Ali S, McGuinness I, de Chavez V, Vuguin PM, Charron MJ, Powers AC, Drucker DJ. Liver-specific disruption of the murine glucagon receptor produces α-cell hyperplasia: evidence for a circulating α-cell growth factor. *Diabetes* 2013; **62**: 1196-1205 [PMID: 23160527 DOI: 10.2337/db11-1605]

69   **Chen M**, Gavrilova O, Zhao WQ, Nguyen A, Lorenzo J, Shen L, Nackers L, Pack S, Jou W, Weinstein LS. Increased glucose tolerance and reduced adiposity in the absence of fasting hypoglycemia in mice with liver-specific Gs alpha deficiency. *J Clin Invest* 2005; **115**: 3217-3227 [PMID: 16239968 DOI: 10.1172/JCI24196]

70   **Shen DM**, Lin S, Parmee ER. A survey of small molecule glucagon receptor antagonists from recent patents (2006 - 2010). *Expert Opin Ther Pat* 2011; **21**: 1211-1240 [PMID: 21635155 DOI: 10.1517/13543776.2011.587001]

71   **Scheen AJ**, Paquot N, Lefèbvre PJ. Investigational glucagon receptor antagonists in Phase I and II clinical trials for diabetes. *Expert Opin Investig Drugs* 2017; **26**: 1373-1389 [PMID: 29052441 DOI: 10.1080/13543784.2017.1395020]

72   **Yang J**, MacDougall ML, McDowell MT, Xi L, Wei R, Zavadoski WJ, Molloy MP, Baker JD, Kuhn M, Cabrera O, Treadway JL. Polyomic profiling reveals significant hepatic metabolic alterations in glucagon-receptor (GCGR) knockout mice: implications on anti-glucagon therapies for diabetes. *BMC Genomics* 2011; **12**: 281 [PMID: 21631939 DOI: 10.1186/1471-2164-12-281]

73   **Solloway MJ**, Madjidi A, Gu C, Eastham-Anderson J, Clarke HJ, Kljavin N, Zavala-Solorio J, Kates L, Friedman B, Brauer M, Wang J, Fiehn O, Kolumam G, Stern H, Lowe JB, Peterson AS, Allan BB. Glucagon Couples Hepatic Amino Acid Catabolism to mTOR-Dependent Regulation of α-Cell Mass. *Cell Rep* 2015; **12**: 495-510 [PMID: 26166562 DOI: 10.1016/j.celrep.2015.06.034]

74   **Kim J**, Okamoto H, Huang Z, Anguiano G, Chen S, Liu Q, Cavino K, Xin Y, Na E, Hamid R, Lee J, Zambrowicz B, Unger R, Murphy AJ, Xu Y, Yancopoulos GD, Li WH, Gromada J. Amino Acid Transporter Slc38a5 Controls Glucagon Receptor Inhibition-Induced Pancreatic α Cell Hyperplasia in Mice. *Cell Metab* 2017; **25**: 1348-1361.e8 [PMID: 28591637 DOI: 10.1016/j.cmet.2017.05.006]

75   **Dean ED**, Li M, Prasad N, Wisniewski SN, Von Deylen A, Spaeth J, Maddison L, Botros A, Sedgeman LR, Bozadjieva N, Ilkayeva O, Coldren A, Poffenberger G, Shostak A, Semich MC, Aamodt KI, Phillips N, Yan H, Bernal-Mizrachi E, Corbin JD, Vickers KC, Levy SE, Dai C, Newgard C, Gu W, Stein R, Chen W, Powers AC. Interrupted Glucagon Signaling Reveals Hepatic α Cell Axis and Role for L-Glutamine in α Cell Proliferation. *Cell Metab* 2017; **25**: 1362-1373.e5 [PMID: 28591638 DOI: 10.1016/j.cmet.2017.05.011]

76   **Larger E**, Wewer Albrechtsen NJ, Hansen LH, Gelling RW, Capeau J, Deacon CF, Madsen OD, Yakushiji F, De Meyts P, Holst JJ, Nishimura E. Pancreatic α-cell hyperplasia and hyperglucagonemia due to a glucagon receptor splice mutation. *Endocrinol Diabetes Metab Case Rep* 2016; **2016** [PMID: 27933176 DOI: 10.1530/EDM-16-0081]

77   **Li H**, Zhao L, Singh R, Ham JN, Fadoju DO, Bean LJH, Zhang Y, Xu Y, Xu HE, Gambello MJ. The first pediatric case of glucagon receptor defect due to biallelic mutations in *GCGR* is identified by newborn screening of elevated arginine. *Mol Genet Metab Rep* 2018; **17**: 46-52 [PMID: 30294546 DOI: 10.1016/j.ymgmr.2018.09.006]

78   **Gild ML**, Tsang V, Samra J, Clifton-Bligh RJ, Tacon L, Gill AJ. Hypercalcemia in Glucagon Cell Hyperplasia and Neoplasia (Mahvash Syndrome): A New Association. *J Clin Endocrinol Metab* 2018; **103**: 3119-3123 [PMID: 30032256 DOI: 10.1210/jc.2018-01074]

79   **Galsgaard KD**, Jepsen SL, Kjeldsen SAS, Pedersen J, Wewer Albrechtsen NJ, Holst JJ. Alanine, arginine, cysteine, and proline, but not glutamine, are substrates for, and acute mediators of, the liver-α-cell axis in female mice. *Am J Physiol Endocrinol Metab* 2020; **318**: E920-E929 [PMID: 32255678 DOI: 10.1152/ajpendo.00459.2019]

80   **Kim J**, Dominguez Gutierrez G, Xin Y, Cavino K, Sung B, Sipos B, Kloeppel G, Gromada J, Okamoto H. Increased SLC38A4 Amino Acid Transporter Expression in Human Pancreatic α-Cells After Glucagon Receptor Inhibition. *Endocrinology* 2019; **160**: 979-988 [PMID: 30938753 DOI: 10.1210/en.2019-00022]

81   **Kaestner KH**. Betatrophin--promises fading and lessons learned. *Cell Metab* 2014; **20**: 932-933 [PMID: 25470542 DOI: 10.1016/j.cmet.2014.11.016]

82   **Abu-Farha M**, Abubaker J, Tuomilehto J. ANGPTL8 (betatrophin) role in diabetes and metabolic diseases. *Diabetes Metab Res Rev* 2017; **33** [PMID: 28722798 DOI: 10.1002/dmrr.2919]

83   **Yi P**, Park JS, Melton DA. Betatrophin: a hormone that controls pancreatic β cell proliferation. *Cell* 2013; **153**: 747-758 [PMID: 23623304 DOI: 10.1016/j.cell.2013.04.008]

84   **Seymour PA**, Serup P. Bulking up on beta cells. *N Engl J Med* 2013; **369**: 777-779 [PMID: 23964941 DOI: 10.1056/NEJMcibr1307038]

85   **Gusarova V**, Alexa CA, Na E, Stevis PE, Xin Y, Bonner-Weir S, Cohen JC, Hobbs HH, Murphy AJ, Yancopoulos GD, Gromada J. ANGPTL8/betatrophin does not control pancreatic beta cell expansion. *Cell* 2014; **159**: 691-696 [PMID: 25417115 DOI: 10.1016/j.cell.2014.09.027]

86   **Wang Y**, Quagliarini F, Gusarova V, Gromada J, Valenzuela DM, Cohen JC, Hobbs HH. Mice lacking ANGPTL8 (Betatrophin) manifest disrupted triglyceride metabolism without impaired glucose homeostasis. *Proc Natl Acad Sci U S A* 2013; **110**: 16109-16114 [PMID: 24043787 DOI: 10.1073/pnas.1315292110]

87   **Cox AR**, Lam CJ, Bonnyman CW, Chavez J, Rios JS, Kushner JA. Angiopoietin-like protein 8 (ANGPTL8)/betatrophin overexpression does not increase beta cell proliferation in mice. *Diabetologia* 2015; **58**: 1523-1531 [PMID: 25917759 DOI: 10.1007/s00125-015-3590-z]

88   **Cox AR**, Barrandon O, Cai EP, Rios JS, Chavez J, Bonnyman CW, Lam CJ, Yi P, Melton DA, Kushner JA. Resolving Discrepant Findings on ANGPTL8 in β-Cell Proliferation: A Collaborative Approach to Resolving the Betatrophin Controversy. *PLoS One* 2016; **11**: e0159276 [PMID: 27410263 DOI: 10.1371/journal.pone.0159276]

89   **Chen J**, Chen S, Huang P, Meng XL, Clayton S, Shen JS, Grayburn PA. In vivo targeted delivery of ANGPTL8 gene for beta cell regeneration in rats. *Diabetologia* 2015; **58**: 1036-1044 [PMID: 25720603 DOI: 10.1007/s00125-015-3521-z]

90   **Liu S**, Smith EM, King TH, Glenn L, Trevino M, Park SH, Machida Y, Villaflor C, Grzesik W, Morris MA, Imai Y, Nadler JL. Host Factors Alter Effects of Angiopoietin-Like Protein 8 on Glucose Homeostasis in Diabetic Mice. *J Diabetes Mellitus* 2016; **6**: 277-290 [PMID: 31741751 DOI: 10.4236/jdm.2016.64029]

91   **Xu J**, Lin Y, Zhou H, Zhao L, Xiang G. The Correlation Between Circulating Betatrophin and Insulin Resistance in General Population: A Meta-Analysis. *Horm Metab Res* 2017; **49**: 760-771 [PMID: 28931172 DOI: 10.1055/s-0043-108911]

92   **Yue S**, Wu J, Zhang J, Liu L, Chen L. The Relationship between Betatrophin Levels in Blood and T2DM: A Systematic Review and Meta-Analysis. *Dis Markers* 2016; **2016**: 9391837 [PMID: 27242389 DOI: 10.1155/2016/9391837]

93   **Kong FJ**, Ma LL, Li G, Chen YX, Zhou JQ. Circulating Betatrophin Levels and Gestational

Diabetes Mellitus: A Systematic Review and Meta-Analysis. *PLoS One* 2017; **12**: e0169941 [PMID: 28081192 DOI: 10.1371/journal.pone.0169941]

94   **Varikasuvu SR**, Panga JR, Satyanarayana MV. Circulating Angiopoietin-like 8 protein (ANGPTL8/Betatrophin) in patients with polycystic ovary syndrome: a systematic review and multi effect size meta-analysis. *Gynecol Endocrinol* 2019; **35**: 190-197 [PMID: 30614305 DOI: 10.1080/09513590.2018.1536736]

95   **Ye J**, Qin Y, Wang D, Yang L, Yuan G. The Relationship between Circulating ANGPTL8/Betatrophin Concentrations and Adult Obesity: A Meta-Analysis. *Dis Markers* 2019; **2019**: 5096860 [PMID: 31772689 DOI: 10.1155/2019/5096860]

96   **Wang H**, Du L, Wu T, Yang G, Hu W, Wang H, Yang M, Liu D, Gu HF, Zhu Z, Zheng H, Li L. Circulating betatrophin is associated with insulin resistance in humans: cross-sectional and interventional studies *in vivo* and *in vitro*. *Oncotarget* 2017; **8**: 96604-96614 [PMID: 29228555 DOI: 10.18632/oncotarget.21852]

97   **Hao Q**, Zheng A, Zhang H, Cao H. Down-regulation of betatrophin enhances insulin sensitivity in type 2 diabetes mellitus through activation of the GSK-3β/PGC-1α signaling pathway. *J Endocrinol Invest* 2021; **44**: 1857-1868 [PMID: 33464548 DOI: 10.1007/s40618-020-01493-1]

98   **Zhang R**. Lipasin, a novel nutritionally-regulated liver-enriched factor that regulates serum triglyceride levels. *Biochem Biophys Res Commun* 2012; **424**: 786-792 [PMID: 22809513 DOI: 10.1016/j.bbrc.2012.07.038]

99   **Gao T**, Jin K, Chen P, Jin H, Yang L, Xie X, Yang M, Hu C, Yu X. Circulating Betatrophin Correlates with Triglycerides and Postprandial Glucose among Different Glucose Tolerance Statuses--A Case-Control Study. *PLoS One* 2015; **10**: e0133640 [PMID: 26247824 DOI: 10.1371/journal.pone.0133640]

100   **Gómez-Ambrosi J**, Pascual-Corrales E, Catalán V, Rodríguez A, Ramírez B, Romero S, Vila N, Ibáñez P, Margall MA, Silva C, Gil MJ, Salvador J, Frühbeck G. Altered Concentrations in Dyslipidemia Evidence a Role for ANGPTL8/Betatrophin in Lipid Metabolism in Humans. *J Clin Endocrinol Metab* 2016; **101**: 3803-3811 [PMID: 27472196 DOI: 10.1210/jc.2016-2084]

101   **Fu Z**, Abou-Samra AB, Zhang R. A lipasin/Angptl8 monoclonal antibody lowers mouse serum triglycerides involving increased postprandial activity of the cardiac lipoprotein lipase. *Sci Rep* 2015; **5**: 18502 [PMID: 26687026 DOI: 10.1038/srep18502]

102   **Jung TW**, Yoo HJ, Choi KM. Implication of hepatokines in metabolic disorders and cardiovascular diseases. *BBA Clin* 2016; **5**: 108-113 [PMID: 27051596 DOI: 10.1016/j.bbacli.2016.03.002]

103   **Jensen-Cody SO**, Potthoff MJ. Hepatokines and metabolism: Deciphering communication from the liver. *Mol Metab* 2021; **44**: 101138 [PMID: 33285302 DOI: 10.1016/j.molmet.2020.101138]

104   **Bourebaba L**, Marycz K. Pathophysiological Implication of Fetuin-A Glycoprotein in the Development of Metabolic Disorders: A Concise Review. *J Clin Med* 2019; **8** [PMID: 31766373 DOI: 10.3390/jcm8122033]

105   **Jahnen-Dechent W**, Heiss A, Schäfer C, Ketteler M. Fetuin-A regulation of calcified matrix metabolism. *Circ Res* 2011; **108**: 1494-1509 [PMID: 21659653 DOI: 10.1161/CIRCRESAHA.110.234260]

106   **Mathews ST**, Rakhade S, Zhou X, Parker GC, Coscina DV, Grunberger G. Fetuin-null mice are protected against obesity and insulin resistance associated with aging. *Biochem Biophys Res Commun* 2006; **350**: 437-443 [PMID: 17011519 DOI: 10.1016/j.bbrc.2006.09.071]

107   **Ren G**, Kim T, Papizan JB, Okerberg CK, Kothari VM, Zaid H, Bilan PJ, Araya-Ramirez F, Littlefield LA, Bowers RL, Mahurin AJ, Nickles MM, Ludvigsen R, He X, Grandjean PW, Mathews ST. Phosphorylation status of fetuin-A is critical for inhibition of insulin action and is correlated with obesity and insulin resistance. *Am J Physiol Endocrinol Metab* 2019; **317**: E250-E260 [PMID: 31084489 DOI: 10.1152/ajpendo.00089.2018]

108   **Ramírez-Vélez R**, García-Hermoso A, Hackney AC, Izquierdo M. Effects of exercise training on Fetuin-a in obese, type 2 diabetes and cardiovascular disease in adults and elderly: a systematic review and Meta-analysis. *Lipids Health Dis* 2019; **18**: 23 [PMID: 30670052 DOI: 10.1186/s12944-019-0962-2]

109   **Pan X**, Wen SW, Bestman PL, Kaminga AC, Acheampong K, Liu A. Fetuin-A in Metabolic syndrome: A systematic review and meta-analysis. *PLoS One* 2020; **15**: e0229776 [PMID: 32134969 DOI: 10.1371/journal.pone.0229776]

110   **Ochi A**, Mori K, Emoto M, Nakatani S, Morioka T, Motoyama K, Fukumoto S, Imanishi Y, Koyama H, Ishimura E, Inaba M. Direct inhibitory effects of pioglitazone on hepatic fetuin-A expression. *PLoS One* 2014; **9**: e88704 [PMID: 24551137 DOI: 10.1371/journal.pone.0088704]

111   **Fisher FM**, Maratos-Flier E. Understanding the Physiology of FGF21. *Annu Rev Physiol* 2016; **78**: 223-241 [PMID: 26654352 DOI: 10.1146/annurev-physiol-021115-105339]

112   **Tezze C**, Romanello V, Sandri M. FGF21 as Modulator of Metabolism in Health and Disease. *Front Physiol* 2019; **10**: 419 [PMID: 31057418 DOI: 10.3389/fphys.2019.00419]

113   **Markan KR**, Naber MC, Ameka MK, Anderegg MD, Mangelsdorf DJ, Kliewer SA, Mohammadi M, Potthoff MJ. Circulating FGF21 is liver derived and enhances glucose uptake during refeeding and overfeeding. *Diabetes* 2014; **63**: 4057-4063 [PMID: 25008183 DOI: 10.2337/db14-0595]

114   **Erickson A**, Moreau R. The regulation of FGF21 gene expression by metabolic factors and nutrients. *Horm Mol Biol Clin Investig* 2016; **30** [PMID: 27285327 DOI: 10.1515/hmbci-2016-0016]

115   **Fazeli PK**, Lun M, Kim SM, Bredella MA, Wright S, Zhang Y, Lee H, Catana C, Klibanski A, Patwari P, Steinhauser ML. FGF21 and the late adaptive response to starvation in humans. *J Clin*

Invest 2015; **125**: 4601-4611 [PMID: 26529252 DOI: 10.1172/JCI83349]

116   **Zhang X**, Yeung DC, Karpisek M, Stejskal D, Zhou ZG, Liu F, Wong RL, Chow WS, Tso AW, Lam KS, Xu A. Serum FGF21 Levels are increased in obesity and are independently associated with the metabolic syndrome in humans. *Diabetes* 2008; **57**: 1246-1253 [PMID: 18252893 DOI: 10.2337/db07-1476]

117   **Habegger KM**, Stemmer K, Cheng C, Müller TD, Heppner KM, Ottaway N, Holland J, Hembree JL, Smiley D, Gelfanov V, Krishna R, Arafat AM, Konkar A, Belli S, Kapps M, Woods SC, Hofmann SM, D'Alessio D, Pfluger PT, Perez-Tilve D, Seeley RJ, Konishi M, Itoh N, Kharitonenkov A, Spranger J, DiMarchi RD, Tschöp MH. Fibroblast growth factor 21 mediates specific glucagon actions. *Diabetes* 2013; **62**: 1453-1463 [PMID: 23305646 DOI: 10.2337/db12-1116]

118   **Adams AC**, Astapova I, Fisher FM, Badman MK, Kurgansky KE, Flier JS, Hollenberg AN, Maratos-Flier E. Thyroid hormone regulates hepatic expression of fibroblast growth factor 21 in a PPARalpha-dependent manner. *J Biol Chem* 2010; **285**: 14078-14082 [PMID: 20236931 DOI: 10.1074/jbc.C110.107375]

119   **Uebanso T**, Taketani Y, Fukaya M, Sato K, Takei Y, Sato T, Sawada N, Amo K, Harada N, Arai H, Yamamoto H, Takeda E. Hypocaloric high-protein diet improves fatty liver and hypertriglyceridemia in sucrose-fed obese rats *via* two pathways. *Am J Physiol Endocrinol Metab* 2009; **297**: E76-E84 [PMID: 19435858 DOI: 10.1152/ajpendo.00014.2009]

120   **Martínez-Garza Ú**, Torres-Oteros D, Yarritu-Gallego A, Marrero PF, Haro D, Relat J. Fibroblast Growth Factor 21 and the Adaptive Response to Nutritional Challenges. *Int J Mol Sci* 2019; **20** [PMID: 31546675 DOI: 10.3390/ijms20194692]

121   **Chen XY**, Li GM, Dong Q, Peng H. MiR-577 inhibits pancreatic β-cell function and survival by targeting fibroblast growth factor 21 (FGF-21) in pediatric diabetes. *Genet Mol Res* 2015; **14**: 15462-15470 [PMID: 26634512 DOI: 10.4238/2015.November.30.24]

122   **Xiao J**, Bei Y, Liu J, Dimitrova-Shumkovska J, Kuang D, Zhou Q, Li J, Yang Y, Xiang Y, Wang F, Yang C, Yang W. miR-212 downregulation contributes to the protective effect of exercise against non-alcoholic fatty liver *via* targeting FGF-21. *J Cell Mol Med* 2016; **20**: 204-216 [PMID: 26648452 DOI: 10.1111/jcmm.12733]

123   **Schaap FG**, Kremer AE, Lamers WH, Jansen PL, Gaemers IC. Fibroblast growth factor 21 is induced by endoplasmic reticulum stress. *Biochimie* 2013; **95**: 692-699 [PMID: 23123503 DOI: 10.1016/j.biochi.2012.10.019]

124   **Gälman C**, Lundåsen T, Kharitonenkov A, Bina HA, Eriksson M, Hafström I, Dahlin M, Amark P, Angelin B, Rudling M. The circulating metabolic regulator FGF21 is induced by prolonged fasting and PPARalpha activation in man. *Cell Metab* 2008; **8**: 169-174 [PMID: 18680716 DOI: 10.1016/j.cmet.2008.06.014]

125   **Vernia S**, Cavanagh-Kyros J, Garcia-Haro L, Sabio G, Barrett T, Jung DY, Kim JK, Xu J, Shulha HP, Garber M, Gao G, Davis RJ. The PPARα-FGF21 hormone axis contributes to metabolic regulation by the hepatic JNK signaling pathway. *Cell Metab* 2014; **20**: 512-525 [PMID: 25043817 DOI: 10.1016/j.cmet.2014.06.010]

126   **Videla LA**, Fernández V, Vargas R, Cornejo P, Tapia G, Varela N, Valenzuela R, Arenas A, Fernández J, Hernández-Rodas MC, Riquelme B. Upregulation of rat liver PPARα-FGF21 signaling by a docosahexaenoic acid and thyroid hormone combined protocol. *Biofactors* 2016; **42**: 638-646 [PMID: 27248050 DOI: 10.1002/biof.1300]

127   **Nishimura T**, Nakatake Y, Konishi M, Itoh N. Identification of a novel FGF, FGF-21, preferentially expressed in the liver. *Biochim Biophys Acta* 2000; **1492**: 203-206 [PMID: 10858549 DOI: 10.1016/s0167-4781(00)00067-1]

128   **Kilkenny DM**, Rocheleau JV. The FGF21 Receptor Signaling Complex: Klothoβ, FGFR1c, and Other Regulatory Interactions. *Vitam Horm* 2016; **101**: 17-58 [PMID: 27125737 DOI: 10.1016/bs.vh.2016.02.008]

129   **Staiger H**, Keuper M, Berti L, Hrabe de Angelis M, Häring HU. Fibroblast Growth Factor 21-Metabolic Role in Mice and Men. *Endocr Rev* 2017; **38**: 468-488 [PMID: 28938407 DOI: 10.1210/er.2017-00016]

130   **Li H**, Wu G, Fang Q, Zhang M, Hui X, Sheng B, Wu L, Bao Y, Li P, Xu A, Jia W. Fibroblast growth factor 21 increases insulin sensitivity through specific expansion of subcutaneous fat. *Nat Commun* 2018; **9**: 272 [PMID: 29348470 DOI: 10.1038/s41467-017-02677-9]

131   **Ge X**, Chen C, Hui X, Wang Y, Lam KS, Xu A. Fibroblast growth factor 21 induces glucose transporter-1 expression through activation of the serum response factor/Ets-like protein-1 in adipocytes. *J Biol Chem* 2011; **286**: 34533-34541 [PMID: 21846717 DOI: 10.1074/jbc.M111.248591]

132   **Minard AY**, Tan SX, Yang P, Fazakerley DJ, Domanova W, Parker BL, Humphrey SJ, Jothi R, Stöckli J, James DE. mTORC1 Is a Major Regulatory Node in the FGF21 Signaling Network in Adipocytes. *Cell Rep* 2016; **17**: 29-36 [PMID: 27681418 DOI: 10.1016/j.celrep.2016.08.086]

133   **Sarruf DA**, Thaler JP, Morton GJ, German J, Fischer JD, Ogimoto K, Schwartz MW. Fibroblast growth factor 21 action in the brain increases energy expenditure and insulin sensitivity in obese rats. *Diabetes* 2010; **59**: 1817-1824 [PMID: 20357365 DOI: 10.2337/db09-1878]

134   **Geng L**, Lam KSL, Xu A. The therapeutic potential of FGF21 in metabolic diseases: from bench to clinic. *Nat Rev Endocrinol* 2020; **16**: 654-667 [PMID: 32764725 DOI: 10.1038/s41574-020-0386-0]

135   **Baruch A**, Wong C, Chinn LW, Vaze A, Sonoda J, Gelzleichter T, Chen S, Lewin-Koh N, Morrow

L, Dheerendra S, Boismenu R, Gutierrez J, Wakshull E, Wilson ME, Arora PS. Antibody-mediated activation of the FGFR1/Klothoβ complex corrects metabolic dysfunction and alters food preference in obese humans. *Proc Natl Acad Sci U S A* 2020; **117**: 28992-29000 [PMID: 33139537 DOI: 10.1073/pnas.2012073117]

136 **Fang J**, Wang SQ, Smiley E, Bonadio J. Genes coding for mouse activin beta C and beta E are closely linked and exhibit a liver-specific expression pattern in adult tissues. *Biochem Biophys Res Commun* 1997; **231**: 655-661 [PMID: 9070865 DOI: 10.1006/bbrc.1997.6162]

137 **Namwanje M**, Brown CW. Activins and Inhibins: Roles in Development, Physiology, and Disease. *Cold Spring Harb Perspect Biol* 2016; **8** [PMID: 27328872 DOI: 10.1101/cshperspect.a021881]

138 **Sugiyama M**, Kikuchi A, Misu H, Igawa H, Ashihara M, Kushima Y, Honda K, Suzuki Y, Kawabe Y, Kaneko S, Takamura T. Inhibin βE (INHBE) is a possible insulin resistance-associated hepatokine identified by comprehensive gene expression analysis in human liver biopsy samples. *PLoS One* 2018; **13**: e0194798 [PMID: 29596463 DOI: 10.1371/journal.pone.0194798]

139 **Hashimoto O**, Funaba M, Sekiyama K, Doi S, Shindo D, Satoh R, Itoi H, Oiwa H, Morita M, Suzuki C, Sugiyama M, Yamakawa N, Takada H, Matsumura S, Inoue K, Oyadomari S, Sugino H, Kurisaki A. Activin E Controls Energy Homeostasis in Both Brown and White Adipose Tissues as a Hepatokine. *Cell Rep* 2018; **25**: 1193-1203 [PMID: 30380411 DOI: 10.1016/j.celrep.2018.10.008]

140 **Sekiyama K**, Ushiro Y, Kurisaki A, Funaba M, Hashimoto O. Activin E enhances insulin sensitivity and thermogenesis by activating brown/beige adipocytes. *J Vet Med Sci* 2019; **81**: 646-652 [PMID: 30880304 DOI: 10.1292/jvms.19-0036]

141 **Dellett M**, Hu W, Papadaki V, Ohnuma S. Small leucine rich proteoglycan family regulates multiple signalling pathways in neural development and maintenance. *Dev Growth Differ* 2012; **54**: 327-340 [PMID: 22524604 DOI: 10.1111/j.1440-169X.2012.01339.x]

142 **Wang Q**, Sharma VP, Shen H, Xiao Y, Zhu Q, Xiong X, Guo L, Jiang L, Ohta K, Li S, Shi H, Rui L, Lin JD. The hepatokine Tsukushi gates energy expenditure *via* brown fat sympathetic innervation. *Nat Metab* 2019; **1**: 251-260 [PMID: 31535079 DOI: 10.1038/s42255-018-0020-9]

143 **Liu D**, Zhang P, Wei X, Deng Y, Liu W, Guo D, Liu J, Xu B, Huang C, Huang J, Lin J, Liu S, Xue Y, Zhang H. Elevated Serum Tsukushi Levels in Patients With Hyperthyroidism. *Front Endocrinol (Lausanne)* 2020; **11**: 580097 [PMID: 33117292 DOI: 10.3389/fendo.2020.580097]

144 **Mouchiroud M**, Camiré É, Aldow M, Caron A, Jubinville É, Turcotte L, Kaci I, Beaulieu MJ, Roy C, Labbé SM, Varin TV, Gélinas Y, Lamothe J, Trottier J, Mitchell PL, Guénard F, Festuccia WT, Joubert P, Rose CF, Karvellas CJ, Barbier O, Morissette MC, Marette A, Laplante M. The Hepatokine TSK does not affect brown fat thermogenic capacity, body weight gain, and glucose homeostasis. *Mol Metab* 2019; **30**: 184-191 [PMID: 31767170 DOI: 10.1016/j.molmet.2019.09.014]

145 **van der Lienden MJC**, Gaspar P, Boot R, Aerts JMFG, van Eijk M. Glycoprotein Non-Metastatic Protein B: An Emerging Biomarker for Lysosomal Dysfunction in Macrophages. *Int J Mol Sci* 2018; **20** [PMID: 30586924 DOI: 10.3390/ijms20010066]

146 **Kuang H**, Lin JD. GPNMB: expanding the code for liver-fat communication. *Nat Metab* 2019; **1**: 507-508 [PMID: 32694853 DOI: 10.1038/s42255-019-0069-0]

147 **Gong XM**, Li YF, Luo J, Wang JQ, Wei J, Xiao T, Xie C, Hong J, Ning G, Shi XJ, Li BL, Qi W, Song BL. Gpnmb secreted from liver promotes lipogenesis in white adipose tissue and aggravates obesity and insulin resistance. *Nat Metab* 2019; **1**: 570-583 [PMID: 32694855 DOI: 10.1038/s42255-019-0065-4]

Current strategies to induce liver remnant hypertrophy before major liver resection

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Abstract

Hepatic resection is the gold standard for patients affected by primary or metastatic liver tumors but is hampered by the risk of post-hepatectomy liver failure. Despite recent impro-vements, liver surgery still requires excellent clinical judgement in selecting patients for surgery and, above all, efficient pre-operative strategies to provide adequate future liver remnant. The aim of this article is to review the literature on the rational, the preliminary assessment, the advantages as well as the limits of each existing technique for preparing the liver for major hepatectomy.

Key Words: Liver regeneration; Major hepatectomy; Liver insufficiency; Future liver remnant; Portal vein embolization

Core Tip: Hepatic resection is the gold standard for patients affected by liver tumors but is hampered by the risk of post-hepatectomy liver failure. We herein review the literature on the rational, the preliminary assessment, the advantages as well as the limits of each existing technique for preparing the liver for major hepatectomy.

reviewed.

INTRODUCTION

Hepatic resection is the gold standard for patients affected by primary or metastatic liver tumors but is hampered by the risk of post hepatectomy liver failure (PHLF). Indeed, PHLF is considered the most frightening complication of liver surgery, representing a major source of severe morbidity and mortality[1]. Despite recent improvements, liver surgery still requires excellent clinical judgement in selecting patients for surgery and, above all, efficient pre-operative tools to provide an adequate future liver remnant (FLR).

The liver has a unique capacity of preserving its volume due to regeneration. The atrophy-hypertrophy phenomenon is a prime example of the liver's pathophysiologic (atrophy) and restorative (hypertrophy) response to injury[2]. It occurs whenever there is impairment of bile or blood flow: the liver reacts with atrophy of the region concerned and with compensatory hypertrophy of the less or not impaired regions, resulting in characteristic gross deformity of the organ and, in some instances, in rotation of the liver around a virtual hilar axis[3]. The mechanisms that induce cellular division are complex and based on different inflammatory cytokines. The Hepatocyte Growth Factor (HGF) seems to be the main mitogenic factor and its role has been established in liver regeneration[4].

The first case of *in vivo* human hepatic regeneration was described by Pack *et al*[5] in 1962. Starting from animal models in the first half of the 20th century, it was recognized that liver regeneration could also be induced by portal vein ligation (PVL)[6]. In 1986, the first cases of percutaneous transhepatic portal vein embolization (PVE) were performed before liver resection in the setting of hepatocellular carcinoma[7], and a few years later Makuuchi *et al*[8] reported the utility of PVE in promoting FLR hypertrophy prior to hepatic resection in patients with hilar cholangiocarcinoma. Since those initial reports, preoperative PVE has been established as the standard procedure for obtaining FRL hypertrophy, increasing the eligibility of patients for major hepatectomy as well as improving postoperative outcomes and safety. However, concerns regarding the insufficient increase of FLR and/or concomitant tumoral progression after PVE have led to the development of recent alternative techniques to push further the limits of liver surgery.

The aim of this article is to review the techniques available for preparing the liver for major hepatectomy, and to depict their advantages and limitations.

LIVER REGENERATION

The liver's unique capacity for regeneration was first recorded in the legend of Prometheus in Greek mythology and it represents the basis of the treatment of many liver diseases. Regeneration of the liver is a pathophysiological process, embracing both hypertrophy (increase in cell size or protein content in the prereplicative phase) and hyperplasia (increase in cell numbers). Both events can take place independently [9]. The mechanisms of liver regeneration have mainly been studied after extensive hepatectomy. The players of regeneration following the different techniques exposed in this article are thought to be similar to those after hepatectomy, but the precise mechanism remains unknown. Basically, the regeneration process is a cytokine- and growth-factor-mediated pathway. The main cytokine-mediated pathways include members of the innate immune system, tumor necrosis factor (TNF)α and interleukin (IL)-6, and growth-factor-mediated pathways are regulated by HGF and transforming growth factor (TGF)α[10]. It is a multi-step process, starting from the "priming" of hepatocytes, the moment they acquire replicative capacity, followed by the proliferative step in which an adequate cell mass is attained, and a termination stage in which liver cell proliferation is ended once the necessary functional mass has been reached[11]. Proliferation of hepatocytes advances from periportal to pericentral areas of the lobule, as a wave of mitoses[12]. Proliferation of biliary epithelial cells occurs a

little later than hepatocytes. The particularity of liver regeneration is that replacement of the lost hepatic mass is not mediated by selected stem cells proliferation but it entirely depends on mature adult hepatocytes and other hepatic cell types. Concerning the time interval, as far as we know, normal liver weight is reestablished within 8-15 d in humans[13].

POST-HEPATECTOMY LIVER FAILURE

Although morbidity and mortality after liver surgery have improved over the past 10 years, PHLF is still reported in up to 8%, ranging from 1.2% to 32%, and depends on the patient's condition and functional reserve of the liver before resection[1]. Different definitions of PHLF are available. In 2011, the International Study Group of Liver Surgery (ISGLS) defined PHLF as "a post-operatively acquired deterioration in the ability of the liver to maintain its synthetic, excretory, and detoxifying functions, which are characterized by an increased International Normalized Ratio (INR) and concomitant hyperbilirubinemia on or after postoperative day 5"[14]. It is worth pointing out that severe PHLF is associated with a mortality rate of 54%.

A related syndrome that results in a transient but sometimes fatal form of liver failure has been described following liver transplantation (LT) but also after extensive liver resection. This is the so-called Small For Size Syndrome (SFSS). In 2005, Dahm *et al*[15] defined SFSS as a graft to recipient weight ratio < 0.8% alongside two of the following for three consecutive days; bilirubin > 100 mmol/L, INR > 2 and enceph-alopathy grade 3 or 4. In this definition, SFSS is a clinical syndrome characterized by post-operative liver dysfunction, prolonged cholestasis and coagulopathy, portal hypertension and ascites. It can lead to a higher rate of hemorrhage, sepsis and gastrointestinal bleeding[16]. The key point of SFSS is the presence of portal hypertension and intra-hepatic portal congestion as the underlying cause of liver failure[17].

PREDICTION OF PHLF RISK

Despite improvements in surgical and postoperative management, parameters determining the degree of possible hepatectomy remain largely uncertain. Different patient related and surgical factors have to be considered to decrease PHLF incidence. Surgical factors include the extent of resection and volume of FLR, duration of intraop-erative liver ischemia during portal pedicle clamping, duration of surgery and the need for blood transfusion. The risk of PHLF is highly influenced by the quality of underlying liver parenchyma. The type of underlying liver parenchyma is frequently assessed by preoperative liver biopsy, but noninvasive methods, such as liver stiffness, are now available. For example, liver stiffness measurement by transient elastography (Fibroscan) predicts persistent hepatic decompensation in patients undergoing resection for hepatocellular carcinoma[18].

It is generally thought that the minimal functional liver mass needed for adequate postoperative liver function is estimated to be 20%-25% in patients with normal liver parenchyma, whereas those with chemotherapy-induced liver injury require a FLR volume of approximately 30%, while those with cirrhosis at least a 40% minimal functional liver mass[19]. Therefore, standardized FLR volume can be easily evaluated by a tridimensional computed tomography (CT) reconstruction method, as FLR/ estimated total liver volume[20]. Estimated total liver volume is generally calculated using a formula based on body surface area[21].

In addition to volume, estimation of FLR function is an important factor. Typical biochemical parameters, such as liver function tests, albumin, and clotting factors must be evaluated. The old but effective Child-Turcotte-Pugh score, which was introduced in 1964, still represents a simple system for grading liver function[22]. The model for end-stage liver disease score, which is mainly used in liver transplantation, can also predict the survival rate of cirrhotic patients to better select ideal candidates for surgery[23]. A recent study also showed that mean serum level of hyaluronic acid can be a useful tool, especially when liver biopsy is not feasible[24].

Dynamic tests of liver function can also be used. The most well-known is indocyanine green (ICG) clearance. ICG is a water soluble, inert, fluorescent tricar-bocyanine dye with protein binding close to 95% (mainly, alpha1- and beta-lipoproteins and albumin), a hepatic extraction rate above 70%, and is almost completely excreted in its unchanged form by the liver. ICG elimination can be

expressed as ICG plasma disappearance rate (ICGPDR) or retention rate at 15 min (ICGR15), reflecting liver function. Use of the ICG test for patient selection has been shown to decrease postoperative mortality[25].

In recent years, there have been several attempts to assess hepatobiliary magnetic resonance imaging (MRI) as a tool to predict liver dysfunction. Since it was first described in 1991 by Weinmann *et al*[26], MRI has been showed to provide both global and segmental liver function information, and postoperative remnant liver function thanks to the measurement of liver signal intensity in the hepatobiliary phase.

Liver function evaluation by nuclear medicine techniques is also more and more used. Dynamic 99mTc-mebrofenin hepatobiliary scintigraphy has been used to provide quantitative information on total and regional liver function. The hepatic uptake of 99mTc-mebrofenin is similar to the uptake of organic anions such as bilirubin[27]. This technique efficiently estimates the risk of postoperative liver failure especially in patients with uncertain quality of liver parenchyma[28]. The 99m Tc-GSA is another recently proposed agent that is not affected by hyperbilirubinemia and can be used for liver function assessment in cholestatic patients[29]. Finally, the LiMAx test allows real-time *in vivo* determination of liver Cytochrome P450 1A2 (CYP1A2) activity. The CYP1A2 is not influenced by cholestasis or drugs and is ubiquitous in liver parenchyma. Intravenous administration of 13C methacetin, a substance exclusively metabolized by CYP1A2, with continuous real-time breath analysis represents the basis of the LiMAx test[30].

PORTAL VEIN EMBOLIZATION

Since the first report in 1986, PVE has progressively become the gold standard for inducing liver hypertrophy with satisfying safety and efficacy[31]. Initially described by laparotomy, the portal system access is now obtained by percutaneous puncture of the portal vein. According to the operator's preference, an ipsilateral or contralateral approach can be chosen, in reference to the segment bearing the tumor. The ipsilateral approach has the main advantage of protecting the FLR from injury[2] whereas the contralateral approach facilitates embolization[32]. Irrespective of the approach chosen, PVE is performed in a retrograde manner (Figure 1). Many embolic materials have been used for PVE without significant differences in terms of hypertrophy. Embolic materials include fibrin glue, N-butyl-2-cyanoacrylate and ethiodized oil, gelatin sponge and thrombin, coils, microparticles [*e.g.,* polyvinyl alcohol (PVA) particles or tris-acryl gelatin microspheres] and absolute alcohol[33]. A non absorbable material is generally used. However, interesting results were reported with the use of an absorbable powder material (Gelfoam® powder, Pfizer, New York, USA) that lasts approximately 2 wk, leading to temporary PVE. In an animal model, this method showed efficient and stable liver regeneration[34]. These results were confirmed in a limited preliminary series in clinical practice[35] and a prospective study is undergoing (EMBORES study, NCT02945059). One of the advantages of temporary PVE is that it can theoretically be repeated several times to boost more liver hypertrophy, as has been suggested in an animal model[36].

PVE is successfully performed in more than 90% of cases[37]. A computed tomography scan with volumetric evaluation is generally performed between 4 and 8 wk after embolization. PVE induces a FLR hypertrophy than can reach 40%[37], with a low 2% morbidity rate and no mortality in the vast majority of studies[37-39]. PVE is considered an efficient method, allowing successful hepatectomy in more than 70% of cases[37,38,40].

Contraindications to PVE are extensive portal thrombus and important portal hypertension[41]. Another potential limit of PVE is the risk of tumor growth during the 4 to 8 wk separating PVE and liver surgery. In addition, several authors have suggested that PVE itself could promote tumor growth within the embolized liver[42-45]. Among others, these reasons have led to the development of alternative strategies.

PORTAL VEIN LIGATION (PVL) AND TWO-STAGE HEPATECTOMY

As it requires a surgical procedure with portal pedicles dissection, PVL is nowadays mainly indicated in the setting of two-stage hepatectomy (TSH) for the treatment of bilobar liver disease[46,47]. In the TSH strategy, the first surgical step includes tumoral clearance of the FLR (usually by parenchymal spearing resections or locoregional treatment like radiofrequency ablation) and concomitant PVL that allows FLR growth.
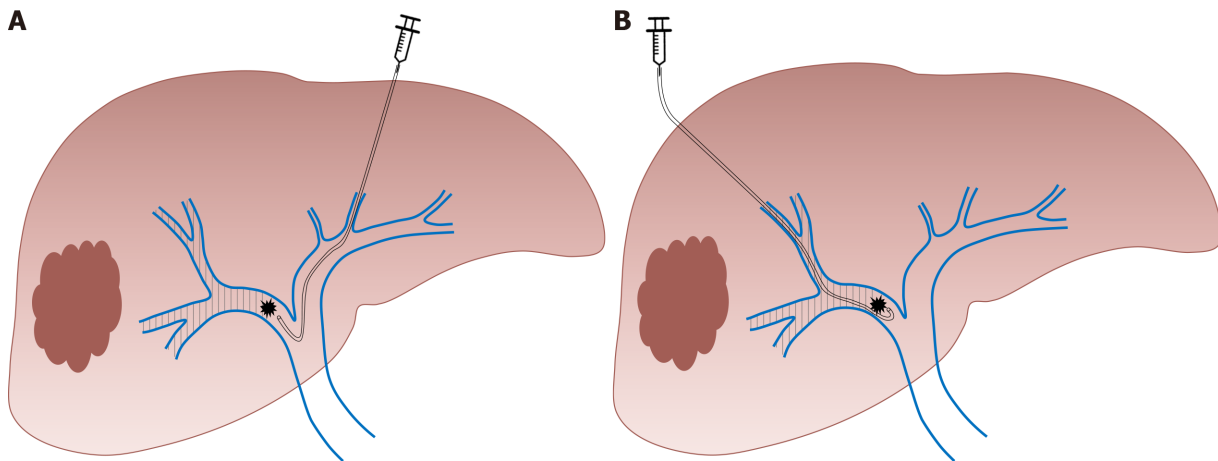
Figure 1 Right portal vein embolization using. A: Contralateral; B: Ipsilateral approach.

In the second step, after liver regeneration (approximately 4 to 8 wk later), major liver resection is performed (usually right or right extended hepatectomy) (Figure 2). Similarly, PVL can be performed for the management of patients presenting synchronous colorectal metastases or neuroendocrine tumors[47]. The first surgical step associates colorectal resection with PVL, followed by major liver surgery in the second procedure. However, many centers have adopted PVE (performed by the percutaneous approach after FLR clearance or colorectal resection) for two-step procedures, avoiding portal pedicle dissection and facilitating the second procedure [48].

It was initially suggested that PVE resulted in superior FLR growth compared to PVL[49] as in theory PVE allows distal portal obstruction which decreases the possibility of intrahepatic collateral development. Several studies demonstrated that the results are globally similar[50,51]. In fact, the debate concerning the efficiency of PVL compared to PVE is no longer relevant. PVL requires a surgical procedure and can appear as an alternative to PVE only when a two-step surgery is planned. In other cases, percutaneous PVE is clearly a simpler and better tolerated approach.

ASSOCIATING LIVER PARTITION AND PORTAL VEIN LIGATION FOR STAGED HEPATECTOMY

The aim of this alternative strategy, described by Schnizbauer *et al*[52] in 2012, is to induce rapid and massive liver hypertrophy, to allow liver surgery in a short period of time in patients with initially very limited FRL volume. The first step of the associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) procedure consists of performing PVL and an *in situ* splitting of the liver parenchyma, leaving the hepatic artery, bile duct, and hepatic vein intact until the subsequent operation. This first surgical step can be associated with tumoral clearance of the FRL. During the second operation (that can be performed one to two weeks later) the remaining hepatic artery, bile duct, and hepatic vein are divided and the liver specimen is extracted (Figure 3).

The first report demonstrated a morbidity rate of 44% and a mortality rate of 12% [52], and triggered an intense debate on the safety of this procedure, limiting its promotion worldwide. The morbi-mortality rate decreased with experience but remains high, with approximately 40% of major postoperative complications and 9% of mortality[53]. Nevertheless, the ALPPS technique induces more than 65% of FLR growth in approximately 7 days[52-55] and the second procedure is feasible in more than 90% of cases[56]. The main advantage of the ALPPS procedure is the rapid increase in FLR volume in a short interval and therefore a shorter interval between the two stages. Although the volumetric results of this technique are impressive, several authors suggested that FLR volume hypertrophy is not correlated to functional improvement[57,58] which could partly explain the high morbidity of the procedure. Besides, concerns have been raised by some authors regarding potentially poorer oncological results comparing to the classical TSH[59]. The results of a meta-analysis comparing ALPPS to TSH showed that the extent of FLR increase was not different
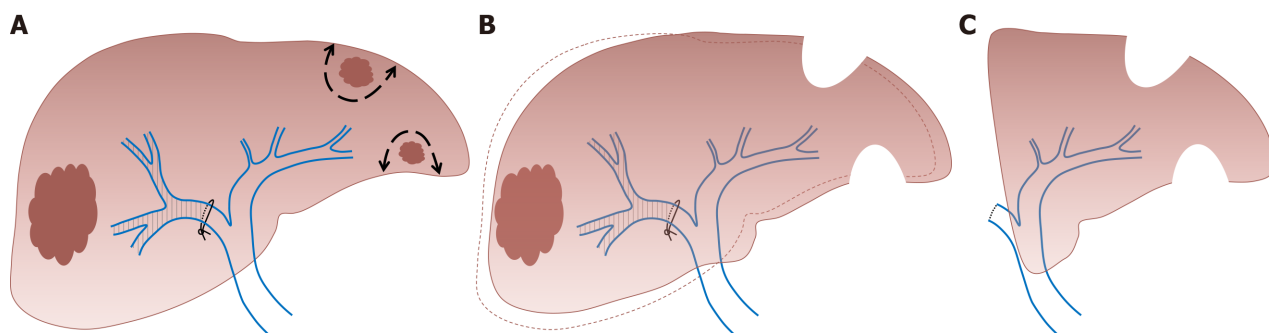
Figure 2 Two-stage hepatectomy procedure starts with tumoral clearance of the future liver remnant. A: Concomitant right portal vein ligation; B: Allowing left liver growth; C: Ends with right hepatectomy.
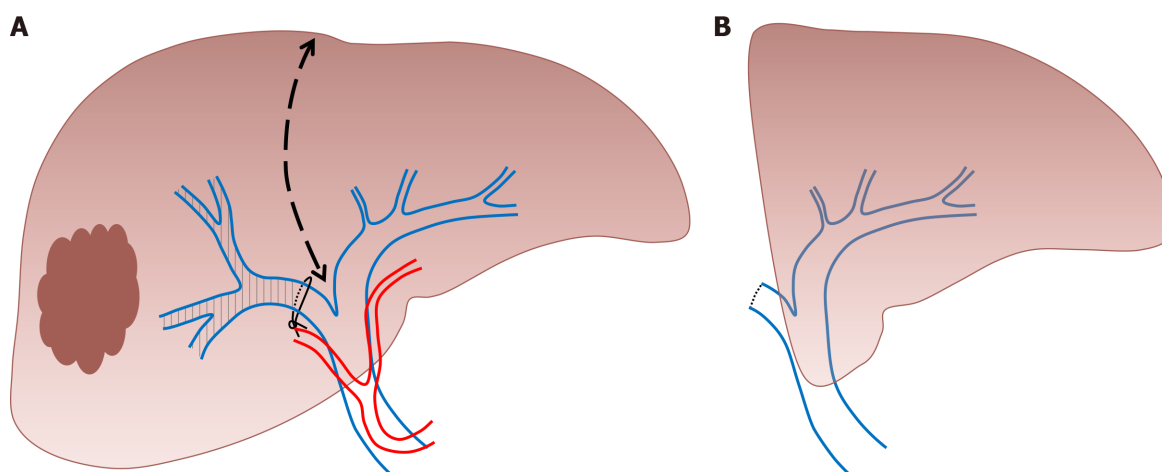
Figure 3 Associating liver partition and portal vein ligation for staged hepatectomy procedure. A: Starts with *in situ* splitting of the liver parenchyma with concomitant right portal vein ligation; B: Ends with right hepatectom.

between the two groups[60]. The time needed to reach final liver volume was shorter in ALPPS than in the TSH approach[60]. In this meta-analysis, ALPPS was associated with a higher incidence of major and overall morbidity and mortality compared to TSH[60]. However, in a recent randomized controlled trial, Hasselgren *et al*[61] observed similar morbidity between ALPPS and classical TSH and an improved survival in the ALPPS group.

To decrease complication rate, a variety of technical modifications have been proposed such as partial-ALPPS, mini-ALPPS, tourniquet-ALPPS, hybrid-ALPPS, microwave ablation-assisted ALPPS and radiofrequency ablation-assisted ALPPS. Huang *et al*[62] suggested in a systematic review that a partial ALPPS technique in which only partial parenchymal sparing is performed during the first surgical step could achieve lower morbidity and mortality rates, reaching the same FLR hypertrophy rate as ALPPS in non-cirrhotic patients.

SEQUENTIAL TRANS-ARTERIAL EMBOLIZATION (TAE) AND PORTAL VEIN EMBOLIZATION

Although PVE remains the gold standard for FLR hypertrophy, two concerns persist with this approach: An insufficient contralateral hypertrophy, particularly in patients with underlying liver disease (steatosis, fibrosis or cirrhosis), and the eventuality of tumor progression while waiting for the non-embolized liver to hypertrophy. In particular, portal flow interruption may induce a compensatory increase in arterial blood flow of embolized segments and result in a paradoxical growth of tumors vascularized by arterial blood flow. In this context, it has been postulated that the addition of trans-arterial embolization (TAE) or trans-arterial chemoembolization (TACE) would produce more rapid and extensive FLR growth (by obtaining

obliteration of intrahepatic arterioportal shunts) and may help to counteract the stimulating effect on tumor growth[63]. Therefore, hepatocellular carcinomas, which are tumors particularly vascularized by arterial blood flow and develop generally in underlying pathological liver parenchyma, are the main target of this combined strategy[64].

During TAE, a catheter is directly inserted *via* either the common femoral or left radial artery and an intra-arterial injection of a combination of microspheres and PVA particles is performed in the arterial branches of the segments to be resected. During TACE, an intra-arterial injection of a cytotoxic drug is performed such as doxorubicin, epirubicin, idarubicin, mitomycin C, or cisplatin, that is emulsified in ethiodized oil (Lipiodol® Ultra-Fluid, Guerbet). This is followed by intra-arterial injection of an embolic agent, such as gelatin sponge, PVA particles, or microspheres[65] (Figure 4). TACE can also be performed using recently developed drug-eluting beads (DEB) that allow the slow release of chemotherapeutic agents, and increase ischemia intensity and duration[65].

A sequential approach, with a time interval of a few days, is recommended to limit the risk of nontumoral liver ischemic necrosis[66] and TAE is mostly performed before PVE[66,67]. Although the number of patients reported in studies that evaluated this approach is limited, observed FLR hypertrophy is generally superior to that observed after isolated PVE. For example, Yoo *et al*[68] reported a statistically significant increase of 7.3% and 5.8% in FLR (over the total liver volume) for sequential TACE/PVE and isolated PVE, respectively.

An important elevation of transaminases is generally observed after this sequential approach without important clinical consequences. In the largest series reporting this approach, Peng *et al*[64] reported 29 procedures without deaths and only one complication and 27 patients (93%) underwent subsequent hepatectomy. Post-hepatectomy morbidity and mortality among these patients was 27.5% and 6.9%, respectively.

Theoretical contraindications of this method include extensive portal thrombus, important portal hypertension or previous biliary surgery (biliodigestive anastomosis) which exposes the patient to hepatic abscess formation after arterial embolization.

LIVER VENOUS DEPRIVATION

This technique consists of performing conventional PVE and ipsilateral hepatic vein obstruction (Figure 5). By associating hepatic vein embolization, the aim is to eliminate any residual portal vein flow and reduce hepatic artery inflow which can further encourage liver regeneration. Initially described as a sequential approach in which hepatic vein embolization is secondarily performed in case of insufficient FLR growth after PVE, it was demonstrated that both procedures (portal and hepatic vein embolization) can be performed simultaneously[69,70]. This novel approach is particularly interesting as it allows important liver regeneration with good tolerance. Although no study comparing ALPPS to LVD is available, it has been suggested that LVD could overcome the limits of ALPPS, abolishing the necessity of two major surgical interventions in close sequence.

Firstly, PVE is performed as previously described. For hepatic vein embolization, a vascular plug is placed in the proximal part of the hepatic vein to avoid migration of embolization agent. The vein is then embolized with a mixture of ethiodized oil and N- butyl cyanoacrylate[71]. The term "extended LVD" is used for concomitant embolization of the right and middle hepatic vein with the right portal branch[57].

The results of this approach on FLR increase are superior to those observed after isolated PVE. In a recent large comparative study, Laurent *et al*[71] observed a FLR volume increase of 28.9% after PVE compared to 61.2% after LVD ($P < 0.0001$). In this study, LVD allowed surgery in 86.4% of patients and no PHLF was reported. Kobayashi *et al*[72] observed similar results with a superior FLR hypertrophy after LVD compared to PVE (35% *vs* 24%, $P = 0.034$). In addition, the tolerance of LVD seems to be similar to the tolerance of isolated PVE[71,72].

RADIATION LOBECTOMY

This recent approach is derived from trans-arterial radioembolization with yttrium-90 [73]. In radiation lobectomy (RL), radioembolization of both the tumor and the non-tumoral liver parenchyma that will be secondarily resected is performed, which
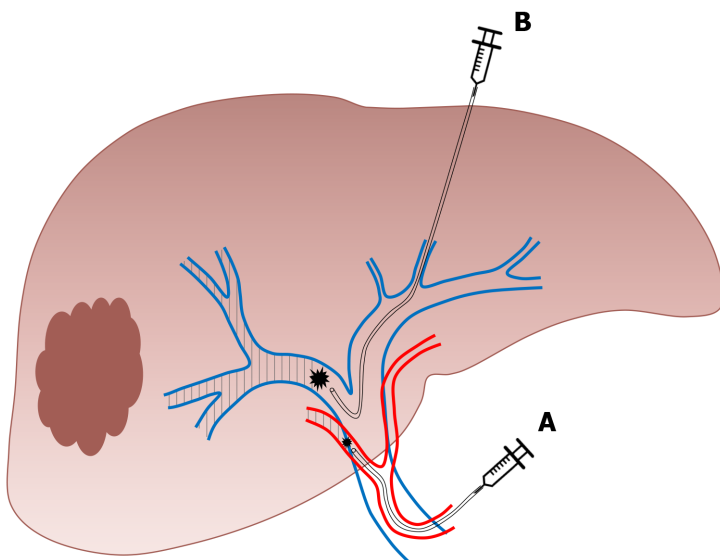
Figure 4 Sequential embolization. A: Trans-arterial embolization; B: Portal vein embolization of the right liver.
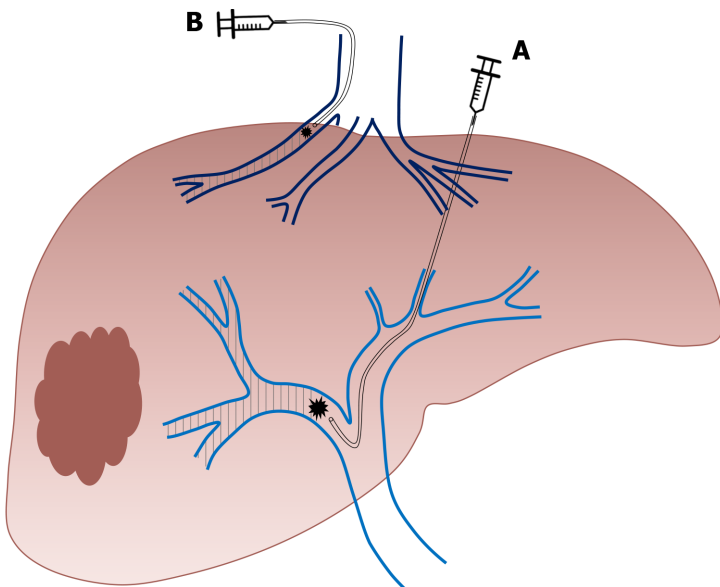
Figure 5 Right liver venous derivation associates in a sequential or concomitant approach. A: Right portal vein embolization; B: Ipsilateral hepatic vein embolization.

requires higher radiation doses[74,75]. This technique allows concomitant tumoral control and FLR increase. One major advantage of this approach is that it could be carried out in patients with portal vein thrombosis[75].

The procedure is well-tolerated[74] with transient moderate adverse events. Results in terms of FLR volume growth are very similar to those observed after PVE. Vouche *et al*[74] reported 45% of FLR hypertrophy and observed a correlation between the presence of a portal vein thrombosis and FLR growth. However, series reporting major liver resection after RL are scarce[76,77]. Andel *et al*[77] recently reported 10 major hepatectomies in patients that were initially treated with RL for insufficient functional FLR. The RL allowed a 41% increase in FLR volume with 84% of FLR function increase (evaluated on scintigraphy). All resections were performed without major intraoperative problems. Only one patient developed a serious complication not directly related to the liver surgery and other complications were mild.

Table 1 Indication, advantages, and disadvantages of existing approaches to induce liver remnant hypertrophy before major liver resection			
Approach	**Indication**	**Advantage**	**Disadvantage**
PVE	Insufficient FLR volume	Percutaneous approach	Contraindicated in patients with extensive portal thrombus and important portal hypertension; Could promote tumoral growth within the embolized liver
PVL and two-stage hepatectomy	Insufficient FLR volume and treatment of bilobar liver disease	PVL is performed during the first surgical step (tumoral clearance of the FLR)	Surgical procedure; Morbidity
Associating liver partition and PVL for staged hepatectomy	Insufficient FLR volume +/- treatment of bilobar liver disease	Liver surgery is performed in a short period of time (15 d); First surgical step (PVL and *in situ* splitting of the liver parenchyma) can be associated with tumoral clearance of the FLR	Surgical procedure; Morbidity
Sequential trans arterial embolization and PVE	Insufficient FLR volume in patients with hepatocellular carcinoma	Percutaneous approachMay help to counteract the stimulating effect of PVE on tumor growth	Sequential approach (two procedures) is recommended to limit the risk of nontumoral liver ischemic necrosis; Contraindicated in patients with extensive portal thrombus, important portal hypertension or previous biliary surgery (biliodigestive anastomosis)
Liver venous deprivation	Insufficient FLR volume	Percutaneous approach	Contraindicated in patients with extensive portal thrombus and important portal hypertension; Could promote tumoral growth within the embolized liver
RL	Insufficient FLR volume	Percutaneous approachConcomitant tumoral control and FLR increaseCan be carried out in patients with portal vein thrombosis	Data reporting liver resection after RL is scarce

PVE: Portal vein embolization; FLR: Future liver remnant; PVL: Portal vein ligation; RL: Radiation lobectomy.

CONCLUSION

Careful initial evaluation of FLR volume and function is crucial before planning major liver resection. When required, several approaches are now available to decrease the risk of PHLF (Table 1) and thus postoperative mortality. Although PVE remains the gold standard, recent techniques that are derived from PVE might play an increasingly important role in future years.

REFERENCES

1   **Schreckenbach T**, Liese J, Bechstein WO, Moench C. Posthepatectomy liver failure. *Dig Surg* 2012; **29**: 79-85 [PMID: 22441624 DOI: 10.1159/000335741]
2   **Black DM**, Behrns KE. A scientist revisits the atrophy-hypertrophy complex: hepatic apoptosis and regeneration. *Surg Oncol Clin N Am* 2002; **11**: 849-864 [PMID: 12607575 DOI: 10.1016/s1055-3207(02)00031-5]
3   **Kim RD**, Kim JS, Watanabe G, Mohuczy D, Behrns KE. Liver regeneration and the atrophy-hypertrophy complex. *Semin Intervent Radiol* 2008; **25**: 92-103 [PMID: 21326550 DOI: 10.1055/s-2008-1076679]
4   **Fajardo-Puerta AB**, Mato Prado M, Frampton AE, Jiao LR. Gene of the month: HGF. *J Clin Pathol* 2016; **69**: 575-579 [PMID: 27072686 DOI: 10.1136/jclinpath-2015-203575]
5   **Pack GT**, Islami AH, Hubbard JC, Brasfield RD. Regeneration of human liver after major hepatectomy. *Surgery* 1962; **52**: 617-623 [PMID: 14483060]
6   **Honjo I**, Suzuki T, Ozawa K, Takasan H, Kitamura O. Ligation of a branch of the portal vein for carcinoma of the liver. *Am J Surg* 1975; **130**: 296-302 [PMID: 170837 DOI: 10.1016/0002-9610(75)90389-x]
7   **Kinoshita H**, Sakai K, Hirohashi K, Igawa S, Yamasaki O, Kubo S. Preoperative portal vein embolization for hepatocellular carcinoma. *World J Surg* 1986; **10**: 803-808 [PMID: 3022488 DOI: 10.1007/BF01655244]
8   **Makuuchi M**, Thai BL, Takayasu K, Takayama T, Kosuge T, Gunvén P, Yamazaki S, Hasegawa H, Ozaki H. Preoperative portal embolization to increase safety of major hepatectomy for hilar bile duct carcinoma: a preliminary report. *Surgery* 1990; **107**: 521-527 [PMID: 2333592]
9   **Palmes D**, Spiegel HU. Animal models of liver regeneration. *Biomaterials* 2004; **25**: 1601-1611 [PMID: 14697862 DOI: 10.1016/s0142-9612(03)00508-8]
10   **Taub R**. Liver regeneration: from myth to mechanism. *Nat Rev Mol Cell Biol* 2004; **5**: 836-847 [PMID: 15459664 DOI: 10.1038/nrm1489]
11   **Markiewski MM**, DeAngelis RA, Lambris JD. Liver inflammation and regeneration: two distinct

biological phenomena or parallel pathophysiologic processes? *Mol Immunol* 2006; **43**: 45-56 [PMID: 16002143 DOI: 10.1016/j.molimm.2005.06.019]

12   **Rabes HM**. Kinetics of hepatocellular proliferation as a function of the microvascular structure and functional state of the liver. *Ciba Found Symp* 1977; 31-53 [PMID: 248005 DOI: 10.1002/9780470720363.ch3]

13   **Michalopoulos GK**. Liver regeneration. *J Cell Physiol* 2007; **213**: 286-300 [PMID: 17559071 DOI: 10.1002/jcp.21172]

14   **Rahbari NN**, Garden OJ, Padbury R, Brooke-Smith M, Crawford M, Adam R, Koch M, Makuuchi M, Dematteo RP, Christophi C, Banting S, Usatoff V, Nagino M, Maddern G, Hugh TJ, Vauthey JN, Greig P, Rees M, Yokoyama Y, Fan ST, Nimura Y, Figueras J, Capussotti L, Büchler MW, Weitz J. Posthepatectomy liver failure: a definition and grading by the International Study Group of Liver Surgery (ISGLS). *Surgery* 2011; **149**: 713-724 [PMID: 21236455 DOI: 10.1016/j.surg.2010.10.001]

15   **Dahm F**, Georgiev P, Clavien PA. Small-for-size syndrome after partial liver transplantation: definition, mechanisms of disease and clinical implications. *Am J Transplant* 2005; **5**: 2605-2610 [PMID: 16212618 DOI: 10.1111/j.1600-6143.2005.01081.x]

16   **Tucker ON**, Heaton N. The 'small for size' liver syndrome. *Curr Opin Crit Care* 2005; **11**: 150-155 [PMID: 15758596 DOI: 10.1097/01.ccx.0000157080.11117.45]

17   **Riddiough GE**, Christophi C, Jones RM, Muralidharan V, Perini MV. A systematic review of small for size syndrome after major hepatectomy and liver transplantation. *HPB (Oxford)* 2020; **22**: 487-496 [PMID: 31786053 DOI: 10.1016/j.hpb.2019.10.2445]

18   **Rajakannu M**, Cherqui D, Ciacio O, Golse N, Pittau G, Allard MA, Antonini TM, Coilly A, Sa Cunha A, Castaing D, Samuel D, Guettier C, Adam R, Vibert E. Liver stiffness measurement by transient elastography predicts late posthepatectomy outcomes in patients undergoing resection for hepatocellular carcinoma. *Surgery* 2017; **162**: 766-774 [PMID: 28711320 DOI: 10.1016/j.surg.2017.06.006]

19   **Adams RB**, Aloia TA, Loyer E, Pawlik TM, Taouli B, Vauthey JN; Americas Hepato-Pancreato-Biliary Association; Society of Surgical Oncology; Society for Surgery of the Alimentary Tract. Selection for hepatic resection of colorectal liver metastases: expert consensus statement. *HPB (Oxford)* 2013; **15**: 91-103 [PMID: 23297719 DOI: 10.1111/j.1477-2574.2012.00557.x]

20   **Shindoh J**, Truty MJ, Aloia TA, Curley SA, Zimmitti G, Huang SY, Mahvash A, Gupta S, Wallace MJ, Vauthey JN. Kinetic growth rate after portal vein embolization predicts posthepatectomy outcomes: toward zero liver-related mortality in patients with colorectal liver metastases and small future liver remnant. *J Am Coll Surg* 2013; **216**: 201-209 [PMID: 23219349 DOI: 10.1016/j.jamcollsurg.2012.10.018]

21   **Vauthey JN**, Abdalla EK, Doherty DA, Gertsch P, Fenstermacher MJ, Loyer EM, Lerut J, Materne R, Wang X, Encarnacion A, Herron D, Mathey C, Ferrari G, Charnsangavej C, Do KA, Denys A. Body surface area and body weight predict total liver volume in Western adults. *Liver Transpl* 2002; **8**: 233-240 [PMID: 11910568 DOI: 10.1053/jlts.2002.31654]

22   **Child CG**, Turcotte JG. Surgery and portal hypertension. *Major Probl Clin Surg* 1964; **1**: 1-85 [PMID: 4950264]

23   **Kong FH**, Miao XY, Zou H, Xiong L, Wen Y, Chen B, Liu X, Zhou JJ. End-stage liver disease score and future liver remnant volume predict post-hepatectomy liver failure in hepatocellular carcinoma. *World J Clin Cases* 2019; **7**: 3734-3741 [PMID: 31799298 DOI: 10.12998/wjcc.v7.i22.3734]

24   **Gudowska M**, Gruszewska E, Panasiuk A, Cylwik B, Flisiak R, Świderska M, Szmitkowski M, Chrostek L. Hyaluronic acid concentration in liver diseases. *Clin Exp Med* 2016; **16**: 523-528 [PMID: 26354758 DOI: 10.1007/s10238-015-0388-8]

25   **Imamura H**, Sano K, Sugawara Y, Kokudo N, Makuuchi M. Assessment of hepatic reserve for indication of hepatic resection: decision tree incorporating indocyanine green test. *J Hepatobiliary Pancreat Surg* 2005; **12**: 16-22 [PMID: 15754094 DOI: 10.1007/s00534-004-0965-9]

26   **Weinmann HJ**, Schuhmann-Giampieri G, Schmitt-Willich H, Vogler H, Frenzel T, Gries H. A new lipophilic gadolinium chelate as a tissue-specific contrast medium for MRI. *Magn Reson Med* 1991; **22**: 233-7; discussion 242 [PMID: 1812351 DOI: 10.1002/mrm.1910220214]

27   **de Graaf W**, van Lienden KP, Dinant S, Roelofs JJ, Busch OR, Gouma DJ, Bennink RJ, van Gulik TM. Assessment of future remnant liver function using hepatobiliary scintigraphy in patients undergoing major liver resection. *J Gastrointest Surg* 2010; **14**: 369-378 [PMID: 19937195 DOI: 10.1007/s11605-009-1085-2]

28   **Erdogan D**, Heijnen BH, Bennink RJ, Kok M, Dinant S, Straatsburg IH, Gouma DJ, van Gulik TM. Preoperative assessment of liver function: a comparison of 99mTc-Mebrofenin scintigraphy with indocyanine green clearance test. *Liver Int* 2004; **24**: 117-123 [PMID: 15078475 DOI: 10.1111/j.1478-3231.2004.00901.x]

29   **Cieslak KP**, Runge JH, Heger M, Stoker J, Bennink RJ, van Gulik TM. New perspectives in the assessment of future remnant liver. *Dig Surg* 2014; **31**: 255-268 [PMID: 25322678 DOI: 10.1159/000364836]

30   **Stockmann M**, Lock JF, Riecke B, Heyne K, Martus P, Fricke M, Lehmann S, Niehues SM, Schwabe M, Lemke AJ, Neuhaus P. Prediction of postoperative outcome after hepatectomy with a new bedside test for maximal liver function capacity. *Ann Surg* 2009; **250**: 119-125 [PMID: 19561474 DOI: 10.1097/SLA.0b013e3181ad85b5]

31   **Shinkawa H**, Takemura S, Tanaka S, Kubo S. Portal Vein Embolization: History and Current Indications. *Visc Med* 2017; **33**: 414-417 [PMID: 29344514 DOI: 10.1159/000479474]

32   **Li D**, Madoff DC. Portal vein embolization for induction of selective hepatic hypertrophy prior to major hepatectomy: rationale, techniques, outcomes and future directions. *Cancer Biol Med* 2016; **13**: 426-442 [PMID: 28154774 DOI: 10.20892/j.issn.2095-3941.2016.0083]

33   **Madoff DC**, Abdalla EK, Vauthey JN. Portal vein embolization in preparation for major hepatic resection: evolution of a new standard of care. *J Vasc Interv Radiol* 2005; **16**: 779-790 [PMID: 15947041 DOI: 10.1097/01.RVI.0000159543.28222.73]

34   **Lainas P**, Boudechiche L, Osorio A, Coulomb A, Weber A, Pariente D, Franco D, Dagher I. Liver regeneration and recanalization time course following reversible portal vein embolization. *J Hepatol* 2008; **49**: 354-362 [PMID: 18387688 DOI: 10.1016/j.jhep.2008.01.034]

35   **Tranchart H**, Catherine L, Maitre S, Lainas P, De Laveaucoupet J, Dagher I. Efficient liver regeneration following temporary portal vein embolization with absorbable gelatin sponge powder in humans. *J Vasc Interv Radiol* 2015; **26**: 507-515 [PMID: 25640643 DOI: 10.1016/j.jvir.2014.11.033]

36   **Tranchart H**, Koffi GM, Gaillard M, Lainas P, Poüs C, Gonin P, Nguyen TH, Dubart-Kupperschmitt A, Dagher I. Liver regeneration following repeated reversible portal vein embolization in an experimental model. *Br J Surg* 2016; **103**: 1209-1219 [PMID: 27256140 DOI: 10.1002/bjs.10153]

37   **van Lienden KP**, van den Esschert JW, de Graaf W, Bipat S, Lameris JS, van Gulik TM, van Delden OM. Portal vein embolization before liver resection: a systematic review. *Cardiovasc Intervent Radiol* 2013; **36**: 25-34 [PMID: 22806245 DOI: 10.1007/s00270-012-0440-y]

38   **Abulkhir A**, Limongelli P, Healey AJ, Damrah O, Tait P, Jackson J, Habib N, Jiao LR. Preoperative portal vein embolization for major liver resection: a meta-analysis. *Ann Surg* 2008; **247**: 49-57 [PMID: 18156923 DOI: 10.1097/SLA.0b013e31815f6e5b]

39   **Isfordink CJ**, Samim M, Braat MNGJA, Almalki AM, Hagendoorn J, Borel Rinkes IHM, Molenaar IQ. Portal vein ligation *vs* portal vein embolization for induction of hypertrophy of the future liver remnant: A systematic review and meta-analysis. *Surg Oncol* 2017; **26**: 257-267 [PMID: 28807245 DOI: 10.1016/j.suronc.2017.05.001]

40   **Wajswol E**, Jazmati T, Contractor S, Kumar A. Portal Vein Embolization Utilizing N-Butyl Cyanoacrylate for Contralateral Lobe Hypertrophy Prior to Liver Resection: A Systematic Review and Meta-Analysis. *Cardiovasc Intervent Radiol* 2018; **41**: 1302-1312 [PMID: 29687262 DOI: 10.1007/s00270-018-1964-6]

41   **May BJ**, Madoff DC. Portal vein embolization: rationale, technique, and current application. *Semin Intervent Radiol* 2012; **29**: 81-89 [PMID: 23729977 DOI: 10.1055/s-0032-1312568]

42   **Hoekstra LT**, van Lienden KP, Doets A, Busch OR, Gouma DJ, van Gulik TM. Tumor progression after preoperative portal vein embolization. *Ann Surg* 2012; **256**: 812-7; discussion 817 [PMID: 23095626 DOI: 10.1097/SLA.0b013e3182733f09]

43   **Hoekstra LT**, van Lienden KP, Verheij J, van der Loos CM, Heger M, van Gulik TM. Enhanced tumor growth after portal vein embolization in a rabbit tumor model. *J Surg Res* 2013; **180**: 89-96 [PMID: 23149224 DOI: 10.1016/j.jss.2012.10.032]

44   **Maggiori L**, Bretagnol F, Sibert A, Paradis V, Vilgrain V, Panis Y. Selective portal vein ligation and embolization induce different tumoral responses in the rat liver. *Surgery* 2011; **149**: 496-503 [PMID: 21167542 DOI: 10.1016/j.surg.2010.10.012]

45   **Pamecha V**, Levene A, Grillo F, Woodward N, Dhillon A, Davidson BR. Effect of portal vein embolisation on the growth rate of colorectal liver metastases. *Br J Cancer* 2009; **100**: 617-622 [PMID: 19209170 DOI: 10.1038/sj.bjc.6604872]

46   **Adam R**, Laurent A, Azoulay D, Castaing D, Bismuth H. Two-stage hepatectomy: A planned strategy to treat irresectable liver tumors. *Ann Surg* 2000; **232**: 777-785 [PMID: 11088072 DOI: 10.1097/00000658-200012000-00006]

47   **Kianmanesh R**, Farges O, Abdalla EK, Sauvanet A, Ruszniewski P, Belghiti J. Right portal vein ligation: a new planned two-step all-surgical approach for complete resection of primary gastrointestinal tumors with multiple bilateral liver metastases. *J Am Coll Surg* 2003; **197**: 164-170 [PMID: 12831938 DOI: 10.1016/S1072-7515(03)00334-X]

48   **Jaeck D**, Oussoultzoglou E, Rosso E, Greget M, Weber JC, Bachellier P. A two-stage hepatectomy procedure combined with portal vein embolization to achieve curative resection for initially unresectable multiple and bilobar colorectal liver metastases. *Ann Surg* 2004; **240**: 1037-49; discussion 1049 [PMID: 15570209 DOI: 10.1097/01.sla.0000145965.86383.89]

49   **Broering DC**, Hillert C, Krupski G, Fischer L, Mueller L, Achilles EG, Schulte am Esch J, Rogiers X. Portal vein embolization *vs* portal vein ligation for induction of hypertrophy of the future liver remnant. *J Gastrointest Surg* 2002; **6**: 905-13; discussion 913 [PMID: 12504230 DOI: 10.1016/s1091-255x(02)00122-1]

50   **Aussilhou B**, Lesurtel M, Sauvanet A, Farges O, Dokmak S, Goasguen N, Sibert A, Vilgrain V, Belghiti J. Right portal vein ligation is as efficient as portal vein embolization to induce hypertrophy of the left liver remnant. *J Gastrointest Surg* 2008; **12**: 297-303 [PMID: 18060468 DOI: 10.1007/s11605-007-0410-x]

51   **Capussotti L**, Muratore A, Baracchi F, Lelong B, Ferrero A, Regge D, Delpero JR. Portal vein ligation as an efficient method of increasing the future liver remnant volume in the surgical treatment of colorectal metastases. *Arch Surg* 2008; **143**: 978-82; discussion 982 [PMID: 18936377 DOI: 10.1001/archsurg.143.10.978]

52   **Schnitzbauer AA**, Lang SA, Goessmann H, Nadalin S, Baumgart J, Farkas SA, Fichtner-Feigl S, Lorf T, Goralcyk A, Hörbelt R, Kroemer A, Loss M, Rümmele P, Scherer MN, Padberg W, Königsrainer A, Lang H, Obed A, Schlitt HJ. Right portal vein ligation combined with in situ splitting

induces rapid left lateral liver lobe hypertrophy enabling 2-staged extended right hepatic resection in small-for-size settings. *Ann Surg* 2012; **255**: 405-414 [PMID: 22330038 DOI: 10.1097/SLA.0b013e31824856f5]

53 **Sandström P**, Røsok BI, Sparrelid E, Larsen PN, Larsson AL, Lindell G, Schultz NA, Bjørnbeth BA, Isaksson B, Rizell M, Björnsson B. ALPPS Improves Resectability Compared With Conventional Two-stage Hepatectomy in Patients With Advanced Colorectal Liver Metastasis: Results From a Scandinavian Multicenter Randomized Controlled Trial (LIGRO Trial). *Ann Surg* 2018; **267**: 833-840 [PMID: 28902669 DOI: 10.1097/SLA.0000000000002511]

54 **Schadde E**, Ardiles V, Robles-Campos R, Malago M, Machado M, Hernandez-Alejandro R, Soubrane O, Schnitzbauer AA, Raptis D, Tschuor C, Petrowsky H, De Santibanes E, Clavien PA; ALPPS Registry Group. Early survival and safety of ALPPS: first report of the International ALPPS Registry. *Ann Surg* 2014; **260**: 829-36; discussion 836 [PMID: 25379854 DOI: 10.1097/SLA.0000000000000947]

55 **Knoefel WT**, Gabor I, Rehders A, Alexander A, Krausch M, Schulte am Esch J, Fürst G, Topp SA. In situ liver transection with portal vein ligation for rapid growth of the future liver remnant in two-stage liver resection. *Br J Surg* 2013; **100**: 388-394 [PMID: 23124776 DOI: 10.1002/bjs.8955]

56 **Schadde E**, Schnitzbauer AA, Tschuor C, Raptis DA, Bechstein WO, Clavien PA. Systematic review and meta-analysis of feasibility, safety, and efficacy of a novel procedure: associating liver partition and portal vein ligation for staged hepatectomy. *Ann Surg Oncol* 2015; **22**: 3109-3120 [PMID: 25448799 DOI: 10.1245/s10434-014-4213-5]

57 **Deshayes E**, Schadde E, Piron L, Quenet F, Guiu B. Extended Liver Venous Deprivation Leads to a Higher Increase in Liver Function that ALPPS in Early Assessment: A comment to "Sparrelid E *et al* ". Dynamic Evaluation of Liver Volume and Function in Associating Liver Partition and Portal Vein Ligation for Staged Hepatectomy. *J Gastrointest Surg* 2017; **21**: 1754-1755 [PMID: 28785933 DOI: 10.1007/s11605-017-3508-9]

58 **Sparrelid E**, Jonas E, Tzortzakakis A, Dahlén U, Murquist G, Brismar T, Axelsson R, Isaksson B. Dynamic Evaluation of Liver Volume and Function in Associating Liver Partition and Portal Vein Ligation for Staged Hepatectomy. *J Gastrointest Surg* 2017; **21**: 967-974 [PMID: 28283924 DOI: 10.1007/s11605-017-3389-y]

59 **Adam R**, Imai K, Castro Benitez C, Allard MA, Vibert E, Sa Cunha A, Cherqui D, Baba H, Castaing D. Outcome after associating liver partition and portal vein ligation for staged hepatectomy and conventional two-stage hepatectomy for colorectal liver metastases. *Br J Surg* 2016; **103**: 1521-1529 [PMID: 27517369 DOI: 10.1002/bjs.10256]

60 **Moris D**, Ronnekleiv-Kelly S, Kostakis ID, Tsilimigras DI, Beal EW, Papalampros A, Dimitroulis D, Felekouras E, Pawlik TM. Operative Results and Oncologic Outcomes of Associating Liver Partition and Portal Vein Ligation for Staged Hepatectomy (ALPPS) Versus Two-Stage Hepatectomy (TSH) in Patients with Unresectable Colorectal Liver Metastases: A Systematic Review and Meta-Analysis. *World J Surg* 2018; **42**: 806-815 [PMID: 28798996 DOI: 10.1007/s00268-017-4181-6]

61 **Hasselgren K**, Røsok BI, Larsen PN, Sparrelid E, Lindell G, Schultz NA, Bjørnbeth BA, Isaksson B, Larsson AL, Rizell M, Björnsson B, Sandström P. ALPPS Improves Survival Compared With TSH in Patients Affected of CRLM: Survival Analysis From the Randomized Controlled Trial LIGRO. *Ann Surg* 2021; **273**: 442-448 [PMID: 32049675 DOI: 10.1097/SLA.0000000000003701]

62 **Huang HC**, Bian J, Bai Y, Lu X, Xu YY, Sang XT, Zhao HT. Complete or partial split in associating liver partition and portal vein ligation for staged hepatectomy: A systematic review and meta-analysis. *World J Gastroenterol* 2019; **25**: 6016-6024 [PMID: 31660037 DOI: 10.3748/wjg.v25.i39.6016]

63 **Kim D**, Cornman-Homonoff J, Madoff DC. Preparing for liver surgery with "Alphabet Soup": PVE, ALPPS, TAE-PVE, LVD and RL. *Hepatobiliary Surg Nutr* 2020; **9**: 136-151 [PMID: 32355673 DOI: 10.21037/hbsn.2019.09.10]

64 **Peng PD**, Hyder O, Bloomston M, Marques H, Corona-Villalobos C, Dixon E, Pulitano C, Hirose K, Schulick RD, Barroso E, Aldrighetti L, Choti M, Shen F, Kamel I, Geschwind JF, Pawlik TM. Sequential intra-arterial therapy and portal vein embolization is feasible and safe in patients with advanced hepatic malignancies. *HPB (Oxford)* 2012; **14**: 523-531 [PMID: 22762400 DOI: 10.1111/j.1477-2574.2012.00492.x]

65 **Raoul JL**, Forner A, Bolondi L, Cheung TT, Kloeckner R, de Baere T. Updated use of TACE for hepatocellular carcinoma treatment: How and when to use it based on clinical evidence. *Cancer Treat Rev* 2019; **72**: 28-36 [PMID: 30447470 DOI: 10.1016/j.ctrv.2018.11.002]

66 **Ogata S**, Belghiti J, Farges O, Varma D, Sibert A, Vilgrain V. Sequential arterial and portal vein embolizations before right hepatectomy in patients with cirrhosis and hepatocellular carcinoma. *Br J Surg* 2006; **93**: 1091-1098 [PMID: 16779884 DOI: 10.1002/bjs.5341]

67 **Aoki T**, Imamura H, Hasegawa K, Matsukura A, Sano K, Sugawara Y, Kokudo N, Makuuchi M. Sequential preoperative arterial and portal venous embolizations in patients with hepatocellular carcinoma. *Arch Surg* 2004; **139**: 766-774 [PMID: 15249411 DOI: 10.1001/archsurg.139.7.766]

68 **Yoo H**, Kim JH, Ko GY, Kim KW, Gwon DI, Lee SG, Hwang S. Sequential transcatheter arterial chemoembolization and portal vein embolization *vs* portal vein embolization only before major hepatectomy for patients with hepatocellular carcinoma. *Ann Surg Oncol* 2011; **18**: 1251-1257 [PMID: 21069467 DOI: 10.1245/s10434-010-1423-3]

69 **Hwang S**, Lee SG, Ko GY, Kim BS, Sung KB, Kim MH, Lee SK, Hong HN. Sequential preoperative ipsilateral hepatic vein embolization after portal vein embolization to induce further liver regeneration in patients with hepatobiliary malignancy. *Ann Surg* 2009; **249**: 608-616 [PMID: 19300228 DOI:

10.1097/SLA.0b013e31819ecc5c]

70   **Le Roy B**, Perrey A, Fontarensky M, Gagnière J, Abergel A, Pereira B, Lambert C, Boyer L, Pezet D, Chabrot P, Buc E. Combined Preoperative Portal and Hepatic Vein Embolization (Biembolization) to Improve Liver Regeneration Before Major Liver Resection: A Preliminary Report. *World J Surg* 2017; **41**: 1848-1856 [PMID: 28417185 DOI: 10.1007/s00268-017-4016-5]

71   **Laurent C**, Fernandez B, Marichez A, Adam JP, Papadopoulos P, Lapuyade B, Chiche L. Radiological Simultaneous Portohepatic Vein Embolization (RASPE) Before Major Hepatectomy: A Better Way to Optimize Liver Hypertrophy Compared to Portal Vein Embolization. *Ann Surg* 2020; **272**: 199-205 [PMID: 32675481 DOI: 10.1097/SLA.0000000000003905]

72   **Kobayashi K**, Yamaguchi T, Denys A, Perron L, Halkic N, Demartines N, Melloul E. Liver venous deprivation compared to portal vein embolization to induce hypertrophy of the future liver remnant before major hepatectomy: A single center experience. *Surgery* 2020; **167**: 917-923 [PMID: 32014304 DOI: 10.1016/j.surg.2019.12.006]

73   **Vilgrain V**, Pereira H, Assenat E, Guiu B, Ilonca AD, Pageaux GP, Sibert A, Bouattour M, Lebtahi R, Allaham W, Barraud H, Laurent V, Mathias E, Bronowicki JP, Tasu JP, Perdrisot R, Silvain C, Gerolami R, Mundler O, Seitz JF, Vidal V, Aubé C, Oberti F, Couturier O, Brenot-Rossi I, Raoul JL, Sarran A, Costentin C, Itti E, Luciani A, Adam R, Lewin M, Samuel D, Ronot M, Dinut A, Castera L, Chatellier G; SARAH Trial Group. Efficacy and safety of selective internal radiotherapy with yttrium-90 resin microspheres compared with sorafenib in locally advanced and inoperable hepatocellular carcinoma (SARAH): an open-label randomised controlled phase 3 trial. *Lancet Oncol* 2017; **18**: 1624-1636 [PMID: 29107679 DOI: 10.1016/S1470-2045(17)30683-6]

74   **Vouche M**, Lewandowski RJ, Atassi R, Memon K, Gates VL, Ryu RK, Gaba RC, Mulcahy MF, Baker T, Sato K, Hickey R, Ganger D, Riaz A, Fryer J, Caicedo JC, Abecassis M, Kulik L, Salem R. Radiation lobectomy: time-dependent analysis of future liver remnant volume in unresectable liver cancer as a bridge to resection. *J Hepatol* 2013; **59**: 1029-1036 [PMID: 23811303 DOI: 10.1016/j.jhep.2013.06.015]

75   **Malhotra A**, Liu DM, Talenfeld AD. Radiation Segmentectomy and Radiation Lobectomy: A Practical Review of Techniques. *Tech Vasc Interv Radiol* 2019; **22**: 49-57 [PMID: 31079710 DOI: 10.1053/j.tvir.2019.02.003]

76   **Lewandowski RJ**, Donahue L, Chokechanachaisakul A, Kulik L, Mouli S, Caicedo J, Abecassis M, Fryer J, Salem R, Baker T. (90) Y radiation lobectomy: Outcomes following surgical resection in patients with hepatic tumors and small future liver remnant volumes. *J Surg Oncol* 2016; **114**: 99-105 [PMID: 27103352 DOI: 10.1002/jso.24269]

77   **Andel D**, Dassen MG, Reinders-Hut MTM, Peters NA, Kranenburg OW, Lam MGEH, Hagendoorn J, Rinkes IHMB. Surgical outcomes of major hepatectomy following "radiation lobectomy" for hepatic malignancies and insufficiently functional future liver remnant: initial experience. *Br J Surg* 2020; **107**: e609-e610 [PMID: 32936446 DOI: 10.1002/bjs.11828]

MINIREVIEWS

Health-related quality of life in autoimmune hepatitis

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Abstract

Autoimmune hepatitis (AIH) is a severe chronic autoimmune disease and has a significant impact on the patient's quality of life, in particular regarding psychological problems such as anxiety and depression. Consistent evidence on which patient-related, disease-related or physician-related factors cause health-related quality of life (HRQoL) impairment in patients with AIH is lacking. Current studies on HRQoL in AIH are mainly single-centered, comprising small numbers of patients, and difficult to compare because of the use of different questionnaires, patient populations, and cutoff values. Literature in the pediatric field is sparse, but suggests that children/adolescents with AIH have a lower HRQoL. Knowledge of HRQoL and cohesive factors in AIH are important to improve healthcare for AIH patients, for example by developing an AIH-specific chronic healthcare model. By recognizing the importance of quality of life beyond the concept of biochemical and histological remission, clinicians allow us to seek enhancements and possible interventions in the management of AIH, aiming at

improved health.

Key Words: Autoimmune hepatitis; Quality of life; Depression; Anxiety; Corticosteroids

Core Tip: Autoimmune hepatitis (AIH) is a severe chronic autoimmune disease and has a significant impact on the patient's quality of life, in particular regarding psychological problems such as anxiety and depression. The health-related quality of life (HRQoL) of patients with AIH can be affected by various patient-related, disease-related, and physician-related factors. In this review we summarized several specific factors that are liable to influence HRQoL in AIH. By recognizing the importance of quality of life beyond the concept of biochemical and histological remission, clinicians allow us to seek enhancements and possible interventions in the management of AIH.

INTRODUCTION

Autoimmune hepatitis (AIH) is a severe chronic autoimmune disease that occurs mainly in women and affects health-related quality of life (HRQoL) worldwide. The diagnosis of AIH is based on the presence of autoantibodies, typical features on liver histology, and increased immunoglobulin G (IgG) levels[1]. The presentation of AIH is variable, ranging from mild and asymptomatic disease to fulminant hepatic failure. Nonspecific symptoms at presentation are fatigue, anorexia, jaundice, and abdominal pain, whereas others are asymptomatic at disease onset[1]. The majority of patients need lifelong treatment to prevent disease progression to cirrhosis and/or decompensation[2]. Current treatment strategies in AIH include administering corticosteroids (mainly prednisolone) and a long-term corticosteroid-saving regime, including azathioprine (AZA) as first-line treatment[3,4]. Second-line immunosuppressants include mycophenolate mofetil (MMF), calcineurin inhibitors (CNIs), and mercaptopurine and have proven to be effective in mainly uncontrolled studies[5].

The main goal of AIH treatment is to achieve complete biochemical and histological remission without the occurrence of side effects. Alanine aminotransferase, aspartate aminotransferase and IgG serum levels are used as parameters to monitor biochemical response, and current guidelines advocate pursuit of normalization of those parameters as the aim of treatment. As a result, treatment failure, defined as absence of normalization of transaminases, triggers clinical actions such as increase of drug dose or change in drug class. A sole focus on biochemical response is insufficient when managing AIH. From a patient perspective, other aspects that affect HRQoL, including but not limited to side effects, psychological health, and implications of the disease, are just as important.

One of the main objectives relating to AIH according to the International Autoimmune Hepatitis Group (IAIHG), is better assessment of HRQoL in patients. However, literature or guidelines on that topic in AIH are scarce and inconsistent. An update on current literature on HRQoL in AIH, is warranted to reveal the most important research gaps[6]. Understanding which potentially treatable factors are associated with reduced quality of life in patients with AIH is essential for development of interventions targeting well-being. The focus of this paper is to review the current knowledge of HRQoL and associated factors in AIH, to comment on the current status, and to identify future perspectives that may influence and benefit disease management of adult patients with AIH.

METHODOLOGY

We searched the titles, abstracts, and MeSH terms of articles indexed in PubMed using the keywords "autoimmune hepatitis," "AIH," "health-related quality of life," and "quality of life." The search was limited to articles published before January 27, 2021. We included articles based on the following criteria: (1) Full-text articles published in peer-reviewed journals; (2) English or Dutch articles; (3) Publication dates within the last 20 years at the time of the search; and (4) Either adult or pediatric AIH. The search retrieved 116 publications; 39 were evaluated in full-text after screening the titles and abstracts (Figure 1). We also checked the reference lists of the included articles to identify other articles. For the purpose of this review, we primarily focused on articles addressing the role of HRQoL in AIH.

HRQOL IN ADULT PATIENTS WITH AIH

Several studies have reported reduced general or liver-specific HRQoL in AIH patients (Tables 1 and 2)[7-15]. The first study published was conducted in the Netherlands and showed a reduced quality of life in 141 patients with AIH compared with healthy controls, using three instruments, the SF-36 for generic HRQoL, the Multidimensional Fatigue Index-20, and the Liver Disease Symptom Index 2.0, which is a liver-specific questionnaire addressing nine topics. In particular, patients had lower scores in subscales measuring physical problems or general health. Patients with AIH mentioned fatigue more often than healthy controls did[13]. A landmark study performed in Germany compared 102 AIH patients to the German general population and to published data of patients with arthritis using the SF-12[12]. They reported lower mental well-being in patients with AIH compared with both groups, but the physical component score (PCS) was unaffected[12]. A Polish single-center study showed that patients with AIH (*n* = 140) scored significantly worse in all subscales of the SF-36, except for one measuring the impact of emotional problems on work and daily activities[15]. The majority of the AIH patients in that cohort had cirrhosis (55%), and as in the previously mentioned study, that did not have a significant effect on well-being. A recent Italian multicenter study of chronic liver disease reported that of a total of seven different chronic liver diseases without cirrhosis, patients with AIH had a lower quality of life measured with the EQ-5D VAS score, and experienced difficulties in the self-care domain, even after adjusting for multiple possible confounders, including age, sex, education, and professional status[10]. That was confirmed in a Cuban study in which AIH patients had lower quality of life scores than hepatitis B patients using the disease-specific Chronic Liver Disease Questionnaire (CLDQ)[7]. Only one meta-analysis was performed, including three studies that evaluated HRQoL measured with the SF-36. The analysis confirmed reduction of the PCS and mild reduction of the mental component score in patients with AIH. However, they included only older studies and compared all AIH patients (including Dutch and German patients) to the United States general population norm [16]. Finally, the largest study conducted so far involved multiple health centers in the United Kingdom and confirmed previous results by finding that the HRQoL of patients with AIH (*n* = 990) was worse than it was in the general population, adjusted for age and gender and using the EQ-5D-5L[14]. Although these studies consistently report a lower HRQoL in AIH, albeit in varying domains, it remains difficult to compare the studies because of the use of different questionnaires (EQ-5D-5L *vs* SF-12 or SF-36 *vs* CLDQ), cutoff values, methodology, and patient populations. Moreover, most studies were conducted at single centers and included small numbers of participants, thereby introducing bias based on the heterogenicity in study populations (*e.g.*, remission status and demographic differences).

HRQOL IN PEDIATRIC PATIENTS WITH AIH

A lower HRQoL was also found in children and adolescents with AIH, although literature in the pediatric field is sparse[17-19]. A study performed in Portugal compared 43 children with AIH to 62 healthy children using the Pediatric Quality of Life Inventory 4.0 (PedsQL 4.0)[17]. They found that especially children with associated comorbidities (*e.g.*, inflammatory bowel disease, hemolytic anemia, and hypothyroidism) had a lower quality of life. That was confirmed in a Brazilian cohort using the same questionnaire[18]. Interestingly, the evaluation of HRQoL in the

Table 1 Overview of the studies assessing aspects of health-related quality of life in autoimmune hepatitis

Ref.	Country	Population (n)	Biochemical remission (%)	Cirrhosis (%)	Questionnaire	Factors/results
van der Plas *et al* [13], 2007	The Netherlands	AIH (142), other liver diseases (776)	-	-	SF-36, MFI-20, LDSI	HRQoL impairment; Association with: Fatigue
Afendy *et al*[8], 2009	United States, Italy	AIH (13), other chronic liver diseases (1090)	-	84.6[1]	SF-36	HRQoL impairment; Negative correlation: Age (every scale), female gender (primary predictor of mental health), cirrhosis (every scale, primary predictor of physical health)
Schramm *et al* [12], 2014	Germany	AIH (103)	77	27	SF-12, PHQ-9, GAD-7	HRQoL impairment (total mental score/mental well-being); Association with: depression and anxiety (positive correlation with female gender, corticosteroid use, and concerns about progression of the liver disease)
Takahashi *et al* [11], 2018	Japan	AIH (265), chronic hepatitis C (88)	-	10.6	CLDQ, SF-36	HRQoL impairment; Negative correlation: Age, cirrhosis, comorbid diseases, corticosteroid use (worry domain), disease duration, AST; Positive correlation: platelet count
Wong *et al*[14], 2018	United Kingdom	AIH (990)	56	33	EQ-5D-5L, FIS, CFQ, HADS	HRQoL impairment; Positive correlation: Biochemical remission; Negative correlation: overlap syndromes, corticosteroid use, and calcineurin inhibitor use
Janik *et al*[15], 2019	Poland	AIH (140)	-	55	SF-36, MFIS, PHQ-9, STAI	HRQoL impairment (every scale, except role emotional[2]); Negative correlation: Female gender, depression, trend toward better HRQoL (physical health) with budesonide *vs* prednisone; Association with: Anxiety, depression, and fatigue
Dirks *et al*[9], 2019	Germany	AIH (27), AIH/PBC (8), other liver diseases (97)	-	0	SF-36, FIS, HADS	HRQoL impairment; Association with: Anxiety, depression, and fatigue
Castellanos-Fern ández *et al*[7], 2021	Cuba	AIH (22), overlap syndrome of AIH and PBC (7), PBC (14), other liver diseases (500)	-	43.9[3]	FACIT-F, WPAI:SHP, CLDQ	HRQoL impairment; Positive correlation: Male gender, exercising > 90 min/wk; Negative correlation: Fatigue, abdominal pain, anxiety, depression, and extrahepatic comorbidity (diabetes mellitus type 2, sleep apnea)
Cortesi *et al*[10], 2020	Italy	AIH (51), other chronic liver diseases (2911)	-	0	EQ-5D-3L	HRQoL impairment in AIH

[1]Eight patients with Child-Pugh class A and three patients with Child-Pugh class C.

[2]Scale measures the impact of emotional problems on work and daily activities.

[3]Cirrhosis in patients with autoimmune liver diseases (*n* = 43). AIH: Autoimmune hepatitis; HRQoL: Health-related quality of life; PBC: Primary biliary cholangitis; AST: Aspartate aminotransferase; CFQ: Cognitive failure questionnaire; CLDQ: Chronic liver disease questionnaire; ECR: Experiences in close relationship scale; EQ-5D-5L/3L: European quality of life 5-dimension 5-level/3-level; FACIT-F: Functional assessment of chronic illness therapy-fatigue; FIS: Fatigue impact scale; GAD-7: Generalized anxiety disorder screener; HADS: Hospital anxiety depression scale; LDSI: Liver disease symptom index 2.0; MFI-20: Multidimensional fatigue index-20; PHQ-9: Patient health questionnaire; SF-12: Short-form 12; SF-36: Short-form 36; STAI: State-trait anxiety inventory; WPAI:SHP: Work productivity and activity-specific health problem.

parents differed from the children's self-reports[18]. Only the physical and total scores were significantly lower in patients with AIH based on the parental reports, whereas in the children's reports the emotional, school, physical, and total scores were significantly lower.

Table 2 Overview of the questionnaires assessing aspects of health-related quality of life in autoimmune hepatitis

Questionnaire	Main function	Domains	Items, total score
CFQ[41]	Cognition	Memory, attention, concentration, forgetfulness, word-finding abilities, and confusion	25 items scored 0-4, total score 0-100
CLDQ[42]	Generic HRQoL	Abdominal symptoms, fatigue, systemic symptoms, activity, emotions, and worry	29 items scored 1-7, total score 29-203
ECR[43]	Relationship styles	ECR-anxiety, and ECR-avoidance	12 items scored 1-7, each scale total score 7-42
EQ-5D-5L/EQ-5D-3L/EQ-VAS [44]	Generic HRQoL, EQ-VAS: participants' self-rated health on a visual analog scale	Mobility, self-care, usual activities, pain/discomfort, and anxiety/depression	EQ-5D: 5 items scored 1-5, total score 5-25; EQ-VAS: total score 0-100
FACIT-F[45]	Fatigue	Physical well-being, social well-being, emotional well-being, functional well-being, and a fatigue-specific domain	40 items scored 0-4, total score 0-160
FIS[46]	Fatigue	Cognitive functioning, physical functioning, and psychosocial functioning	40 items scored 0-4, total score 0-160
GAD-7[47]	Anxiety	-	7 items scored 0-3, total score 0-21
HADS[48]	Anxiety, depression	Anxiety, and depression	14 items scored 0-3, total score 0-42
LDSI[49]	Liver disease symptoms	Itch, joint pain, abdominal pain, daytime sleepiness, worry about family situation, decreased appetite, depression, fear of complications, and jaundice (+ symptom hinderance)	18 items scored 1-5, total score 18-90
MFI-20[50]	Fatigue	General fatigue, physical fatigue, reduction in activity, reduction in motivation, and mental fatigue	20 items scored 1-5, each domain total score 4-20
MFIS[46,51]	Fatigue	Physical, cognitive, and psychosocial functioning	21 items scored 0-4, total score 0-84
PHQ-9[52]	Depression	Anhedonia, feeling down, sleep, feeling tired, appetite, feeling bad about self, concentration, activity, and suicidality	9 items scored 0-3, total score 0-27
SF-12[53]	Generic HRQoL	Physical functioning, role limitations due to physical problems, bodily pain, general health, vitality, social functioning, role limitations due to emotional problems, and mental health	12 items scored 1-5, total score 0-100
SF-36[54]	Generic HRQoL	General health, physical and social functioning, bodily pain, role-physical, mental health, role-emotional, and vitality	36 items, total score 0-100
STAI[55]	Anxiety	State anxiety, and trait anxiety	40 items scored 1-4, total score 0-80
WPAI:SHP[56]	Impairment in daily activities and in work	Work productivity impairment, and activity impairment	6 items scored 0-10, total score -

Included in the table are the questionnaires that were employed in the reviewed studies. CFQ: Cognitive Failure Questionnaire; CLDQ: Chronic Liver Disease Questionnaire; ECR: Experiences in Close Relationship Scale; EQ-5D-5L/EQ-5D-3L/EQ-VAS: European Quality of life 5-Dimension 5-Level/3-Level/EQ-visual analog scale; FACIT-F: Functional Assessment of Chronic Illness Therapy-Fatigue; FIS: Fatigue Impact Scale; GAD-7: Generalized Anxiety Disorder Screener; HADS: Hospital Anxiety Depression Scale; LDSI: Liver Disease Symptom Index 2.0); MFI-20: Multidimensional Fatigue Index-20; MFIS: Modified Fatigue Impact Scale); PHQ-9: Patient Health Questionnaire; SF-12: Short-form 12; SF-36: Short-form 36; STAI: State-Trait Anxiety Inventory; WPAI:SHP: Work Productivity and Activity-Specific Health Problem).

DETERMINANTS OF HRQOL IN AIH

The HRQoL of patients with chronic diseases can be affected by various patient-related, disease-related, and physician-related factors. We have summarized the patient-, disease- and physician-related factors that are liable to influence HRQoL in AIH in Figure 2.

Patient-related factors

Patients with AIH are more often diagnosed with symptoms of depression and anxiety compared with the general population or healthy controls[7,9,10,12,15]. Studies by Schramm and Janik *et al*[15] showed a significantly higher percentage of depression
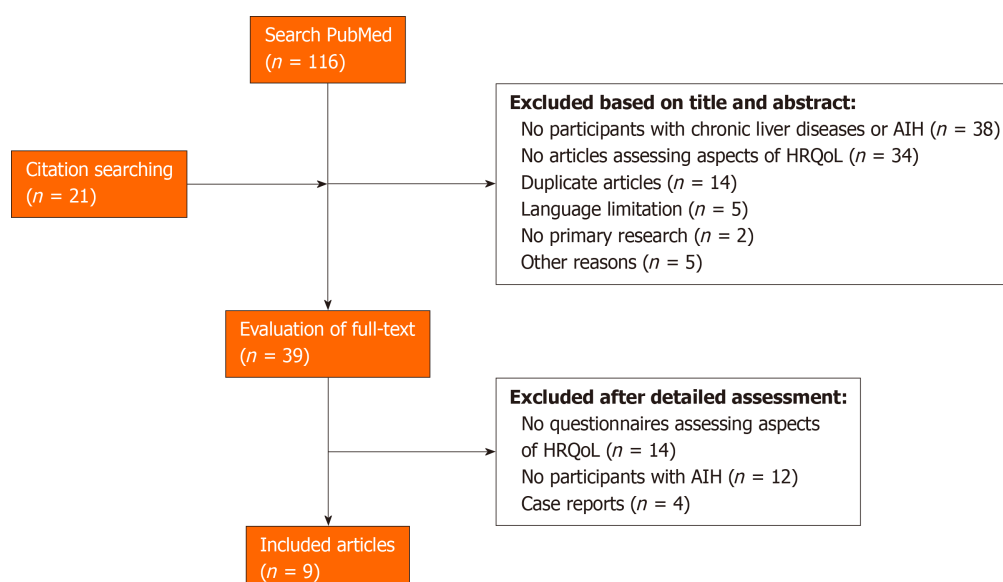
Figure 1 Flowchart of included studies after performing the literature search. AIH: Autoimmune hepatitis; HRQoL: Health-related quality of life.
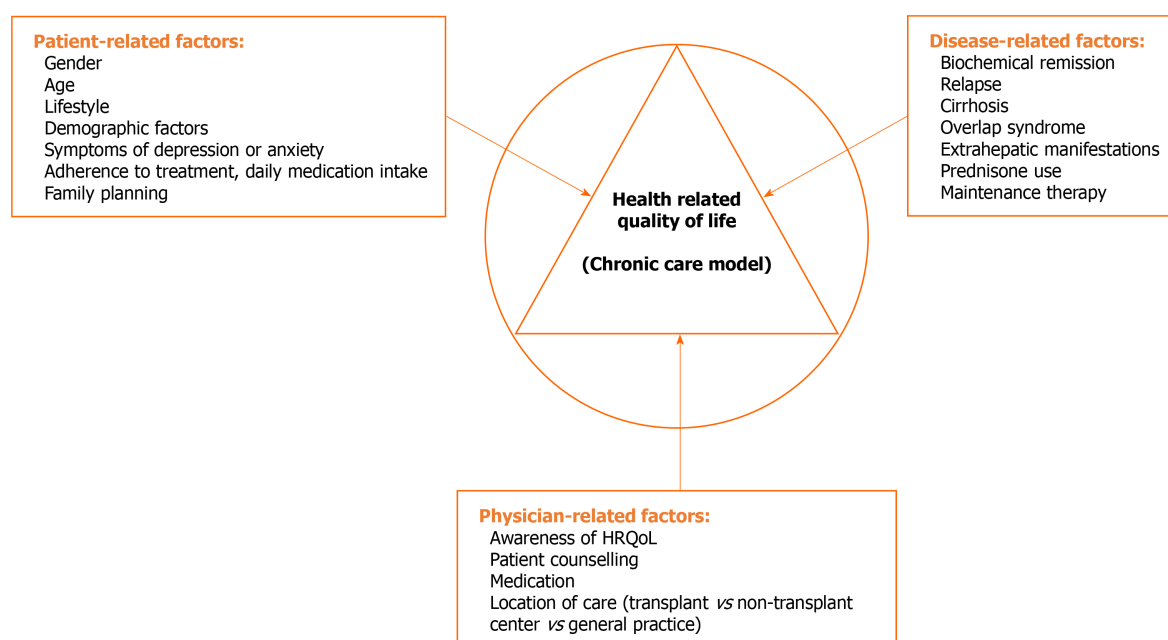
Figure 2 Patient-, disease- and physician-related factors affecting health-related quality of life in patients with autoimmune hepatitis.
HRQoL: Health-related quality of life.

and anxiety symptoms, measured with the PHQ-9, GAD-7, or State-Trait Anxiety Inventory[12,15]. Depression was strongly correlated with both physical and mental components of SF-36. Despite biochemical remission in 77% of the patients (*n* = 103), the occurrence of severe depressive symptoms within the German cohort appeared to be five times as frequent compared with the general population.[12] In addition, even AIH patients without cirrhosis revealed more problems with regard to depression and anxiety compared with the general population[10]. It is interesting to note that psychological stress was also associated with relapses in patients with AIH type 1[20].

Other patient-related factors, particularly age and sex, have been described often in previous studies[7,11,12,14]. Studies in the United Kingdom and Japan reported a negative correlation between age and HRQoL[11,14], but Polish and Cuban studies did not find such a correlation [7,15]. With respect to sex differences, female patients experience more symptoms of depression[12,15] and have a worse quality of life than their male counterparts[7,15]. In our experience, women experience weight increase and other cosmetic changes associated with corticosteroids as a great inconvenience in

particular. In contrast, a study in the United Kingdom study found that the female sex was associated with a higher quality of life, albeit in an unadjusted regression analysis. These inconsistent correlations highlight that we still do not know which patient factors are important when assessing HRQoL in patients with AIH.

For all chronic liver diseases, it holds that lifestyle changes are part of the treatment. While tackling lifestyle is a hot topic in chronic disease, it is infrequently addressed in AIH. However, patients should still be informed about the risk of specific lifestyles, such as overweight, alcohol misuse, and sedentary behavior. Losing weight, more exercise, and a healthier diet contribute to successful management of chronic liver diseases and cirrhosis[21]. Indeed, exercising for more than 90 min/wk is a predictor of a better quality of life in patients with chronic liver diseases (*e.g.*, AIH)[7]. Another study confirmed that an increased body mass index was associated with a lower quality of life in patients with AIH[14]. In addition, alcohol consumption presents a clear risk of the progression of liver fibrosis in chronic liver diseases. Other factors, such as education level, socioeconomic data, smoking, or losing weight, were not frequently mentioned in the described studies. It follows that physicians need to communicate with patients about lifestyle adaptations through motivational interviews.

Coping with chronic conditions and taking medication daily goes hand in hand with discomfort, which potentially results in reduced HRQoL. Patients with more than one chronic disease that take daily medication have a lower quality of life[22]. Adherence to treatment is rarely discussed with patients but has a great impact on well-being and treatment response. A high psychosocial burden has been shown to significantly decrease adherence to treatment and to be associated with poor treatment response[23]. Therefore, prompt recognition of symptoms of depression and anxiety is important to improve patient adherence and lead to better response to treatment. Various factors may influence adherence to drug treatment in adolescents with AIH, particularly depression, anxiety, younger age, sex, prednisone dose, and long-term therapy have been found in previous studies[23-25]. In liver transplant recipients, marital status (if the patient is divorced) and having mental distress are associated with reduced self-reported adherence to immunotherapy[26]. However, information on demographic factors or socioeconomic data, including the status of a relationship and educational level, were not explicitly examined in all previous studies, which would be necessary for more detailed conclusions.

Disease-related factors

As mentioned previously, the main objective in treating AIH is to achieve complete biochemical and histological remission without side effects. While it is plausible that achieving biochemical remission results in better HRQoL, the association has not been studied often. One study found that patients with biochemical remission had a significantly higher quality of life [14]. One could speculate that incomplete biochemical remission causes uncertainty about, and possibly fear of, a relapse, which is understandable given that every relapse increases the risk of decompensated liver failure or the necessity of liver transplantation[27]. Whether this has a role in AIH is unknown at present.

Liver cirrhosis, or an advanced stage of fibrosis in patients with chronic liver disease is a known cause for reduced HRQoL, independent of the underlying liver disease[8, 28,29]. However, studies in patients with AIH demonstrate significant variability regarding the relation between fibrosis and HRQoL. Most studies describe that having liver fibrosis or compensated cirrhosis does not affect patient well-being in general[12, 14,15]. In contrast, another study did find an impaired physical condition in patients with AIH using the same SF-36 questionnaire and an overall lower quality of life using the CLDQ[11]. Plausible explanations for the discrepancy are the use of different general *vs* disease-specific, SF-36 *vs* SF-12 *vs* EQ-5D-5L questionnaires and the inclusion of different AIH populations regarding biochemical remission status and disease duration. Interestingly, none of the cited studies included AIH patients with decompensated cirrhosis in their cohort, which is known to be a major factor for reduced HRQoL in cirrhosis with other etiologies[30,31].

Patients with an overlap syndrome or a variant syndrome of AIH and primary biliary cholangitis (PBC) or primary sclerosing cholangitis (PSC), had a worse quality of life than patients not reporting those comorbidities[7,9,14]. In addition, fatigue is a typical symptom in patients with characteristics of PBC, and is expected to have a negative impact on HRQoL[7,9]. In that context, it is not only essential to treat both AIH and the overlapping syndrome (*i.e.*, PBC or PSC), but also to address associated symptoms (*i.e.*, IBD in PSC, itch in PBC) in the patients[14]. Interestingly, such a correlation was not found in a study in children with autoimmune liver diseases. It

found no differences in HRQoL scores in children with AIH *vs* overlap syndrome or variant syndrome with PSC[19]. Extrahepatic manifestations, for example thyroid disease, insulin-dependent diabetes mellitus, connective tissue disorders, and autoimmune skin disease, are common in AIH and can affect well-being, including fatigue, but the effect on HRQoL is unstudied so far[32].

A large proportion of patients with AIH receive corticosteroid therapy[11,33]. All treatments have specific side effects[34,35], but long-term use of corticosteroids is well-known for its undesirable effects, including osteoporosis, mood swings, depression, obesity, cognitive dysfunction, chronic fatigue, and reduced physical activity[1,5]. The negative impact of the use of corticosteroids on HRQoL was demonstrated in several studies[12,14]. In the United Kingdom cohort, corticosteroids were extensively linked to impaired HRQoL. Even patients who received low-dose of corticosteroids, and independent of their biochemical status, had a lower HRQoL[14]. Schramm *et al*[12] found a significant correlation between corticosteroids and depression. Sockalingam *et al*[23] found that patients with a moderate or high PHQ-9 score of > 10 were administered a significantly higher dose of prednisone compared with patients with a score of < 10. These data give additional support for steroid-free therapy as a treatment goal in every AIH patient to prevent steroid-related complications, and should be attempted within the first year of treatment. Other disease-related factors affecting mental well-being or HRQoL, such as markers of disease activity or disease duration, are so far unknown[12,15].

Currently, AZA is still the primary choice for maintenance therapy, and was not directly associated with a lower quality of life or health utility in a large cross-sectional analysis[14]. It is important to note that the use of AZA is associated with an increased risk of lymphoma and nonmelanoma skin cancer[36,37]. Although lymphoma in the long term is rare, it has to be taken into account that the occurrence of these side effects, or even the patient's concerns, might affect their quality of life. AZA may also cause hair loss that leads to alopecia. The possibility is frequently raised by the female patients and may affect various aspects of quality of life and lead to incompliance. The effect of other prescribed therapies on improving psychosocial outcomes, such as mycophenolate mofetil and mercaptopurine, is unknown. However, calcineurin inhibitors that have undesirable effects may be associated with lower health utility[14].

Physician-related factors

Physician-related factors are usually not addressed in studies and are thus difficult to take into account. Schramm *et al*[12] found that patient concerns about the severity of their disease, and being fearful of cirrhosis (mostly unnecessary) were factors associated with depression and anxiety symptoms. Providing the patient with information on his/her illness or medications and involving the patient in treatment options, can contribute to the patient's well-being. Whether the location of care (*i.e.* transplant *vs* nontransplant center) matters is uncertain. One study showed that there was no difference in health utility between transplant and nontransplant centers[14], and another found that biochemical remission rates were higher in transplant centers compared with nontransplant centers[33]. Both were conducted in the United Kingdom. Extrapolation of the results to other countries is difficult given the differences in health care management among countries.

CONCLUSION

It is clear that patients with AIH experience a lower quality of life and have more psychological problems, such as anxiety and depression, compared with the general population. Consistent evidence on which patient-related, disease-related, or physician-related factors cause HRQoL impairment in patients with AIH is lacking. Most studies did not include information on important socioeconomic, disease behavior, maintenance treatment, or even geographical factors, whereas they are known to affect patient well-being and HRQoL in other chronic liver diseases. In addition, some aspects of AIH are unexplored so far, for example the effect of lifestyle changes, extrahepatic manifestations, and patient counseling on HRQoL. Studies addressing HRQoL in pediatric AIH and their parents/support team are scarce and are desperately needed as a first step to improve their well-being.

Knowledge of HRQoL and associated factors in AIH are important to improve healthcare for AIH patients, for example by incorporating the factors in a chronic healthcare model (CCM). A CCM provides a clear approach for managing chronic diseases, with focus on assessment of the modifiable factors affecting the disease in

order to improve patient well-being. While no studies mentioned a CCM for AIH so far, some studies discussed elements that could be part of a model. For example, Janik *et al*[38] screened AIH patients for moderately severe depression and redirected them to a psychiatrist and psychiatric therapeutic interventions in case of a PHQ ≥ 15 points. Another example are lifestyle interventions for overweight patients[39]. There is also a role for the development of a disease-specific questionnaire for AIH patients, similar to the PBC-40 questionnaire, to measure the patient's perspective of the disease[40]. In what way, a CCM can be developed and implemented that would probably differ from country to country because of differences in health care. However, it is paramount that the AIH-specific CCM incorporate the most important factors of HRQoL in AIH, as discussed in this review.

Finally, HRQoL should not only be targeted in everyday clinical treatment approaches, but also as an important outcome of clinical trials and a research objective per se. Most studies of HRQoL in AIH have been conducted at a single center and comprised small numbers of patients, which underlines the need for collaboration between healthcare centers in different countries. Currently, there is an ongoing multicenter, cross-sectional study of HRQoL in patients with AIH within the European Network for Rare Liver Diseases. Recognizing the importance that quality of life has for the patient beyond the concept of biochemical and histological remission allows us to strive for significant improvements in management of adult and pediatric AIH.

REFERENCES

1 **Manns MP**, Czaja AJ, Gorham JD, Krawitt EL, Mieli-Vergani G, Vergani D, Vierling JM; American Association for the Study of Liver Diseases. Diagnosis and management of autoimmune hepatitis. *Hepatology* 2010; **51**: 2193-2213 [PMID: 20513004 DOI: 10.1002/hep.23584]

2 **Harrison L**, Gleeson D. Stopping immunosuppressive treatment in autoimmune hepatitis (AIH): Is it justified (and in whom and when)? *Liver Int* 2019; **39**: 610-620 [PMID: 30667576 DOI: 10.1111/liv.14051]

3 **Doycheva I**, Watt KD, Gulamhusein AF. Autoimmune hepatitis: Current and future therapeutic options. *Liver Int* 2019; **39**: 1002-1013 [PMID: 30716203 DOI: 10.1111/liv.14062]

4 **Terziroli Beretta-Piccoli B**, Mieli-Vergani G, Vergani D. Autoimmune hepatitis: Standard treatment and systematic review of alternative treatments. *World J Gastroenterol* 2017; **23**: 6030-6048 [PMID: 28970719 DOI: 10.3748/wjg.v23.i33.6030]

5 **European Association for the Study of the Liver**. EASL Clinical Practice Guidelines: Autoimmune hepatitis. *J Hepatol* 2015; **63**: 971-1004 [PMID: 26341719 DOI: 10.1016/j.jhep.2015.06.030]

6 **Dyson JK**, De Martin E, Dalekos GN, Drenth JPH, Herkel J, Hubscher SG, Kelly D, Lenzi M, Milkiewicz P, Oo YH, Heneghan MA, Lohse AW; IAIHG Consortium. Review article: unanswered clinical and research questions in autoimmune hepatitis-conclusions of the International Autoimmune Hepatitis Group Research Workshop. *Aliment Pharmacol Ther* 2019; **49**: 528-536 [PMID: 30671977 DOI: 10.1111/apt.15111]

7 **Castellanos-Fernández MI**, Borges-González SA, Stepanova M, Infante-Velázquez ME, Ruenes-Domech C, González-Suero SM, Dorta-Guridi Z, Arus-Soler ER, Racila A, Younossi ZM. Health-related quality of life in Cuban patients with chronic liver disease: A real-world experience. *Ann Hepatol* 2021; **22**: 100277 [PMID: 33130334 DOI: 10.1016/j.aohep.2020.10.005]

8 **Afendy A**, Kallman JB, Stepanova M, Younoszai Z, Aquino RD, Bianchi G, Marchesini G, Younossi ZM. Predictors of health-related quality of life in patients with chronic liver disease. *Aliment Pharmacol Ther* 2009; **30**: 469-476 [PMID: 19508612 DOI: 10.1111/j.1365-2036.2009.04061.x]

9 **Dirks M**, Haag K, Pflugrad H, Tryc AB, Schuppner R, Wedemeyer H, Potthoff A, Tillmann HL, Sandorski K, Worthmann H, Ding X, Weissenborn K. Neuropsychiatric symptoms in hepatitis C patients resemble those of patients with autoimmune liver disease but are different from those in hepatitis B patients. *J Viral Hepat* 2019; **26**: 422-431 [PMID: 30120896 DOI: 10.1111/jvh.12979]

10 **Cortesi PA**, Conti S, Scalone L, Jaffe A, Ciaccio A, Okolicsanyi S, Rota M, Fabris L, Colledan M, Fagiuoli S, Belli LS, Cesana G, Strazzabosco M, Mantovani LG. Health related quality of life in chronic liver diseases. *Liver Int* 2020; **40**: 2630-2642 [PMID: 32851764 DOI: 10.1111/liv.14647]

11 **Takahashi A**, Moriya K, Ohira H, Arinaga-Hino T, Zeniya M, Torimura T, Abe M, Takaki A, Kang JH, Inui A, Fujisawa T, Yoshizawa K, Suzuki Y, Nakamoto N, Koike K, Yoshiji H, Goto A, Tanaka A, Younossi ZM, Takikawa H; Japan AIH Study Group. Health-related quality of life in patients with autoimmune hepatitis: A questionnaire survey. *PLoS One* 2018; **13**: e0204772 [PMID: 30286131 DOI: 10.1371/journal.pone.0204772]

12 **Schramm C**, Wahl I, Weiler-Normann C, Voigt K, Wiegard C, Glaubke C, Brähler E, Löwe B, Lohse AW, Rose M. Health-related quality of life, depression, and anxiety in patients with autoimmune hepatitis. *J Hepatol* 2014; **60**: 618-624 [PMID: 24240053 DOI: 10.1016/j.jhep.2013.10.035]

13 **van der Plas SM**, Hansen BE, de Boer JB, Stijnen T, Passchier J, de Man RA, Schalm SW. Generic and disease-specific health related quality of life of liver patients with various aetiologies: a survey.

Qual Life Res 2007; **16**: 375-388 [PMID: 17334830 DOI: 10.1007/s11136-006-9131-y]

14   **Wong LL**, Fisher HF, Stocken DD, Rice S, Khanna A, Heneghan MA, Oo YH, Mells G, Kendrick S, Dyson JK, Jones DEJ; UK-AIH Consortium. The Impact of Autoimmune Hepatitis and Its Treatment on Health Utility. *Hepatology* 2018; **68**: 1487-1497 [PMID: 29663477 DOI: 10.1002/hep.30031]

15   **Janik MK**, Wunsch E, Raszeja-Wyszomirska J, Moskwa M, Kruk B, Krawczyk M, Milkiewicz P. Autoimmune hepatitis exerts a profound, negative effect on health-related quality of life: A prospective, single-centre study. *Liver Int* 2019; **39**: 215-221 [PMID: 30204306 DOI: 10.1111/liv.13960]

16   **Honoré LR**, Kjær TW, Kjær MS. Health-related quality of life in patients with autoimmune hepatitis - a systematic review and meta-analysis. *Clin Res Hepatol Gastroenterol* 2018; **42**: e97-e99 [PMID: 30049510 DOI: 10.1016/j.clinre.2018.06.006]

17   **Trevizoli IC**, Pinedo CS, Teles VO, Seixas RBPM, de Carvalho E. Autoimmune Hepatitis in Children and Adolescents: Effect on Quality of Life. *J Pediatr Gastroenterol Nutr* 2018; **66**: 861-865 [PMID: 29470290 DOI: 10.1097/MPG.0000000000001930]

18   **Bozzini AB**, Neder L, Silva CA, Porta G. Decreased health-related quality of life in children and adolescents with autoimmune hepatitis. *J Pediatr (Rio J)* 2019; **95**: 87-93 [PMID: 29331407 DOI: 10.1016/j.jped.2017.10.013]

19   **Gulati R**, Radhakrishnan KR, Hupertz V, Wyllie R, Alkhouri N, Worley S, Feldstein AE. Health-related quality of life in children with autoimmune liver disease. *J Pediatr Gastroenterol Nutr* 2013; **57**: 444-450 [PMID: 23783017 DOI: 10.1097/MPG.0b013e31829ef82c]

20   **Srivastava S**, Boyer JL. Psychological stress is associated with relapse in type 1 autoimmune hepatitis. *Liver Int* 2010; **30**: 1439-1447 [PMID: 20849437 DOI: 10.1111/j.1478-3231.2010.02333.x]

21   **Tandon P**, Berzigotti A. Management of Lifestyle Factors in Individuals with Cirrhosis: A Pragmatic Review. *Semin Liver Dis* 2020; **40**: 20-28 [PMID: 31470455 DOI: 10.1055/s-0039-1696639]

22   **Moussavi S**, Chatterji S, Verdes E, Tandon A, Patel V, Ustun B. Depression, chronic diseases, and decrements in health: results from the World Health Surveys. *Lancet* 2007; **370**: 851-858 [PMID: 17826170 DOI: 10.1016/S0140-6736(07)61415-9]

23   **Sockalingam S**, Blank D, Abdelhamid N, Abbey SE, Hirschfield GM. Identifying opportunities to improve management of autoimmune hepatitis: evaluation of drug adherence and psychosocial factors. *J Hepatol* 2012; **57**: 1299-1304 [PMID: 22871503 DOI: 10.1016/j.jhep.2012.07.032]

24   **Leoni MC**, Amelung L, Lieveld FI, van den Brink J, de Bruijne J, Arends JE, van Erpecum CP, van Erpecum KJ. Adherence to ursodeoxycholic acid therapy in patients with cholestatic and autoimmune liver disease. *Clin Res Hepatol Gastroenterol* 2019; **43**: 37-44 [PMID: 30219692 DOI: 10.1016/j.clinre.2018.08.006]

25   **Gray WN**, Denson LA, Baldassano RN, Hommel KA. Treatment adherence in adolescents with inflammatory bowel disease: the collective impact of barriers to adherence and anxiety/depressive symptoms. *J Pediatr Psychol* 2012; **37**: 282-291 [PMID: 22080456 DOI: 10.1093/jpepsy/jsr092]

26   **Lamba S**, Nagurka R, Desai KK, Chun SJ, Holland B, Koneru B. Self-reported non-adherence to immune-suppressant therapy in liver transplant recipients: demographic, interpersonal, and intrapersonal factors. *Clin Transplant* 2012; **26**: 328-335 [PMID: 21955028 DOI: 10.1111/j.1399-0012.2011.01489.x]

27   **Montano-Loza AJ**, Carpenter HA, Czaja AJ. Consequences of treatment withdrawal in type 1 autoimmune hepatitis. *Liver Int* 2007; **27**: 507-515 [PMID: 17403191 DOI: 10.1111/j.1478-3231.2007.01444.x]

28   **Younossi Z**, Henry L. Overall health-related quality of life in patients with end-stage liver disease. *Clin Liver Dis (Hoboken)* 2015; **6**: 9-14 [PMID: 31040976 DOI: 10.1002/cld.480]

29   **Bonkovsky HL**, Snow KK, Malet PF, Back-Madruga C, Fontana RJ, Sterling RK, Kulig CC, Di Bisceglie AM, Morgan TR, Dienstag JL, Ghany MG, Gretch DR; HALT-C Trial Group. Health-related quality of life in patients with chronic hepatitis C and advanced fibrosis. *J Hepatol* 2007; **46**: 420-431 [PMID: 17196293 DOI: 10.1016/j.jhep.2006.10.009]

30   **van der Plas SM**, Hansen BE, de Boer JB, Stijnen T, Passchier J, de Man RA, Schalm SW. Generic and disease-specific health related quality of life in non-cirrhotic, cirrhotic and transplanted liver patients: a cross-sectional study. *BMC Gastroenterol* 2003; **3**: 33 [PMID: 14617381 DOI: 10.1186/1471-230X-3-33]

31   **Labenz C**, Toenges G, Schattenberg JM, Nagel M, Huber Y, Marquardt JU, Galle PR, Wörns MA. Health-related quality of life in patients with compensated and decompensated liver cirrhosis. *Eur J Intern Med* 2019; **70**: 54-59 [PMID: 31530418 DOI: 10.1016/j.ejim.2019.09.004]

32   **Wong GW**, Yeong T, Lawrence D, Yeoman AD, Verma S, Heneghan MA. Concurrent extrahepatic autoimmunity in autoimmune hepatitis: implications for diagnosis, clinical course and long-term outcomes. *Liver Int* 2017; **37**: 449-457 [PMID: 27541063 DOI: 10.1111/liv.13236]

33   **Dyson JK**, Wong LL, Bigirumurame T, Hirschfield GM, Kendrick S, Oo YH, Lohse AW, Heneghan MA, Jones DEJ; UK-AIH Consortium. Inequity of care provision and outcome disparity in autoimmune hepatitis in the United Kingdom. *Aliment Pharmacol Ther* 2018; **48**: 951-960 [PMID: 30226274 DOI: 10.1111/apt.14968]

34   **Pape S**, Gevers TJG, Vrolijk JM, van Hoek B, Bouma G, van Nieuwkerk CMJ, Taubert R, Jaeckel E, Manns MP, Papp M, Sipeki N, Stickel F, Efe C, Ozaslan E, Purnak T, Nevens F, Kessener DJN, Kahraman A, Wedemeyer H, Hartl J, Schramm C, Lohse AW, Heneghan MA, Drenth JPH. High discontinuation rate of azathioprine in autoimmune hepatitis, independent of time of treatment initiation. *Liver Int* 2020; **40**: 2164-2171 [PMID: 32410363 DOI: 10.1111/liv.14513]

35   **Snijders RJALM**, Pape S, Gevers TJG, Drenth JPH. Discontinuation rate of azathioprine. *Liver Int* 2020; **40**: 2878 [PMID: 32744415 DOI: 10.1111/liv.14615]

36   **Smith MA**, Irving PM, Marinaki AM, Sanderson JD. Review article: malignancy on thiopurine treatment with special reference to inflammatory bowel disease. *Aliment Pharmacol Ther* 2010; **32**: 119-130 [PMID: 20412066 DOI: 10.1111/j.1365-2036.2010.04330.x]

37   **Ngu JH**, Gearry RB, Frampton CM, Stedman CA. Mortality and the risk of malignancy in autoimmune liver diseases: a population-based study in Canterbury, New Zealand. *Hepatology* 2012; **55**: 522-529 [PMID: 21994151 DOI: 10.1002/hep.24743]

38   **Janik MK**, Wunsch E, Moskwa M, Raszeja-Wyszomirska J, Krawczyk M, Milkiewicz P. Depression in patients with autoimmune hepatitis: the need for detailed psychiatric assessment. *Pol Arch Intern Med* 2019; **129**: 645-647 [PMID: 31316046 DOI: 10.20452/pamw.14898]

39   **Rubin RR**, Wadden TA, Bahnson JL, Blackburn GL, Brancati FL, Bray GA, Coday M, Crow SJ, Curtis JM, Dutton G, Egan C, Evans M, Ewing L, Faulconbridge L, Foreyt J, Gaussoin SA, Gregg EW, Hazuda HP, Hill JO, Horton ES, Hubbard VS, Jakicic JM, Jeffery RW, Johnson KC, Kahn SE, Knowler WC, Lang W, Lewis CE, Montez MG, Murillo A, Nathan DM, Patricio J, Peters A, Pi-Sunyer X, Pownall H, Rejeski WJ, Rosenthal RH, Ruelas V, Toledo K, Van Dorsten B, Vitolins M, Williamson D, Wing RR, Yanovski SZ, Zhang P; Look AHEAD Research Group. Impact of intensive lifestyle intervention on depression and health-related quality of life in type 2 diabetes: the Look AHEAD Trial. *Diabetes Care* 2014; **37**: 1544-1553 [PMID: 24855155 DOI: 10.2337/dc13-1928]

40   **Jacoby A**, Rannard A, Buck D, Bhala N, Newton JL, James OF, Jones DE. Development, validation, and evaluation of the PBC-40, a disease specific health related quality of life measure for primary biliary cirrhosis. *Gut* 2005; **54**: 1622-1629 [PMID: 15961522 DOI: 10.1136/gut.2005.065862]

41   **Broadbent DE**, Cooper PF, FitzGerald P, Parkes KR. The Cognitive Failures Questionnaire (CFQ) and its correlates. *Br J Clin Psychol* 1982; **21**: 1-16 [PMID: 7126941 DOI: 10.1111/j.2044-8260.1982.tb01421.x]

42   **Younossi ZM**, Guyatt G, Kiwi M, Boparai N, King D. Development of a disease specific questionnaire to measure health related quality of life in patients with chronic liver disease. *Gut* 1999; **45**: 295-300 [PMID: 10403745 DOI: 10.1136/gut.45.2.295]

43   **Lo C**, Walsh A, Mikulincer M, Gagliese L, Zimmermann C, Rodin G. Measuring attachment security in patients with advanced cancer: psychometric properties of a modified and brief Experiences in Close Relationships scale. *Psychooncology* 2009; **18**: 490-499 [PMID: 18821528 DOI: 10.1002/pon.1417]

44   **Lloyd A**, Pickard AS. The EQ-5D and the EuroQol Group. *Value Health* 2019; **22**: 21-22 [PMID: 30661629 DOI: 10.1016/j.jval.2018.12.002]

45   **Webster K**, Cella D, Yost K. The Functional Assessment of Chronic Illness Therapy (FACIT) Measurement System: properties, applications, and interpretation. *Health Qual Life Outcomes* 2003; **1**: 79 [PMID: 14678568 DOI: 10.1186/1477-7525-1-79]

46   **Fisk JD**, Ritvo PG, Ross L, Haase DA, Marrie TJ, Schlech WF. Measuring the functional impact of fatigue: initial validation of the fatigue impact scale. *Clin Infect Dis* 1994; **18** Suppl 1: S79-S83 [PMID: 8148458 DOI: 10.1093/clinids/18.supplement_1.s79]

47   **Spitzer RL**, Kroenke K, Williams JB, Löwe B. A brief measure for assessing generalized anxiety disorder: the GAD-7. *Arch Intern Med* 2006; **166**: 1092-1097 [PMID: 16717171 DOI: 10.1001/archinte.166.10.1092]

48   **Zigmond AS**, Snaith RP. The hospital anxiety and depression scale. *Acta Psychiatr Scand* 1983; **67**: 361-370 [PMID: 6880820 DOI: 10.1111/j.1600-0447.1983.tb09716.x]

49   **van der Plas SM**, Hansen BE, de Boer JB, Stijnen T, Passchier J, de Man RA, Schalm SW. The Liver Disease Symptom Index 2.0; validation of a disease-specific questionnaire. *Qual Life Res* 2004; **13**: 1469-1481 [PMID: 15503842 DOI: 10.1023/B:QURE.0000040797.17449.c0]

50   **Smets EM**, Garssen B, Bonke B, De Haes JC. The Multidimensional Fatigue Inventory (MFI) psychometric qualities of an instrument to assess fatigue. *J Psychosom Res* 1995; **39**: 315-325 [PMID: 7636775 DOI: 10.1016/0022-3999(94)00125-o]

51   **Kos D**, Kerckhofs E, Nagels G, D'Hooghe BD, Duquet W, Duportail M, Ketelaer P. Assessing fatigue in multiple sclerosis: Dutch modified fatigue impact scale. *Acta Neurol Belg* 2003; **103**: 185-191 [PMID: 15008502]

52   **Kroenke K**, Spitzer RL, Williams JB. The PHQ-9: validity of a brief depression severity measure. *J Gen Intern Med* 2001; **16**: 606-613 [PMID: 11556941 DOI: 10.1046/j.1525-1497.2001.016009606.x]

53   **Ware J Jr**, Kosinski M, Keller SD. A 12-Item Short-Form Health Survey: construction of scales and preliminary tests of reliability and validity. *Med Care* 1996; **34**: 220-233 [PMID: 8628042 DOI: 10.1097/00005650-199603000-00003]

54   **Ware JE Jr**, Sherbourne CD. The MOS 36-item short-form health survey (SF-36). I. Conceptual framework and item selection. *Med Care* 1992; **30**: 473-483 [PMID: 1593914]

55   **Gaudry E**, Vagg P, Spielberger CD. Validation of the State-Trait Distinction in Anxiety Research. *Multivariate Behav Res* 1975; **10**: 331-341 [PMID: 26829634 DOI: 10.1207/s15327906mbr1003_6]

56   **Reilly MC**, Zbrozek AS, Dukes EM. The validity and reproducibility of a work productivity and activity impairment instrument. *Pharmacoeconomics* 1993; **4**: 353-365 [PMID: 10146874 DOI: 10.2165/00019053-199304050-00006]

Fungal infections following liver transplantation

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Abstract

With increasing morbidity and mortality from chronic liver disease and acute liver failure, the need for liver transplantation is on the rise. Most of these patients are extremely vulnerable to infections as they are immune-compromised and have other chronic co-morbid conditions. Despite the recent advances in practice and improvement in diagnostic surveillance and treatment modalities, a major portion of these patients continue to be affected by post-transplant infections. Of these, fungal infections are particularly notorious given their vague and insidious onset and are very challenging to diagnose. This mini-review aims to discuss the incidence of fungal infections following liver transplantation, the different fungi involved, the risk factors, which predispose these patients to such infections, associated diagnostic challenges, and the role of prophylaxis. The population at risk is increasingly old and frail, suffering from various other co-morbid conditions, and needs special attention. To improve care and to decrease the burden of such infections, we need to identify the at-risk population with more robust clinical and diagnostic parameters. A more robust global consensus and stringent guidelines are needed to fight against resistant microbes and maintain the longevity of current antimicrobial therapies.

Key Words: Invasive fungal infections; Liver transplantation; Candidiasis; Antifungal prophylaxis; Aspergillosis; *Cryptococcus*

Core Tip: Fungal infections post liver transplant remains the predominant source of morbidity and mortality despite the incidence being low. This is because of evasive clinical features coupled with difficulty to isolate and culture these pathogens. Therefore, appropriate patients are selected for prophylactic regimen based on specific risk factors to curb the rise of drug-resistant species. Traditional regimens include fluconazole or liposomal amphotericin with a shift towards echinocandins based on recently published and promising data.

INTRODUCTION

Liver transplantation is one of the principal treatment modalities for the treatment of many hepatic diseases, mainly but not limited to chronic and end-stage liver disease. Despite advances in the field of transplantation, invasive fungal infections remain a major source of morbidity and mortality. This is attributed to delay in diagnosis, nonspecific clinical features[1], fastidious nature of these organisms, lack of consensus on prophylactic regimens, and rise of antifungal resistant species.

Moreover, with an increase in the number of grafts being offered, there is a trend towards recipients being older, debilitated, and having more non hepatic comorbidities which contributes to the burden and subsequently leads to a higher rate of fungal infections[2].

In this article, we aim to discuss the incidence and trend of invasive fungal infections (IFI) in liver transplant (LT) patients, associated risk factors, diagnostic challenges, and data on prophylaxis.

IFI DEFINITION

IFIs, according to the Invasive Fungal Infections Cooperative Group in Europe and the Mycoses Study Group in the United States, are divided into 3 categories: proven, probable and possible.

Proven IFI is defined as a positive fungal culture or histological proof of fungal or hyphal elements in a sterile site biopsy. This also includes positive cryptococcal antigen in cerebrospinal fluid.

Probable and possible IFIs have a wider definition and inclusion criteria. This is based on several host factors along with various clinical and mycological criteria[3].

Some studies evaluating prophylactic regimens, in this regard have been a focus of criticism as their IFI's were considered colonization rather than infection[4].

INCIDENCE AND RESPONSIBLE FUNGI

The incidence of IFI after LT has decreased in recent years and this is attributable to advancement and improvement in surgical techniques along with more aggressive post-operative care. Previously, in one study by Fung *et al*[5], the incidence of IFI after LT was reported to be 6.6% with a mortality of 54.5%. The ninety-day cumulative mortality after invasive candidiasis has been reported to be 26% and 1-year survival after invasive aspergillosis is about 59% according to TRANSNET in 2010[6].

More recently, according to some cohort studies, the overall incidence of IFI after solid organ transplant is about 1%-4%[7-9]. 1-year cumulative probability of IFI in LT was 1.8%[7]. This shows a promising trend and is related to improvise surgical techniques and timely recognition of risk factors that make certain patients more susceptible to IFIs.

However, in underdeveloped nations, it remains higher at 14.7% with an in-hospital mortality rate of 77%[10]. A future streamlined approach to the problem with specific guidelines might be one of the ways to improve these numbers.

The three major fungi involved are *Candida* spp., *Cryptococcus*, and *Aspergillus* spp. *Candida* predominates with 81% followed by *Aspergillus* (16%) and *Cryptococcus* (3%). Non-*Albicans Candida* accounted for 68% of all *Candida* infections[11]. The rise of resistant non-*Albicans Candida* especially C. parapsilosis was felt to coincide with the increased use of fluconazole[11]. C. parapsilosis is associated with increased mortality in these patients. This increase in resistant fungal species indicates a dire need for a patient-specific prophylactic regimen based on risk factors *vs* a universal approach.

The distribution of the fungal species remains similar in the East with *Candida* representing 64.1% and *Aspergillus* 35.8% of the IFIs in LT patients.

Despite the highly variable clinical presentation, these pathogens most commonly affect the respiratory system followed by renal and gastrointestinal tract[10]. According to a retrospective study in 2015 by Eschenauer and colleagues, intra-abdominal candidiasis (73%) was the most common IFI[12]. The common clinical manifestations of various fungal organisms are shown in Table 1.

TIMING FROM TRANSPLANT TO INFECTION

There has been a shift in the time duration between the developments of IFIs after LT. It was initially thought to occur in the early post-operative phase most commonly within the first couple of months.

Grauhan *et al*[13] in 1994 reported a median time from LT to IFI of 2 mo.

According to Husain *et al*[14] in 2003, the median time to infection for invasive candidiasis was 13.5 d with 72% of the IFIs happening within the first month after LT.

Aspergillus tends to present later as compared to *Candida*. Results from one study by Singh and colleagues in 2003 reported 55% of their *Aspergillus* IFI occurring after 90 d [15] and Gravalda *et al*[16] also described 43% of their IFIs as late onset *Aspergillus*.

In transplant centers with a higher risk of *Aspergillus* based on epidemiology, this delayed time to presentation is important to consider while deciding on the length of prophylactic regimen in high-risk patients. Moreover, clinicians need to be mindful of this time frame while diagnosing an already difficult-to-diagnose disease.

RISK FACTORS

Multiple factors have been observed over time to be associated with the development of fungal infections in LTs. Identifying patients that are at high risk for developing IFI can be of immense help as that can aide in decreasing the diagnostic delay and assure appropriate prophylaxis. By adopting this targeted method of prophylaxis *vs* universal approach, we can also potentially reduce the incidence of drug-resistant fungi, lower the morbidity due to side effects and interactions of these medications particularly with immunosuppressants, and mitigate the overall cost.

Many scientists over the past few decades have worked on identifying these attributes. These can be categorized into pre-operative, operative, and post-operative factors as shown in Table 2. Risk factors for *Aspergillus* specifically seem to depend more on post-operative factors as highlighted in Figure 1.

Collins *et al*[17] in 1994 identified the following as potential risk factors: renal insufficiency, length of transplant operation, rate of re-transplantation, abdominal or intra-thoracic reoperation, and cytomegalovirus infection.

Other studies showed that model for end-stage liver disease (MELD) scores > 25, post-transplant acute kidney injury (Cr > 2 or risk, injury, failure, loss of kidney function, and end-stage n criteria I- or F-) and pre-transplant fungal colonization seem to be the culprits identified with IFIs[11,18].

One of these was an important and common risk factor of daily prophylactic fluconazole dose of < 200 mg, which was thought to cause a rise in drug-resistant non-*Albicans Candida* spp[11].

Although very rare, a French study also identified contamination during organ procurement as a risk factor with a 1.33% prevalence of *Candida* spp. in preservation fluid. This was associated with a higher rate of IFI and impaired survival[19].

Alongside predictable risk factors like diabetes and hemodialysis dependence, Verma *et al*[10] pointed out prior antibiotic use, cerebral and respiratory organ failures, chronic liver failure (CLIF) organ failure/CLIF-consortium acute-on-chronic liver

Table 1 Common clinical manifestations of invasive fungal infection

	Clinical manifestations
Candida	Intra-abdominal abscesses
	Recurrent cholangitis
	Peritonitis
	Fungemia
Aspergillus	Invasive pulmonary *Aspergillosis*
	Brain abscess
	Endophthalmitis
	Osteomyelitis
	Endocarditis
Cryptococcus	CNS infection
	Focal lesions on imaging
	Meningeal enhancement

CNS: Central nervous system.

Table 2 Risk factors for invasive fungal infections

	Risk factors
Pre-operative	SBP prophylaxis with fluoroquinolone
Operative	Retransplantation
	Long transplantation time
	Long transplantation time
	Class 2 partial or complete match
	Donor from male
Post-operative	Post-transplant HD
	High number of RBC units transfused
	Post-transplant bacterial infection
	Cytomegalovirus infection
	Use of muromonab-CD3
	Aspergillus antigenemia

SBP: Spontaneous bacterial prophylaxis; HD: Hemodialysis; RBC: Red blood cells.

failure as predictors of IFIs. Non-survivors in their study also had higher levels of 1.3-beta D glucan (BDG) levels. BDG levels have been studied as a diagnostic marker and look promising.

There has been a general shift in the trend of risk factors over the last 2 decades, which is attributable to better surgical techniques. Singh *et al*[20] studied 190 liver transplants during 1990 and 2000 and demonstrated improvement in length of operation, intraoperative transfusion requirements, use of roux-en-Y biliary anastomosis, re-transplantation, rate of rejection over time, and cold ischemic time. This led to a decrease in the incidence of invasive candidiasis in this study population from 9%-1.7% without any use of antifungal prophylaxis.

In 2015, Eschenauer and colleagues identified bile leaks within the first 30 d post-transplant and living donor liver transplants as new independent risk factors for IFIs. This is because *Candida* has an affinity for growth in the biliary tract. Moreover, living donor liver transplants are highly technical procedures that are not commonly
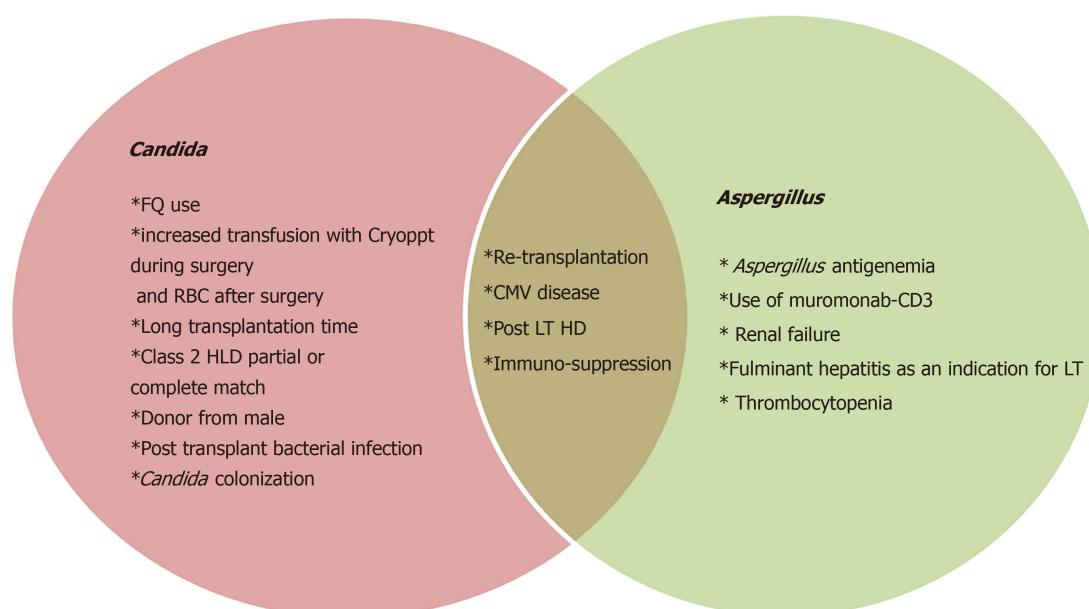
Figure 1 Risk factors for Candida and Aspergillus. FQ: Fluoroquinolone; HLA: Human leukocyte antigen; CMV: Cytomegalovirus; HD: Hemodialysis; LT: Liver transplant.

performed in the United States. The increased length and complexity of these procedures along with higher disruption of the biliary tract is responsible for these findings. The authors recommended instituting antifungal prophylaxis in all living donor liver transplants[12].

A small study recently in 2020 by Jorgenson *et al*[21] studied the effects of pre-transplant roux-en Y gastric bypass on liver transplant outcomes. There were increased rates of fungal infection in patients with bariatric surgery before transplant and might be associated with loss of defense provided by gastric acid. This study is limited by its retrospective nature and its size.

DIAGNOSTIC CHALLENGES

In general, fungal infections do not present themselves vividly and are increasingly difficult to grow in culture media. It makes it even more challenging in patients who have chronic liver disease, are immunosuppressed, or have other underlying comorbidities. They are difficult to detect clinically and also objectively in laboratories. Hence, prevention becomes essential, and it has significantly improved in the last decade with the advancement in surgical techniques, intense pre-operative evaluation, and appropriate use of antifungal prophylactic agents in high-risk patients.

Distinguishing between colonization and true infection can be challenging for the clinician. Apart from 'proven IFI' as discussed above, the other two categories are vague and have plenty of variable factors. In these clinical scenarios, the use of newer diagnostic tools like BDG and galactomannan (GM) can be helpful. Polymerase chain reaction fungal assays are promising but not yet approved by the Food and Drug Administration.

BDG has been studied and looks promising as a diagnostic marker in serum. In a study from 2017, with 271 transplant patients, weekly BDG was tested and monitored for IFIs. 95% of the patients with IFI had positive BDG and a very promising negative predictive value of 96% was seen. The sensitivity of BDG was 75% and specificity was 65%, making it a very good tool to rule out IFIs[22].

The GM test is an enzyme-linked immunosorbent assay that detects the GM antigen released by *Aspergillus* hyphae when they invade host cells.

The patient's epidemiological risk factors should be considered strongly which would help guide better towards increasing clinical suspicion and ordering appropriate tests and guided treatments. Objective risk factors such as the MELD score, the overall duration of need for total parenteral nutrition, length of the operative procedure, and removal of abdominal drains and other catheters or lines should be evaluated[23].

PROPHYLAXIS

Fungal infections in liver transplant recipients are mostly attributed to *Aspergillus* and *Candida*. Three agents are mainly used in prophylaxis–fluconazole, liposomal amphotericin B, and itraconazole. The studies involving these agents have been confounded by the difficulty of differentiating colonization and a true infection, the variability between patient selection, therapeutic agent(s) used in comparison with placebo or each other, and variable duration of treatment.

Data on the effectiveness of antifungal prophylaxis in LT over the past 10 years have been summarized in the Table 3 below.

There have been three meta-analyses as summarized in Table 3. Playford *et al*[24] and Cruciani *et al*[25] published two in 2006, with 10 and 6 studies respectively. These summarized that universal fungal prophylaxis leads to a reduction in proven IFIs without any mortality benefit. This universal approach leads to a significantly higher proportion of episodes of non-*Albicans Candida* infection.

In 2014, Evans *et al*[26] published a meta-analysis of studies on prophylaxis to prevent IFIs after LT and concluded that the odds of proven IFI and IFI related mortality were lower in patients receiving antifungal prophylaxis, even if the overall mortality did not change. It was also demonstrated that the efficacy of fluconazole compared to liposomal amphotericin was similar with the latter having the benefit of not altering the cytochrome P450 system and therefore not affecting the calci-neurin inhibitor levels. However, fluconazole is favored because of its cost-effectiveness and safety profile. This meta-analysis did not reveal any information on echinocandins, however, it was different from their counterparts in that they did a mixed treatment comparison and was more recent of the few meta-analyses already on the subject matter.

Studies since 2014 (after the last meta-analysis) on prophylaxis are summarized in Table 4.

In 2015, Eschenauer and colleagues performed a retrospective study involving liver transplant patients that were divided into three main groups. Group 1 included 145 patients who received targeted prophylaxis with either voriconazole in 54%, fluconazole in 5% or no antifungal which was the case of 38% of these patients. This was compared to a group of 237 patients, who received universal prophylaxis with voriconazole. These regimens were continued for a median time of 11 d in the targeted group and for 6 d in the universal group, with a significant *P* value. There was no statistical difference between incidence of IFI between both groups (6.8% in targeted and 4.2% in universal). Similarly, the *P* value was not statistically significant for the mortality rates over 100 d from IFIs in both groups (10% for targeted and 7% for universal group). They, therefore concluded that targeted approach to antifungal use in liver transplant patients was a safe, cost effective strategy and prevented unnec-essary side effects[12].

With regards to echinocandins, Saliba *et al*[27] in 2015 compared micafungin *vs* standard treatment and found them equally effective. Standard therapy was center-specific and included IV fluconazole, liposomal amphotericin, or IV caspofungin.

Similarly, in a study from Spain in 2016, caspofungin was compared to fluconazole in high-risk patients and similar efficacy was reported to prevent global IFIs. In this study caspofungin was related to decrease in breakthrough IFIs and also led to a lower rate of invasive aspergillosis[28].

Echinocandins should be considered as prophylactic agents, where appropriate, especially in areas of increased prevalence of drug-resistant non-*Albicans Candida*. Unfortunately, these too come with a higher price tag compared to fluconazole which can affect their use, especially in non-affluent countries.

According to the Infectious Disease Society of America guidelines, patients who meet 2 or more of the following risk factors to be considered for prophylaxis: creat-inine more than 2 mg/dL, need for re-transplantation, choledochojejunostomy, more than 11 h of operative time, need to transfuse with ≥ 40 units of blood products, evidence of fungal colonization in immediate pre and post-operative days. Suggested duration of antifungal use is 14-21 d.

However, since the current data suggest that the incidence and risk of fungal infection overall in the general liver transplantation population is low, these agents should be utilized for higher-risk patients as unguided use is associated with drug-resistant non-*Albicans Candida* infection and higher mortality in these patients[23].

Table 3 Effectiveness of antifungal prophylaxis in liver transplant

Ref.	Trials	Patients	Regimens	Infection reduction (95%CI)	Comments
Cruciani *et al*[25], 2006	6	698	AmB *vs* Pla (1)	Total proven fungal infections RR 0.31 (0.21-0.46), IFI RR 0.33 (0.18-0.59)	Patients receiving prophylaxis had higher number of non-*Albicans* proven fungal infections. Mostly C. glabrata.
			Flu *vs* nonsystemic AF (1)		
			Flu *vs* Pla (2)		
			Itra *vs* Pla(1)		
			Amb-Itra *vs* Flu-itra *vs* Pla (1)		
Playford *et al*[24], 2006	7	793	Flu *vs* Pla (2)	Proven IFI RR 0.39 (0.18-0.85), fungal colonization RR 0.51 (0.41-0.62), fungal colonization with C. glabrata/C. krusei, RR 1.57 (0.76-3.24)	Formulated algorithm in which patients with < 2 RF deemed low risk (4%incidence) for IFI and those with ≥ 2 at high risk (25% incidence) for IFI.
			Flu *vs* nonsystemic AF (2)		
			Itra *vs* Pla (2)		
			AmB *vs* Pla (1)		
Evans *et al* [26], 2014	14	1633	Flu *vs* Pla/nonabs AF (4)	Proven IFI OR 0.37 (0.19-0.72), $P = 0.003$, Bayesian MTC, AmB *vs* Pla OR 0.21 (0.05-0.71), Flu *vs* Pla OR 0.21 (0.06-0.57)	Benefit of AmB is of similar magnitude to that previously described for fluconazole.
			Itra *vs* Pla (1)		
			AmB *vs* Pla (1)		
			3 arm study with Pla/AmB/Flu (1)		
			Flu *vs* AmB (3)		
			Liposomal + Flu *vs* standard AmB + Flu		
			Itra *vs* Flu (2)		
			Micafungin *vs* standard care (1)		
			Clo *vs* Nys (1)		

AmB: Amphotericin-B; Pla: Placebo; Flu: Fluconazole; AF: Antifungal; Itra: Itraconazole; Nonabs AF: Nonabsorbable antifungal; Nys: Nystatin; Clo: Clotrimazole.

CONCLUSION

Fungal infections following liver transplantation remain an influential cause of morbidity and mortality in these patients, despite the low incidence. Identification of high-risk patients based on risk factors discussed above and starting an appropriate prophylactic antifungal regimen based on epidemiology, calcineurin inhibitor use, and renal function is the first step in avoiding dealing with this evasive disease.

Prophylactic antifungals are generally well tolerated but can lead to drug-resistant *Candida* spp., hence the importance of selecting the appropriate patient and agent. Using BDG as a negative predictive tool and having a high degree of suspicion, even if the time from transplant exceeds 2 mo, can prevent diagnostic delays.

Further randomized controlled trials comparing azoles, amphotericin, and echinocandins are needed to develop an updated standard of care.

Table 4 Studies since 2014 (after the last meta-analysis) on prophylaxis for liver transplant

Ref.	Design	Regimen	Outcomes
Antunes *et al* [29], 2014	Single center. Retrospective (*n* = 461)	High risk group: AmB *vs* nystatin; Low risk group: nystatin	Higher IFI in high risk patients who did not receive AmB
Winston *et al* [30], 2014	Randomized, double-blind. 2010-2011 (*n* = 200)	Group 1: Andulafugin; Group 2: Flu	1:1 randomized. Similar cumulative IFI occurrence and equal 3 mo mortality
Saliba *et al*[27], 2015	Randomized, open label. 2009-2012 (*n* = 347)	Micafungin *vs* center specific standard care (Flu/AmB/Caspo)	Micafungin was non-inferior to standard of care
Giannella *et al* [31], 2015	Prospective, non-randomized. 2009-2013. Safety of high dose AmB (*n* = 76)	Amb 10 mg/kg Q weekly until hospital discharge for a minimum of 2 wk	10 patients discontinued therapy. (6 for AmB related AEs and 4 for IFI)
Eschenauer *et al*[12], 2015	Single center study. 2008-2012. Effectiveness of targeted prophylaxis (*n* = 381)	Universal ppx: Vori. Targeted: Group1: Vori, 30 d. Group 2: Flu during icu sta. Group3: No ppx	Cumulative IFI occurrence 5.2% (targeted *vs* universal group). Similar 100 day mortality between targeted and universal ppx gp. 40% breakthrough IFI
Balogh *et al* [32], 2016	Single center study. 2008-2014 (*n* = 314)	Voriconazole *vs* oral nystatin or Flu	No episodes of IA occurred. No difference in graft and patient survival curves between the two groups
Perrella *et al* [33], 2016	Single center study. 2006-2012. Comparative observational study for targeted prophylaxis (*n* = 54)	Group 1: AmB 3 mg/kg/day; Group2: Caspofungin 70 mg loading→50 mg/day	No episodes of IFI in both groups
Fortún *et al* [28], 2016	Multicenter. 2005-2012. Comparative observational study for targeted prophylaxis (*n* = 195)	Group 1: Caspofungin 50 mg/d; Group 2: Flu median 200 mg/day	Similar 6 m IFI occurrence [5.2% b (G1) *vs* 12.2% (G2)]. Reduced risk of IA in LT receiving caspofungin. Similar overall mortality
Chen *et al*[34], 2016	Single center study. 2005-2014. Effectiveness of targeted prophylaxis (*n* = 402)	Group 1: Anidulafungin 100 mg/day or micafungin 100 mg/day; Group 2: No prophylaxis	High risk patients MELD > 20; Similar IFI occurrence lower cumulative mortality in group 1 (*P* = 0.001)
Giannella *et al* [35], 2016	Retrospective, single center. 2010-2014. Evaluation of RF for a targeted prophylaxis (*n* = 303)	Group 1: No RF. No prophylaxis; Group 2: 1RF IC, Flu; Group3: High risk, anti mould agent	Antifungal prophylaxis administered to 45.9% patients. Cumulative IFI prevalence 6.3%. Flu independently associated with IFI development
Lavezzo *et al* [36], 2018	Single center study. 2011-2015. Effectiveness of targeted prophylaxis	Group 1 high risk: AmB; Group 2 low risk: No prophylaxis	Overall IFI prevalence 2.8%. 1 yr mortality higher in prophylaxis group (*P* = 0.001). 1 yr mortality higher in IFI patients (*P* < 0.001)
Jorgenson *et al* [37], 2019	Single center study. 2009-2016. Effectiveness of fixed dose prophylaxis (*n* = 189)	Group 1: Flu 400 mg/day for 14 d for high risk patients; Group 2: unsupervised antifungal protocols	Reduction in 1 yr IFI among high risk group (12.5% *vs* 26.6%). Similar 1 yr patient and graft survival
Kang *et al*[38], 2020	Multicenter, randomized, open label. Living donor LT. 2012-2015 (*n* = 144)	Group 1: Micafungin 100 mg/d; Group 2: Flu 100-200 mg/day	Group 1 *vs* Group 2: 69 *vs* 75 pts. IFI occurrence in 3 wk: 1/69 *vs* 0/75. Micafungin was noninferior to Flu

AmB: Amphotericin-b; Flu: Fluconazole; Caspo: Caspofungin; AE: Adverse effects; Vori: Voriconazole; ppx: Prophylaxis; gp: Group; IA: Invasive aspergillosis; IC: Invasive candidiasis.

REFERENCES

1 **Liu X**, Ling Z, Li L, Ruan B. Invasive fungal infections in liver transplantation. *Int J Infect Dis* 2011; **15**: e298-e304 [PMID: 21345708 DOI: 10.1016/j.ijid.2011.01.005]

2 **Toniutto P**, Zanetto A, Ferrarese A, Burra P. Current challenges and future directions for liver transplantation. *Liver Int* 2017; **37**: 317-327 [PMID: 27634369 DOI: 10.1111/liv.13255]

3 **De Pauw B**, Walsh TJ, Donnelly JP, Stevens DA, Edwards JE, Calandra T, Pappas PG, Maertens J, Lortholary O, Kauffman CA, Denning DW, Patterson TF, Maschmeyer G, Bille J, Dismukes WE, Herbrecht R, Hope WW, Kibbler CC, Kullberg BJ, Marr KA, Muñoz P, Odds FC, Perfect JR, Restrepo A, Ruhnke M, Segal BH, Sobel JD, Sorrell TC, Viscoli C, Wingard JR, Zaoutis T, Bennett JE; European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group; National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. *Clin Infect Dis* 2008; **46**: 1813-1821 [PMID: 18462102 DOI: 10.1086/588660]

4 **Huprikar S**. Reply: Effect of prophylaxis on fungal infection and costs for high-risk liver transplant recipients. *Liver Transpl* 2008; **14**: 709 [PMID: 18433053 DOI: 10.1002/lt.21458]

5 **Fung JJ**. Fungal infection in liver transplantation. *Transpl Infect Dis* 2002; **4** Suppl 3: 18-23 [PMID: 12486788 DOI: 10.1034/j.1399-3062.4.s3.3.x]

6 **Pappas PG**, Alexander BD, Andes DR, Hadley S, Kauffman CA, Freifeld A, Anaissie EJ, Brumble

LM, Herwaldt L, Ito J, Kontoyiannis DP, Lyon GM, Marr KA, Morrison VA, Park BJ, Patterson TF, Perl TM, Oster RA, Schuster MG, Walker R, Walsh TJ, Wannemuehler KA, Chiller TM. Invasive fungal infections among organ transplant recipients: results of the Transplant-Associated Infection Surveillance Network (TRANSNET). *Clin Infect Dis* 2010; **50**: 1101-1111 [PMID: 20218876 DOI: 10.1086/651262]

7   **Hosseini-Moghaddam SM**, Ouédraogo A, Naylor KL, Bota SE, Husain S, Nash DM, Paterson JM. Incidence and outcomes of invasive fungal infection among solid organ transplant recipients: A population-based cohort study. *Transpl Infect Dis* 2020; **22**: e13250 [PMID: 31981389 DOI: 10.1111/tid.13250]

8   **Andes DR**, Safdar N, Baddley JW, Alexander B, Brumble L, Freifeld A, Hadley S, Herwaldt L, Kauffman C, Lyon GM, Morrison V, Patterson T, Perl T, Walker R, Hess T, Chiller T, Pappas PG; TRANSNET Investigators. The epidemiology and outcomes of invasive Candida infections among organ transplant recipients in the United States: results of the Transplant-Associated Infection Surveillance Network (TRANSNET). *Transpl Infect Dis* 2016; **18**: 921-931 [PMID: 27643395 DOI: 10.1111/tid.12613]

9   **van Delden C**, Stampf S, Hirsch HH, Manuel O, Meylan P, Cusini A, Hirzel C, Khanna N, Weisser M, Garzoni C, Boggian K, Berger C, Nadal D, Koller M, Saccilotto R, Mueller NJ; Swiss Transplant Cohort Study. Burden and Timeline of Infectious Diseases in the First Year After Solid Organ Transplantation in the Swiss Transplant Cohort Study. *Clin Infect Dis* 2020; **71**: e159-e169 [PMID: 31915816 DOI: 10.1093/cid/ciz1113]

10   **Verma N**, Singh S, Taneja S, Duseja A, Singh V, Dhiman RK, Chakrabarti A, Chawla YK. Invasive fungal infections amongst patients with acute-on-chronic liver failure at high risk for fungal infections. *Liver Int* 2019; **39**: 503-513 [PMID: 30276951 DOI: 10.1111/liv.13981]

11   **Raghuram A**, Restrepo A, Safadjou S, Cooley J, Orloff M, Hardy D, Butler S, Koval CE. Invasive fungal infections following liver transplantation: incidence, risk factors, survival, and impact of fluconazole-resistant Candida parapsilosis (2003-2007). *Liver Transpl* 2012; **18**: 1100-1109 [PMID: 22577087 DOI: 10.1002/lt.23467]

12   **Eschenauer GA**, Kwak EJ, Humar A, Potoski BA, Clarke LG, Shields RK, Abdel-Massih R, Silveira FP, Vergidis P, Clancy CJ, Nguyen MH. Targeted versus universal antifungal prophylaxis among liver transplant recipients. *Am J Transplant* 2015; **15**: 180-189 [PMID: 25359455 DOI: 10.1111/ajt.12993]

13   **Grauhan O**, Lohmann R, Lemmens P, Schattenfroh N, Keck H, Klein E, Raakow R, Jonas S, Langrehr JM, Bechstein W. Fungal infections in liver transplant recipients. *Langenbecks Arch Chir* 1994; **379**: 372-375 [PMID: 7845165 DOI: 10.1007/BF00191586]

14   **Husain S**, Tollemar J, Dominguez EA, Baumgarten K, Humar A, Paterson DL, Wagener MM, Kusne S, Singh N. Changes in the spectrum and risk factors for invasive candidiasis in liver transplant recipients: prospective, multicenter, case-controlled study. *Transplantation* 2003; **75**: 2023-2029 [PMID: 12829905 DOI: 10.1097/01.TP.0000065178.93741.72]

15   **Singh N**, Avery RK, Munoz P, Pruett TL, Alexander B, Jacobs R, Tollemar JG, Dominguez EA, Yu CM, Paterson DL, Husain S, Kusne S, Linden P. Trends in risk profiles for and mortality associated with invasive aspergillosis among liver transplant recipients. *Clin Infect Dis* 2003; **36**: 46-52 [PMID: 12491201 DOI: 10.1086/345441]

16   **Gavalda J**, Len O, San Juan R, Aguado JM, Fortun J, Lumbreras C, Moreno A, Munoz P, Blanes M, Ramos A, Rufi G, Gurgui M, Torre-Cisneros J, Montejo M, Cuenca-Estrella M, Rodriguez-Tudela JL, Pahissa A; RESITRA (Spanish Network for Research on Infection in Transplantation). Risk factors for invasive aspergillosis in solid-organ transplant recipients: a case-control study. *Clin Infect Dis* 2005; **41**: 52-59 [PMID: 15937763 DOI: 10.1086/430602]

17   **Collins LA**, Samore MH, Roberts MS, Luzzati R, Jenkins RL, Lewis WD, Karchmer AW. Risk factors for invasive fungal infections complicating orthotopic liver transplantation. *J Infect Dis* 1994; **170**: 644-652 [PMID: 8077723]

18   **Utsumi M**, Umeda Y, Yagi T, Nagasaka T, Shinoura S, Yoshida R, Nobuoka D, Kuise T, Fuji T, Takagi K, Takaki A, Fujiwara T. Risk Analysis for Invasive Fungal Infection after Living Donor Liver Transplantation: Which Patient Needs Potent Prophylaxis? *Dig Surg* 2019; **36**: 59-66 [PMID: 29649828 DOI: 10.1159/000486548]

19   **Levesque E**, Paugam-Burtz C, Saliba F, Khoy-Ear L, Merle JC, Jung B, Stecken L, Ferrandiere M, Mihaila L, Botterel F. Fungal complications after Candida preservation fluid contamination in liver transplant recipients. *Transpl Int* 2015; **28**: 1308-1316 [PMID: 26147662 DOI: 10.1111/tri.12633]

20   **Singh N**, Wagener MM, Marino IR, Gayowski T. Trends in invasive fungal infections in liver transplant recipients: correlation with evolution in transplantation practices. *Transplantation* 2002; **73**: 63-67 [PMID: 11792979 DOI: 10.1097/00007890-200201150-00011]

21   **Jorgenson MR**, Gracon AS, Hanlon B, Leverson GE, Parajuli S, Smith JA, Al-Adra DP. Pre-transplant bariatric surgery is associated with increased fungal infection after liver transplant. *Transpl Infect Dis* 2021; **23**: e13484 [PMID: 33012079 DOI: 10.1111/tid.13484]

22   **Levesque E**, Rizk F, Noorah Z, Aït-Ammar N, Cordonnier-Jourdin C, El Anbassi S, Bonnal C, Azoulay D, Merle JC, Botterel F. Detection of (1,3)-β-d-Glucan for the Diagnosis of Invasive Fungal Infection in Liver Transplant Recipients. *Int J Mol Sci* 2017; **18** [PMID: 28422065 DOI: 10.3390/ijms18040862]

23   **Eschenauer GA**, Lam SW, Carver PL. Antifungal prophylaxis in liver transplant recipients. *Liver Transpl* 2009; **15**: 842-858 [PMID: 19642130 DOI: 10.1002/lt.21826]

24   **Playford EG**, Webster AC, Sorrell TC, Craig JC. Systematic review and meta-analysis of antifungal agents for preventing fungal infections in liver transplant recipients. *Eur J Clin Microbiol Infect Dis* 2006; **25**: 549-561 [PMID: 16912905 DOI: 10.1007/s10096-006-0182-3]

25   **Cruciani M**, Mengoli C, Malena M, Bosco O, Serpelloni G, Grossi P. Antifungal prophylaxis in liver transplant patients: a systematic review and meta-analysis. *Liver Transpl* 2006; **12**: 850-858 [PMID: 16628697 DOI: 10.1002/lt.20690]

26   **Evans JD**, Morris PJ, Knight SR. Antifungal prophylaxis in liver transplantation: a systematic review and network meta-analysis. *Am J Transplant* 2014; **14**: 2765-2776 [PMID: 25395336 DOI: 10.1111/ajt.12925]

27   **Saliba F**, Pascher A, Cointault O, Laterre PF, Cervera C, De Waele JJ, Cillo U, Langer RM, Lugano M, Göran-Ericzon B, Phillips S, Tweddle L, Karas A, Brown M, Fischer L; TENPIN (Liver Transplant European Study Into the Prevention of Fungal Infection) Investigators; TENPIN Liver Transplant European Study Into the Prevention of Fungal Infection Investigators. Randomized trial of micafungin for the prevention of invasive fungal infection in high-risk liver transplant recipients. *Clin Infect Dis* 2015; **60**: 997-1006 [PMID: 25520332 DOI: 10.1093/cid/ciu1128]

28   **Fortún J**, Muriel A, Martín-Dávila P, Montejo M, Len O, Torre-Cisneros J, Carratalá J, Muñoz P, Fariñas C, Moreno A, Fresco G, Goikoetxea J, Gavaldá J, Pozo JC, Bodro M, Vena A, Casafont F, Cervera C, Silva JT, Aguado JM; Grupo de Estudio de Infección en Pacientes Trasplantados-Grupo de Estudio de Micología Médica (Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica), and Red Española de Investigación en Patología Infecciosa. Caspofungin versus fluconazole as prophylaxis of invasive fungal infection in high-risk liver transplantation recipients: A propensity score analysis. *Liver Transpl* 2016; **22**: 427-435 [PMID: 26709146 DOI: 10.1002/lt.24391]

29   **Antunes AM**, Teixeira C, Corvo ML, Perdigoto R, Barroso E, Marcelino P. Prophylactic use of liposomal amphotericin B in preventing fungal infections early after liver transplantation: a retrospective, single-center study. *Transplant Proc* 2014; **46**: 3554-3559 [PMID: 25498088 DOI: 10.1016/j.transproceed.2014.06.065]

30   **Winston DJ**, Limaye AP, Pelletier S, Safdar N, Morris MI, Meneses K, Busuttil RW, Singh N. Randomized, double-blind trial of anidulafungin versus fluconazole for prophylaxis of invasive fungal infections in high-risk liver transplant recipients. *Am J Transplant* 2014; **14**: 2758-2764 [PMID: 25376267 DOI: 10.1111/ajt.12963]

31   **Giannella M**, Ercolani G, Cristini F, Morelli M, Bartoletti M, Bertuzzo V, Tedeschi S, Faenza S, Puggioli C, Lewis RE, Pinna AD, Viale P. High-dose weekly liposomal amphotericin b antifungal prophylaxis in patients undergoing liver transplantation: a prospective phase II trial. *Transplantation* 2015; **99**: 848-854 [PMID: 25531982 DOI: 10.1097/TP.0000000000000393]

32   **Balogh J**, Gordon Burroughs S, Boktour M, Patel S, Saharia A, Ochoa RA, McFadden R, Victor DW, Ankoma-Sey V, Galati J, Monsour HP Jr, Fainstein V, Li XC, Grimes KA, Gaber AO, Aloia T, Ghobrial RM. Efficacy and cost-effectiveness of voriconazole prophylaxis for prevention of invasive aspergillosis in high-risk liver transplant recipients. *Liver Transpl* 2016; **22**: 163-170 [PMID: 26515643 DOI: 10.1002/lt.24365]

33   **Perrella A**, Esposito C, Amato G, Perrella O, Migliaccio C, Pisaniello D, Calise F, Cuomo O, Santaniello W. Antifungal prophylaxis with liposomal amphotericin B and caspofungin in high-risk patients after liver transplantation: impact on fungal infections and immune system. *Infect Dis (Lond)* 2016; **48**: 161-166 [PMID: 26513601 DOI: 10.3109/23744235.2015.1100322]

34   **Chen YC**, Huang TS, Wang YC, Cheng CH, Lee CF, Wu TJ, Chou HS, Chan KM, Lee WC, Soong RS. Effect of Prophylactic Antifungal Protocols on the Prognosis of Liver Transplantation: A Propensity Score Matching and Multistate Model Approach. *Biomed Res Int* 2016; **2016**: 6212503 [PMID: 27747235 DOI: 10.1155/2016/6212503]

35   **Giannella M**, Bartoletti M, Morelli M, Cristini F, Tedeschi S, Campoli C, Tumietto F, Bertuzzo V, Ercolani G, Faenza S, Pinna AD, Lewis RE, Viale P. Antifungal prophylaxis in liver transplant recipients: one size does not fit all. *Transpl Infect Dis* 2016; **18**: 538-544 [PMID: 27237076 DOI: 10.1111/tid.12560]

36   **Lavezzo B**, Patrono D, Tandoi F, Martini S, Fop F, Ballerini V, Stratta C, Skurzak S, Lupo F, Strignano P, Donadio PP, Salizzoni M, Romagnoli R, De Rosa FG. A simplified regimen of targeted antifungal prophylaxis in liver transplant recipients: A single-center experience. *Transpl Infect Dis* 2018; **20**: e12859 [PMID: 29427394 DOI: 10.1111/tid.12859]

37   **Jorgenson MR**, Descourouez JL, Marka NA, Leverson GE, Smith JA, Andes DR, Fernandez LA, Foley DP. A targeted fungal prophylaxis protocol with static dosed fluconazole significantly reduces invasive fungal infection after liver transplantation. *Transpl Infect Dis* 2019; **21**: e13156 [PMID: 31390109 DOI: 10.1111/tid.13156]

38   **Kang WH**, Song GW, Lee SG, Suh KS, Lee KW, Yi NJ, Joh JW, Kwon CHD, Kim JM, Choi DL, Kim JD, Kim MS. A Multicenter, Randomized, Open-Label Study to Compare Micafungin with Fluconazole in the Prophylaxis of Invasive Fungal Infections in Living-Donor Liver Transplant Recipients. *J Gastrointest Surg* 2020; **24**: 832-840 [PMID: 31066013 DOI: 10.1007/s11605-019-04241-w]

Elastography as a predictor of liver cirrhosis complications after hepatitis C virus eradication in the era of direct-acting antivirals

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Abstract

Chronic inflammation due to hepatitis C virus (HCV) infection leads to liver fibrosis and rearrangement of liver tissue, which is responsible for the development of portal hypertension (PH) and hepatocellular carcinoma (HCC). The advent of direct-acting antiviral drugs has revolutionized the natural history of HCV infection, providing an overall eradication rate of over 90%. Despite a significant decrease after sustained virological response (SVR), the rate of HCC and liver-related complications is not completely eliminated in patients with advanced liver disease. Although the reasons are still unclear, cirrhosis itself has a residual risk for the development of HCC and other PH-related complications. Ultrasound elastography is a recently developed non-invasive technique for the assessment of liver fibrosis. Following the achievement of SVR, liver stiffness (LS) usually decreases, as a consequence of reduced inflammation and, possibly, fibrosis. Recent studies emphasized the application of LS assessment in the management of patients with SVR in order to define the risk for developing the complications of chronic liver disease (functional decompensation, gastrointestinal bleeding, HCC) and to optimize long-term prognostic outcomes in clinical practice.

Key Words: Direct-acting antiviral agents; Liver stiffness; Portal hypertension; Hepatocellular carcinoma

Core Tip: Direct-acting antiviral agents lead to hepatitis C virus eradication and to the regression of liver inflammation. However, they do not eliminate the risk of possible portal hypertension-related complications and hepatocellular carcinoma (HCC), increasing the necessity for post-sustained virological response surveillance and the development of non-invasive predictive models to detect the categories of patients requiring more intensive follow-up. Many studies reported a significant reduction in liver fibrosis markers after treatment with direct-acting antiviral drugs. Ultrasound elastography is gaining growing importance as a predictive element in the assessment of the risk of developing esophageal varices or gastrointestinal bleeding, liver functional decompensation and HCC.

INTRODUCTION

Hepatitis C virus (HCV) infection is one of the major causes of chronic liver disease and a significant cause of morbidity and mortality worldwide[1]. In 2015, it was estimated that over 70 million people were affected, most of whom were unaware of the infection[2]. Chronic inflammation due to HCV infection leads to liver fibrosis and rearrangement of liver tissue, which is responsible for the development of portal hypertension (PH) and other complications. Moreover, inflammation and microenvironmental changes are known risk factors for the occurrence of hepatocellular carcinoma (HCC)[3].

The advent of direct-acting antiviral drugs (DAAs) has revolutionized the natural history of HCV infection, providing an overall eradication rate of over 90% associated with a remarkable safety profile in all stages of chronic liver disease[1].

The achievement of sustained virological response (SVR) prevents the development of cirrhosis in the early stages of the disease and significantly reduces the risk of HCC and PH-related events, such as ascites, hepatic encephalopathy, hepatorenal syndrome, infections and gastrointestinal bleeding, in patients with advanced liver disease[4-6]. However, initial reports have warned of an increased risk of HCC in patients who achieved SVR after treatments with DAAs[7,8]. On the other hand, other studies have shown a protective effect on the development of HCC[9,10]. More recently, a meta-analysis analyzing 41 studies concluded that there is no evidence for increased occurrence or recurrence of HCC in patients treated with DAAs compared with interferon-based therapies[11].

Despite a significant decrease after SVR, the rate of HCC and liver-related complications is not completely eliminated in patients with advanced liver disease. Although the reasons are still unclear, cirrhosis itself has a residual risk for the development of HCC and other PH-related complications[12]. At present, there are no validated predictors to estimate the risk of HCC and PH-related events after HCV eradication.

Ultrasound elastography is a recently developed non-invasive technique for the assessment of liver fibrosis. Vibration controlled transient elastography (VCTE), is the oldest share-wave-based method and the reference standard in this field. The device is equipped with a one-dimensional probe, where a vibrator sends low frequency shear waves through the liver. Wave propagation, evaluated by an ultrasound receiver inside the probe, is directly related to liver tissue elasticity. Since its emergence, this technique has provided a fast point-of-care estimate of liver fibrosis in daily clinical practice, avoiding the complications of liver biopsy[13]. Indeed, several studies using histology as the reference standard defined accurate thresholds that are able to distinguish the different stages of liver fibrosis[14]. In the last few years, new ultrasound based elastographic techniques have been developed. They are embedded into conventional ultrasound devices, allowing visualization of the sampling area. The two main categories are the point shear wave elastography (pSWE) and bidimensional SWE (2D-SWE)[13]. All these devices are able to evaluate the elastic properties of the

liver during real-time B mode imaging. In particular, the ultrasound probe generates short-duration acoustic impulses in a small region of interest that causes soft tissue displacement and shear waves running in the perpendicular plane. Shear wave travelling speed can then be quantified and interpreted as a measurement for liver stiffness (LS)[13].

To date, LS measurement (LSM) is recommended by the European Association for the Study of Liver Disease (EASL) and the American Association for the Study of Liver Disease (AASLD) guidelines for the assessment of liver disease severity in patients with HCV infection eligible for DAAs[1,15]. Following the achievement of SVR, LS usually decreases, as a consequence of reduced inflammation and, possibly, fibrosis [16-19]. Recent studies evaluated the usefulness of LS assessment after HCV eradication and the prediction of HCC and other PH-related complications in patients with advanced liver disease.

In this review, we summarize the current evidence on the role of ultrasound elastography in the prediction of liver-related outcomes of patients with HCV infection treated with DAAs.

DIRECT-ACTING ANTIVIRAL AGENTS AND LIVER FIBROSIS

Despite DAAs being pharmacologically designed only for the eradication of HCV infection and since HCV is directly responsible for liver injury and consequent parenchymal fibrosis, the achievement of both SVR and anti-fibrotic effect results in advantages in terms of prevention of chronic liver disease complications (Table 1).

Different non-invasive methods traditionally used to assess liver fibrosis such as VCTE and the Fibrosis-4 (FIB-4) score (based on patient's age, transaminases levels and platelet count) and aspartate aminotransferase to platelet ratio index (APRI score) have been evaluated for staging chronic liver disease and predicting hepatic fibrosis in patients with HCV infection.

It has been demonstrated that baseline LSM by VCTE together with FIB-4 and APRI score have an important role in the prediction of treatment outcome in the new era of DAAs and could be integrated in pre-treatment assessment as a guide for treatment decisions and optimization of patient management[20,21].

Many authors have documented the improvement of VCTE, FIB-4 and APRI score after DAAs treatment. However, it is not clear if this finding is a true recovery of liver fibrosis or represents only an epiphenomenon of the reduction in liver inflammation resulting in the normalization of blood tests and decrease of LS values[22-25]. The retrospective study by Elsharkawy *et al*[26] analyzed a group of 337 Egyptian patients with chronic genotype 4 HCV infection who underwent sofosbuvir-based treatments. Among the patients evaluated, 29.1% had non-relevant fibrosis (F0-1; VCTE < 7.1 kPa), 17.2% were included in the F2 group (7.1 kPa ≤ VCTE < 9.5 kPa), 8.6% in the F3 group (VCTE ≥ 9.5 kPa) and 45.1% were classified as cirrhotic (F4; ≥ 12.5 kPa). One year after treatment, 77% of responders (with any stage fibrosis) and 81.8% of cirrhotic patients had a valuable recovery in liver fibrosis parameters (measured with FIB-4 and APRI score), due to the increase in platelet count and decrease in transaminase levels together with a reduction in LS values (11.8 ± 8.8 kPa *vs* 14.8 ± 10.7 kPa, *P* = 0.000). A higher number of patients with poor LS improvement after DAAs-therapy was observed in cases with low baseline LS values and infection relapse.

In a group of 42 patients treated with DAAs, Chekuri *et al*[27] demonstrated a significant decrease in LS values at SVR 24 wk after the end of treatment (median values: 10.40 kPa *vs* 7.60 kPa, *P* < 0.01), without significant improvement in the follow-up.

Abdel Alem *et al*[28] used pre-treatment liver fibrosis (measured by VCTE and FIB-4 score) as a predictor of treatment outcome after sofosbuvir-based regimens in 7256 HCV patients (46.6% cirrhotic, 91.4% with SVR12). Both, baseline FIB-4 and VCTE were significantly lower in the group with SVR (2.66 ± 1.98 kPa and 17.8 ± 11.5 kPa, respectively) compared to relapsers (4.02 ± 3.3 kPa and 24.5 ± 13.9 kPa, respectively). Based on these results, the authors concluded that fibrosis stage is a crucial element in the evaluation of treatment outcome and disease prognosis. In particular, a LS value higher than 16.7 kPa resulted as an unfavorable prognostic factor for treatment response (relapse rate 13%), probably related to an impaired immune-mediated HCV clearance that is worsened in advanced liver fibrosis. Similar considerations were drawn by Neukam *et al*[29] in patients treated with pegylated interferon/ribavirin-based therapy associated with NS3/4A protease inhibitor (PR-PI) and patients under DAAs therapy. In the PR-PI group, SVR12 was obtained in 59.6% of patients with LS < 21 kPa and in 46.5% of subjects with LS ≥ 21 kPa (*P* = 0.064); in the DAAs group,

Table 1 Liver stiffness improvement after treatment with direct acting antivirals

Ref.	Study design	Number of Patients	Drugs	Patients with LS improvement (%)	Pre-treatment LS	Post-treatment LS	*P* value	Measurement
Elsharkawy *et al*[26], 2017	Retrospective	337	DAA	81.8% (cirrhotic) 71.7% (non-cirrhotic)	14.8 ± 10.7 kPa	11.8 ± 8.8 kPa	0.000	Fibroscan
Chekuri *et al* [27], 2016	Observational	100	IFN-based and DAA	NA	10.40 kPa	7.60 kPa	< 0.01	Fibroscan
Bachofner *et al* [30], 2017	Multicenter, observational	392	DAA	93%	12.65 kPa	8.55 kPa	< 0.001	Fibroscan
Afdhal *et al* [39], 2017	Prospective	52	DAA	59.6%	15.2 kPa	9.3 kPa (6.7–16.8 kPa)	< 0.0001	Fibroscan
Ravaioli *et al* [68], 2018	Retrospective	139	DAA	44.6% (LS reduction > 30%)	18.6 kPa (15-26.3 kPa)	13.8 kPa (10.4-20.4 kPa)	< 0.001	Fibroscan
Pan *et al*[70], 2018	Retrospective	84	DAA	62%	Fibrosis regression by at least two stages: Cirrhosis group (48%); F3 fibrosis group (39%)	-	Fibroscan

DAA: Direct acting antivirals; IFN: Interferon; LS: Liver stiffness; NA: Not applicable.

SVR12 was reached by 95.3% of patients with LS < 21 kPa and 87.4% of patients with ≥ 21 kPa. Relapse rates after an apparent end-of-treatment response were 4.8% *vs* 17.9% in patients treated with PR-PI and 2.4% *vs* 8.2% in the DAAs group, respectively, for LS < 21 kPa and ≥ 21 kPa. These results suggest that LS evaluation might be useful to avoid HCV-relapse in cirrhotic patients by choosing both the appropriate composition and duration of DAAs-therapy.

Many studies reported a significant reduction in liver fibrosis markers after treatment with DAAs. In particular, Bachofner *et al*[30] highlighted a 32.4% drop in VCTE values from 12.65 kPa to 8.55 kPa (*P* < 0.001), a reduction of FIB-4 from 2.54 to 1.80 (*P* < 0.001) and a decrease of APRI from 1.10 to 0.43 (*P* < 0.001).

DIRECT-ACTING ANTIVIRAL AGENTS AND LIVER CIRRHOSIS RELATED EVENTS

Even though DAA-therapy leads to HCV eradication and to the regression of liver inflammation, it does not eliminate the risk of possible PH-related complications and HCC, increasing the necessity for post-SVR surveillance and the development of non-invasive predictive models to detect the categories of patients requiring more intensive follow-up (Table 2).

To this purpose, Trivedi *et al*[31] suggested a VCTE-based algorithm in order to schedule the controls of patients with SVR after HCV eradication: In the case of mild fibrosis (F1) without liver-related comorbidities, regular monitoring with the primary care physician is indicated; for advanced fibrosis/cirrhosis (F3-4), routine HCC and variceal surveillance is prescribed (six-monthly ultrasound, upper endoscopy every 2-3 years, annual non-invasive fibrosis assessment); for moderate fibrosis (F2) or in the case of concomitant liver-related comorbidities an annual non-invasive fibrosis measurement should be performed.

The importance of liver fibrosis stage in the development of liver-related complications was confirmed by Kozbial *et al*[32], who analyzed 551 patients treated with DAAs for a median period of 65.6 wk: No complications were registered in patients with severe fibrosis, whereas 9.1% of subjects with compensated cirrhosis developed liver-associated complications including HCC (4.1%). Furthermore, the presence of decompensated cirrhosis was markedly associated with the development of complications and mortality.

Even though histology remains the gold standard in evaluating fibrosis, liver biopsy presents some potential obstacles such as patient compliance, severe post-procedural complications, and sampling errors. For this reason, elastography has been proposed as a possible non-invasive alternative to biopsy for patient surveillance after SVR[33-35].

Table 2 Direct-acting antiviral agents and liver cirrhosis related events

Ref.	Study design	Number of patients	Drugs	HCC	Portal hypertension-related complications
Kozbial *et al* [32], 2018	Prospective	551	DAA	16 (4.1%)	Ascites: 3.1%; variceal hemorrhage: 1%; hepatic encephalopathy: 0%
Masuzaki *et al* [36], 2009	Prospective	984	DAA	77 (2.9% *per* 1 person-year); HCC risk: 45.5 times higher in LS > 25 kPa	NA
Afdhal *et al* [39], 2017	Prospective	50	DAA	LS improvement in patients who did not develop HCC during follow-up (42.6% reduction in patients without HCC *vs* 13.6% in HCC group)	24% patients had ≥ 20% decreases in HVPG during treatment (89% subjects with baseline HVPG ≥ 12 mmHg had a ≥ 20% reduction in HVPG after SVR)
Giannini *et al* [51], 2019	Prospective	52	DAA	4 (7.7%)	Clinical decompensation: 0%
Tachi *et al*[58], 2017	Prospective	263	DAA	19 (7.2%)	NA
Foster *et al* [60], 2016	Retrospective, observational	467	DAA	NA	MELD improvement (0.85, SD 2.54); composite adverse outcome in 52.0% (treated) *vs* 61.7% (untreated)
Rinaldi *et al* [63], 2019	Multicenter, prospective	258	DAA	35 (13.6%)	NA
Ravaioli *et al* [68], 2018	Retrospective	139	DAA	20 (14.4%)	NA
Pan *et al*[70], 2018	Retrospective	84	DAA	4 (4.8%)	NA
Toyoda *et al* [75], 2015	Retrospective/prospective	522	IFN-based	18 (1.2% after five yr; 4.3% after ten yr)	NA
D'Ambrosio *et al*[77], 2018	Prospective	38	DAA	5 (13%)	Clinical decompensation: 0%
Lleo *et al*[78], 2019	Prospective	1927	DAA	Previous HCC: 38/161 (recurrence rate: 24.8 *per* 100-yr); No previous HCC: 50/1766 (incidence rate: 2.4 *per* 100-yr)	NA
Hamada *et al* [79], 2018	Retrospective	196	DAA	8 (4.1%)	NA

DAA: Direct acting antivirals; HCC: Hepatocellular carcinoma; HVPG: Hepatic venous pressure gradient; IFN: Interferon; LS: Liver stiffness; MELD: Model for end-stage liver disease; NA: Not applicable; SD: Standard deviation; SVR: Sustained virological response.

VCTE is gaining growing importance as a predictive element in the assessment of the risk of developing esophageal varices or gastrointestinal bleeding, liver functional decompensation and HCC[36]. The retrospective study by Mandorfer *et al*[37] was the first to compare Hepatic Venous Pressure Gradient (HVPG) measurement with VCTE for the assessment of PH and showed a good agreement between the techniques. The authors also observed that a PH decrease after SVR was less likely in subjects with baseline HVPG higher than 16 mmHg and severe liver function impairment.

The review by Garbuzenko *et al*[38] confirmed that staging the severity of PH in cirrhotic subjects and personalized preventive therapy could lead to an increase in both patient survival and treatment effectiveness; particularly, DAAs achieve the amelioration of subclinical PH. In a recent study by Afdhal *et al*[39] of 50 patients with clinically significant PH (presence of esophageal varices, HVPG > 6 mmHg) from different international centers, 89% obtained a HVPG reduction of > 20% and only 3 patients obtained a reduction of portal pressure to less than 12 mmHg.

Paternostro *et al*[40] endorsed spleen stiffness measurement (SSM) through elastography (especially pSWE and 2D-SWE) as an effective tool for high-risk varices assessment in chronic liver disease, especially in distinguishing between small and large varices as confirmed by Sharma *et al*[41]. Previously, both Colecchia *et al*[42] and Fraquelli *et al*[43] had underlined the efficacy of LSM and SSM association in the assessment of HVPG and prediction of gastroesophageal varices in cirrhotic patients, showing a very high sensitivity (98% and 100% in the two studies, respectively), and economic advantages following the implementation of endoscopic screening progr-

ams. However, there are some important limitations related to SSM: It is an operator-dependent measurement and the upper limit of VCTE is fixed to a fibrosis value of 75 kPa that, in the case of severe PH, could be widely exceeded by SSM unlike LSM. Concerning the latter issue, Calvaruso *et al*[44] demonstrated the superior predictive value of SSM for high-risk varices, adopting a modified VCTE unit with a maximum stiffness value of 150 kPa (AUC: 0.80 for SSM *vs* 0.71 for LSM).

It has been demonstrated that the association of LSM with other non-invasive items (*e.g.* platelets, SSM) has a powerful positive predictive value in the detection of esophageal varices: Stefanescu *et al*[45] created a simple diagnostic algorithm with the combination of LSM and SSM (cut-off: 19 kPa and 55 kPa, respectively), thus reaching a 93% sensibility and a 95% positive predictive value.

Wang *et al*[46] observed that the combination of Baveno VI criteria with SSM (with 46 kPa cut-off) might help to avoid 61.6% of esophagogastroduodenoscopies in HBV-related cirrhosis with persistent viral suppression due to antiviral therapy, missing less than 5% high-risk varices.

An interesting analysis by Fofiu *et al*[47] evaluated a score based on the combination of LSM, SSM and spleen size as non-invasive predictors of high-risk varices in compensated cirrhosis, proving a better performance of the association of the three elements compared to each parameter alone. However, a meta-analysis by Ma *et al*[48] found that SSM alone is superior to LSM in predicting any grade esophageal varices, thus turning out to be useful in clinical practice, especially in the case of non-measurable LSM (multifocal HCC, biliary obstruction or liver metastasis).

Semmler *et al*[49] underlined the predictive value of LSM by VCTE included in a non-invasive algorithm together with von Willebrand factor-platelet count ratio as a useful method to define PH, stratify risk categories and predict liver decompensation and HCC development in patients with HCV-related advanced chronic liver disease treated with DAAs. These results could be very interesting in introducing the concept of a tailored follow-up strategy.

It is still not clear if the improvement in non-invasive markers after SVR could be associated to a decline in PH itself. However, in a recent study, Thabut *et al*[50] noted that subjects with previous unfavorable Baveno VI status (LS > 20 kPa, platelets < 150000/mm^3) who experienced platelets increase and/or LS reduction after SVR reached a favorable Baveno VI class, with a subsequent reduction in the probability of PH progression and development of esophageal varices. A decrease of PH has also been demonstrated by Giannini *et al*[51] in a group of 52 patients with advanced fibrosis/cirrhosis at baseline followed for approximately 60 wk after SVR with DAAs. A significant improvement in HVPG was detected, together with a decrease in LS values (from 15.2 kPa at baseline to 9.3 kPa at the end of follow-up), APRI and FIB-4 score, spleen bipolar diameter and an increase in platelet count[37].

As the role of these indices is quite limited, other non-invasive methods have been proposed to detect varices at high risk of bleeding: Considering the worldwide low availability of TE, Jangouk *et al*[52] demonstrated the effectiveness of Baveno VI consensus criteria as a non-invasive method to identify patients with compensated liver cirrhosis and low-risk of varices requiring endoscopic treatment. In particular, the authors highlight the uppermost role of both platelet count (> 150000/mm^3) and MELD score (< 6) in defining a low probability of high-risk varices.

Chen *et al*[53] demonstrated the efficacy and extremely high negative predictive value (97.1% in the study group and 98.1% in the validation cohort) of the association of albumin-bilirubin grade with platelet count (ALBI-PLT score) in the screening of high-risk esophageal varices in subjects with HCC: The 5-year variceal hemorrhage rate was 9.7% in patients with ALBI-PLT score > 2 (decompensated liver disease) as compared to 1.7% in those with a score of 2 (*P* = 0.007).

Baveno VI guidelines indicate platelet count and VCTE as effective elements in the identification of cirrhotic patients who are at high-risk of developing esophageal varices: Due to the not-always easy access to VCTE (for example, in the case of inmates) or to the unavailability of adequate instrumentation in all hepatological centers, Calvaruso *et al*[54] proposed the "Rete Sicilia Selezione Terapia–HCV" algorithm as an effective and simple tool (based only on blood tests: Platelet count and serum albumin level) that could substitute Baveno VI criteria in the identification of HCV-cirrhotic patients with medium/large varices, thus simplifying the diagnosis of the complications of PH, with a reduction of more than 30% of useless endoscopic exams and diminishing the risk of false-negative results.

The implications of HCV eradication on HCC development are even more complex. Despite the widely demonstrated efficacy of DAAs in both achieving SVR and a reduction in liver fibrosis, there is no corresponding decrease in HCC development risk. These data led to an initial alert claiming the possibility of a DAAs-driven

oncogenic mechanism[7], even if this theory was subsequently proved wrong by other studies[11]. The mechanism of HCC development post SVR is probably sustained by a "point of no-return" in HCV pathogenesis that determines the loss of the potential benefits brought by viral eradication[55]. This evidence highlights the necessity for optimizing regular HCC surveillance with a particular focus on patients with advanced fibrosis or cirrhosis[56]. In fact, even though a decrease in LS values from cirrhosis to advanced fibrosis was observed in some cases after DAAs therapy, patients with SVR maintained an elevated HCC risk[57,58].

Whether the HCC risk of patients with SVR coincides with that of viremic subjects is still a matter of debate. In the case of precariously compensated or decompensated liver function, the achievement of SVR could be useful to reduce the risk of HCC because of the decrease in intrahepatic inflammatory processes, despite the persistence of PH and decompensated liver function (that increase the risk of liver cancer in cirrhotic patients)[59,60].

Both EASL and AASLD guidelines recommend continuing ultrasound surveillance in subjects with advanced fibrosis/cirrhosis despite histological response to treatment and suggest accurate definition of the additional baseline risk-factors profile[61,62].

Rinaldi *et al*[63] assessed the importance of both baseline LS evaluation and ultrasound liver surveillance for the risk of HCC in patients with HCV-related cirrhosis, treated with DAAs: Among 258 subjects enrolled, divided into three groups according to liver fibrosis stage (< 20 kPa, from 20 kPa to 30 kPa, > 30 kPa), 35 developed HCC during follow-up. The group with LS higher than 30 kPa had a statistically significant increase in HCC risk [HR (95%CI): 0.329 (0.131-0.830); P = 0.019].

Even though the mechanisms directly involving HCV in both fibrogenesis and oncogenesis have not yet been completely explained, it seems crucial to define the degree of liver fibrosis through VCTE and FIB-4, in order to set appropriate HCC screening and the subsequent therapeutic strategy[64,65].

Many attempts have been made to create prognostic scores to evaluate the risk of HCC development in chronic liver diseases, considering other criteria than PH alone [66]. An interesting example is represented by the King score that includes laboratory parameters (platelet count and bilirubin levels) and gene signature, and classifies cirrhotic patients with HCV infection into three risk categories for functional decompensation, HCC and death. However, it is not clear if this score maintains its predictive efficacy in patients with SVR[67].

Ravaioli *et al*[68] studied 139 cirrhotic patients treated with DAAs, analyzing the difference between LS at baseline and at the end of treatment: They found a lower reduction of LS in patients who developed HCC compared to patients who did not (-18.0% *vs* -28.9%, P = 0.005).

Recent studies demonstrated that LS assessment after SVR could be an inaccurate method to define the grade of fibrosis in patients treated with DAAs. In fact, the fast modifications in LS could be determined by both the reduction of liver inflammatory activity and the narrowing of fibrotic septa, without real histological improvement in fibrosis grading as demonstrated by liver biopsy[69-71]. Notwithstanding, LS evaluation by VCTE remains a cornerstone in the assessment of HCC risk after SVR, especially due to its non-invasiveness.

Masuzaki *et al*[36] demonstrated that HCC risk was 45.5 times higher in patients with LS values higher than 25 kPa.

However, it becomes important in the association to other elements in a more complete non-invasive score. Among them, we can include: Age, alcohol abuse, pre-treatment advanced fibrosis/cirrhosis, platelet count, steatosis, diabetes, alfa fetoprotein (AFP), baseline gamma-glutamyltransferase (GGT) levels together with ethnic and environmental factors. All these factors have been studied in patients treated with interferon-based therapies with interesting results[72-76]. During the pre-DAAs era, studies on the complications of liver cirrhosis after HCV-treatment showed that SVR and fibrosis regression did not prevent hepatic carcinogenesis. D'Ambrosio *et al*[77] found that 13% of patients who responded to interferon-based treatments, developed HCC during an 8-year follow-up (17% cumulative probability and 1.2% annual incidence rate) whereas neither variceal-bleeding nor liver-function decompensation occurred. Higher baseline levels of GGT and glycemia were identified as risk factors for HCC development. Similarly, Toyoda *et al*[75] demonstrated that diabetes mellitus and FIB-4 index increase represent risk factors for HCC after SVR with interferon-based regimens, thus suggesting continuing active surveillance in these groups of patients.

In a prospective analysis of 1927 patients with HCV-related cirrhosis, receiving DAAs in ten tertiary Italian liver centers, Lleo *et al*[78] observed a recurrence rate of HCC of 24.8 *per* 100 patients/year and a *de novo* occurrence rate of 2.4 *per* 100

patients/year. They found that treatment failure and high AFP levels represent independent predictors of HCC development, while SVR and absence of PH are associated with a lower HCC incidence, suggesting that HCC risk stratification should rely on the presence of PH and elevated baseline AFP levels.

It has been suggested that PH as a complication of liver fibrosis (more than fibrosis itself) may represent an independent risk factor for HCC[66]. Afdhal *et al*[39] analyzed 50 patients with HCV-related liver cirrhosis treated with DAAs and observed a significant reduction in HVPG values during long-term follow-up after SVR: 24% of all patients and 89% of subjects with baseline HVPG ≥ 12 mmHg who reached SVR had a ≥ 20% reduction in HVPG. With regard to LS, a more evident improvement was observed in patients who did not develop HCC during follow-up (42.6% reduction in patients without HCC *vs* 13.6% in the HCC group), thus proposing a protective role of HVPG and LS against HCC development.

In a recent retrospective study performed in patients with SVR after DAAs, Hamada *et al*[79], identified six variables that could be included in the HCC prediction model: Age, body mass index, platelet count, albumin, AFP, LS and FIB-4 index. Following multivariate analysis they found that age ≥ 75 years, AFP ≥ 6 ng/mL, and LS ≥ 11 kPa were independent risk factors for hepatocarcinogenesis (risk ratio: 35.16, 43.30 and 28.71, respectively; *P* = 0.001, 0.003 and 0.006, respectively). In particular, patients with LS < 11 kPa had a cumulative HCC incidence of 1.3% at 12 mo, 24 mo, 36 mo and 48 mo, while in the group with LS > 11 kPa the HCC incidence rate was 4.6% at 12 mo and 24 mo, 24.8% at 36 mo and 62.4% at 48 mo.

The role of LSM in the development of a prediction model for HCC has also been emphasized by Feier *et al*[80]. They confirmed that high levels of AFP, transaminases and LS are excellent predictors of HCC but underlined the importance of interquartile range (IQR) in LSMs. This led to the hypothesis of "stiffness shadow" that indicated an inhomogeneous shear stress due to the chaotic tumoral growth in the already hard cirrhotic tissue, with relevant diagnostic repercussions[81,82]. The overall prognostic model combining the four variables demonstrated relevant results both in the training and validation phase with a positive relation with tumor size. The four parameters together showed a 64.5% HCC prediction, with LS alone reaching the highest predictive power. The authors concluded that an elevation in LS values and IQR during follow-up could enhance the diagnostic skill towards early HCC[80].

It is interesting to note that some genetic factors also seem to be involved in hepato-carcinogenesis, despite the lack of clear evidence and the need for further prospective studies.

In their cohort of 200 patients with HCV-related cirrhosis with SVR after DAAs, Simili *et al*[83] noted a strong association of the single-nucleotide polymorphism of interleukin 28 (IL28B–rs12979860) with HCC development (both *de novo* and disease recurrence); furthermore, they observed a relation of HCC with lower levels of serum retinol and the presence of another two polymorphisms: Major histocompatibility complex class I polypeptide-related sequence A gene (*MICA*) and tolloid-like 1. The latter has proven particularly controversial since its oncogenic role was stated by Matsuura *et al*[84] but denied by Degasperi *et al*[85]: The difference between these studies could be ascribed to the different allele frequency or the presence of still unknown cofactors in the two ethnic groups (Japanese and Caucasian) or to discrepancies in the length of the follow-up period.

CONCLUSION

DAAs-therapy has brought about an effective revolution in hepatology resulting in HCV eradication in a wide range of patients and eventually reducing liver fibrosis after SVR. However, these benefits have not erased the risk of developing liver disease-related complications and in particular HCC and PH associated events. For this reason, it is crucial to continue long-term systematic surveillance after HCV eradication focusing on the subjects with a high-risk score.

Due to its accuracy, cost-effectiveness and non-invasiveness, together with specific clinical and laboratory parameters, LSM is gaining a relevant role in the construction of algorithms assessing both liver fibrosis and PH. The potential application of this non-invasive and simple method has been emphasized especially in the management of patients with SVR in order to define the risk to develop the complications of chronic liver disease (functional decompensation, gastrointestinal bleeding, HCC) and optimize long-term prognostic outcomes in clinical practice.

REFERENCES

1   **European Association for the Study of the Liver**; Clinical Practice Guidelines Panel: Chair:; EASL Governing Board representative:; Panel members:. EASL recommendations on treatment of hepatitis C: Final update of the series☆. *J Hepatol* 2020; **73**: 1170-1218 [PMID: 32956768 DOI: 10.1016/j.jhep.2020.08.018]

2   **Polaris Observatory HCV Collaborators**. Global prevalence and genotype distribution of hepatitis C virus infection in 2015: a modelling study. *Lancet Gastroenterol Hepatol* 2017; **2**: 161-176 [PMID: 28404132 DOI: 10.1016/S2468-1253(16)30181-9]

3   **Westbrook RH**, Dusheiko G. Natural history of hepatitis C. *J Hepatol* 2014; **61**: S58-S68 [PMID: 25443346 DOI: 10.1016/j.jhep.2014.07.012]

4   **Bruno S**, Di Marco V, Iavarone M, Roffi L, Crosignani A, Calvaruso V, Aghemo A, Cabibbo G, Viganò M, Boccaccio V, Craxí A, Colombo M, Maisonneuve P. Survival of patients with HCV cirrhosis and sustained virologic response is similar to the general population. *J Hepatol* 2016; **64**: 1217-1223 [PMID: 27059129 DOI: 10.1016/j.jhep.2016.01.034]

5   **van der Meer AJ**, Feld JJ, Hofer H, Almasio PL, Calvaruso V, Fernández-Rodríguez CM, Aleman S, Ganne-Carrié N, D'Ambrosio R, Pol S, Trapero-Marugan M, Maan R, Moreno-Otero R, Mallet V, Hultcrantz R, Weiland O, Rutter K, Di Marco V, Alonso S, Bruno S, Colombo M, de Knegt RJ, Veldt BJ, Hansen BE, Janssen HLA. Risk of cirrhosis-related complications in patients with advanced fibrosis following hepatitis C virus eradication. *J Hepatol* 2017; **66**: 485-493 [PMID: 27780714 DOI: 10.1016/j.jhep.2016.10.017]

6   **Nahon P**, Bourcier V, Layese R, Audureau E, Cagnot C, Marcellin P, Guyader D, Fontaine H, Larrey D, De Lédinghen V, Ouzan D, Zoulim F, Roulot D, Tran A, Bronowicki JP, Zarski JP, Leroy V, Riachi G, Calès P, Péron JM, Alric L, Bourlière M, Mathurin P, Dharancy S, Blanc JF, Abergel A, Serfaty L, Mallat A, Grangé JD, Attali P, Bacq Y, Wartelle C, Dao T, Benhamou Y, Pilette C, Silvain C, Christidis C, Capron D, Bernard-Chabert B, Zucman D, Di Martino V, Thibaut V, Salmon D, Ziol M, Sutton A, Pol S, Roudot-Thoraval F; ANRS CO12 CirVir Group. Eradication of Hepatitis C Virus Infection in Patients With Cirrhosis Reduces Risk of Liver and Non-Liver Complications. *Gastroenterology* 2017; **152**: 142-156.e2 [PMID: 27641509 DOI: 10.1053/j.gastro.2016.09.009]

7   **Reig M**, Mariño Z, Perelló C, Iñarrairaegui M, Ribeiro A, Lens S, Díaz A, Vilana R, Darnell A, Varela M, Sangro B, Calleja JL, Forns X, Bruix J. Unexpected high rate of early tumor recurrence in patients with HCV-related HCC undergoing interferon-free therapy. *J Hepatol* 2016; **65**: 719-726 [PMID: 27084592 DOI: 10.1016/j.jhep.2016.04.008]

8   **Kozbial K**, Moser S, Schwarzer R, Laferl H, Al-Zoairy R, Stauber R, Stättermayer AF, Beinhardt S, Graziadei I, Freissmuth C, Maieron A, Gschwantler M, Strasser M, Peck-Radosalvjevic M, Trauner M, Hofer H, Ferenci P. Unexpected high incidence of hepatocellular carcinoma in cirrhotic patients with sustained virologic response following interferon-free direct-acting antiviral treatment. *J Hepatol* 2016; **65**: 856-858 [PMID: 27318327 DOI: 10.1016/j.jhep.2016.06.009]

9   **ANRS collaborative study group on hepatocellular carcinoma (ANRS CO22 HEPATHER CO12 CirVir and CO23 CUPILT cohorts)**. Lack of evidence of an effect of direct-acting antivirals on the recurrence of hepatocellular carcinoma: Data from three ANRS cohorts. *J Hepatol* 2016; **65**: 734-740 [PMID: 27288051 DOI: 10.1016/j.jhep.2016.05.045]

10 **Romano A**, Angeli P, Piovesan S, Noventa F, Anastassopoulos G, Chemello L, Cavalletto L, Gambato M, Russo FP, Burra P, Vincenzi V, Scotton PG, Panese S, Tempesta D, Bertin T, Carrara M, Carlotto A, Capra F, Carolo G, Scroccaro G, Alberti A. Newly diagnosed hepatocellular carcinoma in patients with advanced hepatitis C treated with DAAs: A prospective population study. *J Hepatol* 2018; **69**: 345-352 [PMID: 29551707 DOI: 10.1016/j.jhep.2018.03.009]

11 **Waziry R**, Hajarizadeh B, Grebely J, Amin J, Law M, Danta M, George J, Dore GJ. Hepatocellular carcinoma risk following direct-acting antiviral HCV therapy: A systematic review, meta-analyses, and meta-regression. *J Hepatol* 2017; **67**: 1204-1212 [PMID: 28802876 DOI: 10.1016/j.jhep.2017.07.025]

12 **Mallet V**, Gilgenkrantz H, Serpaggi J, Verkarre V, Vallet-Pichard A, Fontaine H, Pol S. Brief communication: the relationship of regression of cirrhosis to outcome in chronic hepatitis C. *Ann Intern Med* 2008; **149**: 399-403 [PMID: 18794559 DOI: 10.7326/0003-4819-149-6-200809160-00006]

13 **Dietrich CF**, Bamber J, Berzigotti A, Bota S, Cantisani V, Castera L, Cosgrove D, Ferraioli G, Friedrich-Rust M, Gilja OH, Goertz RS, Karlas T, de Knegt R, de Ledinghen V, Piscaglia F, Procopet B, Saftoiu A, Sidhu PS, Sporea I, Thiele M. EFSUMB Guidelines and Recommendations on the Clinical Use of Liver Ultrasound Elastography, Update 2017 (Long Version). *Ultraschall Med* 2017; **38**: e48 [PMID: 30176678 DOI: 10.1055/a-0641-0076]

14 **Friedrich-Rust M**, Ong MF, Martens S, Sarrazin C, Bojunga J, Zeuzem S, Herrmann E. Performance of transient elastography for the staging of liver fibrosis: a meta-analysis. *Gastroenterology* 2008; **134**: 960-974 [PMID: 18395077 DOI: 10.1053/j.gastro.2008.01.034]

15 **Ghany MG**, Morgan TR; AASLD-IDSA Hepatitis C Guidance Panel. Hepatitis C Guidance 2019 Update: American Association for the Study of Liver Diseases-Infectious Diseases Society of America Recommendations for Testing, Managing, and Treating Hepatitis C Virus Infection. *Hepatology* 2020; **71**: 686-721 [PMID: 31816111 DOI: 10.1002/hep.31060]

16 **Fernandes FF**, Piedade J, Guimaraes L, Nunes EP, Chaves U, Goldenzon RV, Cardoso SW, Duarte J, Grinsztejn B, Veloso VG, Pereira G, Perazzo H. Effectiveness of direct-acting agents for hepatitis C

and liver stiffness changing after sustained virological response. *J Gastroenterol Hepatol* 2019; **34**: 2187-2195 [PMID: 31062880 DOI: 10.1111/jgh.14707]

17   **Pons M**, Rodríguez-Tajes S, Esteban JI, Mariño Z, Vargas V, Lens S, Buti M, Augustin S, Forns X, Mínguez B, Genescà J. Non-invasive prediction of liver-related events in patients with HCV-associated compensated advanced chronic liver disease after oral antivirals. *J Hepatol* 2020; **72**: 472-480 [PMID: 31629779 DOI: 10.1016/j.jhep.2019.10.005]

18   **Alonso López S**, Manzano ML, Gea F, Gutiérrez ML, Ahumada AM, Devesa MJ, Olveira A, Polo BA, Márquez L, Fernández I, Cobo JCR, Rayón L, Riado D, Izquierdo S, Usón C, Real Y, Rincón D, Fernández-Rodríguez CM, Bañares R. A Model Based on Noninvasive Markers Predicts Very Low Hepatocellular Carcinoma Risk After Viral Response in Hepatitis C Virus-Advanced Fibrosis. *Hepatology* 2020; **72**: 1924-1934 [PMID: 33022803 DOI: 10.1002/hep.31588]

19   **Lens S**, Baiges A, Alvarado-Tapias E, LLop E, Martinez J, Fortea JI, Ibáñez-Samaniego L, Mariño Z, Rodríguez-Tajes S, Gallego A, Bañares R, Puente Á, Albillos A, Calleja JL, Torras X, Hernández-Gea V, Bosch J, Villanueva C, García-Pagán JC, Forns X. Clinical outcome and hemodynamic changes following HCV eradication with oral antiviral therapy in patients with clinically significant portal hypertension. *J Hepatol* 2020; **73**: 1415-1424 [PMID: 32535060 DOI: 10.1016/j.jhep.2020.05.050]

20   **Yosry A**, Fouad R, Alem SA, Elsharkawy A, El-Sayed M, Asem N, Hassan E, Ismail A, Esmat G. FibroScan, APRI, FIB4, and GUCI: Role in prediction of fibrosis and response to therapy in Egyptian patients with HCV infection. *Arab J Gastroenterol* 2016; **17**: 78-83 [PMID: 27353055 DOI: 10.1016/j.ajg.2016.05.002]

21   **Bonnard P**, Elsharkawy A, Zalata K, Delarocque-Astagneau E, Biard L, Le Fouler L, Hassan AB, Abdel-Hamid M, El-Daly M, Gamal ME, El Kassas M, Bedossa P, Carrat F, Fontanet A, Esmat G. Comparison of liver biopsy and noninvasive techniques for liver fibrosis assessment in patients infected with HCV-genotype 4 in Egypt. *J Viral Hepat* 2015; **22**: 245-253 [PMID: 25073725 DOI: 10.1111/jvh.12285]

22   **Ogasawara N**, Kobayashi M, Akuta N, Kominami Y, Fujiyama S, Kawamura Y, Sezaki H, Hosaka T, Suzuki F, Saitoh S, Suzuki Y, Arase Y, Ikeda K, Kumada H. Serial changes in liver stiffness and controlled attenuation parameter following direct-acting antiviral therapy against hepatitis C virus genotype 1b. *J Med Virol* 2018; **90**: 313-319 [PMID: 28906010 DOI: 10.1002/jmv.24950]

23   **Tada T**, Kumada T, Toyoda H, Mizuno K, Sone Y, Kataoka S, Hashinokuchi S. Improvement of liver stiffness in patients with hepatitis C virus infection who received direct-acting antiviral therapy and achieved sustained virological response. *J Gastroenterol Hepatol* 2017; **32**: 1982-1988 [PMID: 28299813 DOI: 10.1111/jgh.13788]

24   **Dolmazashvili E**, Abutidze A, Chkhartishvili N, Karchava M, Sharvadze L, Tsertsvadze T. Regression of liver fibrosis over a 24-week period after completing direct-acting antiviral therapy in patients with chronic hepatitis C receiving care within the national hepatitis C elimination program in Georgia: results of hepatology clinic HEPA experience. *Eur J Gastroenterol Hepatol* 2017; **29**: 1223-1230 [PMID: 28857900 DOI: 10.1097/MEG.0000000000000964]

25   **Tachi Y**, Hirai T, Kojima Y, Ishizu Y, Honda T, Kuzuya T, Hayashi K, Ishigami M, Goto H. Liver stiffness reduction correlates with histological characteristics of hepatitis C patients with sustained virological response. *Liver Int* 2018; **38**: 59-67 [PMID: 28557143 DOI: 10.1111/liv.13486]

26   **Elsharkawy A**, Alem SA, Fouad R, El Raziky M, El Akel W, Abdo M, Tantawi O, AbdAllah M, Bourliere M, Esmat G. Changes in liver stiffness measurements and fibrosis scores following sofosbuvir based treatment regimens without interferon. *J Gastroenterol Hepatol* 2017; **32**: 1624-1630 [PMID: 28177543 DOI: 10.1111/jgh.13758]

27   **Chekuri S**, Nickerson J, Bichoupan K, Sefcik R, Doobay K, Chang S, DelBello D, Harty A, Dieterich DT, Perumalswami PV, Branch AD. Liver Stiffness Decreases Rapidly in Response to Successful Hepatitis C Treatment and Then Plateaus. *PLoS One* 2016; **11**: e0159413 [PMID: 27442255 DOI: 10.1371/journal.pone.0159413]

28   **Abdel Alem S**, Elsharkawy A, El Akel W, Abdelaziz AO, Salama RM, El-Sayed MH, El Kassas M, Anees M, Shedeed M, Abdelsalam F, Ziada DH, El Shazly Y, El-Serafy M, Waked I, Esmat G, Doss W. Liver stiffness measurements and FIB-4 are predictors of response to sofosbuvir-based treatment regimens in 7256 chronic HCV patients. *Expert Rev Gastroenterol Hepatol* 2019; **13**: 1009-1016 [PMID: 31418303 DOI: 10.1080/17474124.2019.1653183]

29   **Neukam K**, Morano-Amado LE, Rivero-Juárez A, Macías J, Granados R, Romero-Palacios A, Márquez M, Merino D, Ortega E, Alados-Arboledas JC, Cucurull J, Omar M, Ryan-Murua P, Pineda JA; Grupo de Estudio de Hepatitis Vírica, of the Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica: GEHEP-SEIMC and Grupo de Estudio de Hepatitis Vírica, of the Sociedad Andaluza de Enfermedades Infecciosas y Microbiología Clínica: HEPAVIR/Red de Investigación en SIDA (RIS-HEP07). Liver stiffness predicts the response to direct-acting antiviral-based therapy against chronic hepatitis C in cirrhotic patients. *Eur J Clin Microbiol Infect Dis* 2017; **36**: 853-861 [PMID: 28004322 DOI: 10.1007/s10096-016-2871-x]

30   **Bachofner JA**, Valli PV, Kröger A, Bergamin I, Künzler P, Baserga A, Braun D, Seifert B, Moncsek A, Fehr J, Semela D, Magenta L, Müllhaupt B, Terziroli Beretta-Piccoli B, Mertens JC. Direct antiviral agent treatment of chronic hepatitis C results in rapid regression of transient elastography and fibrosis markers fibrosis-4 score and aspartate aminotransferase-platelet ratio index. *Liver Int* 2017; **37**: 369-376 [PMID: 27678216 DOI: 10.1111/liv.13256]

31   **Trivedi HD**, Lin SC, T Y Lau D. Noninvasive Assessment of Fibrosis Regression in Hepatitis C

Virus Sustained Virologic Responders. *Gastroenterol Hepatol (N Y)* 2017; **13**: 587-595 [PMID: 29391861]

32   **Kozbial K**, Moser S, Al-Zoairy R, Schwarzer R, Datz C, Stauber R, Laferl H, Strasser M, Beinhardt S, Stättermayer AF, Gschwantler M, Zoller H, Maieron A, Graziadei I, Trauner M, Steindl-Munda P, Hofer H, Ferenci P. Follow-up of sustained virological responders with hepatitis C and advanced liver disease after interferon/ribavirin-free treatment. *Liver Int* 2018; **38**: 1028-1035 [PMID: 29136329 DOI: 10.1111/liv.13629]

33   **Kobayashi M**, Suzuki F, Fujiyama S, Kawamura Y, Sezaki H, Hosaka T, Akuta N, Suzuki Y, Saitoh S, Arase Y, Ikeda K, Kumada H. Sustained virologic response by direct antiviral agents reduces the incidence of hepatocellular carcinoma in patients with HCV infection. *J Med Virol* 2017; **89**: 476-483 [PMID: 27531586 DOI: 10.1002/jmv.24663]

34   **Suda T**, Okawa O, Masaoka R, Gyotoku Y, Tokutomi N, Katayama Y, Tamano M. Shear wave elastography in hepatitis C patients before and after antiviral therapy. *World J Hepatol* 2017; **9**: 64-68 [PMID: 28105260 DOI: 10.4254/wjh.v9.i1.64]

35   **Wang JH**, Yen YH, Yao CC, Hung CH, Chen CH, Hu TH, Lee CM, Lu SN. Liver stiffness-based score in hepatoma risk assessment for chronic hepatitis C patients after successful antiviral therapy. *Liver Int* 2016; **36**: 1793-1799 [PMID: 27254286 DOI: 10.1111/liv.13179]

36   **Masuzaki R**, Tateishi R, Yoshida H, Goto E, Sato T, Ohki T, Imamura J, Goto T, Kanai F, Kato N, Ikeda H, Shiina S, Kawabe T, Omata M. Prospective risk assessment for hepatocellular carcinoma development in patients with chronic hepatitis C by transient elastography. *Hepatology* 2009; **49**: 1954-1961 [PMID: 19434742 DOI: 10.1002/hep.22870]

37   **Mandorfer M**, Kozbial K, Schwabl P, Freissmuth C, Schwarzer R, Stern R, Chromy D, Stättermayer AF, Reiberger T, Beinhardt S, Sieghart W, Trauner M, Hofer H, Ferlitsch A, Ferenci P, Peck-Radosavljevic M. Sustained virologic response to interferon-free therapies ameliorates HCV-induced portal hypertension. *J Hepatol* 2016; **65**: 692-699 [PMID: 27242316 DOI: 10.1016/j.jhep.2016.05.027]

38   **Garbuzenko DV**, Arefyev NO. Primary prevention of bleeding from esophageal varices in patients with liver cirrhosis: An update and review of the literature. *J Evid Based Med* 2020; **13**: 313-324 [PMID: 33037792 DOI: 10.1111/jebm.12407]

39   **Afdhal N**, Everson GT, Calleja JL, McCaughan GW, Bosch J, Brainard DM, McHutchison JG, De-Oertel S, An D, Charlton M, Reddy KR, Asselah T, Gane E, Curry MP, Forns X. Effect of viral suppression on hepatic venous pressure gradient in hepatitis C with cirrhosis and portal hypertension. *J Viral Hepat* 2017; **24**: 823-831 [PMID: 28295923 DOI: 10.1111/jvh.12706]

40   **Paternostro R**, Reiberger T, Bucsics T. Elastography-based screening for esophageal varices in patients with advanced chronic liver disease. *World J Gastroenterol* 2019; **25**: 308-329 [PMID: 30686900 DOI: 10.3748/wjg.v25.i3.308]

41   **Sharma P**, Kirnake V, Tyagi P, Bansal N, Singla V, Kumar A, Arora A. Spleen stiffness in patients with cirrhosis in predicting esophageal varices. *Am J Gastroenterol* 2013; **108**: 1101-1107 [PMID: 23629600 DOI: 10.1038/ajg.2013.119]

42   **Colecchia A**, Montrone L, Scaioli E, Bacchi-Reggiani ML, Colli A, Casazza G, Schiumerini R, Turco L, Di Biase AR, Mazzella G, Marzi L, Arena U, Pinzani M, Festi D. Measurement of spleen stiffness to evaluate portal hypertension and the presence of esophageal varices in patients with HCV-related cirrhosis. *Gastroenterology* 2012; **143**: 646-654 [PMID: 22643348 DOI: 10.1053/j.gastro.2012.05.035]

43   **Fraquelli M**, Rigamonti C, Colombo M. Spleen stiffness measured by transient elastography accurately predicts esophageal varices in liver cirrhosis. *Gastroenterology* 2012; **143**: e23; author reply e23-e23; author reply e24 [PMID: 22921672 DOI: 10.1053/j.gastro.2012.07.118]

44   **Calvaruso V**, Bronte F, Conte E, Simone F, Craxì A, Di Marco V. Modified spleen stiffness measurement by transient elastography is associated with presence of large oesophageal varices in patients with compensated hepatitis C virus cirrhosis. *J Viral Hepat* 2013; **20**: 867-874 [PMID: 24304456 DOI: 10.1111/jvh.12114]

45   **Stefanescu H**, Grigorescu M, Lupsor M, Procopet B, Maniu A, Badea R. Spleen stiffness measurement using Fibroscan for the noninvasive assessment of esophageal varices in liver cirrhosis patients. *J Gastroenterol Hepatol* 2011; **26**: 164-170 [PMID: 21175810 DOI: 10.1111/j.1440-1746.2010.06325.x]

46   **Wang H**, Wen B, Chang X, Wu Q, Wen W, Zhou F, Guo Y, Ji Y, Gu Y, Lai Q, He Q, Li J, Chen J, Hou J. Baveno VI criteria and spleen stiffness measurement rule out high-risk varices in virally suppressed HBV-related cirrhosis. *J Hepatol* 2021; **74**: 584-592 [PMID: 33039403 DOI: 10.1016/j.jhep.2020.09.034]

47   **Fofiu R**, Bende F, Popescu A, Şirli R, Lupuşoru R, Ghiuchici AM, Sporea I. Spleen and Liver Stiffness for Predicting High-Risk Varices in Patients with Compensated Liver Cirrhosis. *Ultrasound Med Biol* 2021; **47**: 76-83 [PMID: 33067019 DOI: 10.1016/j.ultrasmedbio.2020.09.004]

48   **Ma X**, Wang L, Wu H, Feng Y, Han X, Bu H, Zhu Q. Spleen Stiffness Is Superior to Liver Stiffness for Predicting Esophageal Varices in Chronic Liver Disease: A Meta-Analysis. *PLoS One* 2016; **11**: e0165786 [PMID: 27829057 DOI: 10.1371/journal.pone.0165786]

49   **Semmler G**, Binter T, Kozbial K, Schwabl P, Hametner-Schreil S, Zanetto A, Gavasso S, Chromy D, Bauer DJM, Simbrunner B, Scheiner B, Bucsics T, Stättermayer AF, Pinter M, Steindl-Munda P, Schöfl R, Russo FP, Simioni P, Trauner M, Ferenci P, Reiberger T, Mandorfer M. Noninvasive Risk Stratification After HCV Eradication in Patients With Advanced Chronic Liver Disease. *Hepatology*

2021; **73**: 1275-1289 [PMID: 32659847 DOI: 10.1002/hep.31462]

50   **Thabut D**, Bureau C, Layese R, Bourcier V, Hammouche M, Cagnot C, Marcellin P, Guyader D, Pol S, Larrey D, De Lédinghen V, Ouzan D, Zoulim F, Roulot D, Tran A, Bronowicki JP, Zarski JP, Goria O, Calès P, Péron JM, Alric L, Bourlière M, Mathurin P, Blanc JF, Abergel A, Serfaty L, Mallat A, Grangé JD, Attali P, Bacq Y, Wartelle-Bladou C, Dao T, Pilette C, Silvain C, Christidis C, Capron D, Bernard-Chabert B, Hillaire S, Di Martino V, Sutton A, Audureau E, Roudot-Thoraval F, Nahon P; ANRS CO12 CirVir group. Validation of Baveno VI Criteria for Screening and Surveillance of Esophageal Varices in Patients With Compensated Cirrhosis and a Sustained Response to Antiviral Therapy. *Gastroenterology* 2019; **156**: 997-1009.e5 [PMID: 30768988 DOI: 10.1053/j.gastro.2018.11.053]

51   **Giannini EG**, Crespi M, Demarzo M, Bodini G, Furnari M, Marabotto E, Torre F, Zentilin P, Savarino V. Improvement in hepatitis C virus patients with advanced, compensated liver disease after sustained virological response to direct acting antivirals. *Eur J Clin Invest* 2019; **49**: e13056 [PMID: 30474209 DOI: 10.1111/eci.13056]

52   **Jangouk P**, Turco L, De Oliveira A, Schepis F, Villa E, Garcia-Tsao G. Validating, deconstructing and refining Baveno criteria for ruling out high-risk varices in patients with compensated cirrhosis. *Liver Int* 2017; **37**: 1177-1183 [PMID: 28160373 DOI: 10.1111/liv.13379]

53   **Chen PH**, Hsieh WY, Su CW, Hou MC, Wang YP, Hsin IF, Yang TC, Liao WC, Lin HC, Lee FY, Wu JC. Combination of albumin-bilirubin grade and platelets to predict a compensated patient with hepatocellular carcinoma who does not require endoscopic screening for esophageal varices. *Gastrointest Endosc* 2018; **88**: 230-239.e2 [PMID: 29317268 DOI: 10.1016/j.gie.2017.12.023]

54   **Calvaruso V**, Cacciola I, Licata A, Madonia S, Benigno R, Petta S, Bronte F, Conte E, Malizia G, Bertino G, Distefano M, Montineri A, Digiacomo A, Alaimo G, Cacopardo B, Davì A, Guarneri L, Scalisi I, Colletti P, Cartabellotta F, Portelli V, Prestileo T, Averna A, Iacobello C, Mondello L, Scifo G, Russello M, Squadrito G, Raimondo G, Cammà C, Craxì A, Di Marco V; RESIST-HCV (Rete Sicilia Selezione Terapia–HCV). Is Transient Elastography Needed for Noninvasive Assessment of High-Risk Varices? *Am J Gastroenterol* 2019; **114**: 1275-1282 [PMID: 31135449 DOI: 10.14309/ajg.0000000000000266]

55   **Hamdane N**, Jühling F, Crouchet E, El Saghire H, Thumann C, Oudot MA, Bandiera S, Saviano A, Ponsolles C, Roca Suarez AA, Li S, Fujiwara N, Ono A, Davidson I, Bardeesy N, Schmidl C, Bock C, Schuster C, Lupberger J, Habersetzer F, Doffoël M, Piardi T, Sommacale D, Imamura M, Uchida T, Ohdan H, Aikata H, Chayama K, Boldanova T, Pessaux P, Fuchs BC, Hoshida Y, Zeisel MB, Duong FHT, Baumert TF. HCV-Induced Epigenetic Changes Associated With Liver Cancer Risk Persist After Sustained Virologic Response. *Gastroenterology* 2019; **156**: 2313-2329.e7 [PMID: 30836093 DOI: 10.1053/j.gastro.2019.02.038]

56   **D'Ambrosio R**, Colombo M. Should surveillance for liver cancer be modified in hepatitis C patients after treatment-related cirrhosis regression? *Liver Int* 2016; **36**: 783-790 [PMID: 26936383 DOI: 10.1111/liv.13106]

57   **D'Ambrosio R**, Aghemo A, Fraquelli M, Rumi MG, Donato MF, Paradis V, Bedossa P, Colombo M. The diagnostic accuracy of Fibroscan for cirrhosis is influenced by liver morphometry in HCV patients with a sustained virological response. *J Hepatol* 2013; **59**: 251-256 [PMID: 23528378 DOI: 10.1016/j.jhep.2013.03.013]

58   **Tachi Y**, Hirai T, Kojima Y, Ishizu Y, Honda T, Kuzuya T, Hayashi K, Ishigami M, Goto H. Liver stiffness measurement predicts hepatocellular carcinoma development in patients treated with direct-acting antivirals. *JGH Open* 2017; **1**: 44-49 [PMID: 30483532 DOI: 10.1002/jgh3.12007]

59   **Terrault NA**, Zeuzem S, Di Bisceglie AM, Lim JK, Pockros PJ, Frazier LM, Kuo A, Lok AS, Shiffman ML, Ben Ari Z, Akushevich L, Vainorius M, Sulkowski MS, Fried MW, Nelson DR; HCV-TARGET Study Group. Effectiveness of Ledipasvir-Sofosbuvir Combination in Patients With Hepatitis C Virus Infection and Factors Associated With Sustained Virologic Response. *Gastroenterology* 2016; **151**: 1131-1140.e5 [PMID: 27565882 DOI: 10.1053/j.gastro.2016.08.004]

60   **Foster GR**, Irving WL, Cheung MC, Walker AJ, Hudson BE, Verma S, McLauchlan J, Mutimer DJ, Brown A, Gelson WT, MacDonald DC, Agarwal K; HCV Research, UK. Impact of direct acting antiviral therapy in patients with chronic hepatitis C and decompensated cirrhosis. *J Hepatol* 2016; **64**: 1224-1231 [PMID: 26829205 DOI: 10.1016/j.jhep.2016.01.029]

61   **European Association for the Study of the Liver**. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. *J Hepatol* 2018; **69**: 182-236 [PMID: 29628281 DOI: 10.1016/j.jhep.2018.03.019]

62   **Marrero JA**, Kulik LM, Sirlin CB, Zhu AX, Finn RS, Abecassis MM, Roberts LR, Heimbach JK. Diagnosis, Staging, and Management of Hepatocellular Carcinoma: 2018 Practice Guidance by the American Association for the Study of Liver Diseases. *Hepatology* 2018; **68**: 723-750 [PMID: 29624699 DOI: 10.1002/hep.29913]

63   **Rinaldi L**, Guarino M, Perrella A, Pafundi PC, Valente G, Fontanella L, Nevola R, Guerrera B, Iuliano N, Imparato M, Trabucco A, Sasso FC, Morisco F, Ascione A, Piai G, Adinolfi LE. Role of Liver Stiffness Measurement in Predicting HCC Occurrence in Direct-Acting Antivirals Setting: A Real-Life Experience. *Dig Dis Sci* 2019; **64**: 3013-3019 [PMID: 30937719 DOI: 10.1007/s10620-019-05604-8]

64   **Lai MM**. Hepatitis C virus proteins: direct link to hepatic oxidative stress, steatosis, carcinogenesis and more. *Gastroenterology* 2002; **122**: 568-571 [PMID: 11832470 DOI: 10.1053/gast.2002.31474]

65   **Okuda M**, Li K, Beard MR, Showalter LA, Scholle F, Lemon SM, Weinman SA. Mitochondrial

injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. *Gastroenterology* 2002; **122**: 366-375 [PMID: 11832451 DOI: 10.1053/gast.2002.30983]

66   **Ripoll C**, Groszmann RJ, Garcia-Tsao G, Bosch J, Grace N, Burroughs A, Planas R, Escorsell A, Garcia-Pagan JC, Makuch R, Patch D, Matloff DS; Portal Hypertension Collaborative Group. Hepatic venous pressure gradient predicts development of hepatocellular carcinoma independently of severity of cirrhosis. *J Hepatol* 2009; **50**: 923-928 [PMID: 19303163 DOI: 10.1016/j.jhep.2009.01.014]

67   **King LY**, Canasto-Chibuque C, Johnson KB, Yip S, Chen X, Kojima K, Deshmukh M, Venkatesh A, Tan PS, Sun X, Villanueva A, Sangiovanni A, Nair V, Mahajan M, Kobayashi M, Kumada H, Iavarone M, Colombo M, Fiel MI, Friedman SL, Llovet JM, Chung RT, Hoshida Y. A genomic and clinical prognostic index for hepatitis C-related early-stage cirrhosis that predicts clinical deterioration. *Gut* 2015; **64**: 1296-1302 [PMID: 25143343 DOI: 10.1136/gutjnl-2014-307862]

68   **Ravaioli F**, Conti F, Brillanti S, Andreone P, Mazzella G, Buonfiglioli F, Serio I, Verrucchi G, Bacchi Reggiani ML, Colli A, Marasco G, Colecchia A, Festi D. Hepatocellular carcinoma risk assessment by the measurement of liver stiffness variations in HCV cirrhotics treated with direct acting antivirals. *Dig Liver Dis* 2018; **50**: 573-579 [PMID: 29567413 DOI: 10.1016/j.dld.2018.02.010]

69   **Martínez-Campreciós J**, Bonis Puig S, Pons Delgado M, Salcedo Allende MT, Mínguez Rosique B, Genescà Ferrer J. Transient elastography in DAA era. Relation between post-SVR LSM and histology. *J Viral Hepat* 2020; **27**: 453-455 [PMID: 31816146 DOI: 10.1111/jvh.13245]

70   **Pan JJ**, Bao F, Du E, Skillin C, Frenette CT, Waalen J, Alaparthi L, Goodman ZD, Pockros PJ. Morphometry Confirms Fibrosis Regression From Sustained Virologic Response to Direct-Acting Antivirals for Hepatitis C. *Hepatol Commun* 2018; **2**: 1320-1330 [PMID: 30411079 DOI: 10.1002/hep4.1228]

71   **Tachi Y**, Hirai T, Toyoda H, Tada T, Hayashi K, Honda T, Ishigami M, Goto H, Kumada T. Predictive Ability of Laboratory Indices for Liver Fibrosis in Patients with Chronic Hepatitis C after the Eradication of Hepatitis C Virus. *PLoS One* 2015; **10**: e0133515 [PMID: 26214180 DOI: 10.1371/journal.pone.0133515]

72   **Iwasaki Y**, Takaguchi K, Ikeda H, Makino Y, Araki Y, Ando M, Kobashi H, Kobatake T, Tanaka R, Tomita M, Senoh T, Kawaguchi M, Shimoe T, Manabe K, Kita K, Shimamura J, Sakaguchi K, Shiratori Y. Risk factors for hepatocellular carcinoma in Hepatitis C patients with sustained virologic response to interferon therapy. *Liver Int* 2004; **24**: 603-610 [PMID: 15566511 DOI: 10.1111/j.1478-3231.2004.0956.x]

73   **Arase Y**, Kobayashi M, Suzuki F, Suzuki Y, Kawamura Y, Akuta N, Sezaki H, Saito S, Hosaka T, Ikeda K, Kumada H, Kobayashi T. Effect of type 2 diabetes on risk for malignancies includes hepatocellular carcinoma in chronic hepatitis C. *Hepatology* 2013; **57**: 964-973 [PMID: 22991257 DOI: 10.1002/hep.26087]

74   **Nagaoki Y**, Aikata H, Nakano N, Shinohara F, Nakamura Y, Hatooka M, Morio K, Kan H, Fujino H, Kobayashi T, Fukuhara T, Masaki K, Ono A, Nakahara T, Kawaoka T, Miki D, Tsuge M, Hiramatsu A, Imamura M, Takahashi S, Kawakami Y, Ochi H, Chayama K; Hiroshima Liver Study Group. Development of hepatocellular carcinoma in patients with hepatitis C virus infection who achieved sustained virological response following interferon therapy: A large-scale, long-term cohort study. *J Gastroenterol Hepatol* 2016; **31**: 1009-1015 [PMID: 26584407 DOI: 10.1111/jgh.13236]

75   **Toyoda H**, Kumada T, Tada T, Kiriyama S, Tanikawa M, Hisanaga Y, Kanamori A, Kitabatake S, Ito T. Risk factors of hepatocellular carcinoma development in non-cirrhotic patients with sustained virologic response for chronic hepatitis C virus infection. *J Gastroenterol Hepatol* 2015; **30**: 1183-1189 [PMID: 25678094 DOI: 10.1111/jgh.12915]

76   **Fouad R**, Elsharkawy A, Abdel Alem S, El Kassas M, Alboraie M, Sweedy A, Afify S, Abdellatif Z, Khairy M, Esmat G. Clinical impact of serum α-fetoprotein and its relation on changes in liver fibrosis in hepatitis C virus patients receiving direct-acting antivirals. *Eur J Gastroenterol Hepatol* 2019; **31**: 1129-1134 [PMID: 30896550 DOI: 10.1097/MEG.0000000000001400]

77   **D'Ambrosio R**, Aghemo A, Rumi MG, Degasperi E, Sangiovanni A, Maggioni M, Fraquelli M, Perbellini R, Rosenberg W, Bedossa P, Colombo M, Lampertico P. Persistence of hepatocellular carcinoma risk in hepatitis C patients with a response to IFN and cirrhosis regression. *Liver Int* 2018; **38**: 1459-1467 [PMID: 29377616 DOI: 10.1111/liv.13707]

78   **Lleo A**, Aglitti A, Aghemo A, Maisonneuve P, Bruno S, Persico M; collaborators. Predictors of hepatocellular carcinoma in HCV cirrhotic patients treated with direct acting antivirals. *Dig Liver Dis* 2019; **51**: 310-317 [PMID: 30473220 DOI: 10.1016/j.dld.2018.10.014]

79   **Hamada K**, Saitoh S, Nishino N, Fukushima D, Horikawa Y, Nishida S, Honda M. Shear wave elastography predicts hepatocellular carcinoma risk in hepatitis C patients after sustained virological response. *PLoS One* 2018; **13**: e0195173 [PMID: 29672518 DOI: 10.1371/journal.pone.0195173]

80   **Feier D**, Lupsor Platon M, Stefanescu H, Badea R. Transient elastography for the detection of hepatocellular carcinoma in viral C liver cirrhosis. Is there something else than increased liver stiffness? *J Gastrointestin Liver Dis* 2013; **22**: 283-289 [PMID: 24078985]

81   **Kobayashi M**, Suzuki F, Akuta N, Suzuki Y, Sezaki H, Yatsuji H, Kawamura Y, Hosaka T, Kobayashi M, Arase Y, Ikeda K, Mineta R, Iwasaki S, Watahiki S, Miyakawa Y, Kumada H. Development of hepatocellular carcinoma in elderly patients with chronic hepatitis C with or without elevated aspartate and alanine aminotransferase levels. *Scand J Gastroenterol* 2009; **44**: 975-983 [PMID: 19521923 DOI: 10.1080/00365520802588125]

82   **Miyakawa K**, Tarao K, Ohshige K, Morinaga S, Ohkawa S, Okamoto N, Shibuya A, Adachi S,

Miura Y, Fujiyama S, Miyase S, Tomita K. High serum alanine aminotransferase levels for the first three successive years can predict very high incidence of hepatocellular carcinoma in patients with Child Stage A HCV-associated liver cirrhosis. *Scand J Gastroenterol* 2009; **44**: 1340-1348 [PMID: 19891585 DOI: 10.3109/00365520903222681]

83   **Simili A**, Mazzella G, Ravaioli F, Festi D, Bacchi-Reggiani ML, Porro A, Bazzoli F, Azzaroli F. Interleukin 28 Polymorphisms and Hepatocellular Carcinoma Development after Direct Acting Antiviral Therapy for Chronic Hepatitis C. *J Gastrointestin Liver Dis* 2019; **28**: 449-456 [PMID: 31826071 DOI: 10.15403/jgld-309]

84   **Matsuura K**, Sawai H, Ikeo K, Ogawa S, Iio E, Isogawa M, Shimada N, Komori A, Toyoda H, Kumada T, Namisaki T, Yoshiji H, Sakamoto N, Nakagawa M, Asahina Y, Kurosaki M, Izumi N, Enomoto N, Kusakabe A, Kajiwara E, Itoh Y, Ide T, Tamori A, Matsubara M, Kawada N, Shirabe K, Tomita E, Honda M, Kaneko S, Nishina S, Suetsugu A, Hiasa Y, Watanabe H, Genda T, Sakaida I, Nishiguchi S, Takaguchi K, Tanaka E, Sugihara J, Shimada M, Kondo Y, Kawai Y, Kojima K, Nagasaki M, Tokunaga K, Tanaka Y; Japanese Genome-Wide Association Study Group for Viral Hepatitis. Genome-Wide Association Study Identifies TLL1 Variant Associated With Development of Hepatocellular Carcinoma After Eradication of Hepatitis C Virus Infection. *Gastroenterology* 2017; **152**: 1383-1394 [PMID: 28163062 DOI: 10.1053/j.gastro.2017.01.041]

85   **Degasperi E**, Galmozzi E, Facchetti F, Farina E, D'Ambrosio R, Soffredini R, Iavarone M, Lampertico P. TLL1 variants do not predict hepatocellular carcinoma development in HCV cirrhotic patients treated with direct-acting antivirals. *J Viral Hepat* 2019; **26**: 1233-1236 [PMID: 31177595 DOI: 10.1111/jvh.13155]

MINIREVIEWS

Role of immune dysfunction in drug induced liver injury

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Abstract

Drug-induced liver injury (DILI) is one of the leading causes of liver failure and withdrawal of drugs from the market. A poor understanding of the precipitating event aetiology and mechanisms of disease progression has rendered the prediction and subsequent treatment intractable. Recent literature suggests that some drugs can alter the liver's repair systems resulting in injury. The pathophysiology of DILI is complex, and immune dysfunction plays an important role in determining the course and severity of the disease. Immune dysfunction is influenced by the host response to drug toxicity. A deeper understanding of these processes may be beneficial in the management of DILI and aid in drug development. This review provides a structured framework presenting DILI in three progressive stages that summarize the interplay between drugs and the host defence networks.

Key Words: Immune dysfunction; Liver damage; Hepatotoxic drugs; Drug-induced liver injury; High mobility group box 1

Core Tip: This review demonstrates the critical role of the immune system in the progression of drug-induced liver injury and also in determining the severity of the damage. Drugs affect the normal functioning of hepatocytes through several direct and indirect mechanisms leading to the dysfunctional immune response. The major effector cells in amplifying liver damage are Kupffer cells, monocytes and neutrophils. Genetic predispositions and environmental factors also make individuals vulnerable to immune dysfunction.

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INTRODUCTION

The liver plays a central role in the complex process of metabolism and elimination of drugs from the body. The liver is equipped with a wide array of detoxification systems that have evolved over time with exposure to xenobiotics. The primary role of this system is to convert a drug to a more hydrophilic form so that it can be eliminated through bile or urine. Despite the liver's detox potential, certain drugs can still cause hepatotoxicity that can range from mild asymptomatic liver damage to liver failure[1, 2].

A study showed that, out of the 462 pharmaceuticals withdrawn due to adverse drug reactions between 1953 and 2013, hepatotoxicity ranked first with 81 cases (18%). It is estimated that over 1000 drugs currently available on the market that cause liver damage[3] despite these drugs passing the safety measures of clinical trials before entering the market. Some drugs that are hepatotoxic at doses higher than the therapeutic range can also cause drug-induced liver injury (DILI) at doses within the therapeutic range[2,4-6]. This implies that the dose may not be the only contributing factor.

Despite large number of drugs known to cause liver injury, the incidence of DILI is rare. DILI is reported in 1 in every 10000 to 100000 individuals annually. This suggests that drug-host interactions in these susceptible individuals may play an important role in DILI[7-9]. Recent data shows that this interaction can result in an imbalance between damage and repair mechanisms resulting in DILI with immune dysfunction being cited as an important precipitating event in the pathophysiology of DILI[10-12]. This is supported by evidence from experimental studies. Some drugs that are hepatotoxic in humans do not cause liver damage in animal models, but the adminis-tration of these drugs along with low doses of lipopolysaccharide (LPS) result in a similar pattern of liver injury as observed in humans. For example, Trovafloxacin (TVX) is a broad-spectrum fluoroquinolone antibiotic, and a study reported that TVX use caused 140 severe hepatic reactions resulting in 14 cases of liver failure. Examination of the case reports suggest that the duration of TVX therapy in patients does not correlate with the toxic response, so TVX hepatotoxicity is classified as idiosyncratic. In rodent models, TVX did not cause liver damage, even at high doses. However, further studies with a normally nontoxic dose of TVX coupled with LPS induced inflammatory stress caused acute liver injury[13,14].

The upcoming sections provide a structured framework presenting DILI in three progressive stages, summarizing the interplay between drugs and the host defence networks that lead to immune system dysfunction.

STAGES OF DILI

Initiation of DILI

Direct initiation: The metabolism of drugs by phase 1 enzymes results in the prod-uction of intermediary metabolites and free radicals, in some instances. These intermediary metabolites may also be unstable and reactive, but they are subsequently neutralized by phase 2 conjugation. DILI is initiated when there is an imbalance between the production of reactive metabolites and their subsequent detoxification[2, 5] (Figure 1).

Certain drugs and reactive metabolites can bind to cellular organelles resulting in loss of function and likely cell death. One such case is the damage caused by drugs acting on the endoplasmic reticulum (ER). The ER plays an important role in protein synthesis, folding, assembly, trafficking, and regulation of intracellular calcium homeostasis. Drug related oxidative stress can disturb ER function and lead to the accumulation of unfolded proteins in the ER. This process is termed ER stress. A variety of common drugs cause ER stress, including paracetamol, lopinavir, ritonavir, saquinavir, nelfinavir, atazanavir, and amprenavir[15].

During drug metabolism, free radicals are released that are normally detoxified by cell defence mechanisms. Excessive free radical generation can be caused by enzyme induction or genetic defects in enzyme systems. Free radicals damage the cellular organelles and the lipid bilayer, which results in amplification of damage. Lipid
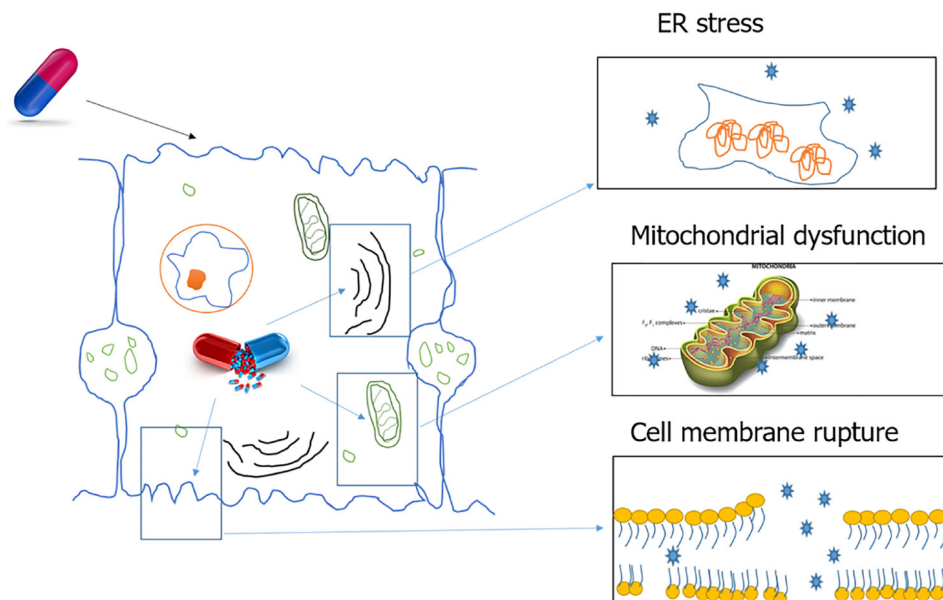
Figure 1 Initiation of drug-induced liver injury - Direct damage by drug and metabolite. Drugs and their metabolites damage organelles and cell membrane of liver cells causing damage. ER: Endoplasmic reticulum.

bilayer damage can lead to the release of cytosolic components and alarmins that attract the liver's resident immune cells. This initial immune response can amplify the sterile damage. Some of the alarmins associated with DILI are high mobility group box 1, S100 proteins, hepatoma-derived growth factor and heat shock proteins[16-20].

Free radicals can also damage the mitochondrial membrane leading to cell dysfunction and death. Mitochondrial dysfunction includes disruption or disturbance to different metabolic pathways and damage to mitochondrial components. In addition, these mitochondrial alterations can have several deleterious consequences, such as oxidative stress, ATP depletion, triglycerides accumulation, and necrotic cell death[21].

Indirect initiation of DILI: There are two main mechanisms of indirect initiation of DILI. Inhibition of efflux transporters. Bile salt export pump (BSEP) is a member of the ABC transporter superfamily located in the canalicular membrane of hepatocytes. BSEP is responsible for the biliary excretion of bile acids. Drug metabolites inhibit BSEP function, resulting in toxicity. One such metabolite, Troglitazone sulphate, a metabolite of troglitazone, inhibits BSEP mediated taurocholate transport which contributes to troglitazone toxicity. Other potent BSEP inhibitors with the potential to cause DILI include cyclosporin A, bosentan, sulindac, rifamycin, and glibenclamide[2, 22].

Enzyme induction: Paracetamol is known to cause liver injury through enzyme induction due to CYP2E1 induction by ethanol. A minor percentage of ethanol is metabolised by CYP2E1. When ethanol and paracetamol are taken simultaneously, ethanol slows the degradation of the CYP enzyme increasing its half-life from 7 h to 37 h. Until ethanol is present in the body more CYP2E1 is induced and a portion is blocked from paracetamol for ethanol metabolism. Once ethanol is completely removed, CYP2E1 enhances paracetamol metabolism resulting in the excess production of toxic intermediary metabolite, NAPQI, causing liver injury[2,23] (Figure 2).

PROGRESSION

The initiation of DILI does not necessarily result in adverse outcomes. In experimental models, the progression of DILI mainly depends on the persistent and recurrent assault by the toxins that deplete the liver's resources leading to irreversible damage. This is unlikely at the therapeutic dose of most drugs, as the liver has highly developed protective and regenerative mechanisms. Experimental and clinical data suggest that a myriad of host and drug-related factors contribute to the progressive dysfunction of survival mechanisms that lead to DILI. This is further complicated by
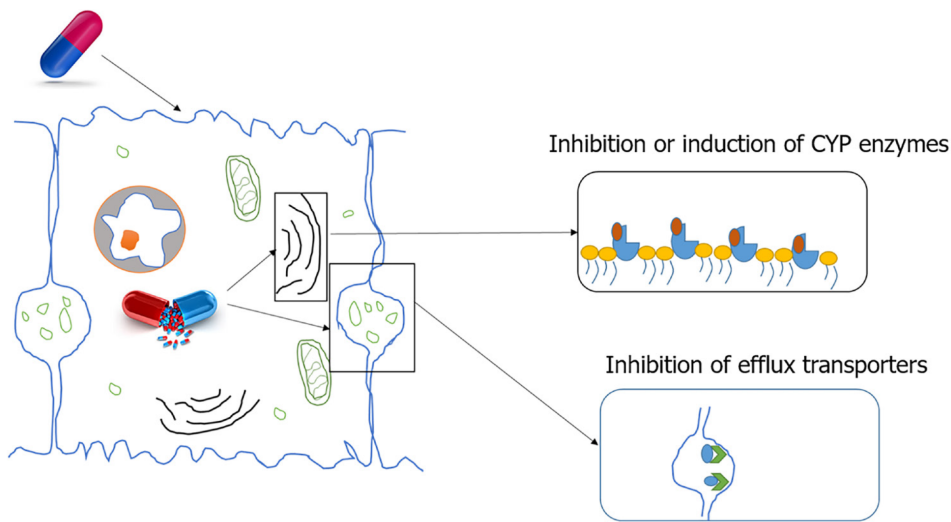
Figure 2 Initiation of drug-induced liver injury - Indirect damage by drugs. Drugs can modulate the functioning of enzymes and transporters involved in drug metabolism and elimination that may lead to toxicity.

the fact that each drug can cause multiple patterns of liver disease, implying an important role for host-drug interactions in the progression of DILI. Immune dysfunction is a major determinant of hepatic cell death and DILI progression[2,4,6,24-26].

This section covers the two main mechanisms of immune reactions induced by drugs and the influence of host factors on them.

Immune allergic DILI

A drug or its metabolites alone cannot activate an immune response due to their small size, but a drug's reactive metabolites or the drug itself can bind to cellular proteins and form protein-drug adducts that elicit an immune response. In normal individuals, this complex is degraded by cellular detoxification but in susceptible individuals, these adducts act as immunogens and are taken up by antigen-presenting cells and presented by major histocompatibility complexes to helper T cells, and further activation by cytokines stimulates an immune response and anti-drug antibodies are also produced, resulting in extensive death of cells where the drug has accumulated[6, 27-29] (Figure 3).

It is hypothesized that ER stress is a contributing factor for this type of reaction. Accumulation of drug/metabolite causes ER stress, which results in misfolding of proteins. These misfolded proteins are more susceptible to drug-protein adduct formations that elicit an immune response[15].

An example of this type of reaction is abacavir, a reverse transcriptase inhibitor employed in the treatment of AIDS, which causes a rare, but serious hypersensitivity reaction that resembles an immune allergic drug reaction. Several genetic variants in the HLA regions are identified as risk factors for DILI, the incidence of hypersensitivity reactions to abacavir is markedly elevated in subjects who carry the B*57:01 variant in the human leukocyte antigen B (*HLA-B*) gene. Furthermore, carriers of this genotype are at increased risk of flucloxacillin-induced DILI. Studies have shown an association between HLA-B1*15:01 and amoxicillin/clavulanate DILI. The HLA-B* 35:02 allele is reported to have a significant association with minocycline DILI[10,25,30, 31]. DILI caused by other drugs such as amoxicillin-clavulanate, lumiracoxib, ticlopidine, lapatinib, and ximelagatran is also associated with HLA genotypes, suggesting an important role of the immune system in DILI[25,31].

Autoimmune DILI

Autoimmune DILI is caused by the release of alarmins from necrotic cells or cells with leaky cell membranes. This results in the activation of innate immune cells. Alarmins are rapidly released following necrotic cell death that are not released by apoptotic cells. The immune system also can be induced to produce and release alarmins to recruit and activate innate immune cells[19,32] (Figure 4).

Mitochondrial dysfunction is reported to play a critical role in the pathogenesis of autoimmune DILI. NSAIDs, such as diclofenac and nimesulide, and other drugs can cause mitochondrial dysfunction that leads to the formation of the mitochondrial
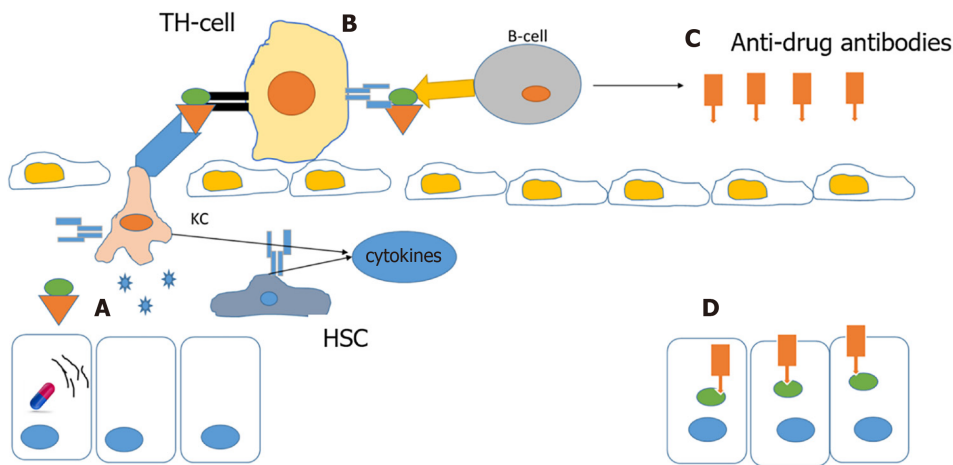
Figure 3 Immune allergic drug-induced liver injury. A: Endoplasmic reticulum stress by drug, causes misfolded protein resulting in cell death and release of stress signals and drug-protein complex. Kupffer cells ingest the drug-protein complex to T-helper cells; B: T-helper cells process it and present it to B-cells; C: B-cells produce anti-drug antibodies; D: These antibodies target the tissues, where drug is accumulated. KC: Kupffer cell; HSC: Hepatic stellate cells.
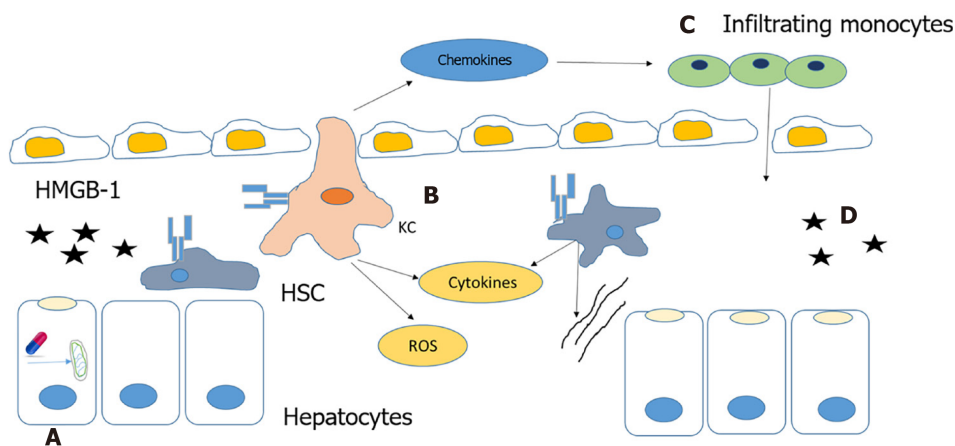
Figure 4 Mechanism of autoimmune drug-induced liver injury. A: Drug causes mitochondrial dysfunction resulting in cell death and release of HMGB-1 and other stress signals; B: Kupffer cells and Stellate cells get activated. Release cytokines, chemokines and toxins; C: Chemokines attract monocytes; D: Amplification of injury and cell death. KC: Kupffer cell; HSC: Hepatic stellate cells; ROS: Reactive oxygen species.

permeability transition pore (MPTP). MPTP formation is induced by increased oxidative stress that results in a dissipation of membrane potential, uncoupling of oxidative phosphorylation leading to necrotic cell death and the release of alarmins[18, 21,33].

HMGB-1 is an alarmin released by necrotic cells that binds to TLR4 receptors of kupffer cells (KCs) and hepatic stellate cells (HSC), and activates them. Activated KCs produce mediators that directly induce cell death, such as tumor necrosis factor (TNF)-α, Fas ligand and reactive oxygen species, or indirectly cause death through the recruitment of neutrophils by cytokines and chemokines like IL-1β and CXCL2. Production of chemokine, CCL2 (MCP-1) recruits monocytes from the bone marrow to the liver. These infiltrating monocytes produce inflammatory chemokines resulting in the activation of HSCs and the promotion of fibrosis[18,34].

Host sex and sex hormones influence immune response. Studies have shown that female patients with DILI are at higher risk of developing acute liver failure (ALF) with more severe hepatitis and higher levels of pro-inflammatory cytokines. In a halothane-induced experimental DILI model, oestrogen reduced liver injury while progesterone increased liver damage, both hormones influenced immune response. Another important factor affecting DILI is race. A study reported that African-Americans are at a higher risk of developing chronic DILI, while Asian individuals are at increased risk of ALF, liver-related death, or damage that precipitates a need for liver transplantation[4,7,10,24,35].

ADVERSE OUTCOMES

In normal individuals, DILI resolves completely without any residual liver injury. But there are three major exceptions. They are ALF, cirrhosis and acute-on-chronic liver failure (ACLF). These conditions are relatively rare but severe and may result in death or require a liver transplant.

ALF

Even in the absence of pre-existing liver disease, drugs can cause a rapid loss of liver function either directly, as seen in overdoses, or through inflammatory cell mediated mechanisms such as cytokine overproduction. Drug-induced ALF is defined by the signs or symptoms of hepatic failure and encephalopathy during the course of acute DILI. The time to onset of ALF after the start of a medication can vary from a few days to months, but not exceeding six months[4,24,36-38].

In Western countries, paracetamol overdose is the most common reason behind ALF. In India, anti-TB regimens with isoniazid, rifampicin and pyrazinamide are reported as the leading cause of ALF. Other drugs that are reported to cause ALF include phenytoin, carbamazepine, valproate, nitrofurantoin, propylthiouracil, disulfiram, diclofenac, ketoconazole, flutamide, sulphonamides, terbinafine, fluoroquinolone antibiotics and macrolide antibiotics. Drug-induced ALF is a major cause for withdrawal from the market or restricted use of a medication (troglitazone, bromfenac, nefazodone, halothane, telithromycin). ALF occurs in cases with acute hepatocellular injury with characteristics similar to acute viral hepatitis[10,23,39-41].

Paracetamol is responsible for more than 50% of drug related ALF and about 20% of liver transplant cases in the United States[42]. In case of paracetamol overdose, the drug metabolite NAPQ1 depletes GSH and causes organelle damage, the most significant resulting in mitochondrial stress. Thereby the NAPQ1 accumulation triggers necrosis[43,44]. Hepatocyte necrosis passively releases various DAMPs such as HMGB-1, HSP and DNA fragments. These DAMPs activate the resident immune cells such as Kupffer cells and natural killer (NK) cells. Cytokines and chemokines such as TNF-α, IL-1β and CCL2 produced by the activated immune cells and the DAMPs enter systemic circulation and cause infiltration of neutrophils and monocytes into the liver. In conditions of sterile injury, the immune cells function to clear the dead cells by producing chemokines and free radicals to digest it. Once the cellular debris is cleared the immune cells undergo phenotypic change and support in liver regeneration. However, in case of paracetamol overdose, the overwhelming amount of cellular debris and DAMPs causes excess immune activation, whose products such as superoxide, nitric oxide and peroxynitrite result in further amplification of liver injury leading to massive necrosis and organ failure[45-48].

Cirrhosis

Cirrhosis is characterized by islands or nodules of regenerative parenchymal cells surrounded by excessive deposition of fibrous tissue and portal hypertension. Cirrhosis is rarely the initial manifestation of DILI and is most often a cumulative response to long-term exposure to hepatotoxic drugs. It usually occurs at least six months after starting the drug treatment. The time to onset of cirrhosis due to medications is typically long; at least 6 month after starting the medication but usually several years afterwards. The drugs that are most commonly cause cirrhosis are vitamin A, amiodarone, statins, tamoxifen, valproic acid, fibrates, and methotrexate[4, 25,26,49-51]. Drugs such as dantrolene, phenytoin, trazadone and nitrofurantoin are also associated with chronic hepatitis with autoimmune features that may lead to cirrhosis[52-54].

Amiodarone is a benzofuran derivative mainly used in the treatment of arrhythmia. The safety of long-term use of amiodarone is well established however there are several reports of reversible and irreversible liver injury from its long-term use. Even though rare amiodarone can cause asymptomatic continuous liver injury that has histological features similar to alcoholic hepatitis such as nodular formation, fibrosis, steatosis and neutrophil infiltration[55-61]. Due to its lipophilic nature and long half-life, amiodarone accumulates in the hepatocytes affecting cellular organelles such as ER and mitochondria causing misfolding of proteins. Amiodarone affects the cholesterol metabolism by blocking enzymes emopamil binding protein and dehydrocholesterol reductase 24. As cholesterol plays an important role in maintaining membrane fluidity and composition this affects the function of potassium channels and other membrane proteins resulting in "lipid traffic jam"[62-67]. The immune cells in the liver get activated in response to cellular debris, misfolded

proteins and accumulating cholesterol precursors such as desmosterol[63,66,68]. Unless diagnosed in an early stage, this leads to irreversible end stage liver disease[62, 69].

Acute-on-chronic liver failure

Acute-on-chronic liver failure (ACLF) as the name suggests is characterized by ALF due to a different cause in patients with chronic liver disease (compensated) resulting in short term mortality. It consists of two components: a chronic underlying liver disease and an acute trigger[70,71]. Devarbhavi *et al*[72] reported that drugs contributed to 10.5% cases in the Asia-Pacific region. Among these drugs, the most common culprits were complementary and alternative medications (71.7%), followed by anti-TB drug combination therapies (27.3%). Anti-TB drug isoniazid is also observed to cause severe hepatitis that leads to liver failure[72-74].

Studies suggest that excessive focal liver and systemic inflammatory response play a significant role in the development of ACLF. Reports have shown high levels of cytokines in patients with ACLF. This may be due to the activation of monocytes and macrophages in response to DAMPs, microbial toxins or drug adducts[19,75,76].

Paracetamol induced liver failure in patients with alcoholic hepatitis is a typical example of drug induced ACLF. Alcoholic hepatitis is reported in approximately 25% of the cases of ACLF. The trigger due to paracetamol toxicity can occur in two ways-the first is due to direct toxicity by paracetamol and the second due to immune response that is secondary to the hepatocellular damage due to the direct toxicity. The activation of innate immune response due to the paracetamol acute toxicity results in upregulation of cytokine and chemokine production that initiates severe systemic inflammation, liver damage and mortality[70,75,77,78].

The dysregulation in innate immune response plays important roles in disease progression as well as disease severity. In the liver, systemic inflammation plays a significant role in the development and course of chronic alcoholic hepatitis. Similar to the acute toxicity, immune activation in alcoholic liver disease results in activation of resident Kupffer cells and dendritic cells as well as the infiltrating immune cells-monocytes and neutrophils lead to progression towards fibrosis and cirrhosis. This disrupts the liver architecture and function setting stage for liver failure, that can be actuated by an acute trigger[75,78,79].

CONCLUSION

Drugs and their metabolic products can cause liver damage through multiple mechanisms. Under normal conditions, the liver is well equipped to neutralize potential drug-related damage, but in susceptible individuals, this same drug use can result in severe liver injury. This is further amplified by a dysfunctional immune responses that is influenced by host factors like genetics, age and sex. The severe adverse outcomes of DILI are ALF, cirrhosis and acute-on-chronic liver injury. All these injuries are associated with concurrent immune dysfunction. A better understanding of immune mediators may offer new targets for the management of DILI. Individualized therapy that focuses on early detection of risk factors, triggers and stage of the liver injury may play a significant role in effectively attenuating this disorder.

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REFERENCES

1   **Kirchain WR**, Allen RE. Drug-Induced Liver Disease. In: DiPiro JT, Talbert RL, Yee GC, Matzke GR, Wells BG, Posey L. eds. Pharmacotherapy: A pathophysiologic approach, 10e. McGraw-Hill, 2016

2   **Lee WM**. Drug-induced hepatotoxicity. *N Engl J Med* 2003; **349**: 474-485 [PMID: 12890847 DOI: 10.1056/NEJMra021844]

3   **Onakpoya IJ**, Heneghan CJ, Aronson JK. Post-marketing withdrawal of 462 medicinal products because of adverse drug reactions: a systematic review of the world literature. *BMC Med* 2016; **14**: 10 [PMID: 26843061 DOI: 10.1186/s12916-016-0553-2]

4   **Kaplowitz N**. Avoiding idiosyncratic DILI: two is better than one. *Hepatology* 2013; **58**: 15-17 [PMID: 23390057 DOI: 10.1002/hep.26295]

5   **Kaplowitz N**. Drug-induced liver injury. *Clin Infect Dis* 2004; **38** Suppl 2: S44-S48 [PMID: 14986274 DOI: 10.1086/381446]

6   **Waddington JC**, Meng X, Naisbitt DJ, Park BK. Immune drug-induced liver disease and drugs. *Curr Opin Toxicol* 2018; **10**: 46-53 [DOI: 10.1016/j.cotox.2017.12.006]

7   **Bell LN**, Chalasani N. Epidemiology of idiosyncratic drug-induced liver injury. *Semin Liver Dis* 2009; **29**: 337-347 [PMID: 19826967 DOI: 10.1055/s-0029-1240002]

8   **Björnsson ES**. Incidence and outcomes of DILI in Western patients. *Clin Liver Dis (Hoboken)* 2014; **4**: 9-11 [PMID: 30992911 DOI: 10.1002/cld.368]

9   **Devarbhavi H**, Dierkhising R, Kremers WK, Sandeep MS, Karanth D, Adarsh CK. Single-center experience with drug-induced liver injury from India: causes, outcome, prognosis, and predictors of mortality. *Am J Gastroenterol* 2010; **105**: 2396-2404 [PMID: 20648003 DOI: 10.1038/ajg.2010.287]

10   **Chen M**, Suzuki A, Borlak J, Andrade RJ, Lucena MI. Drug-induced liver injury: Interactions between drug properties and host factors. *J Hepatol* 2015; **63**: 503-514 [PMID: 25912521 DOI: 10.1016/j.jhep.2015.04.016]

11   **Williams CD**, Jaeschke H. Role of innate and adaptive immunity during drug-induced liver injury. *Toxicol Res* 2012; **1**: 161 [DOI: 10.1039/C2TX20032E]

12   **Liu ZX**, Kaplowitz N. Role of innate immunity in acetaminophen-induced hepatotoxicity. *Expert Opin Drug Metab Toxicol* 2006; **2**: 493-503 [PMID: 16859400 DOI: 10.1517/17425255.2.4.493]

13   **Shaw PJ**, Ganey PE, Roth RA. Idiosyncratic drug-induced liver injury and the role of inflammatory stress with an emphasis on an animal model of trovafloxacin hepatotoxicity. *Toxicol Sci* 2010; **118**: 7-18 [PMID: 20538741 DOI: 10.1093/toxsci/kfq168]

14   **Oda S**, Yokoi T. [Establishment of animal models of drug-induced liver injury and analysis of possible mechanisms]. *Yakugaku Zasshi* 2015; **135**: 579-588 [PMID: 25832838 DOI: 10.1248/yakushi.14-00249-2]

15   **Kraskiewicz H**, FitzGerald U. InterfERing with endoplasmic reticulum stress. *Trends Pharmacol Sci* 2012; **33**: 53-63 [PMID: 22112465 DOI: 10.1016/j.tips.2011.10.002]

16   **Zhang L**, Wang X, Cueto R, Effi C, Zhang Y, Tan H, Qin X, Ji Y, Yang X, Wang H. Biochemical basis and metabolic interplay of redox regulation. *Redox Biol* 2019; **26**: 101284 [PMID: 31400697 DOI: 10.1016/j.redox.2019.101284]

17   **Stanger BZ**. Cellular homeostasis and repair in the mammalian liver. *Annu Rev Physiol* 2015; **77**: 179-200 [PMID: 25668020 DOI: 10.1146/annurev-physiol-021113-170255]

18   **Yang R**, Tonnesseen TI. DAMPs and sterile inflammation in drug hepatotoxicity. *Hepatol Int* 2019; **13**: 42-50 [PMID: 30474802 DOI: 10.1007/s12072-018-9911-9]

19   **Bianchi ME**. DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol* 2007; **81**: 1-5 [PMID: 17032697 DOI: 10.1189/jlb.0306164]

20   **Zanger UM**, Schwab M. Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacol Ther* 2013; **138**: 103-141 [PMID: 23333322 DOI: 10.1016/j.pharmthera.2012.12.007]

21   **Smith RA**, Hartley RC, Cochemé HM, Murphy MP. Mitochondrial pharmacology. *Trends Pharmacol Sci* 2012; **33**: 341-352 [PMID: 22521106 DOI: 10.1016/j.tips.2012.03.010]

22   **Schuetz JD**, Swaan PW, Tweedie DJ. The role of transporters in toxicity and disease. *Drug Metab Dispos* 2014; **42**: 541-545 [PMID: 24598705 DOI: 10.1124/dmd.114.057539]

23   **Larson AM**, Polson J, Fontana RJ, Davern TJ, Lalani E, Hynan LS, Reisch JS, Schiødt FV, Ostapowicz G, Shakil AO, Lee WM; Acute Liver Failure Study Group. Acetaminophen-induced acute liver failure: results of a United States multicenter, prospective study. *Hepatology* 2005; **42**: 1364-1372 [PMID: 16317692 DOI: 10.1002/hep.20948]

24   **Tujios SR**, Lee WM. Acute liver failure induced by idiosyncratic reaction to drugs: Challenges in diagnosis and therapy. *Liver Int* 2018; **38**: 6-14 [PMID: 28771932 DOI: 10.1111/liv.13535]

25   **Russmann S**, Kullak-Ublick GA, Grattagliano I. Current concepts of mechanisms in drug-induced hepatotoxicity. *Curr Med Chem* 2009; **16**: 3041-3053 [PMID: 19689281 DOI: 10.2174/092986709788803097]

26   **Kuzu UB**, Öztaş E, Turhan N, Saygili F, Suna N, Yildiz H, Kaplan M, Akpinar MY, Akdoğan M, Kaçar S, Kiliç ZM, Köksal AŞ, Ödemiş B, Kayaçetin E. Clinical and histological features of idiosyncratic liver injury: Dilemma in diagnosis of autoimmune hepatitis. *Hepatol Res* 2016; **46**: 277-291 [PMID: 25926402 DOI: 10.1111/hepr.12530]

27   **Liu ZX**, Kaplowitz N.   Immune Mechanisms in Drug-Induced Hepatotoxicity. In: Gershwin ME, Vierling JM, Manns MP, eds. Liver Immunology. Totowa, NJ: Humana Press; 2007: 363–374 [DOI: 10.1007/978-1-59745-518-3_29]

28   **Uetrecht J**. Mechanistic Studies of Idiosyncratic DILI: Clinical Implications. *Front Pharmacol* 2019; **10**: 837 [PMID: 31402866 DOI: 10.3389/fphar.2019.00837]

29   **Fontana RJ**. Pathogenesis of idiosyncratic drug-induced liver injury and clinical perspectives. *Gastroenterology* 2014; **146**: 914-928 [PMID: 24389305 DOI: 10.1053/j.gastro.2013.12.032]

30   **Mallal S**, Phillips E, Carosi G, Molina JM, Workman C, Tomazic J, Jägel-Guedes E, Rugina S,

Kozyrev O, Cid JF, Hay P, Nolan D, Hughes S, Hughes A, Ryan S, Fitch N, Thorborn D, Benbow A; PREDICT-1 Study Team. HLA-B*5701 screening for hypersensitivity to abacavir. *N Engl J Med* 2008; **358**: 568-579 [PMID: 18256392 DOI: 10.1056/NEJMoa0706135]

31   **Russmann S**, Jetter A, Kullak-Ublick GA. Pharmacogenetics of drug-induced liver injury. *Hepatology* 2010; **52**: 748-761 [PMID: 20607838 DOI: 10.1002/hep.23720]

32   **Sebode M**, Schulz L, Lohse AW. "Autoimmune(-Like)" Drug and Herb Induced Liver Injury: New Insights into Molecular Pathogenesis. *Int J Mol Sci* 2017; **18** [PMID: 28895915 DOI: 10.3390/ijms18091954]

33   **Han D**, Dara L, Win S, Than TA, Yuan L, Abbasi SQ, Liu ZX, Kaplowitz N. Regulation of drug-induced liver injury by signal transduction pathways: critical role of mitochondria. *Trends Pharmacol Sci* 2013; **34**: 243-253 [PMID: 23453390 DOI: 10.1016/j.tips.2013.01.009]

34   **Lotze MT**, Tracey KJ. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat Rev Immunol* 2005; **5**: 331-342 [PMID: 15803152 DOI: 10.1038/nri1594]

35   **Kullak-Ublick GA**, Andrade RJ, Merz M, End P, Benesic A, Gerbes AL, Aithal GP. Drug-induced liver injury: recent advances in diagnosis and risk assessment. *Gut* 2017; **66**: 1154-1164 [PMID: 28341748 DOI: 10.1136/gutjnl-2016-313369]

36   **Larrey D**, Pageaux GP. Drug-induced acute liver failure. *Eur J Gastroenterol Hepatol* 2005; **17**: 141-143 [PMID: 15674089 DOI: 10.1097/00042737-200502000-00002]

37   **Lee WM**. Drug-induced acute liver failure. *Clin Liver Dis* 2013; **17**: 575-586, viii [PMID: 24099019 DOI: 10.1016/j.cld.2013.07.001]

38   **Argo CK**, Caldwell SH. Editorial: Severe Acute Liver Injury: Cause Connects to Outcome. *Am J Gastroenterol* 2017; **112**: 1397-1399 [PMID: 28874859 DOI: 10.1038/ajg.2017.221]

39   **Koch DG**, Speiser JL, Durkalski V, Fontana RJ, Davern T, McGuire B, Stravitz RT, Larson AM, Liou I, Fix O, Schilsky ML, McCashland T, Hay JE, Murray N, Shaikh OS, Ganger D, Zaman A, Han SB, Chung RT, Brown RS, Munoz S, Reddy KR, Rossaro L, Satyanarayana R, Hanje AJ, Olson J, Subramanian RM, Karvellas C, Hameed B, Sherker AH, Lee WM, Reuben A. The Natural History of Severe Acute Liver Injury. *Am J Gastroenterol* 2017; **112**: 1389-1396 [PMID: 28440304 DOI: 10.1038/ajg.2017.98]

40   **Antoniades CG**, Berry PA, Wendon JA, Vergani D. The importance of immune dysfunction in determining outcome in acute liver failure. *J Hepatol* 2008; **49**: 845-861 [PMID: 18801592 DOI: 10.1016/j.jhep.2008.08.009]

41   **Yang Q**, Shi Y, He J, Chen Z. The evolving story of macrophages in acute liver failure. *Immunol Lett* 2012; **147**: 1-9 [PMID: 22820147 DOI: 10.1016/j.imlet.2012.07.002]

42   **Yoon E**, Babar A, Choudhary M, Kutner M, Pyrsopoulos N. Acetaminophen-Induced Hepatotoxicity: a Comprehensive Update. *J Clin Transl Hepatol* 2016; **4**: 131-142 [PMID: 27350943 DOI: 10.14218/JCTH.2015.00052]

43   **Tittarelli R**, Pellegrini M, Scarpellini MG, Marinelli E, Bruti V, di Luca NM, Busardò FP, Zaami S. Hepatotoxicity of paracetamol and related fatalities. *Eur Rev Med Pharmacol Sci* 2017; **21**: 95-101 [PMID: 28379590]

44   **Rotundo L**, Pyrsopoulos N. Liver injury induced by paracetamol and challenges associated with intentional and unintentional use. *World J Hepatol* 2020; **12**: 125-136 [PMID: 32685105 DOI: 10.4254/wjh.v12.i4.125]

45   **Krenkel O**, Mossanen JC, Tacke F. Immune mechanisms in acetaminophen-induced acute liver failure. *Hepatobiliary Surg Nutr* 2014; **3**: 331-343 [PMID: 25568858 DOI: 10.3978/j.issn.2304-3881.2014.11.01]

46   **Mossanen JC**, Krenkel O, Ergen C, Govaere O, Liepelt A, Puengel T, Heymann F, Kalthoff S, Lefebvre E, Eulberg D, Luedde T, Marx G, Strassburg CP, Roskams T, Trautwein C, Tacke F. Chemokine (C-C motif) receptor 2-positive monocytes aggravate the early phase of acetaminophen-induced acute liver injury. *Hepatology* 2016; **64**: 1667-1682 [PMID: 27302828 DOI: 10.1002/hep.28682]

47   **Yan M**, Huo Y, Yin S, Hu H. Mechanisms of acetaminophen-induced liver injury and its implications for therapeutic interventions. *Redox Biol* 2018; **17**: 274-283 [PMID: 29753208 DOI: 10.1016/j.redox.2018.04.019]

48   **Guo H**, Chen S, Xie M, Zhou C, Zheng M. The complex roles of neutrophils in APAP-induced liver injury. *Cell Prolif* 2021; **54**: e13040 [PMID: 33942422 DOI: 10.1111/cpr.13040]

49   **Dakhoul L**, Ghabril M, Chalasani N. Drug-induced chronic liver injury. *J Hepatol* 2018; **69**: 248-250 [PMID: 29598958 DOI: 10.1016/j.jhep.2018.01.001]

50   **Ortega-Alonso A**, Stephens C, Lucena MI, Andrade RJ. Case Characterization, Clinical Features and Risk Factors in Drug-Induced Liver Injury. *Int J Mol Sci* 2016; **17** [PMID: 27187363 DOI: 10.3390/ijms17050714]

51   **Vaja R**, Ghuman N. Drugs and the liver. *Anaesth Intensive Care Med* 2018; **19**: 30-34 [DOI: 10.1016/j.mpaic.2020.07.001]

52   **Chang CC**, Petrelli M, Tomashefski JF Jr, McCullough AJ. Severe intrahepatic cholestasis caused by amiodarone toxicity after withdrawal of the drug: a case report and review of the literature. *Arch Pathol Lab Med* 1999; **123**: 251-256 [PMID: 10086516 DOI: 10.5858/1999-123-0251-SICCBA]

53   **Fisher K**, Vuppalanchi R, Saxena R. Drug-Induced Liver Injury. *Arch Pathol Lab Med* 2015; **139**: 876-887 [PMID: 26125428 DOI: 10.5858/arpa.2014-0214-RA]

54   **Fernandes NF**, Martin RR, Schenker S. Trazodone-induced hepatotoxicity: a case report with comments on drug-induced hepatotoxicity. *Am J Gastroenterol* 2000; **95**: 532-535 [PMID: 10685763

DOI: 10.1111/j.1572-0241.2000.t01-1-01780.x]

55   **Oikawa H**, Maesawa C, Sato R, Oikawa K, Yamada H, Oriso S, Ono S, Yashima-Abo A, Kotani K, Suzuki K, Masuda T. Liver cirrhosis induced by long-term administration of a daily low dose of amiodarone: a case report. *World J Gastroenterol* 2005; **11**: 5394-5397 [PMID: 16149155 DOI: 10.3748/wjg.v11.i34.5394]

56   **Raja K**, Thung SN, Fiel MI, Chang C. Drug-induced steatohepatitis leading to cirrhosis: long-term toxicity of amiodarone use. *Semin Liver Dis* 2009; **29**: 423-428 [PMID: 19826976 DOI: 10.1055/s-0029-1240011]

57   **Buggey J**, Kappus M, Lagoo AS, Brady CW. Amiodarone-Induced Liver Injury and Cirrhosis. *ACG Case Rep J* 2015; **2**: 116-118 [PMID: 26157932 DOI: 10.14309/crj.2015.23]

58   **Dees A**. A Late Presentation of Amiodarone -Induced Hepatotoxicity. *MOJ Clin Med Case Rep* 2016; **4**

59   **Tsuda T**, Tada H, Tanaka Y, Nishida N, Yoshida T, Sawada T, Sakata K, Hayashi K, Kawashiri MA, Oyama T, Sasaki M, Kurose N, Yamagishi M. Amiodarone-induced reversible and irreversible hepatotoxicity: two case reports. *J Med Case Rep* 2018; **12**: 95 [PMID: 29653592 DOI: 10.1186/s13256-018-1629-8]

60   **Kocak MZ**. Oral Amiodarone-induced liver Injury, especially Gamma Glutamyl Transferase Elevation: A Case Report. *EJMO* 2018; **2**: 117-119 [DOI: 10.14744/ejmo.2017.82474]

61   **Bratton H**, Alomari M, Al Momani LA, Aasen T, Young M. Prolonged Jaundice Secondary to Amiodarone Use: A Case Report and Literature Review. *Cureus* 2019; **11**: e3850 [PMID: 30891390 DOI: 10.7759/cureus.3850]

62   **Erez N**, Hubel E, Avraham R, Cohen R, Fishman S, Bantel H, Manns M, Tirosh B, Zvibel I, Shibolet O. Hepatic Amiodarone Lipotoxicity Is Ameliorated by Genetic and Pharmacological Inhibition of Endoplasmatic Reticulum Stress. *Toxicol Sci* 2017; **159**: 402-412 [PMID: 28962527 DOI: 10.1093/toxsci/kfx143]

63   **Rutkowski DT**. Liver function and dysfunction - a unique window into the physiological reach of ER stress and the unfolded protein response. *FEBS J* 2019; **286**: 356-378 [PMID: 29360258 DOI: 10.1111/febs.14389]

64   **Mansouri A**, Gattolliat CH, Asselah T. Mitochondrial Dysfunction and Signaling in Chronic Liver Diseases. *Gastroenterology* 2018; **155**: 629-647 [PMID: 30012333 DOI: 10.1053/j.gastro.2018.06.083]

65   **Maiers JL**, Malhi H. Endoplasmic Reticulum Stress in Metabolic Liver Diseases and Hepatic Fibrosis. *Semin Liver Dis* 2019; **39**: 235-248 [PMID: 30912096 DOI: 10.1055/s-0039-1681032]

66   **Simonen P**, Li S, Chua NK, Lampi AM, Piironen V, Lommi J, Sinisalo J, Brown AJ, Ikonen E, Gylling H. Amiodarone disrupts cholesterol biosynthesis pathway and causes accumulation of circulating desmosterol by inhibiting 24-dehydrocholesterol reductase. *J Intern Med* 2020; **288**: 560-569 [PMID: 32415867 DOI: 10.1111/joim.13095]

67   **Allen LB**, Genaro-Mattos TC, Anderson A, Porter NA, Mirnics K, Korade Z. Amiodarone Alters Cholesterol Biosynthesis through Tissue-Dependent Inhibition of Emopamil Binding Protein and Dehydrocholesterol Reductase 24. *ACS Chem Neurosci* 2020; **11**: 1413-1423 [PMID: 32286791 DOI: 10.1021/acschemneuro.0c00042]

68   **Njoku DB**. Drug-induced hepatotoxicity: metabolic, genetic and immunological basis. *Int J Mol Sci* 2014; **15**: 6990-7003 [PMID: 24758937 DOI: 10.3390/ijms15046990]

69   **European Association for the Study of the Liver**. Clinical Practice Guideline Panel: Chair:; Panel members; EASL Governing Board representative:. EASL Clinical Practice Guidelines: Drug-induced liver injury. *J Hepatol* 2019; **70**: 1222-1261 [PMID: 30926241 DOI: 10.1016/j.jhep.2019.02.014]

70   **Hernaez R**, Solà E, Moreau R, Ginès P. Acute-on-chronic liver failure: an update. *Gut* 2017; **66**: 541-553 [PMID: 28053053 DOI: 10.1136/gutjnl-2016-312670]

71   **Blasco-Algora S**, Masegosa-Ataz J, Gutiérrez-García ML, Alonso-López S, Fernández-Rodríguez CM. Acute-on-chronic liver failure: Pathogenesis, prognostic factors and management. *World J Gastroenterol* 2015; **21**: 12125-12140 [PMID: 26576097 DOI: 10.3748/wjg.v21.i42.12125]

72   **Devarbhavi H**, Choudhury AK, Sharma MK, Maiwall R, Al Mahtab M, Rahman S, Chawla YK, Dhiman RK, Duseja A, Taneja S, Ning Q, Jia JD, Duan Z, Yu C, Eapen CE, Goel A, Tan SS, Hamid SS, Butt AS, Jafri W, Kim DJ, Hu J, Sood A, Midha V, Shukla A, Ghazinian H, Sahu MK, Treeprasertsuk S, Lee GH, Lim SG, Lesmana LA, Lesmana CR, Shah S, Kalal C, Abbas Z, Sollano JD, Prasad VGM, Payawal DA, Dokmeci AK, Rao PN, Shrestha A, Lau GK, Yuen MF, Saraswat VA, Shiha G, Yokosuka O, Kedarisetty CK, Jain P, Bhatia P, Sarin SK; APASL ACLF working party. Drug-Induced Acute-on-Chronic Liver Failure in Asian Patients. *Am J Gastroenterol* 2019; **114**: 929-937 [PMID: 31021832 DOI: 10.14309/ajg.0000000000000201]

73   **Wang P**, Pradhan K, Zhong XB, Ma X. Isoniazid metabolism and hepatotoxicity. *Acta Pharm Sin B* 2016; **6**: 384-392 [PMID: 27709007 DOI: 10.1016/j.apsb.2016.07.014]

74   **Metushi I**, Uetrecht J, Phillips E. Mechanism of isoniazid-induced hepatotoxicity: then and now. *Br J Clin Pharmacol* 2016; **81**: 1030-1036 [PMID: 26773235 DOI: 10.1111/bcp.12885]

75   **Khanam A**, Kottilil S. Abnormal Innate Immunity in Acute-on-Chronic Liver Failure: Immunotargets for Therapeutics. *Front Immunol* 2020; **11**: 2013 [PMID: 33117329 DOI: 10.3389/fimmu.2020.02013]

76   **Chen P**, Wang YY, Chen C, Guan J, Zhu HH, Chen Z. The immunological roles in acute-on-chronic liver failure: An update. *Hepatobiliary Pancreat Dis Int* 2019; **18**: 403-411 [PMID: 31303562 DOI: 10.1016/j.hbpd.2019.07.003]

77   **Kamath PS**. Acute on chronic liver failure. *Clin Liver Dis (Hoboken)* 2017; **9**: 86-88 [PMID: 30992966 DOI: 10.1002/cld.625]

78   **Gustot T**, Jalan R. Acute-on-chronic liver failure in patients with alcohol-related liver disease. *J Hepatol* 2019; **70**: 319-327 [PMID: 30658733 DOI: 10.1016/j.jhep.2018.12.008]

79   **Arroyo V**, Moreau R, Kamath PS, Jalan R, Ginès P, Nevens F, Fernández J, To U, García-Tsao G, Schnabl B. Acute-on-chronic liver failure in cirrhosis. *Nat Rev Dis Primers* 2016; **2**: 16041 [PMID: 27277335 DOI: 10.1038/nrdp.2016.41]

Abnormal liver enzymes: A review for clinicians

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Abstract

Liver biochemical tests are some of the most commonly ordered routine tests in the inpatient and outpatient setting, especially with the automatization of testing in this technological era. These tests include aminotransferases, alkaline phosphatase, gamma-glutamyl transferase, bilirubin, albumin, prothrombin time and international normalized ratio (INR). Abnormal liver biochemical tests can be categorized based on the pattern and the magnitude of aminotransferases elevation. Generally, abnormalities in aminotransferases can be classified into a hepatocellular pattern or cholestatic pattern and can be further sub-classified based on the magnitude of aminotransferase elevation to mild [< 5 × upper limit of normal (ULN)], moderate (> 5-< 15 × ULN) and severe (> 15 × ULN). Hepatocellular pattern causes include but are not limited to; non-alcoholic fatty liver disease/non-alcoholic steatohepatitis, alcohol use, chronic viral hepatitis, liver cirrhosis (variable), autoimmune hepatitis, hemochromatosis, Wilson's disease, alpha-1 antitrypsin deficiency, celiac disease, medication-induced and ischemic hepatitis. Cholestatic pattern causes include but is not limited to; biliary pathology (obstruction, autoimmune), other conditions with hyperbilirubinemia (conjugated and unconjugated). It is crucial to interpret these commonly ordered tests accurately as appropriate further workup, treatment and referral can greatly benefit the patient due to prompt treatment which can improve the natural history of several of the diseases mentioned and possibly reduce the risk of progression to the liver cirrhosis.

Key Words: Liver function test; Liver enzymes; Hepatitis; Liver biochemical studies; Non-alcoholic steatohepatitis; Hyperbilirubenemia

Core Tip: Liver function test are one of the most commonly ordered tests. With the automation of test and its inclusion in the complete metabolic profile, the knowledge as it pertains to its interpretation is of paramount importance. It is also important for the clinician to understand the difference between cholestatic and hepatocellular abnormalities. This can be of help for the clinician to formulate appropriate further diagnostic workup and plan the treatment.

INTRODUCTION

Liver biochemical tests are some of the most commonly ordered tests in the United States due to the automation of routine laboratory tests. A United States population-based study of 6823 subjects from 1999 to 2002 showed elevated alanine aminotransferase (ALT) in 8.9% of subjects and aspartate aminotransferase (AST) in 4.9% of subjects.

Another population-based study consisting of 15676 subjects was done from 1988 to 1994 which showed elevation in aminotransferases (either ALT or AST) in 7.9%. In that study, 69% of the elevated aminotransferases results were unexplained[1].

Laboratory tests normal ranges are calculated based on the mean value found amongst a group of healthy individuals +/- 2 standard deviations. Hence 5% of healthy individuals' results lie outside the reference range[2].

As a result of the prevalence of liver biochemical tests ordered and abnormal results, we will be writing this review to increase the knowledge about liver tests to clinicians and improve the interpretation of these tests.

Liver function tests (LFTs) are a term commonly used for aminotransferases, alkaline phosphatase (ALP), bilirubin, and albumin which is somewhat of a misnomer as only bilirubin and albumin represent a synthetic function by the liver[3]. Besides, the liver is crucial in clotting factors production and decreased synthetic function of the liver can result in prothrombin time (PT) prolongation and an increase in the international normalized ratio (INR). Consequently, some of the most widely used scores for predicting mortality in cirrhotic patients such as the Child-Pugh score and model for end stage liver disease-Na (MELD-Na) score do not include AST, ALT, or ALP but rather use INR, bilirubin, and albumin in Child-Pugh score and INR and bilirubin in MELD-Na score.

LIVER BIOCHEMICAL STUDIES

Liver biochemical studies include; ALT, AST, ALP, gamma-glutamyl transferase (GGT), 5'nucleotidase, lactate dehydrogenase (LDH), bilirubin, albumin, PT/INR (Table 1).

Enzymes

ALT is an enzyme that is found primarily in hepatocytes (lower concentrations in cardiac, renal, and muscle tissue) and thus is specific to the hepatocellular injury. ALT levels often fluctuate throughout the d. ALT facilitates the formation of glutamate and pyruvate in the hepatocyte which is important for energy production[4]. The normal range for ALT in males is between 29-33 IU/L and 19-25 IU/L for females.

Table 1 Liver biochemical tests and their respective sites and functions

Interpretation	Test	Site (s)	Function
Hepatocellular integrity	ALT	Hepatocyte (main), cardiac, renal and muscle tissue to smaller extent	Amino acid catabolism. Glutamate and pyruvate production for ATP production
	AST	Hepatocyte, cardiac, muscle and brain tissue	
	LDH	Nonspecific, present widely in the body	Anaerobic glycolysis major enzyme in addition to NADH production. Significant in ischemic hepatitis
Cholestatic pattern	ALP	Hepatobiliary tract, bone, placenta and intestines	Dephosphorylation reactions. Role in bile production
	GGT	Mainly in hepatobiliary tract, present in multiple other organs (nonspecific as an isolate test)	Aids in identification of elevated ALP of biliary origin
	5'nucleotidase	Nonspecific, present widely in the body	Clinical value in hepatobiliary and cholestatic disease specifically when paired with ALP and GGT
	Bilirubin	Serum and liver	End product of heme breakdown. Exists in conjugated and unconjugated form. Elevation in conjugated suggestive of possible cholestasis
Synthetic function	Albumin	Serum	Main protein in the serum, maintains oncotic pressure. Produced by the liver
	PT/INR	Test to measure extrinsic coagulation pathway	Clotting factors primarily produced in the liver. Helpful however does not reflect true coagulation status

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; LDH: Lactate dehydrogenase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyl transferase; ATP: Adenosine-triphosphate; PT: Prothrombin time; INR: International normalized ratio.

ALT levels have been a point of debate recently as newer studies are suggesting the need for a lower ALT cutoff to increase the sensitivity of the test. It's believed that the current ALT cutoffs were defined by using patients with possible underlying subclinical liver disease and hence decrease the sensitivity of the test. A retrospective study in 2002 evaluated 6835 patients and hypothesized that undiagnosed hepatitis C and non-alcoholic fatty liver disease (NAFLD) are likely to have skewed the studies previously used to determine normal ALT levels based on the 95th or the 97.5th percentile.

Suggested new cut-offs from this study are ALT < 30 in men and < 19 in women. It was found that the sensitivity in detecting hepatitis C virus viremia with the lower cut offs was higher than that of the traditional cut-offs. Nonetheless these values should be cautiously interpreted as body mass index, cholesterol levels and age can affect ALT levels[5].

It is important to note that the reference ranges for labs differs across countries and sometimes even between different centers in the same country.

AST is an enzyme which like ALT is also found in the liver however has also other sites where its presence is not as minimal as ALT. These sites are primarily skeletal muscle, cardiac muscle, renal tissue, and brain. It occurs as 2 isoenzymes that are not differentiated on standard testing and hold little clinical value. AST facilitates amino acid metabolism[6]. When it comes to AST, caution must be practiced when evaluating abnormal levels due to its presence in other tissues. The normal range for AST is < 35 IU/L[7].

ALP is an enzyme that is primarily found in the hepatobiliary tract, bone, placenta, and to a smaller extent in intestinal tissue. ALP is involved in multiple dephos-phorylating reactions. The normal range for ALP is between 30-120 IU/L. ALP is generally higher in children and adolescents due to the increased osteoblastic activity associated with the bone growth[8].

GGT is an enzyme that is found in multiple organs in the body including the pancreas, seminal vesicles, kidneys, biliary tract, and liver. Its elevation is usually considered significant for a hepatobiliary disease when accompanied by an elevation in other liver biochemical tests. It is generally elevated in biliary disease, cytochrome-inducing medications, and alcohol abuse. GGT is involved in the glutathione metabolism and production in multiple tissues in the body. Normal GGT levels range between 0-30 IU/L. GGT levels are generally 6-8 times higher in infants[9].

5'nucleotidase is an enzyme that is present in many organs however its clinical value holds significance primarily in hepatobiliary or cholestatic disease. It is generally used as a test to help in evaluating whether an isolated elevated ALP is from a hepatobiliary source *vs* an osseous source. Its primary function is in nucleotide hydrolysis reactions. The normal range for 5'nucleotidase 0.3-3.2 Bodansky units (levels need to be corrected with elevated serum ALP)[10].

LDH is an enzyme that is widely present in the body, it has multiple isoenzymes of which one is primarily excreted/taken up by Kupffer cells in the liver[11]. Hence liver disease/injury can result in elevated LDH. This is non-specific and is rarely used as means of evaluating liver disease. Normal LDH ranges between 140-280 U/L (ranges vary slightly between different labs).

Markers of liver synthetic function

Albumin is one of the major protein constituents in the blood and comprises 50%-60% of total protein in the serum. Albumin synthesis occurs in the liver hence it is considered a marker of the liver's synthetic function. Albumin levels can be influenced by other causes such as systemic inflammation as albumin is a negative inflammatory marker, protein malnutrition, nephrotic syndrome, fluid overload, or protein-losing enteropathy. Albumin has multiple functions such as maintaining serum oncotic pressure and endogenous (*i.e.*, bilirubin) and exogenous (*i.e.*, drugs) substances transport in the blood[12]. Normal albumin levels range between 3.5-5 g/dL.

PT and INR reflect the coagulation cascade and in specific, the extrinsic pathway of the coagulation cascade. The liver is involved in the synthesis of multiple clotting factors including, factors I, II, V, VII, IX, X, XI, and XIII, in addition to protein C, protein S, and anti-thrombin. The reason why PT and INR are primarily elevated rather than activated partial thromboplastin time (aPTT) is due to factor VIII and von Willebrand factor being produced in multiple organs around the body and conceals the aPTT prolongation *in vitro*. Due to deficiency of both pro-coagulant and anticoagulant factors, PT/INR and aPTT are not reliable measures of bleeding risk in cirrhotic patients. Moreover, PT/INR and aPTT are measures of pro-coagulant activity and do not take into consideration defects in anticoagulant pathways. Besides, patients with chronic liver diseases or cirrhosis are likely to have thrombocytopenia due to splenic sequestration and decreased thrombopoietin levels which further increases the risk of bleeding[13].

Bilirubin itself is not a marker of liver synthetic function per se however its excretion and conjugation are closely linked to the liver's conjugating and excreting function. Bilirubin is the end product of heme breakdown and is initially bound to albumin in the serum. In the liver, it is conjugated and excreted in the bile. Elevations in bilirubin levels are further classified as direct hyperbilirubinemia and indirect hyperbilirubinemia. Direct hyperbilirubinemia is generally due to an excretion defect in the liver such as cholestasis or Dubin-Johnson and Rotor syndrome. Indirect hyperbilirubinemia can be due to intrinsic liver injury or hemolysis[14].

PATTERN RECOGNITION AND INTERPRETATION

Pattern recognition and interpretation are crucial in the evaluation of abnormal liver biochemical tests. Patterns can be primarily divided into hepatocellular and cholestatic. These can be subdivided further into; acute (< 6 wk), subacute (6 wk-6 mo), or chronic (> 6 mo).

In hepatocellular pattern, there is a disproportionate rise in ALT and AST in contrast to ALP and GGT. In hepatocellular injury, there is release of aminotransferases from the hepatocytes resulting in elevated serum levels. *R* value is a proposed score aimed to aid physicians in determining the pattern of liver injury based on the upper limit of normal (ULN) of certain enzymes. *R* value = (ALT ÷ ULN ALT)/(ALP ÷ ULN ALP). *R* value > 5 is suggestive of hepatocellular pattern, > 2 to < 5 is suggestive of a mixed pattern, and < 2 suggestive of cholestatic pattern (Table 2)[15].

Hepatocellular pattern

Aminotransferase elevations can be divided into mild, moderate, and severe even though the values for this classification are variable, in this review we will be taking mild as > 2 × - < 5 × ULN lab value, moderate > 5 × - < 15 ×, severe as > 15 × ULN and massive > 10000 IU/L[16]. These values are not accurate measures of the extent of liver injury however can aid in initial workup.

Table 2 R-value calculation and interpretation

R value = (ALT ULN ALT)/(ALP ÷ ULN ALP)

R value	Interpretation
> 5	Hepatocellular pattern
> 2 but < 5	Mixed pattern
< 2	Cholestatic pattern

ALT: Alanine aminotransferase; ULN: Upper limit of normal; ALP: Alkaline phosphatase.

One of the most commonly known and used ratios is AST:ALT and is generally helpful only for an alcoholic liver disease where AST:ALT > 2. A study done in 1979 among patients with histologic evidence of liver disease demonstrated that 90% of patients with AST:ALT > 2 had alcoholic liver disease and > 96% of patients with AST:ALT > 3 had alcoholic liver disease[17]. This ratio can be explained due to alcohol being a mitochondrial toxin and low pyridoxal phosphate absorption as a result of heavy alcohol use. AST is found in mitochondria and cytoplasm, while ALT is found in cytoplasm but not mitochondria. ALT synthesis is more dependent on pyridoxal phosphate when compared to AST. In alcoholic liver disease, ALT is generally < 300 IU/L and is rarely > 500 IU/L. In situations where ALT > 500 IU/L, even if AST: ALT > 2, other etiologies should be explored. AST:ALT > 1 can be seen in cases of liver cirrhosis. GGT > 2 × the ULN is suggestive of alcohol abuse specifically when paired with AST:ALT > 2, GGT on its own is not a specific indicator of alcohol abuse[1].

Mild elevations in aminotransferases are common to be seen in clinical practice and are generally caused by medications (nontoxic ingestions), alcohol use, and chronic liver diseases such as liver cirrhosis, NAFLD, chronic hepatitis infections (B and C), hemochromatosis, Wilson's disease, autoimmune hepatitis, alpha-1 antitrypsin deficiency (AATD) and celiac disease (CD)[16]. It is advisable in patients with a mild increase in AST and ALT to undergo repeat testing in addition to the investigation of the aforementioned causes.

Moderate and severe elevations of aminotransferases are generally attributed to acute exacerbations of chronic liver diseases (such as exacerbations of hepatitis B virus, Wilson's disease, acute viral hepatitis, autoimmune hepatitis), drug-induced liver injury (DILI), and ischemic liver injury[16]. Also, they can occur in cases of acute biliary obstruction and tend to resolve soon after the obstruction is relieved.

Cholestatic pattern

Elevation of ALP and bilirubin levels often indicate a cholestatic pattern[18]. ALP can be elevated in the presence of liver or bone disease, additionally, it can be elevated due to pregnancy (placenta production). GGT is often used to clarify the origin of ALP elevation. Since ALP is produced in the bile duct epithelia, cholestasis or biliary pathology elevates the enzyme. Both anatomic and autoimmune conditions that affect the biliary system cause a cholestatic pattern. When obstruction of the common bile duct (CBD) is the cause of ALP elevation, the aminotransferases can also be elevated [18].

GGT elevation is also caused by biliary or hepatocyte disease but not bone disease. However, other causes may elevate this enzyme such as drugs (anticonvulsants and oral contraceptives), pulmonary and renal disease. As a marker, it has a high sensitivity for liver disease but low specificity[19,20].

Elevations in bilirubin levels are further classified as direct (conjugated) hyperbilirubinemia and indirect (unconjugated) hyperbilirubinemia. Hemolysis is the most common cause of indirect hyperbilirubinemia followed by Gilbert's syndrome. On the other hand, direct hyperbilirubinemia indicates liver pathology including cholestatic drug reactions, autoimmune cholestatic disease, and biliary obstruction[21].

Further laboratory and imaging studies are essential to work up the causes of a cholestatic pattern[18]. When autoimmune cholestatic liver disease is suspected the presence of anti-neutrophil cytoplasmic antibodies (for primary sclerosing cholangitis) or anti-mitochondrial antibodies (for primary biliary cirrhosis) among other studies help aid in the diagnosis.

COMMON CONDITIONS ASSOCIATED WITH ABNORMAL LIVER ENZYMES

NAFLD is one of the most common liver diseases, a meta-analysis was done in 2016 demonstrated the global prevalence of NAFLD to be approximately 25.24%[22]. Common condition associated with abnormal liver enzyme is shown in Table 3.

Nonalcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH) are diseases in the same spectrum where NAFL can progress to NASH and subsequently liver cirrhosis if no intervention or modification of risk factors was done[23]. These terms are often used interchangeably however it is important to note that the management is different and accurate assessment should be made. The difference between the two is primarily seen on histology as NAFL has only fatty infiltration without inflammation whereas NASH has marked inflammation. AST and ALT levels can be normal in NAFL and are generally mildly elevated in NASH (ALT > AST). NAFL and NASH are diseases of exclusion and general risk factors are metabolic, such as obesity, dyslipidemia, and diabetes mellitus[23]. It is important to note that NAFL is generally reversible with lifestyle modifications in contrast to NASH (Table 4).

Viral hepatitis can result in a mild increase in aminotransferases, specifically chronic viral hepatitis. Hepatitis B and Hepatitis C infections can generally cause chronic infections and also have a risk for developing liver cirrhosis. In a study done in 1988, patients with chronic viral hepatitis without liver cirrhosis had an AST:ALT < 1 (0.59 average), however those with chronic viral hepatitis and liver cirrhosis had an AST:ALT > 1. This was found to be significant and is important to identify in cases of chronic viral hepatitis to aid in recognizing possible concomitant liver cirrhosis[24]. Nonetheless, caution must be practiced when looking at AST:ALT specifically when alcohol use cannot be excluded. Acute viral hepatitis on the other hand can result in moderate to severe elevation in aminotransferases, often with ALT elevations higher than that of AST. Acute hepatitis C virus can result in marked elevations in aminotransferases however generally the elevation is modest compared to hepatitis A and B. Acutely, elevation in aminotransferases levels peak before bilirubin levels, however, begins declining gradually after in contrast to bilirubin[25]. Acute hepatitis A and B in adults are associated with elevations in bilirubin resulting in jaundice (more common with hepatitis A infection) and ALP. The risk of progression to chronic hepatitis is approximately 10% in hepatitis B patients above the age of 6, hepatitis A is not associated with chronic infection[26].

Hereditary hemochromatosis is an autosomal recessive disease caused by over absorption of iron secondary to abnormal iron sensing in the gastrointestinal tract resulting in iron overload[27]. The 2 most common mutations identified are C282Y and H63D on the hemochromatosis (*HFE*) gene. Non-*HFE* hemochromatosis exists, however in this review we will talk only about *HFE* hemochromatosis.

Hemochromatosis causes mild elevations in aminotransferases (ALT > AST), elevations in ALP and bilirubin can also be seen however liver biochemical tests are non-specific in cases of hemochromatosis[27]. Bilirubin elevation is thought to be a protective mechanism to help mitigate oxidative damage caused by excess iron in the liver. Moreover, a study done in 2004 demonstrated that bilirubin level elevation was found to have a positive correlation with serum iron level[28]. In cases of elevated aminotransferases without a clear cause, it would be wise to check iron studies including iron level, ferritin level, total iron-binding capacity, and transferrin saturation. If results suggestive of iron overload, genetic testing and liver biopsy should be considered.

Wilson's disease is an autosomal recessive disease due to mutations in the ATP7B gene with a prevalence of approximately 1:30000 worldwide, studies have suggested higher prevalence based on gene mutation frequency. The difference between the 2 reported prevalence could be related to the disease's possible low penetrance[29]. Wilson's disease liver presentation is variable and can be from asymptomatic elevation in aminotransferases to acute liver failure (ALF). Aminotransferase elevation is mild in the majority of cases however can be moderate to severe in patients with Wilson's presenting with ALF. 6%-12% of emergent liver transplant referrals are due to Wilson's disease ALF[30]. Markers that aid in the diagnosis of ALF secondary to Wilson's disease are non-immune hemolytic anemia, acute renal failure, AST:ALT > 2.2, and ALP: Bilirubin < 4. Almost all patients presenting with ALF secondary to Wilson's have underlying liver fibrosis or cirrhosis[31,32].

AATD is an autosomal co-dominant disease with an expected prevalence of 3.4 million globally with combinations for severe AATD[33]. However, this number is thought to be under-representative of the actual prevalence[33]. A study done in 1989

Table 3 Common condition with abnormal liver biochemical tests

Condition	AST/ALT	ALP	GGT	Bilirubin	Other
Alcoholic hepatitis	↑↑ AST:ALT > 2	↑	↑	↑	AST/ALT < 500
NAFLD	-/↑ ALT > AST	-/Mild ↑	-/Mild ↑	↑ If progress to cirrhosis	-
Viral hepatitis	↑↑ In acute/↑ in chronic	↑	↑	↑ In chronic	AST:ALT > 1 suggestive of cirrhosis
Hemochromatosis	↑ ALT > AST	↑	↑	↑ Higher levels = higher iron load	↑ Ferritin and transferrin saturation
Wilson's disease	↑/↑↑↑ AST:ALT > 2.2 in ALF	↑	↑	↑	ALP:Bilirubin < 4
AATD	↑ AST > ALT	-	-	-	-
Celiac disease	↑ ALT > AST	-	-	-	-
Autoimmune hepatitis	↑↑	↑	↑	↑	ALP:AST/ALT < 3
DILI	↑↑/↑↑	↑	↑	↑	↑ PT/INR
Cholestasis	↑	↑↑	↑↑	↑	AST:ALT < 1.5 – ExtrahepaticAST:ALT > 1.5 - Intrahepatic

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyl transferase; NAFLD: Non-alcohol fatty liver disease; AATD: Alpha-1 antitrypsin deficiency; DILI: Drug induced liver injury; PT: Prothrombin time; INR: International normalized ratio.

Table 4 Non-alcoholic fatty liver disease spectrum

Non-alcoholic fatty liver disease spectrum		
NAFL	Steatosis changes. No cellular ballooning, hepatocyte inflammation or fibrosis	Prevalence of 25% approximately. Reversible
NASH	Steatosis changes. Cellular ballooning and hepatocyte inflammation. No fibrosis	Prevalence of 1.5%-6.45% approximately. Generally irreversible (has been found to be reversible in some patients)
NASH related liver cirrhosis	Hepatocyte destruction and fibrosis	Prevalence of 1%-2% approximately. Irreversible
Healthy liver ←→ NAFL → NASH → NASH related cirrhosis		

NAFL: Non-alcoholic fatty liver; NASH: Non-alcoholic steatohepatitis.

in St. Louis examined 20000 blood bank samples, 700 blood samples came back positive for homozygous PI∗Z mutation, however, only 28 of those individuals have been diagnosed with AATD[34]. AATD involves multiple alleles however the alleles thought to be contributing to liver disease are M (maltron) and Z allele. In adults with homozygous PI∗Z mutation, 40% were found to have evidence of injury and cirrhosis histologically. Aminotransferases are generally mildly elevated with ALT predominance. Bilirubin levels are elevated in later stages (cirrhosis) along with a decrease in albumin[35].

CD is an autoimmune disease characterized by gluten intolerance which often leads to malabsorption. A study was done where 158 adults recently diagnosed with CD were followed, 42% of patients were found to have mild elevations in aminotransferases. Patients were started on a gluten-free diet and in 95% of cases, the aminotransferases levels normalized at 1 year[36]. Another study was done evaluating patients with chronically elevated aminotransferases, workup on those patients revealed that 9.3% of patients had serological evidence of CD and all but one of the 9.3% had duodenal biopsy findings of CD[37]. Aminotransferases elevation is mild with an AST:ALT < 1, bilirubin levels are generally normal. ALP can be slightly elevated in a subset of patients but is generally normal. Albumin and PT/INR values are not very reliable indicators of hepatic synthetic function in cases of CD as CD is an autoimmune disease, and a state of inflammation could cause a decrease in albumin levels. Moreover, PT/INR values can be elevated due to concomitant vitamin K deficiency secondary to malabsorption[38].

Autoimmune hepatitis is an inflammatory disorder with a female predilection and a prevalence of approximately 1:5000-1:10000 in Europe. At the time of diagnosis, almost 50% of patients have jaundice and approximately 30% have cirrhosis[39,40]. Autoimmune hepatitis affects aminotransferases variably depending on acute *vs* chronic presentations. Acutely, elevations in aminotransferases can be moderate to severe and tend to gradually decline as the disease becomes chronic and/or liver cirrhosis ensues. Bilirubin, ALP, and gamma globulins elevations are also seen in autoimmune hepatitis. ALP:AST or ALT ratio < 3 which is calculated by using the following equation (ALP/ALP ULN)/(AST/AST ULN) (ALT can be used in place of AST for this calculation) and this ratio is thought to be helpful as disproportionate elevation of ALP should prompt exploration of other differentials such as primary biliary cholangitis[41]. Furthermore, it was found that patients with higher elevations in aminotransferases had a better prognosis when compared to those with milder aminotransferase elevations[42].

DILI can cause a multitude of effects on aminotransferases and elevations of aminotransferases can be mild, moderate, or severe. A wide range of medications can cause mild elevations of aminotransferases and those include antibiotics (such as amoxicillin-clavulanic acid, macrolides (cholestatic pattern), ceftriaxone), anticonvulsants (such as Carbamazepine, Phenytoin, Valproic acid, Gabapentin), statins, anti-tuberculosis medications, and herbal supplements. Hence, a thorough history of medication history is crucial in patients with elevated aminotransferases. More commonly, DILI is ALT predominant.

Drugs can also be a cause of moderate to severe aminotransferase elevation with the most commonly implicated drug being acetaminophen. Acetaminophen is advertised as safe with a daily dose < 4000 mg/d[43]. Acetaminophen-induced hepatotoxicity has a prevalence of approximately 30000 cases a year in the United States[44]. Up to 50% of overdoses were found to be unintentional[44]. Studies have been done which showed 6% of acetaminophen prescriptions to be > 4000 mg/d. A study evaluating AST:ALT ratio found that in cases of severe toxicity, an AST:ALT < 0.4 is suggestive of resolving hepatitis and is a positive prognostic marker[45]. Bilirubin, ALP, and PT/INR can all rise in cases of acetaminophen overdose. It is important to note that aminotransferases generally rise 2-3 d after an initial overdose and that an initial normal liver biochemical test does not exclude acetaminophen toxicity[45].

Acute cholecystitis (AC) usually presents as a cholestatic pattern or mixed. The biochemical test abnormalities are associated with obstruction from CBD, reactive hepatitis, fatty liver, direct gallbladder pressure on the biliary tract, or portal tract inflammation[19-21]. Patients with calculous AC may have CBD stones in up to 15% [17]. Gallbladder ultrasound and computed tomography (CT) is not entirely reliable for the diagnosis of CBD stones. Therefore, LFTs may be used for the identification of patients with suspected CBD stones who would benefit from endoscopic retrograde cholangiopancreatography (ERCP) or magnetic resonance cholangiopancreatography (MRCP) which are more sensitive and specific for this condition[18]. Multiple studies have shown mean values of LFTs higher in patients with AC plus CBD stones[18,22]. Bilirubin, AST, ALP, and GGT are the variables mostly studied to predict CBD stones. Ahn *et al*[18] found GGT to be the most reliable variable for CBD stones prediction with a sensitivity of 80.6% and specificity of 75%. Another study found an elevation in ALP to be the most important predictor for CBD stones[21]. Elevated LFTs in patients with AC without CBD stones are more likely to be transient and resolve within 2-7 d after surgery[18].

Ischemic hepatitis (often also referred to as hypoxic liver injury, shock liver, and hypoxic hepatitis) is a clinical condition characterized by acute liver injury causing severe elevation of aminotransferases secondary to hypoperfusion with a prevalence of approximately 2:1000 admissions and 2.5:100 in intensive care unit admissions. Moreover, it was found that approximately 4 out of 10 admissions with severe elevations in aminotransferases had ischemic hepatitis diagnosis. After further analysis, 78.2% of patients with ischemic hepatitis had a preceding acute cardiac event, 23.4% of patients with ischemic hepatitis had a diagnosis of sepsis and 52.9% of patients had a documented episode of hypotension (unspecified duration)[46].

The aminotransferase elevation is generally severe with level > 75 × ULN being suggestive of ischemic hepatitis, AST:ALT > 1 usually due to the location of AST (zone 3) in the liver and ischemic effect on zone 3. Bilirubin rise is not uncommon yet it can bemild and typically < 3 mg/dL. ALP is usually normal and PT/INR can be mildly elevated[47]. Another ratio that was found to be useful is AST:LDH < 1.5 which helps in differentiating ischemic hepatitis from viral hepatitis[48]. The AST:LDH ratio is thought to be due to the rapid and severe rise of LDH in cases of ischemic hepatitis due to hypoperfusion.

ALF is another potential cause of severe elevation in aminotransferases and cautious identification of this condition is crucial as mortality risk is approximately 40%-80% [49]. ALF is defined as the presence of severe liver injury in addition to clinical and laboratory features of liver failure such as hepatic encephalopathy and elevation in INR specifically in an individual with no prior history of liver cirrhosis or liver disease. Etiologies of ALF include but are not limited to Ischemic hepatitis, Budd Chiari syndrome, Wilson's disease, autoimmune hepatitis, acute viral hepatitis, and drug-induced liver disease. Biochemical test evaluation in ALF can be hepatocellular initially and progress to cholestatic in later stages. Labs are typically significant for severe elevation in aminotransferases, mild to moderate elevation in bilirubin and ALP in addition to INR ≥ 1.5, and in some cases LDH elevation[49]. While declining aminotransferases can be suggestive of recovery, this is not an accurate measure of recovery as it could be indicative of worsening liver failure and severe loss of liver mass. It is more appropriate to follow bilirubin, INR, and clinical features (hepatic encephalopathy) in patients with ALF for possible recovery[49].

DIAGNOSTIC TESTS

The initial evaluation of abnormal biochemical tests will be guided by the pattern (hepatocellular, cholestatic, or mixed). As a first step, the clinician should inquire about the use of medication, herbal therapies, drugs, or alcohol consumption. If a hepatocellular pattern is identified, initial serology should be obtained to rule out infectious and autoimmune etiologies. A right upper quadrant ultrasound (RUQ US) is also justified to evaluate for fatty liver. If the previous workup is unrevealing uncommon causes should be worked up (such as Wilson disease, AATD, *etc.*). If the serologic studies and imaging are unremarkable and ALT/AST is persistently elevated, consider a liver biopsy. When ALP is elevated, GGT and 5' nucleotidase tests are important to identify the source of ALP elevation. If the latter is elevated ALP likely is elevated from hepatobiliary origin. The RUQ US will help to identify ductal dilation or the absence of it. Further workup includes either an MRCP or an ERCP (when ductal dilation is present) or serological studies including AMA if no dilation is identified. Cholestasis can be further divided into intrahepatic or extrahepatic both usually seen with marked elevation of ALP. The workup for extrahepatic cholestasis should aim to rule out choledocholithiasis, malignant obstruction, and biliary strictures. For intrahepatic cholestasis, laboratory works up should aim to rule out primary biliary cholangitis, primary sclerosing cholangitis, sickle cell disease among other causes. In intrahepatic cholestasis imaging or laboratory, workup may not yield a definitive diagnosis and other causes should be considered (*i.e.*, total parenteral nutrition, drugs associated with cholestasis, ischemic, cholestasis of pregnancy, *etc.*)

CONCLUSION

The elevation of liver biochemical studies is a common encounter of all clinicians. The multiple markers used to identify liver injury may be also elevated due to other sources (bone, placenta, kidney, muscle, *etc.*). The biochemical knowledge helps to better understand the behavior of these markers in specific conditions. The proper recognition of hepatocellular or cholestatic pattern prompts further investigations that include imaging and laboratory studies. Other factors highly important to consider when evaluating abnormal liver biochemical patterns are signs and symptoms, medications, degree of liver tests elevation, and other laboratory abnormalities present. Unfortunately, despite the use of additional tests (imaging and laboratory) in some causes the diagnostic is unclear and liver biopsy is recommended.

REFERENCES

1   **Ioannou GN**, Boyko EJ, Lee SP. The prevalence and predictors of elevated serum aminotransferase activity in the United States in 1999-2002. *Am J Gastroenterol* 2006; **101**: 76-82 [PMID: 16405537 DOI: 10.1111/j.1572-0241.2005.00341.x]
2   **Pratt DS**, Kaplan MM. Evaluation of abnormal liver-enzyme results in asymptomatic patients. *N Engl J Med* 2000; **342**: 1266-1271 [PMID: 10781624 DOI: 10.1056/NEJM200004273421707]
3   **Hall P**, Cash J. What is the real function of the liver 'function' tests? *Ulster Med J* 2012; **81**: 30-36

[PMID: 23536736]

4   **Aulbach AD**, Amuzie CJ.   Chapter 17 - Biomarkers in Nonclinical Drug Development. In: Faqi AS. A Comprehensive Guide to Toxicology in Nonclinical Drug Development (Second Edition). Second Edition. Boston: Academic Press, 2017: 447-471

5   **Prati D**, Taioli E, Zanella A, Della Torre E, Butelli S, Del Vecchio E, Vianello L, Zanuso F, Mozzi F, Milani S, Conte D, Colombo M, Sirchia G. Updated definitions of healthy ranges for serum alanine aminotransferase levels. *Ann Intern Med* 2002; **137**: 1-10 [PMID: 12093239 DOI: 10.7326/0003-4819-137-1-200207020-00006]

6   **Sparling DW**.   Chapter 3 - Bioindicators of Contaminant Exposure. In: Sparling DW. Ecotoxicology Essentials. San Diego: Academic Press, 2016: 45-66

7   **Pagana K**, Pagana T.   Mosby's Diagnostic & Laboratory Test Reference. 14th ed. St. Louis, MO: Elsevier

8   **Lowe D**, Sanvictores T, John S.   Alkaline Phosphatase. In: StatPearls. Treasure Island. StatPearls Publishing, 2021

9   **Cabrera-Abreu JC**, Green A. Gamma-glutamyltransferase: value of its measurement in paediatrics. *Ann Clin Biochem* 2002; **39**: 22-25 [PMID: 11853185 DOI: 10.1258/0004563021901685]

10   **YOUNG II**. Serum 5-nucleotidase; characterization and evaluation in disease states. *Ann N Y Acad Sci* 1958; **75**: 357-362 [PMID: 13627835 DOI: 10.1111/j.1749-6632.1958.tb36883.x]

11   **Smit MJ**, Duursma AM, Bouma JM, Gruber M. Receptor-mediated endocytosis of lactate dehydrogenase M4 by liver macrophages: a mechanism for elimination of enzymes from plasma. Evidence for competition by creatine kinase MM, adenylate kinase, malate, and alcohol dehydrogenase. *J Biol Chem* 1987; **262**: 13020-13026 [PMID: 2820961]

12   **Moman RN**, Gupta N, Varacallo M.   Physiology, Albumin. In: StatPearls. Treasure Island. StatPearls Publishing, 2021

13   **Thachil J**. Relevance of clotting tests in liver disease. *Postgrad Med J* 2008; **84**: 177-181 [PMID: 18424573 DOI: 10.1136/pgmj.2007.066415]

14   **Gopal DV**, Rosen HR. Abnormal findings on liver function tests. Interpreting results to narrow the diagnosis and establish a prognosis. *Postgrad Med* 2000; **107**: 100-102, 105 [PMID: 10689411 DOI: 10.3810/pgm.2000.02.869]

15   **Chalasani NP**, Hayashi PH, Bonkovsky HL, Navarro VJ, Lee WM, Fontana RJ; Practice Parameters Committee of the American College of Gastroenterology. ACG Clinical Guideline: the diagnosis and management of idiosyncratic drug-induced liver injury. *Am J Gastroenterol* 2014; **109**: 950-66; quiz 967 [PMID: 24935270 DOI: 10.1038/ajg.2014.131]

16   **Green RM**, Flamm S. AGA technical review on the evaluation of liver chemistry tests. *Gastroenterology* 2002; **123**: 1367-1384 [PMID: 12360498 DOI: 10.1053/gast.2002.36061]

17   **Cohen JA**, Kaplan MM. The SGOT/SGPT ratio--an indicator of alcoholic liver disease. *Dig Dis Sci* 1979; **24**: 835-838 [PMID: 520102 DOI: 10.1007/BF01324898]

18   **Ahn KS**, Yoon YS, Han HS, Cho JY. Use of Liver Function Tests as First-line Diagnostic Tools for Predicting Common Bile Duct Stones in Acute Cholecystitis Patients. *World J Surg* 2016; **40**: 1925-1931 [PMID: 27094560 DOI: 10.1007/s00268-016-3517-y]

19   **Chang CW**, Chang WH, Lin CC, Chu CH, Wang TE, Shih SC. Acute transient hepatocellular injury in cholelithiasis and cholecystitis without evidence of choledocholithiasis. *World J Gastroenterol* 2009; **15**: 3788-3792 [PMID: 19673021 DOI: 10.3748/wjg.15.3788]

20   **Thapa PB**, Maharjan DK, Suwal B, Byanjankar B, Singh DR. Serum gamma glutamyl transferase and alkaline phosphatase in acute cholecystitis. *J Nepal Health Res Counc* 2010; **8**: 78-81 [PMID: 21876567]

21   **Zgheib H**, Wakil C, Shayya S, Mailhac A, Al-Taki M, El Sayed M, Tamim H. Utility of liver function tests in acute cholecystitis. *Ann Hepatobiliary Pancreat Surg* 2019; **23**: 219-227 [PMID: 31501809 DOI: 10.14701/ahbps.2019.23.3.219]

22   **Younossi ZM**, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* 2016; **64**: 73-84 [PMID: 26707365 DOI: 10.1002/hep.28431]

23   **Chalasani N**, Younossi Z, Lavine JE, Charlton M, Cusi K, Rinella M, Harrison SA, Brunt EM, Sanyal AJ. The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. *Hepatology* 2018; **67**: 328-357 [PMID: 28714183 DOI: 10.1002/hep.29367]

24   **Williams AL**, Hoofnagle JH. Ratio of serum aspartate to alanine aminotransferase in chronic hepatitis. Relationship to cirrhosis. *Gastroenterology* 1988; **95**: 734-739 [PMID: 3135226 DOI: 10.1016/s0016-5085(88)80022-2]

25   **Giannini EG**, Testa R, Savarino V. Liver enzyme alteration: a guide for clinicians. *CMAJ* 2005; **172**: 367-379 [PMID: 15684121 DOI: 10.1503/cmaj.1040752]

26   **Thuener J**. Hepatitis A and B Infections. *Prim Care* 2017; **44**: 621-629 [PMID: 29132524 DOI: 10.1016/j.pop.2017.07.005]

27   **Lin E**, Adams PC. Biochemical liver profile in hemochromatosis. A survey of 100 patients. *J Clin Gastroenterol* 1991; **13**: 316-320 [PMID: 2066547 DOI: 10.1097/00004836-199106000-00013]

28   **Alizadeh BZ**, Njajou OT, Houwing-Duistermaat JJ, de Jong G, Vergeer JM, Hofman A, Pols HA, van Duijn CM. Does bilirubin protect against hemochromatosis gene (HFE) related mortality? *Am J Med Genet A* 2004; **129A**: 39-43 [PMID: 15266614 DOI: 10.1002/ajmg.a.30163]

29   **Coffey AJ**, Durkie M, Hague S, McLay K, Emmerson J, Lo C, Klaffke S, Joyce CJ, Dhawan A,

Hadzic N, Mieli-Vergani G, Kirk R, Elizabeth Allen K, Nicholl D, Wong S, Griffiths W, Smithson S, Giffin N, Taha A, Connolly S, Gillett GT, Tanner S, Bonham J, Sharrack B, Palotie A, Rattray M, Dalton A, Bandmann O. A genetic study of Wilson's disease in the United Kingdom. *Brain* 2013; **136**: 1476-1487 [PMID: 23518715 DOI: 10.1093/brain/awt035]

30   **European Association for Study of Liver**. EASL Clinical Practice Guidelines: Wilson's disease. *J Hepatol* 2012; **56**: 671-685 [PMID: 22340672 DOI: 10.1016/j.jhep.2011.11.007]

31   **Korman JD**, Volenberg I, Balko J, Webster J, Schiodt FV, Squires RH Jr, Fontana RJ, Lee WM, Schilsky ML; Pediatric and Adult Acute Liver Failure Study Groups. Screening for Wilson disease in acute liver failure: a comparison of currently available diagnostic tests. *Hepatology* 2008; **48**: 1167-1174 [PMID: 18798336 DOI: 10.1002/hep.22446]

32   **Schilsky ML**. Wilson Disease: Diagnosis, Treatment, and Follow-up. *Clin Liver Dis* 2017; **21**: 755-767 [PMID: 28987261 DOI: 10.1016/j.cld.2017.06.011]

33   **de Serres FJ**. Worldwide racial and ethnic distribution of alpha1-antitrypsin deficiency: summary of an analysis of published genetic epidemiologic surveys. *Chest* 2002; **122**: 1818-1829 [PMID: 12426287 DOI: 10.1378/chest.122.5.1818]

34   **Silverman EK**, Miletich JP, Pierce JA, Sherman LA, Endicott SK, Broze GJ Jr, Campbell EJ. Alpha-1-antitrypsin deficiency. High prevalence in the St. Louis area determined by direct population screening. *Am Rev Respir Dis* 1989; **140**: 961-966 [PMID: 2679271 DOI: 10.1164/ajrccm/140.4.961]

35   **Bals R**. Alpha-1-antitrypsin deficiency. *Best Pract Res Clin Gastroenterol* 2010; **24**: 629-633 [PMID: 20955965 DOI: 10.1016/j.bpg.2010.08.006]

36   **Bardella MT**, Fraquelli M, Quatrini M, Molteni N, Bianchi P, Conte D. Prevalence of hypertransaminasemia in adult celiac patients and effect of gluten-free diet. *Hepatology* 1995; **22**: 833-836 [PMID: 7657290]

37   **Bardella MT**, Vecchi M, Conte D, Del Ninno E, Fraquelli M, Pacchetti S, Minola E, Landoni M, Cesana BM, De Franchis R. Chronic unexplained hypertransaminasemia may be caused by occult celiac disease. *Hepatology* 1999; **29**: 654-657 [PMID: 10051464 DOI: 10.1002/hep.510290318]

38   **Rubio-Tapia A**, Murray JA. Liver involvement in celiac disease. *Minerva Med* 2008; **99**: 595-604 [PMID: 19034257]

39   **Ngu JH**, Bechly K, Chapman BA, Burt MJ, Barclay ML, Gearry RB, Stedman CA. Population-based epidemiology study of autoimmune hepatitis: a disease of older women? *J Gastroenterol Hepatol* 2010; **25**: 1681-1686 [PMID: 20880179 DOI: 10.1111/j.1440-1746.2010.06384.x]

40   **Werner M**, Prytz H, Ohlsson B, Almer S, Björnsson E, Bergquist A, Wallerstedt S, Sandberg-Gertzén H, Hultcrantz R, Sangfelt P, Weiland O, Danielsson A. Epidemiology and the initial presentation of autoimmune hepatitis in Sweden: a nationwide study. *Scand J Gastroenterol* 2008; **43**: 1232-1240 [PMID: 18609163 DOI: 10.1080/00365520802130183]

41   **Alvarez F**, Berg PA, Bianchi FB, Bianchi L, Burroughs AK, Cancado EL, Chapman RW, Cooksley WG, Czaja AJ, Desmet VJ, Donaldson PT, Eddleston AL, Fainboim L, Heathcote J, Homberg JC, Hoofnagle JH, Kakumu S, Krawitt EL, Mackay IR, MacSween RN, Maddrey WC, Manns MP, McFarlane IG, Meyer zum Büschenfelde KH, Zeniya M. International Autoimmune Hepatitis Group Report: review of criteria for diagnosis of autoimmune hepatitis. *J Hepatol* 1999; **31**: 929-938 [PMID: 10580593 DOI: 10.1016/s0168-8278(99)80297-9]

42   **Al-Chalabi T**, Underhill JA, Portmann BC, McFarlane IG, Heneghan MA. Effects of serum aspartate aminotransferase levels in patients with autoimmune hepatitis influence disease course and outcome. *Clin Gastroenterol Hepatol* 2008; **6**: 1389-95; quiz 1287 [PMID: 18840547 DOI: 10.1016/j.cgh.2008.08.018]

43   **Yoon E**, Babar A, Choudhary M, Kutner M, Pyrsopoulos N. Acetaminophen-Induced Hepatotoxicity: a Comprehensive Update. *J Clin Transl Hepatol* 2016; **4**: 131-142 [PMID: 27350943 DOI: 10.14218/JCTH.2015.00052]

44   **Blieden M**, Paramore LC, Shah D, Ben-Joseph R. A perspective on the epidemiology of acetaminophen exposure and toxicity in the United States. *Expert Rev Clin Pharmacol* 2014; **7**: 341-348 [PMID: 24678654 DOI: 10.1586/17512433.2014.904744]

45   **McGovern AJ**, Vitkovitsky IV, Jones DL, Mullins ME. Can AST/ALT ratio indicate recovery after acute paracetamol poisoning? *Clin Toxicol (Phila)* 2015; **53**: 164-167 [PMID: 25652258 DOI: 10.3109/15563650.2015.1006399]

46   **Tapper EB**, Sengupta N, Bonder A. The Incidence and Outcomes of Ischemic Hepatitis: A Systematic Review with Meta-analysis. *Am J Med* 2015; **128**: 1314-1321 [PMID: 26299319 DOI: 10.1016/j.amjmed.2015.07.033]

47   **Lightsey JM**, Rockey DC. Current concepts in ischemic hepatitis. *Curr Opin Gastroenterol* 2017; **33**: 158-163 [PMID: 28346236 DOI: 10.1097/MOG.0000000000000355]

48   **Cassidy WM**, Reynolds TB. Serum lactic dehydrogenase in the differential diagnosis of acute hepatocellular injury. *J Clin Gastroenterol* 1994; **19**: 118-121 [PMID: 7963356 DOI: 10.1097/00004836-199409000-00008]

49   **Gill RQ**, Sterling RK. Acute liver failure. *J Clin Gastroenterol* 2001; **33**: 191-198 [PMID: 11500606 DOI: 10.1097/00004836-200109000-00005]

MINIREVIEWS

Hepatopulmonary syndrome: An update

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Abstract

Hepatopulmonary syndrome (HPS) is characterized by defects in oxygenation caused by intra-pulmonary vasodilation occurring because of chronic liver disease, portal hypertension, or congenital portosystemic shunts. Clinical implications of portal hypertension are very well-known, however, awareness of its effect on multiple organs such as the lungs are less known. The presence of HPS in chronic liver disease is associated with increased mortality. Medical therapies available for HPS have not been proven effective and definitive treatment for HPS is mainly liver transplantation (LT). LT improves mortality for patients with HPS drastically. This article provides a review on the definition, clinical presentation, diagnosis, and management of HPS.

Key Words: Hepatopulmonary syndrome; Chronic liver disease; Hypoxemia; Intrapulmonary vasodilatation; Liver failure

Core Tip: Hepatopulmonary syndrome (HPS) is a progressive disease, the presence of which in cirrhotic patients worsens their prognosis. Patients with HPS have an increase rate of mortality compared to those without HPS when matched for severity of liver disease, age, sex, and liver transplantation (LT). HPS should be identified in all patients with chronic liver disease and supportive management should be provided until definitive treatment, *e.g.*, LT could be done.

INTRODUCTION

HPS is a progressive disease associated with worsen prognosis in patients with chronic liver disease. Patients with HPS have an increase rate of mortality compared to those without HPS when matched for severity of liver disease, age, sex, and liver transplantation (LT)[1]. Hepatopulmonary syndrome (HPS) was first described in 1884 by Fluckiger based on observation in a woman with cyanosis, clubbing, and cirrhosis. Later, HPS was coined in 1977 after multiple post-mortem studies showing pulmonary vascular dilation in cirrhotic patients. These studies showed marked peripheral dilation of pulmonary arteries at precapillary and capillary levels, without any obvious lung parenchymal disease. These studies were also remarkable for multiple pleural spider naevi[2].

DEFINITION

HPS is defined as hypoxemia due to pulmonary vascular dilation in the setting of liver disease with or without portal hypertension. Definition and staging of HPS are shown in Table 1 and Table 2.

INCIDENCE/PREVALENCE

HPS has been reported in 5%-35% of patients with end-stage liver disease[3,4]. Studies have shown the presence of HPS in various liver etiologies including cirrhosis, non-cirrhotic portal fibrosis, and extra-hepatic portal vein obstruction[5,6]. Studies showed an increasing prevalence of intrapulmonary shunt in patients with increased severity of cirrhotic disease such as pretransplant patients with Child-Pugh Class C when compared with class A or B[7]. It has also been found to be associated with liver disease severity assessed by MELD score[3].

PATHOPHYSIOLOGY

Chronic liver disease can lead to hypoxemia due to a variety of underlying pathologies. Thus, it is imperative to differentiate between them. For example, HPS is caused by pulmonary vasodilation in the setting of liver disease whereas Portopulmonary hypertension, which is very similar in clinical presentation, is defined by pulmonary vasoconstriction causing hypoxemia due to resultant pulmonary hypertension.

The hypoxemia associated with HPS is secondary ventilation-perfusion mismatch caused mainly by diffusion defect in the dilated pulmonary bed: (1) Increased blood flow through the intra-pulmonary vasodilatation (IPVD) through the well-ventilated alveoli results in the passage of mixed venous blood in the pulmonary veins; and (2) Diffusion of oxygen is limited through the dilated pulmonary vessels due to their increased diameters resulting in disequilibrium. Supplemental oxygen increases the partial pressure of oxygen by providing the driving pressure for the oxygen to diffuse across the dilated vessels. Thus, IPVDs act as physiologic shunts more than anatomic shunts as oxygenation improves with external supplementation[8].

The unique pathological feature of HPS is dilatation of pulmonary precapillary and capillary vessels (15-100 μm diameter) along with an absolute increase in the number of dilated vessels. Paraumbilical vein and hepatic artery diameters are significant larger in cirrhotic patients with HPS compared to non-HPS[9]. Lungs and pleural spider nevi are the terms used when these vessels are noted in the lungs and along the pleural surface. Intrahepatic vasculature changes which were reported in HPS include thrombosis in intrahepatic portal venules, fibrous septa with vessels proliferation, and

Table 1 Hepatopulmonary syndrome definition

Index	
Oxygenation	PaO2 < 80 mmHg or A-a gradient (corrected for age) > 15 mmHg or 20 mmHg if age > 64 years while breathing room air
Intrapulmonary vasodilation	Confirmed by contrast-enhance echocardiography or lung perfusion scanning showing brain shunt fraction > 6%
Liver disease	Cirrhosis and/or portal hypertension

Table 2 Staging based on severity of hepatopulmonary syndrome

Stage	Partial pressure of oxygen (mmHg) on room air
Mild	≥ 80
Moderate	≥ 60 to < 80
Severe	≥ 50 to < 60
Very severe	< 50 on room air or < 300 while breathing 100% oxygen

centrilobular venous thickening[9]. Doppler ultrasonography in HPS reveals hepato-jugular flow and portal blood flow of less than 10 cm/s[9].

The underlying pathophysiology is not fully proven, however, is thought to be caused by loss of pulmonary capillary vessel tone and inhibition of pulmonary vasoconstrictors. Enhanced production of nitric oxide (NO) is the major factor for pulmonary vasodilatation. NO is produced by the action of NO synthase on l-arginine. NO synthase had three isoforms of which endothelial NO synthase (eNOS) produced by pulmonary endothelial cells is the major source of NO production[10].

In experimental rat models of HPS with common bile duct ligation, proliferating cholangiocytes produces endothelin-1 (ET-1) which activates pulmonary vascular endothelin-B (ETB) receptor which in turn mediates eNOS activation and pulmonary macrophages accumulation. These animal models also showed overall increased expression of ETB receptors and increased circulation of ET-1[11,12].

In humans with HPS, exhaled NO is elevated which is a result of pulmonary vascular production and it normalizes after LT[13,14]. Acute administration of methylene blue, an inhibitor of NOS, transiently improves oxygenation[15].

Bacterial translocation from the gut in the setting of portal hypertension results in pulmonary vascular macrophages has been proposed as a mechanism causing pulmonary vasodilatation[16,17]. A study shows the decrease in this bacterial translocation by norfloxacin and thus, decreasing the severity of HPS[18]. Heme-oxygenase-derived carbon monoxide and tumor necrosis factor-alpha are also observed to contribute to pulmonary vasodilatation and angiogenesis[19,20].

CLINICAL PRESENTATION

Dyspnea on exertion or rest is the most common presenting symptom of HPS. However, dyspnea is very non-specific given it can be present in chronic liver disease due to ascites, volume overload, anemia, or muscle weakness. The presence of platypnea and orthodeoxia are specific for HPS, but not pathognomonic. Platypnea means dyspnea in an upright position which is relieved in the supine position. Orthodeoxia refers to a decrease in partial pressure of oxygen by greater than 4 mmHg or a decrease in oxygen saturation by more than 5% from a supine to upright position [21]. Both platypnea and orthodeoxia are attributed to the ventilation-perfusion mismatch.

Physical signs such as the presence of spider nevi, clubbing, cyanosis along hypoxia are strongly suggestive of HPS. Of these signs, patients with the chronic liver disease having spider nevi have a higher prevalence of HPS compared to those without spider nevi[22].

DIAGNOSIS

Patients with chronic liver disease who has dyspnea, or signs of clubbing, cyanosis, spider nevi should undergo screening and evaluation for HPS. All patients who are candidates for LT are also screened for HPS. Evaluation of HPS includes assessment of hypoxemia and intrapulmonary vasodilation. Exhaled NO is found to be higher in HPS than non-HPS patients which may help with the diagnosis.

ASSESSMENT FOR HYPOXEMIA

Pulse oximetry is used for screening purposes in chronic liver diseases to assess for HPS. All the patients with oxygen saturation < 96% should further undergo arterial blood gas analysis (ABG) to evaluate for underlying hypoxemia[23]. ABG should be drawn in the upright position to evaluate for orthodeoxia. A-a gradient > 15 mmHg or PaO_2 < 80 mmHg is used for evaluation of hypoxemia. A-a gradient is more reliable than the partial pressure of oxygen as it accounts for hyperventilation, which is common in chronic liver disease[24].

The establishment of hypoxemia alone is not enough for the diagnosis of HPS, as it can be seen in other diseases such as Porto-pulmonary hypertension. Diagnosis requires confirmation of intrapulmonary vasodilation.

ASSESSMENT FOR INTRAPULMONARY VASCULAR DILATATIONS

Transthoracic contrast echocardiography (TTCE) is first-line diagnostic tool for IPVDs. IPVDs create a shut wherein 5%-6% of the cardiac output gets shunted. TTCE is performed by injecting the agitated saline into the venous system during the echocardiogram. Agitated saline leads to the formation of bubbles in the right atrium which is then filtered by the pulmonary capillary bed. Pulmonary capillary diameter varies from 8 to 15 μm which does not allow the passage of the microbubbles. The presence of intra-cardiac or intra-pulmonary shunt leads to visualization of microbubbles/contrast in the left heart chambers. The timing of the appearance of these bubbles in the left atrium varies with heart rate, cardiac output, and shunt size. With the intra-pulmonary shunt, the microbubbles or opacification of the left atrium occurs in three to six cardiac cycles after their first appearance in the right atrium. Whereas with the intra-cardiac shunt, this opacification of the left atrium is visualized within the first three cardiac cycles after its first appearance in the right atrium. Thus, TTCE is a sensitive tool for the diagnosis of pulmonary shunt[25].

Transesophageal echocardiography is a more specific alternative to TTCE, however, is generally avoided due to the high risk associated with bleeding from esophageal varices in this patient population[26].

Technetium-99m-labeled macro aggregated albumin is also filtered by the pulmonary capillary bed and can be used to measure shunt fraction by identifying its uptake in the brain and/or kidneys. Under normal circumstances, macro aggregated albumin should not pass the pulmonary capillary bed. However, in presence of right-to-left shunt, the radionuclide is taken up by the brain and kidneys and the percentage uptake can be used to quantify the shunt. In contrast to TTCE, this method does not distinguish between intra-pulmonary and intra-cardiac shunts[27].

Contrast pulmonary angiography is rarely used to visualize the IPVD due to the invasive nature of this procedure. It is generally indicated in patients with suspicion for pulmonary arteriovenous malformations, which rarely occurs in HPS[28]. Contrast-enhanced triple phase multi-detector computed tomography abdominal portosystemic shunts of more than 10 mm in diameter[9].

MANAGEMENT

LT

The only definitive management for HPS is LT. All the patients with the partial pressure of oxygen less than 60 mmHg should be evaluated for LT. Mortality is significantly higher in patients with HPS who do not undergo LT compared to those who undergo LT. A study showed 78% mortality in HPS patients who did not undergo LT compared to 21% mortality in patients who underwent LT[29]. Thus,

patients with HPS are given higher priority for liver transplants compared to other factors. LT has been shown to improve oxygenation and shunt within the first year of transplant[30,31]. A retrospective study with 74 patients showed improvement in PaO_2 from 89% to 94% and a decrease in A-a gradient from 16 to 8 mmHg after transplantation, without significant change in DLCO[32]. A study showed a 76% 5-year survival rate in HPS who underwent LT, which is similar to liver transplant patients without HPS[33].

Oxygen supplementation

All the patients with mild to moderate HPS should be evaluated every 3 to 6 mo with ABG. All patients with oxygen saturation less than 89% or partial pressure of oxygen less than 55 mmHg at rest, exercise and while sleep should be provided supplemental oxygen.

Investigational therapies

Pentoxifylline, a tumor necrosis factor-alpha inhibitor, vasodilator with anti-angiogenesis, showed variable results in oxygenation improvement in HPS[34-36]. Early-stage HPS patients seem to have a favorable outcome, while patients with advanced-stage HPS had unimproved oxygenation and difficulty tolerating pentoxi-fylline due to gastrointestinal adverse effects. Randomized placebo-controlled trial is needed to prove its result.

Garlic, has allicin which is a potent vasodilator and anti-angiogenesis. It shows significant improvement in gas exchange in small studies, which include one randomized controlled trial[37,38]. Large trials are still required to prove its benefit. Inhaled NO, a vasodilator, showed an improvement of PaO_2 in a recent physiologic study even though prior findings were contradicting[39,40]. Vascular dilatations, pulmonary capillary arteriovenous communication, and blood flow shunting in HPS are thought to be more prominent in lower lung zones due to gravitation and the vasodilators use in HPS are believed to be more potent in upper and mid lung zones. Therefore, ventilation-perfusion mismatch decreased.

Methylene blue causes vasoconstriction by inhibiting NO and may also decrease angiogenesis. It has shown some benefits in improving oxygenation; however, no randomized clinical trial is available to support its use[15]. Another agent that has been shown to reduce pulmonary NO is N(G)-nitro-L-arginine methyl ester. However, it didn't improve arterial oxygenation or ventilation-perfusion mismatch[41].

Sorafenib is a tyrosine kinase inhibitor that can reduce angiogenesis. It significantly decreased alveolar-arterial oxygen gradient in rat model but failed to show benefit in patients with HPS in a randomized-controlled trial[42]. Octreotide, a somatostatin analogue that can inhibit angiogenesis, also showed no benefit in HPS patients in few studies[43].

Mycophenolate mofetil only showed benefit in one case report[44]. Norfloxacin decreases bacterial translocation and reveals benefit in an animal study and a human case report but not in a randomized controlled trial[45]. Other medications including iloprost (vasodilator), paroxetine (NO synthase inhibitor), almitrine bismesylate (pulmonary vasoconstrictor) have been tried without any clear benefit. Letrozole is undergoing an ongoing phase two trial.

The transjugular intrahepatic portosystemic shunt has been proposed to decrease portal hypertension in HPS. A small prospective study showed improvement in gas exchanged, but limited data are available[46,47]. Few case reports regarding embolization of pulmonary vasodilatation have shown improvement in oxygen[28]. All these studies do not have clear establish benefits.

CONCLUSION

All the patients with chronic liver disease with dyspnea should be screened for HPS using ABG. There is no definitive proven treatment plan for HPS except LT. Thus, all patients with HPS should undergo expedited evaluation of LT.

REFERENCES

1   **Yi HM**, Wang GS, Yi SH, Yang Y, Cai CJ, Chen GH. Prospective evaluation of postoperative outcome after liver transplantation in hepatopulmonary syndrome patients. *Chin Med J (Engl)* 2009;

122: 2598-2602 [PMID: 19951576]

2   **Rodríguez-Roisin R**, Agustí AG, Roca J. The hepatopulmonary syndrome: new name, old
       complexities. *Thorax* 1992; **47**: 897-902 [PMID: 1465744 DOI: 10.1136/thx.47.11.897]

3   **Ferreira PP**, Camara EJ, Paula RL, Zollinger CC, Cavalcanti AR, Bittencourt PL. Prevalence of
       hepatopulmonary syndrome in patients with decompensated chronic liver disease and its impact on
       short-term survival. *Arq Gastroenterol* 2008; **45**: 34-37 [PMID: 18425226 DOI:
       10.1590/s0004-28032008000100007]

4   **Lv Y**, Fan D. Hepatopulmonary Syndrome. *Dig Dis Sci* 2015; **60**: 1914-1923 [PMID: 25732713 DOI:
       10.1007/s10620-015-3593-0]

5   **Gupta D**, Vijaya DR, Gupta R, Dhiman RK, Bhargava M, Verma J, Chawla YK. Prevalence of
       hepatopulmonary syndrome in cirrhosis and extrahepatic portal venous obstruction. *Am J
       Gastroenterol* 2001; **96**: 3395-3399 [PMID: 11774955 DOI: 10.1111/j.1572-0241.2001.05274.x]

6   **Anand AC**, Mukherjee D, Rao KS, Seth AK. Hepatopulmonary syndrome: prevalence and clinical
       profile. *Indian J Gastroenterol* 2001; **20**: 24-27 [PMID: 11206870]

7   **Lee JM**, Choi MS, Lee SC, Park SW, Bae MH, Lee JH, Koh KC, Paik SW, Rhee PL, Kim JJ, Rhee
       JC. [Prevalence and risk factors of significant intrapulmonary shunt in cirrhotic patients awaiting liver
       transplantation]. *Taehan Kan Hakhoe Chi* 2002; **8**: 271-276 [PMID: 12499784]

8   **Davis HH 2nd**, Schwartz DJ, Lefrak SS, Susman N, Schainker BA. Alveolar-capillary oxygen
       disequilibrium in hepatic cirrhosis. *Chest* 1978; **73**: 507-511 [PMID: 630968 DOI:
       10.1378/chest.73.4.507]

9   **Lejealle C**, Paradis V, Bruno O, de Raucourt E, Francoz C, Soubrane O, Lebrec D, Bedossa P, Valla
       D, Mal H, Vilgrain V, Durand F, Rautou PE. Evidence for an Association Between Intrahepatic
       Vascular Changes and the Development of Hepatopulmonary Syndrome. *Chest* 2019; **155**: 123-136
       [PMID: 30292761 DOI: 10.1016/j.chest.2018.09.017]

10   **Rolla G**. Hepatopulmonary syndrome: role of nitric oxide and clinical aspects. *Dig Liver Dis* 2004;
       36: 303-308 [PMID: 15191196 DOI: 10.1016/j.dld.2003.12.016]

11   **Fallon MB**, Abrams GA, McGrath JW, Hou Z, Luo B. Common bile duct ligation in the rat: a model
       of intrapulmonary vasodilatation and hepatopulmonary syndrome. *Am J Physiol* 1997; **272**: G779-
       G784 [PMID: 9142908 DOI: 10.1152/ajpgi.1997.272.4.G779]

12   **Zhang J**, Ling Y, Tang L, Luo B, Pollock DM, Fallon MB. Attenuation of experimental
       hepatopulmonary syndrome in endothelin B receptor-deficient rats. *Am J Physiol Gastrointest Liver
       Physiol* 2009; **296**: G704-G708 [PMID: 19196949 DOI: 10.1152/ajpgi.90627.2008]

13   **Rolla G**, Brussino L, Colagrande P, Scappaticci E, Morello M, Bergerone S, Ottobrelli A, Cerutti E,
       Polizzi S, Bucca C. Exhaled nitric oxide and impaired oxygenation in cirrhotic patients before and
       after liver transplantation. *Ann Intern Med* 1998; **129**: 375-378 [PMID: 9735065 DOI:
       10.7326/0003-4819-129-5-199809010-00005]

14   **Rolla G**, Brussino L, Colagrande P, Dutto L, Polizzi S, Scappaticci E, Bergerone S, Morello M,
       Marzano A, Martinasso G, Salizzoni M, Bucca C. Exhaled nitric oxide and oxygenation abnormalities
       in hepatic cirrhosis. *Hepatology* 1997; **26**: 842-847 [PMID: 9328302 DOI:
       10.1053/jhep.1997.v26.pm0009328302]

15   **Schenk P**, Madl C, Rezaie-Majd S, Lehr S, Müller C. Methylene blue improves the hepatopulmonary
       syndrome. *Ann Intern Med* 2000; **133**: 701-706 [PMID: 11074903 DOI:
       10.7326/0003-4819-133-9-200011070-00012]

16   **Zhang HY**, Han DW, Su AR, Zhang LT, Zhao ZF, Ji JQ, Li BH, Ji C. Intestinal endotoxemia plays a
       central role in development of hepatopulmonary syndrome in a cirrhotic rat model induced by
       multiple pathogenic factors. *World J Gastroenterol* 2007; **13**: 6385-6395 [PMID: 18081228 DOI:
       10.3748/wjg.v13.i47.6385]

17   **Sztrymf B**, Libert JM, Mougeot C, Lebrec D, Mazmanian M, Humbert M, Herve P. Cirrhotic rats
       with bacterial translocation have higher incidence and severity of hepatopulmonary syndrome. *J
       Gastroenterol Hepatol* 2005; **20**: 1538-1544 [PMID: 16174071 DOI:
       10.1111/j.1440-1746.2005.03914.x]

18   **Rabiller A**, Nunes H, Lebrec D, Tazi KA, Wartski M, Dulmet E, Libert JM, Mougeot C, Moreau R,
       Mazmanian M, Humbert M, Hervé P. Prevention of gram-negative translocation reduces the severity
       of hepatopulmonary syndrome. *Am J Respir Crit Care Med* 2002; **166**: 514-517 [PMID: 12186830
       DOI: 10.1164/rccm.200201-027OC]

19   **Arguedas MR**, Drake BB, Kapoor A, Fallon MB. Carboxyhemoglobin levels in cirrhotic patients
       with and without hepatopulmonary syndrome. *Gastroenterology* 2005; **128**: 328-333 [PMID:
       15685544 DOI: 10.1053/j.gastro.2004.11.061]

20   **Luo B**, Liu L, Tang L, Zhang J, Ling Y, Fallon MB. ET-1 and TNF-alpha in HPS: analysis in
       prehepatic portal hypertension and biliary and nonbiliary cirrhosis in rats. *Am J Physiol Gastrointest
       Liver Physiol* 2004; **286**: G294-G303 [PMID: 14715521 DOI: 10.1152/ajpgi.00298.2003]

21   **Gómez FP**, Martínez-Pallí G, Barberà JA, Roca J, Navasa M, Rodríguez-Roisin R. Gas exchange
       mechanism of orthodeoxia in hepatopulmonary syndrome. *Hepatology* 2004; **40**: 660-666 [PMID:
       15349905 DOI: 10.1002/hep.20358]

22   **Younis I**, Sarwar S, Butt Z, Tanveer S, Qaadir A, Jadoon NA. Clinical characteristics, predictors, and
       survival among patients with hepatopulmonary syndrome. *Ann Hepatol* 2015; **14**: 354-360 [PMID:
       25864216]

23   **Deibert P**, Allgaier HP, Loesch S, Müller C, Olschewski M, Hamm H, Maier KP, Blum HE.
       Hepatopulmonary syndrome in patients with chronic liver disease: role of pulse oximetry. *BMC*

Gastroenterol 2006; **6**: 15 [PMID: 16638132 DOI: 10.1186/1471-230X-6-15]

24   **Voiosu A**, Voiosu T, Stănescu CM, Chirilă L, Băicuș C, Voiosu R. Novel predictors of intrapulmonary vascular dilatations in cirrhosis: extending the role of pulse oximetry and echocardiography. *Acta Gastroenterol Belg* 2013; **76**: 241-245 [PMID: 23898563]

25   **Abrams GA**, Jaffe CC, Hoffer PB, Binder HJ, Fallon MB. Diagnostic utility of contrast echocardiography and lung perfusion scan in patients with hepatopulmonary syndrome. *Gastroenterology* 1995; **109**: 1283-1288 [PMID: 7557096 DOI: 10.1016/0016-5085(95)90589-8]

26   **Aller R**, Moya JL, Moreira V, Boixeda D, Cano A, Picher J, García-Rull S, de Luis DA. Diagnosis of hepatopulmonary syndrome with contrast transesophageal echocardiography: advantages over contrast transthoracic echocardiography. *Dig Dis Sci* 1999; **44**: 1243-1248 [PMID: 10389704 DOI: 10.1023/a:1026657114256]

27   **Mimidis KP**, Vassilakos PI, Mastorakou AN, Spiropoulos KV, Lambropoulou-Karatza CA, Thomopoulos KC, Tepetes KN, Nikolopoulou VN. Evaluation of contrast echocardiography and lung perfusion scan in detecting intrapulmonary vascular dilatation in normoxemic patients with early liver cirrhosis. *Hepatogastroenterology* 1998; **45**: 2303-2307 [PMID: 9951913]

28   **Ryu JK**, Oh JH. Hepatopulmonary syndrome: angiography and therapeutic embolization. *Clin Imaging* 2003; **27**: 97-100 [PMID: 12639774 DOI: 10.1016/s0899-7071(02)00511-9]

29   **Swanson KL**, Wiesner RH, Krowka MJ. Natural history of hepatopulmonary syndrome: Impact of liver transplantation. *Hepatology* 2005; **41**: 1122-1129 [PMID: 15828054 DOI: 10.1002/hep.20658]

30   **Eriksson LS**, Söderman C, Ericzon BG, Eleborg L, Wahren J, Hedenstierna G. Normalization of ventilation/perfusion relationships after liver transplantation in patients with decompensated cirrhosis: evidence for a hepatopulmonary syndrome. *Hepatology* 1990; **12**: 1350-1357 [PMID: 2258151 DOI: 10.1002/hep.1840120616]

31   **Gupta S**, Castel H, Rao RV, Picard M, Lilly L, Faughnan ME, Pomier-Layrargues G. Improved survival after liver transplantation in patients with hepatopulmonary syndrome. *Am J Transplant* 2010; **10**: 354-363 [PMID: 19775311 DOI: 10.1111/j.1600-6143.2009.02822.x]

32   **Battaglia SE**, Pretto JJ, Irving LB, Jones RM, Angus PW. Resolution of gas exchange abnormalities and intrapulmonary shunting following liver transplantation. *Hepatology* 1997; **25**: 1228-1232 [PMID: 9141442 DOI: 10.1002/hep.510250527]

33   **Deberaldini M**, Arcanjo AB, Melo E, da Silva RF, Felício HC, Arroyo PC Jr, Duca WJ, Cordeiro JA, da Silva RC. Hepatopulmonary syndrome: morbidity and survival after liver transplantation. *Transplant Proc* 2008; **40**: 3512-3516 [PMID: 19100426 DOI: 10.1016/j.transproceed.2008.08.134]

34   **Zhang J**, Ling Y, Tang L, Luo B, Chacko BK, Patel RP, Fallon MB. Pentoxifylline attenuation of experimental hepatopulmonary syndrome. *J Appl Physiol (1985)* 2007; **102**: 949-955 [PMID: 17110505 DOI: 10.1152/japplphysiol.01048.2006]

35   **Gupta LB**, Kumar A, Jaiswal AK, Yusuf J, Mehta V, Tyagi S, Tempe DK, Sharma BC, Sarin SK. Pentoxifylline therapy for hepatopulmonary syndrome: a pilot study. *Arch Intern Med* 2008; **168**: 1820-1823 [PMID: 18779471 DOI: 10.1001/archinte.168.16.1820]

36   **Tanikella R**, Philips GM, Faulk DK, Kawut SM, Fallon MB. Pilot study of pentoxifylline in hepatopulmonary syndrome. *Liver Transpl* 2008; **14**: 1199-1203 [PMID: 18668653 DOI: 10.1002/lt.21482]

37   **Abrams GA**, Fallon MB. Treatment of hepatopulmonary syndrome with Allium sativum L. (garlic): a pilot trial. *J Clin Gastroenterol* 1998; **27**: 232-235 [PMID: 9802451 DOI: 10.1097/00004836-199810000-00010]

38   **De BK**, Dutta D, Pal SK, Gangopadhyay S, Das Baksi S, Pani A. The role of garlic in hepatopulmonary syndrome: a randomized controlled trial. *Can J Gastroenterol* 2010; **24**: 183-188 [PMID: 20352147 DOI: 10.1155/2010/349076]

39   **Jounieaux V**, Leleu O, Mayeux I. Cardiopulmonary effects of nitric oxide inhalation and methylene blue injection in hepatopulmonary syndrome. *Intensive Care Med* 2001; **27**: 1103-1104 [PMID: 11497151 DOI: 10.1007/s001340100967]

40   **Gupta S**, Tang R, Al-Hesayen A. Inhaled nitric oxide improves the hepatopulmonary syndrome: a physiologic analysis. *Thorax* 2021; **76**: 1142-1145 [PMID: 33859047 DOI: 10.1136/thoraxjnl-2020-216128]

41   **Gómez FP**, Barberà JA, Roca J, Burgos F, Gistau C, Rodríguez-Roisin R. Effects of nebulized N(G)-nitro-L-arginine methyl ester in patients with hepatopulmonary syndrome. *Hepatology* 2006; **43**: 1084-1091 [PMID: 16628648 DOI: 10.1002/hep.21141]

42   **Kawut SM**, Ellenberg SS, Krowka MJ, Goldberg D, Vargas H, Koch D, Sharkoski T, Al-Naamani N, Fox A, Brown R, Levitsky J, Oh JK, Lin G, Song N, Mottram C, Doyle MF, Kaplan DE, Gupta S, Fallon MB. Sorafenib in Hepatopulmonary Syndrome: A Randomized, Double-Blind, Placebo-Controlled Trial. *Liver Transpl* 2019; **25**: 1155-1164 [PMID: 30816637 DOI: 10.1002/lt.25438]

43   **Söderman C**, Juhlin-Dannfelt A, Lagerstrand L, Eriksson LS. Ventilation-perfusion relationships and central haemodynamics in patients with cirrhosis. Effects of a somatostatin analogue. *J Hepatol* 1994; **21**: 52-57 [PMID: 7963422 DOI: 10.1016/s0168-8278(94)80136-3]

44   **Moreira Silva H**, Reis G, Guedes M, Cleto E, Vizcaíno JR, Kelly D, Gennery AR, Santos Silva E. A case of hepatopulmonary syndrome solved by mycophenolate mofetil (an inhibitor of angiogenesis and nitric oxide production). *J Hepatol* 2013; **58**: 630-633 [PMID: 23104163 DOI: 10.1016/j.jhep.2012.10.021]

45   **Gupta S**, Faughnan ME, Lilly L, Hutchison S, Fowler R, Bayoumi AM. Norfloxacin therapy for hepatopulmonary syndrome: a pilot randomized controlled trial. *Clin Gastroenterol Hepatol* 2010; **8**:

1095-1098 [PMID: 20816858 DOI: 10.1016/j.cgh.2010.08.011]

46   **Tsauo J**, Zhao H, Zhang X, Ma H, Jiang M, Weng N, Li X. Effect of Transjugular Intrahepatic Portosystemic Shunt Creation on Pulmonary Gas Exchange in Patients with Hepatopulmonary Syndrome: A Prospective Study. *J Vasc Interv Radiol* 2019; **30**: 170-177 [PMID: 30717947 DOI: 10.1016/j.jvir.2018.09.017]

47   **Lasch HM**, Fried MW, Zacks SL, Odell P, Johnson MW, Gerber DA, Sandhu FS, Fair JH, Shrestha R. Use of transjugular intrahepatic portosystemic shunt as a bridge to liver transplantation in a patient with severe hepatopulmonary syndrome. *Liver Transpl* 2001; **7**: 147-149 [PMID: 11172400 DOI: 10.1053/jlts.2001.21287]

MINIREVIEWS

Mitochondrial hepatopathy: Respiratory chain disorders- 'breathing in and out of the liver'

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Abstract

Mitochondria, the powerhouse of a cell, are closely linked to the pathophysiology of various common as well as not so uncommon disorders of the liver and beyond. Evolution supports a prokaryotic descent, and, unsurprisingly, the organelle is worthy of being labeled an organism in itself. Since highly metabolically active organs require a continuous feed of energy, any dysfunction in the structure and function of mitochondria can have variable impact, with the worse end of the spectrum producing catastrophic consequences with a multisystem predisposition. Though categorized a hepatopathy, mitochondrial respiratory chain defects are not limited to the liver in time and space. The liver involvement is also variable in clinical presentation as well as in age of onset, from acute liver failure, cholestasis, or chronic liver disease. Other organs like eye, muscle, central and peripheral nervous system, gastrointestinal tract, hematological, endocrine, and renal systems are also variably involved. Diagnosis hinges on recognition of subtle clinical clues, screening metabolic investigations, evaluation of the extrahepatic involvement, and role of genetics and tissue diagnosis. Treatment is aimed at both circumventing the acute metabolic crisis and long-term management including nutritional rehabilitation. This review lists and discusses the burden of mitochondrial respiratory chain defects, including various settings when to suspect, their evolution with time, including certain specific disorders, their tiered evaluation with diagnostic algorithms, management dilemmas, role of liver transplantation, and the future research tools.

Key Words: Mitochondrial hepatopathy; Respiratory chain defects; Maternal inheritance; Neonatal liver failure; DNA depletion syndrome; Pearson syndrome

Core Tip: Liver disease with multi-system involvement should arouse the suspicion for mitochondrial respiratory chain hepatopathies. These disorders are predominantly autosomal recessive with some having a maternal inheritance. Presence of lactic acidosis without hypoglycemia is an important clue. A tiered evaluation yields the most data, with the final step being a genetic and enzyme analysis from tissue of interest. Treatment is largely supportive with blood transfusions, correction of acidosis and shock, providing cofactors and salvage therapies, with liver transplantation in a select group. A periodic follow-up is mandatory for monitoring evolution of disease including "migration" to other systems.

INTRODUCTION

Mitochondria are intracellular organelles, with a double lamellar covering outer membrane serving as a corset that holds the highly convoluted inner membrane in place (Figure 1). The inter-membrane space is the first of two liquid components within the mitochondria mainly participating in the exchange of lipids, proteins, and metal ions and also signaling cascades[1]. The second space is the soluble matrix, lying within the inner membrane and the hub of various metabolically active processes, most notably the tricarboxylic acid cycle, fatty acid oxidation, and urea synthesis. The inner membrane is folded into multiple cristae, which are shelf like projections into the matrix. The number of cristae are reflective of a metabolically active state, with a more active tissue having numerous mitochondria with many cristae, an ideal comparison being striated muscle tissue against adipocytes. This inner membrane of the mitochondria houses the respiratory chain comprised of electron carriers (complexes I, II, III, and IV, cytochrome c, coenzyme Q) and complex V, which is the hydrogen adenosine triphosphatases complex (Figure 2). All metabolic processes within the matrix generate reducing equivalents in the form of electrons (carried as NADPH2), which pass through these complexes, entering it at various points. While doing so, from one complex to another, it also results in proton (H^+) flow from matrix to intermembrane space leading to its pooling up and a chemical gradient that then flows down the potential *via* the complex V, which utilizes the energy to generate adenosine triphosphate from adenosine diphosphate, the ultimate objective of this intricately woven complex process called oxidative phosphorylation[2].

MITOCHONDRIAL GENOME AND ITS IMPLICATIONS

From the perspective of evolution, the classical endosymbiont theory proposes that mitochondria are actually prokaryotes within eukaryotic cells and hence have a genome of their own[3]. Mitochondrial genome consists of a circular double stranded DNA made of 16569 base pairs organized to make up 37 genes. Of these, 13 genes are exclusively for synthesis of proteins that are part of the respiratory chain. The other 24 genes are required for mitochondrial DNA (mtDNA) translation process (22 genes for an equal number of transfer RNA and two for ribosomal RNA synthesis). There are three major differences between mitochondrial and Mendelian inheritance: Maternal inheritance, heteroplasmy and threshold effect, and mitotic segregation. Maternal inheritance in simple terms means that the mtDNA and its aberrations are transferred from mother (ovum) to its offspring (zygote), as there is hardly any mitochondria left in the sperm, which concentrates itself to fill its entire cytoplasm with the energy dense nucleus. However, there are a few exceptions, as reported in skeletal muscle defects linked to mitochondrial inheritance that are transmitted by father to offspring[4]. It is essential to understand that all characteristics encoded by mtDNA are maternally inherited but all mitochondrial diseases are not maternally inherited.
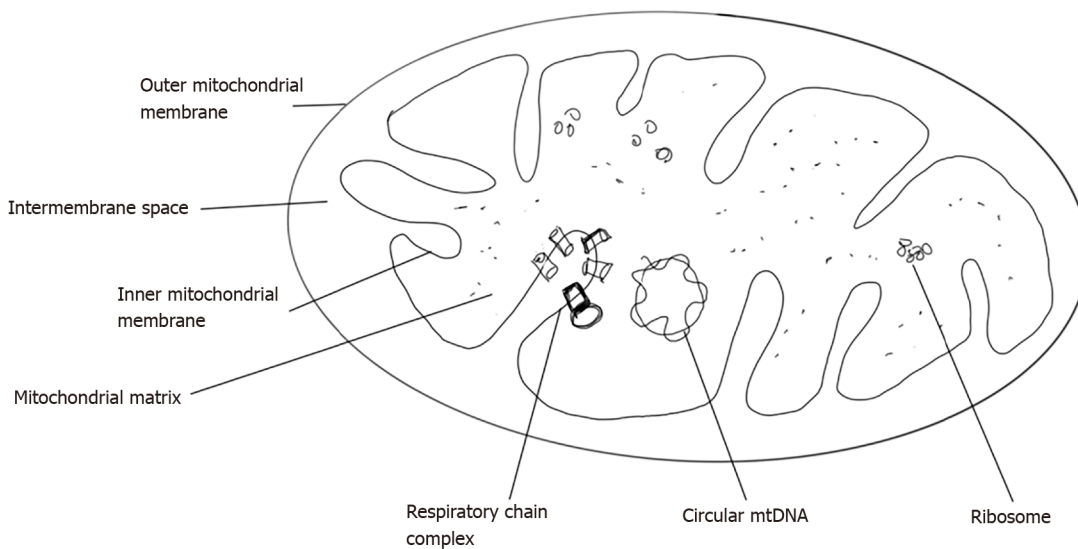
Figure 1 Diagrammatic representation of structure of mitochondria. mtDNA: Mitochondrial DNA.
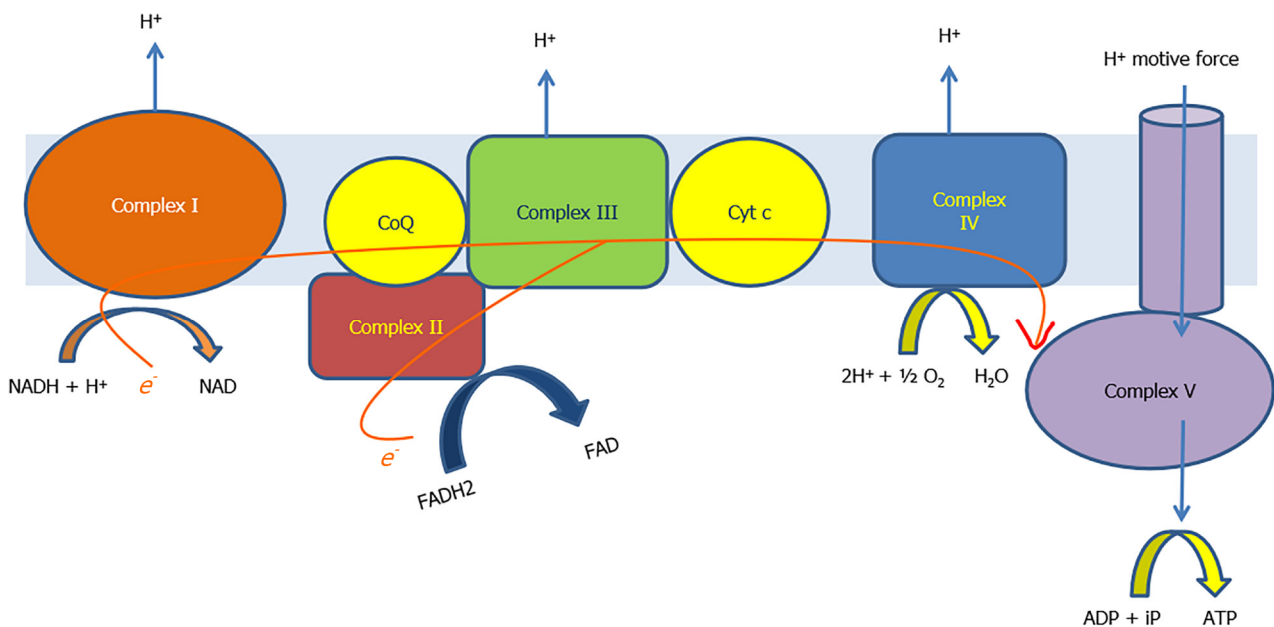
Figure 2 The electron transport chain formed by the respiratory chain complexes and process of oxidative phosphorylation. ADP: Adenosine diphosphate; ATP: Adenosine triphosphate; CoQ: Coenzyme Q; Cyt c: Cytochrome c; FAD: Flavin adenine dinucleotide; FADH2: Reduced form of FAD; NAD: Nicotinamide adenine dinucleotide; NADH: Reduced form of NAD.

Nuclear DNA (nuDNA) encodes most of the metabolic processes occurring in the mitochondria. NuDNA also encodes many enzymes and cofactors required for maintenance of mtDNA as well as approximately 70 respiratory chain subunits[5]. Figures 3 and 4 describe the way of inheritance and the mathematics of genetics in mitochondrial diseases. In normal persons, all mtDNA are identical, a state known as homoplasmy. Presence of both mutated and non-mutated wild type mtDNA containing mitochondria together in a cell is cellular heteroplasmy, while having 2 types of mtDNA within a single mitochondrion is organellar heteroplasmy. A particular number of abnormal mtDNA burden should exist for disease phenotype to manifest, a phenomenon known as threshold effect. This effect is seen at different levels of mutated mtDNA in various organs, the lowest threshold (and hence maximum susceptibility) being in organs dependent highly on oxidative metabolism like brain, heart, skeletal muscle, retina, and endocrine organs. Another interesting phenomenon is "skewed heteroplasmy" where some organs selectively have a higher burden of abnormal mitochondria, exemplified by mitochondrial diabetes, cardiomy-

Mitochondrial diseases

Maternal inheritance

Sporadic

Autosomal recessive

Point mutations in mtDNA

Deletions/rearrangements of mtDNA

Nuclear DNA mutations affecting mtDNA
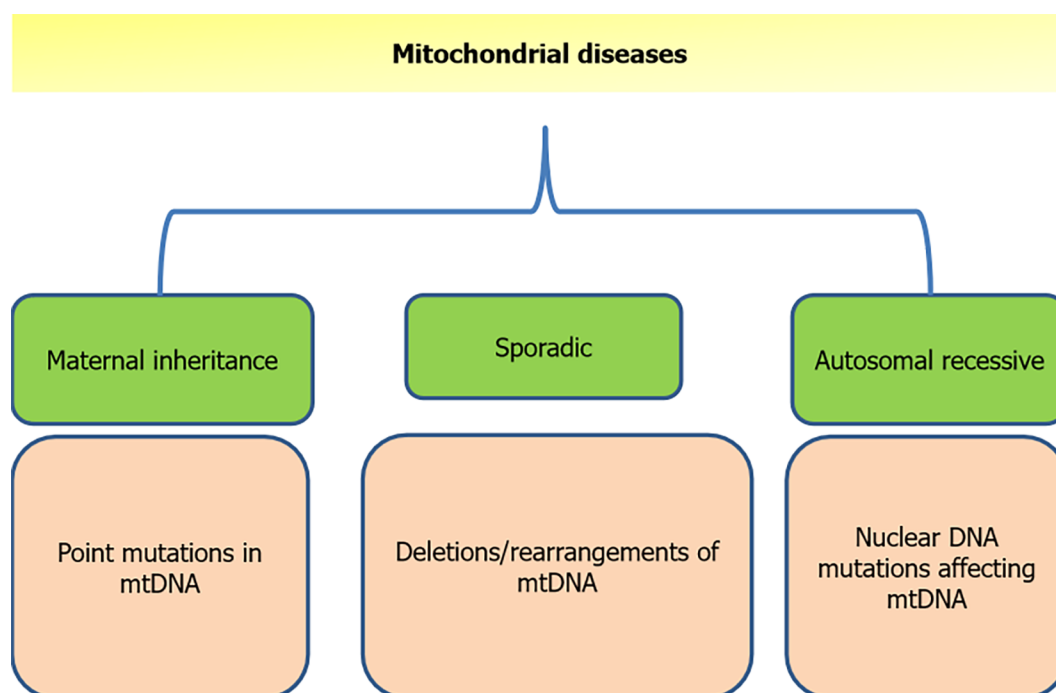
Figure 3 Various modes of inheritance of mitochondrial disease. mtDNA: Mitochondrial DNA.

The numbers game

No. of mitochondrial genes: 37

No. of genes encoding proteins required for translation of mtDNA : 24

  tRNA – 22

  rRNA – 2

No. of genes encoding for final structuro-functional proteins in mitochondria: 13

  Complex 1 subunits – 7

  Complex 3 subunit –1

  Cyt c oxidase subunits – 3

  ATP synthase subunits - 2

No. of gene products in mitochondria: 900

Gene products encoded by nuDNA: 863

Figure 4 Comparison of mitochondrial and nuclear DNA influence in genetics of mitochondria. mtDNA: Mitochondrial DNA; rRNA: Ribosomal RNA; tRNA: Transfer RNA.

opathies, and deafness[6-8]. Mitotic segregation effect refers to the random distribution of mitochondria at end of cell division, which can segregate mutated and non-mutated mtDNA in a variable manner into the two daughter cells. This may result in a daughter cell phenotype that is diseased (due to presence of more abnormal mitochondria), *i.e.* more abnormal than the originator cell in the subsequent divisions. With age, abnormal cells may predominate, explaining age related unmasking of diseases.

THE PROBLEM STATEMENT: EPIDEMIOLOGY

Prevalence of respiratory chain defects is variable across geographical lines as well as across eras. A large study examining birth prevalence of mitochondrial respiratory

chain disorders (RCDs) up to 16 years of age puts the figure at 5/100000 births[9]. This would mean that for every 20000 births in a particular time period, 1 child has the probability of getting affected by a respiratory chain defect of any type till he or she reaches the age of 16. The same study extrapolated the prevalence as 13.1/100000 births with onset at any age when seen together in the light of another study by Chinnery *et al*[10]. According to the Swedish registry, in a population study identifying mitochondrial encephalomyopathies, 20% had liver involvement[11]. In a 5 year French study of 1041 children, 22 (10%) of the 234 patients with respiratory chain defects had hepatopathy[12]. We would, however, add a word of caution that these figures can be an underrepresentation of true values in view of the heterogeneity of presentation and difficulty in diagnosis of mitochondrial respiratory chain defects.

CLASSIFICATION OF MITOCHONDRIAL HEPATOPATHIES AND STATUS OF RESPIRATORY CHAIN DISORDERS

Mitochondrial disorders are characterized by their variability in presentation and predilection for more than one organ system simultaneously or separated in time.

Sokol and Treem proposed classifying these disorders as primary and secondary depending on whether defect is inherently present in the mitochondria and leads to liver dysfunction or there is secondary involvement of mitochondria in the form of injury or alteration in non-mitochondrial genetics. There are two broad types of mitochondrial hepatopathies, one which affects the respiratory chain present on the inner mitochondrial membrane and the other includes fatty acid oxidation defects, which are related to the process within the mitochondrial matrix. The RCDs can also be divided into those arising due to defective mtDNA and those due to defect/ mutation in nuDNA. Among the diseases affecting mtDNA, the affliction can be in the form of either mutations or an overall depletion of quantity of mtDNA compared to nuDNA in a cell/tissue. Figure 5 shows a simplified way of classification of mitochondrial hepatopathies (MH), and a tabular representation of primary MH individual disorders is shown in Table 1. It is worthwhile to note that usually mitochondrial disorders with primary myopathic involvement have mutations in mtDNA, while those with primary hepatic involvement have mutations in nuDNA affecting mitochondrial processes, with some exceptions[13]. Since we are discussing respiratory chain disorders not confined to liver but to include the gastrointestinal tract, we will include one prototype non-hepatic RCD affecting the gastrointestinal (GI) tract, mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), in our review. This review does not cover non-RCD mitochondrial hepatopathies (fatty acid oxidation disorders and others) and GI manifestations of non-RCD, non-hepatic mitochondrial disorders.

CLINICAL PRESENTATION

Mitochondrial disorders are often called mitochondrial multiorgan disorder syndrome (MIMODS) in view of their heterogenous presentation affecting the nervous system (central and peripheral), eyes, ears, endocrine system, kidneys, heart and blood vessels, bone marrow, lungs, and also the intestinal tract, apart from affecting the liver (hepatopathy). The liver involvement is also variable in clinical presentation as well as in age of onset, from acute liver failure, cholestasis, or as chronic liver disease. A graphical summary of all mitochondrial RCDs affecting the liver is represented in Figure 6. Each of the individual disorders is briefly discussed.

Neonatal liver failure

Neonatal liver failure is a catastrophic event, and there are few disorders that present in the first few months as liver failure. Neonatal acute liver failure (ALF) is distinct from pediatric and adult liver failures in that it can include causes that have underlying cirrhosis. Also, the cut-off for coagulopathy is proposed as an international normalized ratio (INR) of ≥ 3 for newborns, as normal INR can be up to 2 in this age [14]. The four main causes of neonatal liver failure are: (1) Gestational alloimmune liver disease (neonatal hemochromatosis); (2) Viral infections (herpes simplex); (3) Hemophagocytic lymphohistiocytosis (primary-familial/secondary to infections); and (4) Mitochondrial hepatopathies (respiratory chain defects).

Table 1 Various mitochondrial primary respiratory chain disorders

Disorder	Mutation/defective gene	Location of defect	Affected proteins/consequence
Neonatal liver failure: (1) Complex I deficiency; (2) Complex III deficiency; (3) Complex IV deficiency; and (4) Multiple complex deficiencies	ACAD9; BCS1L; SCO1	nuDNA	Respective complexes deficiency as per name
Delayed onset liver failure: Alper's Huttenlocher syndrome	POLG mutation	nuDNA	Defective mtDNA polymerase; mtDNA depletion
MtDNA depletion syndrome	DGUOK; TK-2; MPV 17; POLG	All nuDNA	Decreased deoxyribonucleotide concentrations within mitochondria
Mitochondrial neuro-gastrointestinal encephalomyelopathy	TYMP	nuDNA	Markedly low levels of thymidine phosphorylase activity
Pearson marrow pancreas syndrome	4000-5000 bp deletions in mtDNA; tRNA gene of mtDNA	Both mtDNA	Complex I, IV, V
Navajo neurohepatopathy	MPV 17 mutations	nuDNA	mtDNA depletion
Villous atrophy with hepatic involvement	Rearrangement defect/deletion-duplications in mtDNA	mtDNA	Complex III deficiency

nuDNA: Nuclear DNA; mtDNA: Mitochondrial DNA.
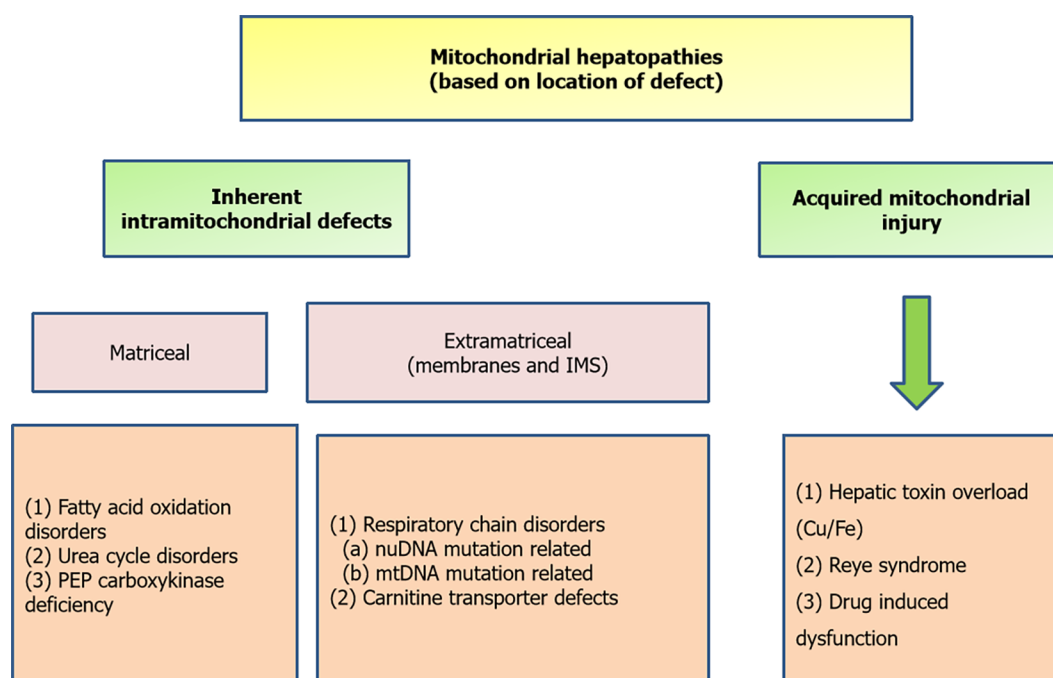
Figure 5 Simplified way of classification of mitochondrial hepatopathies based on location of defect. IMS: Intermembrane space; mtDNA: Mitochondrial DNA; PEP: Phosphoenolpyruvate.

Apart from these, galactosemia, tyrosinemia, and hereditary fructose intolerance can present as ALF in early infantile period and rarely in neonatal age[15]. The key here is to keep mitochondrial hepatopathy (RCD and non-RCD) as one of the differentials of acute liver failure in a newborn/early infantile period, though it accounts for < 5% in neonatal ALF series[14]. Multi-system involvement, especially with neurological symptoms in form of lethargy, floppy tone, vomiting, poor suck, and seizures, are diagnostic clues. Some patients are apparently normal until a viral illness or an unknown inciting event seems to trigger a downhill course either hepatic or neurological or both. Infants with mitochondrial hepatopathies are seen to have a low birth weight in up to 23%, and associated intrauterine growth retardation is seen in 16%, likely due to insult beginning from intrauterine period[16]. Laboratory findings of metabolic acidosis, elevated lactate levels, high lactate to pyruvate ratio often more than 30 mol/mol, elevated ketone bodies betahydroxybutyrate, and betahydroxybu-
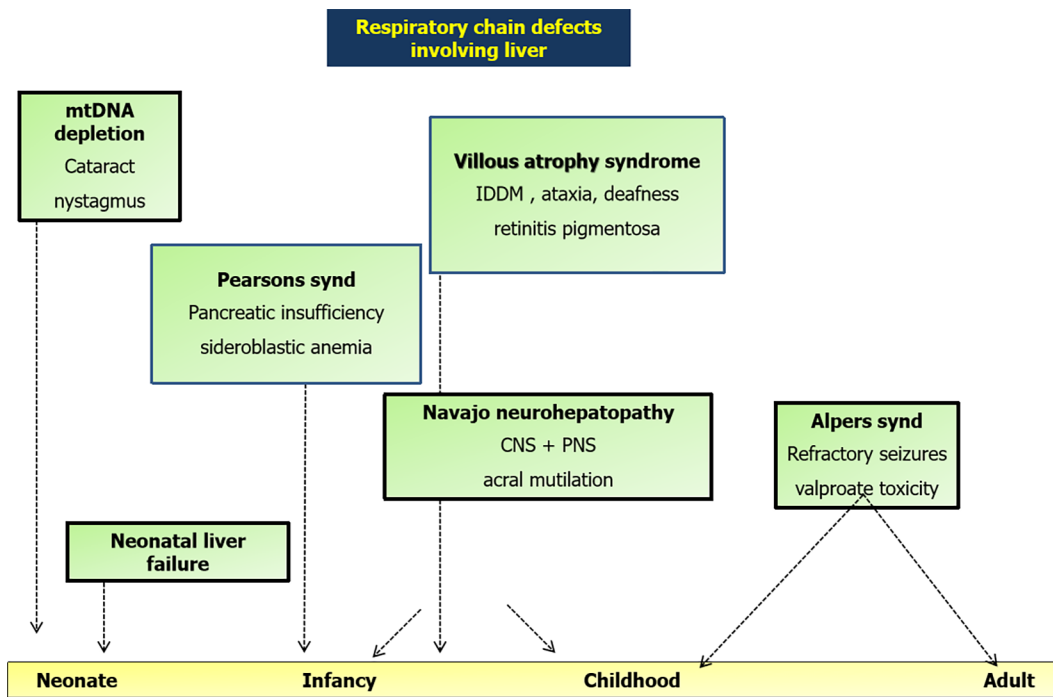
Figure 6 Graphical summary of various respiratory chain disorders involving liver on a timeline with key features. CNS: Central nervous system; IDDM: Insulin dependent diabetes mellitus; mtDNA: Mitochondrial DNA; PNS: Peripheral nervous system.

tyrate to acetoacetate ratio > 2 mol/mol are corroborative, but absence does not rule out the diagnosis. Liver biopsy findings may yield micro or macrovesicular steatosis, which reflects impaired energy metabolism. Liver or muscle tissue respiratory chain analysis shows decreased levels of complexes I, III, or IV. Liver biopsy is often done post-mortem due to the inability to do so percutaneously in view of coagulopathy. Treatment including liver transplant is discussed subsequently.

Delayed onset liver disease: Alpers Huttenlocher syndrome

This syndrome presents anywhere from 2 mo to 8 years of age, predominantly in late infancy to childhood (Figure 7 graphical summary). The diagnostic criteria include[17]: (1) Presence of refractory seizures including focal seizures; (2) Infection triggered psychomotor regression that is episodic in nature; and (3) Liver dysfunction with or without liver failure. Liver involvement is in the form of hepatomegaly, jaundice, coagulopathy, and episodes of hypoglycemia. Gastrointestinal involvement mainly due to the muscle impairment results in progressive feeding difficulty and gastroeso-phageal reflux, progressing to intractable vomiting. One series of 5 patients with Alpers Huttenlocher syndrome (AHS) showed mean age of liver disease presentation of 35 mo, and all died over a mean 4.6 wk period, due to progressive liver failure[18]. Autopsy findings across series show macrovesicular steatosis, massive hepatocyte dropout, proliferating bile ductular elements replacing hepatocytes, and often cirrhosis [17,18]. Valproate is known to precipitate liver failure in these patients when given for the frequently associated seizure disorder, which often demands use of more than one anticonvulsant. This is possibly because of depletion of respiratory chain enzyme activity by the drug and inability to increase metabolic rate by the DNA polymerase subunit gamma (POLG) deficient cells[19]. Valproate increases glycolysis, likely an indirect clue of impaired mitochondrial function as shown in yeast and mouse liver models[20]. POLG mutation subtype and zygosity influence outcome, with worst outcomes shown in compound heterozygous mutations for A467T and W748S[21].

Liver failure management, addressing feeding issues often mandating percutaneous endoscopic gastrostomy tube insertion, seizure control, and use of respiratory aids like continuous positive airway pressure in view of progressive motor impairment are cornerstones of management. Liver transplantation is often contraindicated in view of the multisystem involvement.

MtDNA depletion syndrome

DNA depletion is distinct from DNA deletion. MtDNA depletion refers to a state when a cell contains less than normal mtDNA per unit nuDNA. Depletion diseases are
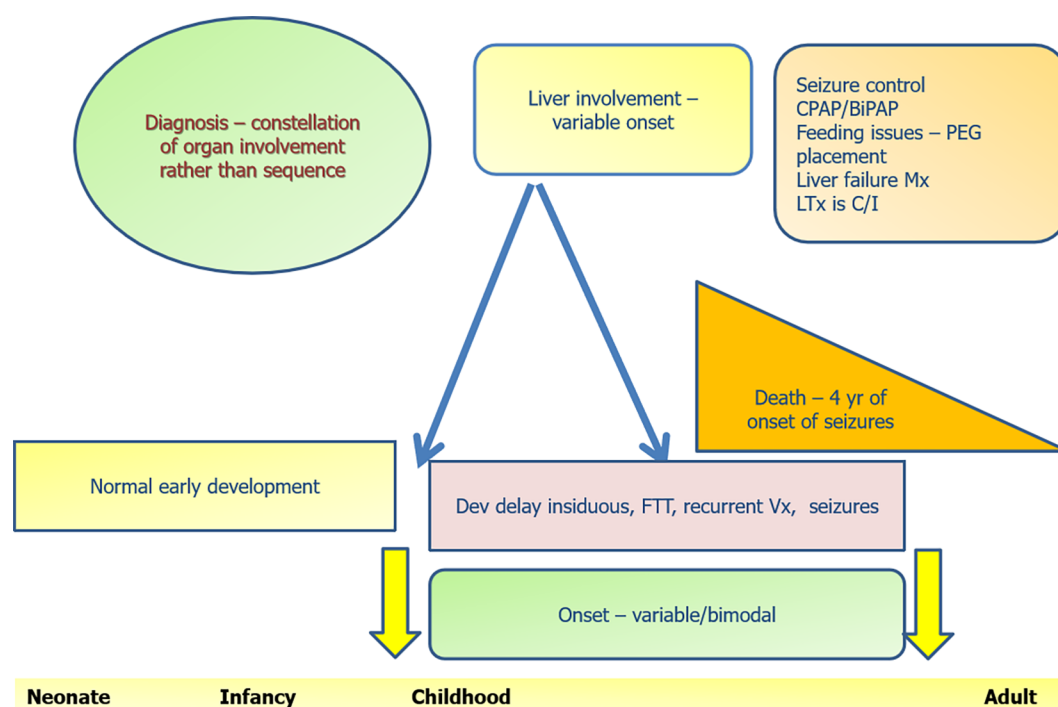
Figure 7 Graphical summary of Alpers Huttenlocher syndrome and its natural history. BiPAP: Bilevel positive airway pressure; C/I: Contraindicated; CPAP: Continuous positive airway pressure; FTT: Failure to thrive; LTx: Liver transplantation; Mx: Management; PEG: Percutaneous endoscopic gastrostomy; Vx: Vomiting.

much more severe and earlier in onset compared to deletion diseases[22]. NuDNA encodes for processes within the mitochondria including production and stability of mtDNA. Mutations in nuDNA may result in low levels of or increased destruction of DNA pool essential for mtDNA synthesis[23], thereby reducing the concentration of mtDNA in the cell or tissue as a whole. The end result is suboptimal mitochondrial function. DGUOK mutations lead to predominant neuro-hepatopathy, while TK2 mutations lead to predominant myopathy[23]. As an illustration to above statements, TK2 induced depletions present early in the first few years with myopathy, feeding difficulty, hypotonia, and respiratory failure as a terminal event. However, multiple TK2 deletions present as proximal myopathy and chronic progressive external ophthalmoplegia later in life[24]. Two additional genes, POLG coding for mtDNA polymerase and MPV17, have been described in hepatocerebral form of MDS. POLG mutations in older children have been associated with AHS as already described. It should be understood that MDS and AHS both have mtDNA depletion. AHS got its name earlier and was later found to have its molecular basis as mtDNA depletion, and it characteristically refers to a comparatively delayed onset (> 2 mo age), compared to MDS, which has its onset in the first few weeks of life. The other mutation in nuclear gene MPV17 leads to decreased synthesis of an unknown inner mitochondrial membrane protein that possibly has a role in oxidative phosphorylation, and knockout mice (-/-) have shown impaired oxidative phosphorylation and also mtDNA depletion [25].

Liver failure in infancy is the common presentation of the hepatopathic form. There is notably an overlap between the hepatopathic form of MDS and neonatal liver failure presentation of RCD. The difference exists in the fact that the former has mtDNA quantity that is < 10% of nuDNA, and there is no sequence alteration in mtDNA.

Pearson syndrome

Pearson syndrome is one among three mitochondrial diseases (Kearne Sayre syndrome and chronic progressive external ophthalmoplegia being the other two) associated with a single large deletion in mtDNA[26]. This is a multi-systemic fatal disorder with involvement of exocrine pancreas, eyes, skin, hematological system, liver, and kidneys[22]. MtDNA rearrangements form the etiological basis, and it is associated with large 4-5 kbp deletions in a large proportion of cases. All respiratory chain complexes can suffer a decreased synthesis, with complex I most severely affected. Refractory anemia with ring sideroblasts occurs in infancy with vacuolization in bone marrow of myeloid and erythroid precursors[27]. Elevated plasma alanine and

fumaric acid levels are discriminating from other non-mitochondrial bone marrow failure syndromes[28], though neither specific for Pearson syndrome nor distinguishing it from other mitochondrial disorders. Hematological manifestations may occur alone or in combination with renal tubular dysfunction (Fanconi syndrome) and hepatic failure. If the patient survives this phase of hematological symptoms, its intensity begins to decrease[29], and symptoms change from hematological to a phenotype of severe pancreatic insufficiency in late infancy to early childhood, during the same time which villous atrophy is found to appear. Eye involvement is in form of pigmentary retinopathy and external ophthalmoplegia and appears in early to late childhood. Liver involvement is in form of hepatomegaly with cirrhosis, cholestatic jaundice, elevated liver enzymes, and progressive liver failure leading to death in early childhood similar to what is seen in MDS[30]. Recent series have shown age of death ranging from 5 to 11 years, and mortality is worse in Pearson syndrome compared to other single large mitochondrial deletions[28,31]. A graphical summary outlining the natural history is shown in Figure 8. Supportive therapy with packed red cell transfusions for anemia, granulocyte colony stimulated factor for neutropenia, and bicarbonate for metabolic acidosis forms the basis of care.

Navajo neurohepatopathy

This is an autosomal recessive disease prevalent in southwestern United States. The genetic defect is a nuclear gene MPV17 (chromosome 2p24)[32], whose product is located on the inner mitochondrial membrane and is responsible for mtDNA maintenance and regulation of oxidative phosphorylation. Hence, there is impaired pool of mtDNA and disrupted oxidative phosphorylation. While earlier only neurological manifestations were known and this entity was called Navajo neuropathy, liver manifestations in form of jaundice, failure to thrive, and liver failure were recognized to be part of the same disease spectrum prompting a change in name to Navajo neurohepatopathy[33]. Clinical features are outlined in Figure 9 graphical summary. All the three subtypes have occurred in same kindred, underscoring the pattern of mitochondrial inheritance.

Villous atrophy syndrome

This disorder was described in 1994 by Cormier-Daire *et al*[34] in 2 unrelated children presenting as chronic diarrhea in infancy with villous atrophy. The defect was identified as mtDNA rearrangements in the form of deletion-duplications. Hepatomegaly and steatosis on biopsy with mildly deranged transaminases was the liver manifestation. Both children survived the diarrheal phase, which subsided by early childhood, including a reversal in histology (Figure 10: Graphical summary). However, the phenotype then changed to neuromuscular and ophthalmic involvement and death by the end of first decade. Complex III defect was detected on muscle biopsy after the advent of neuromuscular symptoms and was normal in lymphocytes. Intravenous dextrose for resuscitation should not be used in high rates as it may lead to worsening of metabolic acidosis.

Mitochondrial neurogastrointestinal encephalomyopathy

This entity is discussed purely as a prototype for GI (non-hepatic) manifestations of mitochondrial disorders, and also since it is a respiratory chain disorder, though not classically a "hepatopathy", and additionally as it is rewarding to diagnose in view of available therapy[35]. It is to be understood that RCD and non RCD mitochondrial diseases can have some or the other GI manifestation (Table 2). MNGIE was earlier known as polyneuropathy, ophthalmoplegia, leukoencephalopathy and intestinal pseudo-obstruction, oculogastrointestinal encephalopathy syndrome, or oculogastrointestinal muscular distrophy[36]. The current nomenclature was given by Hirano *et al*[37].

MNGIE occurs due to mutation in a nuclear gene encoding TYMP, encoding thymidine phosphorylase, deficiency of which leads to toxic accumulation of pyrimidine nucleosides thymidine and deoxyuridine. This impairs mtDNA synthesis thereby leading to a mtDNA depletion state. Clinical symptoms of MNGIE usually begin between the first and fifth decades of life and before 20 years of age in approximately 60%. GI dysmotility is one of the most important features in form of dysphagia, gastroparesis, and pseudo-obstruction leading to consequences like small bowel bacterial overgrowth, nutritional deficiencies, and severe weight loss[38]. Hepatic steatosis, hepatomegaly, elevated transaminases, and cirrhosis have also been described[38,39].

Table 2 Gastrointestinal manifestations of mitochondrial respiratory chain defects	
Site	**Manifestation**
Oral cavity and esophagus	Sicca syndrome; Dry mouth; Dysphagia
Stomach	Vomiting; Reflux; Pseudo-obstruction
Small bowel and large bowel	Pseudo-obstruction; Diarrhea; Megacolon; Constipation
Extra-luminal/miscellaneous	Poor appetite; Pancreatitis; Pancreatic cysts
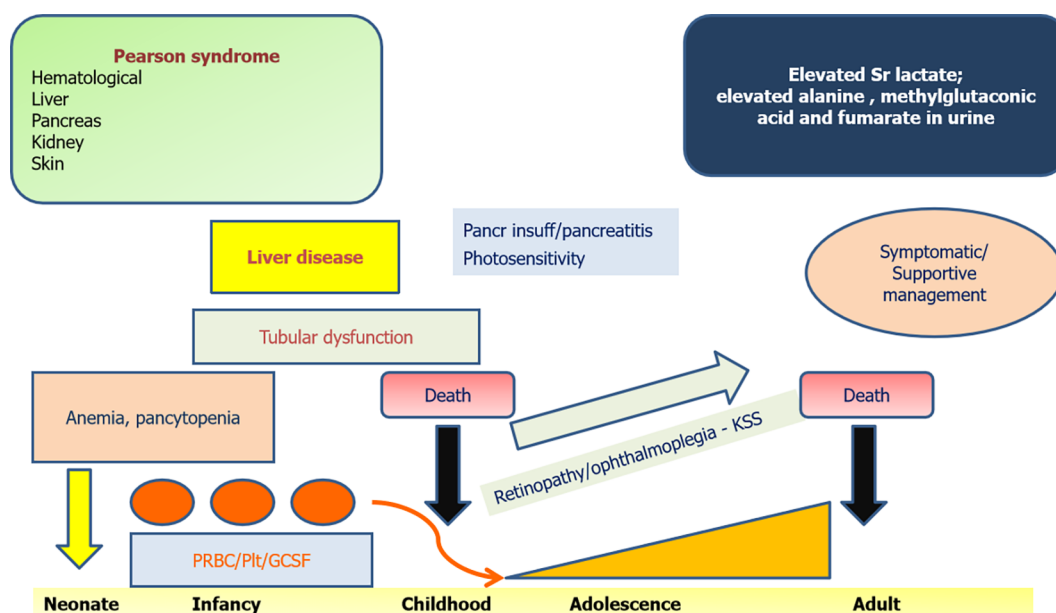
Figure 8 Graphical summary of Pearson marrow pancreas syndrome and its natural history. GCSF: Granulocyte colony stimulation factor; insuff: Insufficiency; KSS: Kearns Sayre syndrome; Pancr: Pancreatic; Plt: Platelets; PRBC: Packed red blood cells.

A diagnostic delay of about 5 to 10 years can occur in view of multisystem and complex clinical presentation[40,41]. Often there are unnecessary exploratory surgeries for the GI symptoms before being diagnosed as pseudo-obstruction[36]. Neurological involvement is mainly in form of peripheral neuropathy (demyelination with or without axonal neuropathy)[38], oculoparesis, with subtle central nervous system manifestations due to subcortical white matter involvement, and magnetic resonance imaging changes showing leukoencephalopathy. Muscle biopsies may show ragged red fibers due to proliferation of abnormal mitochondria. Current diagnostic methods employ testing for plasma thymidine and deoxyuridine levels (> 3 µmol/L and > 5 µmol/L, respectively)[42] or elevated urinary concentrations[43] and thymidine phosphorylase activity in leucocytes (< 10% of healthy controls)[43]. TYMP gene (nuDNA) mutations and also consequent mtDNA abnormalities can be identified on Sanger sequencing and Southern blot assays[44]. A graphical summary is as shown in Figure 11.

Symptomatic management remains the cornerstone. Experimental therapies include hemodialysis and peritoneal dialysis[43], platelet transfusions, hematopoietic stem cell transplant, enzyme replacement, and liver transplant[45,46]. All above therapies concentrate on 2 aspects: To reduce the toxic load of nucleosides and to replace the enzyme thymidine phosphorylase.

SETTINGS TO SUSPECT RCD AND DIAGNOSTIC EVALUATION

The settings of when to suspect a mitochondrial hepatopathy are shown in Figure 12.

Individual disorders discussed above and their graphical summaries outlined give specific information. The diagnostic evaluation of mitochondrial disorders follows once a clinical suspicion is raised, and in this section we highlight general steps towards approaching to diagnose a mitochondrial RCD[30]. Parallel evaluation of
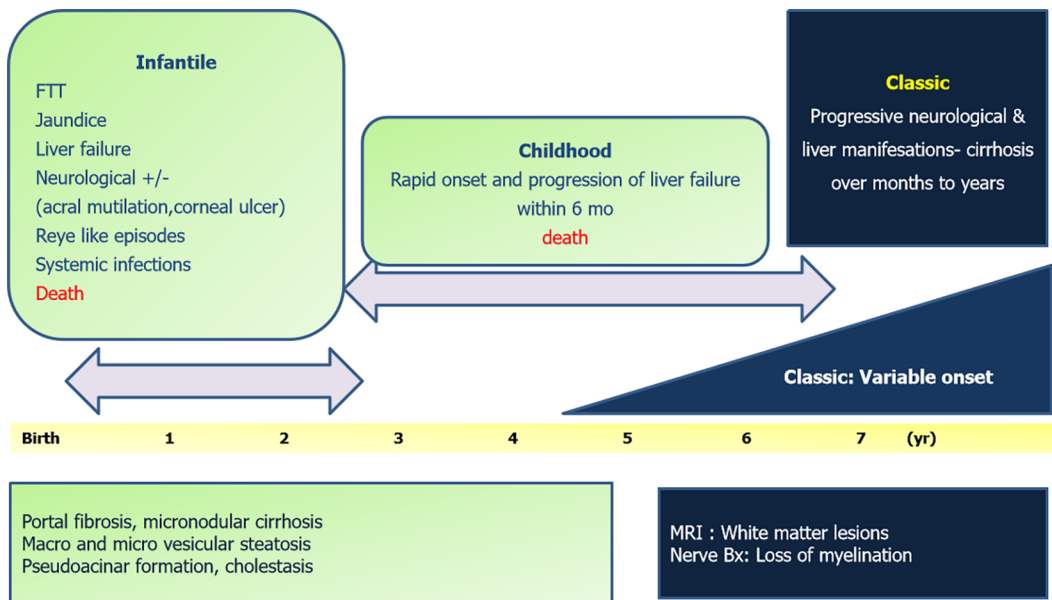
Figure 9 Graphical summary of Navajo neurohepatopathy. Bx: Biopsy; FTT: Failure to thrive.
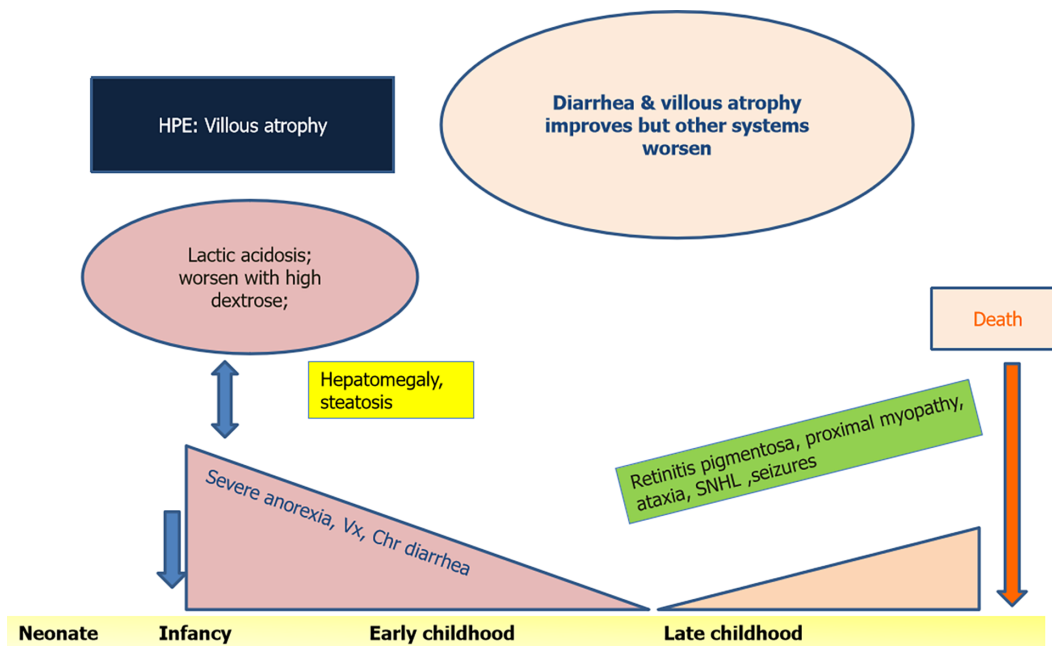
Figure 10 Graphical summary of villous atrophy syndrome and its natural history. HPE: Histopathological examination; SNHL: Sensorineural hearing loss; Vx: Vomiting.

extra-hepatic and extra-GI symptoms, if present, need to be carried out for "mapping" the disease and for aid in management and improving quality of life. Table 3 elucidates a stepwise evaluation algorithm[30]. Diagnostic steps proceed from non-invasive, easily available, and less expensive investigations to more complex elaborate tests, some of which are available in the research setting only. Level-1 entails workup for basic metabolic causes including checking for hypoglycemia and, if present, whether it is ketotic or non-ketotic. Fatty acid oxidation defects (but not RCDs) are known to have non-ketotic hypoglycemic episodes. Lactate levels more than 2.1 mmol/L (mmol) are significant, and this may often not be observed when not in a metabolic crisis. Notably, lactate may not be elevated much in POLG1 mutations[47]. Normal lactate to pyruvate ratio is less than 20 mol/mol. However, the value often rises above 30 and is typical though not exclusive to RCDs and is discriminatory from pyruvate metabolism defects[48]. Similarly, 3-hydroxy butyrate to acetoacetate ratio is normally less than 4, and values above this should arouse a suspicion of mitochondrial

Table 3 Stepwise evaluation of mitochondrial hepatopathies (respiratory chain disorder/non- respiratory chain disorders)

Steps	Description	Additional action
Level-1 (body fluids)	**Basic:** CBC, INR, AFP, CPK, NH3, sugars, phosphorous, urine ketones. **Advanced:** Lactate: Pyruvate (1 h post feeds); Ketone Body ratio, 3OH-butyrate: Acetoacetate; Serum acylcarnitine profile; Urine organic acidogram; Serum aminoacidogram; 3 Methyl Glutaconic acid in serum/urine; CSF lactate: Pyruvate, CSF alanine, protein; Plasma thymidine (MNGIE); Leucocyte CoQ levels	**Parallel level-1:** Evaluate other involved systems: CNS: MRI/MR-Spectroscopy, EEG; Eye: Fundus evaluation, clinical evaluation for ophthalmoplegias; Hearing screen; Heart: 2D-Echo, ECG; Renal: urine electrolytes, proteins, amino acids; Muscle: Muscle biopsy (Level-1 in case of primary muscle involvement, level-3 otherwise); Endocrine: HbA1c, 8 AM cortisol; Pancreas: Fecal elastase
Level-2 (genetics)	**Common genes genotyping:** POLG-1; DGUOK; MPV-17; SUCLG-1; TRMU; C10ORF2/Twinkle; CPT-1; mtDNA point mutations	**Alternative level-2:** Next generation sequencing/clinical exome sequencing for simultaneous evaluation of all mitochondrial DNA and nuclear DNA
Level-3 (invasive)	**Tissue diagnosis:** (1) Liver biopsy: Light microscopy including oil red O stain for steatosis; Electron microscopy for structural mitochondrial alterations; Frozen tissue analysis for respiratory chain enzymes, DNA quantification. (2) Muscle biopsy: Frozen tissue analysis as above; Blue native page analysis. (3) Skin biopsy: Same as muscle biopsy	**Key points to note during level-3 evaluation:** Biopsy specimens for electron microscopy need to be preserved in glutaraldehyde and not formalin; It is possible that one invasive test may not give a clue and one has to proceed for an additional invasive test. This is usually because of heteroplasmy. Often liver biopsy molecular analysis provides a final definitive answer; Combination of level-1, level-2 and level-3 studies are sometimes needed to provide comprehensive management and for prognostication

2D Echo: Two-dimensional echocardiography; AFP: Alpha-fetoprotein; CBC: Complete blood count; CNS: Central nervous system; CoQ: Coenzyme Q; CPK: Creatine phosphokinase; CSF: Cerebrospinal fluid; EEG: Electroencephalogram; HbA1c: Glycosylated hemoglobin; INR: International normalized ratio; MRI: Magnetic resonance imaging; NH3: Serum ammonia levels; POLG: DNA polymerase subunit gamma; RCD: Respiratory chain disorders.
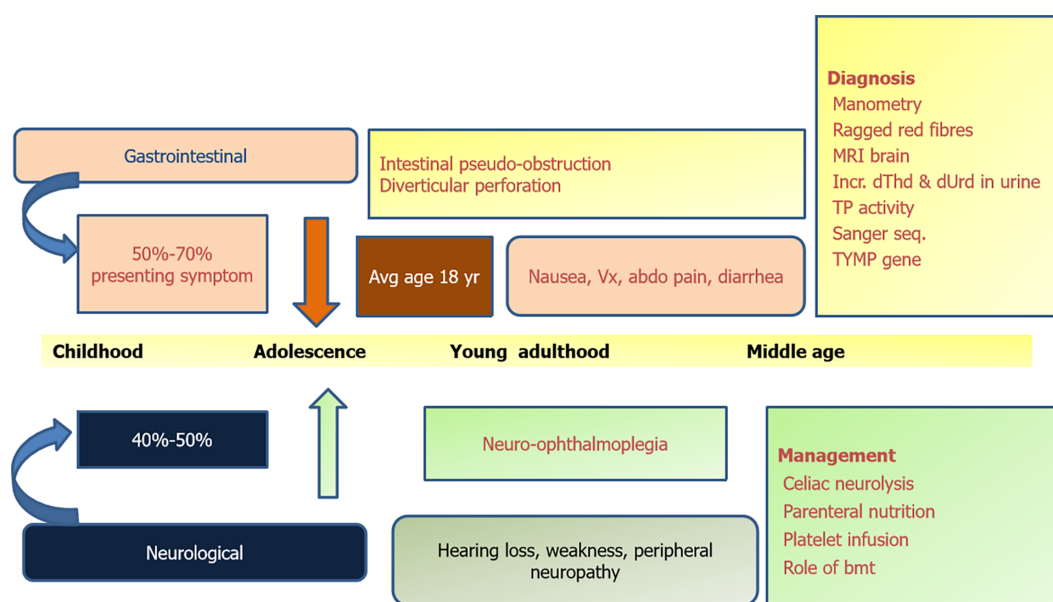
Figure 11 Graphical summary of mitochondrial neurogastrointestinal encephalomyopathy. BMT: Bone marrow transplantation; dThd: Thymidine; dUrd: Deoxy uridine levels; MRI: Magnetic resonance imaging; TP: Thymidine phosphorylase. Vx: Vomiting.

dysfunction. Urine organic acids like lactate, succinate, fumarate, malate, and 3-methyl-glutaconic are seen elevated in Pearson syndrome. Serum alanine elevation is also a clue; however, it is often more elevated in pyruvate dehydrogenase deficiency than in RCDs[48]. Creatine kinase elevation and concomitant low levels of phospho-creatine in brain and muscle tissue are seen in RCDs[49]. Branched chain amino acid to glutamine ratios were highest in RCDs and lowest in pyruvate dehydrogenase deficiency compared to controls, according to one study[48].

Table 4 helps differentiate the common metabolic disorders encountered in the pediatric patient and how to filter out RCD.

Role of genetic testing

Genetic studies are confirmatory but have a high turnaround time of 4-6 wk. They may not also be available freely at all centers or in resource poor settings. Timely referral to tertiary care centers for management is advisable. A major limitation is selection of the gene panel testing for the phenotypic presentation. In products of consanguineous

Table 4 Biochemical differentiation between various metabolic hepatopathies (respiratory chain disorder *vs* non respiratory chain disorder comparison)

	Acidosis	Urine ketones	Blood sugar	Serum lactate	Serum ammonia
RCD	++	++	Normal	++++	±
FAOD	++	Nil (non-ketotic)	Low (hypoglycemia)	+	+
OA	+++ (persistent)	++/+++	Low/normal/high	Normal	++
UCD	Normal	Normal	Normal	Normal	++++

FAOD: Fatty acid oxidation defects; OA: Organic acidemias; RCD: Respiratory chain defects; UCD: Urea cycle defects.

When to suspect mitochondrial hepatopathy ?

Core tip: Multisystem involvement – brain/eye/heart/muscle

Scenario 1: Neonate/infant/young child: Acute liver failure
➢ **Suspect mitochondrial if pre-existing/disproportionate CNS symptoms**

Scenario 2: Infant/child: Chronic liver disease (cirrhosis/cholestasis)
➢ **If steatosis on Bx, hypoglycemia, lactic acidosis/ketonemia**

Scenario 3 (general scenario): Any age: Liver disease (acute/chronic)
➢ **If chronic neuromuscular disease /eye/heart involvement**

Figure 12 Scenarios when to suspect mitochondrial hepatopathy. Bx: Biopsy; CNS: Central nervous system.

union and multiple affected siblings, genetic evaluation is better guided, and it is possible to identify the index patient's chromosomal region containing the abnormality by linkage analysis. Targeted gene analysis is performed if the phenotype matches the previous cases in a family and there is an already identified mutation responsible for the clinical features in those particular kindred. Whole exome analysis for nuDNA and mtDNA is preferred otherwise as an alternative step in case there are no previously affected siblings or if the phenotype does not classically match previously described entities[50]. Targeted analysis can be performed using Sanger method of few genes, while whole exome sequencing refers to a massive parallel sequencing technique of multiple genes or the entire exome using next generation sequencing[51]. Another method to short-list genes for analysis is to study the expression profiles of RNA or specific proteins or polypeptides levels encoded by the gene(s) of interest, especially after a biochemical diagnosis is made. As an example, complex I deficiency can be caused by any of the multiple mtDNA and nuDNA responsible for each of its subunits. To identify a particular gene (of the multiple encoding ones) responsible for causing overall complex I deficiency in an index patient, analyzing the distribution or expression of proteins or RNA and its deviation from healthy controls or known standards can help pinpoint which gene may be defective. Once a specific change is identified, either in RNA expression, which can be detected by microarray assays, or in enzyme levels or stress protein expression, which can be identified by immunoblotting using an antibody panel, indirectly "reverse identification" of the causative gene(s) is facilitated[52]. This of course is only possible when, with time, all genes encoding each subunit of large proteins are identified so

that such indirect, simpler, and time saving methods may be employed. Tables 1 and 3 list genes implicated in mitochondrial RCDs. Figure 13 suggests a two-step strategy of genetic evaluation in mitochondrial RCDs and also a few phenotypes for which specific genes should be tested.

It is pertinent to note that mitochondrial hepatopathies, unlike other metabolic disorders, require analysis of mtDNA in addition to nuDNA defects. Hence, when screening genetics do not yield the diagnosis and the next step of whole exome sequencing is being undertaken, it is essential to specify to the testing lab that mtDNA analysis be included in addition to the wide gamut of nuDNA being tested. While for nuDNA analysis, the tissue of interest may not be specific and whole blood sample may serve the purpose, for mtDNA molecular analysis, specific tissues (liver, muscle) may be required, mainly because of the phenomenon of heteroplasmy.

MtDNA depletions are diagnosed by first isolating the DNA of the tissue biopsied, which is then subjected to electrophoresis and blotting followed by hybridization with probes specific for mtDNA and nuDNA both. The relative levels of autoradiographic signals emitted post hybridization are detected for mtDNA and nuDNA, and this helps in diagnosing mtDNA depletions. MtDNA deletions and point mutations on the other hand can be detected by single strand conformational polymorphisms[53,54].

Next generation sequencing by parallel exome sequencing undertaken with blood or any tissue is limited by its inability to detect mutations in the non-exonic region, like untranslated regions or intronic splice sites. It is also not adept in diagnosing trinucleotide repeat sequences, complex genetic inheritance like synergistic contribution of nuDNA and mtDNA to cause a particular disease, and epigenetic effects[51].

Role of tissue biopsies

Tissue biopsies are important despite having readily evident biochemical abnormalities; only one-third to one-half of mitochondrial disorders have identifiable mutations despite extensive exome sequencing of known genetic defects[51,55]. That is to say, all genes related to mitochondrial disorders have not yet been identified. Biopsies from the most involved site are more likely to yield the diagnosis[56]. Respiratory chain enzymes can be analyzed and activity quantified on tissue biopsy specimens. Quantitative Southern blot analysis or real-time quantitative polymerase chain reaction to detect mtDNA depletion can be done in liver biopsy specimens. Skin biopsy for cultured skin fibroblasts can be stored indefinitely and retrieved for re-culture once newer diagnostic modalities are available. It is simpler to perform and less invasive compared to muscle biopsy. However, the downside is that not all diseases are detectable on skin fibroblast analysis[47].

How to select which tissue to test is an important question that the clinician must be aware. Most mitochondrial disorders involve the muscle, and hence muscle is one of the most useful sites for analysis of enzymes, metabolites, and even molecular DNA studies. While earlier 1-5 g of muscle tissue was required for respiratory chain enzyme assays, now even 100-200 mg of skeletal muscle tissue (usually quadriceps or soleus) is sufficient especially in young children, which then yields a mitochondrial enriched fraction of 400-500 μg of protein, enough to characterize the respiratory chain enzyme deficiencies[57]. Muscle biopsies may be analyzed either as frozen or fresh samples. Samples once collected should be snap frozen immediately bedside or in the procedure room at -80 °C till analysis of mitochondrial enzymes[53]. Fresh muscle samples should not be frozen and transported in cool buffer solution, which offers the advantage of analysis of the entire mitochondrial energy generation system in addition to mitochondrial enzymes being studied in frozen samples[58].

Those diseases that have primary liver involvement and no apparent muscle involvement, especially the ones with liver failure phenotype, liver tissue of up to 10 mg can be more yielding than muscle. Cardiac tissue requirement, when indicated, is even less, about 1-2 mg, obtained by endomyocardial biopsy[53]. These invasive techniques may be gradually substituted by molecular DNA techniques done on whole blood and cater to only research purpose over time as cost and availability of next generation sequencing is eased.

MANAGEMENT OF MITOCHONDRIAL RESPIRATORY CHAIN DEFECTS

There are three aspects to management: Firstly, acute management of crisis, second is general management of children with metabolic liver disease, followed by specific treatment if available including the role of liver transplant. An additional important component relates to parental counseling and to bust myths and avoid patients to
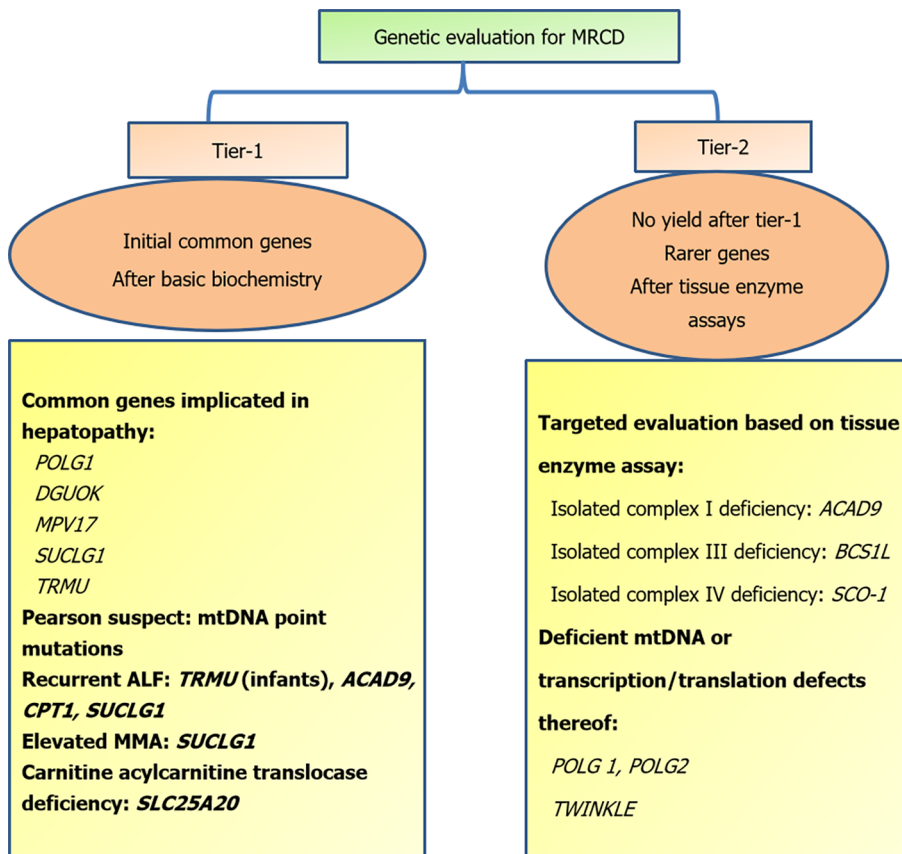
Figure 13 Step-wise strategy of genetic evaluation in mitochondrial respiratory chain defects. MRCD: Mitochondrial respiratory chain disorders; ALF: Acute liver failure; MMA: Methylmalonic acid.

resort to non-scientific therapies and refraining from standard of care.

Acute crisis management

Treatment of acute liver failure and progressive liver disease remains unsatisfactory. The aim of therapy is mainly mitigating, postponing, or circumventing damage to the respiratory chain. The basic steps and precautions are outlined as in Table 5. Bicarbonate infusions when used for a longer time may itself worsen cerebral function. An alternative is dichloroacetate, which inhibits pyruvate dehydrogenase kinase, hence favoring persistent levels of active pyruvate dehydrogenase and hence preventing pyruvate accumulation and pyruvate to lactate conversion.

Other supportive therapy is to give packed red cell and platelet transfusions for anemia and thrombocytopenia and pancreatic enzyme replacement in case of insufficiency.

General considerations for managing a child with mitochondrial hepatopathy

Once the acute crisis is settled, it is important to improve the nutrition of the child as malnutrition itself can lead to secondary mitochondrial dysfunction[59]. These children often have increased caloric needs and an inability to maintain it owing to either repeated sickness bouts or a general anorexia associated with liver disorders. The issues of swallowing difficulties, impaired gut motility, and gastro-esophageal reflux need to be addressed often to the extent of placement of feeding tubes (orogastric or nasogastric), percutaneous endoscopic feeding gastrostomy, or using parenteral nutrition therapy. Nutritional improvement has led to improved quality of life and an increase in developmental quotients in these children[60]. Ketogenic diet may be useful in some mitochondrial disorders but may worsen fatty acid oxidation defects and should be avoided in them. Exercise helps in reducing the burden of abnormal mitochondria[61]. It is useful to do so regularly, under supervision in a graded manner, and to have a meal prior to exercise[62].

Pharmacotherapy

A combination of drugs is often empirically administered to suspected mitochondrial

Table 5 Management during evaluation in acute phase
Following thumb rules while attending to a patient with suspected mitochondrial disorder
Monitor closely for hypoglycemia and acidosis
Avoid lactated ringer's solution for fluid administration: Worsens acidosis
Bicarbonate infusions as 1st line of defense
Avoid propofol for sedation/anesthesia
Avoid fasting > 12 h; avoid high rate glucose only infusions
Avoid drugs that are toxic to mitochondria: Chloramphenicol, valproate, aminoglycosides, phenytoin, carbamazapine, phenobarbital, statins, linezolid
Avoid drugs precipitating hepatopathy/liver dysfunction

disorders and comprises: Coenzyme Q, carnitine, thiamine, riboflavin, vitamins C and E, and creatine. Of all, Coenzyme Q shows promise and along with B vitamins remains the most common combination as part of cocktail therapy[63]. The various drugs and their pediatric dosages are outlined in Table 6[13,63,63].

Role of organ transplant

Multisystem involvement in mitochondrial hepatopathies often precludes performing a liver transplant. However, in hepatocerebral form of DGUOK defects when detected in infancy without neurological involvement, liver transplant has shown to be effective. Those with neurological involvement do not benefit from liver transplant [65]. Overall post-transplant survival is less with RCDs than non-RCDs. Sokal *et al*[66] reported 8 cases with a survival of 50% post transplantation for RCDs. In an elaborate compilation of 40 cases with mitochondrial RCDs across various centers at different time points, it was noted that 22 (55%) patients died within 24 mo post-transplant[67]. Early postoperative multi-organ failure and neuro-degeneration followed by respiratory complications and severe pulmonary hypertension were the cause of death in these patients. The same group recognized that those diagnosed pre-transplant had a higher survival (58%) than those recognized to have RCD after transplant (29%). Thus, the emphasis is on early recognition of the diagnosis and a thorough evaluation for extra-hepatic manifestations, adding investigations like magnetic resonance imaging of the brain and echocardiography.

MNGIE stands out as the single mitochondrial disorder for which replacement of the missing enzyme thymidine phosphorylase by stem cell transplantation can be curative and lead to improvement in long term outcomes. While earlier enzyme levels were artificially increased using repeated platelet transfusions[40], stem cell transplant has come up as a definitive modality[68,69].

Myths in mitochondrial disorders

Immunizations are not contraindicated in children with mitochondrial diseases. This is to be emphasized because of certain misconceptions that immunization may lead to autism in children with mitochondrial diseases for which there is no evidence[64]. The other important aspect that needs to be clarified is that there is no role of hyperbaric therapy in treatment of MH and in fact may lead to oxygen toxicity. Vagus nerve stimulation may not be very helpful in controlling refractory seizures in children with MH[63].

CONCLUSION

In a nutshell: (1) Liver along with other system involvement may not be just sepsis – think of mitochondrial respiratory chain hepatopathy; (2) Lactic acidosis without hypoglycemia is an important clue, avoid ringer lactate and drugs causing hepatopathy; (3) Evaluation should be done in a tiered manner – genetic evaluation and enzyme analysis from tissue of interest; (4) Treatment is largely supportive with transfusions, correction of acidosis, shock, and providing cofactors/salvage therapies; (5) Liver transplantation needs to be considered in only a select group and may worsen disease despite adequate precautions; and (6) Periodic follow-up is mandatory for monitoring evolution of disease including "migration" to other organ systems.

Table 6 Pharmacotherapy used for mitochondrial diseases

Drug	Pediatric dose	Remark
Coenzyme Q: (1) Ubiquinol form; (2) Ubiquinone form	2-8 mg/kg/d in BD dosing; 10-30 mg/kg/d BD dosing	Preferably had after meals; Most effective and most used therapy; Free radical scavenger; Bypasses complex I
Idebenone	5 mg/kg/d	Synthetic form of CoQ; Penetrates blood-brain barrier
L-carnitine	10-100 mg/kg/d IV or oral divided 3 times/d	Avoid in long chain FAO-Ds: May lead to cardiac arrhythmias
Creatine	0.1 g/kg PO, OD	Used for repletion of muscle phosphocreatine levels
L-arginine	500 mg/kg IV per day for 1-3 d followed by 150-300 mg/kg oral daily in BD dosing	Used for acute stroke; Watch for hypotension while infusion; Evidence is anecdotal
Thiamine	100 mg/d	Cofactor of PDH; useful for thiamine responsive PDH deficiency; Helpful in leigh disease
Riboflavin	50-400 mg/d	Give at night time before sleep; Shown to be useful in ACAD9 mutations; Flavin precursor for complex I & II
Vitamin C	5 mg/kg/d OD	Antioxidant; Artificial electron acceptor
Vitamin E	Variable dosing, up to 25 IU/kg/d OD (avoid > 400 IU/d)	Absorption better when taken with meals
Dichloroacetate	25-50 mg/kg/d	Improves lactic acidosis

BD: Twice daily; CoQ: Coenzyme Q; FAO-D: Fatty acid oxidation defects; IV: Intravenous; PDH: Pyruvate dehydrogenase; PO: Per oral; OD: Once daily.

REFERENCES

1   **Herrmann JM**, Riemer J. The intermembrane space of mitochondria. *Antioxid Redox Signal* 2010; **13**: 1341-1358 [PMID: 20367280 DOI: 10.1089/ars.2009.3063]

2   **Lee WS**, Sokol RJ. Mitochondrial hepatopathies: advances in genetics and pathogenesis. *Hepatology* 2007; **45**: 1555-1565 [PMID: 17538929 DOI: 10.1002/hep.21710]

3   **Gray MW**. Mitochondrial evolution. *Cold Spring Harb Perspect Biol* 2012; **4**: a011403 [PMID: 22952398 DOI: 10.1101/cshperspect.a011403]

4   **Schwartz M**, Vissing J. Paternal inheritance of mitochondrial DNA. *N Engl J Med* 2002; **347**: 576-580 [PMID: 12192017 DOI: 10.1056/NEJMoa020350]

5   **Di Mauro S**, Schon EA. Mitochondrial Respiratory-Chain Diseases. *N Engl J Med* 2003; **348**: 2656-2668 [DOI: 10.1056/NEJMra022567]

6   **Kadowaki T**, Kadowaki H, Mori Y, Tobe K, Sakuta R, Suzuki Y, Tanabe Y, Sakura H, Awata T, Goto Y. A subtype of diabetes mellitus associated with a mutation of mitochondrial DNA. *N Engl J Med* 1994; **330**: 962-968 [PMID: 8121460 DOI: 10.1056/NEJM199404073301403]

7   **Meyers DE**, Basha HI, Koenig MK. Mitochondrial cardiomyopathy: pathophysiology, diagnosis, and management. *Tex Heart Inst J* 2013; **40**: 385-394 [PMID: 24082366]

8   **Fischel-Ghodsian N**. Mitochondrial mutations and hearing loss: paradigm for mitochondrial genetics. *Am J Hum Genet* 1998; **62**: 15-19 [PMID: 9443888 DOI: 10.1086/301695]

9   **Skladal D**, Halliday J, Thorburn DR. Minimum birth prevalence of mitochondrial respiratory chain disorders in children. *Brain* 2003; **126**: 1905-1912 [PMID: 12805096 DOI: 10.1093/brain/awg170]

10   **Chinnery PF**, Johnson MA, Wardell TM, Singh-Kler R, Hayes C, Brown DT, Taylor RW, Bindoff LA, Turnbull DM. The epidemiology of pathogenic mitochondrial DNA mutations. *Ann Neurol* 2000; **48**: 188-193 [PMID: 10939569]

11   **Darin N**, Oldfors A, Moslemi AR, Holme E, Tulinius M. The incidence of mitochondrial encephalomyopathies in childhood: clinical features and morphological, biochemical, and DNA abnormalities. *Ann Neurol* 2001; **49**: 377-383 [PMID: 11261513 DOI: 10.1002/ana.75]

12   **Cormier-Daire V**, Chretien D, Rustin P, Rötig A, Dubuisson C, Jacquemin E, Hadchouel M, Bernard O, Munnich A. Neonatal and delayed-onset liver involvement in disorders of oxidative phosphorylation. *J Pediatr* 1997; **130**: 817-822 [PMID: 9152294 DOI: 10.1016/s0022-3476(97)80027-3]

13   **Lee WS**, Sokol RJ. Liver disease in mitochondrial disorders. *Semin Liver Dis* 2007; **27**: 259-273 [PMID: 17682973 DOI: 10.1055/s-2007-985071]

14   **Taylor SA**, Whitington PF. Neonatal acute liver failure. *Liver Transpl* 2016; **22**: 677-685 [PMID: 26946058 DOI: 10.1002/lt.24433]

15   **Durand P**, Debray D, Mandel R, Baujard C, Branchereau S, Gauthier F, Jacquemin E, Devictor D. Acute liver failure in infancy: a 14-year experience of a pediatric liver transplantation center. *J Pediatr* 2001; **139**: 871-876 [PMID: 11743517 DOI: 10.1067/mpd.2001.119989]

16   **von Kleist-Retzow JC**, Cormier-Daire V, Viot G, Goldenberg A, Mardach B, Amiel J, Saada P, Dumez Y, Brunelle F, Saudubray JM, Chrétien D, Rötig A, Rustin P, Munnich A, De Lonlay P. Antenatal manifestations of mitochondrial respiratory chain deficiency. *J Pediatr* 2003; **143**: 208-212

[PMID: 12970634 DOI: 10.1067/S0022-3476(03)00130-6]

17   **Harding BN**. Progressive neuronal degeneration of childhood with liver disease (Alpers-Huttenlocher syndrome): a personal review. *J Child Neurol* 1990; **5**: 273-287 [PMID: 2246481 DOI: 10.1177/088307389000500402]

18   **Narkewicz MR**, Sokol RJ, Beckwith B, Sondheimer J, Silverman A. Liver involvement in Alpers disease. *J Pediatr* 1991; **119**: 260-267 [PMID: 1861211 DOI: 10.1016/s0022-3476(05)80736-x]

19   **Sitarz KS**, Elliott HR, Karaman BS, Relton C, Chinnery PF, Horvath R. Valproic acid triggers increased mitochondrial biogenesis in POLG-deficient fibroblasts. *Mol Genet Metab* 2014; **112**: 57-63 [PMID: 24725338 DOI: 10.1016/j.ymgme.2014.03.006]

20   **Salsaa M**, Pereira B, Liu J, Yu W, Jadhav S, Hüttemann M, Greenberg ML. Valproate inhibits mitochondrial bioenergetics and increases glycolysis in Saccharomyces cerevisiae. *Sci Rep* 2020; **10**: 11785 [PMID: 32678210 DOI: 10.1038/s41598-020-68725-5]

21   **Tzoulis C**, Engelsen BA, Telstad W, Aasly J, Zeviani M, Winterthun S, Ferrari G, Aarseth JH, Bindoff LA. The spectrum of clinical disease caused by the A467T and W748S POLG mutations: a study of 26 cases. *Brain* 2006; **129**: 1685-1692 [PMID: 16638794 DOI: 10.1093/brain/awl097]

22   **Rötig A**, Cormier V, Blanche S, Bonnefont JP, Ledeist F, Romero N, Schmitz J, Rustin P, Fischer A, Saudubray JM. Pearson's marrow-pancreas syndrome. A multisystem mitochondrial disorder in infancy. *J Clin Invest* 1990; **86**: 1601-1608 [PMID: 2243133 DOI: 10.1172/JCI114881]

23   **Mandel H**, Szargel R, Labay V, Elpeleg O, Saada A, Shalata A, Anbinder Y, Berkowitz D, Hartman C, Barak M, Eriksson S, Cohen N. The deoxyguanosine kinase gene is mutated in individuals with depleted hepatocerebral mitochondrial DNA. *Nat Genet* 2001; **29**: 337-341 [PMID: 11687800 DOI: 10.1038/ng746]

24   **Tyynismaa H**, Sun R, Ahola-Erkkilä S, Almusa H, Pöyhönen R, Korpela M, Honkaniemi J, Isohanni P, Paetau A, Wang L, Suomalainen A. Thymidine kinase 2 mutations in autosomal recessive progressive external ophthalmoplegia with multiple mitochondrial DNA deletions. *Hum Mol Genet* 2012; **21**: 66-75 [PMID: 21937588 DOI: 10.1093/hmg/ddr438]

25   **Spinazzola A**, Viscomi C, Fernandez-Vizarra E, Carrara F, D'Adamo P, Calvo S, Marsano RM, Donnini C, Weiher H, Strisciuglio P, Parini R, Sarzi E, Chan A, DiMauro S, Rötig A, Gasparini P, Ferrero I, Mootha VK, Tiranti V, Zeviani M. MPV17 encodes an inner mitochondrial membrane protein and is mutated in infantile hepatic mitochondrial DNA depletion. *Nat Genet* 2006; **38**: 570-575 [PMID: 16582910 DOI: 10.1038/ng1765]

26   **Saneto RP**. Mitochondrial diseases: expanding the diagnosis in the era of genetic testing. *J Transl Genet Genom* 2020; **4**: 384-428 [PMID: 33426505 DOI: 10.20517/jtgg.2020.40]

27   **Dror Y**.   Inherited Bone Marrow Failure Syndromes. In: Hoffman R, Benz Jr E, Silberstein L, Weitz J, Heslop H, Anastasi J, Salama M, Abutalib SA, editors. Hematology: Basic Principles and Practice. Elsevier Science, 2017: 350-393 [DOI: 10.1016/B978-0-323-35762-3.00029-9]

28   **Broomfield A**, Sweeney MG, Woodward CE, Fratter C, Morris AM, Leonard JV, Abulhoul L, Grunewald S, Clayton PT, Hanna MG, Poulton J, Rahman S. Paediatric single mitochondrial DNA deletion disorders: an overlapping spectrum of disease. *J Inherit Metab Dis* 2015; **38**: 445-457 [PMID: 25352051 DOI: 10.1007/s10545-014-9778-4]

29   **Muraki K**, Nishimura S, Goto Y, Nonaka I, Sakura N, Ueda K. The association between haematological manifestation and mtDNA deletions in Pearson syndrome. *J Inherit Metab Dis* 1997; **20**: 697-703 [PMID: 9323565 DOI: 10.1023/a:1005378527077]

30   **Molleston JP**, Sokol RJ, Karnsakul W, Miethke A, Horslen S, Magee JC, Romero R, Squires RH, Van Hove JL; Childhood Liver Disease Research Education Network (ChiLDREN). Evaluation of the child with suspected mitochondrial liver disease. *J Pediatr Gastroenterol Nutr* 2013; **57**: 269-276 [PMID: 23783016 DOI: 10.1097/MPG.0b013e31829ef67a]

31   **Farruggia P**, Di Cataldo A, Pinto RM, Palmisani E, Macaluso A, Valvo LL, Cantarini ME, Tornesello A, Corti P, Fioredda F, Varotto S, Martire B, Moroni I, Puccio G, Russo G, Dufour C, Pillon M. Pearson Syndrome: A Retrospective Cohort Study from the Marrow Failure Study Group of A.I.E.O.P. (Associazione Italiana Emato-Oncologia Pediatrica). *JIMD Rep* 2016; **26**: 37-43 [PMID: 26238250 DOI: 10.1007/8904_2015_470]

32   **Karadimas CL**, Vu TH, Holve SA, Chronopoulou P, Quinzii C, Johnsen SD, Kurth J, Eggers E, Palenzuela L, Tanji K, Bonilla E, De Vivo DC, DiMauro S, Hirano M. Navajo neurohepatopathy is caused by a mutation in the MPV17 gene. *Am J Hum Genet* 2006; **79**: 544-548 [PMID: 16909392 DOI: 10.1086/506913]

33   **Holve S**, Hu D, Shub M, Tyson RW, Sokol RJ. Liver disease in Navajo neuropathy. *J Pediatr* 1999; **135**: 482-493 [PMID: 10518083 DOI: 10.1016/s0022-3476(99)70172-1]

34   **Cormier-Daire V**, Bonnefont JP, Rustin P, Maurage C, Ogler H, Schmitz J, Ricour C, Saudubray JM, Munnich A, Rötig A. Mitochondrial DNA rearrangements with onset as chronic diarrhea with villous atrophy. *J Pediatr* 1994; **124**: 63-70 [PMID: 8283377 DOI: 10.1016/s0022-3476(94)70255-1]

35   **Finsterer J**, Frank M. Gastrointestinal manifestations of mitochondrial disorders: a systematic review. *Therap Adv Gastroenterol* 2017; **10**: 142-154 [PMID: 28286566 DOI: 10.1177/1756283X16666806]

36   **Pacitti D**, Levene M, Garone C, Nirmalananthan N, Bax BE. Mitochondrial Neurogastrointestinal Encephalomyopathy: Into the Fourth Decade, What We Have Learned So Far. *Front Genet* 2018; **9**: 669 [PMID: 30627136 DOI: 10.3389/fgene.2018.00669]

37   **Hirano M**, Silvestri G, Blake DM, Lombes A, Minetti C, Bonilla E, Hays AP, Lovelace RE, Butler I, Bertorini TE. Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE): clinical,

biochemical, and genetic features of an autosomal recessive mitochondrial disorder. *Neurology* 1994; **44**: 721-727 [PMID: 8164833 DOI: 10.1212/wnl.44.4.721]

38   **Garone C**, Tadesse S, Hirano M. Clinical and genetic spectrum of mitochondrial neurogastrointestinal encephalomyopathy. *Brain* 2011; **134**: 3326-3332 [PMID: 21933806 DOI: 10.1093/brain/awr245]

39   **Finkenstedt A**, Schranz M, Bösch S, Karall D, Bürgi SS, Ensinger C, Drach M, Mayr JA, Janecke AR, Vogel W, Nachbaur D, Zoller H. MNGIE Syndrome: Liver Cirrhosis Should Be Ruled Out Prior to Bone Marrow Transplantation. *JIMD Rep* 2013; **10**: 41-44 [PMID: 23430799 DOI: 10.1007/8904_2012_199]

40   **Lara MC**, Weiss B, Illa I, Madoz P, Massuet L, Andreu AL, Valentino ML, Anikster Y, Hirano M, Martí R. Infusion of platelets transiently reduces nucleoside overload in MNGIE. *Neurology* 2006; **67**: 1461-1463 [PMID: 16971699 DOI: 10.1212/01.wnl.0000239824.95411.52]

41   **Taanman JW**, Daras M, Albrecht J, Davie CA, Mallam EA, Muddle JR, Weatherall M, Warner TT, Schapira AH, Ginsberg L. Characterization of a novel TYMP splice site mutation associated with mitochondrial neurogastrointestinal encephalomyopathy (MNGIE). *Neuromuscul Disord* 2009; **19**: 151-154 [PMID: 19056268 DOI: 10.1016/j.nmd.2008.11.002]

42   **Martí R**, Nishigaki Y, Hirano M. Elevated plasma deoxyuridine in patients with thymidine phosphorylase deficiency. *Biochem Biophys Res Commun* 2003; **303**: 14-18 [PMID: 12646159 DOI: 10.1016/s0006-291x(03)00294-8]

43   **Spinazzola A**, Marti R, Nishino I, Andreu AL, Naini A, Tadesse S, Pela I, Zammarchi E, Donati MA, Oliver JA, Hirano M. Altered thymidine metabolism due to defects of thymidine phosphorylase. *J Biol Chem* 2002; **277**: 4128-4133 [PMID: 11733540 DOI: 10.1074/jbc.M111028200]

44   **Nishino I**, Spinazzola A, Hirano M. MNGIE: from nuclear DNA to mitochondrial DNA. *Neuromuscul Disord* 2001; **11**: 7-10 [PMID: 11166160 DOI: 10.1016/s0960-8966(00)00159-0]

45   **De Giorgio R**, Pironi L, Rinaldi R, Boschetti E, Caporali L, Capristo M, Casali C, Cenacchi G, Contin M, D'Angelo R, D'Errico A, Gramegna LL, Lodi R, Maresca A, Mohamed S, Morelli MC, Papa V, Tonon C, Tugnoli V, Carelli V, D'Alessandro R, Pinna AD. Liver transplantation for mitochondrial neurogastrointestinal encephalomyopathy. *Ann Neurol* 2016; **80**: 448-455 [PMID: 27421916 DOI: 10.1002/ana.24724]

46   **D'Angelo R**, Rinaldi R, Pironi L, Dotti MT, Pinna AD, Boschetti E, Capristo M, Mohamed S, Contin M, Caporali L, Carelli V, De Giorgio R. Liver transplant reverses biochemical imbalance in mitochondrial neurogastrointestinal encephalomyopathy. *Mitochondrion* 2017; **34**: 101-102 [PMID: 28263873 DOI: 10.1016/j.mito.2017.02.006]

47   **Mitochondrial Medicine Society's Committee on Diagnosis**, Haas RH, Parikh S, Falk MJ, Saneto RP, Wolf NI, Darin N, Wong LJ, Cohen BH, Naviaux RK. The in-depth evaluation of suspected mitochondrial disease. *Mol Genet Metab* 2008; **94**: 16-37 [PMID: 18243024 DOI: 10.1016/j.ymgme.2007.11.018]

48   **Clarke C**, Xiao R, Place E, Zhang Z, Sondheimer N, Bennett M, Yudkoff M, Falk MJ. Mitochondrial respiratory chain disease discrimination by retrospective cohort analysis of blood metabolites. *Mol Genet Metab* 2013; **110**: 145-152 [PMID: 23920046 DOI: 10.1016/j.ymgme.2013.07.011]

49   **Atkuri KR**, Cowan TM, Kwan T, Ng A, Herzenberg LA, Enns GM. Inherited disorders affecting mitochondrial function are associated with glutathione deficiency and hypocitrullinemia. *Proc Natl Acad Sci U S A* 2009; **106**: 3941-3945 [PMID: 19223582 DOI: 10.1073/pnas.0813409106]

50   **ACMG Board of Directors**. Points to consider in the clinical application of genomic sequencing. *Genet Med* 2012; **14**: 759-761 [PMID: 22863877 DOI: 10.1038/gim.2012.74]

51   **McCormick E**, Place E, Falk MJ. Molecular genetic testing for mitochondrial disease: from one generation to the next. *Neurotherapeutics* 2013; **10**: 251-261 [PMID: 23269497 DOI: 10.1007/s13311-012-0174-1]

52   **Thorburn DR**, Sugiana C, Salemi R, Kirby DM, Worgan L, Ohtake A, Ryan MT. Biochemical and molecular diagnosis of mitochondrial respiratory chain disorders. *Biochim Biophys Acta* 2004; **1659**: 121-128 [PMID: 15576043 DOI: 10.1016/j.bbabio.2004.08.006]

53   **Rustin P**, Chretien D, Bourgeron T, Gérard B, Rötig A, Saudubray JM, Munnich A. Biochemical and molecular investigations in respiratory chain deficiencies. *Clin Chim Acta* 1994; **228**: 35-51 [PMID: 7955428 DOI: 10.1016/0009-8981(94)90055-8]

54   **Munnich A**, Rötig A, Chretien D, Saudubray JM, Cormier V, Rustin P. Clinical presentations and laboratory investigations in respiratory chain deficiency. *Eur J Pediatr* 1996; **155**: 262-274 [PMID: 8777918 DOI: 10.1007/BF02002711]

55   **Calvo SE**, Compton AG, Hershman SG, Lim SC, Lieber DS, Tucker EJ, Laskowski A, Garone C, Liu S, Jaffe DB, Christodoulou J, Fletcher JM, Bruno DL, Goldblatt J, Dimauro S, Thorburn DR, Mootha VK. Molecular diagnosis of infantile mitochondrial disease with targeted next-generation sequencing. *Sci Transl Med* 2012; **4**: 118ra10 [PMID: 22277967 DOI: 10.1126/scitranslmed.3003310]

56   **Munnich A**, Rustin P. Clinical spectrum and diagnosis of mitochondrial disorders. *Am J Med Genet* 2001; **106**: 4-17 [PMID: 11579420 DOI: 10.1002/ajmg.1391]

57   **Janssen AJ**, Smeitink JA, van den Heuvel LP. Some practical aspects of providing a diagnostic service for respiratory chain defects. *Ann Clin Biochem* 2003; **40**: 3-8 [PMID: 12542905 DOI: 10.1258/000456303321016114]

58   **Lian C**, Cao S, Zeng W, Li Y, Su J, Li J, Zhao S, Wu L, Tao J, Zhou J, Chen X, Peng C. RJT-101, a novel camptothecin derivative, is highly effective in the treatment of melanoma through DNA damage by targeting topoisomerase 1. *Biochem Pharmacol* 2020; **171**: 113716 [PMID: 31751535 DOI:

10.1016/j.bcp.2019.113716]

59   **Wortmann SB**, Zweers-van Essen H, Rodenburg RJ, van den Heuvel LP, de Vries MC, Rasmussen-Conrad E, Smeitink JA, Morava E. Mitochondrial energy production correlates with the age-related BMI. *Pediatr Res* 2009; **65**: 103-108 [PMID: 19096353 DOI: 10.1203/PDR.0b013e31818d1c8a]

60   **Choi HS**, Lee YM. Enteral Tube Feeding in Paediatric Mitochondrial Diseases. *Sci Rep* 2017; **7**: 16909 [PMID: 29203845 DOI: 10.1038/s41598-017-17256-7]

61   **Tarnopolsky MA**. Mitochondrial DNA shifting in older adults following resistance exercise training. *Appl Physiol Nutr Metab* 2009; **34**: 348-354 [PMID: 19448697 DOI: 10.1139/H09-022]

62   **Jeppesen TD**, Schwartz M, Olsen DB, Wibrand F, Krag T, Dunø M, Hauerslev S, Vissing J. Aerobic training is safe and improves exercise capacity in patients with mitochondrial myopathy. *Brain* 2006; **129**: 3402-3412 [PMID: 16815877 DOI: 10.1093/brain/awl149]

63   **Parikh S**, Saneto R, Falk MJ, Anselm I, Cohen BH, Haas R, Medicine Society TM. A modern approach to the treatment of mitochondrial disease. *Curr Treat Options Neurol* 2009; **11**: 414-430 [PMID: 19891905 DOI: 10.1007/s11940-009-0046-0]

64   **Dimauro S**, Rustin P. A critical approach to the therapy of mitochondrial respiratory chain and oxidative phosphorylation diseases. *Biochim Biophys Acta* 2009; **1792**: 1159-1167 [PMID: 19026744 DOI: 10.1016/j.bbadis.2008.10.015]

65   **Dimmock DP**, Dunn JK, Feigenbaum A, Rupar A, Horvath R, Freisinger P, Mousson de Camaret B, Wong LJ, Scaglia F. Abnormal neurological features predict poor survival and should preclude liver transplantation in patients with deoxyguanosine kinase deficiency. *Liver Transpl* 2008; **14**: 1480-1485 [PMID: 18825706 DOI: 10.1002/lt.21556]

66   **Sokal EM**, Sokol R, Cormier V, Lacaille F, McKiernan P, Van Spronsen FJ, Bernard O, Saudubray JM. Liver transplantation in mitochondrial respiratory chain disorders. *Eur J Pediatr* 1999; **158** Suppl 2: S81-S84 [PMID: 10603105 DOI: 10.1007/pl00014328]

67   **De Greef E**, Christodoulou J, Alexander IE, Shun A, O'Loughlin EV, Thorburn DR, Jermyn V, Stormon MO. Mitochondrial respiratory chain hepatopathies: role of liver transplantation. A case series of five patients. *JIMD Rep* 2012; **4**: 5-11 [PMID: 23430890 DOI: 10.1007/8904_2011_29]

68   **Hirano M**, Martí R, Casali C, Tadesse S, Uldrick T, Fine B, Escolar DM, Valentino ML, Nishino I, Hesdorffer C, Schwartz J, Hawks RG, Martone DL, Cairo MS, DiMauro S, Stanzani M, Garvin JH Jr, Savage DG. Allogeneic stem cell transplantation corrects biochemical derangements in MNGIE. *Neurology* 2006; **67**: 1458-1460 [PMID: 16971696 DOI: 10.1212/01.wnl.0000240853.97716.24]

69   **Rahman S**, Hargreaves IP. Allogeneic stem cell transplantation corrects biochemical derangements in MNGIE. *Neurology* 2007; **68**: 1872; author reply 1872; discussion 1872-1872; author reply 1872; discussion 1873 [PMID: 17515557 DOI: 10.1212/01.wnl.0000265356.98295.be]

MINIREVIEWS

Cystic fibrosis associated liver disease in children

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Abstract

Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations in the CF transmembrane conductance regulator gene. CF liver disease develops in 5%-10% of patients with CF and is the third leading cause of death among patients with CF after pulmonary disease or lung transplant complications. We review the pathogenesis, clinical presentations, complications, diagnostic evaluation, effect of medical therapies especially CF transmembrane conductance regulator modulators and liver transplantation in CF associated liver disease.

Key Words: Cystic fibrosis liver disease; Portal hypertension; Cirrhosis; Liver transplantation; Cystic fibrosis transmembrane conductance regulator modulators; Distal intestinal obstructive syndrome

Core Tip: Cystic fibrosis(CF) liver disease is caused by abnormal cholangiocyte function, altered biliary secretion and abnormal innate immune response with abnormal response to endotoxins. CF liver disease can present with a wide variety of clinical features from a heterogenous liver on ultrasound, to life threatening gastrointestinal bleeds secondary to portal hypertension. Novel treatment strategies directly targeting the ion channel abnormality-cystic fibrosis transmembrane conductance regulator modulators are available and has significantly improved the clinical status and life expectancy of the cystic fibrosis patients.

INTRODUCTION

Cystic fibrosis (CF) the most frequent fatal autosomal recessive disorder in Caucasians, is caused by autosomal recessive disease caused by mutations in the CF transmembrane conductance regulator (*CFTR*) gene on the long arm of chromosome 7 with more than 2000 variants reported[1]. F508del variant resulting from deletion of three nucleotides that leads to loss of a single phenylalanine residue at codon 508, accounts for approximately 70% mutations[2]. CFTR protein is found in the epithelial cells of lungs, sweat glands, liver pancreas and intestine. Liver disease is one of the classic phenotypes of CF.

CF liver disease (CFLD) usually develops within the first 20 years of life and has a stable non-progressive or mildly progressive course in later life[3,4]. Most children with CF will have some degree of steatosis but clinically significant liver disease develops in < 10% of pediatric CF patients usually by 10 years of age. CF related cirrhosis is a disease of the childhood and adolescence while predominant biliary involvement mimicking sclerosing cholangitis mostly occurs in adulthood[5]. The diagnosis of liver disease has profound implications in short and long term prognosis in CF patients and is the third leading cause of mortality in CF. Analysis of a large cohort of patients from the CF Foundation Patient Registry database showed that in CF patients with liver disease, the estimated 10-year cumulative rate of any adverse liver-related outcomes was approximately 20%[2]. Liver disease with cirrhosis and or portal hypertension has been classified as severe CFLD.

PATHOPHYSIOLOGY

CFLD is a genetic disorder of cholangiocyte transport protein defect, resulting in chronic cholangiopathy caused by reduced ductal bile flow generation and reduction in biliary chloride and bicarbonate secretion caused by the dysfunction of CFTR[6,7]. But this mechanism alone cannot explain CFLD, because CFTR deficiency is present in all patients while CFLD occurs only in a small population of CF patients and has varying clinical manifestations and severity. As described below, a combination of factors including CFTR genotype, non-CFTR genetic variability, abnormal intracellular interactions, abnormal cholangiocyte function, altered biliary secretion, pathologic stimulation of innate immune response with abnormal response to endotoxins lead to CFLD.

Abnormal cholangiocyte function and altered biliary secretion

Abnormal CFTR results in inhibition of cyclic adenosine monophosphate dependent chloride and bicarbonate secretion. This reduces the bile flow and alkalinity resulting in the biliary epithelial damages deriving from the retention of cytotoxic bile acids and xenobiotics and from the reduction in natural defenses against microbiologic pathogens. The response to chronic epithelial damage and the progression in the liver damage depends on the immunogenetic response of the individual and on other modifier genes.

Abnormal protein-protein interactions

CFTR mediated liver injury is also postulated to be caused by ability to regulate the function of other proteins by physically associating in macromolecular complexes at the membrane (protein-protein interaction)[8,9]. CFTR interacting proteins are located not only in the plasma membrane but also in nucleus, endoplasmic reticulum, Golgi apparatus, trafficking vesicles, proteasomes and cytoskeleton[9]. For example, the interaction of CFTR with proteins regulating the function of non-receptor tyrosine kinase Rous sarcoma oncogene cellular homologue can modulate innate immune responses in cholangiocytes[8]. Dysfunction of interactions can have systemic consequences resulting from the perturbation of the interconnected cellular networks accounting for some of the phenotypic variation in CF[8].

Abnormal innate inflammatory response

The conventional theory of CFLD postulates that biliary epithelial CFTR dysfunction causes alterations in the volume and composition of bile, resulting in loss of protective effect of biliary bicarbonate and mucus and an accumulation of toxic bile acids causing damage to the epithelium by initiating an inflammatory response[8]. But it is now postulated that the abnormal inflammatory response is due to lack of tolerance in the innate immune system[7]. CFTR is a now thought as a regulator of cholangiocyte

innate immune responses and defective CFTR results in aberrant activation of Src tyrosine kinase causing upregulation of innate inflammatory responses *via* the Toll-like receptor 4/NF-κB axis[7,10]. This results in lack of tolerance of biliary epithelium to endotoxin (*e.g.* pathogen-associated molecular patterns) from bile and intestine, leading to a para-inflammatory process in the biliary epithelium with the release of cyto/chemokines and the infiltration of the portal spaces with inflammatory cells[7, 10].

Gut dysbiosis and role of gut-liver axis

There is a substantial reduction in the richness and diversity of gut bacteria in patients with CF from early childhood until late adolescence and the changes deviate progressively farther from the path of healthy controls with increasing age[11]. Gut dysbiosis results in reduction in anti-inflammatory short-chain fatty acids, altered ratios of arachidonic acid/Linoleic acid and arachidonic acid/docosahexaenoic acid leading to increased gut inflammation[8,12]. This causes increased permeability of intestinal epithelia, increasing the exposure of biliary epithelial cholangiocytes to endotoxins, perpetuating the inflammatory cascade[8,12]. But it is not certain if intestinal inflammation is caused by the altered microbiota in CF or is the consequence of an altered environment[8,12].

Genetics

There is massive heterogenicity in CFTR phenotype among patients with CFLD and CFTR genotype-phenotype correlations are generally weak. The functional consequences of CF-causing variants have been grouped into six classes[1,13] (Figure 1). Mutations in classes I and II are also known as minimal function mutations since they demonstrate no to very little CFTR function, while those in classes IV, V, and VI are known as residual function mutations since they demonstrate some CFTR function, although it is lower compared to the wild type CFTR[14]. CFLD is mostly occurs in pancreatic insufficient patients with biallelic loss-of-function mutations in CFTR (class I, II, or III mutations on both allele)[1,3]. It has been shown that non-CFTR genetic variability also contributes to risk for severe liver disease[15]. This might be one of the reasons in variability of phenotype even between siblings inheriting the same mutations. Though many candidate genes have been postulated, in a large study *SERPINA1* (coding for alpha1-antitrypsin) Z allele was significantly associated with CFLD and portal hypertension[16].

CLINICAL FEATURES

The prevalence of CFLD varies widely in children and adolescents, based upon the diagnostic criteria used ranging from < 5% to 68%[17,18]. CFLD is more common and the median age of diagnosis is earlier in males[19]. Liver involvement in CF may be subclinical until diffuse liver damage occurs. Liver involvement can vary from mild elevation of aminotransferases to cirrhosis with synthetic failure and portal hypertension. The degree of liver involvement and the rate of progression of liver disease varies significantly among individuals. The awareness of CFLD and its clinical implications has increased as evidenced by an early diagnosis and a drop in the median time at diagnosis from adolescence to < 3 years of age[17,18].

Risk factors for CFLD include male sex, presence of severe mutations, presence of *SERPINA 1Z* allele, history of meconium ileus, exocrine pancreatic insufficiency and CF-related diabetes[20]. The most common clinical feature is asymptomatic hepatomegaly detected by clinical examination or ultrasonography[18]. Pancreatic insufficiency occurs in 99% of patients with CFLD[19]. Liver involvement in CF can be classified into two broad categories based on the presence of cirrhosis/portal hypertension (Table 1).

Liver disease without portal hypertension

Cholestasis: Neonatal/infantile cholestasis is the earliest manifestation of liver involvement in CF, but is very rare (< 2%). It is important to exclude other common causes of neonatal cholestasis like biliary atresia and also to consider the diagnosis of CF in infants who present with cholestasis[21].

Abnormal liver enzymes: The commonly noticed abnormalities include intermittent rise in serum transaminases (aspartate aminotransferase (AST) and alanine aminotransferase (ALT)) and/or increased serum levels of alkaline phosphatase (ALP)

Table 1 Spectrum of cystic fibrosis liver disease in children
Spectrum of cystic fibrosis liver disease in children
Liver
Neonatal cholestasis
Pre-clinical
Elevated aminotransferases
Increased GGT
Steatosis
Portal hypertension including non-cirrhotic portal hypertension
Cirrhosis
Focal biliary
Multi-lobular
Gallbladder and biliary system
Cholelithiasis
Abnormal size/function
Intra and extrahepatic biliary strictures (sclerosing cholangitis)

GGT: Gamma glutamyl transferase.

and gamma glutamyl transferase (GGT). Elevated liver enzymes can precede clinical and radiological abnormalities by several years. Bile duct damages can be demonstrated even in asymptomatic cases[22]. About 53%–93% of patients with CF have at least one abnormal value of AST/ALT, while over one-third have abnormal levels of GGT by 21 years of age[23]. CFLD patients with cirrhosis with portal hypertension can have normal liver biochemistry and synthetic function. Fluctuations in liver biochemistry is common and can be due to medications, infection or malnutrition.

Steatosis: Steatosis is common in CF patients, seen in upto 70% children undergoing liver biopsies[24]. The etiology is uncertain, but postulated to be due to malnutrition, deficiencies of essential fatty acid, carnitine and choline[24,25]. Steatosis in CF patients can also be caused by impaired glucose tolerance, diabetes mellites, hypertrigly-ceridemia and obesity[23]. Significant steatosis has become uncommon due to earlier diagnosis of CFLD and appropriate nutritional management. Alcohol consumption should be considered in adolescent CF patients with steatosis. Steatosis in CF was previously thought to be a benign condition, but with the emergence of nonalcoholic steatohepatitis as a leading cause of cirrhosis and understanding of the pathology, this might no longer be the case. Other signs of chronic liver disease or portal hypertension are usually not present.

Gallbladder and biliary tract involvement: Abnormalities of gallbladder (GB) can be present in children with CF. Micro-GB has been described in up to 33% of patients and GB might even be absent in CF patients[26]. Abnormal function of gallbladder and gallstones can also present. Black pigmented stones are more commonly found in patients with CF compared to cholesterol gallstones which are common in general population[26]. Symptomatic GB disease (4%) and need for cholecystectomy is common in adults[26].

Intra- or extrahepatic biliary strictures and segmental dilation has been reported in children with CF. Bile duct strictures and associated complications frequently occur even in patients with mild variants of CF. Magnetic resonance (MR) cholangiography data has shown that up to 70% of patients can have abnormalities of biliary tree regardless of biochemical or clinical evidence of liver disease and can mimic primary sclerosing cholangitis[24,26]. There is no correlation between severity of liver disease, abnormal liver tests and the presence of biliary strictures[24,26].

	WT-CFTR	I	II	III	IV	V	VI
Mutation examples		G542X, R553X, R1162X, W1282X	G85E, I507del, F508del, N1303K	S549R, G551D, G1349d	R117H, R334W, D1152H	A455E, 2789+5G>A, 3849+10kbC>T	r.F508del, Q1411X
Defect types		Protein synthesis	Protein traffic	Channel gating	Channel conductance	Protein abundance	PM Protein stability
		Minimal function			Residual function		
Clinical features (global aspects)		More severe phenotype			Milder phenotype		

Figure 1 The functional consequences of cystic fibrosis-causing variants have been grouped into six classes. Class I mutations lead to no protein synthesis or translation of shortened, truncated forms. They result from splice site abnormalities, frameshifts due to deletions or insertions, or nonsense mutations, which generate premature termination codons. Class II mutations lead to a misfolding protein that fails to achieve conformational stability in the endoplasmic reticulum and then does not traffic to the plasma membrane (PM), being instead prematurely degraded by proteasomes. Class III mutations lead to a gating channel defect due to impaired response to agonists, although the protein is present at the PM. Class IV mutations lead to a channel conductance defect with a significant reduction in cystic fibrosis transmembrane conductance regulator (CFTR)-dependent chloride transport. Class V mutations lead to a reduction in protein abundance of functional CFTR due to reduced synthesis or inefficient protein maturation. They result from alternative splicing, promoter or missense mutations. Class VI mutations lead to reduced protein stability at the PM, which results in increased endocytosis and degradation by lysosomes, and reduced recycling to the PM. PM: Plasma membrane.

Liver disease with cirrhosis/portal hypertension (severe CFLD)

Portal hypertension: Variceal bleed can occur with or without cirrhosis and frequently occurs in the context of preserved hepatic synthetic function. Varices can be seen in 10-70% with CFLD and may be present at diagnosis of CFLD in 25%[19,27,28]. Isolated gastric varices may be seen in 15%[19]. Variceal bleed can be the sentinel event in CFLD leading to the diagnosis of portal hypertension/cirrhosis in up to 50% and may also be fatal, either from bleed itself or by precipitating liver failure. The age at first bleed can range from 10-30 years and recurrent bleeds can also occur[4]. Variceal bleed is associated with 5 fold risk of liver transplantation (LT)[2]. Thrombocytopenia has been postulated as a marker of severe CFLD with portal hypertension, so decreasing or persistently low platelet counts should prompt evaluation for portal hypertension [19]. Non cirrhotic portal hypertension can also occur in CFLD[28,29]. This has been postulated to be due to perisinusoidal portal venopathy caused by inflammation and fibrosis[24,29].

Focal biliary cirrhosis: Focal biliary cirrhosis is characterized by focal areas of scarring and furrowing in the liver with large areas of normal preserved hepatic architecture in between. Histologically, it is characterized by cholestasis, significant focal fibrosis, plugging of bile ducts with eosinophilic material, bile duct proliferation and expansion of portal tract leading to the postulation that bile duct plugging is the causative factor.

Focal biliary cirrhosis is clinically silent without any abnormalities on physical examination and normal liver biochemistry. Radiological imaging is also frequently noncontributory. Postmortem studies have shown that the incidence of focal biliary cirrhosis increases with advanced age- 11% in infants, 27% at 1 year and 25%–70% of adults[24]. Only a small subset of patients will progress to more severe liver disease and eventually multilobular cirrhosis, but the factors causing this is not known.

Multilobular cirrhosis: Biliary cirrhosis with portal hypertension is the most severe clinical manifestation of CFLD. Clinically, liver is multilobulated and firm- extensive lobulation is characteristic of CF cirrhosis. Signs of chronic liver disease such as clubbing, spider angioma, and palmar erythema may be present but is uncommon and

often occurs late in the disease course. There are no clinical or biochemical abnormalities or radiological features that consistently predict the presence of cirrhosis or risk of development of portal hypertension[28]. Majority of the morbidity due cirrhosis is caused by complications arising from portal hypertension. Hepatic encephalopathy is rare complication of cirrhosis per se in CFLD and mostly has occurred after therapeutic portosystemic shunting for management of portal hypertension[24]. Hepatic decompensation as evidenced by progressive decrease in albumin levels and development of ascites represents poor prognosis and necessitates LT evaluation.

Patients with cirrhosis are at risk of significant malnutrition as compared to CF patients without liver disease. This is due to anorexia, micronutrient deficiency, early satiety due to organomegaly and increased catabolism. In a study comparing CFLD patients with CF patients without liver disease, body fat measurements, including triceps, subscapular, and supra-iliac skinfold measures, were significantly less in the CFLD patients[27]. However, weight, height and mid upper arm circumference were not different between the two groups[27].

EVALUATION

Liver enzymes (AST, ALT, GGT) are poor predictors or indicators of cirrhosis or the risk of development of cirrhosis or CFLD and are neither sensitive or specific. There is poor correlation of liver enzymes with histologic findings, with 25% of CFLD patients with biopsy proven severe liver fibrosis having normal ALT levels[28]. But patients presenting with significant or persistently elevated liver biochemistries warrant further investigation for evidence of CFLD and other etiologies (Table 2). Persistently elevated GGT might be a pointer to biliary disease (*e.g.,* sclerosing cholangitis). Thrombocytopenia with splenomegaly is suggestive of development of portal hypertension. The synthetic function of liver (clotting, albumin) should be checked in all patients with suspected CFLD. If deranged after correcting nutritional (poor diet, vitamin deficiency) defects, should be thoroughly investigated.

Imaging
Ultrasound (US) of the hepatobiliary system with Doppler measurements of hepatic vasculature is non-invasive and may be a valuable marker of early CFLD[30]. Partial or complete hyper echogenicity liver, suggestive of steatosis is the most common US finding in CF[31]. Another fatty infiltration pattern, pseudomasses, seen as lobulated fatty structures of 1–2cm causing heterogeneity in the liver parenchyma is typical of CF[31]. Focal biliary cirrhosis appears sonographically as regions of increased echogenicity in periportal areas[31,32]. Cirrhotic liver has a nodular appearance with a coarsened echotexture[32]. Right hepatic lobe atrophy and hypertrophy of the caudate and lateral segments of the left lobe may be seen[32]. Splenomegaly, portosystemic shunts, hepatofugal flow in portal vein, and ascites can be seen with portal hypertension.

Abnormal echogenicity frequently precedes biochemical/clinical evidence of liver disease, with one study showing that two thirds of the children with abnormal liver echotexture and 50% with portal hypertension had no biochemical/clinical evidence of CFLD at the time when US changes were first noted[30]. Heterogeneous pattern of liver has been shown to be associated with higher risk of development of advanced liver disease in CF patients[30,33]. However, there is significant intra/ interobserver variability in US imaging and children with normal hepatic US can have advanced fibrosis, so a normal US does not exclude significant liver fibrosis or CFLD[3].

Assessment of the intra and extrahepatic biliary tree is better with MR cholangiography. The typical appearances include strictures, beading, narrowing, or dilatation of the intrahepatic ducts; diffuse narrowing or focal stricture of the common bile duct; and calculi[32].

Liver biopsy
Liver biopsy (LB) the gold standard in diagnosing fibrosis and cirrhosis, but is difficult to perform in CF patients because of the invasive nature and presence of associated comorbidities. Also because of the patchy distribution of lesions in CFLD, LB may underestimate the severity of lesions[25]. LB should be reserved for evaluation for other potential causes of fibrosis (autoimmune hepatitis, Wilson's disease, hepatotrophic infections) or drug-induced liver injury.

Table 2 Causes of acute or chronic liver disease in cystic fibrosis patients showing hepatic abnormalities

Condition	Investigation
Acute/chronic viral hepatitis	Serology for HAV, HBV, HCV, EBV, CMV, adenovirus, HHV 6, parvovirus
α1 antitrypsin deficiency	Serum α1 antitrypsin level, including phenotype
Autoimmune hepatitis	Non-organ specific autoantibodies (SMA, anti-LKM1, LC1)
Celiac disease	Total IgA, IgA anti-tissue transglutaminase
Wilson disease	Ceruloplasmin, serum copper, 24 h urinary copper
Drug induced liver injury	Antibiotics (cyclines, macrolides, amoxicillin-based, and cephalosporins) & antifungals (azoles and polyenes)
Genetic hemochromatosis (adults)	Iron, Ferritin, Transferrin binding capacity
Other causes of steatosis	Malnutrition, diabetes, obesity

This table is modified from Debray *et al*[25]. HAV: Hepatitis A virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; CMV: Cytomegalovirus; EBV: Epstein-Barr virus; HHV6: Herpes hominis virus type 6; SMA: Smooth muscle antibody; LKM1: Liver kidney microsomal type 1; LC: Liver cytosol type 1; IgA: Immunoglobulin A.

Noninvasive tests of fibrosis and liver disease

The early detection and monitoring of fibrosis, assessment of stage of fibrosis and progression to CFLD is challenging because routinely available tests to measure liver damage can often be normal even in advanced cirrhosis and liver biopsy is invasive with potential risk of complications. Non-invasive tests are divided into direct and indirect markers of liver fibrosis and imaging modalities as outlined in Table 3.

Direct markers are components of extracellular matrix degradation or fibrogenesis in serum include Matrix Metalloproteinase-9, tissue inhibitor of metalloproteinase-1 and 2, procollagen III peptide, collagen type-IV, hyaluronic acid, laminin, prolyl hydroxylase and YKL-40. These are not readily available in the routine clinical setting, are costly and are not validated in large scale studies. Indirect markers are serum-based tests and consist of readily available biochemical surrogates and clinical risk factors (AST, ALT, platelet count, age) for liver fibrosis. These include aspartate aminotransferase to platelet ratio index (APRI) and Fibrosis-4 (Fib-4). Stonebraker *et al* [19] demonstrated in a large pediatric cohort (n = 497) with CFLD and portal hypertension that APRI and Fib-4 values could differentiate patients who developed complications of portal hypertension and were significantly different in CFLD patients with and without oesophageal varices.

Advanced imaging modalities which quantify liver stiffness as a marker of fibrosis such as transient elastography (TE, Fibroscan®), acoustic radiation force impulse and MR elastography have been shown to accurately reflect advanced liver disease/end-stage fibrosis in CF. Liver stiffness as measured by TE had high diagnostic accuracy and was increased in CFLD compared to CF patients without liver disease[34]. Serial monitoring using TE is more useful as progressive enhancement of liver stiffness as this might reflect progression of liver disease thereby facilitating early detection[34, 35]. MR elastography is currently the most accurate noninvasive method across the spectrum of liver fibrosis and offers promise in the assessment of response to antifibrotic drugs but is not well studied in the context of CF liver disease[36].

Noninvasive methods are valuable for excluding advanced fibrosis or cirrhosis, but are not sufficiently predictive when used in isolation and have not yet been demonstrated to accurately reflect fibrosis change in response to treatment, limiting their role in disease monitoring[36]. Combination of serum markers with liver stiffness analysis might improve the sensitivity and negative predictive value without altering the specificity[34]. The negative predictive value of noninvasive tests is generally very high, allowing the clinician to be confident that advanced fibrosis or cirrhosis has been excluded.

DIFFERENTIAL DIAGNOSIS

The wide spectrum, variability of presentation at different age groups, presence of confounding factors and the absence of specific markers or tests makes it difficult to diagnose CFLD. The common differential diagnosis to be considered in CFLD are

Table 3 Examples of noninvasive monitoring of liver fibrosis in pediatric cystic fibrosis liver disease

Non-invasive marker	Ref.	Outcome measured	AUC	Sensitivity	Specificity	Comments
Indirect markers of liver fibrosis						
APRI	Leung *et al*[37]	CFLD diagnosis and severe CFLD	0.81	73%	70%	APRI score cut-off > 0.264; Predict CFLD and significant fibrosis in CFLD with a high degree of accuracy
FIB-4	Leung *et al*[37]	Portal hypertension	0.91	78%	93%	FIB-4 cutoff 0.358
Direct markers of liver fibrosis						
TIMP-1	Pereira *et al*[38]	CFLD diagnosis	0.76	64%	83%	Significantly increased in CFLD *vs* no-CFLD
Prolyl hydroxylase	Pereira *et al*[38]	CFLD diagnosis		60%	91%	Negative correlation between serum TIMP-1 levels and the stage of histological fibrosis; Prolyl hydroxylase useful in distinguishing CFLD patients with early fibrogenesis *vs* extensive fibrosis; Not able to differentiate CFLD versus no-CFLD
TIMP-2	Rath *et al* [38]	CFLD diagnosis	0.69	-	-	
m-RNA's	Cook *et al* [39]	CFLD diagnosis	0.78	47%	94%	Able to differentiate between CFLD versus no-CFLD but quantify not fibrosis stage; Pathological significance not yet certain, more studies needed
Imaging methods						
Transient elastography	Witters *et al*[40]	Liver stiffness	0.86	63%	87%	Less inter and intra-observer variability; Easy to learn and perform; Regular measurements for serial follow-up feasible
	Rath *et al* [34]	Liver stiffness	0.68	-	-	Few centres have access to technology
MR elastography	Palermo *et al*[41]	Liver stiffness	-	100%	100%	Small study, paucity of data; Shear stiffness significantly elevated in CF patients with cirrhosis; Costly with limited availability

AUC: Area under the curve; APRI: Aspartate aminotransferase to platelet ratio index; CFLD: Cystic fibrosis associated liver disease; Fib-4: Fibrosis-4; TIMP: Tissue inhibitor of metalloproteinase; m-RNA: Messenger ribonucleic acid; MR: Magnetic resonance.

listed in Table 2.

DIAGNOSTIC CRITERIA OF CFLD

The commonly used diagnostic criteria are described in Table 4.

MANAGEMENT

Management of CFLD should be done by a multi-disciplinary team and is mainly supportive since there is no effective therapy to treat or prevent progression of fibrosis, portal hypertension, or cirrhosis in CFLD. The CF foundation guidelines recommends annual screening for CFLD in children with examination of abdomen (hepatospleno-megaly), biochemical evaluation (bilirubin, AST, ALT, GGT, ALP, albumin, prothrombin time, platelet count), abdominal US and pulse oximetry (screening for hepatopulmonary syndrome)[25]. Salicylic acid and non-steroid anti-inflammatory drugs are contraindicated once CFLD is diagnosed and vaccination against hepatitis A and B should be done.

Ursodeoxycholic acid (UDCA) is recommended for all children diagnosed with CFLD at 20 mg/kg/d divided twice daily initially and increased up to 30 mg/kg/d [25]. A Cochrane review[42] had shown that there were only few trials assessing the effectiveness of UDCA with poor quality of evidence and there was no data on the effect of UDCA on long term outcomes including need for LT or mortality. Hence, the long term continuation of UDCA should be individualized.

Table 4 Diagnostic criteria of cystic fibrosis liver disease

Debray *et al*[25]	CF foundation classification[24]
Hepatomegaly and/or splenomegaly- increased liver span at midclavicular line and spleen size in longitudinal coronal plane for age and sex, confirmed by ultrasonography	CF related liver disease with cirrhosis/portal hypertension (based on clinical exam/imaging, histology, laparoscopy)
Abnormalities of liver function tests-elevated AST and ALT and GGT levels above the upper limit of normal with at least at 3 consecutive determinations over 12 months after excluding other causes of liver diseases	Liver involvement without cirrhosis/portal hypertension consisting of at least one of the following: (1) Persistent AST, ALT, GGT > 2 times upper limit of normal; (2) Intermittent elevations of the above laboratory values; (3) Steatosis (histologic determination); (4) Fibrosis (histologic determination); (5) Cholangiopathy (based on ultrasound, MRI, CT, ERCP); and (6) Ultrasound abnormalities not consistent with cirrhosis
Ultrasonographic evidence of coarseness, nodularity, increased echogenicity, or portal hypertension	Preclinical: No evidence of liver disease on clinical examination, imaging or laboratory values
Liver biopsy showing cirrhosis	

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma glutamyl transpeptidase; CF: Cystic fibrosis; MRI: Magnetic resonance imaging; CT: Computed tomography; ERCP: Endoscopic retrograde cholangiopancreatography.

Nutrition

Optimal nutrition is the cornerstone of CFLD management. Malnutrition in CF is multifactorial including malabsorption due to pancreatic insufficiency, recurrent infections, chronic inflammation, chronic liver disease and anorexia. Nutrition should be managed by experienced CF dietetic team. It is recommended that CFLD patients increase energy intake to 150% of Recommended Daily Allowance preferably by increasing proportion of fat to 40%–50% of the energy content of the feed or diet, with supplementation in medium chain triglycerides and special attention to polyunsaturated fatty acids[25].

About 3 g/kg/d of protein and sufficient pancreatic enzymes to allow optimal absorption of long-chain triglycerides and essential fatty acids is also recommended. High dose oral fat soluble vitamin supplements is recommended- vitamin A (5000–15000 international units daily), vitamin E (alpha tocopherol 100–500 mg daily), vitamin D (alphacalcidiol 50 ng/kg to maximum of 1 µg) and vitamin K (1–10 mg daily)[25]. Plasma levels of vitamins (A, D and E) and prothrombin time needs to be closely monitored to prevent toxicity or deficiencies.

Salt supplementation should be avoided in CF patients with cirrhosis and portal hypertension due to the risk of development of ascites. If adequate caloric intake cannot be achieved orally, nasogastric feeding may be required to ensure adequate caloric intake. CFTR modulator therapy has resulted in less pulmonary exacerbations, decrease in levels of inflammatory makers, better body mass index and pancreatic function resulting in better overall nutritional status[14].

Management of esophageal varices

Management of varices in CFLD is complicated by the fact that non-selective beta-blocker (propranolol or carvedilol) might be contraindicated due to the associated lung disease and repeated general anesthesia required for screening of therapeutic endoscopic procedures may also reduce lung function and predispose to infections. Primary variceal prophylaxis in CFLD most commonly involves endoscopic variceal band ligation, but there is lack of quality evidence in children[24].

Variceal bleeding in the absence of decompensated cirrhosis in CFLD is most commonly managed by therapeutic endoscopy (band ligation +/- sclerotherapy)[4]. Sclerotherapy is useful if variceal band ligation is unsuccessful or gastric varices are present. Patients with refractory life threatening bleeds might require transjugular intrahepatic portosystemic shunt (TIPSS) or in rare circumstances surgical portosystemic shunting as an lifesaving procedure. Careful evaluation of liver disease and lung disease is necessary before proceeding with an elective TIPSS procedure. In a study[4] specifically analyzing outcomes of variceal bleeds in CFLD, out of 35 bleeding episodes, 30 were controlled by endoscopic procedures, while 11% (4 episodes) required either TIPSS, surgical shunts procedures.

Liver transplantation

LT evaluation should be offered for CFLD patients with intractable complications of portal hypertension and/or end stage liver disease since LT confers significant

survival advantage[43]. The main indications of isolated LT in CFLD is listed in Table 5. Poor growth and nutrition as an indication remains controversial because studies have not shown consistent improvement after LT[43]. LT should be considered when nutritional deficiencies are believed to be sequelae of advanced liver disease and portal hypertensive enteropathy impacting clinical outcomes[43]. Lung function may improve, remain stable or deteriorate after LT and any short term advantage with improvement of lung function is lost within 3 years of LT[44,45]. So, rapidly deteriorating lung function alone should not be an indication for isolated LT in stable CFLD [46].

Long term outcomes after LT are lower in children with CFLD as compared to other etiologies[44]. Table 6 illustrates details of few published series on LT in CFLD in children. For those patients with end-stage liver disease and significant pulmonary complications, combined liver-lung or liver-heart-lung transplantation may be considered, but outcomes are worse compared to isolated LT[45,46].

Pre-transplant considerations

Careful assessment of liver disease, pulmonary function, nutritional status and type of transplant to be performed should be done by an experienced multidisciplinary team. Concomitant causes or other etiologies of liver injury as listed in Table 3 should be ruled out before LT is considered. Alpha-1-antitrypsin level and genotype, screening for autoimmune hepatitis and Wilson's disease should be done as a part of the workup especially if the child is seen for the first time in a LT center. CFLD patients being considered for LT should have endoscopic variceal surveillance and possibly coordinated with bronchoscopy and dental procedures as part of the LT evaluation to minimize the number anaesthetic procedures[43]. Careful evaluation of cardiac function should be done since patients with cardiomyopathy or severe pulmonary hypertension may require combined heart, lung, and liver transplantation.

A thorough evaluation by a pediatric pulmonologist with CF and lung transplantation expertise should be a part of the LT assessment, irrespective of the forced expiratory volume in one second (FEV1). Analysis of United Network for Organ Sharing data from 1987 through 2009 suggested that patients with a predicted forced vital capacity (FVC) > 75% and FEV1 > 60% (possibly even ≥ 40%) may be safely offered isolated LT[50]. The possibility of progressive deterioration in lung function after LT should be communicated to the family. The most difficult group to decide is patients who require LT but present with borderline (FEV1 40%-60% predicted) and/or rapidly declining (10% FEV1 predicted/year) pulmonary function[43].

Microbial considerations, such as multidrug resistant bacterial infections and history of recurrent/ invasive fungal infections are critical since post-transplant sepsis is a leading cause of mortality[43,50]. Flexible bronchoscopy with bronchioalveolar lavage with cultures for mycobacteria, fungus, and quantitative bacterial analysis from at least 2 locations within each lung is recommended[43]. The presence of multidrug resistant *Mycobacterium abscessus* in the lungs, even with well-preserved pulmonary function, carries a high risk of mortality in the first year after transplant and needs to be considered carefully before recommendation for LT[43].

Patients should be evaluated for nasal polyps and chronic sinusitis and treated immediately if identified[43]. CF-related diabetes should be evaluated and well controlled prior to LT. Dietetic and nutritional assessment is an integral part of the evaluation.

Post-transplant considerations

Immunosuppression after LT in patients with CF will vary from center to center but typically consists of triple drug therapy with tacrolimus, steroids and mycophenolate mofetil/azathioprine. Close collaboration between the CF, transplant and infectious diseases teams is crucial because of the increased risk of mortality from infections. Early mortality (< 6 mo) post-LT is due to disseminated aspergillosis/candidiasis, and sepsis with gram-negative enteric bacteria and staphylococcus aureus while later deaths are a result of progressive pulmonary disease[43]. Post-transplant antibiotic prophylaxis in our unit consists of fluconazole for candida species, acyclovir for herpes simplex virus, valganciclovir for cytomegalovirus and trimethoprim-sulfamethoxazole for *Pneumocystis jiroveci*. Distal intestinal obstructive syndrome (DIOS) causing acute potentially life-threatening intestinal obstruction can develop post- transplant in >20% of pediatric patients[49]. In the pre-transplant period, DIOS occurs typically in older CF patients in adolescence and adulthood, in those with advanced liver disease, severe CFTR mutations, pancreatic insufficiency and diabetes mellitus. In our unit, patients are categorized into low risk (no episodes of DIOS in previous 5 years) and high risk (episodes of DIOS in previous 5 years and previous abdominal surgery) before LT.

Table 5 Indications and contraindications for liver transplantation in cystic fibrosis liver disease (Modified from Freeman *et al*[43])

Indications and contraindications	
Indications	
Strong	(1) Progressive hepatic dysfunction with hypoalbuminemia and coagulopathy (Coagulopathy not corrected by vitamin K, cholestasis not attributed to other causes); (2) Complications of portal hypertension (Intractable/recurrent variceal bleeding which is not controlled by medical or endoscopic management); (3) Hepatopulmonary and porto-pulmonary syndrome; (4) Overt hepatic encephalopathy; and (5) Hepatorenal syndrome
Controversial	(1) Deteriorating pulmonary function (FEV1/FVC <50%) with increased frequency and severity of pulmonary infective episodes requiring hospitalization; and (2) Severe malnutrition, unresponsive to intensive nutritional support
Contraindications	
Absolute	(1) Extrahepatic malignancies not amenable to curative therapy; (2) Multiorgan disease for which transplant would not be considered life-sustaining; (3) Uncontrolled systemic or pulmonary infection, active exacerbation, or veno-arterial extracorporeal membrane oxygenation; and (4) Severe porto-pulmonary hypertension nonresponsive to medical management
Relative	(1) Hepatocellular carcinoma; (2) Noncompliance or psychosocial concerns unamenable to transplant; (3) Uncontrollable CF-related diabetes; (4) Substance abuse; (5) Severe cardiopulmonary disease; and (6) Infection/colonization with multi-resistant organism (*e.g.*, *Burkholderia cenocepacia* and *Mycobacterium abscessus*)

FEV1: Forced expiratory volume in one second; FVC: Forced vital capacity.

Table 6 Liver transplantation in cystic fibrosis liver disease - data from few published series

Ref.	Type	Number of pediatric recipients	Type of transplants	Males	Mean age at isolated liver transplantation (yr)	Lung function after Liver transplantation	5-year survival
Milkiewicz *et al*[45], 2002	Single center	9	Liver; Liver-lung -heart	Not available	15	Improved	Not available
Fridell *et al*[21], 2003	Single center	12	Liver	83%	10 ± 4.5	Improved or remained unchanged	75%
Molmenti *et al*[47], 2003	Single center	10	Liver	90%	9.7 (1.23–19)	Not available	60%
Mendizabal *et al*[44], 2011	Analysis of United Network for Organ Sharing database	148	Liver; Liver-lung (3.4%)	62%	11 ± 4.7	Not available	86%
Miguel *et al*[48], 2011	Single center	11	Liver	67%	12 (5.4–17)	Worsened or remained unchanged	> 85%
Dowman *et al*[49], 2012	Single center	19	Liver	Not available	11.8 (9.5–16.5)	Stable/improved initially, deteriorated > 5 years after transplant	> 60%

Our pre and post-LT protocol for prevention and treatment of DIOS is given in Table 7. High risk patients should be counselled for loop ileostomy formation at transplant assessment.

CFTR modulators

CFTR modulator drugs enhance or even restore the expression, function, and stability of a defective CFTR by different mechanisms[14,51] (Table 8). These treatments target the underlying cause of CF and is classified into five main groups depending on their effects on CFTR mutations[14,51] (Table 8). Different CFTR genetic variants can benefit from the same type of modulator and this is the base of a new system recently introduced to classify and group common and rare CFTR variants based on their response to modulators called 'theratyping'.

The first United States Food and Drug Administration (FDA) approved drug was ivacaftor (Kalydeco, Vertex Pharmaceuticals)[14,51]. Other FDA approved CF modulators combinations are lumacaftor/ivacaftor (Orkambi®, Vertex Pharmaceuticals), tezacaftor/ivacaftor (Symdeko® or Symkevi®, Vertex Pharmaceuticals) for patients aged ≥ 12 years who are F508del-homozygous or F508del-heterozygous with a residual function mutation[14,20]. Lumacaftor/ivacaftor has been approved for F508del homozygous patients aged ≥ 2 years[14]. The triple combination elexacaftor/ ivacaftor/tezacaftor (Trikafta™, Vertex Pharmaceuticals) has been by the FDA for the

Table 7 Pre and post-transplant protocol for prevention and treatment of distal intestinal obstructive syndrome

Pre and post-transplant protocol	
Low risk	(1) 600 mg N-acetyl-cysteine in 120 mL water orally/nasogastric tube twice/day. Senna twice daily; (2) 2 liters of Klean prep per day post-transplant; (3) Consider early nasogastric tube in patients with delayed gastric emptying studies pre-operatively; (4) All patients in intensive care unit should receive only elemental feed *via* nasogastric tube as this does not require pancreatic enzyme replacement. Once transferred to ward, can be restarted on regular feeding and pancreatic enzyme supplements; (5) Try and reduce opiates early during hospital stay; and (6) Treat all patients with proton pump inhibitors.
High risk	(1) As per low risk management; and (2) High risk of developing DIOS and subsequent surgical gut decompression is associated with a high mortality. So these patients should receive a prophylactic loop ileostomy.
Treatment of DIOS	(1) Stop feeding, nasogastric tube on free drainage and intravenous fluids; (2) 100 mL gastrografin in 400 mL water enterally and repeat after 6 h; (3) Subsequent management is with Klean prep in 1 L water over 1 h *via* oral/nasogastric tube and can be repeated up to 4 times every 24 h until bowel movement is achieved; and (4) If no improvement after 48 h, then it is unlikely to resolve without surgery to decompress the gut and also consider total parenteral nutrition.

DIOS: Distal intestinal obstructive syndrome.

Table 8 Cystic fibrosis transmembrane conductance regulator modulators

Type of modulator	Mechanism of action	Mutation class in which drug is effective	Example	Clinical effects/present status of modulator
Potentiators	Restore or even enhance the channel open probability, thus allowing for CFTR-dependent anion conductance	Classes III and IV	Ivacaftor	Improvement in lung function, pancreatic function and body mass index
Correctors	Rescue folding, processing and trafficking to the plasma membrane of a CFTR mutant. Enhance protein conformational stability during the endoplasmic reticulum folding process	Class II	Lumacaftor; Tezacaftor; Posenacaftor; Elexacaftor	Significant improvement in lung function when used with Ivacaftor
Stabilizers	Anchor CFTR at the plasma membrane, thus preventing its removal and degradation by lysosomes	Class VI	Cavosonstat	First CFTR stabilizer studied in clinical trials- studies terminated because of lack of clinical efficacy
Read-through agents	Induce ribosomal over-reading of premature termination codon, enabling the incorporation of a foreign amino acid in place and continued translation to the normal end of the transcript	Class I	Ataluren (PTC124)	Clinical trials terminated
Amplifiers	Increase expression of CFTR mRNA and thus biosynthesis of the CFTR protein	Class V	Nesolicaftor (PTI-428)	Clinical trial planned

CFTR: Cystic fibrosis transmembrane conductance regulator; mRNA: Messenger RNA.

treatment of CF patients aged ≥ 12 years with F508del mutation in at least one allele, benefiting 90% of CF population[14,51].

CF MODULATORS AND LIVER

Abnormal elevation aminotransaminases (> 8 times upper limit of normal, more commonly in pediatric patients) and bilirubin (> 3 times upper limit of normal) has been reported 3%-15% of patients on CFTR modulators[52-54]. Lumacaftor/ivacaftor was shown to have less hepatic steatosis as assessed by MR imaging proton density fat fraction in a small cohort[55]. In a study[56] of 117 patients with CFTR gating mutations (partially F508 del heterozygous) treatment with Ivacaftor partially restored disrupted FGF19-regulated bile acid homeostasis. Worsening of liver function and liver failure leading to death has been reported in CF patients with pre-existing cirrhosis and portal hypertension receiving lumacaftor/ivacaftor.

Recommendations for dose adjustment are based on Child Pugh classification: no dose adjustment for Child-Pugh Class A but dose reduction is recommended for Child-Pugh Class B and C. This is applicable to adults and no specific recommendations exist in the literature for children with CFLD. Lumacaftor/ivacaftor should be used with caution in patients with advanced liver disease and only if the benefits are expected to outweigh the risks.

Because an association with liver injury cannot be excluded, assessments of liver function tests (ALT, AST and bilirubin) are recommended before initiation, a month after starting the treatment and every 3 mo during the first year of treatment, and annually thereafter. For patients with a history of ALT, AST, or bilirubin elevations, more frequent monitoring should be considered in collaboration with a pediatric hepatology centre. In the event of significant elevation of ALT or AST, with or without elevated bilirubin [either ALT or AST > 5× the upper limit of normal (ULN), or ALT or AST > 3× ULN with bilirubin > 2× ULN and/or clinical jaundice], dosing with CFTR modulators should be discontinued and closely followed up until the abnormalities resolve. A thorough investigation of potential causes should be conducted and patients should be followed closely for clinical progression. Following resolution of transaminase elevations, the benefits and risks of resuming CFTR modulators should be considered.

Metabolism of CFTR inhibitors is by the CYP450 enzyme pathway. Hence concomitant use of lumacaftor/ivacaftor with these immunosuppressants is not recommended at present as they may reduce efficacy of immunosuppressants by induction of the CYP3A pathway. Given the fact that respiratory function may eventually worsen after LT, CFTR modulators might need to be initiated post-transplant due to significant beneficial effects on lung function, nutritional status and decreased pulmonary exacerbations[43].

CONCLUSION

CFLD is the most important non-pulmonary cause of death in CF. CFLD is has a wide spectrum from asymptomatic elevation of liver enzymes to severe disease with portal hypertension and cirrhosis with synthetic failure. The degree of liver involvement and the rate of progression of liver disease varies significantly among individuals. There are no specific clinical features or tests for prediction or early detection of CFLD, so regular screening is essential for CF patients. Currently, there is no medical therapy to prevent or treat or CFLD. With the advent of CFTR modulators, improvement in medical management has resulted in significantly improved life expectancy in patients with CF and this will have implications in the management of CFLD in future. The long term effects of CFTR modulators on CFLD and liver function is not known, but will hopefully have a beneficial effect. LT is indicated in patients with CFLD with severe portal hypertension or impaired synthetic function of liver either alone or in combination with lung transplantation.

REFERENCES

1   **Cutting GR**, Engelhardt J, Zeitlin PL.   Genetics and pathophysiology of Cystic Fibrosis. In: Wilmott R, Bush A, Deterding R, Ratjen F, Sly P, Zar H, Li AP, editors. Kendig's Disorders of the Respiratory Tract in Children. Philadelphia: Elsevier, 2019: 757-768

2   **Ye W**, Narkewicz MR, Leung DH, Karnsakul W, Murray KF, Alonso EM, Magee JC, Schwarzenberg SJ, Weymann A, Molleston JP; CFLDnet research group. Variceal Hemorrhage and Adverse Liver Outcomes in Patients With Cystic Fibrosis Cirrhosis. *J Pediatr Gastroenterol Nutr* 2018; **66**: 122-127 [PMID: 28906321 DOI: 10.1097/MPG.0000000000001728]

3   **Lindblad A**, Glaumann H, Strandvik B. Natural history of liver disease in cystic fibrosis. *Hepatology* 1999; **30**: 1151-1158 [PMID: 10534335 DOI: 10.1002/hep.510300527]

4   **Gooding I**, Dondos V, Gyi KM, Hodson M, Westaby D. Variceal hemorrhage and cystic fibrosis: outcomes and implications for liver transplantation. *Liver Transpl* 2005; **11**: 1522-1526 [PMID: 16258952 DOI: 10.1002/Lt.20465]

5   **Leung DH**, Narkewicz MR. Cystic Fibrosis-related cirrhosis. *J Cyst Fibros* 2017; **16** Suppl 2: S50-S61 [PMID: 28986027 DOI: 10.1016/j.jcf.2017.07.002]

6   **Staufer K**, Halilbasic E, Trauner M, Kazemi-Shirazi L. Cystic fibrosis related liver disease--another black box in hepatology. *Int J Mol Sci* 2014; **15**: 13529-13549 [PMID: 25093717 DOI: 10.3390/ijms150813529]

7   **Strazzabosco M**, Fiorotto R, Cadamuro M, Spirli C, Mariotti V, Kaffe E, Scirpo R, Fabris L. Pathophysiologic implications of innate immunity and autoinflammation in the biliary epithelium. *Biochim Biophys Acta Mol Basis Dis* 2018; **1864**: 1374-1379 [PMID: 28754453 DOI: 10.1016/j.bbadis.2017.07.023]

8   **Fiorotto R**, Strazzabosco M. Pathophysiology of Cystic Fibrosis Liver Disease: A Channelopathy Leading to Alterations in Innate Immunity and in Microbiota. *Cell Mol Gastroenterol Hepatol* 2019; **8**: 197-207 [PMID: 31075352 DOI: 10.1016/j.jcmgh.2019.04.013]

9   **Lim SH**, Legere EA, Snider J, Stagljar I. Recent Progress in CFTR Interactome Mapping and Its

Importance for Cystic Fibrosis. *Front Pharmacol* 2017; **8**: 997 [PMID: 29403380 DOI: 10.3389/fphar.2017.00997]

10   **Fiorotto R**, Scirpo R, Trauner M, Fabris L, Hoque R, Spirli C, Strazzabosco M. Loss of CFTR affects biliary epithelium innate immunity and causes TLR4-NF-κB-mediated inflammatory response in mice. *Gastroenterology* 2011; **141**: 1498-1508, 1508.e1 [PMID: 21712022 DOI: 10.1053/j.gastro.2011.06.052]

11   **Nielsen S**, Needham B, Leach ST, Day AS, Jaffe A, Thomas T, Ooi CY. Disrupted progression of the intestinal microbiota with age in children with cystic fibrosis. *Sci Rep* 2016; **6**: 24857 [PMID: 27143104 DOI: 10.1038/srep24857]

12   **Garg M**, Ooi CY. The Enigmatic Gut in Cystic Fibrosis: Linking Inflammation, Dysbiosis, and the Increased Risk of Malignancy. *Curr Gastroenterol Rep* 2017; **19**: 6 [PMID: 28155088 DOI: 10.1007/s11894-017-0546-0]

13   **Ratjen F**, Bell SC, Rowe SM, Goss CH, Quittner AL, Bush A. Cystic fibrosis. *Nat Rev Dis Primers* 2015; **1**: 15010 [PMID: 27189798 DOI: 10.1038/nrdp.2015.10]

14   **Lopes-Pacheco M**. CFTR Modulators: The Changing Face of Cystic Fibrosis in the Era of Precision Medicine. *Front Pharmacol* 2019; **10**: 1662 [PMID: 32153386 DOI: 10.3389/fphar.2019.01662]

15   **Debray D**, Corvol H, Housset C. Modifier genes in cystic fibrosis-related liver disease. *Curr Opin Gastroenterol* 2019; **35**: 88-92 [PMID: 30585791 DOI: 10.1097/MOG.0000000000000508]

16   **Bartlett JR**, Friedman KJ, Ling SC, Pace RG, Bell SC, Bourke B, Castaldo G, Castellani C, Cipolli M, Colombo C, Colombo JL, Debray D, Fernandez A, Lacaille F, Macek M Jr, Rowland M, Salvatore F, Taylor CJ, Wainwright C, Wilschanski M, Zemková D, Hannah WB, Phillips MJ, Corey M, Zielenski J, Dorfman R, Wang Y, Zou F, Silverman LM, Drumm ML, Wright FA, Lange EM, Durie PR, Knowles MR; Gene Modifier Study Group. Genetic modifiers of liver disease in cystic fibrosis. *JAMA* 2009; **302**: 1076-1083 [PMID: 19738092 DOI: 10.1001/jama.2009.1295]

17   **Lamireau T**, Monnereau S, Martin S, Marcotte JE, Winnock M, Alvarez F. Epidemiology of liver disease in cystic fibrosis: a longitudinal study. *J Hepatol* 2004; **41**: 920-925 [PMID: 15582124 DOI: 10.1016/j.jhep.2004.08.006]

18   **Dos Santos ALM**, de Melo Santos H, Nogueira MB, Távora HTO, de Lourdes Jaborandy Paim da Cunha M, de Melo Seixas RBP, de Freitas Velloso Monte L, de Carvalho E. Cystic Fibrosis: Clinical Phenotypes in Children and Adolescents. *Pediatr Gastroenterol Hepatol Nutr* 2018; **21**: 306-314 [PMID: 30345244 DOI: 10.5223/pghn.2018.21.4.306]

19   **Stonebraker JR**, Ooi CY, Pace RG, Corvol H, Knowles MR, Durie PR, Ling SC. Features of Severe Liver Disease With Portal Hypertension in Patients With Cystic Fibrosis. *Clin Gastroenterol Hepatol* 2016; **14**: 1207-1215.e3 [PMID: 27062904 DOI: 10.1016/j.cgh.2016.03.041]

20   **Staufer K**. Current Treatment Options for Cystic Fibrosis-Related Liver Disease. *Int J Mol Sci* 2020; **21** [PMID: 33202578 DOI: 10.3390/ijms21228586]

21   **Fridell JA**, Bond GJ, Mazariegos GV, Orenstein DM, Jain A, Sindhi R, Finder JD, Molmenti E, Reyes J. Liver transplantation in children with cystic fibrosis: a long-term longitudinal review of a single center's experience. *J Pediatr Surg* 2003; **38**: 1152-1156 [PMID: 12891484 DOI: 10.1016/s0022-3468(03)00260-4]

22   **Strazzabosco M**, Fabris L, Spirli C. Pathophysiology of cholangiopathies. *J Clin Gastroenterol* 2005; **39**: S90-S102 [PMID: 15758666 DOI: 10.1097/01.mcg.0000155549.29643.ad]

23   **Sakiani S**, Kleiner DE, Heller T, Koh C. Hepatic Manifestations of Cystic Fibrosis. *Clin Liver Dis* 2019; **23**: 263-277 [PMID: 30947876 DOI: 10.1016/j.cld.2018.12.008]

24   **Flass T**, Narkewicz MR. Cirrhosis and other liver disease in cystic fibrosis. *J Cyst Fibros* 2013; **12**: 116-124 [PMID: 23266093 DOI: 10.1016/j.jcf.2012.11.010]

25   **Debray D**, Kelly D, Houwen R, Strandvik B, Colombo C. Best practice guidance for the diagnosis and management of cystic fibrosis-associated liver disease. *J Cyst Fibros* 2011; **10** Suppl 2: S29-S36 [PMID: 21658639 DOI: 10.1016/S1569-1993(11)60006-4]

26   **Assis DN**, Debray D. Gallbladder and bile duct disease in Cystic Fibrosis. *J Cyst Fibros* 2017; **16** Suppl 2: S62-S69 [PMID: 28986023 DOI: 10.1016/j.jcf.2017.07.006]

27   **Rowland M**, Gallagher CG, O'Laoide R, Canny G, Broderick A, Hayes R, Greally P, Slattery D, Daly L, Durie P, Bourke B. Outcome in cystic fibrosis liver disease. *Am J Gastroenterol* 2011; **106**: 104-109 [PMID: 20736939 DOI: 10.1038/ajg.2010.316]

28   **Lewindon PJ**, Shepherd RW, Walsh MJ, Greer RM, Williamson R, Pereira TN, Frawley K, Bell SC, Smith JL, Ramm GA. Importance of hepatic fibrosis in cystic fibrosis and the predictive value of liver biopsy. *Hepatology* 2011; **53**: 193-201 [PMID: 21254170 DOI: 10.1002/hep.24014]

29   **Witters P**, Libbrecht L, Roskams T, Boeck KD, Dupont L, Proesmans M, Vermeulen F, Strandvik B, Lindblad A, Stéphenne X, Sokal E, Gosseye S, Heye S, Maleux G, Aerts R, Monbaliu D, Pirenne J, Hoffman I, Nevens F, Cassiman D. Noncirrhotic presinusoidal portal hypertension is common in cystic fibrosis-associated liver disease. *Hepatology* 2011; **53**: 1064-1065 [PMID: 21374682 DOI: 10.1002/hep.24183]

30   **Lenaerts C**, Lapierre C, Patriquin H, Bureau N, Lepage G, Harel F, Marcotte J, Roy CC. Surveillance for cystic fibrosis-associated hepatobiliary disease: early ultrasound changes and predisposing factors. *J Pediatr* 2003; **143**: 343-350 [PMID: 14517517 DOI: 10.1067/S0022-3476(03)00329-9]

31   **Akata D**, Akhan O. Liver manifestations of cystic fibrosis. *Eur J Radiol* 2007; **61**: 11-17 [PMID: 17174503 DOI: 10.1016/j.ejrad.2006.11.008]

32   **Fields TM**, Michel SJ, Butler CL, Kriss VM, Albers SL. Abdominal manifestations of cystic fibrosis in older children and adults. *AJR Am J Roentgenol* 2006; **187**: 1199-1203 [PMID: 17056906 DOI:

10.2214/AJR.05.0327]

33   **Siegel MJ**, Freeman AJ, Ye W, Palermo JJ, Molleston JP, Paranjape SM, Stoll J, Leung DH, Masand P, Karmazyn B, Harned R, Ling SC, Navarro OM, Karnsakul W, Alazraki A, Schwarzenberg SJ, Seidel FG, Towbin A, Alonso EM, Nicholas JL, Murray KF, Otto RK, Sherker AH, Magee JC, Narkewicz MR; CFLD Network. Heterogeneous Liver on Research Ultrasound Identifies Children with Cystic Fibrosis at High Risk of Advanced Liver Disease: Interim Results of a Prospective Observational Case-Controlled Study. *J Pediatr* 2020; **219**: 62-69.e4 [PMID: 32061406 DOI: 10.1016/j.jpeds.2019.12.033]

34   **Rath T**, Menendez KM, Kügler M, Hage L, Wenzel C, Schulz R, Graf J, Nährlich L, Roeb E, Roderfeld M. TIMP-1/-2 and transient elastography allow non invasive diagnosis of cystic fibrosis associated liver disease. *Dig Liver Dis* 2012; **44**: 780-787 [PMID: 22652148 DOI: 10.1016/j.dld.2012.04.008]

35   **Gominon AL**, Frison E, Hiriart JB, Vergniol J, Clouzeau H, Enaud R, Bui S, Fayon M, de Ledinghen V, Lamireau T. Assessment of Liver Disease Progression in Cystic Fibrosis Using Transient Elastography. *J Pediatr Gastroenterol Nutr* 2018; **66**: 455-460 [PMID: 29112089 DOI: 10.1097/MPG.0000000000001822]

36   **Loomba R**, Adams LA. Advances in non-invasive assessment of hepatic fibrosis. *Gut* 2020; **69**: 1343-1352 [PMID: 32066623 DOI: 10.1136/gutjnl-2018-317593]

37   **Leung DH**, Khan M, Minard CG, Guffey D, Ramm LE, Clouston AD, Miller G, Lewindon PJ, Shepherd RW, Ramm GA. Aspartate aminotransferase to platelet ratio and fibrosis-4 as biomarkers in biopsy-validated pediatric cystic fibrosis liver disease. *Hepatology* 2015; **62**: 1576-1583 [PMID: 26223427 DOI: 10.1002/hep.28016]

38   **Pereira TN**, Lewindon PJ, Smith JL, Murphy TL, Lincoln DJ, Shepherd RW, Ramm GA. Serum markers of hepatic fibrogenesis in cystic fibrosis liver disease. *J Hepatol* 2004; **41**: 576-583 [PMID: 15464237 DOI: 10.1016/j.jhep.2004.06.032]

39   **Cook NL**, Pereira TN, Lewindon PJ, Shepherd RW, Ramm GA. Circulating microRNAs as noninvasive diagnostic biomarkers of liver disease in children with cystic fibrosis. *J Pediatr Gastroenterol Nutr* 2015; **60**: 247-254 [PMID: 25625579 DOI: 10.1097/MPG.0000000000000600]

40   **Witters P**, De Boeck K, Dupont L, Proesmans M, Vermeulen F, Servaes R, Verslype C, Laleman W, Nevens F, Hoffman I, Cassiman D. Non-invasive liver elastography (Fibroscan) for detection of cystic fibrosis-associated liver disease. *J Cyst Fibros* 2009; **8**: 392-399 [PMID: 19733131 DOI: 10.1016/j.jcf.2009.08.001]

41   **Palermo J**, Kotyk JJ, Siegel MJ. Magnetic resonance elastography for assessment of cystic fibrosis liver disease. *Gastroenterology* 2011; **140**: S-686

42   **Cheng K**, Ashby D, Smyth RL. Ursodeoxycholic acid for cystic fibrosis-related liver disease. *Cochrane Database Syst Rev* 2014; CD000222 [PMID: 25501301 DOI: 10.1002/14651858.CD000222.pub3]

43   **Freeman AJ**, Sellers ZM, Mazariegos G, Kelly A, Saiman L, Mallory G, Ling SC, Narkewicz MR, Leung DH. A Multidisciplinary Approach to Pretransplant and Posttransplant Management of Cystic Fibrosis-Associated Liver Disease. *Liver Transpl* 2019; **25**: 640-657 [PMID: 30697907 DOI: 10.1002/lt.25421]

44   **Mendizabal M**, Reddy KR, Cassuto J, Olthoff KM, Faust TW, Makar GA, Rand EB, Shaked A, Abt PL. Liver transplantation in patients with cystic fibrosis: analysis of United Network for Organ Sharing data. *Liver Transpl* 2011; **17**: 243-250 [PMID: 21384506 DOI: 10.1002/lt.22240]

45   **Milkiewicz P**, Skiba G, Kelly D, Weller P, Bonser R, Gur U, Mirza D, Buckels J, Stableforth D, Elias E. Transplantation for cystic fibrosis: outcome following early liver transplantation. *J Gastroenterol Hepatol* 2002; **17**: 208-213 [PMID: 11966953 DOI: 10.1046/j.1440-1746.2002.02671.x]

46   **Morrell MR**, Kiel SC, Pilewski JM. Organ Transplantation for Cystic Fibrosis. *Semin Respir Crit Care Med* 2019; **40**: 842-856 [PMID: 31887769 DOI: 10.1055/s-0039-3399554]

47   **Molmenti EP**, Squires RH, Nagata D, Roden JS, Molmenti H, Fasola CG, Prestidge C, D'Amico L, Casey D, Sanchez EQ, Goldstein RM, Levy MF, Benser M, McPhail W, Andrews W, Andersen JA, Klintmalm GB. Liver transplantation for cholestasis associated with cystic fibrosis in the pediatric population. *Pediatr Transplant* 2003; **7**: 93-97 [PMID: 12654048 DOI: 10.1034/j.1399-3046.2003.00021.x]

48   **Miguel M**, Andres AM, Lopez-Santamaria M, Barrena S, Hierro L, Hernandez F, Ramírez M, Frauca E, Encinas JL, Lopez-Fernandez S, Jara P, Tovar JA. Liver transplantation in children with cystic fibrosis: experience in our centre and preliminary results with a combined en bloc liver-pancreas graft. *Eur J Pediatr Surg* 2012; **22**: 60-66 [PMID: 22161075 DOI: 10.1055/s-0031-1291288]

49   **Dowman JK**, Watson D, Loganathan S, Gunson BK, Hodson J, Mirza DF, Clarke J, Lloyd C, Honeybourne D, Whitehouse JL, Nash EF, Kelly D, van Mourik I, Newsome PN. Long-term impact of liver transplantation on respiratory function and nutritional status in children and adults with cystic fibrosis. *Am J Transplant* 2012; **12**: 954-964 [PMID: 22225648 DOI: 10.1111/j.1600-6143.2011.03904.x]

50   **Arnon R**, Annunziato RA, Miloh T, Padilla M, Sogawa H, Batemarco L, Willis A, Suchy F, Kerkar N. Liver and combined lung and liver transplantation for cystic fibrosis: analysis of the UNOS database. *Pediatr Transplant* 2011; **15**: 254-264 [PMID: 21219560 DOI: 10.1111/j.1399-3046.2010.01460.x]

51   **Cuevas-Ocaña S**, Laselva O, Avolio J, Nenna R. The era of CFTR modulators: improvements made and remaining challenges. *Breathe (Sheff)* 2020; **16**: 200016 [PMID: 33304402 DOI: 10.1183/20734735.0016-2020]

52   **Davies JC**, Cunningham S, Harris WT, Lapey A, Regelmann WE, Sawicki GS, Southern KW, Robertson S, Green Y, Cooke J, Rosenfeld M; KIWI Study Group. Safety, pharmacokinetics, and pharmacodynamics of ivacaftor in patients aged 2-5 years with cystic fibrosis and a CFTR gating mutation (KIWI): an open-label, single-arm study. *Lancet Respir Med* 2016; **4**: 107-115 [PMID: 26803277 DOI: 10.1016/S2213-2600(15)00545-7]

53   **Keating D**, Marigowda G, Burr L, Daines C, Mall MA, McKone EF, Ramsey BW, Rowe SM, Sass LA, Tullis E, McKee CM, Moskowitz SM, Robertson S, Savage J, Simard C, Van Goor F, Waltz D, Xuan F, Young T, Taylor-Cousar JL; VX16-445-001 Study Group. VX-445-Tezacaftor-Ivacaftor in Patients with Cystic Fibrosis and One or Two Phe508del Alleles. *N Engl J Med* 2018; **379**: 1612-1620 [PMID: 30334692 DOI: 10.1056/NEJMoa1807120]

54   **Ratjen F**, Hug C, Marigowda G, Tian S, Huang X, Stanojevic S, Milla CE, Robinson PD, Waltz D, Davies JC; VX14-809-109 investigator group. Efficacy and safety of lumacaftor and ivacaftor in patients aged 6-11 years with cystic fibrosis homozygous for F508del-CFTR: a randomised, placebo-controlled phase 3 trial. *Lancet Respir Med* 2017; **5**: 557-567 [PMID: 28606620 DOI: 10.1016/S2213-2600(17)30215-1]

55   **Kutney K**, Donnola SB, Flask CA, Gubitosi-Klug R, O'Riordan M, McBennett K, Sferra TJ, Kaminski B. Lumacaftor/ivacaftor therapy is associated with reduced hepatic steatosis in cystic fibrosis patients. *World J Hepatol* 2019; **11**: 761-772 [PMID: 31966908 DOI: 10.4254/wjh.v11.i12.761]

56   **van de Peppel IP**, Doktorova M, Berkers G, de Jonge HR, Houwen RHJ, Verkade HJ, Jonker JW, Bodewes FAJA. IVACAFTOR restores FGF19 regulated bile acid homeostasis in cystic fibrosis patients with an S1251N or a G551D gating mutation. *J Cyst Fibros* 2019; **18**: 286-293 [PMID: 30279125 DOI: 10.1016/j.jcf.2018.09.001]

ORIGINAL ARTICLE

Case Control Study

Tumor characteristics of hepatocellular carcinoma after direct-acting antiviral treatment for hepatitis C: Comparative analysis with antiviral therapy-naive patients

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Author contributions: Fouad M and El Kassas M conceptualized the idea and design; El Sheemy R drafted, revised and edited the manuscript; all authors participated in and supervised patients' treatment and follow-up, revised and approved the final version of the manuscript.

Institutional review board statement: The study protocol was approved by the Institutional Research Board at the Faculty of Medicine, Minia University, Egypt. Informed written consent was obtained from all patients of the study. This research was performed in agreement with the guidelines of the 1975 Declaration of Helsinki.

Informed consent statement: Informed consent was obtained from all individual participants included in the study

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Abstract

BACKGROUND
Insufficient and contradictory data are available about the relation between direct-acting antivirals (DAAs) and hepatocellular carcinoma (HCC) development in patients with hepatitis C virus (HCV).

AIM
To analyze differences in basic clinical, radiological, and laboratory characteristics in addition to tumor behavior upon HCC diagnosis between patients with and without a previous history of DAAs exposure.

METHODS
This multicenter case-control study included 497 patients with chronic HCV-related HCC, allocated into one of two groups according to their history of antiviral treatment for their HCV.

RESULTS
Group I included 151 HCC patients with a history of DAAs, while 346 patients who had never been treated with DAAs were assigned to group II. A significant difference was observed between both groups regarding basic assessment scores (Child, MELD, and BCLC), which tended to have more advanced liver disease and HCC stage upon diagnosis in group I. However, serum albumin was

significantly affected, and serum α-fetoprotein was significantly higher in group II ($P < 0.001$). In addition, group I showed significant HCC multicentricity than group II, while the incidence of portal vein thrombosis was significantly higher in group I ($P < 0.001$).

CONCLUSION
The basic clinical scores and laboratory characteristics of HCC patients are advanced in patients who are naïve to DAAs treatment; however, HCC behavior is more aggressive in DAA-treated patients.

Key Words: Hepatocellular carcinoma; Direct-acting antiviral treatment; Hepatitis C; Tumor behavior; Occurrence

Core Tip: Despite the introduction of newer direct-acting antivirals (DAAs), hepatitis C virus (HCV)-related hepatocellular carcinoma (HCC) will continue to be a significant public health concern in the coming decades. Post-treatment HCV-related HCC has been discovered to be an emerging issue due to unmet needs for early HCC identification and intervention. In addition, we found that aggressive tumors were more common in DAAs exposed patients, which needs to be investigated further in prospective studies with larger cohorts and necessitates proactive screening for HCC in HCV-treated patients *via* public or private pharmacovigilance programs.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third most common cause of cancer-related mortality worldwide[1]. In Egypt, HCC is a significant public health problem responsible for 33.63% and 13.54% of all cancers in males and females, respectively[2]. On the other hand, hepatitis C virus infection (HCV) is considered a leading cause of chronic liver disease in Europe, the United States, and many other countries, including Egypt[3,4]. The risk of HCC development in HCV-related liver cirrhosis is 2% to 8% per year[5]. Multiple studies and meta-analyses demonstrated during the era of interferon (IFN)-based therapy that HCV eradication decreased the risk of hepatocarcinogenesis regardless of fibrosis stage[6,7]. Furthermore, these studies showed that the achievement of sustained virologic response (SVR) after IFN based treatment is directly related to reduced incidence of HCC and increased survival rates[8].

In 2014, the introduction of the more effective direct-acting antivirals (DAAs) for HCV treatment was generally expected to benefit all patients, including those who were not permitted to be treated with IFN-based therapy[7]. However, unexpectedly, the clinical use of DAAs has evoked a significant dilemma about the relationship between DAAs and the development of HCC. Some studies have suggested a direct relation between DAAs and the development of HCC, while others have insisted that DAAs are protective against HCC development[7].

In 2016, the first report in this context showed an unexpectedly high recurrence rate of previously treated HCC after DAAs exposure[6]. This initial report was followed by another retrospective study conducted in Italy which included 344 patients with HCV-related cirrhosis who received different DAA regimens; 91% achieved SVR. The patients were followed for 24 wk. The study revealed a 29% recurrence rate for those with a history of HCC and a 3.16% incidence rate (de novo HCCs) in those without a history of prior HCC irrespective of the used DAA regimen[9].

In addition to HCC recurrence, the different biological behavior of HCC in DAAs exposed patients, and the pattern of recurrence after DAA treatment has also been reported in studies coming from various countries. For example, in 2017, Reig and his colleagues reported more aggressive HCC recurrence after DAA treatment, as defined by an advanced Barcelona Clinic Liver Cancer (BCLC) stage[6]. Moreover, Renzulli *et al*[10] found a more aggressive HCC recurrence pattern with vascular invasion evidence after DAA therapy.

This study aimed to analyze differences in basic clinical, radiological, and laboratory characteristics and tumor behavior upon HCC diagnosis between patients with and without a previous history of DAAs exposure.

MATERIALS AND METHODS

Study design

The current study is a multicenter retrospective case-control study designed to compare the basic demographic, laboratory, and radiological criteria of HCC in patients with a history of DAAs treatment for their chronic HCV infection compared to HCC patients with no previous history of HCV antiviral treatment. Patients were recruited from December 2016 to April 2019 from Minia university hospital and Minia fever hospital, Minia, Egypt. Study patients were assigned to one of 2 groups according to previous DAAs exposure. The first group included 151 HCC patients who were previously treated with DAAs (Group I). According to a standardized treatment protocol, all patients were treated in one of the specialized viral hepatitis treatment centers affiliated to the Egyptian National Committee for Control of Viral Hepatitis. Group II included 346 HCC patients with the first presentation as HCC and no history of antiviral treatment for their HCV infection. Patients with combined HBV or HIV infections and patients with extrahepatic malignancies were excluded from the study.

Methods

All patients were recruited and diagnosed according to EASL guidelines and updated AASLD practice guidelines for managing HCC and BCLC guidelines[11-13]. In addition, baseline demographic, clinical, laboratory, and radiological criteria were studied. The Child-Turcotte Pugh score (CTP), Model for End-stage Liver Disease (MELD) score, BCLC score, and FIB 4 as a non-invasive marker for fibrosis were calculated and presented.

Lines of treatment for HCV have been verified as well as the viral response. In addition, all baseline characteristics, laboratory, radiological and medical scores were compared between the two groups.

The study was performed according to the ethical guidelines of the 1975 Declaration of Helsinki after approval from the Research Ethics Committee for human subject research at the Faculty of Medicine, Minia University (Serial: 165: 2/2019) on Feb 25, 2019. In addition, written informed consent was obtained from all participants before enrolment in the study.

Statistical analysis

Statistical analyses were performed using IBM SPSS advanced statistics, version 26 (SPSS Inc., Chicago, Illinois, USA. Numerical data were presented as mean ± SD and median (range), whereas categorical data were presented as number (percent). The Mann-Whitney U-test and the χ^2-test are used when appropriate. Statistical significance is considered if *P* value is less than or equal to 0.05.

RESULTS

This study included 497 patients with chronic HCV-related HCC, allocated into one of two groups according to their history of antiviral treatment for their HCV. Group I included 151 patients with chronic HCV and HCC who were previously treated with DAAs. Group II included 346 patients representing all patients recruited in the same period with HCV-related HCC and age and sex-matched with group I. Most of the studied patients in both groups were males: (76.2%) and (72.0%) (*P* value 0.33), with a mean age of 60.2 years and 59.8 years in groups I and II, respectively (*P* value 0.70) (Table 1). Regarding the received DAAs regimen in group I patients, 44.4% of patients

Table 1 Basic demographic data and underlying liver status in both groups			
	Group I	Group II	*P* value
	HCC with previous DAAs (*n* = 151)	HCC without previous DAAS (*n* = 346)	
Age (mean ± SD)	60.17 ± 7.75	59.84 ± 9.12	0.70
Gender			0.33
Female	36 (23.8)	97 (28.0)	
Male	115 (76.2)	249 (72.0)	
Residence			0.28
Rural	131 (86.8)	287 (82.9)	
Urban	20 (13.2)	59 (17.1)	
BCLC			< 0.001[a]
0	5 (3.3)	15 (4.3)	
A	47 (31.1)	134 (38.7)	
B	17 (11.3)	68 (19.7)	
C	49 (32.5)	50 (14.5)	
D	33 (21.9)	79 (22.8)	
MELD (mean ± SD)	14.35 ± 5.041	36.10 ± 30.22	< 0.001[a]
CTP score			0.04
A	50 (33.1)	88 (25.4)	
B	65(43.0)	138 (39.9)	
C	36 (23.8)	120 (34.7)	
FIB4			< 0.001[c]
mean ± SD	3.25 ± 9.87	7.11 ± 7.68	
Median	0.023	4.49	
IQR	4.51	6.2	
HCC detection time after stop of DAAS	Range: 1-72 moMedian: 8 mo	-	-

[a]$P < 0.05$.

[c]$P < 0.001$.

HCC: Hepatocellular carcinoma; MELD: Model for end stage liver disease; CTP: Child Turcotte-pough; BCLC: Barcelona cancer liver clinic.

received sofosbuvir/daclatasvir (SOF/DAC), 40.1% received SOF/DAC/RBV, 13.2% received SOF/RBV, and only 2% received SOF/RBV/PEG IFN. Figure 1 shows patients' distribution among various treatment regimens and treatment duration, in addition to treatment viral response.

Notably, significant differences were observed between the two groups regarding the case assessment scores that reflect the severity of the underlying liver condition upon HCC discovery. A total of 34.7% of patients in group II were CTP class C, and only 23.8% of group I patients were class C. Mean MELD score in group I was 14, while the mean MELD in group II was 36 (*P* value < 0.001). Moreover, a significant difference was observed in the BCLC score (*P* value < 0.001). A significant difference was encountered in FIB4 as a method for non-invasive fibrosis assessment with a mean FIB4 of 3.25 in group I, compared to 7.11 in group II (*P* value < 0.001). Basic demographic data and underlying liver status in both groups are detailed in Table 1. The time between stopping DAAs and the development of HCC ranged from 1 to 72 mo with a median of 8 mo.

When comparing both groups' clinical data, no significant differences were observed except in the current smoking status, which was significantly increased in group I compared to the other group (*P* value 0.005). On the other hand, a significant history of blood transfusion was observed in patients with no previous history of DAAs (*P* value 0.01); cellular decompensation in the form of hepatic encephalopathy is
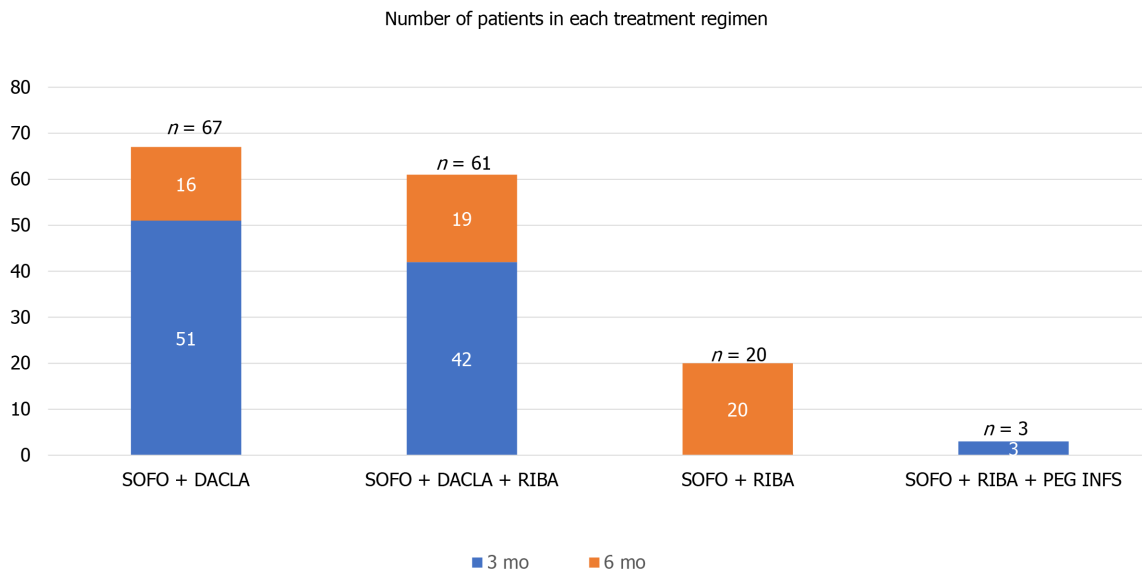
Number of patients in each treatment regimen

Figure 1 Patients in group I distribution among different treatment regimen. SOFO: Sofosbuvir; DACLA: Daclatasvir; RIBA: Ribavirin; PEG INFS: Pegylated interferon.

significantly observed in patients with no previous history of DAAs (*P* value 0.01). Detailed clinical data of the two studied groups are well presented in Table 2.

Comparing laboratory data in both groups, hemoglobin level and total leukocytic count were significantly different (*P* values are 0.02 and 0.004, respectively). Median ALT in group I was 77 IU in comparison to 54 IU in group II (*P* value 0.001). Mean albumin in groups I and II was (3.3 and 2.9 respectively) (*P* value 0.004), and mean urea in groups I and II was (40 and 54 respectively) (*P* value 0.04). Median AFP in group I was 184 in comparison to 60 in group II (*P* value < 0.001). An illustrated comparison of all laboratory data is presented in Table 3.

Regarding the radiological characters of HCC in both groups, HCC in group I patients was more multifocal (53%) in comparison to (25%) in group II (*P* value < 0.001). Moreover, HCCs in group I patients tended to present with a bigger tumor size at the initial presentation than group II patients. More precisely, less than 1% of group I patients were presented with tumors less than 2 cm, while more than 15% of group II patients presented with tumors less than 2 cm (*P* value < 0.001), indicating more aggressive tumor behavior associated with the previous history of DAAs. The right lobe was the dominant victim in both groups. Early vascular invasion was significantly higher in group I compared to group II as evidenced radiologically by portal vein thrombosis (PVT), which present in 45% of group I patients and only 21% of group II patients (*P* value < 0.001), all radiological data for HCCs in the studied patients are detailed in Table 4.

DISCUSSION

Chronic HCV infection is a significant risk factor for developing liver cirrhosis in approximately 20%-30% of patients with subsequent increased risk for HCC development in those patients with an estimated annual incidence of 3.5%[14]. This risk is shown to be lower in patients with chronic HCV infection without cirrhosis and in patients who succeeded in achieving eradication, as proved by their SVR[15]. Despite the notable decrease in the overall incidence of HCV infection, its prevalence in HCC patients is still high[16]. Surprisingly, HCC development's risk is continuous in HCV-induced liver cirrhosis even after viral eradication and SVR achievement[16]. During the interferon-based treatment era, successful viral eradication decreases the risk for HCC and improvement in the fibrosis stage[9].

The emergence of DAAs with their extended patient spectrum, improved efficacy, and safety profile increased our expectations regarding a decrease in HCC occurrence and recurrence. However, unpleasant data from new studies showed that DAAs might encourage tumor occurrence in patients with cirrhosis or recurrence in patients with previously treated HCC[9,17]. The same was reported in some studies regarding HCC

Table 2 Clinical presentation in both groups

	Group I	Group II	*P* value
	HCC with previous DAAs (*n* = 151)	HCC without previous DAAS (*n* = 346)	
Hypertension	56 (37.1)	135 (39.0)	0.68
DM	56 (37.1)	113 (32.7)	0.33
Smoking	73 (48.3)	121 (35.0)	0.005[a]
Surgical operations	32 (21.2)	101 (29.2)	0.06
Blood transfusion	23 (15.2)	87 (25.1)	0.01[a]
Jaundice	60 (39.7)	154 (44.5)	0.32
Ascites	90 (59.6)	197 (56.9)	0.58
LL edema	48 (31.8)	143 (41.3)	0.07
Hepatic encephalopathy	14 (9.3)	61 (17.6)	0.01[a]

[a]$P < 0.05$.

HCC: Hepatocellular carcinoma; DM: Diabetes mellitus; LL: Lower limb.

Table 3 Comparison of laboratory data in both groups

	Group I	Group II	*P* value
	HCC with previous DAAs (*n* = 151)	HCC without previous DAAS (*n* = 346)	
HB (mean ± SD)	10.41 ± 1.88	10.78 ± 1.99	0.02[a]
TLC (mean ± SD)	6.55 ± 6.20	7.74 ± 8.60	0.004[b]
PLATELETS (mean ± SD)	147.28 ± 79.76	135.95 ± 61.17	0.22
TBIL (median)	3.07	2.5	0.93
DBIL (median)	0.7	0.9	0.84
ALB (mean ± SD)	3.32 ± 1.47	2.98 ± 0.85	0.004[b]
INR	1.31 ± 0.35	1.44 ± 0.47	0.4
ALT (median)	77	54	0.001[c]
AST (median)	76	70	0.62
CREAT (mean ± SD)	1.21 ± 0.45	1.43 ± 3.67	0.15
UREA (mean ± SD)	40.81 ± 16.01	54.93 ± 46.17	0.04[a]
AFP (median)	184.0	60.0	< 0.001[c]

[a]$P < 0.05$.

[b]$P < 0.01$.

[c]$P < 0.001$.

HCC: Hepatocellular carcinoma; CBC: Complete blood picture; TLC: Total leucocytic count; TBIL: Total bilirubin; DBIL: Direct bilirubin; ALB: Albumin, ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AFP: Alfa feto protein.

recurrence after initial management upon treatment with DAAs[18].

This study stands at the current dilemma between DAAs' benefits and drawbacks; studying the basic characteristics of HCC patients previously treated with DAAs and comparing them with HCC patients never treated with DAAs provides the central part of this controversy.

In our study, significant differences were found in the CTP, MELD, and BCLC scores in HCC patients without DAAs and those who received DAAs; these findings are contrary to what proved by Abdelaziz *et al*[19], who found matching between patients with HCC and previous DAAs and HCC without DAAs regarding CTP score. In accordance with our results, a large study from Pakistan reported a raised neutrophil to lymphocyte ratio and younger patient age with more aggressive tumor

Table 4 Radiological characters of hepatocellular carcinoma in both groups

	Group I	Group II	P value[c]
	HCC with previous DAAs (*n* = 151)	HCC without previous DAAS (*n* = 346)	
Number			< 0.001
Single	71 (47.0)	259 (74.9)	
Multiple	80 (53.0)	87 (25.1)	
Size			< 0.001
Less than 2 cm	1 (0.7)	54 (15.6)	
2.5 cm	91(60.3)	177 (51.2)	
Greater than 5 cm	59 (39.1)	115 (33.2)	
Site			0.001
Bilobar	21 (13.9)	29 (8.4)	
Lt lobe	24 (15.9)	23 (6.6)	
RT lobe	106 (70.2)	294 (84.9)	
PVT	68 (45.0)	75 (21.7)	< 0.001
Splenomegaly			< 0.001
Average	38 (25.2)	98 (28.3)	
Mild	113 (74.8)	214 (61.8)	
Moderate	0 (0.0)	34 (9.8)	

[c]*P* < 0.001.

HCC: Hepatocellular carcinoma; DAAs: Directly acting antiviral agents; PVT: Portal vein thrombosis.

behavior in HCV-treated HCC patients[20].

The pattern of HCC invasion either locally inside the liver manifested by multiplicity and larger size or vascularly manifested by PVT is significantly increased with the previous history of DAAs, suggesting a possible DAAs role in such aggressive behavior. In accordance with the current study, Reig *et al*[6] stated the increased aggressiveness of HCC, but in recurrent cases, he omitted de novo HCC in his study. Also, Renzulli *et al*[10] noticed a faster rate of development of HCC after DAA therapy with an aggressive course of microvascular invasion. Similarly, Faillaci *et al*[21] proved that DAAs are associated with increased aggressiveness and tumor recurrence growth. Another study done by Romano *et al*[21] demonstrated an aggressive behavior of tumors after DAA in the form of a higher number of nodules and extrahepatic metastases, suggesting that such patients' tumor growth is faster than usual. Many theories have been proposed to explain this unexpected event; some researchers have related the development of HCC to baseline risk factors such as advanced fibrosis grade, HBV co-infection, or age[7]. Another theory proposes that DAAs cause immune surveillance mechanisms to become dysregulated due to the rapid viral clearance, and this behavior has been confirmed by several investigations [16,19]. With the downregulation of type II and III IFNs, their receptors, and IFN-stimulated genes, this dysregulation may result in the re-establishment of innate immunity. Due to the anti-angiogenic and anti-proliferative capabilities of IFN, which DAAs lack, a reduction in IFN activation may promote the proliferation of malignant cells. Furthermore, after HCV eradication, one of the immune system alterations observed is a decrease in the number of cytotoxic activity of natural killer cells in the liver, favoring a faster progression of HCC foci[7,22].

A significant difference was observed in AFP levels between the two groups, explained mainly by the invasive pattern and prominent vascular invasion in group I, and this is in agreement with Abdelaziz *et al*[20].

The strengths of our study include its design and the large number of included subjects. Limitations include the exclusive existence of genotype four patients because of its prevalence in Egypt and lack of tight evaluation of other risk factors for HCC, like non-alcoholic fatty liver disease and aflatoxin effect, and the lack of further longitudinal follow up of the studied cohort.

CONCLUSION

In conclusion, despite the introduction of newer DAAs, HCV-related HCC will continue to be a significant public health concern in the coming period. Post-treatment HCV-related HCC has been discovered to be an emerging issue due to unmet needs for early HCC identification and intervention. In this study, more aggressive tumor behavior was encountered in DAAs exposed patients. Such finding needs to be investigated further in prospective studies with larger cohorts and more longitudinal follow-up for comparing survival and necessitates proactive screening for HCC in HCV-treated patients *via* public or private pharmacovigilance programs. Furthermore, anti-HCV therapy in HCC patients should be postponed until a consistent risk-benefit ratio is established through further research.

ARTICLE HIGHLIGHTS

Research background

The evidence on the link between direct-acting antivirals (DAAs) and the development of hepatocellular carcinoma (HCC) in hepatitis C virus (HCV) patients is insufficient and conflicting.

Research motivation

Due to unmet needs for early HCC detection and care, post-treatment HCV-related HCC is an increasing concern.

Research objectives

To compare fundamental clinical, radiographic, and laboratory features and tumor behavior in individuals with and without a history of DAAs exposure after HCC diagnosis.

Research methods

A multicenter case-control study including 497 patients with chronic HCV-related HCC, allocated into one of two groups according to their history of antiviral treatment for their HCV.

Research results

Group I consisted of 151 HCC patients who had previously been treated with DAAs, while group II included 346 patients who had never been treated with DAAs. Regarding basic assessment scores (Child, MELD, and BCLC), there was a substantial difference between the two groups, with group I showing a tendency for more advanced liver disease and HCC stage at diagnosis. However, serum albumin levels were considerably lower in group II, and serum-fetoprotein levels were significantly greater ($P = 0.001$). In addition, HCC multicentricity was substantially higher in group I than in group II, and the rate of portal vein thrombosis was significantly higher in group I ($P = 0.001$).

Research conclusions

HCC patients who are naïve to DAAs have more advanced clinical scores and laboratory features than those who have never been treated with DAAs; yet, HCC behavior is more aggressive in DAA-treated patients.

Research perspectives

The findings of this study warrant additional investigation in prospective trials with larger cohorts and longer follow-up for comparing survival and proactive screening for HCC in HCV-treated patients through public or private pharmacovigilance programs.

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REFERENCES

1   **Tabrizian P**, Roayaie S, Schwartz ME. Current management of hepatocellular carcinoma. *World J Gastroenterol* 2014; **20**: 10223-10237 [PMID: 25132740 DOI: 10.3748/wjg.v20.i30.10223]

2   **Holah NS**, El-Azab DS, Aiad HAE, Sweed DMM. The Diagnostic Role of SPINK1 in Differentiating Hepatocellular Carcinoma From Nonmalignant Lesions. *Appl Immunohistochem Mol Morphol* 2017; **25**: 703-711 [PMID: 27028242 DOI: 10.1097/PAI.0000000000000363]

3   **Messina JP**, Humphreys I, Flaxman A, Brown A, Cooke GS, Pybus OG, Barnes E. Global distribution and prevalence of hepatitis C virus genotypes. *Hepatology* 2015; **61**: 77-87 [PMID: 25069599 DOI: 10.1002/hep.27259]

4   **Kandeel A**, Genedy M, El-Refai S, Funk AL, Fontanet A, Talaat M. The prevalence of hepatitis C virus infection in Egypt 2015: implications for future policy on prevention and treatment. *Liver Int* 2017; **37**: 45-53 [PMID: 27275625 DOI: 10.1111/liv.13186]

5   **Ioannou GN**, Splan MF, Weiss NS, McDonald GB, Beretta L, Lee SP. Incidence and predictors of hepatocellular carcinoma in patients with cirrhosis. *Clin Gastroenterol Hepatol* 2007; **5**: 938-945, 945.e1 [PMID: 17509946 DOI: 10.1016/j.cgh.2007.02.039]

6   **Reig M**, Mariño Z, Perelló C, Iñarrairaegui M, Ribeiro A, Lens S, Díaz A, Vilana R, Darnell A, Varela M, Sangro B, Calleja JL, Forns X, Bruix J. Unexpected high rate of early tumor recurrence in patients with HCV-related HCC undergoing interferon-free therapy. *J Hepatol* 2016; **65**: 719-726 [PMID: 27084592 DOI: 10.1016/j.jhep.2016.04.008]

7   **El Kassas M**, Elbaz T, Salaheldin M, Abdelsalam L, Kaseb A, Esmat G. Impact of treating chronic hepatitis C infection with direct-acting antivirals on the risk of hepatocellular carcinoma: The debate continues - A mini-review. *J Adv Res* 2019; **17**: 43-48 [PMID: 31193326 DOI: 10.1016/j.jare.2019.03.001]

8   **Tanimoto Y**, Tashiro H, Aikata H, Amano H, Oshita A, Kobayashi T, Kuroda S, Tazawa H, Takahashi S, Itamoto T, Chayama K, Ohdan H. Impact of pegylated interferon therapy on outcomes of patients with hepatitis C virus-related hepatocellular carcinoma after curative hepatic resection. *Ann Surg Oncol* 2012; **19**: 418-425 [PMID: 21710324 DOI: 10.1245/s10434-011-1866-1]

9   **Conti F**, Buonfiglioli F, Scuteri A, Crespi C, Bolondi L, Caraceni P, Foschi FG, Lenzi M, Mazzella G, Verucchi G, Andreone P, Brillanti S. Early occurrence and recurrence of hepatocellular carcinoma in HCV-related cirrhosis treated with direct-acting antivirals. *J Hepatol* 2016; **65**: 727-733 [PMID: 27349488 DOI: 10.1016/j.jhep.2016.06.015]

10 **Renzulli M**, Buonfiglioli F, Conti F, Brocchi S, Serio I, Foschi FG, Caraceni P, Mazzella G, Verucchi G, Golfieri R, Andreone P, Brillanti S. Imaging features of microvascular invasion in hepatocellular carcinoma developed after direct-acting antiviral therapy in HCV-related cirrhosis. *Eur Radiol* 2018; **28**: 506-513 [PMID: 28894901 DOI: 10.1007/s00330-017-5033-3]

11 **Bruix J**, Sherman M, Llovet JM, Beaugrand M, Lencioni R, Burroughs AK, Christensen E, Pagliaro L, Colombo M, Rodés J; EASL Panel of Experts on HCC. Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. *J Hepatol* 2001; **35**: 421-430 [PMID: 11592607 DOI: 10.1016/s0168-8278(01)00130-1]

12 **Bruix J**, Sherman M; American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma: an update. *Hepatology* 2011; **53**: 1020-1022 [PMID: 21374666 DOI: 10.1002/hep.24199.]

13 **Llovet JM**, Bustamante J, Castells A, Vilana R, Ayuso Mdel C, Sala M, Brú C, Rodés J, Bruix J. Natural history of untreated nonsurgical hepatocellular carcinoma: rationale for the design and evaluation of therapeutic trials. *Hepatology* 1999; **29**: 62-67 [PMID: 9862851 DOI: 10.1002/hep.510290145]

14 **Lok AS**, Seeff LB, Morgan TR, di Bisceglie AM, Sterling RK, Curto TM, Everson GT, Lindsay KL, Lee WM, Bonkovsky HL, Dienstag JL, Ghany MG, Morishima C, Goodman ZD; HALT-C Trial Group. Incidence of hepatocellular carcinoma and associated risk factors in hepatitis C-related advanced liver disease. *Gastroenterology* 2009; **136**: 138-148 [PMID: 18848939 DOI: 10.1053/j.gastro.2008.09.014.]

15 **Swain MG**, Lai MY, Shiffman ML, Cooksley WG, Zeuzem S, Dieterich DT, Abergel A, Pessôa MG, Lin A, Tietz A, Connell EV, Diago M. A sustained virologic response is durable in patients with chronic hepatitis C treated with peginterferon alfa-2a and ribavirin. *Gastroenterology* 2010; **139**: 1593-1601 [PMID: 20637202 DOI: 10.1053/j.gastro.2010.07.009]

16 **Robert S B Jr**. The Possible Association Between DAA Treatment for HCV Infection and HCC Recurrence. *Gastroenterol Hepatol (N Y)* 2016; **12**: 776-779 [PMID: 28035203]

17 **Tayyab GUN**, Rasool S, Nasir B, Rubi G, Abou-Samra AB, Butt AA. Hepatocellular carcinoma occurs frequently and early after treatment in HCV genotype 3 infected persons treated with DAA regimens. *BMC Gastroenterol* 2020; **20**: 93 [PMID: 32252635 DOI: 10.1186/s12876-020-01249-4]

18 **Sapena V**, Enea M, Torres F, Celsa C, Rios J, Rizzo GEM, Nahon P, Mariño Z, Tateishi R, Minami T, Sangiovanni A, Forns X, Toyoda H, Brillanti S, Conti F, Degasperi E, Yu ML, Tsai PC, Jean K, El Kassas M, Shousha HI, Omar A, Zavaglia C, Nagata H, Nakagawa M, Asahina Y, Singal AG, Murphy C, Kohla M, Masetti C, Dufour JF, Merchante N, Cavalletto L, Chemello LL, Pol S, Crespo J, Calleja JL, Villani R, Serviddio G, Zanetto A, Shalaby S, Russo FP, Bielen R, Trevisani F, Cammà C, Bruix J, Cabibbo G, Reig M. Hepatocellular carcinoma recurrence after direct-acting antiviral therapy: an individual patient data meta-analysis. *Gut* 2021 [DOI: 10.1136/gutjnl-2020-323663]

19 **Abdelaziz AO**, Nabil MM, Abdelmaksoud AH, Shousha HI, Hashem MB, Hassan EM, Salah A,

Omran DA, Elbaz TM. Tumor behavior of hepatocellular carcinoma after hepatitis C treatment by direct-acting antivirals: comparative analysis with non-direct-acting antivirals-treated patients. *Eur J Gastroenterol Hepatol* 2019; **31**: 75-79 [PMID: 30199473 DOI: 10.1097/MEG.0000000000001264]

20 **Khalid J**, Umar M, Ur-Rehman T, Ali M, Khan GM. Tumor aggression among hepatitis-C related hepatocellular carcinoma patients: an observational study regarding the impact of anti-HCV therapy. *Infect Agent Cancer* 2020; **15**: 35 [PMID: 32508980 DOI: 10.1186/s13027-020-00300-z]

21 **Faillaci F**, Marzi L, Critelli R, Milosa F, Schepis F, Turola E, Andreani S, Vandelli G, Bernabucci V, Lei B, D'Ambrosio F, Bristot L, Cavalletto L, Chemello L, Sighinolfi P, Manni P, Maiorana A, Caporali C, Bianchini M, Marsico M, Turco L, de Maria N, Del Buono M, Todesca P, di Lena L, Romagnoli D, Magistri P, di Benedetto F, Bruno S, Taliani G, Giannelli G, Martinez-Chantar ML, Villa E. Liver Angiopoietin-2 Is a Key Predictor of De Novo or Recurrent Hepatocellular Cancer After Hepatitis C Virus Direct-Acting Antivirals. *Hepatology* 2018; **68**: 1010-1024 [PMID: 29604220 DOI: 10.1002/hep.29911]

22 **Innes H**, Barclay ST, Hayes PC, Fraser A, Dillon JF, Stanley A, Bathgate A, McDonald SA, Goldberg D, Valerio H, Fox R, Kennedy N, Bramley P, Hutchinson SJ. The risk of hepatocellular carcinoma in cirrhotic patients with hepatitis C and sustained viral response: Role of the treatment regimen. *J Hepatol* 2018; **68**: 646-654 [PMID: 29155019 DOI: 10.1016/j.jhep.2017.10.033]

Circulating microRNA 9-3p and serum endocan as potential biomarkers for hepatitis C virus-related hepatocellular carcinoma

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Abstract

BACKGROUND
The high mortality rate of hepatocellular carcinoma (HCC) in Egypt is due mainly to the increasing prevalence of hepatitis C virus infection (HCV) and late diagnosis of the carcinoma. MicroRNAs (miRNA), which regulate tumor proliferation and metastasis in HCC, may serve as a useful diagnostic approach for the early detection of HCC, thus decreasing its mortality. Meanwhile, endocan is a protein with angiogenic and inflammatory properties that are associated with tumor progression and poor outcomes.

AIM
To analyze the levels of miRNA 9-3p and endocan in HCV-infected HCC patients and correlate them with clinicopathological parameters.

METHODS
We compared levels of endocan and circulating miRNA 9-3p from 35 HCV-related HCC patients to 33 patients with HCV-induced chronic liver disease and 32 age and gender matched healthy controls recruited from inpatient and outpatient clinics of the National Liver Institute, Menoufia University, Egypt in the period from January to March 2021 in a case-control study. Serum samples from all groups were analyzed for HCV. Endocan was measured by enzyme-

linked immunosorbent assays, and the expression levels of circulating miRNA 9-3p were measured by real-time quantitative reverse transcriptase PCR.

RESULTS
The levels of circulating miRNA 9-3p were significantly lower in the HCC group compared to the chronic liver disease ($P < 0.001$) and control ($P < 0.001$) groups, while levels in the chronic liver disease were significantly lower than those in the control group ($P < 0.001$). The levels of serum endocan were significantly higher in the HCC group compared to the chronic liver disease ($P < 0.001$) and control ($P < 0.001$) groups. Moreover miRNA 9-3p and endocan performed better than α-fetoprotein in discriminating HCC patients from cirrhosis and healthy patients. The levels of miRNA 9-3p were significantly inversely correlated to vascular invasion ($P = 0.002$), stage of advancement of Barcelona Clinical Liver Cancer ($P < 0.001$) and the metastatic site ($P < 0.001$) of the HCC group.

CONCLUSION
Circulating miRNA 9-3p and endocan can be used as novel biomarkers for the early diagnosis of HCV-related HCC.

Key Words: MicroRNA 9-3p; Hepatocellular carcinoma; Endocan; Diagnostic; Biomarker; Egypt

Core Tip: The level of circulating microRNA 9-3p was significantly decreased in hepatocellular carcinoma (HCC) patients than in chronic liver disease and control groups. The level of serum endocan was significantly increased in HCC patients than in the cirrhotic and control groups, and there was better diagnostic performance of microRNA 9-3p and endocan than α-fetoprotein. The levels of microRNA 9-3p have a significant inverse correlation with endocan and vascular invasion and advanced stage of Barcelona Clinical Liver Cancer in the HCC group. Circulating microRNA 9-3p and endocan could be novel biomarkers for early diagnosis of hepatitis C virus-related HCC patients.

INTRODUCTION

Worldwide, hepatocellular carcinoma (HCC) is the sixth most prevalent cancer and the third leading cause of cancer deaths[1]. In Egypt, HCC is a significant health problem, as it is the most prevalent and second most prevalent cancer in males and females, respectively. It is the most prevalent malignancy in general, accounting for 32.35% of the total cancer deaths[2,3]. One reason for these high prevalence rates is the high prevalence of hepatitis C in Egypt[4].

Moreover, HCC has been attributed to molecular aberrations, such as errors in regulation of gene expression, which may result in translational repression and/or degradation[5,6]. To improve the overall survival from HCC, extensive research is needed, focusing particularly on more accurate and monitored management of the disease[7].

MicroRNAs (miRNAs) are small (approximately 22 nucleotides long), endogenous, non-protein coding RNAs that are key post-transcriptional regulators of gene expression[8]. miRNAs regulate different cellular pathways, including the cell cycle, cell proliferation and apoptosis. Dysregulation of miRNAs can therefore impact cellular processes involved in tumorigenesis and cancer. Thus, serum miRNAs may serve as non-invasive biomarkers for the diagnosis of cancer[9].

Three genes encode miRNA 9-3p: *MIR9-1*, *MIR9-2* and *MIR9-3*, located on chromosomes 1 (1q22), 5 (5q14.3) and 15 (15q26.1), respectively[10]. miRNA 9-3p is expressed abnormally in various types of human cancer, suggesting that it is functionally versatile[11]. miRNA 9-3p has been identified to have a tumor suppressive role by targeting an oncogenic tafazzin expression in HCC cells[12].

Endocan is a 50 kDa soluble proteoglycan that circulates freely in the bloodstream of healthy individuals and is expressed by the vascular endothelium. It has angiogenic and inflammatory properties that may affect vascular permeability, thus it plays crucial roles in regulating major physiological and pathophysiological processes, such as cell adhesion, inflammation and tumor progression[13]. Endocan expression is upregulated in cancer cells derived from the lung, kidney, brain, astrocytes and liver [14].

A single study has reported that miRNA 9-3p expression decreases in bladder cancer patients, resulting in reduced inhibition of endocan, thus increasing its expression, which promotes cell proliferation[15]. These results indicate that the gene encoding endocan is a target of miRNA 9-3p. To the best of our knowledge, simultaneous assessment of the roles of both miRNA 9-3p and inflammatory role of endocan that may induce tumorigenesis and tumor progression have not been evaluated in HCV-related HCC, which is triggered by the viral inflammation. Thus, the current work aimed to study the diagnostic value of circulating levels of miRNA 9-3p and endocan in HCV-related HCC patients and to correlate them with clinicopathological parameters.

MATERIALS AND METHODS

Study subjects

This case-control study included a total of 100 subjects recruited from inpatient and outpatient clinics of the National Liver Institute, Menoufia University, Egypt in the period from January to March 2021. Participants were categorized into three groups: Group I: 35 patients with HCV-related HCC; Group II: 33 patients with chronic liver disease due to chronic HCV; and Group III: 32 healthy and free of viral infection volunteers of matched age and gender. Patients were selected based on restrictive inclusion criteria including patients whose age was more than 18 years with confirmed HCV infection by both HCV antibody (anti-HCV) detection and positive HCV RNA. In Group I, HCC was diagnosed (triphasic spiral computed tomography or dynamic magnetic resonance imaging together with elevated α-fetoprotein and/or liver biopsy), and its stage was identified according to the Barcelona Clinical Liver Cancer (BCLC) system[16]. In Group II, chronic liver disease was diagnosed based on history, clinical examination, laboratory results and imaging that included abdominal ultrasonography and computed tomography. Liver disease severity was assessed by the Child-Pugh score. Patients with positive hepatitis B surface antigen and/or hepatitis B c antibody, secondary liver cancer, other malignancies, chronic hepatitis or cirrhosis due to any cause other than HCV infection, significant associated comorbidities (such as renal failure or heart failure) and those receiving chemotherapy, radiotherapy or on immunosuppression medication were excluded. Detailed histories of all participants were taken, and they all underwent physical examination, liver imaging (abdominal ultrasound) and routine laboratory tests that included complete blood counts, kidney and liver function tests [albumin, total bilirubin, direct bilirubin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST)], serum levels of α-fetoprotein, and serological tests for hepatitis B virus and HCV. Serum samples from Groups I and II were analyzed for HCV by reverse transcriptase PCR (RT-PCR).

Laboratory procedures

Seven milliliters of venous blood were withdrawn by venipuncture; of this, 5 mL were transferred into a plain tube, left to clot and centrifuged for 10 min at 4000 rpm. The serum obtained was stored at -80 ℃ until subsequent analyses for serum α-fetoprotein levels, liver function tests, hepatitis viral markers and endocan levels. The remaining 2 mL of blood were placed into an EDTA containing tube for HCV RT-PCR and miRNA 9-3p expression analysis. Anti-HCV levels were determined by electrochemiluminescence immunoassay using the Cobas immunoassay analyzer.

The hepatitis B surface antigen in serum was determined using (Sorin Biomedica Co. kits, Italy). Serum levels of ALT and AST were determined by the kinetic UV optimized method of the IFCC (ELTEC Kit, England). Serum levels of total bilirubin were measured using the DIAMOND diagnostics Kit, Germany. Serum albumin levels

were quantified using a colorimetric method of enhanced specificity of bromocresol green (DIAMOND diagnostics Kit, Germany). Prothrombin time was determined by the STA-Stago Compact computed tomography autoanalyzer. Serum α-fetoprotein levels were measured by enzyme-linked immunosorbent assays using the IMMULITE 1000 system (Siemens Medical Solutions Diagnostics, United States).

Endocan detection

Serum endocan levels were measured by enzyme-linked immunosorbent assay using the PicokineTM ELISA Kit for human ESMI/Endocan (Boster Biological Technology Co., Ltd., CA, United States, cat# EK0752).

RT-PCR for HCV

Nucleic acids were extracted using the Qiagen viral RNA Mini Extraction Kit.

Expression assay for miRNA 9-3p

miRNA was isolated from plasma using the Qiagen™ RNA extraction Kit MiRNeasy Kit (QIAGEN). miRNA was purified and then its concentration and purity were quantified using a NanoDrop® N50 nanophotometer (Implant GmbH and Implen, Inc. Schatzbogen 52 81829 München, Germany). Purified miRNA was stored at -80 °C until reverse transcription, which was accomplished using the Qiagen®miScript II RT Kit (QIAGEN) following the manufacturer's instructions. Each 20-μl reaction tube contained 4 μL 5 × miScript HiSpec Buffer, 2 μL 10 × miScript Nuclease Mix, 2 μL RNase-free water, 2 μL miScript Reverse Transcriptase Mix, and 10 μL template RNA. Reverse transcription was carried out at 37 °C for 60 min and 95 °C for 5 min on an Applied Biosystems 2720 thermal cycler (Bioline, Singapore, United States). The cDNA product was diluted to 5 ng/ul before determining the transcript levels by real-time quantitative PCR. Real-time quantitative PCR was performed using the miScript SYBR Green PCR Kit (QIAGEN) according to the manufacturer's instructions. The reaction mixture contained 12.5 μL 2x QuantiTect SYBR Green PCR Master Mix, 2.5 μL 10x miScript Universal Primer based on mRNA sequences obtained from the miRBase database for miRNA 9-3p, 2.5 μL template cDNA and 3.5 μL RNase-free water. The Applied Biosystems®7500 real-time thermal cycler (Applied Biosystems, Foster City, CA, United States) was programmed to run 40 cycles of the following steps: 95 °C for 15 min (initial denaturation step), denaturation at 94 °C for 15 s, annealing for 30 s at 55 °C and extension for 30 s at 70 °C. U6 snRNA was used as an endogenous control. Relative quantification expression levels were calculated using the comparative 2−ΔΔCt method with Applied Biosystems 7500 software version 2.0.1. Each run was completed using melting curve analysis to confirm the specificity of the amplification and absence of primer dimers.

All procedures involving human participants were performed according to the ethical standards of the institutional and/or national research committee and the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the Menoufia University Faculty of Medicine Research Ethics Committee. Every patient in this research provided their written consent to participate in the research, provided that they were not identified in the paper.

Statistical analysis

Data were analyzed using IBM SPSS statistics software version 20 (SPSS Inc. Released 2011. IBM SPSS statistics for windows, version 20.0, Armonk, NY, United States: IBM Corp). Quantitative data are presented as means, standard deviations, medians and interquartile ranges, while qualitative data are presented as frequencies and percentages. The relationship between qualitative variables was evaluated by the χ^2 test. Pairs of groups of non-normally distributed quantitative data were compared by the Mann–Whitney test, while three groups were compared by the Kruskal–Wallis test (non-parametric analysis of variance). Based on Kruskal–Wallis distribution, a post-hoc test was performed for pairwise comparisons. Correlations were assessed using the Spearman correlation test. The diagnostic values of serum miRNA 9-3p and endocan in HCC patients were evaluated by the receiver operating characteristic (ROC) curve analysis. The most independent factor associated with metastasis was identified by logistic regression analysis. *P* values of < 0.05 were considered statistically significant.

RESULTS

The patients in all three groups did not differ statistically in terms of age and gender. Biochemical analyses results (Table 1) showed that the HCC group and the chronic liver disease group differed in the following: ALT, direct bilirubin, international normalized ratio, endocan and miRNA 9-3p ($P = 0.005$, $P = 0.003$, $P = 0.002$, $P < 0.001$ and $P < 0.001$, respectively).

The HCC group differed significantly from the control group in terms of ALT, AST, platelet count, serum albumin, direct bilirubin, international normalized ratio, α-fetoprotein level, endocan and miRNA 9-3p ($P < 0.001$). The chronic liver disease group differed significantly from the control group in terms of ALT, AST, hemoglobin level, platelet count, international normalized ratio, α-fetoprotein level, endocan and miRNA 9-3p ($P = 0.003$, $P < 0.001$, $P = 0.019$, $P = 0.022$, $P = 0.001$, $P < 0.001$, $P < 0.001$ and $P < 0.001$ respectively).

Serum endocan levels in the HCC group were significantly higher than those in the chronic liver disease and control groups ($P < 0.001$). Furthermore, serum miRNA 9-3p expression levels in the HCC group were significantly lower than those in the chronic liver disease and control groups ($P < 0.001$), while levels in the chronic liver disease group were significantly lower than those in the control group ($P < 0.001$).

In the HCC group, 62.9% of patients (22 patients) were classified as grade A and (37.1%) of patients (13 patients) were classified as grade B according to Child-Pugh classifications; 4 (11.4%) patients were classified as grade A in BCLC stage, 18 (51.4 %) patients in stage B and 13 (37.1%) patients were in stage C. Detailed tumor characteristics of the HCC group are shown in Table 2.

The correlations between serum miRNA 9-3p levels and clinical data in HCC patients are shown in Table 3. miRNA 9-3p expression levels were significantly inversely correlated to vascular invasion, BCLC classification and metastatic site. Moreover, miRNA 9-3p expression levels were also significantly inversely correlated to serum endocan levels (Figure 1).

Univariate and multivariate logistic regression analyses on the HCC group indicated that miRNA 9-3p is an independent predictor factor of metastasis ($P = 0.041$; 95% confidence interval: 0.089-0.951) (Table 4).

ROC analysis of miRNA 9-3p and endocan levels indicated that at a cutoff point of 0.26, miRNA 9-3p can discriminate between patients with HCC and those with chronic liver disease with a sensitivity of 91.43%, a specificity of 87.88%, a positive predictive value of 88.90% and a negative predictive value of 90.60%. Meanwhile, at a cutoff point of 2370 pg/mL, endocan can discriminate between HCC and chronic liver disease patients with a sensitivity of 82.86%, a specificity of 84.85%, a positive predictive value of 85.30% and a negative predictive value of 82.40%. In comparison, α-fetoprotein was less sensitive and specific (60.00% and 33.30%, respectively).

At a cutoff point of 1.01, miRNA 9-3p can discriminate between HCC and control group patients with a sensitivity of 91.43%, a specificity of 87.50%, a positive predictive value of 88.90% and a negative predictive value of 90.30%. Meanwhile, at a cutoff point of 1510 pg/mL, endocan can discriminate between HCC and chronic liver disease patients with a sensitivity of 85.71%, a specificity of 87.50%, a positive predictive value of 88.20% and a negative predictive value of 84.80%. In comparison, α-fetoprotein was less sensitive and specific (80.00% and 71.87%, respectively). Diagnostically, both miRNA 9-3p and endocan performed better than α-fetoprotein at discriminating HCC patients from both chronic liver disease and healthy patients (Figures 2 and 3).

ROC analysis of miRNA 9-3p levels in the HCC group indicated that at a cutoff point of 0.02, miRNA 9-3p can discriminate between metastatic and non-metastatic HCC patients with a sensitivity of 91.67%, a specificity of 82.61%, a positive predictive value of 73.30% and a negative predictive value of 95.00%.

DISCUSSION

The increasing prevalence of HCC worldwide and its associated poor prognosis make it a global health problem. Studies in Egypt shows the increasing role of HCV infection in liver cancer etiology, and among all cancer deaths in Egypt, HCC is the primary cause[17,18].

HCC is often detected late, when it is no longer operable, which limits curative surgical treatment to only a few cases involving small HCC malignancies. Moreover, as a diagnostic tool, α-fetoprotein is limited in its accuracy[19]. In contrast, circulating

Table 1 Demographic and laboratory data of the study participants

Variables	Group I	Group II	Group III	P value	
	HCC	Chronic liver disease	Control		
	n = 35	*n* = 33	*n* = 32		
Gender					
Male, *n* (%)	30 (85.7)	23 (69.7)	27 (84.4)	NS	
Female, *n* (%)	5 (14.3)	10 (30.3)	5 (15.6)		
Age (yr)					
mean ± SD	55.2 ± 5.2	52.7 ± 5.3	52.8 ± 5.6	NS	
ALT (IU/L), median (IQR)	50.0 (35.0-55.0)	34.0 (28.0-50.0)	29.7 (23.5-31.7)	$P < 0.001$	[a]$P = 0.005$; [b]$P < 0.001$; [c]$P = 0.003$
AST (IU/L), median (IQR)	52.0 (39.0-70.0)	42.0 (32.0-57.0)	32.8 (30.0-36.0)	< 0.001	[a]$P = 0.060$; [b]$P < 0.001$; [c]$P < 0.001$
Hb (mg/dL), mean ± SD	13.1 ± 1.7	12.5 ± 1.6	13.5 ± 1.0	0.025	[a]$P = 0.260$; [b]$P = 0.438$; [c]$P = 0.019$
Platelets, (× 10³/μL), median (IQR)	141.0 (104.5-193.5)	162.0 (134.0-213.0)	197.5 (180.5-246.0)	0.002	[a]$P = 0.225$; [b]$P < 0.001$; [c]$P = 0.022$
Serum ALB (g/dL), mean ± SD	3.6 ± 0.7	3.8 ± 0.6	4.1 ± 0.4	0.001	[a]$P = 0.382$; [b]$P = 0.001$; [c]$P = 0.050$
INR, mean ± SD	1.3 ± 0.2	1.1 ± 0.4	0.8 ± 0.2	< 0.001	[a]$P = 0.002$; [b]$P = 0.001$; [c]$P = 0.001$
α-fetoprotein (ng/mL), median (IQR)	240.0 (28.2-635.0)	124.0 (108.9-166.0)	17.4 (14.0-24.0)	< 0.001	[a]$P = 0.895$; [b]$P < 0.001$; [c]$P < 0.001$
Endocan (pg/mL), median (IQR)	3450.0 (3188.5-4135.0)	1934.0 (1450.0-2257.0)	878.5 (850.0-1188.0)	< 0.001	[a]$P < 0.001$; [b]$P < 0.001$; [c]$P = 0.001$
microRNA 9-3p, median (IQR)	0.03 (0.02-0.05)	0.42 (0.29-1.35)	1.70 (1.40-2.15)	< 0.001	[a]$P < 0.001$; [b]$P < 0.001$; [c]$P < 0.001$

[a]P: P value for comparing between HCC and chronic liver disease.

[b]P: P value for comparing between HCC and control.

[c]P: P value for comparing between chronic liver disease and control. Statistically significant at $P ≤ 0.05$. SD: Standard deviation; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; Hb: Hemoglobin; ALB: Albumin; INR: International normalized ratio; HCC: Hepatocellular carcinoma; NS: Not significant; IQR: Interquartile range.

miRNAs may serve as biomarkers and a useful diagnostic approach for the early detection of HCC[20].

In the present study, clinical and laboratory data from the three different groups of patients revealed that serum α-fetoprotein levels of HCC and chronic liver disease patients was significantly different from those of control patients. α-fetoprotein is known to be overexpressed in HCC[21-23], and the severity of cirrhosis is a significant predictor of elevated serum α-fetoprotein levels; higher serum α-fetoprotein levels are significantly correlated with advanced cirrhosis in patients with chronic HCV[24].

We found that serum α-fetoprotein levels in the HCC group did not differ significantly from those of the chronic liver disease group. This agrees with the results of Massironi *et al*[25], who reported similar findings in HCC and liver cirrhosis subjects. In our study, at a cutoff value of 23 ng/dL, α-fetoprotein discriminates between HCC and control patients at a sensitivity of 80.00% and a specificity of 71.87%. These results are similar to those of Massironi *et al*[25] and Metwaly *et al*[26], who reported a sensitivity of 75% and a specificity of 80% at a cutoff value of 16.9 ng/dL.

Our findings show higher serum endocan levels in HCC patients than in chronic liver disease patients, which agrees with previous studies by Nault *et al*[27] and Ozaki *et al*[28].

Recent studies on HCC show that elevated serum endocan levels and endocan expression by stromal endothelial cells in HCC tissues are correlated with poor survival[29]. Endocan expression in tumors undergoing angiogenesis reflects the

Table 2 Clinical characteristics of tumors in the hepatocellular carcinoma group, *n* = 35	
Number of the focal lesions	***n* (%)**
Single	16 (45.7)
Multiple	19 (54.3)
Tumor size in cm	
Small < 3	7 (20.0)
Medium 3-5	15 (42.9)
Large > 5	13 (37.1)
Location of the focal lesions	
Rt. Lobe	19 (54.3)
Lt. Lobe	8 (22.9)
Both	7 (20.0)
Caudate lobe	1 (2.9)
BCLC stage	
A	4 (11.4)
B	18 (51.4)
C	13 (37.1)
Vascular invasion	
Negative	25 (71.4)
Positive	10 (28.6)
LN metastasis	
Negative	28 (80.0)
Positive	7 (20.0)
Ascites	
No	25 (73.5)
Mild	8 (23.5)
Moderate	1 (2.9)
Child Pugh classA	22 (62.9)
B	13 (37.1)
C	0 (0)
Distant metastasis	
No	23 (65.7)
Yes	12 (34.3)

Rt.: Right; Lt.: Left; BCLC: Barcelona Clinic Liver Cancer; LN: Lymph node.

processes of angiogenesis and tumor invasion. Structurally, the glycan form and phenylalanine-rich region of endocan are its key effective sections through the nuclear factor-κB/IκB pathway[30]. However, the involvement of endocan in HCC development remains unclear.

We found that plasma miRNA 9-3p levels are significantly lower in HCC patients compared to chronic liver disease and control patients. Overall, the order of miRNA 9-3p expression among the different groups is as follows: HCC < chronic liver disease < control.

This supports the concept of the antitumor function of miRNA 9-3p as reported by Higashi *et al*[12], Yang *et al*[31] and Tang *et al*[32]. In contrast, Sun *et al*[33] showed that miR-9 increases the levels of migration and invasion of HCC cell lines. It is possible that miR-9 (*i.e.* miR-9-5p) and miR-9* (miR-9-3p) are two different miRNAs that

Table 3 Correlations between microRNA 9-3p levels and clinical data in hepatocellular carcinoma group

	n	microRNA 9-3p	
		Median (IQR)	P value
Vascular invasion			
Negative	25	0.04 (0.02-0.26)	0.002
Positive	10	0.02 (0.02-0.02)	
LN metastasis			
Negative	28	0.04 (0.02-0.17)	0.072
Positive	7	0.02 (0.02-0.03)	
Distant metastasis			
No	23	0.04 (0.03-0.26)	< 0.001
Yes	12	0.02 (0.02-0.02)	
Child Pugh class			
A	22	0.03 (0.02-0.04)	0.389
B	13	0.04 (0.02-0.26)	
Tumor number			
Single	16	0.03 (0.02-0.17)	0.935
Multiple	19	0.03 (0.02-0.05)	
Tumor size in cm			
Small < 3	7	0.03 (0.02-0.15)	0.852
Medium 3-5	15	0.03 (0.02-0.06)	
Large > 5	13	0.04 (0.02-0.04)	
Tumor site			
Rt lobe	19	0.04 (0.02-0.06)	0.432
Lt lobe	8	0.04 (0.03-0.15)	
Both	7	0.02 (0.02-0.04)	
Caudate lobe	1		
BCLC stage			
A	4	0.26 (0.17-0.26)	< 0.001
B	18	0.04 (0.03-0.05)	
C	13	0.02 (0.02-0.02)	

Rt.: Right; Lt.: Left; BCLC: Barcelona Clinic Liver Cancer; IQR: Interquartile range; LN: Lymph node.

originate from the same precursor, and they can play either synergistic or opposite roles within one malignancy[34].

Interestingly, we observed significantly lower levels of miRNA 9-3p expression and vascular invasion at the advanced stage of BCLC and at the metastatic site of the HCC group.

In cervical adenocarcinoma, miRNA 9-3p is downregulated and acts as a tumor suppressor. Ectopic expression of miR-9-3p inhibits the JAK/STAT3 pathway by targeting interleukin 6, leading to the upregulation of vascular endothelial growth factor and increased angiogenesis. This results in decreased proliferation and migration and reduced tumor growth *in vivo*[35]. Moreover, Tang *et al*[32] reported that exosomal miRNA 9-3p suppresses the development and progression of HCC.

Cai *et al*[15] reported that increased exosomal miR-9-3p counteracts bladder cancer growth and metastasis and decreases endocan protein expression in nude mice. We similarly observed that miR-9-3p expression is inversely correlated to serum endocan levels in the HCC group.

Table 4 Univariate and multivariate regression analyses for the parameters affecting metastasis in hepatocellular carcinoma group

	Univariate		Multivariate	
	P value	OR (95%CI)	*P* value	OR (95%CI)
microRNA 9-3p	0.008	0.193 (0.057-0.653)	0.041	0.291 (0.089-0.951)
Endocan	0.023	1.002 (1.000-1.003)	0.358	1.001 (0.999-1.002)

Statistically significant at $P \leq 0.05$. OR: Odds ratio; CI: Confidence interval.
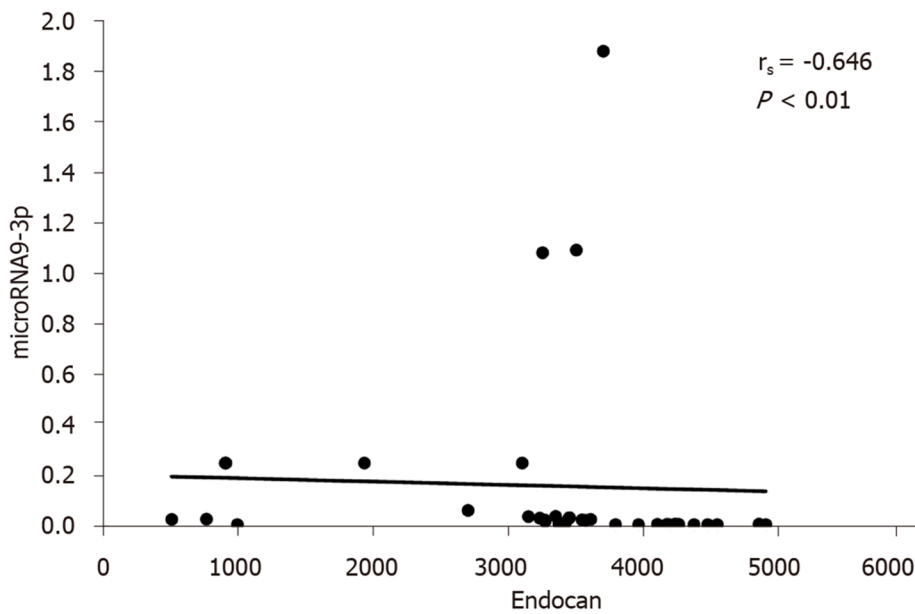
Figure 1 Correlation between microRNA 9-3p and endocan levels in the hepatocellular carcinoma group.

We performed ROC analysis to compare the diagnostic accuracies of miRNA 9-3p, endocan and the traditional HCC tumor marker, α-fetoprotein. Diagnostically, both miRNA 9-3p and endocan perform better than α-fetoprotein in discriminating patients with HCC from those with or without (*i.e.* healthy) chronic liver disease. Furthermore, ROC analysis revealed that miRNA 9-3p performed well at discriminating between metastatic and non-metastatic patients in the HCC group. Statistically, miRNA 9-3p is an independent predictor factor of metastasis. This study could be the nucleus of a larger study working on a larger number of patients that may include those with other causes of chronic liver disease like alcoholism as our study was limited to HCV-induced chronic liver disease as it is highly prevalent in Egypt.

CONCLUSION

Endocan and miRNA 9-3p could be biomarkers with potential use for the early diagnosis of HCV-related HCC. In this regard, they are more valuable than α-fetoprotein. Moreover, miRNA 9-3p is an independent predictor of metastasis in HCC patients.
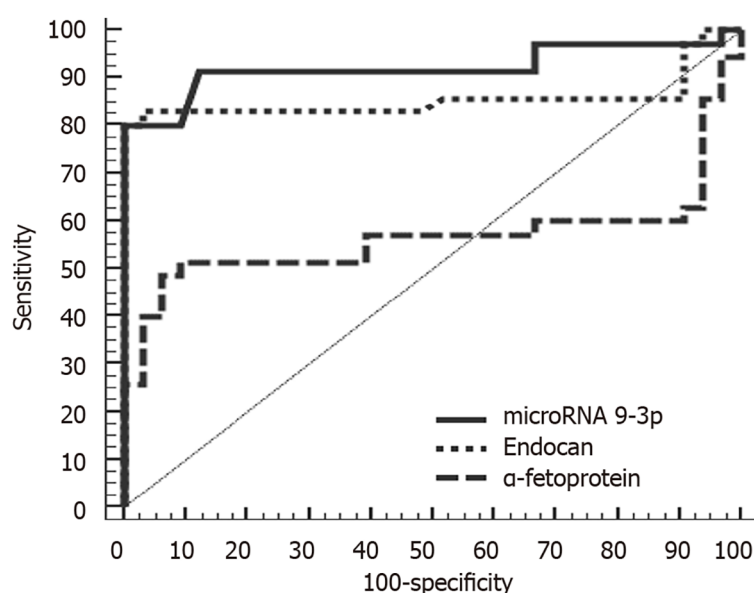
Figure 2 Receiver operating characteristic curve analysis of microRNA 9-3p, endocan and α-fetoprotein for discriminating between hepatocellular carcinoma and chronic liver disease.
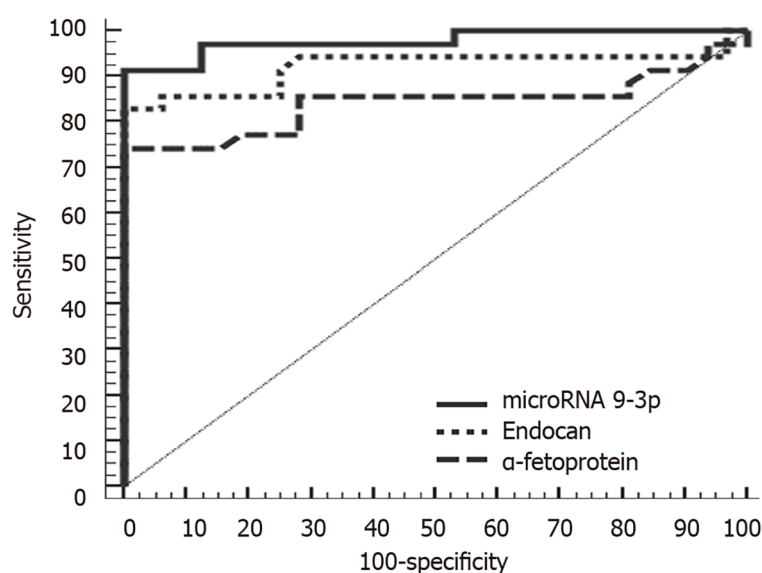
Figure 3 Receiver operating characteristic curve analysis of microRNA 9-3p, endocan and α-fetoprotein for discriminating between hepatocellular carcinoma and control.

ARTICLE HIGHLIGHTS

Research background

The high mortality rate of hepatocellular carcinoma (HCC) in Egypt is due mainly to the increasing prevalence of hepatitis C virus infection (HCV) and late diagnosis of the carcinoma.

Research motivation

MicroRNAs (miRNA), which regulate tumor proliferation and metastasis in HCC, may serve as a useful diagnostic approach for the early detection of HCC, thus decreasing its mortality. Meanwhile, endocan is a protein with angiogenic and inflammatory properties that are associated with tumor progression and poor outcomes.

Research objectives

To analyze the levels of miRNA 9-3p and endocan in HCV-infected HCC patients and correlate them with clinicopathological parameters.

Research methods

We compared levels of endocan and circulating miRNA 9-3p from 35 HCV-related HCC patients to 33 patients with HCV-induced chronic liver disease and 32 age and gender matched healthy controls.

Research results

The levels of circulating miRNA 9-3p were significantly lower in the HCC group compared to the chronic liver disease ($P < 0.001$) and control ($P < 0.001$) groups, while levels in the chronic liver disease were significantly lower than those in the control group ($P < 0.001$). While the levels of serum endocan were significantly higher in the HCC group compared to the chronic liver disease ($P < 0.001$) and control ($P < 0.001$) groups. Moreover, miRNA 9-3p and endocan performed better than α-fetoprotein in discriminating HCC patients from cirrhosis and healthy patients. The levels of miRNA 9-3p are significantly inversely correlated to vascular invasion ($P = 0.002$), stage of advancement of Barcelona Clinical Liver Cancer ($P < 0.001$ and the metastatic site ($P < 0.001$) of the HCC group.

Research conclusions

Endocan and miRNA 9-3p could be biomarkers with potential use for the early diagnosis of HCV-related HCC. In this regard, they are more valuable than α-fetoprotein. Moreover, miRNA 9-3p is an independent predictor of metastasis in HCC patients.

Research perspectives

The findings of this study warrant additional investigation in prospective trials with larger cohorts and longer follow-up for confirming our results and validating the potential clinical use of these markers in early HCC detection.

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REFERENCES

1   **Bray F**, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; **68**: 394-424 [PMID: 30207593 DOI: 10.3322/caac.21492]

2   **Ferlay J**, Colombet M, Soerjomataram I, Mathers C, Parkin DM, Piñeros M, Znaor A, Bray F. Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods. *Int J Cancer* 2019; **144**: 1941-1953 [PMID: 30350310 DOI: 10.1002/ijc.31937]

3   **Rashed WM**, Kandeil MAM, Mahmoud MO, Ezzat S. Hepatocellular Carcinoma (HCC) in Egypt: A comprehensive overview. *J Egypt Natl Canc Inst* 2020; **32**: 5 [PMID: 32372179 DOI: 10.1186/s43046-020-0016-x]

4   **El Kassas M**, Elbaz T, Elsharkawy A, Omar H, Esmat G. HCV in Egypt, prevention, treatment and key barriers to elimination. *Expert Rev Anti Infect Ther* 2018; **16**: 345-350 [PMID: 29506418 DOI: 10.1080/14787210.2018.1448709]

5   **Zucman-Rossi J**, Villanueva A, Nault JC, Llovet JM. Genetic Landscape and Biomarkers of Hepatocellular Carcinoma. *Gastroenterology* 2015; **149**: 1226-1239.e4 [PMID: 26099527 DOI: 10.1053/j.gastro.2015.05.061]

6   **Cancer Genome Atlas Research Network**; Comprehensive and Integrative Genomic Characterization of Hepatocellular Carcinoma. *Cell* 2017; **169**: 1327-1341.e23 [PMID: 28622513 DOI: 10.1016/j.cell.2017.05.046]

7   **El-Gebaly F**, Abou-Saif S, Elkadeem M, Helmy A, Abd-Elsalam S, Yousef M, Elkhouly RA, Amer IF, El-Demerdash T. Study of Serum Soluble Programmed Death Ligand 1 as a Prognostic Factor in Hepatocellular Carcinoma in Egyptian Patients. *Curr Cancer Drug Targets* 2019; **19**: 896-905 [PMID: 31538897 DOI: 10.2174/1568009619666190718141647]

8   **Catalanotto C**, Cogoni C, Zardo G. MicroRNA in Control of Gene Expression: An Overview of Nuclear Functions. *Int J Mol Sci* 2016; **17** [PMID: 27754357 DOI: 10.3390/ijms17101712]

9   **Hu Q**, Du K, Mao X, Ning S. miR-197 is downregulated in cervical carcinogenesis and suppresses cell proliferation and invasion through targeting forkhead box M1. *Oncol Lett* 2018; **15**: 10063-10069 [PMID: 29928375 DOI: 10.3892/ol.2018.8565]

10 **Coolen M**, Katz S, Bally-Cuif L. miR-9: a versatile regulator of neurogenesis. *Front Cell Neurosci* 2013; **7**: 220 [PMID: 24312010 DOI: 10.3389/fncel.2013.00220]

11 **Ma L**, Young J, Prabhala H, Pan E, Mestdagh P, Muth D, Teruya-Feldstein J, Reinhardt F, Onder TT, Valastyan S, Westermann F, Speleman F, Vandesompele J, Weinberg RA. miR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis. *Nat Cell Biol* 2010; **12**: 247-256 [PMID: 20173740 DOI: 10.1038/ncb2024]

12 **Higashi T**, Hayashi H, Ishimoto T, Takeyama H, Kaida T, Arima K, Taki K, Sakamoto K, Kuroki H, Okabe H, Nitta H, Hashimoto D, Chikamoto A, Beppu T, Baba H. miR-9-3p plays a tumour-suppressor role by targeting TAZ (WWTR1) in hepatocellular carcinoma cells. *Br J Cancer* 2015; **113**: 252-258 [PMID: 26125451 DOI: 10.1038/bjc.2015.170]

13 **Sarrazin S**, Adam E, Lyon M, Depontieu F, Motte V, Landolfi C, Lortat-Jacob H, Bechard D, Lassalle P, Delehedde M. Endocan or endothelial cell specific molecule-1 (ESM-1): a potential novel endothelial cell marker and a new target for cancer therapy. *Biochim Biophys Acta* 2006; **1765**: 25-37 [PMID: 16168566 DOI: 10.1016/j.bbcan.2005.08.004]

14 **Leroy X**, Aubert S, Zini L, Franquet H, Kervoaze G, Villers A, Delehedde M, Copin MC, Lassalle P. Vascular endocan (ESM-1) is markedly overexpressed in clear cell renal cell carcinoma. *Histopathology* 2010; **56**: 180-187 [PMID: 20102396 DOI: 10.1111/j.1365-2559.2009.03458.x]

15 **Cai H**, Yang X, Gao Y, Xu Z, Yu B, Xu T, Li X, Xu W, Wang X, Hua L. Exosomal MicroRNA-9-3p Secreted from BMSCs Downregulates ESM1 to Suppress the Development of Bladder Cancer. *Mol Ther Nucleic Acids* 2019; **18**: 787-800 [PMID: 31734559 DOI: 10.1016/j.omtn.2019.09.023]

16 **Llovet JM**, Brú C, Bruix J. Prognosis of hepatocellular carcinoma: the BCLC staging classification. *Semin Liver Dis* 1999; **19**: 329-338 [PMID: 10518312 DOI: 10.1055/s-2007-1007122]

17 **Patel M**, Shariff MI, Ladep NG, Thillainayagam AV, Thomas HC, Khan SA, Taylor-Robinson SD. Hepatocellular carcinoma: diagnostics and screening. *J Eval Clin Pract* 2012; **18**: 335-342 [PMID: 21114800 DOI: 10.1111/j.1365-2753.2010.01599.x]

18 **Abudeif A**. Epidemiology and Risk Factors of Hepatocellular Carcinoma in Egypt. *Sohag Medical Journal* 2019; **23**: 8-12 [DOI: 10.21608/SMJ.2019.13376.1019]

19 **Sun S**, Poon RT, Lee NP, Yeung C, Chan KL, Ng IO, Day PJ, Luk JM. Proteomics of hepatocellular carcinoma: serum vimentin as a surrogate marker for small tumors (<or=2 cm). *J Proteome Res* 2010; **9**: 1923-1930 [PMID: 20121168 DOI: 10.1021/pr901085z]

20 **Chen X**, Ba Y, Ma L, Cai X, Yin Y, Wang K, Guo J, Zhang Y, Chen J, Guo X, Li Q, Li X, Wang W, Wang J, Jiang X, Xiang Y, Xu C, Zheng P, Zhang J, Li R, Zhang H, Shang X, Gong T, Ning G, Zen K, Zhang CY. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res* 2008; **18**: 997-1006 [PMID: 18766170 DOI: 10.1038/cr.2008.282]

21 **Köberle V**, Kronenberger B, Pleli T, Trojan J, Imelmann E, Peveling-Oberhag J, Welker MW, Elhendawy M, Zeuzem S, Piiper A, Waidmann O. Serum microRNA-1 and microRNA-122 are prognostic markers in patients with hepatocellular carcinoma. *Eur J Cancer* 2013; **49**: 3442-3449 [PMID: 23810247 DOI: 10.1016/j.ejca.2013.06.002]

22 **El-Abd NE**, Fawzy NA, El-Sheikh SM, Soliman ME. Circulating miRNA-122, miRNA-199a, and miRNA-16 as Biomarkers for Early Detection of Hepatocellular Carcinoma in Egyptian Patients with Chronic Hepatitis C Virus Infection. *Mol Diagn Ther* 2015; **19**: 213-220 [PMID: 26133725 DOI: 10.1007/s40291-015-0148-1]

23 **Naz Z**, Usman S, Saleem K, Ahmed S, Bashir H, Bilal M, Sumrin A. Alpha-fetoprotein: A fabulous biomarker in hepatocellular, gastric and rectal cancer diagnosis. *Biomed Res* 2018; **29**: 2478-2483 [DOI: 10.4066/biomedicalresearch.29-17-1550]

24 **Chu CW**, Hwang SJ, Luo JC, Lai CR, Tsay SH, Li CP, Wu JC, Chang FY, Lee SD. Clinical, virologic, and pathologic significance of elevated serum alpha-fetoprotein levels in patients with chronic hepatitis C. *J Clin Gastroenterol* 2001; **32**: 240-244 [PMID: 11246354 DOI: 10.1097/00004836-200103000-00014]

25 **Massironi S**, Fraquelli M, Paggi S, Sangiovanni A, Conte D, Sciola V, Ciafardini C, Colombo M, Peracchi M. Chromogranin A levels in chronic liver disease and hepatocellular carcinoma. *Dig Liver Dis* 2009; **41**: 31-35 [PMID: 18762462 DOI: 10.1016/j.dld.2008.05.002]

26 **Metwaly K**, Sameea EA, El-Azab G, Assem M, Abbas M, Zakareya T, Abo Raia G. Mean platelet volume and mean platelet volume/platelet count ratio as markers for hepatocellular carcinoma in patients with chronic hepatitis C virus related cirrhosis. *J Cancer Res Clin Oncol* 2016; **8**: 33-40 [DOI: 10.5897/JCREO2016.0135]

27 **Nault JC**, Guyot E, Laguillier C, Chevret S, Ganne-Carrie N, N'Kontchou G, Beaugrand M, Seror O, Trinchet JC, Coelho J, Lasalle P, Charnaux N, Delehedde M, Sutton A, Nahon P. Serum proteoglycans as prognostic biomarkers of hepatocellular carcinoma in patients with alcoholic cirrhosis. *Cancer Epidemiol Biomarkers Prev* 2013; **22**: 1343-1352 [PMID: 23780836 DOI: 10.1158/1055-9965.EPI-13-0179]

28 **Ozaki K**, Toshikuni N, George J, Minato T, Matsue Y, Arisawa T, Tsutsumi M. Serum endocan as a novel prognostic biomarker in patients with hepatocellular carcinoma. *J Cancer* 2014; **5**: 221-230 [PMID: 24665346 DOI: 10.7150/jca.7691]

29 **Huang GW**, Tao YM, Ding X. Endocan expression correlated with poor survival in human hepatocellular carcinoma. *Dig Dis Sci* 2009; **54**: 389-394 [PMID: 18592377 DOI:

10.1007/s10620-008-0346-3]

30   **Chen LY**, Liu X, Wang SL, Qin CY. Over-expression of the Endocan gene in endothelial cells from hepatocellular carcinoma is associated with angiogenesis and tumour invasion. *J Int Med Res* 2010; **38**: 498-510 [PMID: 20515564 DOI: 10.1177/147323001003800213]

31   **Yang L**, Mu Y, Cui H, Liang Y, Su X. MiR-9-3p augments apoptosis induced by H2O2 through down regulation of Herpud1 in glioma. *PLoS One* 2017; **12**: e0174839 [PMID: 28430789 DOI: 10.1371/journal.pone.0174839]

32   **Tang J**, Li Y, Liu K, Zhu Q, Yang WH, Xiong LK, Guo DL. Exosomal miR-9-3p suppresses HBGF-5 expression and is a functional biomarker in hepatocellular carcinoma. *Minerva Med* 2018; **109**: 15-23 [PMID: 28750499 DOI: 10.23736/S0026-4806.17.05167-9]

33   **Sun Z**, Han Q, Zhou N, Wang S, Lu S, Bai C, Zhao RC. MicroRNA-9 enhances migration and invasion through KLF17 in hepatocellular carcinoma. *Mol Oncol* 2013; **7**: 884-894 [PMID: 23684102 DOI: 10.1016/j.molonc.2013.04.007]

34   **Schraivogel D**, Weinmann L, Beier D, Tabatabai G, Eichner A, Zhu JY, Anton M, Sixt M, Weller M, Beier CP, Meister G. CAMTA1 is a novel tumour suppressor regulated by miR-9/9∗ in glioblastoma stem cells. *EMBO J* 2011; **30**: 4309-4322 [PMID: 21857646 DOI: 10.1038/emboj.2011.301]

35   **Zhang J**, Jia J, Zhao L, Li X, Xie Q, Chen X, Wang J, Lu F. Down-regulation of microRNA-9 Leads to activation of IL-6/Jak/STAT3 pathway through directly targeting IL-6 in HeLa cell. *Mol Carcinog* 2016; **55**: 732-742 [PMID: 25809226 DOI: 10.1002/mc.22317]

ORIGINAL ARTICLE

Retrospective Cohort Study

Do peripartum and postmenopausal women with primary liver cancer have a worse prognosis? A nationwide cohort in Taiwan

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Abstract

BACKGROUND
While primary liver cancer (PLC) is one of the most common cancers around the world, few large-scale population-based studies have been reported that evaluated the clinical survival outcomes among peripartum and postmenopausal women with PLC.

AIM
To investigate whether peripartum and postmenopausal women with PLC have lower overall survival rates compared with women who were not peripartum and postmenopausal.

METHODS
The Taiwan National Health Insurance claims data from 2000 to 2012 was used for this propensity-score-matched study. A cohort of 40 peripartum women with PLC and a reference cohort of 160 women without peripartum were enrolled. In the women with PLC with/without menopause study, a study cohort of 10752

menopausal females with PLC and a comparison cohort of 2688 women without menopause were enrolled.

RESULTS
Patients with peripartum PLC had a non-significant risk of death compared with the non-peripartum cohort [adjusted hazard ratios (aHR) = 1.40, 95% confidence intervals (CI): 0.89-2.20, P = 0.149]. The survival rate at different follow-up durations between peripartum PLC patients and those in the non-peripartum cohort showed a non-significant difference. Patients who were diagnosed with PLC younger than 50 years old (without menopause) had a significant lower risk of death compared with patients diagnosed with PLC at or older than 50 years (postmenopausal) (aHR = 0.64, 95%CI: 0.61-0.68, P < 0.001). The survival rate of women < 50 years with PLC was significantly higher than older women with PLC when followed for 0.5 (72.44% *vs* 64.16%), 1 (60.57% *vs* 51.66%), 3 (42.92% *vs* 31.28%), and 5 year(s) (37.02% *vs* 21.83%), respectively (P < 0.001).

CONCLUSION
Peripartum females with PLC have no difference in survival rates compared with those patients without peripartum. Menopausal females with PLC have worse survival rates compared with those patients without menopause.

Key Words: Primary liver cancer; Peripartum and postmenopausal women; Prognosis; Nationwide cohort; Peripartum women; Postmenopausal women

Core Tip: This is the first nationwide study to evaluate the survival rate of peripartum and postmenopausal women with primary liver cancer (PLC) using the National Health Insurance Research Database in Taiwan. The results showed that patients with peripartum PLC had a non-significant risk of death compared with those in the non-peripartum cohort. Patients who were diagnosed with PLC younger than 50 years (without menopause) had a significantly lower risk of death compared with patients diagnosed with PLC at 50 years or older (after menopause). We believe that the results presented in this study provide important information on clinical applications.

INTRODUCTION

Primary liver cancer (PLC), the sixth most common cancer, and the fourth leading cause of cancer-related death around the world in 2018, put a heavy burden on global health[1,2]. Hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma, account for 70%-75% and 15% of cases, respectively, and comprise most primary liver malignancies[3]. The common risk factors of PLC are male gender, excess body fat, type II diabetes mellitus, chronic infection with hepatitis B virus (HBV) and/or hepatitis C virus (HCV), cigarette smoking, aflatoxin, and heavy alcohol consumption [4,5]. Men appear to have a higher occurrence and worse outcomes, with two to three times higher incidence and mortality compared with women[1,6]. Thus, most studies have included too few women to draw accurate conclusions.

Animal studies indicated that the primary etiology behind the protective effect of the female sex hormone might involve the anti-inflammatory modulation of estrogen, as chronic inflammation was a major contributor to carcinogenic processes[7-9]. Nevertheless, controversial results were obtained in research targeting women of reproductive age. Despite the rarity, PLC diagnosed during pregnancy generally caused a shorter survival compared with non-pregnant patients with inoperable PLC

[10-12]. Several early reports suggested that the adverse influence of pregnancy for the development of PLC was probably due to an alteration of the hormonal milieu[13,14]. In contrast, other recent papers attributed the consequence to delayed diagnosis[11, 15]. However, the latest cohort analysis needs further interpretation, as most of the published articles were case reports, with the largest including 48 cases published in 2011[12,16]. In addition, evidence implied that the downturn in ovarian function in menopause is related to the spontaneous elevation in pro-inflammatory cytokines[17-19], which may have an undesirable effect on PLC development and progression. While there were limited epidemiologic statistics with the survival outcome among females, the research indicated that there was a reduced risk. It increased overall survival times of PLCs in postmenopausal patients receiving hormone replacement therapy (HRT)[20]. It is estimated that 1.2 billion women worldwide will be menopausal or postmenopausal by the year 2030[21]. Therefore, there is a growing necessity to make a thorough exploration of the morbidity and mortality of PLCs among this sector of the population.

To date, few large-scale population-based studies have been conducted to elucidate the relationship between pregnancy, menopause, and survival outcomes among women with PLCs. Our primary aim was to determine if pregnant and postmenopausal female patients with PLCs have a lower survival rate relative to population-based controls using a nationwide database in Taiwan.

MATERIALS AND METHODS

Data source
Taiwan government built a nationwide health record-related database named the National Health Insurance Research Database (NHIRD) in 1995. The database contains comprehensive health information, representative study subjects, and long-term follow-up periods. This study was conducted using the population-based hospital-ization file, including all hospitalization records of Taiwan citizens. The identification was encrypted before the database released the records for medical research to protect the privacy of each patient.

All previous diagnoses in the database were coded according to the International Classification of Disease, Ninth Revision, Clinical Modification (ICD-9-CM). The Research Ethics Committee of China Medical University and Hospital in Taiwan approved the study (CMUH-104-REC2-115-R3).

Study population
According to the study objective, we would like to confirm the association between peripartum PLC and survival. We selected patients with peripartum PLC (ICD-9-CM: 155) who were diagnosed between 10 mo before and six months after delivery, during 2000-2012, as the exposed cohort. We defined the date of newly diagnosed PLC as the index date. The unexposed group was defined as patients with PLC who were diagnosed outside of the pregnancy period and selected by 4:1 propensity score matching with the exposed cohort. The matching variables included age, index year, and comorbidities, such as HBV, unspecified chronic hepatitis, alcoholic liver disease, cirrhosis, biliary stones, cholecystitis, and cholangitis. To further realize the correlation between menopause and PLC prognosis, we defined women aged 50 and beyond as postmenopausal period. While natural menopause may occur from 45 to 55 of age[22], a recent cohort analysis including 36931 postmenopausal women indicated that the mean age at menopause is 50.2 years in Taiwan[23]. Propensity score matching and matching variables mentioned above were applied. Patients with PLC before the index date were excluded from the study. The study population was followed up until death, withdrawn from NHIRD, or until December 31, 2013.

The comorbidities of concern in this study were HBV (ICD-9-CM: 070.2, 070.3, and V02.61), unspecified chronic hepatitis (ICD-9-CM: 070.9, 571.4, 571.8, 571.9), alcoholic liver disease (ICD-9-CM: 571.0, 571.1, 571.2, 571.3), cirrhosis (ICD-9-CM: 571.5, 571.6), biliary stones (ICD-9-CM: 574), cholecystitis (ICD-9-CM: 575), and cholangitis (ICD-9-CM: 576). The comorbidities above were defined as at least one hospitalization before the index date.

Statistical analysis
This study included demographic and comorbidities variables. The continuous and the categorical variables were shown by mean ± SD and number (%), and to compare the difference of each variable in two groups, a *t*-test and chi-square test were used,

respectively. To calculate the risk of death in the exposed and the unexposed cohorts, Cox proportional hazard models were used and presented using hazard ratios, adjusted hazard ratios (aHR) and 95% confidence intervals (CIs). The survival rate of death in the two cohorts was presented by the Kaplan-Meier method. The log-rank test was used to compare the difference between two survival curves. All statistical analyses were performed with SAS statistical software version 9.4 (SAS Institute Inc., Cary, NC). The Figure of the cumulative incidence curve was plotted by R software. The significance criteria were set up as a two-sided test with a *P* value of less than 0.05.

RESULTS

Of 200 eligible subjects in this study (Table 1), 40 were diagnosed with peripartum PLC, and the other 160 were selected as the unexposed cohort. Among patients with peripartum PLC, the dominant age group was younger than 30 years old (47.5%), 11 (27.5%) with HBV, one (2.5%) with unspecified chronic hepatitis, one with alcoholic liver disease, three (7.5%) with cirrhosis, two (5%) with biliary stone, four (10%) with cholecystitis, and three (7.5%) with cholangitis. The mean age of the exposed and unexposed cohort was 30.9 and 31.3 years, respectively. The characteristics and comorbidities showed a non-significant difference between the two cohorts after propensity matching (*P* > 0.05).

Table 2 presents the risk factors of death associated with and without peripartum PLC. Patients with peripartum PLC had a non- significant risk of death compared with the unexposed cohort (aHR = 1.40, 95%CI: 0.89-2.20, *P* = 0.149). Considering their older age and comorbidities, patients with HBV (aHR = 0.48, 95%CI: 0.30-0.77, *P* = 0.002) and cholecystitis (aHR = 0.30, 95%CI: 0.12-0.75) showed a decreased risk of death; patients with cholangitis showed a significantly higher risk of death (aHR = 3.34, 95%CI: 1.49-7.47, *P* = 0.003). Figure 1 illustrates the non-significant difference in the survival curves between the two cohorts (*P* = 0.1649).

The survival rate at different follow-up durations between patients with peripartum PLC and the unexposed cohort (Table 3) revealed a non-significant difference. When followed for less than 0.5 years, 1 year, 3 years, or 5 years, the survival rate in patients with peripartum PLC was lower than that in the unexposed cohort (71.79% *vs* 78.94%; 60.84 *vs* 63.61%; 30.42 *vs* 44.85%; 27.38 *vs* 39.59%), but without a significant difference between the two cohorts (*P* > 0.05).

We enrolled 13440 study subjects to learn more about the influence of age and menopause on survival outcomes. Of these women, 2688 were diagnosed with PLC, younger than 50 years, and without menopause (Table 4). The other group comprised 10752 women who were PLC patients, aged 50 years and older, and with menopause (postmenopausal). The mean ages were 39.7 and 69.1 years, respectively. The percentage of comorbidities had no significant difference between the two cohorts after propensity score matching by age and comorbidities (*P* > 0.05), except alcoholic liver disease (*P* = 0.041).

Table 5 shows the risk factors for developing death. Patients who were diagnosed with PLC at less than 50 years old had a substantially lower risk of death compared with patients diagnosed with PLC at 50 years or older (aHR = 0.64, 95%CI: 0.61-0.68, *P* < 0.001). Patients with HBV (aHR = 0.76, 95%CI: 0.72–0.80, *P* < 0.001), HCV (aHR = 0.72, 95%CI: 0.67-0.78, *P* < 0.001) and cholecystitis (aHR = 0.71, 95%CI: 0.64-0.78, *P* < 0.001) showed a significantly lower risk of developing death. patients with comorbidities such as cirrhosis (aHR = 1.18, 95%CI: 1.13-1.24, *P* < 0.001), and cholangitis (aHR = 1.77, 95%CI: 1.63-1.92, *P* < 0.001) had a notably higher risk of death. Figure 2 shows that the survival rate was significantly higher in women younger than 50 years old with PLC than in the older cohort (*P* < 0.001).

Table 6 presents the survival rates at different follow-up durations. The survival rate in women < 50 years with PLC was significantly higher than in older women with PLC when followed for 0.5 year (72.44% *vs* 64.16%), 1 year (60.57% *vs* 51.66%), 3 years (42.92% *vs* 31.28%), and 5 years (37.02% *vs* 21.83%), respectively (*P* < 0.001).

DISCUSSION

To our knowledge, this large-scale, population-based, cohort study is one of the pioneering research investigations that focused on women under different conditions to determine the relationship between peripartum and postmenopause and the risk of death from liver cancer. Based on our results, despite no significant difference, overall

Table 1 Demographic characteristics and comorbidities of patients with newly diagnosed peripartum primary liver cancer in Taiwan during 1996-2012

Characteristics	Total, N	Peripartum primary liver cancer		P value
		No, *n* = 160	Yes, *n* = 40	
		n (%) / mean ± SD	*n* (%) / mean ± SD	
Age				0.788
< 30	88	69 (43.1)	19 (47.5)	
30-34	64	53 (33.1)	11 (27.5)	
35-49	48	38 (23.8)	10 (25)	
mean ± SD[1]		31.3 ± 5.1	30.9 ± 4.8	0.673
Baseline comorbidity				
HBV	58	47 (29.4)	11 (27.5)	0.815
Unspecified chronic hepatitis	2	1 (0.6)	1 (2.5)	0.286
Alcoholic liver disease	6	5 (3.1)	1 (2.5)	0.836
Cirrhosis	9	6 (3.8)	3 (7.5)	0.306
Biliary stone	6	4 (2.5)	2 (5)	0.407
Cholecystitis	12	8 (5)	4 (10)	0.234
Cholangitis	10	7 (4.4)	3 (7.5)	0.417

[1]*t*-test, Chi-square test.

HBV: Hepatitis B virus; SD: Standard deviation.

low survival was found in PLCs diagnosed either within or outside of the peripartum period among women of reproductive age (15-49 years old). Our data revealed that five-year survival rates in non-peripartum and peripartum PLCs were 39.59% and 27.38% (aHR = 1.40, 95%CI: 0.89-2.20, *P* = 0.149), respectively. However, postmenopausal women (> 50 years old) with PLCs have a considerable decrease in survival rates (five-year survival rates in fertile and postmenopausal women were 37.02% and 21.83%, respectively), compared with a significantly higher risk of death in premenopausal female patients (aHR = 0.64; 95%CI: 0.61-0.68). Although the molecular mechanisms underlying this protective effect are complicated, previous research suggested that the inhibitory role of estrogen was responsible for the gender disparity of PLCs partly *via* micro RNA, DNA repair, and obesity-associated pathways[7]. Moreover, the number of estrogen receptors (ERs) correlated with the risk of tumor occurrence and invasion. Some research proposed that ERs suppressed the proliferation and progression of liver cancer by decreasing the peroxisome proliferator-activated receptor γ and transcription of metastatic tumor antigen 1[24,25]. In the time of limited estrogen supply (*e.g.*, Postmenopause), sex hormone binding globulin (SHBG), a plasma protein that involved in the maintenance of a reservoir of sex steroid hormones, played a crucial role in potentiating estrogenic action[26].

We focused on women of childbearing age to gain a deeper understanding of the influence of reproductive hormones. Because of the elevation of estrogen and progesterone during pregnancy, the diagnosis of PLCs within this period is rare. Nevertheless, among the 62 cases reported to date worldwide, all ended with poor outcomes when compared with non-pregnant women with PLCs[10]. As early as in 1995, Lau and his colleague[27] concluded that pregnancy has an adverse effect on the prognosis of patients with HCC, and therefore measurement of AFP level is recommended for screening HCC in pregnant women at high risk. The largest retrospective review published by Choi *et al*[12] demonstrated poor yet improving survival rates over time (median survivals of the groups before and during/after 1995 were 18 and 25.5 mo, respectively) among all 48 HCC cases in pregnancy. Contrary to prior research, our analysis of the nationwide database revealed an overall unpleasant prognosis among women of childbearing age. There was no significant difference in survival rates between parous and non-parous women with PLCs. This could probably be explained by the limited number of cases and the nationwide coverage of health insurance. Since almost all women received check-ups during the prenatal and

Table 2 Cox model measured hazard ratios and 95% confidence intervals of death associated non-peripartum primary liver cancer and peripartum primary liver cancer patients

Characteristics	Event, *n* = 124	Person, yr	IR	Crude		Adjusted	
				HR (95%CI)	*P* value	HR (95%CI)	*P* value
Peripartum primary liver cancer							
No	97	587	16.53	Ref.		Ref.	
Yes	27	99	27.17	1.35 (0.88-2.08)	0.166	1.40 (0.89-2.20)	0.149
Age at baseline							
< 30	54	350	15.44	Ref.		Ref.	
30-34	39	169	23.08	1.21 (0.80-1.84)	0.359	1.47 (0.95-2.28)	0.083
35-49	31	167	18.52	1.29 (0.83-2.00)	0.266	1.13 (0.69-1.85)	0.617
Baseline comorbidity							
HBV	27	230	11.72	0.52 (0.34-0.80)	0.003	0.48 (0.30-0.77)	0.002
Unspecified chronic hepatitis	1	11	9.04	0.86 (0.12-6.17)	0.882	0.56 (0.08-4.10)	0.565
Alcoholic liver disease	6	8	73.37	2.85 (1.25-6.49)	0.013	2.15 (0.73-6.36)	0.165
Cirrhosis	7	40	17.55	1.12 (0.52-2.40)	0.773	1.49 (0.57-3.90)	0.411
Biliary stone	4	11	35.47	1.23 (0.45-3.33)	0.686	0.64 (0.17-2.35)	0.499
Cholecystitis	5	80	6.26	0.43 (0.18-1.06)	0.066	0.30 (0.12-0.75)	0.010
Cholangitis	7	4	179.30	3.76 (1.71-8.26)	< 0.001	3.34 (1.49-7.47)	0.003

Adjusted HR: Adjusted for gender, age, and all comorbidities in Cox proportional hazards regression; CI: Confidence interval; HR: Hazard ratios; HBV: Hepatitis B virus; IR: Incidence rate.

Table 3 Survival rates of different follow-up durations between non-peripartum primary liver cancer and peripartum primary liver cancer patients

Follow-up duration	Survival rate (%)		*P* value
	Non-peripartum primary liver cancer	Peripartum primary liver cancer	
≤ 0.5	78.94	71.79	0.254
≤ 1	63.61	60.84	0.611
≤ 3	44.85	30.42	0.111
≤ 5	39.59	27.38	0.117

postnatal period under the national health insurance program, proper management could be provided in time to improve outcomes.

Because menopause represents a state of gradual estrogen deficiency in the setting of physiologic aging, we also divided the study population into two groups by age, either younger or older than 50 years. According to Yang's research[28] investigating patients with HCC, women of 18 years old to 64 years old were noted as having longer survival than men of the same age, with the largest difference in survival among women aged 18 years to 44 years. Furthermore, Shimizu *et al*[29] reported that hepatic ER levels, which were inversely related to the progression of HCC, were significantly higher in premenopausal women compared with postmenopausal women. While El Mahdy Korah *et al*[30] stated that there was no clear relationship between sex hormone and HCC development or progression by analyzing total testosterone, estrogen, progesterone and prolactin levels among 40 selected HCC patients, Petrick's cohort study in 2019[31] indicated that higher levels of SHBG and circulating estradiol were associated with an increased risk of HCC and ICC, respectively, among women after menopause. These data suggest that climacteric status may adversely mediate the outcomes of PLCs. Our results are consistent with those of previous studies, that

Table 4 Demographic characteristics and comorbidities of female patients newly diagnosed with and without menopause primary liver cancer patients in Taiwan during 1996-2012

Characteristics	Total, N = 13440	Liver cancer		P value
		≥ 50 yr, n = 10752	< 50 yr, n = 2688	
		n (%)/mean ± SD	n (%)/mean ± SD	
Age				
mean ± SD[1]		69.1 ± 9.6	39.7 ± 10.5	< 0.001
Baseline comorbidity				
HBV	2971	2358 (21.9)	613 (22.8)	0.329
HCV	1168	931 (8.7)	237 (8.8)	0.795
Unspecified chronic hepatitis	780	619 (5.8)	161 (6)	0.645
Alcoholic liver disease	211	157 (1.5)	54 (2)	0.041
Cirrhosis	4142	3321 (30.9)	821 (30.5)	0.730
Biliary stone	1269	1012 (9.4)	257 (9.6)	0.813
Cholecystitis	626	489 (4.5)	137 (5.1)	0.227
Cholangitis	818	649 (6)	169 (6.3)	0.626

[1]*t*-test, Chi-square test.

HBV: Hepatitis B virus; HCV: Hepatitis C virus; SD: Standard deviation.

Table 5 Cox model measured hazard ratios and 95% confidence intervals of death associated with and without menopause primary liver cancer patients

Characteristics	Event, N = 9982	Person, yr	IR	Crude		Adjusted	
				HR (95%CI)	P value	HR (95%CI)	P value
Liver cancer							
≥ 50 yr	8279	23410	35.37	Ref.		Ref.	
< 50 yr	1703	9149	18.61	0.65 (0.61-0.68)	< 0.001	0.64 (0.61-0.68)	< 0.001
Baseline comorbidity							
HBV	2049	7552	27.13	0.81 (0.77-0.85)	< 0.001	0.76 (0.72-0.80)	< 0.001
HCV	831	3513	23.65	0.75 (0.70-0.81)	< 0.001	0.72 (0.67-0.78)	< 0.001
Unspecified chronic hepatitis	584	2224	26.25	0.90 (0.83-0.98)	0.015	0.96 (0.88-1.05)	0.349
Alcoholic liver disease	165	449	36.72	1.04 (0.89-1.21)	0.640	1.07 (0.91-1.25)	0.408
Cirrhosis	3186	9924	32.10	1.01 (0.97-1.05)	0.739	1.18 (1.13-1.24)	< 0.001
Biliary stone	955	2730	34.98	1.08 (1.01-1.15)	0.024	0.98 (0.91-1.05)	0.562
Cholecystitis	416	2162	19.25	0.70 (0.63-0.77)	< 0.001	0.71 (0.64-0.78)	< 0.001
Cholangitis	687	989	69.45	1.76 (1.63-1.91)	< 0.001	1.77 (1.63-1.92)	< 0.001

Adjusted HR: Adjusted for comorbidities in Cox proportional hazards regression; CI: Confidence interval; HR: Hazard ratios; HBV: Hepatitis B virus; HCV: Hepatitis C virus; IR: Incidence rate.

implied a negative interplay between age and hormonal factors in the disease course since women beyond reproductive age (> 50 years old) with PLCs were found to have lower half-year, one-year, three-year, and five-year survival rates. Although it is difficult to distinguish how the two factors account for the consequence individually, it is certain that they interact with each other. This interaction results in diminishing immunologic responses to injury, and the imbalance between antioxidant formation and oxidative stress.

Table 6 Survival rates of different follow-up durations between primary liver cancer patients with and without menopause

Follow-up duration	Survival rate (%)		*P* value
	≥ 50 yr	< 50 yr	
≤ 0.5	64.16	72.44	< 0.001
≤ 1	51.66	60.57	< 0.001
≤ 3	31.28	42.92	< 0.001
≤ 5	21.83	37.02	< 0.001
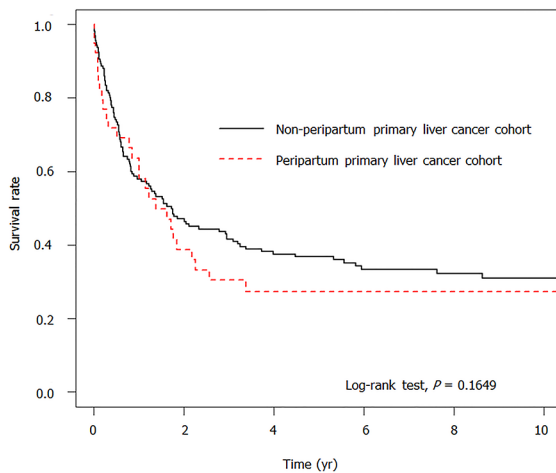
Figure 1 The estimated survival rates between non-peripartum primary liver cancer and peripartum primary liver cancer patients by Kaplan-Meier analysis.
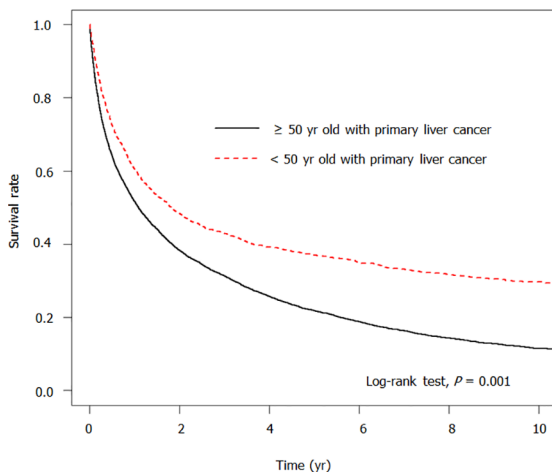
Figure 2 The estimated survival rates between patients younger than 50 years old with primary liver cancer (without menopause) and those older with primary liver cancer (with menopause) by Kaplan-Meier analysis.

The use of a broad, representative, nationwide, population-based sample to observe the survival outcome of PLC in reproductive and postmenopausal female patients increased the validity of the results. Nevertheless, these results should be interpreted with caution because of several limitations in this study. First, detailed information related to the risk of PLC is not available. This information includes data on body mass index, smoking and alcohol use, high-fat diet, lower physical activity lifestyle, history of receiving HRT, and family history of PLC. Second, tumor burden, staging, and management strategies of PLC are not accessible from the NHIRD and therefore cannot be analyzed. Third, defining menopause by age alone may not be comprehensive enough since it is hard to make an optimal covariate adjustment. Fourth, the

generalization of the findings to Western or non-Taiwanese populations is a concern. For instance, the high incidence of PLC warrants further follow-up in other populations. Fifth, the small number of cases during the peripartum period may lead to biased findings. Hence, future studies with an improved design, larger sample sizes, and better control of confounding factors are required to enable a more thorough understanding.

CONCLUSION

In summary, among female patients with PLC, we found a trend for older age to be associated with increased risk for both incidence and mortality of PLC. In contrast, no apparent relationship was noted between pregnancy and prognosis. Even though subsequent clinical studies are necessary for further validation, the present research demonstrates that age and hormonal factors have a protective influence on the occurrence and deterioration of PLCs. Moreover, patients with more risk factors are recommended to follow up regularly to achieve a better prognosis.

ARTICLE HIGHLIGHTS

Research background

Primary liver cancer (PLC), the sixth most common cancer, accounts for the fourth leading cause of cancer-related death worldwide. Given the continuous rise of the global burden, there are increasing concerns about PLC outcomes in different populations.

Research motivation

For a long time, most studies about PLC put their focus on men due to higher incidence and riskier morbidities compared to women. Even with growing evidence on the protective effects of female sex hormones in animal research, few clinical cohorts pay attention to women with PLCs. Therefore, we are interested in the issue of how female reproductive status is related to the prognosis of PLCs.

Research objectives

This study aimed to assess whether peripartum and postmenopausal women with PLC have lower overall survival rates in a large cohort of subjects in Taiwan.

Research methods

This is a retrospective cohort of the PLC prognosis among peripartum, non-peripartum, premenopausal, and postmenopausal women using the Taiwan National Health Insurance Research Database from 2000-2012. There were 200 eligible subjects enrolled in the study of peripartum PLC, whereas 13440 subjects enrolled in the research of menopausal PLC. 4:1 Propensity score matching was applied to adjust the covariates.

Research results

While the survival rate was overall lower in patients with peripartum PLC, there was no significant difference in the risk of death and the survival rate at different follow-up durations among patients with/without peripartum PLC. In the menopausal PLC cohort, significantly lower risk of death (aHR = 0.64, 95%CI: 0.61-0.68, $P < 0.001$) and higher survival rate when followed for 0.5 year (72.44% *vs* 64.16%), 1 year (60.57% *vs* 51.66%), 3 years (42.92% *vs* 31.28%), and 5 years were seen in patients diagnosed with PLC younger than 50 years old (without menopause) compared with patients diagnosed with PLC at or older than 50 years (with menopause).

Research conclusions

According to our dataset, it is concluded that younger age and female hormonal factors may reduce the occurrence and deterioration of PLCs. Females with paripartum PLC have no difference in survival rates compared with those patients without peripartum. Menopausal females with PLC have worse survival rates compared with those patients without menopause.

Research perspectives

To further clarify the association between sexual hormone and PLC outcome, future studies with more detailed information and better-controlled confounders are required.

REFERENCES

1   **Bray F**, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; **68**: 394-424 [PMID: 30207593 DOI: 10.3322/caac.21492]

2   **Lin L**, Yan L, Liu Y, Qu C, Ni J, Li H. The Burden and Trends of Primary Liver Cancer Caused by Specific Etiologies from 1990 to 2017 at the Global, Regional, National, Age, and Sex Level Results from the Global Burden of Disease Study 2017. *Liver Cancer* 2020; **9**: 563-582 [PMID: 33083281 DOI: 10.1159/000508568]

3   **Massarweh NN**, El-Serag HB. Epidemiology of Hepatocellular Carcinoma and Intrahepatic Cholangiocarcinoma. *Cancer Control* 2017; **24**: 1073274817729245 [PMID: 28975830 DOI: 10.1177/1073274817729245]

4   **Rawla P**, Sunkara T, Muralidharan P, Raj JP. Update in global trends and aetiology of hepatocellular carcinoma. *Contemp Oncol (Pozn)* 2018; **22**: 141-150 [PMID: 30455585 DOI: 10.5114/wo.2018.78941]

5   **Petrick JL**, Freedman ND, Demuth J, Yang B, Van Den Eeden SK, Engel LS, McGlynn KA. Obesity, diabetes, serum glucose, and risk of primary liver cancer by birth cohort, race/ethnicity, and sex: Multiphasic health checkup study. *Cancer Epidemiol* 2016; **42**: 140-146 [PMID: 27148890 DOI: 10.1016/j.canep.2016.04.009]

6   **Global Burden of Disease Liver Cancer Collaboration**, Akinyemiju T, Abera S, Ahmed M, Alam N, Alemayohu MA, Allen C, Al-Raddadi R, Alvis-Guzman N, Amoako Y, Artaman A, Ayele TA, Barac A, Bensenor I, Berhane A, Bhutta Z, Castillo-Rivas J, Chitheer A, Choi JY, Cowie B, Dandona L, Dandona R, Dey S, Dicker D, Phuc H, Ekwueme DU, Zaki MS, Fischer F, Fürst T, Hancock J, Hay SI, Hotez P, Jee SH, Kasaeian A, Khader Y, Khang YH, Kumar A, Kutz M, Larson H, Lopez A, Lunevicius R, Malekzadeh R, McAlinden C, Meier T, Mendoza W, Mokdad A, Moradi-Lakeh M, Nagel G, Nguyen Q, Nguyen G, Ogbo F, Patton G, Pereira DM, Pourmalek F, Qorbani M, Radfar A, Roshandel G, Salomon JA, Sanabria J, Sartorius B, Satpathy M, Sawhney M, Sepanlou S, Shackelford K, Shore H, Sun J, Mengistu DT, Topór-Mądry R, Tran B, Ukwaja KN, Vlassov V, Vollset SE, Vos T, Wakayo T, Weiderpass E, Werdecker A, Yonemoto N, Younis M, Yu C, Zaidi Z, Zhu L, Murray CJL, Naghavi M, Fitzmaurice C. The Burden of Primary Liver Cancer and Underlying Etiologies From 1990 to 2015 at the Global, Regional, and National Level: Results From the Global Burden of Disease Study 2015. *JAMA Oncol* 2017; **3**: 1683-1691 [PMID: 28983565 DOI: 10.1001/jamaoncol.2017.3055]

7   **Li Y**, Xu A, Jia S, Huang J. Recent advances in the molecular mechanism of sex disparity in hepatocellular carcinoma. *Oncol Lett* 2019; **17**: 4222-4228 [PMID: 30988804 DOI: 10.3892/ol.2019.10127]

8   **Sukocheva OA**. Estrogen, estrogen receptors, and hepatocellular carcinoma: Are we there yet? *World J Gastroenterol* 2018; **24**: 1-4 [PMID: 29358876 DOI: 10.3748/wjg.v24.i1.1]

9   **Ma WL**, Lai HC, Yeh S, Cai X, Chang C. Androgen receptor roles in hepatocellular carcinoma, fatty liver, cirrhosis and hepatitis. *Endocr Relat Cancer* 2014; **21**: R165-R182 [PMID: 24424503 DOI: 10.1530/ERC-13-0283]

10   **Matsuo M**, Furukawa K, Shimizu H, Yoshitomi H, Takayashiki T, Kuboki S, Takano S, Suzuki D, Sakai N, Kagawa S, Nojima H, Ohsuka M. Novel treatment strategy with radiofrequency ablation and surgery for pregnant patients with hepatocellular carcinoma: a case report. *Surg Case Rep* 2018; **4**: 43 [PMID: 29721779 DOI: 10.1186/s40792-018-0434-3]

11   **Li AJ**, Zhou WP, Lu JH, Cui LJ, Yang XY, Yin L, Wu MC. Surgery for pregnancy-associated primary hepatocellular carcinoma: Report of four cases. *Int J Surg Case Rep* 2014; **5**: 882-885 [PMID: 25462058 DOI: 10.1016/j.ijscr.2014.06.003]

12   **Choi KK**, Hong YJ, Choi SB, Park YN, Choi JS, Lee WJ, Kim KS. Hepatocellular carcinoma during pregnancy: is hepatocellular carcinoma more aggressive in pregnant patients? *J Hepatobiliary Pancreat Sci* 2011; **18**: 422-431 [PMID: 21116657 DOI: 10.1007/s00534-010-0345-6]

13   **Giannitrapani L**, Soresi M, La Spada E, Cervello M, D'Alessandro N, Montalto G. Sex hormones and risk of liver tumor. *Ann N Y Acad Sci* 2006; **1089**: 228-236 [PMID: 17261770 DOI: 10.1196/annals.1386.044]

14   **Hsing AW**, McLaughlin JK, Hoover RN, Co Chien HT, Blot WJ, Fraumeni JF Jr. Parity and primary liver cancer among young women. *J Natl Cancer Inst* 1992; **84**: 1118-1119 [PMID: 1619686 DOI: 10.1093/jnci/84.14.1118]

15   **Garko SB**, David OS, Mohammed T, Isah MS, Bakari AG, Oguntayo AO, Shehu MS, Aminu SM. Hepatocellular carcinoma in pregnancy. *Ann Afr Med* 2009; **8**: 284-286 [PMID: 20139556 DOI: 10.4103/1596-3519.59588]

16   **Russell P**, Sanjay P, Dirkzwager I, Chau K, Johnston P. Hepatocellular carcinoma during pregnancy: case report and review of the literature. *N Z Med J* 2012; **125**: 141-145 [PMID: 22522274]

17   **Shimizu I**. Impact of oestrogens on the progression of liver disease. *Liver Int* 2003; **23**: 63-69 [PMID: 12640729 DOI: 10.1034/j.1600-0676.2003.00811.x]

18   **Brady CW**. Liver disease in menopause. *World J Gastroenterol* 2015; **21**: 7613-7620 [PMID: 26167064 DOI: 10.3748/wjg.v21.i25.7613]

19   **Zhong GC**, Liu Y, Chen N, Hao FB, Wang K, Cheng JH, Gong JP, Ding X. Reproductive factors, menopausal hormone therapies and primary liver cancer risk: a systematic review and dose-response meta-analysis of observational studies. *Hum Reprod Update* 2016; **23**: 126-138 [PMID: 27655589 DOI: 10.1093/humupd/dmw037]

20   **Hassan MM**, Botrus G, Abdel-Wahab R, Wolff RA, Li D, Tweardy D, Phan AT, Hawk E, Javle M, Lee JS, Torres HA, Rashid A, Lenzi R, Hassabo HM, Abaza Y, Shalaby AS, Lacin S, Morris J, Patt YZ, Amos CI, Khaderi SA, Goss JA, Jalal PK, Kaseb AO. Estrogen Replacement Reduces Risk and Increases Survival Times of Women With Hepatocellular Carcinoma. *Clin Gastroenterol Hepatol* 2017; **15**: 1791-1799 [PMID: 28579181 DOI: 10.1016/j.cgh.2017.05.036]

21   Research on the menopause in the 1990s: report of a WHO scientific group. [cited 27 December 2020]. Available from: https://apps.who.int/iris/handle/10665/41841

22   **Feasey R**. Infertility and Non-Traditional Family Building: From Assisted Reproduction to Adoption in the Media, 1st ed.; Palgrave Macmillan: London, U.K., 2019 [DOI: 10.1007/978-3-030-17787-4]

23   **Shen TY**, Strong C, Yu T. Age at menopause and mortality in Taiwan: A cohort analysis. *Maturitas* 2020; **136**: 42-48 [PMID: 32386665 DOI: 10.1016/j.maturitas.2020.04.008]

24   **Lin YM**, Velmurugan BK, Yeh YL, Tu CC, Ho TJ, Lai TY, Tsai CH, Tsai FJ, Huang CY. Activation of estrogen receptors with E2 downregulates peroxisome proliferator-activated receptor γ in hepatocellular carcinoma. *Oncol Rep* 2013; **30**: 3027-3031 [PMID: 24126791 DOI: 10.3892/or.2013.2793]

25   **Deng L**, Yang H, Tang J, Lin Z, Yin A, Gao Y, Wang X, Jiang R, Sun B. Inhibition of MTA1 by ERα contributes to protection hepatocellular carcinoma from tumor proliferation and metastasis. *J Exp Clin Cancer Res* 2015; **34**: 128 [PMID: 26503703 DOI: 10.1186/s13046-015-0248-0]

26   **Lee SR**, Lee YH, Yang H, Lee HW, Lee GS, An BS, Jeung EB, Park BK, Hong EJ. Sex hormone-binding globulin suppresses NAFLD-triggered hepatocarcinogenesis after menopause. *Carcinogenesis* 2019; **40**: 1031-1041 [PMID: 31168625 DOI: 10.1093/carcin/bgz107]

27   **Lau WY**, Leung WT, Ho S, Lam SK, Li CY, Johnson PJ, Williams R, Li AK. Hepatocellular carcinoma during pregnancy and its comparison with other pregnancy-associated malignancies. *Cancer* 1995; **75**: 2669-2676 [PMID: 7743468 DOI: 10.1002/1097-0142(19950601)75:11<2669::aid-cncr2820751105>3.0.co;2-b]

28   **Yang D**, Hanna DL, Usher J, LoCoco J, Chaudhari P, Lenz HJ, Setiawan VW, El-Khoueiry A. Impact of sex on the survival of patients with hepatocellular carcinoma: a Surveillance, Epidemiology, and End Results analysis. *Cancer* 2014; **120**: 3707-3716 [PMID: 25081299 DOI: 10.1002/cncr.28912]

29   **Shimizu I**, Inoue H, Yano M, Shinomiya H, Wada S, Tsuji Y, Tsutsui A, Okamura S, Shibata H, Ito S. Estrogen receptor levels and lipid peroxidation in hepatocellular carcinoma with hepatitis C virus infection. *Liver* 2001; **21**: 342-349 [PMID: 11589771 DOI: 10.1034/j.1600-0676.2001.210507.x]

30   **El Mahdy Korah T**, Abd Elfatah Badr E, Mohamed Emara M, Ahmed Samy Kohla M, Gamal Saad Michael G. Relation between sex hormones and hepatocellular carcinoma. *Andrologia* 2016; **48**: 948-955 [PMID: 26791111 DOI: 10.1111/and.12536]

31   **Petrick JL**, Florio AA, Zhang X, Zeleniuch-Jacquotte A, Wactawski-Wende J, Van Den Eeden SK, Stanczyk FZ, Simon TG, Sinha R, Sesso HD, Schairer C, Rosenberg L, Rohan TE, Purdue MP, Palmer JR, Linet MS, Liao LM, Lee IM, Koshiol J, Kitahara CM, Kirsh VA, Hofmann JN, Guillemette C, Graubard BI, Giovannucci E, Gaziano JM, Gapster SM, Freedman ND, Engel LS, Chong DQ, Chen Y, Chan AT, Caron P, Buring JE, Bradwin G, Beane Freeman LE, Campbell PT, McGlynn KA. Associations Between Prediagnostic Concentrations of Circulating Sex Steroid Hormones and Liver Cancer Among Postmenopausal Women. *Hepatology* 2020; **72**: 535-547 [PMID: 31808181 DOI: 10.1002/hep.31057]

ORIGINAL ARTICLE

Retrospective Cohort Study

Nonalcoholic fatty liver disease is associated with worse intestinal complications in patients hospitalized for *Clostridioides difficile* infection

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Author contributions: Jiang Y and Pyrsopoulos N planned and designed the study; Chowdhury S, Xu BH, Meybodi MA, Damiris K and Devalaraju S conducted the data collection and interpretation; Jiang Y, Chowdhury S, Xu BH, Damiris K and Devalaraju S contributed to the manuscript preparation; All authors contributed to the manuscript revisions, reviewed, and approved the final submitted manuscript.

Institutional review board statement: This retrospective cohort study did not directly involve any patients in the data collection process and the National Inpatient Sample (NIS) database is de-identified and available for the public. Therefore, Institutional Review Board approval was not required.

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Abstract

BACKGROUND

Nonalcoholic fatty liver disease (NAFLD) has become the leading cause of chronic liver disease with increasing prevalence worldwide. *Clostridioides difficile* infection (CDI) remains the most common cause of nosocomial diarrhea in developed countries.

AIM

To assess the impact of NAFLD on the outcomes of hospitalized patients with CDI.

METHODS

This study was a retrospective cohort study. The Nationwide Inpatient Sample database was used to identify a total of 7239 adults admitted as inpatients with a primary diagnosis of CDI and coexisting NAFLD diagnosis from 2010 to 2014 using ICD-9 codes. Patients with CDI and coexisting NAFLD were compared to those with CDI and coexisting alcoholic liver disease (ALD) and viral liver disease (VLD), individually. Primary outcomes included mortality, length of stay, and total hospitalization charges. Secondary outcomes were in-hospital complications. Multivariate regression was used for outcome analysis after adjusting for possible

confounders.

RESULTS
CDI with NAFLD was independently associated with lower rates of acute respiratory failure (2.7% *vs* 4.2%, $P < 0.01$; 2.7% *vs* 4.2%, $P < 0.05$), shorter length of stay (days) (5.75 ± 0.16 *vs* 6.77 ± 0.15, $P < 0.001$; 5.75 ± 0.16 *vs* 6.84 ± 0.23, $P < 0.001$), and lower hospitalization charges (dollars) (38150.34 ± 1757.01 *vs* 46326.72 ± 1809.82, $P < 0.001$; 38150.34 ± 1757.01 *vs* 44641.74 ± 1660.66, $P < 0.001$) when compared to CDI with VLD and CDI with ALD, respectively. CDI with NAFLD was associated with a lower rate of acute kidney injury (13.0% *vs* 17.2%, $P < 0.01$), but a higher rate of intestinal perforation ($P < 0.01$) when compared to VLD. A lower rate of mortality (0.8% *vs* 2.7%, $P < 0.05$) but a higher rate of intestinal obstruction (4.6% *vs* 2.2%, $P = 0.001$) was also observed when comparing CDI with NAFLD to ALD.

CONCLUSION
Hospitalized CDI patients with NAFLD had more intestinal complications compared to CDI patients with VLD and ALD. Gut microbiota dysbiosis may contribute to the pathogenesis of intestinal complications.

Key Words: Nonalcoholic fatty liver disease; *Clostridioides difficile* infection; Gut microbiota; Intestinal complications; Alcoholic liver disease; Viral liver disease

Core Tip: This study demonstrated that patients hospitalized with *Clostridioides difficile* infection (CDI) and coexisting nonalcoholic fatty liver disease (NAFLD) had more favorable overall outcomes but higher rates of intestinal complications when compared to those with alcoholic liver disease and viral liver disease individually, which suggests altering gut microbiota may play an essential role in the pathogenesis of both CDI and NAFLD. NAFLD-associated metabolic syndrome may contribute significantly to gut dysbiosis and increase risk for CDI and its complications. This study provides potential directions for future prospective clinical research to identify the clinical meaningfulness of interactions between the gut microbiota, gut immunity and systemic inflammation.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a heterogeneous disease with a spectrum from simple steatosis to nonalcoholic steatohepatitis (NASH), cirrhosis, and hepato-cellular carcinoma[1,2]. With a prevalence of 10 to 46 percent in the United States and 6% to 35% worldwide[3,4], NAFLD has become the leading cause of chronic liver disease, and its prevalence continues to increase, paralleled by the increase of obesity and type 2 diabetes[5].

Clostridioides difficile (*C. difficile*) is a gram-positive, spore-forming bacterium, known as the most common pathogen causing nosocomial diarrhea in developed countries [6]. Symptoms of *C. difficile* infection (CDI) range from mild to severe diarrhea, which can progress to sepsis, fulminant colitis, and bowel perforation[7]. Severe colitis may also present as ileus and megacolon, which are characterized by symptoms of intestinal obstruction[8,9]. Gut microbiota dysbiosis due to the administration of antibiotics is the most prominent risk factor for the development of CDI. Advanced age, prolonged hospitalization and gastric acid suppression are some common additional risk factors for CDI[10,11].

Recently, a number of animal and human studies have revealed the role of the gut microbiota in the pathophysiology of NAFLD. It is proposed that dysbiosis-induced dysregulation of the gut barrier function and translocation of the bacteria link the gut microbiome to NAFLD[12,13]. In addition, it has been well documented that patients with chronic liver disease are more susceptible to CDI due to frequent hospitalization and antibiotics use. Specifically, recent studies have observed that NAFLD is an independent risk factor for CDI by single-centered retrospective design[14,15].

Although a strong association between NAFLD and CDI has been observed, gut microbiota dysbiosis likely plays a vital role in the pathogenesis of both aforementioned diseases. However, the inpatient outcomes of CDI in the NAFLD population, have not been well studied in large populations. The aim of this nationwide study was to assess the impact of NAFLD on the outcomes of hospitalized patients with CDI.

MATERIALS AND METHODS

Data source and study population

The largest all-payer inpatient care database in the United States, the Nationwide Inpatient Sample (NIS) database was accessed. The NIS database represents approximately 20% of all inpatient hospitalizations. Weighted, it estimates more than 35 million hospitalizations nationally[16]. It includes demographic information (age, sex, race, income), hospital characteristics (*e.g.*, bed size, type), insurance status, discharge status, diagnoses and procedures (identified by The International Classification of Diseases-Ninth Edition Revision Clinical Modification (ICD-9 CM) codes), total hospitalization charges, length of stay (LOS), severity and other comorbidity measures. Yearly sampling weights are applied to generate national estimates.

This retrospective cohort study examined all adult (18-90 years old) patients hospitalized with CDI as the primary diagnosis from 2010 to 2014. Within this CDI population, patients with NAFLD were selected to compare to those with viral liver disease (VLD) (including hepatitis B infection and hepatitis C infection) and those with alcoholic liver disease (ALD). Notably, CDI was identified by ICD-9 CM code 008.45. NAFLD was identified by ICD-9 CM code 571.80 with the exclusion of all diagnostic codes for previous organ recipients and donors as well as other causes of chronic liver disease including hepatitis B and hepatitis C infection, ALD, hemochromatosis, primary biliary cholangitis, autoimmune hepatitis, and other unspecified liver diseases. The diagnosis of VLD was identified by the ICD-9 CM codes for hepatitis B and C caused liver diseases with the exclusion of previous organ recipients and donors, as well as other causes of chronic liver disease including NAFLD, ALD, hemochromatosis, primary biliary cholangitis, autoimmune hepatitis, and other unspecified liver diseases. Similarly, ALD was identified by the ICD-9 CM codes for ALD with the exclusion of previous organ recipients and donors as well as other causes of chronic liver disease including NAFLD, VLD, hemochromatosis, primary biliary cholangitis, autoimmune hepatitis, and other unspecified liver diseases (see Supplementary Table 1, supplemental digital content 1, which demonstrates ICD-9 diagnostic and procedure codes). VLD and ALD were assessed as separate groups which excluded patients with concomitant diagnoses of VLD and ALD. Information such as patients' demographics, comorbidities, disposition, selected outcomes and surgical interventions were extracted from the NIS database. Elixhauser Comorbidity Index (ECI)[17], which measures 29 general medical conditions, then assigns different weights to compile a longitudinal score, allowing for further description of comorbidity burden.

Primary outcomes included mortality, length of stay, and total hospitalization charges. Secondary outcomes were CDI related complications and interventions.

Statistical analysis

SAS Survey Procedures (SAS 9.4, SAS Institute Inc, Cary, NC, United States) was utilized for all statistical analyses. The national estimates were calculated after accounting for sample design elements (clusters, strata, and trend weights) provided by the NIS. Continuous variables were reported as weighted mean ± SE; categorical variables were reported as weighted numbers (*n*) and percentages (%). The SEs of weighted means were estimated using the Taylor linearization method that incorporated the sample design. Weighted Student's *t*-tests were used to analyze the normally distributed continuous variables, while Rao-Scott modified chi-square tests were used to test the difference of distribution for categorical variables. Wilcoxon Rank-Sum Tests were used to test the variables that are not normally distributed.

Multivariate linear regression was used to estimate the average change in LOS and total hospitalization charges after adjusting for patient demographics, hospital characteristics, insurance type, median household income, ECI score, obesity, diabetes, tobacco use disorder, hypertension, dyslipidemia, cirrhosis and its complications, numbers of cirrhosis complications, and hepatocellular carcinoma. Multivariate logistic regression was used to estimate the odds ratio (OR) of mortality, CDI complications and interventions after adjusting for the same confounding variables as noted above.

The statistical methods of this study were reviewed by Dr. Chunyi Wu, PhD of Epidemiology from University of Michigan Medical School.

RESULTS

Patient demographics and baseline characteristics

From 2010 to 2014, the numbers of patients hospitalized for CDI with coexisting NAFLD, VLD and ALD were 7239, 11857 and 5938, respectively. The CDI with NAFLD cohort in this study was predominantly Caucasian with an average age 56.3 years old. In the aforementioned cohort, 69.4% of the patients were female, 41.6% were admitted to southern hospitals, and 58.6% were admitted to large hospitals (Table 1). Compared to CDI with VLD or ALD individually, the CDI with NAFLD group had significantly more patients in the 18-39 and greater than 70-year-old age groups ($P <$ 0.0001), were more likely to be female ($P <$ 0.0001), from the southern hospital region ($P <$ 0.0001), and less likely to be Medicaid insured ($P <$ 0.0001). Additionally, the CDI with coexisting VLD group was associated with a higher percentage of African American patients and had less patients with a high household income (Q3 and Q4, median household income for ZIP code between 51th and 100th percentile) compared to the CDI with NAFLD group.

In regard to comorbidities (Table 2), when compared to the CDI with VLD or ALD groups individually, CDI patients with NAFLD had a greater prevalence of obesity ($P < 0.0001$, $P < 0.0001$), diabetes ($P < 0.0001$, $P < 0.0001$), hypertension ($P = 0.0006$, $P < 0.0001$) and dyslipidemia ($P < 0.0001$, $P < 0.0001$). CDI with NAFLD was also associated with a significantly lower rate of cirrhosis ($P < 0.0001$, $P < 0.0001$) when compared to the other two groups. None of the patients in the CDI with NAFLD group had cirrhosis-related ascites, esophageal varices bleeding, spontaneous bacterial peritonitis or hepatorenal syndrome. Moreover, a lower rate of hepatocellular carcinoma ($P < 0.0001$, $P = 0.0217$) was observed in the CDI with NAFLD group compared to the CDI with VLD or ALD groups individually.

Outcomes and regression analysis of CDI patients with NAFLD vs VLD

When compared to the CDI with NAFLD group, the CDI with VLD group was associated with higher rates of acute kidney injury (AKI) [adjusted OR (aOR) = 1.35, 95%CI: 1.10-1.67, $P = 0.0041$], respiratory failure (RF) (aOR = 1.83, 95%CI: 1.22-2.76, $P = 0.0036$), longer LOS (adjusted LOS ratio = 1.12, 95%CI: 1.06-1.18, $P < 0.0001$) and higher hospitalization charges (adjusted cost ratio = 1.13, 95%CI :1.06-1.2, $P < 0.0001$). However, a lower rate of intestinal perforation rate was observed in the CDI with VLD group (aOR = 0.12, 95%CI: 0.03-0.57, $P = 0.0075$). CDI with VLD was initially associated with higher rates of mortality, colectomy and ileostomy, however this difference no longer existed after adjusting for confounding factors (Table 3).

Outcomes and regression analysis of CDI patients with NAFLD vs ALD

When compared to CDI patients with NAFLD, CDI patients with ALD had higher rates of RF (aOR = 1.72, 95%CI: 1.09-2.72, $P = 0.0201$), mortality (aOR = 2.63, 95%CI: 1.25-5.51, $P = 0.0107$), longer LOS (adjusted LOS ratio = 1.18, 95%CI: 1.10-1.25, $P < 0.0001$) and higher hospitalization charges (adjusted cost ratio = 1.17, 95%CI: 1.09-1.26, $P < 0.0001$). However, a lower rate of intestinal obstruction (aOR = 0.45, 95%CI: 0.28-0.72, $P = 0.0010$) was found in the CDI with ALD group when compared to the CDI with NAFLD group. Higher rates of AKI and septic shock, and a lower rate of colectomy were initially observed in CDI with ALD group, but the difference no longer existed after adjusting for the aforementioned confounders (Table 4).

Table 1 Comparison of demographic data for patients hospitalized with *Clostridioides difficile* infection with coexisting nonalcoholic fatty liver disease, viral liver disease and alcoholic liver disease

Variables	CDI with NAFLD	CDI with VLD	CDI with ALD	*P* value	
				CDI with NAFLD *vs* CDI with VLD	CDI with NAFLD *vs* CDI with ALD
n (weighted)	7239	11857	5938		
Age (yr)	56.32 ± 0.42	57 ± 0.26	56.13 ± 0.37	0.15	0.73
18-39	1133 (15.6%)	791 (6.7%)	557 (9.4%)	< 0.0001	< 0.0001
40-49	1290 (17.8%)	1811 (15.3%)	1051 (17.7%)		
50-59	1618 (22.4%)	4873 (41.1%)	2021 (34%)		
60-69	1620 (22.4%)	2791 (23.5%)	1439 (24.2%)		
≥ 70	1578 (21.8%)	1591 (13.4%)	870 (14.7%)		
Sex				< 0.0001	< 0.0001
Female	5023 (69.4%)	5795 (48.9%)	2300 (38.7%)		
Race				< 0.0001	0.17
Caucasian	5427 (75%)	6920 (58.4%)	4358 (73.4%)		
African American	482 (6.5%)	2773 (23.4%)	525 (8.8%)		
Hispanic	648 (9%)	1144 (9.6%)	515 (8.7%)		
Hospital bed size				0.033	0.9
Large	4241 (58.6%)	7414 (62.6%)	3461 (58.3%)		
Hospital region				< 0.0001	< 0.0001
Northeast	1091 (15.1%)	2618 (22.1%)	1243 (20.9%)		
Midwest	1618 (22.3%)	2514 (21.1%)	1584 (26.7%)		
South	3008 (41.6%)	4208 (35.5%)	1671 (28.1%)		
West	1522 (21%)	2517 (21.2%)	1440 (24.3%)		
Hospital type				< 0.0001	0.22
Urban teaching	3401 (47%)	7207 (60.8%)	3065 (51.6%)		
Insurance				< 0.0001	< 0.0001
Medicare	3086 (42.6%)	5493 (46.3%)	2239 (37.7%)		
Medicaid	914 (12.6%)	3329 (28.1%)	1261 (21.2%)		
Private	2526 (34.9%)	1835 (15.5%)	1391 (23.4%)		
Median household income for ZIP Code, %				< 0.0001	0.61
Q1	1790 (24.7%)	4205 (35.5%)	1592 (26.8%)		
Q2	1824 (25.2%)	3128 (26.4%)	1407 (23.7%)		
Q3	1926 (26.6%)	2353 (19.8%)	1503 (25.3%)		
Q4	1511 (20.9%)	1657 (14%)	1252 (21.1%)		

Values reported as weighted mean ± SE and weighted number [*n* (%)]. CDI: *Clostridioides difficile* infection; NAFLD: Nonalcoholic fatty liver disease; VLD: Viral liver disease; ALD: Alcoholic liver disease; Q1: Quartile 1, 0-25th percentile; Q2: Quartile 2, 26th-50th percentile; Q3: Quartile 3, 51th-75th percentile; Q4: Quartile 4, 76th-100th percentile.

DISCUSSION

This nationwide retrospective cohort study investigated the inpatient clinical characteristics and outcomes of CDI in hospitalized patients with coexisting liver diseases, with comparisons between NAFLD, VLD and ALD. We demonstrated that patients hospitalized with CDI and coexisting NAFLD had overall more favorable outcomes including a lower rate of RF, lower hospitalization charges and a shorter LOS when

Table 2 Comparison of comorbid conditions and complications for patients hospitalized with *Clostridioides difficile* infection with coexisting nonalcoholic fatty liver disease, viral liver disease and alcoholic liver disease

Variables	CDI with NAFLD	CDI with VLD	CDI with ALD	*P* value	
				CDI with NAFLD *vs* CDI with VLD	CDI with NAFLD *vs* CDI with ALD
n (weighted)	7239	11857	5938		
Number of Elixhauser comorbidities				< 0.0001	< 0.0001
0	0 (0%)	114 (1%)	-		
1	244 (3.4%)	574 (4.8%)	116 (2%)		
2	656 (9.1%)	1409 (11.9%)	354 (6%)		
≥ 3	6338 (87.6%)	9760 (82.3%)	5463 (92%)		
Obesity	2012 (27.8%)	850 (7.2%)	372 (6.3%)	< 0.0001	< 0.0001
Diabetes	2750 (38%)	3451 (29.1%)	1170 (19.7%)	< 0.0001	< 0.0001
Hypertension	4300 (59.4%)	6347 (53.5%)	2980 (50.2%)	0.00058	< 0.0001
Dyslipidemia	2619 (36.2%)	1868 (15.8%)	905 (15.2%)	< 0.0001	< 0.0001
Hepatocellular carcinoma	-	253 (2.1%)	45 (0.8%)	< 0.0001	0.0217
Cirrhosis related comorbidities[1]					
Cirrhosis	401 (5.5%)	2508 (21.2%)	3407 (57.4%)	< 0.0001	< 0.0001
Number of cirrhosis complications				0.0013	< 0.0001
0	137 (34.2%)	1773 (70.7%)	2105 (61.8%)		
1	244 (60.8%)	688 (27.4%)	1104 (32.4%)		
v ≥ 2	20 (5.0%)	47 (1.9%)	198 (5.8%)		
Ascites	0 (0%)	0 (0%)	0 (0%)	NA	NA
Esophageal varices bleeding	0 (0%)	-	20 (0.6%)	NA	NA
Hepatic encephalopathy	110 (27.4%)	60 (2.4%)	569 (16.7%)	0.003338	< 0.0001
Hepatorenal syndrome	0 (0%)	15 (0.6%)	33 (1.0%)	NA	NA
Portal hypertension	175 (43.6%)	661 (26.4%)	843 (24.7%)	< 0.0001	< 0.0001
Spontaneous bacterial peritonitis	0 (0%)	38 (1.5%)	40 (1.2%)	NA	NA

[1]Value reported as percentage of all cirrhotic patients.

Values reported as weighted number [*n* (%)].-: Numbers were not displayed due to extremely small numbers were associated with increased risk for identification of persons; CDI: *Clostridioides difficile* infection; NAFLD: Nonalcoholic fatty liver disease; VLD: Viral liver disease; ALD: Alcoholic liver disease; NA: Not available.

compared to those with ALD and VLD individually. Interestingly, higher rates of intestinal complications were observed in the CDI with NAFLD group when compared to the CDI with ALD or VLD groups. Specifically, a significantly higher rate of intestinal obstruction was seen in the CDI with NAFLD group when compared to the CDI with ALD group, and a higher rate of intestinal perforation was seen when compared to CDI patients with concomitant VLD.

Our findings of worse intestinal complications in patients hospitalized with CDI and coexisting NAFLD compared to CDI patients with VLD and ALD, linked the gut pathology to the liver. The crosstalk between the gut and liver is increasingly recognized as the gut-liver axis[18]. Receiving more than 70% of the blood supply from the intestinal venous outflow, the liver represents the first line of defense against gut derived antigens with a broad array of immune cells[19]. The liver also releases many bioactive mediators into the systemic circulation, allowing for communication with the intestine. In the intestine, the endogenous and exogenous products from host and microbial metabolism translocate to the liver through the portal venous system, ultimately influencing liver function[20].

Table 3 Multivariate regression analysis of outcomes for patients hospitalized for *Clostridioides difficile* infection with coexisting nonalcoholic fatty liver disease *vs* viral liver disease

Outcomes	CDI with NAFLD	CDI with VLD	Unadjusted ratio (95%CI)	*P* value	Adjusted ratio[1] (95%CI)	*P* value
n (weighted)	7239	11857				
Hospital mortality	59 (0.8%)	186 (1.6%)	1.94 (1.44, 2.6)	< 0.0001	1.87 (0.95, 3.7)	0.071
Acute kidney injury	938 (13%)	2035 (17.2%)	1.39 (1.28, 1.51)	< 0.0001	1.35 (1.1, 1.67)	0.0041
Respiratory failure	192 (2.7%)	504 (4.2%)	1.63 (1.37, 1.92)	< 0.0001	1.83 (1.22, 2.76)	0.0036
Septic shock	39 (0.5%)	115 (1%)	1.8 (1.25, 2.59)	0.0015	1.64 (0.67, 4.02)	0.27
Intestinal perforation	-	-	0.3 (0.1, 0.89)	0.03	0.12 (0.03, 0.57)	0.0075
Intestinal obstruction	331 (4.6%)	527 (4.4%)	0.97 (0.84, 1.12)	0.67	0.94 (0.66, 1.33)	0.725
Peritonitis	61 (0.8%)	106 (0.9%)	1.06 (0.77, 1.45)	0.71	0.72 (0.35, 1.52)	0.39
Colectomy	45 (0.6%)	105 (0.9%)	1.43 (1.01, 2.03)	0.044	1.38 (0.6, 3.15)	0.44
Ileostomy	-	41 (0.3%)	2.47 (1.24, 4.92)	0.01	2.62 (0.66, 10.41)	0.17
LOS (days)	5.75 ± 0.16	6.77 ± 0.15	1.11 (1.06, 1.16)	< 0.0001	1.12 (1.06, 1.18)	< 0.0001
Total hospitalizationcharges (dollars)	38150.34 ± 1757.01	46326.72 ± 1809.82	1.14 (1.07, 1.2)	< 0.0001	1.13 (1.06, 1.2)	< 0.0001

[1]Adjusted for age, sex, race, primary insurance payer, hospital type, hospital bed size, hospital region, income quartile, Elixhauser Comorbidity Index score, obesity, diabetes, tobacco use disorder, hypertension, dyslipidemia, cirrhosis and its complications, numbers of cirrhosis complications, and hepatocellular carcinoma.

-: Numbers were not displayed due to extremely small numbers were associated with increased risk for identification of persons. Values reported as weighted mean ± SE and weighted numbers [*n* (%)]; CDI: *Clostridioides difficile* infection; NAFLD: Nonalcoholic fatty liver disease; VLD: Viral liver disease; CI: Confidence interval; LOS: Length of stay.

How does NAFLD influence the intestinal complications of CDI through the gut-liver axis? Convincing evidence has shown that NAFLD is associated with significantly increased gut permeability and inflammation in both animal[21] and human models. Miele *et al*[22] found that NAFLD patients had significantly increased gut permeability measured by urine radiolabeled markers and immunohistochemical analysis of zona occludens -1 expression in intestinal biopsy specimens, compared with healthy volunteers. They also discovered that both gut permeability and the prevalence of small intestinal bacterial overgrowth are correlated with the severity of steatosis. Verdam *et al*[23] found that plasma immunoglobulin G levels against endotoxin were increased in NASH patients, which positively correlated with the severity of inflammation. Furthermore, transmission electron microscopy observed irregular microvilli and widened tight junctions in the gut mucosa of the NAFLD patients[24]. In addition, decreased numbers of CD4+ and CD8+ T lymphocytes and increased levels of TNF-α, IL-6 and IFN-γ were detected in the NAFLD patient group compared to healthy control. All of these results suggested impaired gut permeability and increased levels of inflammation at both the tissue and cellular levels in NAFLD disease models.

The gut microbiota-mediated inflammation, the related disturbance of the intestinal integrity and the impairment in mucosal immune function have been reported to play important roles, not only in the pathophysiology of CDI[25] but also in the pathogenesis of NAFLD[13,24,26]. The gut microbiota normally exerts significant influence on intestinal epithelial cell health, nutrient metabolism and mucosal defense [19,27]. Early evidence in animal studies demonstrated that altered gut microbiota composition[28] independently contributed to the development of NAFLD in mice. In addition, altered interaction between the gut and the host (produced by defective inflammasome sensing in inflammasome-deficient mouse models) may govern the rate of progression of multiple metabolic syndrome-associated abnormalities[29]. With the recent developments in genome sequencing technologies, bioinformatics, and culturomics; it has been recognized that NAFLD and NASH are associated with decreased richness of the gut flora and increased risk of pathogenic flora in pediatric and adult patients[30-34], which are both well known risk factors for CDI. Although it is still unclear which specific microorganisms are harmful given conflicting results in

Table 4 Multivariate regression analysis of outcomes for patients hospitalized for Clostridioides difficile infection with coexisting nonalcoholic fatty liver disease *vs* alcoholic liver disease

Outcomes	CDI with NAFLD	CDI with ALD	Unadjusted ratio (95%CI)	*P* value	Adjusted ratio[1] (95%CI)	*P* value
n (weighted)	7239	5938				
Hospital mortality	59 (0.8%)	159 (2.7%)	3.34 (2.48, 4.52)	< 0.0001	2.63 (1.25, 5.51)	0.0107
Acute kidney injury	938 (13%)	935 (15.8%)	1.26 (1.14, 1.39)	< 0.0001	1.2 (0.93, 1.54)	0.15
Respiratory failure	192 (2.7%)	249 (4.2%)	1.61 (1.33, 1.94)	< 0.0001	1.72 (1.09, 2.72)	0.0201
Septic shock	39 (0.5%)	79 (1.3%)	2.48 (1.69, 3.64)	< 0.0001	2.14 (0.84, 5.46)	0.109
Intestinal perforation	-	0 (0%)	NA	NA	NA	NA
Intestinal obstruction	331 (4.6%)	133 (2.2%)	0.48 (0.39, 0.59)	< 0.0001	0.45 (0.28, 0.72)	0.0010
Peritonitis	61 (0.8%)	69 (1.2%)	1.38 (0.97, 1.95)	0.071	0.54 (0.25, 1.18)	0.12
Colectomy	45 (0.6%)	15 (0.3%)	0.42 (0.23, 0.74)	0.003	0.44 (0.14, 1.39)	0.16
Ileostomy	-	-	0.65 (0.23, 1.85)	0.42	0.99 (0.15, 6.61)	0.98
LOS (days)	5.75 ± 0.16	6.84 ± 0.23	1.14 (1.08, 1.21)	< 0.0001	1.18 (1.1, 1.25)	< 0.0001
Totalhospitalizationcharges (dollars)	38150.34 ± 1757.01	44641.74 ± 1660.66	1.14 (1.07, 1.22)	< 0.0001	1.17 (1.09, 1.26)	< 0.0001

[1]Adjusted for age, sex, race, primary insurance payer, hospital type, hospital bed size, hospital region, income quartile, Elixhauser Comorbidity Index score, obesity, diabetes, tobacco use disorder, hypertension, dyslipidemia, cirrhosis and its complications, numbers of cirrhosis complications, and hepatocellular carcinoma.

Values reported as weighted mean ± SE and weighted numbers [*n* (%)]. -: Numbers were not displayed due to extremely small numbers were associated with increased risk for identification of persons. CDI: *Clostridioides difficile* infection; NAFLD: Nonalcoholic fatty liver disease; ALD: Alcoholic liver disease; LOS: Length of stay.

human and animal studies[35], it is believed that gut microbiota-derived signatures extracted by whole-genome shotgun sequencing of DNA can be used for diagnosis of advanced fibrosis in NAFLD[36], and modification of gut microbiota analyzed by 16S ribosomal RNA pyrosequencing can be used for therapeutic purposes in NASH patients[37]. Additionally, increased pathogenic flora in NAFLD and NASH further disturb the immune balance and cause worsened dysbiosis through various mechanisms involving short-chain fatty acids[38], lipopolysaccharide[21], choline metabolism[39], bile acid metabolism[40] and bacteria-derived ethanol[41]. Collectively, NAFLD and NASH related alterations of gut microbiota and its downstream dysbiosis pathways may contribute to CDI risk and worse intestinal complications.

On the other end, we sought to identify the characteristics of gut microbiota changes in ALD and VLD. Compared to NAFLD, ALD is remarkably similar histologically[42] and initiated directly from the gut by alcohol intake or binges. It has been well documented that alcohol intake can lead to changes in gut microbiota composition[43] and gut permeability[44] early on, even before the development of liver disease. These alterations involve multiple physical and biochemical layers of defense in the intestinal barrier[19]. In VLD, the gut microbiome works as an effective tool early on for immunity against the hepatitis virus, and helps with viral clearance[45]. In chronic VLD, large translocations of intestinal microbiota were observed and thought to contribute to not only dysregulation of immune cells and dysfunction of the intestinal barrier, but also viral replication[27]. Comparison analysis revealed that, compared to other cirrhosis etiologies, alcoholic cirrhosis is associated with worse gut dysbiosis after adjusting for Model For End-Stage Liver Disease score and body mass index[46]. In two other studies[47,48], which primarily compared the gut microbiota composition in HBV/HCV related and alcoholic cirrhosis, no difference was observed at the phylum and class level.

Intriguingly, in our study, the majority (94.5%) of patients in CDI with NAFLD group were non-cirrhotic; the percentage of cirrhotic patients in CDI with NAFLD group was significantly less than those in CDI with ALD or VLD group. CDI with NAFLD group was associated with a higher rate of intestinal complications after adjusting for cirrhosis and its complications. These results suggested that NAFLD is

associated with altered gut microbiota that is predisposed to CDI and its complications, likely independent from the liver disease severity. In fact, NAFLD has been reported as an independent risk factor for CDI[14]. Although ALD and VLD cirrhosis was previously found to be associated with worse gut dysbiosis than NAFLD cirrhosis, this finding should be treated cautiously for non-cirrhotic patients, because the alteration of the gut microbiome is associated with the severity of liver disease, as significant differences in gut microbiota have been found between non-cirrhotic, compensated and decompensated cirrhotic patients[49,50]. Importantly, the standard of care therapies in cirrhotic patients such as lactulose, rifaximin, antibiotics and acid-suppressants that can affect the gut microbiota, may be playing a critical role[51]. In summary, our study suggested that NAFLD may be associated with worse dysbiosis in early liver disease stages and therefore a higher risk for CDI and its complications compared to ALD and VLD.

Aside from aforementioned gut microbiota changes that directly link NAFLD to CDI and intestinal complications, NAFLD related metabolic syndrome and systemic inflammation also play crucial roles in intestinal pathology. Recently, metabolic dysfunction-associated fatty liver disease has been proposed as a more appropriate name to replace NAFLD by an international panel of experts, with emphasis on the underlying metabolic dysfunction[52,53]. Clinical evidence has demonstrated that NAFLD, along with other components of metabolic syndrome, such as diabetes and obesity, are associated with an increased prevalence of small intestinal bacterial overgrowth (SIBO)[54,55] by insulin resistance, oxidative stress and chronic low grade inflammation[56]. Subsequently, the dysmotility induced by SIBO can further promote SIBO in NAFLD patients, causing a vicious cycle[57]. In fact, dysmotility itself is associated with NAFLD and may be a potential therapeutic target for NAFLD from a Japanese study[58,59]. Moreover, diabetes, a component of metabolic syndrome which may cause vasculopathies and neuropathies in the intestines, also contributes to dysmotility[60]. Additionally, diverticular disease, irritable bowel disease[61] and inflammatory bowel disease[62], together with SIBO and dysmotility have all been shown to have increased prevalence in NAFLD patients. Not surprisingly, the structural and functional abnormalities in the gut associated with NAFLD and metabolic dysfunction further increase the risk of CDI and its complications.

The strengths of this study include the utilization of the NIS database to provide a unique opportunity to investigate a nationwide population hospitalized for CDI. To the best of our knowledge, this study is a leading clinical research analysis that provided a comprehensive nationwide comparison of outcomes between NAFLD and other common chronic liver diseases, ALD and VLD, in hospitalized CDI patients. There are also limitations in this study. Particularly, NIS data acquisition relies on the accuracy ICD-9-CM codes for medical diagnoses and no lab results, biopsy or image studies were available for NAFLD diagnosis and severity stratification. It is also difficult to determine which cases of CDI were hospital acquired or community acquired because ICD-9 codes are assigned at discharge. To strengthen the validity of ICD-9 codes for NAFLD, VLD and ALD, we used not only diagnostic codes but also excluded the codes for all other chronic liver diseases (Supplementary Table 1)[63]. The ICD-9 codes for CDI were validated previously with good diagnostic accuracy[64, 65].

CONCLUSION

In conclusion, this study found more favorable overall outcomes but higher rates of intestinal complications in patients hospitalized with CDI and coexisting NAFLD, compared to CDI with coexisting ALD and VLD, individually. These results suggested that NAFLD may be associated with a higher risk of CDI associated intestinal complications through alteration of gut microbiota. Our study also suggested that NAFLD associated metabolic syndrome may contribute significantly to the gut dysbiosis even in the early liver disease stages and cause increased risk for CDI and its complications. During the last few years, the novel and rapidly evolving research technologies for the gut microbiome have been opening up an exciting era in the microbiota therapeutics for different disease models[66]. Tremendous progress has been observed in the treatment of NAFLD and CDI through gut microbiome manipulation. Our study may help increase awareness and diagnose intestinal complications in patients with two common diseases: CDI and NAFLD. Unraveling the significance of interactions between gut microbiota, gut immunity and systemic metabolic impact of NAFLD with prospective studies will provide more insights into the future microbiota therapeutics

for CDI and NAFLD.

ARTICLE HIGHLIGHTS

Research background

The ongoing exploration of liver-gut axis has discovered strong association between gut dysbiosis and nonalcoholic fatty liver disease (NAFLD) in both basic science and clinical research. Small-scaled studies have observed that NAFLD is an independent risk factor for *Clostridioides difficile* infection (CDI).

Research motivation

CDI, as the most common cause of nosocomial diarrhea in developed countries, carries high hospitalization burden. NAFLD, as the leading cause of chronic liver disease, is commonly seen in hospitalized patients with CDI. So far the inpatient outcomes of CDI in the NAFLD population have not been well studied.

Research objectives

The authors aimed to examine the impact of NAFLD on the inpatient outcomes of hospitalized patients with CDI, by comparing the effect of NAFLD with alcoholic liver disease (ALD) and viral liver disease (VLD) individually.

Research methods

This nationwide retrospective cohort study was conducted according to STROBE statement using the National Inpatient Sample database. Inpatient CDI with coexisting NAFLD cases were selected using ICD-9 codes. Multivariate regression analysis was used with adjustment for a large group of possible confounders. Elixhauser Comorbidity Index (ECI) was used for a full description of comorbidity burden.

Research results

CDI with NAFLD was independently associated with lower rates of acute respiratory failure, shorter length of stay and lower hospitalization charges when compared to CDI with VLD and CDI with ALD. However, CDI with NAFLD was associated with a higher rate of intestinal perforation when compared to VLD, and a higher rate of intestinal obstruction when compared to ALD.

Research conclusions

CDI and coexisting NAFLD is associated with favorable overall outcomes, but higher rates of intestinal complications compared to CDI with coexisting ALD and VLD, individually.

Research perspectives

This finding suggests that alteration of gut microbiota may play an important role in the pathogenesis of both CDI and NAFLD. NAFLD associated metabolic syndrome may contribute significantly to the gut dysbiosis and cause increased risk for CDI and its complications. This study provides potential directions for future prospective clinical research to identify the clinical meaningfulness of interactions between gut microbiota, gut immunity and systemic inflammation. The study may open the door for potential microbiota therapeutic targets and manipulation as future treatment options for chronic liver diseases.

ACKNOWLEDGEMENTS

REFERENCES

1   **Younossi ZM**, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes.

Hepatology 2016; **64**: 73-84 [PMID: 26707365 DOI: 10.1002/hep.28431]

2   **Huang DQ**, El-Serag HB, Loomba R. Global epidemiology of NAFLD-related HCC: trends, predictions, risk factors and prevention. *Nat Rev Gastroenterol Hepatol* 2021; **18**: 223-238 [PMID: 33349658 DOI: 10.1038/s41575-020-00381-6]

3   **Williams CD**, Stengel J, Asike MI, Torres DM, Shaw J, Contreras M, Landt CL, Harrison SA. Prevalence of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis among a largely middle-aged population utilizing ultrasound and liver biopsy: a prospective study. *Gastroenterology* 2011; **140**: 124-131 [PMID: 20858492 DOI: 10.1053/j.gastro.2010.09.038]

4   **Vernon G**, Baranova A, Younossi ZM. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. *Aliment Pharmacol Ther* 2011; **34**: 274-285 [PMID: 21623852 DOI: 10.1111/j.1365-2036.2011.04724.x]

5   **Younossi ZM**. Non-alcoholic fatty liver disease - A global public health perspective. *J Hepatol* 2019; **70**: 531-544 [PMID: 30414863 DOI: 10.1016/j.jhep.2018.10.033]

6   **Evans CT**, Safdar N. Current Trends in the Epidemiology and Outcomes of Clostridium difficile Infection. *Clin Infect Dis* 2015; **60** Suppl 2: S66-S71 [PMID: 25922403 DOI: 10.1093/cid/civ140]

7   **Rupnik M**, Wilcox MH, Gerding DN. Clostridium difficile infection: new developments in epidemiology and pathogenesis. *Nat Rev Microbiol* 2009; **7**: 526-536 [PMID: 19528959 DOI: 10.1038/nrmicro2164]

8   **McDonald LC**, Gerding DN, Johnson S, Bakken JS, Carroll KC, Coffin SE, Dubberke ER, Garey KW, Gould CV, Kelly C, Loo V, Shaklee Sammons J, Sandora TJ, Wilcox MH. Clinical Practice Guidelines for Clostridium difficile Infection in Adults and Children: 2017 Update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA). *Clin Infect Dis* 2018; **66**: e1-e48 [PMID: 29462280 DOI: 10.1093/cid/cix1085]

9   **Nwachuku E**, Shan Y, Senthil-Kumar P, Braun T, Shadis R, Kirton O, Vu TQ. Toxic Clostridioides (formerly Clostridium) difficile colitis: No longer a diarrhea associated infection. *Am J Surg* 2021; **221**: 240-242 [PMID: 32680621 DOI: 10.1016/j.amjsurg.2020.06.026]

10   **Kistangari G**, Lopez R, Shen B. Frequency and Risk Factors of Clostridium difficile Infection in Hospitalized Patients With Pouchitis: A Population-based Study. *Inflamm Bowel Dis* 2017; **23**: 661-671 [PMID: 28296825 DOI: 10.1097/MIB.0000000000001057]

11   **Czepiel J**, Dróżdż M, Pituch H, Kuijper EJ, Perucki W, Mielimonka A, Goldman S, Wultańska D, Garlicki A, Biesiada G. Clostridium difficile infection: review. *Eur J Clin Microbiol Infect Dis* 2019; **38**: 1211-1221 [PMID: 30945014 DOI: 10.1007/s10096-019-03539-6]

12   **Safari Z**, Gérard P. The links between the gut microbiome and non-alcoholic fatty liver disease (NAFLD). *Cell Mol Life Sci* 2019; **76**: 1541-1558 [PMID: 30683985 DOI: 10.1007/s00018-019-03011-w]

13   **Aron-Wisnewsky J**, Vigliotti C, Witjes J, Le P, Holleboom AG, Verheij J, Nieuwdorp M, Clément K. Gut microbiota and human NAFLD: disentangling microbial signatures from metabolic disorders. *Nat Rev Gastroenterol Hepatol* 2020; **17**: 279-297 [PMID: 32152478 DOI: 10.1038/s41575-020-0269-9]

14   **Nseir WB**, Hussein SHH, Farah R, Mahamid MN, Khatib HH, Mograbi JM, Peretz A, Amara AE. Nonalcoholic fatty liver disease as a risk factor for Clostridium difficile-associated diarrhea. *QJM* 2020; **113**: 320-323 [PMID: 31688897 DOI: 10.1093/qjmed/hcz283]

15   **Papić N**, Jelovčić F, Karlović M, Marić LS, Vince A. Nonalcoholic fatty liver disease as a risk factor for Clostridioides difficile infection. *Eur J Clin Microbiol Infect Dis* 2020; **39**: 569-574 [PMID: 31782025 DOI: 10.1007/s10096-019-03759-w]

16   **Agency for Healthcare Research and Quality**.   Healthcare Cost and Utilization Project (HCUP). 2019. Available from: https://www.ahrq.gov/data/hcup/index.html

17   **Moore BJ**, White S, Washington R, Coenen N, Elixhauser A. Identifying Increased Risk of Readmission and In-hospital Mortality Using Hospital Administrative Data: The AHRQ Elixhauser Comorbidity Index. *Med Care* 2017; **55**: 698-705 [PMID: 28498196 DOI: 10.1097/MLR.0000000000000735]

18   **Tripathi A**, Debelius J, Brenner DA, Karin M, Loomba R, Schnabl B, Knight R. The gut-liver axis and the intersection with the microbiome. *Nat Rev Gastroenterol Hepatol* 2018; **15**: 397-411 [PMID: 29748586 DOI: 10.1038/s41575-018-0011-z]

19   **Son G**, Kremer M, Hines IN. Contribution of gut bacteria to liver pathobiology. *Gastroenterol Res Pract* 2010; **2010** [PMID: 20706692 DOI: 10.1155/2010/453563]

20   **Stärkel P**, Schnabl B. Bidirectional Communication between Liver and Gut during Alcoholic Liver Disease. *Semin Liver Dis* 2016; **36**: 331-339 [PMID: 27997973 DOI: 10.1055/s-0036-1593882]

21   **Mao JW**, Tang HY, Zhao T, Tan XY, Bi J, Wang BY, Wang YD. Intestinal mucosal barrier dysfunction participates in the progress of nonalcoholic fatty liver disease. *Int J Clin Exp Pathol* 2015; **8**: 3648-3658 [PMID: 26097546]

22   **Miele L**, Valenza V, La Torre G, Montalto M, Cammarota G, Ricci R, Mascianà R, Forgione A, Gabrieli ML, Perotti G, Vecchio FM, Rapaccini G, Gasbarrini G, Day CP, Grieco A. Increased intestinal permeability and tight junction alterations in nonalcoholic fatty liver disease. *Hepatology* 2009; **49**: 1877-1887 [PMID: 19291785 DOI: 10.1002/hep.22848]

23   **Verdam FJ**, Rensen SS, Driessen A, Greve JW, Buurman WA. Novel evidence for chronic exposure to endotoxin in human nonalcoholic steatohepatitis. *J Clin Gastroenterol* 2011; **45**: 149-152 [PMID: 20661154 DOI: 10.1097/MCG.0b013e3181e12cc24]

24   **Jiang W**, Wu N, Wang X, Chi Y, Zhang Y, Qiu X, Hu Y, Li J, Liu Y. Dysbiosis gut microbiota

associated with inflammation and impaired mucosal immune function in intestine of humans with non-alcoholic fatty liver disease. *Sci Rep* 2015; **5**: 8096 [PMID: 25644696 DOI: 10.1038/srep08096]

25   **Theriot CM**, Koenigsknecht MJ, Carlson PE Jr, Hatton GE, Nelson AM, Li B, Huffnagle GB, Z Li J, Young VB. Antibiotic-induced shifts in the mouse gut microbiome and metabolome increase susceptibility to Clostridium difficile infection. *Nat Commun* 2014; **5**: 3114 [PMID: 24445449 DOI: 10.1038/ncomms4114]

26   **Campo L**, Eiseler S, Apfel T, Pyrsopoulos N. Fatty Liver Disease and Gut Microbiota: A Comprehensive Update. *J Clin Transl Hepatol* 2019; **7**: 56-60 [PMID: 30944821 DOI: 10.14218/JCTH.2018.00008]

27   **Sehgal R**, Bedi O, Trehanpati N. Role of Microbiota in Pathogenesis and Management of Viral Hepatitis. *Front Cell Infect Microbiol* 2020; **10**: 341 [PMID: 32850467 DOI: 10.3389/fcimb.2020.00341]

28   **Le Roy T**, Llopis M, Lepage P, Bruneau A, Rabot S, Bevilacqua C, Martin P, Philippe C, Walker F, Bado A, Perlemuter G, Cassard-Doulcier AM, Gérard P. Intestinal microbiota determines development of non-alcoholic fatty liver disease in mice. *Gut* 2013; **62**: 1787-1794 [PMID: 23197411 DOI: 10.1136/gutjnl-2012-303816]

29   **Henao-Mejia J**, Elinav E, Jin C, Hao L, Mehal WZ, Strowig T, Thaiss CA, Kau AL, Eisenbarth SC, Jurczak MJ, Camporez JP, Shulman GI, Gordon JI, Hoffman HM, Flavell RA. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. *Nature* 2012; **482**: 179-185 [PMID: 22297845 DOI: 10.1038/nature10809]

30   **Tsai MC**, Liu YY, Lin CC, Wang CC, Wu YJ, Yong CC, Chen KD, Chuah SK, Yao CC, Huang PY, Chen CH, Hu TH, Chen CL. Gut Microbiota Dysbiosis in Patients with Biopsy-Proven Nonalcoholic Fatty Liver Disease: A Cross-Sectional Study in Taiwan. *Nutrients* 2020; **12** [PMID: 32204538 DOI: 10.3390/nu12030820]

31   **Schwimmer JB**, Johnson JS, Angeles JE, Behling C, Belt PH, Borecki I, Bross C, Durelle J, Goyal NP, Hamilton G, Holtz ML, Lavine JE, Mitreva M, Newton KP, Pan A, Simpson PM, Sirlin CB, Sodergren E, Tyagi R, Yates KP, Weinstock GM, Salzman NH. Microbiome Signatures Associated With Steatohepatitis and Moderate to Severe Fibrosis in Children With Nonalcoholic Fatty Liver Disease. *Gastroenterology* 2019; **157**: 1109-1122 [PMID: 31255652 DOI: 10.1053/j.gastro.2019.06.028]

32   **Del Chierico F**, Nobili V, Vernocchi P, Russo A, De Stefanis C, Gnani D, Furlanello C, Zandonà A, Paci P, Capuani G, Dallapiccola B, Miccheli A, Alisi A, Putignani L. Gut microbiota profiling of pediatric nonalcoholic fatty liver disease and obese patients unveiled by an integrated meta-omics-based approach. *Hepatology* 2017; **65**: 451-464 [PMID: 27028797 DOI: 10.1002/hep.28572]

33   **Shen F**, Zheng RD, Sun XQ, Ding WJ, Wang XY, Fan JG. Gut microbiota dysbiosis in patients with non-alcoholic fatty liver disease. *Hepatobiliary Pancreat Dis Int* 2017; **16**: 375-381 [PMID: 28823367 DOI: 10.1016/S1499-3872(17)60019-5]

34   **Wang B**, Jiang X, Cao M, Ge J, Bao Q, Tang L, Chen Y, Li L. Altered Fecal Microbiota Correlates with Liver Biochemistry in Nonobese Patients with Non-alcoholic Fatty Liver Disease. *Sci Rep* 2016; **6**: 32002 [PMID: 27550547 DOI: 10.1038/srep32002]

35   **Jennison E**, Byrne CD. The role of the gut microbiome and diet in the pathogenesis of non-alcoholic fatty liver disease. *Clin Mol Hepatol* 2021; **27**: 22-43 [PMID: 33291863 DOI: 10.3350/cmh.2020.0129]

36   **Loomba R**, Seguritan V, Li W, Long T, Klitgord N, Bhatt A, Dulai PS, Caussy C, Bettencourt R, Highlander SK, Jones MB, Sirlin CB, Schnabl B, Brinkac L, Schork N, Chen CH, Brenner DA, Biggs W, Yooseph S, Venter JC, Nelson KE. Gut Microbiome-Based Metagenomic Signature for Non-invasive Detection of Advanced Fibrosis in Human Nonalcoholic Fatty Liver Disease. *Cell Metab* 2017; **25**: 1054-1062.e5 [PMID: 28467925 DOI: 10.1016/j.cmet.2017.04.001]

37   **Wong VW**, Tse CH, Lam TT, Wong GL, Chim AM, Chu WC, Yeung DK, Law PT, Kwan HS, Yu J, Sung JJ, Chan HL. Molecular characterization of the fecal microbiota in patients with nonalcoholic steatohepatitis--a longitudinal study. *PLoS One* 2013; **8**: e62885 [PMID: 23638162 DOI: 10.1371/journal.pone.0062885]

38   **Cani PD**, Possemiers S, Van de Wiele T, Guiot Y, Everard A, Rottier O, Geurts L, Naslain D, Neyrinck A, Lambert DM, Muccioli GG, Delzenne NM. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. *Gut* 2009; **58**: 1091-1103 [PMID: 19240062 DOI: 10.1136/gut.2008.165886]

39   **Dumas ME**, Barton RH, Toye A, Cloarec O, Blancher C, Rothwell A, Fearnside J, Tatoud R, Blanc V, Lindon JC, Mitchell SC, Holmes E, McCarthy MI, Scott J, Gauguier D, Nicholson JK. Metabolic profiling reveals a contribution of gut microbiota to fatty liver phenotype in insulin-resistant mice. *Proc Natl Acad Sci U S A* 2006; **103**: 12511-12516 [PMID: 16895997 DOI: 10.1073/pnas.0601056103]

40   **Parséus A**, Sommer N, Sommer F, Caesar R, Molinaro A, Ståhlman M, Greiner TU, Perkins R, Bäckhed F. Microbiota-induced obesity requires farnesoid X receptor. *Gut* 2017; **66**: 429-437 [PMID: 26740296 DOI: 10.1136/gutjnl-2015-310283]

41   **Aragonès G**, González-García S, Aguilar C, Richart C, Auguet T. Gut Microbiota-Derived Mediators as Potential Markers in Nonalcoholic Fatty Liver Disease. *Biomed Res Int* 2019; **2019**: 8507583 [PMID: 30719448 DOI: 10.1155/2019/8507583]

42   **Brunt EM**, Tiniakos DG. Histopathology of nonalcoholic fatty liver disease. *World J Gastroenterol* 2010; **16**: 5286-5296 [PMID: 21072891 DOI: 10.3748/wjg.v16.i42.5286]

43   **Bajaj JS**. Alcohol, liver disease and the gut microbiota. *Nat Rev Gastroenterol Hepatol* 2019; **16**: 235-246 [PMID: 30643227 DOI: 10.1038/s41575-018-0099-1]

44   **Parlesak A**, Schäfer C, Schütz T, Bode JC, Bode C. Increased intestinal permeability to macromolecules and endotoxemia in patients with chronic alcohol abuse in different stages of alcohol-induced liver disease. *J Hepatol* 2000; **32**: 742-747 [PMID: 10845660 DOI: 10.1016/S0168-8278(00)80242-1]

45   **Xu D**, Huang Y, Wang J. Gut microbiota modulate the immune effect against hepatitis B virus infection. *Eur J Clin Microbiol Infect Dis* 2015; **34**: 2139-2147 [PMID: 26272175 DOI: 10.1007/s10096-015-2464-0]

46   **Bajaj JS**, Heuman DM, Hylemon PB, Sanyal AJ, White MB, Monteith P, Noble NA, Unser AB, Daita K, Fisher AR, Sikaroodi M, Gillevet PM. Altered profile of human gut microbiome is associated with cirrhosis and its complications. *J Hepatol* 2014; **60**: 940-947 [PMID: 24374295 DOI: 10.1016/j.jhep.2013.12.019]

47   **Chen Y**, Yang F, Lu H, Wang B, Chen Y, Lei D, Wang Y, Zhu B, Li L. Characterization of fecal microbial communities in patients with liver cirrhosis. *Hepatology* 2011; **54**: 562-572 [PMID: 21574172 DOI: 10.1002/hep.24423]

48   **Kakiyama G**, Pandak WM, Gillevet PM, Hylemon PB, Heuman DM, Daita K, Takei H, Muto A, Nittono H, Ridlon JM, White MB, Noble NA, Monteith P, Fuchs M, Thacker LR, Sikaroodi M, Bajaj JS. Modulation of the fecal bile acid profile by gut microbiota in cirrhosis. *J Hepatol* 2013; **58**: 949-955 [PMID: 23333527 DOI: 10.1016/j.jhep.2013.01.003]

49   **Qin N**, Yang F, Li A, Prifti E, Chen Y, Shao L, Guo J, Le Chatelier E, Yao J, Wu L, Zhou J, Ni S, Liu L, Pons N, Batto JM, Kennedy SP, Leonard P, Yuan C, Ding W, Hu X, Zheng B, Qian G, Xu W, Ehrlich SD, Zheng S, Li L. Alterations of the human gut microbiome in liver cirrhosis. *Nature* 2014; **513**: 59-64 [PMID: 25079328 DOI: 10.1038/nature13568]

50   **Qin N**, Le Chatelier E, Guo J, Prifti E, Li L, Ehrlich SD. Qin *et al*. reply. *Nature* 2015; **525**: E2-E3 [PMID: 26381989 DOI: 10.1038/nature14852]

51   **Bajaj JS**, Betrapally NS, Gillevet PM. Decompensated cirrhosis and microbiome interpretation. *Nature* 2015; **525**: E1-E2 [PMID: 26381988 DOI: 10.1038/nature14851]

52   **Eslam M**, Newsome PN, Sarin SK, Anstee QM, Targher G, Romero-Gomez M, Zelber-Sagi S, Wai-Sun Wong V, Dufour JF, Schattenberg JM, Kawaguchi T, Arrese M, Valenti L, Shiha G, Tiribelli C, Yki-Järvinen H, Fan JG, Grønbæk H, Yilmaz Y, Cortez-Pinto H, Oliveira CP, Bedossa P, Adams LA, Zheng MH, Fouad Y, Chan WK, Mendez-Sanchez N, Ahn SH, Castera L, Bugianesi E, Ratziu V, George J. A new definition for metabolic dysfunction-associated fatty liver disease: An international expert consensus statement. *J Hepatol* 2020; **73**: 202-209 [PMID: 32278004 DOI: 10.1016/j.jhep.2020.03.039]

53   **Eslam M**, Sanyal AJ, George J; International Consensus Panel. MAFLD: A Consensus-Driven Proposed Nomenclature for Metabolic Associated Fatty Liver Disease. *Gastroenterology* 2020; **158**: 1999-2014.e1 [PMID: 32044314 DOI: 10.1053/j.gastro.2019.11.312]

54   **Ferolla SM**, Armiliato GN, Couto CA, Ferrari TC. The role of intestinal bacteria overgrowth in obesity-related nonalcoholic fatty liver disease. *Nutrients* 2014; **6**: 5583-5599 [PMID: 25479248 DOI: 10.3390/nu6125583]

55   **Ghoshal UC**, Goel A, Quigley EMM. Gut microbiota abnormalities, small intestinal bacterial overgrowth, and non-alcoholic fatty liver disease: An emerging paradigm. *Indian J Gastroenterol* 2020; **39**: 9-21 [PMID: 32291578 DOI: 10.1007/s12664-020-01027-w]

56   **Augustyn M**, Grys I, Kukla M. Small intestinal bacterial overgrowth and nonalcoholic fatty liver disease. *Clin Exp Hepatol* 2019; **5**: 1-10 [PMID: 30915401 DOI: 10.5114/ceh.2019.83151]

57   **Wu WC**, Zhao W, Li S. Small intestinal bacteria overgrowth decreases small intestinal motility in the NASH rats. *World J Gastroenterol* 2008; **14**: 313-317 [PMID: 18186574 DOI: 10.3748/wjg.14.313]

58   **Kessoku T**, Imajo K, Kobayashi T, Ozaki A, Iwaki M, Honda Y, Kato T, Ogawa Y, Tomeno W, Kato S, Higurashi T, Yoneda M, Kirikoshi H, Kubota K, Taguri M, Yamanaka T, Usuda H, Wada K, Kobayashi N, Saito S, Nakajima A. Lubiprostone in patients with non-alcoholic fatty liver disease: a randomised, double-blind, placebo-controlled, phase 2a trial. *Lancet Gastroenterol Hepatol* 2020; **5**: 996-1007 [PMID: 32805205 DOI: 10.1016/S2468-1253(20)30216-8]

59   **Schattenberg JM**. Intestinal motility: a therapeutic target for NAFLD? *Lancet Gastroenterol Hepatol* 2020; **5**: 957-958 [PMID: 32805206 DOI: 10.1016/S2468-1253(20)30204-1]

60   **Gotfried J**, Priest S, Schey R. Diabetes and the Small Intestine. *Curr Treat Options Gastroenterol* 2017; **15**: 490-507 [PMID: 28913777 DOI: 10.1007/s11938-017-0155-x]

61   **Weaver MJ**, McHenry SA, Sayuk GS, Gyawali CP, Davidson NO. Bile Acid Diarrhea and NAFLD: Shared Pathways for Distinct Phenotypes. *Hepatol Commun* 2020; **4**: 493-503 [PMID: 32258945 DOI: 10.1002/hep4.1485]

62   **Reddy SK**, Zhan M, Alexander HR, El-Kamary SS. Nonalcoholic fatty liver disease is associated with benign gastrointestinal disorders. *World J Gastroenterol* 2013; **19**: 8301-8311 [PMID: 24363521 DOI: 10.3748/wjg.v19.i45.8301]

63   **Bush H**, Golabi P, Otgonsuren M, Rafiq N, Venkatesan C, Younossi ZM. Nonalcoholic Fatty Liver is Contributing to the Increase in Cases of Liver Disease in US Emergency Departments. *J Clin Gastroenterol* 2019; **53**: 58-64 [PMID: 29608451 DOI: 10.1097/MCG.0000000000001026]

64   **Dubberke ER**, Reske KA, McDonald LC, Fraser VJ. ICD-9 codes and surveillance for Clostridium difficile-associated disease. *Emerg Infect Dis* 2006; **12**: 1576-1579 [PMID: 17176576 DOI: 10.3201/eid1210.060016]

65   **Scheurer DB**, Hicks LS, Cook EF, Schnipper JL. Accuracy of ICD-9 coding for Clostridium difficile infections: a retrospective cohort. *Epidemiol Infect* 2007; **135**: 1010-1013 [PMID: 17156501 DOI: 10.1017/S0950268806007655]

66   **Khoruts A**, Staley C, Sadowsky MJ. Faecal microbiota transplantation for Clostridioides difficile: mechanisms and pharmacology. *Nat Rev Gastroenterol Hepatol* 2021; **18**: 67-80 [PMID: 32843743 DOI: 10.1038/s41575-020-0350-4]

Observational Study

Six-minute walking test performance is associated with survival in cirrhotic patients

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Abstract

BACKGROUND
Patients with cirrhosis are at risk of cirrhotic cardiomyopathy, with resulting cardiac dysfunction and exercise limitations. Six minute walking test (6MWT) assesses functional status and predicts morbidity and mortality in cardiopulmonary diseases.

AIM
To determine if it associates with mortality by analyzing 6MWT performance in patients with liver cirrhosis.

METHODS
A cohort of 106 cirrhotic patients was evaluated in the outpatient setting with echocardiogram and 6MWT and follow up for one year to document hepatic decompensation and mortality. The distance in meters was recorded at the end of

6 min (6MWD).

RESULTS

This cohort had a mean age of 51 years and 56% male; patients were staged as Child A in 21.7%, B 66% and C 12.3%. Walk distance inversely correlated with Child scores, and was significantly reduced as Child stages progresses. Patients who died (10.4%) showed shorter mean 6MWD ($P = 0.006$). Low 6MWD was an independent predictor of mortality ($P = 0.01$).

CONCLUSION

6MWT is a noninvasive inexpensive test whose result is related to Child scores and mortality. It is useful to identify patients with liver cirrhosis at high risk of mortality for closer monitoring and potential early intervention.

Key Words: Six-minute walking test; Liver cirrhosis; Hospital admission and mortality; Child score

Core Tip: Our study proposes that six-minute walking test, a simple exercise test, can be applicable in the evaluation of cirrhotic patients. This is a well-none routine assessment in patients with cardiopulmonary diseases, where it is used to predict mortality in this population. Its use in liver cirrhosis is limited. Patients with chronic hepatic insufficient are at risk of progressively muscle loss, frailty, and exercise limitation, all factors directly associated with poor survival. We propose by using six-minute walk test a practical and simple manner of assess this risks and provide a better understanding of how exercise limitation can directly affect survival.

INTRODUCTION

Liver cirrhosis is related to functional impairment leading to reduction in physical fitness[1,2]. Some possible factors implicated in this process are profound muscle wasting (or cirrhotic myopathy)[3], cardiac dysfunction (cirrhotic cardiomyopathy)[4], autonomic dysfunction (chronotropic incompetence) and concurrent pulmonary disease (portopulmonary hypertension and hepatopulmonary syndrome). Recently studies reinforce the importance of frailty scores as a prediction of mortality in liver transplantation list[5,6], giving emphasis in sarcopenia and physical fitness as important factors associated with mortality[7].

The six-minute walk test (6MWT) is a practical simple inexpensive test that provides a global assessment of all systems involved during exercise[8]. Although it does not give information about specific organ impairment, it evaluates overall exercise capacity and has been shown, in patients with cardiac disease, to correlate with the maximal oxygen consumption (VO_2) and survival[9].

Some studies demonstrated that short distance during 6MWT (6MWD) predicted poorer prognosis and disease outcome in patients with heart failure[10] and chronic obstructive pulmonary disease[11]. In addition, this test can be used to assess the overall functional status and quantify response to a certain intervention[8] in a variety of other chronic diseases and in the elderly population[9-12].

Previous studies highlight the importance of 6MWD in predicting survival in cirrhotic and non-cirrhotic patients[13-16]. There are also evidences suggesting an association between exercise performance and increase risk of death on the waiting liver transplantation list[15-18]. Despite its role in long term survival in different chronic diseases, the impact in mortality prediction in cirrhotic patients is underes-

timated over years.

The aim of this study was to analyze the association between 6MWT and long-term mortality in a cohort of cirrhotic patients.

MATERIALS AND METHODS

A total of 106 outpatients with liver cirrhosis (57 male, mean age 51.2 ± 12.9 years) was included in the present study. Cirrhosis was defined by clinical history, physical examination, laboratory analysis and at least one imaging data. Disease prognosis and severity were established based on Child and MELD scores, according to original scores definitions[19,20]. Exclusion criteria were any previous or current cardiovascular or pulmonary disease, heart failure or diagnosis of hemochromatosis (when cardiac involvement was documented). Patients who had a history of alcohol abuse (more than 20 g and 60 g of ethanol per day for women and men, respectively)[21] were included if they had abstained from alcohol use for at least 6 mo prior to enrollment. Patients with non-sinus rhythm, decompensated arterial hypertension, low peripheral oxygen saturation ($SpO_2 < 90\%$), recent history (less than 3 mo) of new liver related decompensation or hospitalizations were also excluded (patients with previous ascites or encephalopathy were included, those characterized with chronic decompensated patients). Patients with neuromuscular diseases, myopathy, balance deficits or orthopedic disorders were also excluded. Patients who have previously received a liver transplant were not included. No paracentesis was performed within at least one week prior to exercise, avoiding volume depletion or electrolyte imbalances.

One hundred and sixty-four patients were consecutively screened from two liver transplantation centers between October 2014 and December 2014, 58 out of 164 were excluded according to previous criteria, most of the due to cardiovascular disorders (26%) or active alcohol consumption (19%). On the day of enrollment, patients provided written informed consent and had blood samples collected and 6MWT done. Electrocardiogram and transthoracic bubble echocardiogram were performed within 1 mo of enrollment.

Patients were followed-up by clinical visits, hospital records or telephone calls to patients to capture deaths and their causes. Patients were stratified according to their ability to complete 6MWT, whether they achieved or not predicted distance according to gender and age, and pattern of symptom secondary to physical effort due to the test. Patients included were follow-up to one year, main outcomes were defined as death or liver transplantation.

The study has been performed in accordance with the Declaration of Helsinki (2000) and approved by the Ethics Committee of our institution.

6MWT

The 6MWT was conducted according to American Thoracic Society guidelines[8] and supervised by a qualified physician. The test was performed indoors, along a 30 m flat, straight corridor with a hard surface and free of any type of obstacles. Before starting the test, all patients were provided instructions by the evaluator, encouraged to walk as far as possible within 6 min, and instructed to stop if pain, dyspnea, or other symptoms. The distance in meters was recorded at the end of the six minutes (6MWD). Predicted distances were computed according to specific equations for gender, weight, height and age[22]. Predicted distance achieved percentage (%6MWD) is then derived by dividing the actual 6MWD divided by the predicted distance.

Statistical analysis

Data were analyzed using a statistical software program (IBM® SPSS® Statistics, version 22.0). Logistic regressions were performed to evaluate the independent association between 6MWD and death. Receiver operating curves (ROC) and the area under ROC (AUROC) were computed to estimate sensitivity, specificity and cut-off points for 6MWD used in regression models, selected by Youden's index. COX regression analysis and Kaplan-Meier curves were performed and significant differences between the later were assessed by means of the log-rank test. We performed subgroup analysis according achievement of liver transplantation in order to evaluated 6MWT distance as a predictor of death.

RESULTS

Patient characteristics

The main demographic, clinical, and laboratory characteristics of the patients are presented in Table 1. One hundred and six patients were selected from two liver transplantation centers in Sao Paulo, Brazil. The majority was male (56%), and non-alcoholic etiology of the liver disease was the most common (69.8%). The mean MELD was 11.1, Child B more common (66%), and 74% of patients presented a history of at least one liver related decompensation. Ascites was identified in 32.1% and hepatic encephalopathy in 10.4% of patients on the day of the test.

All patients were followed until death, time of transplantation or end of study follow-up (12 mo). During the study period, 11 patients died and 3 underwent liver transplantation. All deaths were related to hepatic decompensation.

The majority of this cohort (71.7%) did not achieve the predicted distance adjusted for age and gender according to standardized equations[22] (678 ± 131m, 402-890 m) (see Figure 1). 6MWT performance is demonstrated in Table 2. The mean 6MWD of this cohort was 515 ± 138 m, 180-960 m. Not surprisingly, older patients with higher Child score, worse hepatic synthetic function (lower albumin) and anemia performed worse. It was found to be inversely correlated with age (r = -0.391, P < 0.001) and Child score (r = -0.228, P = 0.019), and positively correlated with albumin (r = 0.242, P = 0.012), creatinine (r = 0.242, P = 0.018) and hemoglobin (r = 0.192, P = 0.048). Patients with a history of at least one hepatic decompensation in the past (74.5%) presented with significant shorter 6MWD (496 ± 141 m *vs* 571 ± 115 m, P = 0.015).

The mean 6MWD was progressively shorter among Child classes (A = 570 ± 144 m, B = 504 ± 137 m and C = 471 ± 115 m) and statistical significance was demonstrated between Child A and C (P = 0.04) and when Child A was compared with more advanced stages (B and C), P = 0.02. 6MWD was different among compensated (Child Pugh A) and decompensated (Child Pugh B and C) patients (P = 0.031) (see Figure 2). Patients decompensated with ascites or hepatic encephalopathy on the day of the test achieved shorter distances than those who did not have ascites or hepatic encephalopathy (472 *vs* 534m, P = 0.03; 440 *vs* 525m, P = 0.04, respectively). All patients previously included were submitted to 6MWT, even those with hepatic decompensation at the moment of evaluation, ascites or encephalopathy. 6MWD did not differ according to the etiologies of cirrhosis (P = 0.08), past history of alcohol abuse (P = 0.58), use of beta-blocker (P = 0.19), tobacco (P = 0.97) and presence of anemia (P = 0.84).

None of the patient presented with liver related decompensation within 2 wk following the exercise, meaning no detectable clinically significant portal hypertension increase induced by exercise. All patients were able to perform exercise adequately, without help, interruptions, or any significantly adverse effect.

To emphasize the role of 6MWD and %6MWD in the prediction mortality, as an additional factor besides liver disease severity, logistic regression models were designed to evaluate if the inclusion of 6MWT parameters improves the model performance and increases the AUROC computed using regression models. MELD and Child score were used to quantify the severity of liver disease. When 6MWT parameters were added to the models designed to predicted mortality using MELD or Child score, we observed an improvement in model performance, defined as a significant difference according to Omnibus Chi-square test (P = 0.01) and higher AUROCs in combining models (see Figure 3).

Cutoff points associated with mortality was 387 m for 6MWD (sensibility 90.9 and specificity 88.4) and 0.82 for %6MWD (sensibility 100 and specificity 83.2). After exclusion of patients who were submitted to liver transplantation, patients who died (11, 10.4%) had a shorter mean 6MWD (423 m *vs* 526 m, P = 0.006) and lower %6MWD (0.72 *vs* 0.92, P = 0.004). Just one of them achieved the predicted distance during 6MWT. 6MWD and %6MWD were independent predictors of mortality, after adjusted for Child scores, according to multivariate regression model analysis (Table 3). Patients who achieved distances shorter than 387 m or %6MWD < 0.82 presented higher mortality, and statistical difference according to Kaplan-Meier and log-rank analysis (P = 0.004 and P = 0.006, respectively) (Figure 4).

DISCUSSION

6MWT is a safe, easy-to-administer, and inexpensive test to determine the functional capacity of cirrhotic patients and also has prognostic value. We found that a decreased

Table 1 Patients' characteristics (*n* = 106), *n* (%)

Characteristic	*n* (%) or means ± SD
Gender M/F	59/47 (56/44)
Age (yr)	51 ± 13
BMI (kg/m^2)	25.7 ± 4.7
PASP (mmHg)	25.4 ± 8.0
Cirrhosis etiology	
Virus	36 (33.9)
Alcohol	32 (30.2)
NASH	8 (7.5)
Others	30 (28.4)
Child-Pugh class *n*	7.1 ± 1.8
A	23 (21.7)
B	70 (66)
C	13 (12.3)
MELD	11.1 ± 3.1
Previous history of liver related decompensation	76 (73.8)
Hypertension	19 (17.9)
Diabetes	26 (24.5)
Tobacco smoking	12 (11.4)
Beta-blocker use	32 (30.2)
Hepatic decompensation on the day of the test	
Ascites	34 (32.1)
(Grade 1, 2, and 3)	(11.3, 17, 5)
Peripheral edema	13 (12.3)
Hepatic encephalopathy	13 (12.3)
(Grade 1, 2, 3, and 4)	(10.4, 1.9, 0, 0)
Hepatocellular carcinoma	5 (4.7)
Patient on the liver transplantation waiting list	35 (33)
Baseline laboratory[1]	
Hemoglobin (mg/dL)	13.1 ± 1.9
Hematocrit (%)	39.3 ± 5.4
Albumin (g/dL)Bilirubin (mg/dL)INR	3.5 ± 0.62.0 ± 1.51.2 ± 0.2
Creatinine (mg/dL)	0.8 ± 0.3
Na (mmol/L)	137.8 ± 2.1
K (mmol/L)	4.1 ± 0.5
Mg (mg/dL)	1.8 ± 0.2
Ca (mmol/L)	1.2 ± 0.1

[1]Continuous variables are shown as means ± SD.

Reference range values: Na (136-145); K (3.5-5.0); Mg (1.6-2.6) and Ca (1.15-1.29).

M: Male; F: Female; PASP: Pulmonary arterial systolic pressure.

Table 2 Six minute walking test performance in 106 patients with liver cirrhosis

Variable	6MWD (m)	P (*t*-test when applicable)	6MWD (%)	P (*t*-test when applicable)
Mean 6MWD (m)	515 ± 138			
Mean 6MWD (%)			0.91 ± 2.3	
6MWD according to Child classes				
A	570 ± 144		0.97 ± 0.22	
B	504 ± 137		0.88 ± 0.21	
C	471 ± 115		0.82 ± 0.25	
6MWD according to Liver decompensation				
Ascites (w vs wo)	473 ± 20 *vs* 535 ± 17	0.03	0.86 ± 0.22 *vs* 0.95 ± 0.21	0.028
Hepatic encephalopathy (w *vs* wo)	435 ± 34 *vs* 525 ± 14	0.04	0.87 ± 0.25 *vs* 0.91 ± 0.21	0.87
History of previous hepatic decompensation (w *vs* wo)	496 ± 141 *vs* 571 ± 115	0.02	0.86 ± 0.22 *vs* 1.02 ± 0.17	0.004
Hospital admission during follow-up (w *vs* wo)	444 ± 172 *vs* 531 ± 125	0.01	0.77 ± 0.25 *vs* 0.92 ± 0.20	0.004
Survival (died *vs* survived)	423 ± 122 *vs* 526 ± 137	0.02	0.72 ± 0.21 *vs* 0.93 ± 0.21	

6MWT: Six-minute walking test; 6MWD: Six-minute walking distance; 6MW (%): Predicted distance achieved percentage; w: With; wo: Without.

6MWD, as a marker of impaired exercise capacity, is associated with hepatic dysfunction. In addition, 6MWD and %6MWD performed as independent predictors of mortality, becoming an important tool during risk evaluation of severe complications and death in liver cirrhosis. Also, this study reinforces the key importance of physical evaluation during cirrhotic patients, especially those referred to liver transplantation team.

Basal exercise capacity was significantly impaired in our patients, as only 28.3% achieved the pre-test predicted distance. The 6MWD results in our cohort of patients was similar to previous studies in patients with cirrhosis which found a significantly lower 6MWD values than expected for healthy population[22]. Our cohort had a mean 89.7% (34.8%-149%) of predicted 6MWD (*vs* 63% found by Román *et al*[18], and a mean 6MWD of 515 m (180-960 m), compared to 306 m in Alameri *et al*[14]'s cohort of 98 patients with cirrhosis. The poor performance during 6MWT meets with the current knowledge about the abnormal exercise capacity in cirrhotic patients. Future studies should verify those findings and evaluate if 6MWD can be used as a more general tool able to evaluate outcomes and quality of life in this group[15].

We reported a weak inverse correlation between 6MWD and Child scores (*r* = -0.228, *P* = 0.019), although it was clear the tendency in walk distance reduction along Child classes. Carey *et al*[15], studying 121 cirrhotic patients, showed a strong correlation with MELD. In this particular study, all patients were listed for liver transplant, denoting a population with more advanced disease, making us understand that this stronger correlation reflects a major prevalence of their patient's overall disability when comparing to our study group. In the same way, by comparing subjects with advanced disease (Child B and C) and those without it (Child A), we detected a significant difference between these groups (*P* = 0.02), supporting the previous interpretation. Furthermore, patients with a history of at least one hepatic decompensation in the past, presented shorter 6MWD (*P* = 0.015) and subjects presenting with ascites or encephalopathy at the moment of evaluation performed worse, these facts highlight the relationship between shorter distances and severity of liver disease in our study. Similarly, Wong *et al*[23] reported that patients with decompensated cirrhosis with ascites performed worse during cycle ergometer evaluation when compared to well compensated patients, however, no specific data is

Table 3 Association between six-minute walking test parameters and unfavorable clinical outcomes (hospital admissions and mortality) using logistic regression models

Predictors	Hospital Admission						Mortality					
	Univariate			Multivariate			Univariate			Multivariate		
	b	p	OR	b	p	OR	b	p	OR	b	p	OR
Child score	0.74	< 0.01	2.1	0.72	< 0.01	2.05	1.01	< 0.01	2.75	1.03	< 0.01	2.8
6MWD	-0.005	< 0.01	0.99	-0.005	0.24	0.99	-0.007	0.01	0.99	-0.007	0.04	0.99
%6MWD	-0.04	0.01	0.96	-0.03	0.03	0.96	-0.05	0.02	0.95	-0.05	0.03	0.95
6MWD ≤ 444 m	-1.395	0.007	0.3	-1.462	0.01	0.2	-	-	-	-	-	-
6MWD ≤ 387 m	-	-	-	-	-	-	1.659	0.004	5.25	-1.17	0.2	0.3[1]

[1]Confidential intervals for odds ratio are not represented but consider adequate for all analysis except for odds ratio.

OR: Odds ratio.

available regarding 6MWT.

Although the gold standard measurement of exercise capacity is maximal VO_2[24] measurement during treadmill or cycle ergometer tests, 6MWT is a cheap and simple test found to correlate with oxygen consumption that can be administered without special equipment or skilled staff that you can perform in clinic to give an immediate result. Noticeable that all patients in our study completed the full test, independently of the presence of ascites or encephalopathy, demonstrating one great advantage above other exercise tests, that sometimes require a more complex adaptation and comprehension about the technique. Cahalin *et al*[9] performed 6MWT and symptom-limited cardiopulmonary exercise testing in patients with heart failure during cardiac transplant evaluation. The authors described a significant correlation between 6MWD and peak VO_2 ($r = 0.64$, $P < 0.001$), concluding that 6MWT is a valuable tool to predict VO_2 and short-term survival. These results should be validated in cirrhotic population, but represent a good evidence that 6MWT could be introduced in routine practice without loss of diagnostic accuracy in exercise capacity estimation. While our study did not evaluate the association between VO_2 and 6MWD, it did show the safety and practicality of this procedure. García-Pagàn *et al*[25] reported that moderate exercise (30% of the maximum) significantly increases portal pressure in patients with portal hypertension, and, therefore, could increase the risk of variceal bleeding, ascites and encephalopathy. Although 6MWT is a submaximal exercise, we did not identify any clinical event directly associated with it during the period following the test. Recent studies do not mention the prevalence of adverse events induced by exercise, and more studies designed to respond this issue should be carried out.
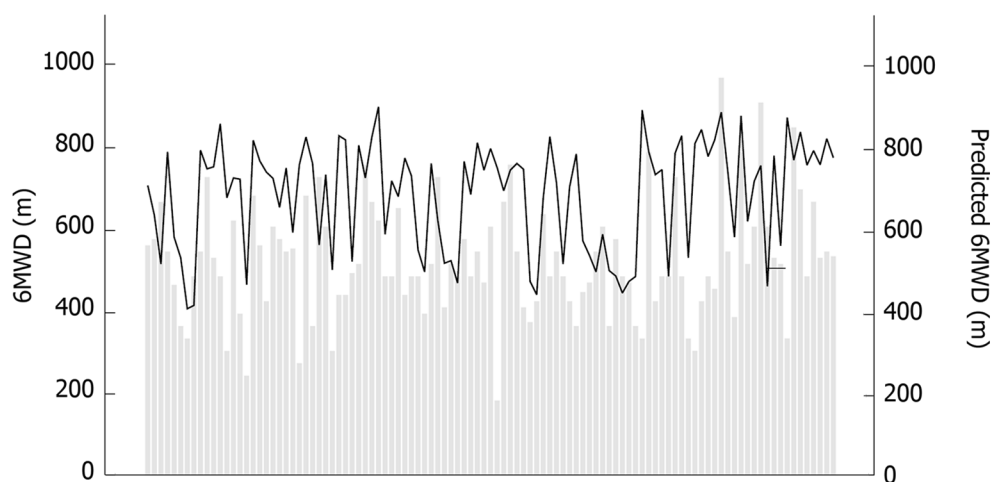
Figure 1 Relationship between predicted (line) and performed (bars) walking distance during six minute walking test. 6MWD: Six minute walking distance.
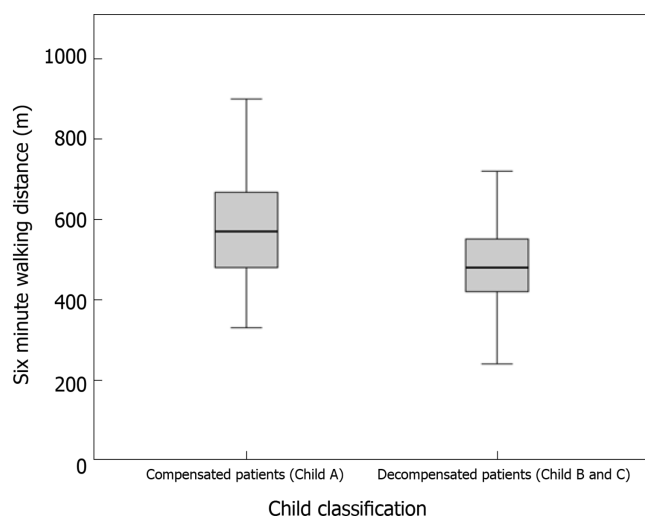

Figure 2 Distance in meters was recorded at the end of the six minutes among compensated (Child Pugh A) and decompensated (Child Pugh B and C) patients

Previously studies who reported the relationship between 6MWT and mortality were conducted with small populations and during a short period of followup[11,12]. Poor performance during 6MWT may warrant that the at-risk patients should be followed more closely due to the risk of adverse events. Notwithstanding, 6MWT has been proposed as a tool during frailty status evaluation, giving emphasis in this role as a practical and cheap method for this proposal. This study reinforces this importance, adding more powerful results due to our long period of follow-up, demonstrating how physical exercise evaluation may be an interesting long predictor of prognosis in cirrhotic patients.

In our study, 6MWD was an independent predictor of death, consistent with findings from previous studies by Alameri *et al*[14], and Carey *et al*[15]. In the first study, mortality was evaluated in the whole group, including patients with non-cirrhotic chronic hepatitis, which may bias the interpretation about causality between 6MWD and cirrhosis. Also, Carey *et al*[15] studied a population with more advanced disease, all of them on the liver transplant waiting list with a high frequency of liver transplantation (50.4%) performed in a short period of time (5-6 mo). The statistical power of 6MWT in predicting mortality could be affected by pulling out so many patients after transplant from this cohort.

The role of 6MWD and %6MWD in the prediction of mortality were independently of Child scores as demonstrated by multivariate logistic regression analysis. These facts highlight the association of 6MWT parameters with disease progression and adverse outcomes, despite the severity of liver disease.
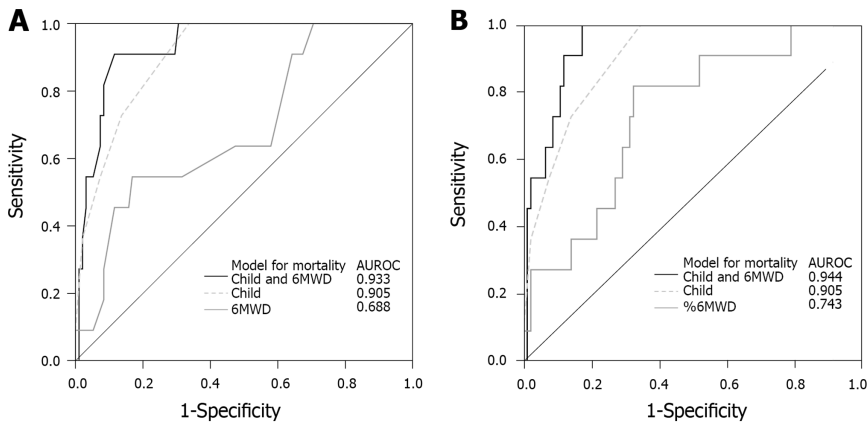
Figure 3 Progressive improvement in prediction of mortality using models combing six minute walking test parameters and Child scores.
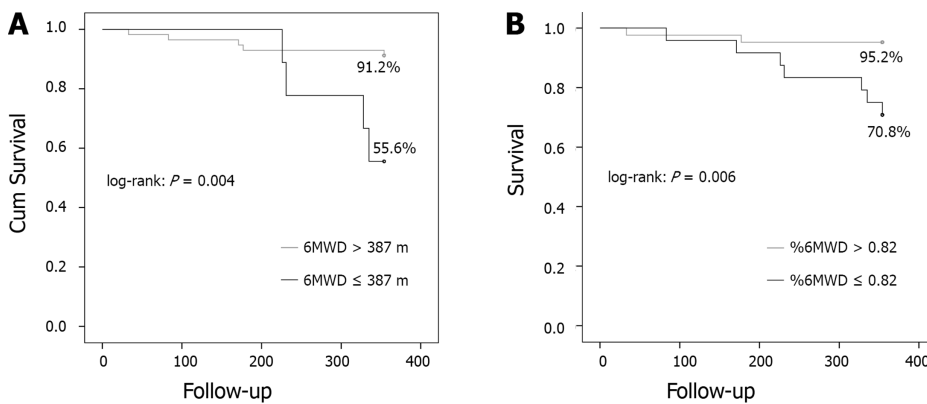6MWD: Six minute walking distance.

Figure 4 Kaplan Meier analysis for overall survival.

There are several limitations to our study. First, we did not proceed an external validation of 6MWD cutoffs used in our study, although our main objectives were focused in the transversal and descriptive characterization of study population. Second, we did neither evaluated nutritional status nor calculate the Frailty score of our patients. When study was designed there were no clear parameters specific settle for this diagnosis and a retrospective evaluation was not possible due to lack of complete data. Although recent studies suggest a close relationship between malnourished patients and physical capacity, in order to better evaluate this relationship, another specific protocol must be designed, which was not in accordance with our main objectives. Finally, we did not submit this cohort to a second phase 6MWT to evaluate the relationship between test performance and disease progression. Maybe this analysis could enhance the comprehension about the association of shorter 6MWD and severity of liver disease and its role as a marker of liver decompensation episodes. As we proposed a sectional evaluation of cirrhotic patients with 6MWT, future prospective studies should be able to better answer the previous questions.

CONCLUSION

In summary, 6MWT is a very simple, inexpensive, well tolerated, noninvasive test to assess exercise capacity and the result of which is related to MELD and Child scores. The present study showed that 6MWD is an independent predictor of mortality in this population. 6MWT is a promising prognostic marker in patients with liver cirrhosis and should be considered as part of liver transplantation evaluation especially in those referred for the liver transplantation team.

ARTICLE HIGHLIGHTS

Research background

Patients with cirrhosis are at risk of exercise limitations due to progressive limitations related to liver dysfunction. Sarcopenia and cirrhotic cardiomyopathy may be possible related factors. The six-minute walking test (6MWT) is a known simple and practical tool used to evaluate patients with cardiopulmonary disease.

Research motivation

In face of limited diagnosis tools focused on exercise capacity, we purposed to evaluate the role of 6MWT in this population.

Research objectives

The aim of our study was to analyzed 6MWT performance in patients with liver cirrhosis to determine if it associates with mortality.

Research methods

We analyzed 6MWT performance in 106 cirrhotic patients. They were evaluated in the outpatient setting with 6MWT and follow up for one year. Hepatic decompensation and mortality were documented.

Research results

This cohort had a mean age of 51 years and 56% male; patients were staged as Child A in 21.7%, B 66%, and C 12.3%. Walk distance inversely correlated with Child scores, and was significantly reduced as Child stages progress. Patients who died (10.4%) showed a shorter mean 6MWD ($P = 0.006$). Low 6MWD was an independent predictor of mortality ($P = 0.01$).

Research conclusions

6MWT is a noninvasive inexpensive test whose result is related to Child scores and mortality.

Research perspectives

It is a useful, simple, practical test that can be incorporated into cirrhotic evaluation due to its relation with mortality for closer monitoring and potential early intervention.

ACKNOWLEDGEMENTS

REFERENCES

1   **Jones JC**, Coombes JS, Macdonald GA. Exercise capacity and muscle strength in patients with cirrhosis. *Liver Transpl* 2012; **18**: 146-151 [PMID: 22139897 DOI: 10.1002/lt.22472]

2   **Campillo B**, Fouet P, Bonnet JC, Atlan G. Submaximal oxygen consumption in liver cirrhosis. Evidence of severe functional aerobic impairment. *J Hepatol* 1990; **10**: 163-167 [PMID: 2332586 DOI: 10.1016/0168-8278(90)90046-t]

3   **Bunchorntavakul C**, Reddy KR. Review article: malnutrition/sarcopenia and frailty in patients with cirrhosis. *Aliment Pharmacol Ther* 2020; **51**: 64-77 [PMID: 31701570 DOI: 10.1111/apt.15571]

4   **Izzy M**, VanWagner LB, Lin G, Altieri M, Findlay JY, Oh JK, Watt KD, Lee SS; Cirrhotic Cardiomyopathy Consortium. Redefining Cirrhotic Cardiomyopathy for the Modern Era. *Hepatology* 2020; **71**: 334-345 [PMID: 31342529 DOI: 10.1002/hep.30875]

5   **Montano-Loza AJ**. Muscle wasting: a nutritional criterion to prioritize patients for liver transplantation. *Curr Opin Clin Nutr Metab Care* 2014; **17**: 219-225 [PMID: 24613858 DOI: 10.1097/MCO.0000000000000046]

6   **Lai JC**, Volk ML, Strasburg D, Alexander N. Performance-Based Measures Associate With Frailty in Patients With End-Stage Liver Disease. *Transplantation* 2016; **100**: 2656-2660 [PMID: 27495749 DOI: 10.1097/TP.0000000000001433]

7   **Bhanji RA**, Montano-Loza AJ, Watt KD. Sarcopenia in Cirrhosis: Looking Beyond the Skeletal

Muscle Loss to See the Systemic Disease. *Hepatology* 2019; **70**: 2193-2203 [PMID: 31034656 DOI: 10.1002/hep.30686]

8   **ATS Committee on Proficiency Standards for Clinical Pulmonary Function Laboratories**. ATS statement: guidelines for the six-minute walk test. *Am J Respir Crit Care Med* 2002; **166**: 111-117 [PMID: 12091180 DOI: 10.1164/ajrccm.166.1.at1102]

9   **Cahalin LP**, Mathier MA, Semigran MJ, Dec GW, DiSalvo TG. The six-minute walk test predicts peak oxygen uptake and survival in patients with advanced heart failure. *Chest* 1996; **110**: 325-332 [PMID: 8697828 DOI: 10.1378/chest.110.2.325]

10 **Roul G**, Germain P, Bareiss P. Does the 6-minute walk test predict the prognosis in patients with NYHA class II or III chronic heart failure? *Am Heart J* 1998; **136**: 449-457 [PMID: 9736136 DOI: 10.1016/S0002-8703(98)70219-4]

11 **Celli BR**, Cote CG, Marin JM, Casanova C, Montes de Oca M, Mendez RA, Pinto Plata V, Cabral HJ. The body-mass index, airflow obstruction, dyspnea, and exercise capacity index in chronic obstructive pulmonary disease. *N Engl J Med* 2004; **350**: 1005-1012 [PMID: 14999112 DOI: 10.1056/NEJMoa021322]

12 **Troosters T**, Gosselink R, Decramer M. Six minute walking distance in healthy elderly subjects. *Eur Respir J* 1999; **14**: 270-274 [PMID: 10515400 DOI: 10.1034/j.1399-3003.1999.14b06.x]

13 **Enright PL**, McBurnie MA, Bittner V, Tracy RP, McNamara R, Arnold A, Newman AB; Cardiovascular Health Study. The 6-min walk test: a quick measure of functional status in elderly adults. *Chest* 2003; **123**: 387-398 [PMID: 12576356 DOI: 10.1378/chest.123.2.387]

14 **Alameri HF**, Sanai FM, Al Dukhayil M, Azzam NA, Al-Swat KA, Hersi AS, Abdo AA. Six Minute Walk Test to assess functional capacity in chronic liver disease patients. *World J Gastroenterol* 2007; **13**: 3996-4001 [PMID: 17663517 DOI: 10.3748/wjg.v13.i29.3996]

15 **Carey EJ**, Steidley DE, Aqel BA, Byrne TJ, Mekeel KL, Rakela J, Vargas HE, Douglas DD. Six-minute walk distance predicts mortality in liver transplant candidates. *Liver Transpl* 2010; **16**: 1373-1378 [PMID: 21117246 DOI: 10.1002/lt.22167]

16 **Dharancy S**, Lemyze M, Boleslawski E, Neviere R, Declerck N, Canva V, Wallaert B, Mathurin P, Pruvot FR. Impact of impaired aerobic capacity on liver transplant candidates. *Transplantation* 2008; **86**: 1077-1083 [PMID: 18946345 DOI: 10.1097/TP.0b013e318187758b]

17 **Veloso-Guedes CA**, Rosalen ST, Thobias CM, Andreotti RM, Galhardo FD, Oliveira da Silva AM, Araujo O, Boin IF. Validation of 20-meter corridor for the 6-minute walk test in men on liver transplantation waiting list. *Transplant Proc* 2011; **43**: 1322-1324 [PMID: 21620120 DOI: 10.1016/j.transproceed.2011.03.057]

18 **Román E**, Torrades MT, Nadal MJ, Cárdenas G, Nieto JC, Vidal S, Bascuñana H, Juárez C, Guarner C, Córdoba J, Soriano G. Randomized pilot study: effects of an exercise programme and leucine supplementation in patients with cirrhosis. *Dig Dis Sci* 2014; **59**: 1966-1975 [PMID: 24599772 DOI: 10.1007/s10620-014-3086-6]

19 **Child CG**, Turcotte JG. Surgery and portal hypertension. *Major Probl Clin Surg* 1964; **1**: 1-85 [PMID: 4950264]

20 **Malinchoc M**, Kamath PS, Gordon FD, Peine CJ, Rank J, ter Borg PC. A model to predict poor survival in patients undergoing transjugular intrahepatic portosystemic shunts. *Hepatology* 2000; **31**: 864-871 [PMID: 10733541 DOI: 10.1053/he.2000.5852]

21 **O'Shea RS**, Dasarathy S, McCullough AJ; Practice Guideline Committee of the American Association for the Study of Liver Diseases; Practice Parameters Committee of the American College of Gastroenterology. Alcoholic liver disease. *Hepatology* 2010; **51**: 307-328 [PMID: 20034030 DOI: 10.1002/hep.23258]

22 **Enright PL**, Sherrill DL. Reference equations for the six-minute walk in healthy adults. *Am J Respir Crit Care Med* 1998; **158**: 1384-1387 [PMID: 9817683 DOI: 10.1164/ajrccm.158.5.9710086]

23 **Wong F**, Girgrah N, Graba J, Allidina Y, Liu P, Blendis L. The cardiac response to exercise in cirrhosis. *Gut* 2001; **49**: 268-275 [PMID: 11454805 DOI: 10.1136/gut.49.2.268]

24 **McArdle W**, Katch F, Katch V.   Exercise Physiology. 7th ed. The Point, editor. Lippincott Williams & Wilkins; 2010: 192–247

25 **García-Pagàn JC**, Santos C, Barberá JA, Luca A, Roca J, Rodriguez-Roisin R, Bosch J, Rodés J. Physical exercise increases portal pressure in patients with cirrhosis and portal hypertension. *Gastroenterology* 1996; **111**: 1300-1306 [PMID: 8898644 DOI: 10.1053/gast.1996.v111.pm8898644]

Incidence of umbilical vein catheter-associated thrombosis of the portal system: A systematic review and meta-analysis

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Abstract

BACKGROUND
The use of umbilical venous catheters (UVCs) in the perinatal period may be associated with severe complications, including the occurrence of portal vein thrombosis (PVT).

AIM
To assess the incidence of UVC-related PVT in infants with postnatal age up to three months.

METHODS
A systematic and comprehensive database searching (PubMed, Cochrane Library, Scopus, Web of Science) was performed for studies from 1980 to 2020 (the search was last updated on November 28, 2020). We included in the final analyses all

peer-reviewed prospective cohort studies, retrospective cohort studies and case-control studies. The reference lists of included articles were hand-searched to identify additional studies of interest. Studies were considered eligible when they included infants with postnatal age up to three months with UVC-associated PVT. Incidence estimates were pooled by using random effects meta-analyses. The quality of included studies was assessed using the Newcastle-Ottawa scale. The systematic review was performed according to the Preferred Reporting Items for Systematic reviews and Meta-Analysis (PRISMA) guidelines.

RESULTS
Overall, 16 studies were considered eligible and included in the final analyses. The data confirmed the relevant risk of UVC-related thrombosis. The mean pooled incidence of such condition was 12%, although it varied across studies (0%-49%). In 15/16 studies (94%), diagnosis of thrombosis was made accidentally during routine screening controls, whilst in 1/16 study (6%) targeted imaging assessments were carried out in neonates with clinical concerns for a thrombus. Tip position was investigated by abdominal ultrasound (US) alone in 1/16 (6%) studies, by a combination of radiography and abdominal US in 14/16 (88%) studies and by a combination of radiography, abdominal US and echocardiography in 1/16 (6%) studies.

CONCLUSION
To the best of our knowledge, this is the first systematic review specifically investigating the incidence of UVC-related PVT. The use of UVCs requires a high index of suspicion, because its use is significantly associated with PVT. Well-designed prospective studies are required to assess the optimal approach to prevent UVC-related thrombosis of the portal system.

Core Tip: Portal vein thrombosis (PVT) is a dreadful complication that can occur after umbilical vein catheterization in neonates. Although previous observational studies have provided a general overview about the risk of this complication, the present systematic review specifically investigates the incidence catheter-related PVT and identifies relevant gaps in knowledge about the optimal diagnostic approach highlighting the need for prospective randomized studies and updated guidelines.

INTRODUCTION
The placement of an umbilical venous catheter (UVC) is a common procedure in neonatology and has multiple clinical indications driven by the need for quick and secure access for medication administration[1]. During placement, the UVC should run through the umbilical vein, pass the medial portion of the left portal vein at the umbilico-portal confluence, join the direct communication existing between the umbilical vein and the ductus venosus and, through it, bypass the liver and join the inferior vena cava[2,3]. The UVC has to be placed in a central position, ideally at the junction between the inferior vena cava and the right atrium. If a central position is not achieved, then the tip of the catheter can be left below the liver, *i.e.*, below the level of umbilical-portal confluence (peripheral position). The UVC in peripheral position can be used as an emergency access, but it has to be replaced as soon as possible by a

central venous catheter. To prevent UVC-related complications, a proper assessment of catheter tip position is mandatory before its use. In fact, if the tip of the catheter is too deep, it can cause complications such as thrombo-embolic disorders, arrhythmias, and pericardial effusion. On the other hand, if the tip of the UVC is too low, then it can be associated with necrotizing enterocolitis, colon perforation, hepatic abscess, and portal vein thrombosis (PVT)[1,4-9]. Furthermore, if the ductus venosus is not perfectly aligned to the umbilical vein, the UVC may unintentionally enter the portal system through the left portal vein during placement and possibly lead to severe complications involving both the hepatic vasculature and parenchyma[1,2,5-8,10-16]. Such liver complications may arise from multiple mechanisms including thrombosis of the portal system vasculature, infusion of irritating drugs and/or hypertonic solutions within the UVC leading to hepatic necrotizing direct mechanical injury[3,17-19]. Besides individual hereditary or acquired predisposing factors (such as prematurity, hereditary prothrombotic disorders, sepsis, the need of transfusions, hyper-viscosity syndrome, dehydration, asphyxia, congenital malformations *etc*.), whose actual role is still debated[3,10,19-26], umbilical venous catheterization itself represents a risk factor for the development of PVT[18]. In fact, multiple factors may explain the association between UVC and PVT: The introduction of a foreign surface with thrombogenic properties in a small diameter vessel, endothelial damage, and the well-known pro-thrombotic predisposition typical of the neonatal period[27-29]. Symptoms/signs suggestive of PVT may include unexplained thrombocytopenia, catheter-obstructed fluid delivery, increased UVC in-line pressure, impaired lower body/extremity perfusion, although PVT may remain completely asymptomatic[30,31]. When persisting, PVT may inflict substantial damage to the liver leading to portal hypertension, mainly related to the increased vascular resistance in the portal venous system, and to liver atrophy[11,19,32].

In the present systematic review, we specifically focused our search attention on the risk of UVC-related PVT. Although multiple observational studies have provided an overview about the risk of PVT after UVC positioning, to the best of our knowledge no reviews explored systematically this issue. Our aim was to investigate the most accurate information about the actual incidence of UVC-related PVT in the neonatal setting, and to assess if any particular risk factor was systematically associated with the development of such complication.

MATERIALS AND METHODS

The review followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) reporting guidelines[33].

The PICOS strategy was used, which comprised the following (PRISMA): Population: Infants with less than three months of postnatal age; Intervention (or exposure): Umbilical venous catheter; Comparison: No catheter; Outcome (primary): Incidence of PVT; Outcome (secondary): Association with a specific risk factor; Study type: Peer-reviewed observational, cohort and case-control studies.

There was no funding agency for this study. The systematic review did not require ethical approval/informed consent since there was no direct contact with individual patients, and only previously published data were included in the analyses.

Outcomes

The primary outcome was the incidence of PVT related to the use of UVCs (UVC only/attempted UVC/UVC + umbilical artery catheters) in infants with postnatal age up to three months. The secondary outcome was the identification of any risk factor associated with the development of UVC-related PVT.

Search strategy and selection criteria

The following search strategy was used: (portal OR vein OR system OR hepatic) AND (thrombosis) AND (neonat∗ OR newborn OR pediatric∗) AND (catheter∗ OR umbilical). For reliability, three review authors (Bersani I, Iacona G and Piersigilli F) independently analyzed the currently available literature through systematic and comprehensive database searching (PubMed, Cochrane Library, Scopus, Web of Science) from 1980 to 2020 (the search was last updated on November 28, 2020). Reviews, *in vitro* studies, animal studies, autopsy studies and conference abstracts were excluded. The reference lists of the included articles were hand-searched to identify additional studies of interest. We obtained the full texts of all the potentially eligible studies.

Eligibility criteria

Three review authors independently undertook eligibility assessment (Bersani I, Iacona G and Piersigilli F). Any disagreement about study eligibility was resolved by discussion with a fourth review author (Garcovich M) until consensus. We considered the studies eligible if they investigated the incidence of UVC-related PVT in infants with postnatal age up to three months. For articles resulting eligible based on the title or abstract, the full paper was retrieved. Case reports were considered not eligible for the final analyses being the calculation of an incidence not possible for such study design. Non-English studies were considered not eligible for the final analyses. We finally included all peer-reviewed, English-language, prospective/retrospective cohort studies and case-control studies.

Study quality assessment

To assess the risk of bias, two authors (Bersani I and Garcovich M) independently used the Newcastle-Ottawa Scale for comparative nonrandomized studies corresponding to each study's design (cohort/cross-sectional)[34]. Such scale is a validated quality assessment instrument for non-randomized trials which evaluates three parameters of study quality: selection, comparability and exposure assessment. The scale assigns a maximum score of 4 for selection, 2 for comparability, and 3 for exposure, for a maximum total score of 9. Studies with a total score of ≥ 5 or ≥ 7 were considered to be of moderate or high quality, whereas those with a score of less than 5 were considered low-quality studies with high risk of bias. The scale results were tabulated in Table 1.

Data extraction

Three review authors independently performed data extraction (Bersani I, Iacona G and Piersigilli F). Disagreements about data extraction were resolved by discussion with a fourth review author (Garcovich M) until consensus. Pertinent findings from the included studies were tabulated in Table 2 and assessed according to pre-specified subgroups analyses: (1) Year of publication: 1980-2000 or 2001-2020; (2) Indication for thrombosis assessment: Abdominal US as systematic screening or abdominal ultrasound (US) only in case of a clinical concern for thrombosis; (3) Type of diagnostic technique to detect tip position: Radiography or/and (US) evaluation; (4) UVC model: UVC material, size (French), single or double lumen; (5) Thrombosis localization and type: Exact localization within the portal system, complete or partial; (6) Dwell time: Mean UVC in situ persistence (in days); and (7) Prophylaxis: None or heparin infusion or other.

Statistical analysis

Because of high heterogeneity, pooled data on the incidence of UVC-related PVT were analyzed using a random effects (DerSimonian and Laird method) model approach. Statistical heterogeneity among studies was assessed with Cochran's Q and quantified with Higgins I^2 statistic[35,36]. We considered an I^2 of < 25% as low heterogeneity, I^2 of 25% to 75% as moderate heterogeneity and I^2 > 75% as high heterogeneity. Publication bias was assessed graphically using funnel plots and qualitatively using Egger's regression and Begg rank correlation method. Statistical analysis was performed by using the Statistical Package for Social Science (SPSS 22.0; SPSS Inc, Chicago, IL, United States) and Microsoft Excel (Version 16.45).

RESULTS

The searches identified 2460 potentially relevant papers, 1835 after duplicates were removed. After title and abstract screening, 53 full-text studies were considered potentially eligible for inclusion and 37 studies were then excluded for the following reasons: (1) Not relevant comparators (n = 23); (2) Non-English language (n = 3); and (3) Wrong study design (n = 11) (Figure 1). Since the design/methodologies varied among different studies, information was not uniformly available for all analyses. For example, some studies could not be considered eligible, although pertinent, since the exact incidence UVC-associated PVT and/or the exact site of a catheter-related thrombosis and/or the exact age of patients with PVT could not be clearly extrapolated from the results.

According to the Newcastle-Ottawa Scale assessing the risk of bias, all the included studies were of moderate-high quality (Table 1). The characteristics and most relevant findings of the included studies are summarized in Table 2[5,21,30-32,37-45]. Of the 16

Table 1 Risk of bias assessment (Newcastle-Ottawa scale for non-randomized studies)

Ref.	Selection	Comparability	Outcome	Total score
Levit *et al*[42], 2020	4	2	3	9
Dubbink-Verheij *et al*[31], 2020	4	2	3	9
Chen *et al*[15], 2020	4	0	3	7
Hwang *et al*[46], 2020	4	2	3	9
Çakır *et al*[38], 2020	4	0	3	7
Cabannes *et al*[32], 2018	4	2	3	9
Derinkuyu *et al*[5], 2018	4	0	3	7
Chandrashekhar *et al*[45], 2015	4	0	3	7
Michel *et al*[37], 2012	4	2	3	9
Gharehbaghi *et al*[39], 2011	4	2	3	9
Sakha *et al*[41], 2007	4	2	3	9
Turebylu *et al*[21], 2007	4	2	3	9
Kim *et al*[30], 2001	4	2	3	9
Boo *et al*[44], 1999	4	2	3	9
Schwartz *et al*[40], 1997	4	0	3	7
Yadav *et al*[43], 1993	4	0	2	6

included studies, 14 were prospective and 2 were retrospective[15,46]. In some cases, the information about the clinical features of the included population was generically related to the overall cohort rather than specifically to neonates with UVC-related PVT and could not be extrapolated.

In the present review a total pooled sample of 4509 of neonates aged less than three months with UVC was included, 195 of whom experienced UVC-related PVT. The sample sizes ranged widely across studies (median, 83 patients; range, 22-2017). Mean gestational age and birth weight were 30.9 wk and 1738 g respectively, but it was not possible to extrapolate these data from each study, since neonates with PVT sometimes only represented a subgroup, whilst the available data mostly referred to the overall cohort. Figure 2 presents the results of overall meta-analysis with a random effects overall pooled-estimated incidence of UVC-related PVT of 12% [95% confidence interval (CI): 5.91-20.16], with high heterogeneity [I^2 = 97.5% (95%CI: 97.1%-97.9%)]. Figure 3 shows evidence of publication bias, as indicated by visual inspection of the funnel plot and by the Egger test for small study effects for the primary outcome [bias coefficient for the main analysis, 3.5309 (95%CI: 1.983176-5.078624); P = 0.0002].

When investigating the pre-specified subgroups analyses, we found the following data (Table 2): (1) Year of publication: Overall, 3/16 (19%) studies were published between 1980 and 2000, whereas 13/16 (81%) between 2001 and 2020; (2) Indication for thrombosis assessment: In 15/16 studies (94%), the diagnosis of thrombosis was made accidentally during routine screening controls, whilst in 1/16 study (6%) targeted imaging assessments were carried out in neonates with clinical concerns for a thrombus. In most studies it was not possible to extrapolate mean age at the time of PVT diagnosis (Table 2); (3) Type of diagnostic technique used to assess tip position: Tip position was never assessed exclusively by radiography or echocardiography alone, while it was investigated by abdominal US alone in 1/16 (6%) studies, by a combination of radiography and abdominal US in 14/16 (88%) studies and by a combination of radiography, abdominal US and echocardiography in 1/16 (6%) studies. Only a minority of studies (3/16 studies, with a total number of 39/195 neonates) explicitly specified wrong tip position at the first imaging assessment, in UVC-related PVT cases[32,37,39]. However, most of the studies did not provide such information specifically for neonates who developed PVT, but rather for the overall population. Follow-up imaging controls were scheduled differently across studies; (4) UVC model: Information about UVC material, size and lumen number was only specified by a minority of studies. When the information was available, the studies reported the use of polyvinyl UVCs (*n* = 3/16) or polyurethane (*n* = 3/16) UVCs. When described, UVC size varied from 2.5 French to 5 French; (5) Thrombosis

Table 2 Characteristics of included studies

Ref.	Study design	UVC with PVT	UVC without PVT	Dwel time UVC with PVT	Dwel time UVC without PVT	Indication to UVC control	Type of imaging	Country/territory
Levit *et al*[42], 2020	Prospective	1	2016	N/A	N/A	Clinical Suspicion	X-ray + US	United States
Dubbink-Verheij *et al*[31], 2020	Prospective	13	27	N/A	N/A	Screening	X-ray + US	The Netherlands
Chen *et al*[15], 2020	Retrospective	7	1320	N/A	N/A	Screening	X-ray + US	Taiwan
Hwang *et al*[46], 2020	Retrospective	15	54	N/A	N/A	Screening	X-ray + US	South Korea
Çakır *et al*[38], 2020	Prospective	13	83	10.5 ± 4.3[1]	12.2 ± 4.1[1]	Screening	X-ray + US	Turkey
Cabannes *et al*[32], 2018	Prospective	51	53	N/A	N/A	Screening	X-ray + US	France
Derinkuyu *et al*[5], 2018	Prospective	15	229	N/A	N/A	Screening	X-ray + US	Turkey
Chandrashekhar *et al*[45], 2015	Prospective	3	27	N/A	N/A	Screening	X-ray + US	India
Michel *et al*[37], 2012	Prospective	2	59	N/A	N/A	Screening	X-ray + US + Echocardiography	France
Gharehbaghi *et al*[39], 2011	Prospective	5	159	N/A	N/A	Screening	X-ray + US	Iran
Sakha *et al*[41], 2007	Prospective	17	33	2 ± 1.12[1]	N/A	Screening	US	Iran
Turebylu *et al*[21], 2007	Prospective	2	26	N/A	6	Screening	X-ray + US	United States
Kim *et al*[30], 2001	Prospective	43	57	> 6 d in 23/43	> 6 d in 6/57	Screening	X-ray + US	South Korea
Boo *et al*[44], 1999	Prospective	0	57	N/A	N/A	Screening	X-ray + US	Malaysia
Schwartz *et al*[40], 1997	Prospective	1	99	3	4 (0-12)[2]	Screening	X-ray + US	United States
Yadav *et al*[43], 1993	Prospective	7	15	N/A	N/A	Screening	X-ray + US	India

[1]Results are expressed as mean ± SD, if reported.

[2]Results are expressed as median (range), if reported.

UVC: umbilical venous catheter; PVT: portal vein thrombosis; N/A: Not applicable; US: Ultrasound (abdominal).

localization and type: Only a minority of studies specified PVT exact localization within the portal system. When reported, the left portal vein was the most frequently involved. Similarly, only a minority of studies (in a total number of 84/195 neonates) specified if PVT was complete or partial[5,30,38-41]. According to the available data, PVT was complete in 27/84 (32%) cases and partial in 57/84 (68%) cases; (6) Dwell time: Only a minority of studies reported explicitly the mean UVC dwelling time in neonates with PVT (since most of the studies provided mean dwelling time for the overall population); and (7) Prophylaxis: Only 6/16 (37%) studies reported a prophylactic administration of heparin[21,38,39,42,44,46].

DISCUSSION

To the best of our knowledge, this is the first systematic review specifically investigating the issue of UVC-related PVT. One of the most important limitations that emerged when reviewing the scientific literature was the extreme heterogeneity of study designs across the investigated studies (Table 2 and Figure 3).

As a whole, the data achieved by our systematic review confirmed the relevant risk of PVT associated with umbilical catheterization. The mean reported pooled incidence of neonatal UVC-related PVT among studies was 12%, with a range which varied from 0% to 49% from study to study (Figure 2). Such large difference might be attributed to
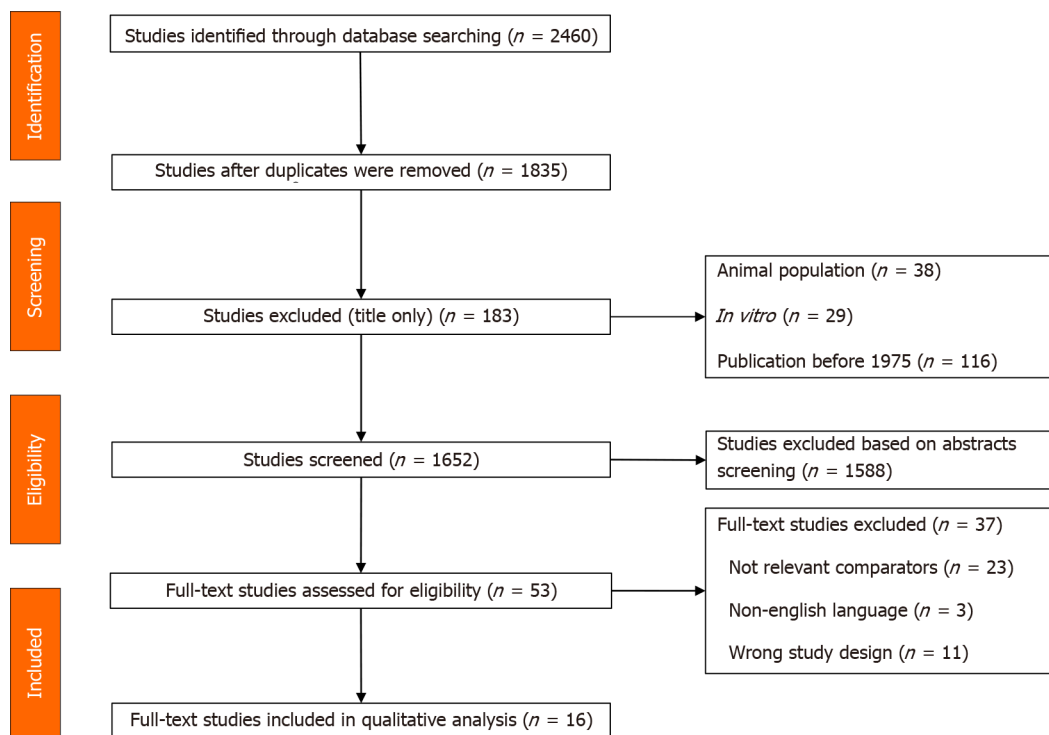
Figure 1 Flow-chart of study selection process.

multiple factors, including the different indication to imaging diagnostics, the different imaging time schedules, the heterogeneous UVC size/position/duration, and the proportion of preterm/term neonates[30,40,43]. Moreover, the time frame of research and publication may have influenced the incidence of UVC-related PVT as well. In fact, across literature, PVT was more frequently reported in the most recent studies. For example, a large multicenter registry assessing all thrombotic events occurring between 1989 and 1992 in 22 Canadian and 42 international centers from Europe, Australia and United States, recorded only 97 thrombotic events but did not explicitly report any case of PVT at all[47]. In contrast, a more recent large multicenter survey which included 187 children with a diagnosis of PVT (mean age at diagnosis: 4 years) reported a history of neonatal UVC placement in 65% of cases[19]. The higher incidence of PVT in recent years might be explained by the fact that clinicians are more aware of the thrombotic risk associated with the use of UVC and are more attentive to its detection. Furthermore, advances in US techniques make the detection of PVT easier.

The scientific literature emphasizes that UVC-related PVT is mostly related to improper tip position. Considering the small distance required for an UVC to become dislodged, UVC may migrate into the portal vein even following an initial proper positioning[2,15,16,42,48-52]. Therefore, tip location must be verified with accuracy not only soon after placement but also at regular intervals throughout time[30,31]. For this purpose, US is the ideal tool to check the position of the tip, since it is easy to perform for clinicians, it can be done at bedside and is not invasive for the patient.

When reviewing the literature, we found differences regarding the indication for US assessment, *i.e.*, systematic surveillance in asymptomatic neonates with history of UVCs *vs* targeted diagnostic test in neonates with clinical concerns for a thrombus. However, in the studies which were finally included in the analyses, UVC-related PVT was mostly asymptomatic and only detected thanks to systematic imaging surveillance. Levit *et al*[42] found that in their neonatal unit, where routine US screening for PVT was not conducted, the rate of clinically identified thrombi was only 0.15% of all UVCs placed and 1.1% of all UVC-associated complications. On the other hand, Kim *et al*[30] found clinically silent PVT after UVC placement in 43% of critically ill neonates undergoing systematic US assessment. This indicates that UVC-related PVT might be largely underestimated if not properly investigated[42], once more confirming the need for routine imaging screenings in all neonates with UVC to exactly determine the incidence of UVC related PVT. Notably, PVT might also be associated with short- and long-term severe complications, deserving meticulous clinical evaluation[5,15].
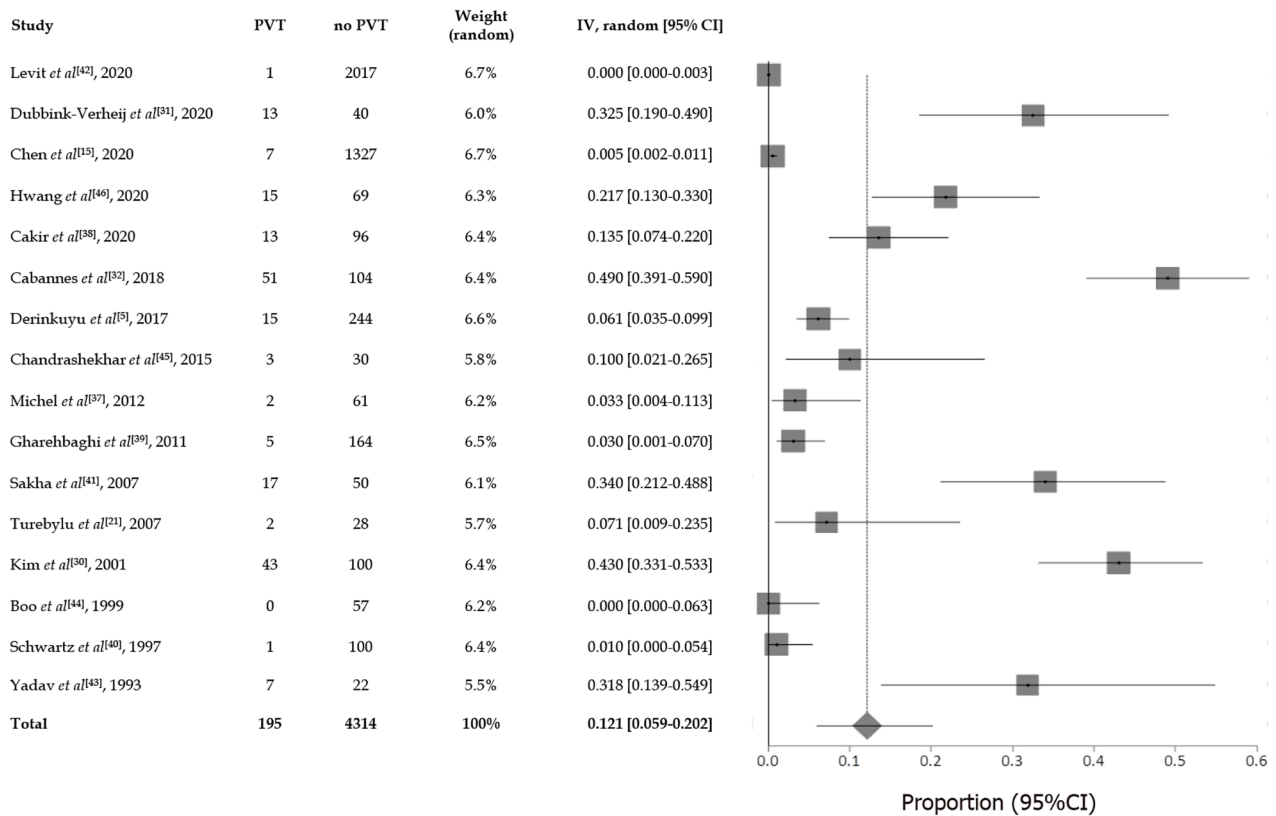
Study	PVT	no PVT	Weight (random)	IV, random [95% CI]
Levit *et al*[42], 2020	1	2017	6.7%	0.000 [0.000-0.003]
Dubbink-Verheij *et al*[31], 2020	13	40	6.0%	0.325 [0.190-0.490]
Chen *et al*[15], 2020	7	1327	6.7%	0.005 [0.002-0.011]
Hwang *et al*[46], 2020	15	69	6.3%	0.217 [0.130-0.330]
Cakir *et al*[38], 2020	13	96	6.4%	0.135 [0.074-0.220]
Cabannes *et al*[32], 2018	51	104	6.4%	0.490 [0.391-0.590]
Derinkuyu *et al*[5], 2017	15	244	6.6%	0.061 [0.035-0.099]
Chandrashekhar *et al*[45], 2015	3	30	5.8%	0.100 [0.021-0.265]
Michel *et al*[37], 2012	2	61	6.2%	0.033 [0.004-0.113]
Gharehbaghi *et al*[39], 2011	5	164	6.5%	0.030 [0.001-0.070]
Sakha *et al*[41], 2007	17	50	6.1%	0.340 [0.212-0.488]
Turebylu *et al*[21], 2007	2	28	5.7%	0.071 [0.009-0.235]
Kim *et al*[30], 2001	43	100	6.4%	0.430 [0.331-0.533]
Boo *et al*[44], 1999	0	57	6.2%	0.000 [0.000-0.063]
Schwartz *et al*[40], 1997	1	100	6.4%	0.010 [0.000-0.054]
Yadav *et al*[43], 1993	7	22	5.5%	0.318 [0.139-0.549]
Total	195	4314	100%	0.121 [0.059-0.202]

Figure 2 Forest plot showing the incidence of umbilical venous catheter-related portal vein thrombosis. PVT: Portal vein thrombosis; CI: Confidence interval.
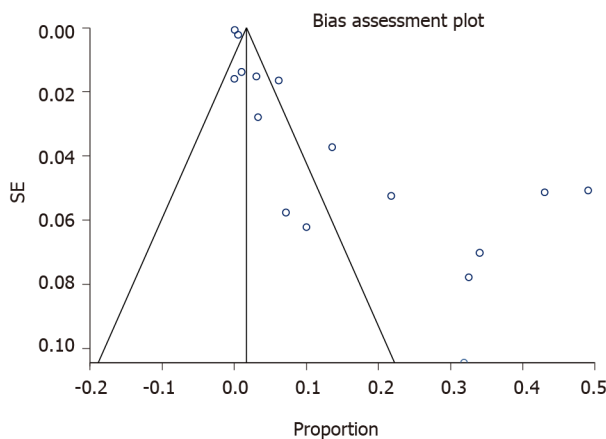

Figure 3 Funnel plot.

According to the results of our systematic review, UVC-related PVT was reliably investigated by US assessment. Nevertheless, we found large discrepancies across studies concerning data presentation. As described above in the text, only a minority of studies reported the exact thrombus position/extension within the portal system and if the occlusion was partial/complete. After PVT detection, imaging follow-up controls were performed with heterogeneous time schedules across studies. As a whole, however, the data confirmed that US is a valid, non-invasive, bed-side diagnostic technique for PVT detection. But whereas assessment of tip position is easy, requires a minimal training, and can be performed by the neonatologist bedside, detection of PVT at an early stage usually warrants a higher degree of US expertise. Besides the skill level of the radiologist/neonatologist, correct US examination might also depend on further technical factors (neonatal cooperation, abdominal gas distension, clinical instability, small-sized anatomical structures *etc*.) which may

influence the assessment.

A meticulous assessment of UVC tip position is needed to decrease catheter-related complications. Radiography is the most widely used technique to assess and follow-up UVC tip location[53,54]. However, most of the studies used only the anteroposterior view to assess tip location, although such view alone is not able to safely define the correct UVC tip position[54]. In case of wrong tip position within the portal system, radiography may show: (1) The tip below the diaphragm (below the vertebral body T10), overlying the liver; (2) Portal venous gas; and (3) Hypodiaphan lesions in the liver if fluid extravasation into liver parenchyma occurred[2,9,10,12,13]. However, radiographic assessments expose neonates to repeated ionizing radiations. US evaluation can be used in daily practice to check UVC tip position as well as the possible occurrence of UVC-associated hepatic complications. In fact, point-of-care US is able to assess in real-time UVC navigation and tip position during catheter placement[55]. Once UVC is correctly in place, US is the technique of choice to detect the development of UVC-related liver complications[5,30,31,53,56,57]. US and Doppler findings demonstrating hepatic complications include: (1) Detection of air in the portal venous system; (2) Portal venous thrombosis with impaired vascular patency; and (3) Liver parenchymal lesions presenting as nodular echogenic lesions/branched echogenic lesions/wide irregular heterogeneous lesions with laceration and the presence of peri-hepatic fluid[2,5,9,10,32]. Data exist comparing the ability of radiography and sonography to assess UVC positioning. A recent study found that US testing of UVC placement was able to identify catheter location in 100% of cases when compared to radiographic assessment[57]. Moreover, US is more accurate in the assessment of tip position compared to an estimation of catheter position achieved by its relationship to external structures on a radiograph[9,37,54,58]. Echocardiographic evaluation of UVC tip position was also assessed with success in recent years, although most studies focused on its ability to detect intra-cardiac abnormal tip position or atrial/inferior vena cava thrombosis, considering its limited ability to detect thrombi outside of the thoracic great vessels[24,59-62].

To date, the latest guidelines recommend the removal of UVCs after 7-10 d, although some authors reported an UVC *in situ* duration up to 28 d, once more proving how the management of UVCs is highly heterogeneous[4,22,24,38,42,61,63,64]. Unfortunately, the mean UVC dwell time in neonates with PVT was explicitly reported only by a minority of the included studies. Some authors found comparable UVC duration both in neonates with or without PVT[38-40], whilst in a large prospective study Kim *et al*[30] found an increased risk of PVT with a dwell time longer than 6 d. Noteworthy, PVT occurrence may develop soon after UVC position, as demonstrated by studies describing its detection already 12 h after placement[37]. It could be put forward that the presence of an UVC may itself represent a trigger for PVT development, presumably by raising vascular pressure in the ductus venosus and slowing down blood flow[18], and that such risk may eventually increase if catheterization persists. Such hypothesis deserves proper validation and large randomized controlled trials are warranted to achieve conclusive data about the benefits of early UVC removal.

Only a minority of studies described the occurrence of difficult or failed umbilical catheterization[30,65]. Considering that traumatic catheterization and/or failed insertion may induce vasculature injury and predispose to PVT by damaging the endothelial wall and decreasing portal flow[8], also the occurrence and number of failed attempts to UVC placement may play a role in PVT development and should be therefore considered either when programming diagnostic/follow-up controls for PVT or in the design of future studies.

The studies included in the final analyses reported the use of different models of UVCs, but unfortunately several studies did not specify the UVC model at all. Today, the most used UVC are dedicated catheters in polyurethane or in polyvinyl chloride but in the past several units used nasogastric tubes for venous umbilical catheterization. Furthermore, most of the studies did not specify the size and the number of lumens of the catheters that have been used. The use of different UVC models/materials may have influenced the incidence of UVC-related PVT in each study.

Concerning the presence of hereditary risk factors, the literature is, once more, quite vague and inconclusive. Turebylu *et al*[21] evaluated prospectively the prevalence of hereditary prothrombotic mutations in neonates with umbilical catheterization developing thrombotic lesions (including two cases of PVT). Interestingly, the authors found no increase in the risk of catheter-related thrombosis in patients carrying such prothrombotic mutations. In contrast, Heller *et al*[25] found that among 65 neonates, 24 of whom had PVT, the rate of genetic prothrombotic risk factors was higher than

healthy, age-/sex-matched controls.

Sepsis was suggested as possible risk factor for pediatric PVT development[3,66,67]. However, only a minority of patients affected by PVT presented with infection[3]. Furthermore, as for the studies included in the present review, only a minority of authors explicitly reported the presence of sepsis in case of PVT.

Recently, Hwang *et al*[46] reported for the first time significantly higher serum calcium concentrations in infants with umbilical catheter-related thrombosis. The authors assessed that such finding may reflect a possible role of calcium as a clotting factor leading to a hypercoagulable state. Further evidence is however required to confirm these results.

Only a minority of the studies included in our review reported a prophylactic treatment with heparin which, moreover, varied in terms of dosage[21,38,39,42,46]. After UVC-related PVT development, spontaneous resolution may often occur in UVC-related PVT, but this warrants close monitoring to determine either progression or resolution of the thrombus[21,30,32,40,46,64,68-70]. However, in case of thrombus extension with occlusion of the portal venous tract or clinical deterioration, antithrombotic therapy with unfractionated or low molecular weight heparin can be considered[64,68,70,71]. Kim *et al*[30] investigated prospectively the occurrence of UVC-related PVT in 100 neonates by subsequent US assessment. The authors found that 43% of neonates had a clinically silent PVT and reported complete resolution in 56% of neonates at follow-up controls, with recanalization being more frequent in neonates with partial rather than occlusive thrombi. Cabannes *et al*[32] investigated prospectively the occurrence of PVT in a cohort of patients including preterm neonates. PVT occurred in 53/123 of which 51 had an UVC. In these cases, the authors reported a spontaneous favorable evolution of left PVT in 95% of cases. In a prospective observational study, Dubbink-Verheij *et al*[31] investigated by serial US evaluations the incidence of catheter-related thrombosis in neonates with UVCs compared to a control group of neonates without UVC. The authors found the presence of thrombotic lesions in the UVC route in 30/40 cases (75%), of which 13 in the portal vein system. Most of the thrombotic lesions were asymptomatic and regressed spontaneously, whilst a minority required treatment with heparin. In contrast, Derinkuyu *et al*[5] treated with low-molecular-weight heparin all neonates with a diagnosis of UVC-related PVT (all described as asymptomatic). This heterogeneous approach may reflect the absence of solid evidence about safety/efficacy of antithrombotic therapy specifically addressing the neonatal period.

Our systematic review has multiple limitations, mostly attributable to the heterogeneity across studies. First, the intrinsic limitation of having included either retrospective studies or "old" studies (from 1980 onwards), *i.e.*, performed at time-points during which clinical approach to patients and awareness about PVT was presumably different compared to more recent studies. Second, the lack of correlation between PVT and UVC tip position in most studies. Third, the different study designs regarding the indication and time schedule for imaging assessment. Fourth, the different approach of clinicians about the use of prophylactic/therapeutic treatment in neonates with indwelling UVCs.

CONCLUSION

In conclusion, the use of umbilical lines requires a high index of suspicion for PVT development, especially if considering that the need for an UVC obviously preselects ill newborns in whom multiple risk factors for the development of thrombotic disorders may coexist. To avoid or minimize the risk of PVT, some crucial key-points have to be followed, as checking the correct position before infusing in the catheter, checking again the correct tip position every 48 h, and removing the UVC after a maximum of 7 d.

As a whole, this systematic review revealed relevant gaps also in knowledge about the optimal diagnostic approach and treatment for UVC-related PVT, maybe related to the lack of updated, evidence-based guidelines addressing step-by-step all the aspects of what the best approach to the management of this complication should be. According to our opinion, this represents a call to action addressed to researchers and clinicians to design large prospective randomized studies and to draft specific, concrete and updated guidelines.

ARTICLE HIGHLIGHTS

Research background

The use of umbilical venous catheters (UVCs) in the perinatal period may be associated with severe complications, including the occurrence of portal vein thrombosis (PVT).

Research motivation

Although multiple observational studies have provided an overview about the risk of PVT after UVC positioning, no studies/reviews explored systematically this issue.

Research objectives

The main goal was to investigate the most accurate information about the actual incidence of UVC-related PVT in the neonatal setting, and to assess if any particular risk factor was systematically associated with the development of such complication.

Research methods

A systematic and comprehensive database searching (PubMed, Cochrane Library, Scopus, Web of Science) was performed for prospective cohort studies, retrospective cohort studies and case-control studies from 1980 to 2020. Incidence estimates were pooled by using random effects meta-analyses. The quality of included studies was assessed using the Newcastle-Ottawa scale.

Research results

Sixteen studies were considered eligible and included in the final analyses. The data confirmed the relevant risk of UVC-related thrombosis with a mean pooled incidence of 12%, although it varied across studies (0%-49%).

Research conclusions

This is the first systematic review specifically investigating the incidence of UVC-related PVT. The use of UVCs requires a high index of suspicion, because its use is significantly associated with PVT.

Research perspectives

Large prospective randomized studies and updated guidelines are warranted in order to define the best management of this dreaded complication.

REFERENCES

1   **El Ters N**, Claassen C, Lancaster T, Barnette A, Eldridge W, Yazigi F, Brar K, Herco M, Rogowski L, Strand M, Vachharajani A. Central *vs* Low-Lying Umbilical Venous Catheters: A Multicenter Study of Practices and Complications. *Am J Perinatol* 2019; **36**: 1198-1204 [PMID: 30566998 DOI: 10.1055/s-0038-1676482]

2   **Hagerott HE**, Kulkarni S, Restrepo R, Reeves-Garcia J. Clinical-radiologic features and treatment of hepatic lesions caused by inadvertent infusion of parenteral nutrition in liver parenchyma due to malposition of umbilical vein catheters. *Pediatr Radiol* 2014; **44**: 810-815 [PMID: 24557484 DOI: 10.1007/s00247-014-2895-2]

3   **Williams S**, Chan AK. Neonatal portal vein thrombosis: diagnosis and management. *Semin Fetal Neonatal Med* 2011; **16**: 329-339 [PMID: 21925985 DOI: 10.1016/j.siny.2011.08.005]

4   **Gordon A**, Greenhalgh M, McGuire W. Early planned removal of umbilical venous catheters to prevent infection in newborn infants. *Cochrane Database Syst Rev* 2017; **10**: CD012142 [PMID: 29017005 DOI: 10.1002/14651858.CD012142.pub2]

5   **Derinkuyu BE**, Boyunaga OL, Damar C, Unal S, Ergenekon E, Alimli AG, Oztunali C, Turkyilmaz C. Hepatic Complications of Umbilical Venous Catheters in the Neonatal Period: The Ultrasound Spectrum. *J Ultrasound Med* 2018; **37**: 1335-1344 [PMID: 29034490 DOI: 10.1002/jum.14443]

6   **Goh SSM**, Kan SY, Bharadwaj S, Poon WB. A review of umbilical venous catheter-related complications at a tertiary neonatal unit in Singapore. *Singapore Med J* 2019 [PMID: 31680179 DOI: 10.11622/smedj.2019140]

7   **Mutlu M**, Aslan Y, Kul S, Yılmaz G. Umbilical venous catheter complications in newborns: a 6-year single-center experience. *J Matern Fetal Neonatal Med* 2016; **29**: 2817-2822 [PMID: 26452458 DOI: 10.3109/14767058.2015.1105952]

8   **Sulemanji M**, Vakili K, Zurakowski D, Tworetzky W, Fishman SJ, Kim HB. Umbilical Venous Catheter Malposition Is Associated with Necrotizing Enterocolitis in Premature Infants. *Neonatology* 2017; **111**: 337-343 [PMID: 28092913 DOI: 10.1159/000451022]

9   **Selvam S**, Humphrey T, Woodley H, English S, Kraft JK. Sonographic features of umbilical catheter-related complications. *Pediatr Radiol* 2018; **48**: 1964-1970 [PMID: 30078110 DOI: 10.1007/s00247-018-4214-9]

10   **Schlesinger AE**, Braverman RM, DiPietro MA. Pictorial essay. Neonates and umbilical venous catheters: normal appearance, anomalous positions, complications, and potential aid to diagnosis. *AJR Am J Roentgenol* 2003; **180**: 1147-1153 [PMID: 12646473 DOI: 10.2214/ajr.180.4.1801147]

11   **Morag I**, Epelman M, Daneman A, Moineddin R, Parvez B, Shechter T, Hellmann J. Portal vein thrombosis in the neonate: risk factors, course, and outcome. *J Pediatr* 2006; **148**: 735-739 [PMID: 16769378 DOI: 10.1016/j.jpeds.2006.01.051]

12   **Grizelj R**, Vukovic J, Bojanic K, Loncarevic D, Stern-Padovan R, Filipovic-Grcic B, Weingarten TN, Sprung J. Severe liver injury while using umbilical venous catheter: case series and literature review. *Am J Perinatol* 2014; **31**: 965-974 [PMID: 24590868 DOI: 10.1055/s-0034-1370346]

13   **Auriti C**, Ronchetti MP, Bersani I, Gennari F, Piersigilli F. Intrahepatic Administration of Liposomal Amphotericin B (Ambisome) for the Management of a Liver Abscess from *Candida albicans* in a Preterm Infant. *Antimicrob Agents Chemother* 2018; **62** [PMID: 30224526 DOI: 10.1128/AAC.01239-18]

14   **Türk E**, Soylar R, Akca T, Serter S, Karaca İ. Venobiliary fistula related to umbilical venous catheter in a newborn. *Pediatr Int* 2015; **57**: 478-480 [PMID: 26011554 DOI: 10.1111/ped.12608]

15   **Chen HJ**, Chao HC, Chiang MC, Chu SM. Hepatic extravasation complicated by umbilical venous catheterization in neonates: A 5-year, single-center experience. *Pediatr Neonatol* 2020; **61**: 16-24 [PMID: 31186169 DOI: 10.1016/j.pedneo.2019.05.004]

16   **Yiğiter M**, Arda IS, Hiçsönmez A. Hepatic laceration because of malpositioning of the umbilical vein catheter: case report and literature review. *J Pediatr Surg* 2008; **43**: E39-E41 [PMID: 18485935 DOI: 10.1016/j.jpedsurg.2008.01.018]

17   **Macartney CA**, Chan AK. Thrombosis in children. *Semin Thromb Hemost* 2011; **37**: 763-761 [PMID: 22187399 DOI: 10.1055/s-0031-1297167]

18   **Sulemanji MN**, Azpurua H, Suh M, Potanos K, Cauley R, Kunisaki SM, Modi B, Zurakowski D, Fishman SJ, Kim HB. Ductus venosus closure results in transient portal hypertension--is this the silent trigger for necrotizing enterocolitis? *J Pediatr Surg* 2013; **48**: 2067-2074 [PMID: 24094959 DOI: 10.1016/j.jpedsurg.2013.01.022]

19   **Di Giorgio A**, De Angelis P, Cheli M, Vajro P, Iorio R, Cananzi M, Riva S, Maggiore G, Indolfi G, Calvo PL, Nicastro E, D'Antiga L. Etiology, presenting features and outcome of children with non-cirrhotic portal vein thrombosis: A multicentre national study. *Dig Liver Dis* 2019; **51**: 1179-1184 [PMID: 30928422 DOI: 10.1016/j.dld.2019.02.014]

20   **Ferri PM**, Rodrigues Ferreira A, Fagundes ED, Xavier SG, Dias Ribeiro D, Fernandes AP, Borges KB, Liu SM, de Melo Mdo C, Roquete ML, Penna FJ. Evaluation of the presence of hereditary and acquired thrombophilias in Brazilian children and adolescents with diagnoses of portal vein thrombosis. *J Pediatr Gastroenterol Nutr* 2012; **55**: 599-604 [PMID: 22684349 DOI: 10.1097/MPG.0b013e318261814d]

21   **Turebylu R**, Salis R, Erbe R, Martin D, Lakshminrusimha S, Ryan RM. Genetic prothrombotic mutations are common in neonates but are not associated with umbilical catheter-associated thrombosis. *J Perinatol* 2007; **27**: 490-495 [PMID: 17625574 DOI: 10.1038/sj.jp.7211786]

22   **Revel-Vilk S**, Ergaz Z. Diagnosis and management of central-line-associated thrombosis in newborns and infants. *Semin Fetal Neonatal Med* 2011; **16**: 340-344 [PMID: 21807572 DOI: 10.1016/j.siny.2011.07.003]

23   **Albisetti M**, Moeller A, Waldvogel K, Bernet-Buettiker V, Cannizzaro V, Anagnostopoulos A, Balmer C, Schmugge M. Congenital prothrombotic disorders in children with peripheral venous and arterial thromboses. *Acta Haematol* 2007; **117**: 149-155 [PMID: 17159337 DOI: 10.1159/000097462]

24   **Narang S**, Roy J, Stevens TP, Butler-O'Hara M, Mullen CA, D'Angio CT. Risk factors for umbilical venous catheter-associated thrombosis in very low birth weight infants. *Pediatr Blood Cancer* 2009; **52**: 75-79 [PMID: 18680150 DOI: 10.1002/pbc.21714]

25   **Heller C**, Schobess R, Kurnik K, Junker R, Günther G, Kreuz W, Nowak-Göttl U. Abdominal venous thrombosis in neonates and infants: role of prothrombotic risk factors - a multicentre case-control study. For the Childhood Thrombophilia Study Group. *Br J Haematol* 2000; **111**: 534-539 [PMID: 11122096 DOI: 10.1046/j.1365-2141.2000.02349.x]

26   **Avila ML**, Amiri N, Stanojevic S, Vu TT, Barron K, Krol P, Yue N, Williams S, Brandão LR. Can thrombophilia predict recurrent catheter-related deep vein thrombosis in children? *Blood* 2018; **131**: 2712-2719 [PMID: 29724900 DOI: 10.1182/blood-2017-10-811216]

27   **Sobczak A**, Kruczek P, Homa M, Kwinta P. A new microscopic insight into the thrombogenicity of umbilical catheters. *Thromb Res* 2018; **168**: 80-82 [PMID: 29936402 DOI: 10.1016/j.thromres.2018.06.007]

28   **Pottecher T**, Forrler M, Picardat P, Krause D, Bellocq JP, Otteni JC. Thrombogenicity of central venous catheters: prospective study of polyethylene, silicone and polyurethane catheters with phlebography or post-mortem examination. *Eur J Anaesthesiol* 1984; **1**: 361-365 [PMID: 6536520]

29   **Thornburg C**, Pipe S. Neonatal thromboembolic emergencies. *Semin Fetal Neonatal Med* 2006; **11**: 198-206 [PMID: 16520103 DOI: 10.1016/j.siny.2006.01.005]

30   **Kim JH**, Lee YS, Kim SH, Lee SK, Lim MK, Kim HS. Does umbilical vein catheterization lead to portal venous thrombosis? *Radiology* 2001; **219**: 645-650 [PMID: 11376248 DOI: 10.1148/radiology.219.3.r01jn17645]

31   **Dubbink-Verheij GH**, Visser R, Roest AA, van Ommen CH, Te Pas AB, Lopriore E. Thrombosis after umbilical venous catheterisation: prospective study with serial ultrasound. *Arch Dis Child Fetal Neonatal Ed* 2020; **105**: 299-303 [PMID: 31391204 DOI: 10.1136/archdischild-2018-316762]

32   **Cabannes M**, Bouissou A, Favrais G, Sembély-Taveau C, Morales L, Favreau A, Bertrand P, Saliba E, Sirinelli D, Morel B. Systematic ultrasound examinations in neonates admitted to NICU: evolution of portal vein thrombosis. *J Perinatol* 2018; **38**: 1359-1364 [PMID: 30082773 DOI: 10.1038/s41372-018-0132-9]

33   **Moher D**, Liberati A, Tetzlaff J, Altman DG; PRISMA Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PLoS Med* 2009; **6**: e1000097 [PMID: 19621072 DOI: 10.1371/journal.pmed.1000097]

34   **Wells GA**, Shea B, O'Connell D, Peterson J, Welch V, Losos M, Tugwell P.   The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomized studies in meta-analyses. [cited 28 November 2020]. In: The Ottawa Hospital Research Institute [Internet]. Available from: http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp

35   **Higgins JP**, Thompson SG. Quantifying heterogeneity in a meta-analysis. *Stat Med* 2002; **21**: 1539-1558 [PMID: 12111919 DOI: 10.1002/sim.1186]

36   **Higgins JP**, Thompson SG, Deeks JJ, Altman DG. Measuring inconsistency in meta-analyses. *BMJ* 2003; **327**: 557-560 [PMID: 12958120 DOI: 10.1136/bmj.327.7414.557]

37   **Michel F**, Brevaut-Malaty V, Pasquali R, Thomachot L, Vialet R, Hassid S, Nicaise C, Martin C, Panuel M. Comparison of ultrasound and X-ray in determining the position of umbilical venous catheters. *Resuscitation* 2012; **83**: 705-709 [PMID: 22155219 DOI: 10.1016/j.resuscitation.2011.11.026]

38   **Çakır SÇ**, Özkan H, Dorum BA, Köksal N, Kudretoğlu P, Baytan B, Sezgin M, Güneş AM. The danger awaiting premature babies: Portal vein thrombosis. *Turk Pediatri Ars* 2020; **55**: 257-262 [PMID: 33061753 DOI: 10.14744/TurkPediatriArs.2020.65289]

39   **Gharehbaghi MM**, Nemati M, Hosseinpour SS, Taei R, Ghargharechi R. Umbilical vascular catheter associated portal vein thrombosis detected by ultrasound. *Indian J Pediatr* 2011; **78**: 161-164 [PMID: 21063811 DOI: 10.1007/s12098-010-0223-x]

40   **Schwartz DS**, Gettner PA, Konstantino MM, Bartley CL, Keller MS, Ehrenkranz RA, Jacobs HC. Umbilical venous catheterization and the risk of portal vein thrombosis. *J Pediatr* 1997; **131**: 760-762 [PMID: 9403662 DOI: 10.1016/s0022-3476(97)70109-4]

41   **Sakha SH**, Rafeey M, Tarzamani MK. Portal venous thrombosis after umbilical vein catheterization. *Indian J Gastroenterol* 2007; **26**: 283-284 [PMID: 18431012]

42   **Levit OL**, Shabanova V, Bizzarro MJ. Umbilical catheter-associated complications in a level IV neonatal intensive care unit. *J Perinatol* 2020; **40**: 573-580 [PMID: 31911645 DOI: 10.1038/s41372-019-0579-3]

43   **Yadav S**, Dutta AK, Sarin SK. Do umbilical vein catheterization and sepsis lead to portal vein thrombosis? *J Pediatr Gastroenterol Nutr* 1993; **17**: 392-396 [PMID: 8145094 DOI: 10.1097/00005176-199311000-00010]

44   **Boo NY**, Wong NC, Zulkifli SS, Lye MS. Risk factors associated with umbilical vascular catheter-associated thrombosis in newborn infants. *J Paediatr Child Health* 1999; **35**: 460-465 [PMID: 10571759 DOI: 10.1046/j.1440-1754.1999.355392.x]

45   **Chandrashekhar C**, Krishnegowda S, Vikas VM, Bhaktavatsala HR. Portal vein thrombosis following umbilical vein catheterization in neonates. *Pediatr Rev: Int J Pediatr Res* 2015; **2**: 135-137 [DOI: 10.17511/ijpr.2015.i04.18]

46   **Hwang JH**, Chung ML, Lim YJ. Incidence and risk factors of subclinical umbilical catheter-related thrombosis in neonates. *Thromb Res* 2020; **194**: 21-25 [PMID: 32563060 DOI: 10.1016/j.thromres.2020.05.034]

47   **Schmidt B**, Andrew M. Neonatal thrombosis: report of a prospective Canadian and international registry. *Pediatrics* 1995; **96**: 939-943 [PMID: 7478839]

48   **Hoellering A**, Tshamala D, Davies MW. Study of movement of umbilical venous catheters over time. *J Paediatr Child Health* 2018; **54**: 1329-1335 [PMID: 29806878 DOI: 10.1111/jpc.14073]

49   **Abiramalatha T**, Kumar M, Shabeer MP, Thomas N. Advantages of being diligent: lessons learnt from umbilical venous catheterisation in neonates. *BMJ Case Rep* 2016; **2016** [PMID: 26843419]

50   **Plooij-Lusthusz AM**, van Vreeswijk N, van Stuijvenberg M, Bos AF, Kooi EMW. Migration of Umbilical Venous Catheters. *Am J Perinatol* 2019; **36**: 1377-1381 [PMID: 30620943 DOI: 10.1055/s-0038-1677016]

51   **Dubbink-Verheij GH**, Visser R, Tan RNGB, Roest AAW, Lopriore E, Te Pas AB. Inadvertent Migration of Umbilical Venous Catheters Often Leads to Malposition. *Neonatology* 2019; **115**: 205-210 [PMID: 30645997 DOI: 10.1159/000494369]

52   **Franta J**, Harabor A, Soraisham AS. Ultrasound assessment of umbilical venous catheter migration in preterm infants: a prospective study. *Arch Dis Child Fetal Neonatal Ed* 2017; **102**: F251-F255 [PMID: 28424358 DOI: 10.1136/archdischild-2016-311202]

53   **Sharma D**, Farahbakhsh N, Tabatabaii SA. Role of ultrasound for central catheter tip localization in neonates: a review of the current evidence. *J Matern Fetal Neonatal Med* 2019; **32**: 2429-2437 [PMID: 29397784 DOI: 10.1080/14767058.2018.1437135]

54   **Guimarães AF**, Souza AA, Bouzada MC, Meira ZM. Accuracy of chest radiography for positioning of the umbilical venous catheter. *J Pediatr (Rio J)* 2017; **93**: 172-178 [PMID: 27424226 DOI: 10.1016/j.jped.2016.05.004]

55   **Kishigami M**, Shimokaze T, Enomoto M, Shibasaki J, Toyoshima K. Ultrasound-Guided Umbilical Venous Catheter Insertion With Alignment of the Umbilical Vein and Ductus Venosus. *J Ultrasound Med* 2020; **39**: 379-383 [PMID: 31400014 DOI: 10.1002/jum.15106]

56   **Simanovsky N**, Ofek-Shlomai N, Rozovsky K, Ergaz-Shaltiel Z, Hiller N, Bar-Oz B. Umbilical venous catheter position: evaluation by ultrasound. *Eur Radiol* 2011; **21**: 1882-1886 [PMID: 21533866 DOI: 10.1007/s00330-011-2129-z]

57   **Saul D**, Ajayi S, Schutzman DL, Horrow MM. Sonography for Complete Evaluation of Neonatal Intensive Care Unit Central Support Devices: A Pilot Study. *J Ultrasound Med* 2016; **35**: 1465-1473 [PMID: 27229130 DOI: 10.7863/ultra.15.06104]

58   **Greenberg M**, Movahed H, Peterson B, Bejar R. Placement of umbilical venous catheters with use of bedside real-time ultrasonography. *J Pediatr* 1995; **126**: 633-635 [PMID: 7699547 DOI: 10.1016/s0022-3476(95)70366-7]

59   **Ades A**, Sable C, Cummings S, Cross R, Markle B, Martin G. Echocardiographic evaluation of umbilical venous catheter placement. *J Perinatol* 2003; **23**: 24-28 [PMID: 12556923 DOI: 10.1038/sj.jp.7210851]

60   **Unal S**, Ekici F, Cetin İİ, Bilgin L. Heparin infusion to prevent umbilical venous catheter related thrombosis in neonates. *Thromb Res* 2012; **130**: 725-728 [PMID: 22901699 DOI: 10.1016/j.thromres.2012.07.018]

61   **Butler-O'Hara M**, Buzzard CJ, Reubens L, McDermott MP, DiGrazio W, D'Angio CT. A randomized trial comparing long-term and short-term use of umbilical venous catheters in premature infants with birth weights of less than 1251 g. *Pediatrics* 2006; **118**: e25-e35 [PMID: 16785289 DOI: 10.1542/peds.2005-1880]

62   **Wever ML**, Liem KD, Geven WB, Tanke RB. Urokinase therapy in neonates with catheter related central venous thrombosis. *Thromb Haemost* 1995; **73**: 180-185 [PMID: 7792727]

63   **O'Grady NP**, Alexander M, Burns LA, Dellinger EP, Garland J, Heard SO, Lipsett PA, Masur H, Mermel LA, Pearson ML, Raad II, Randolph AG, Rupp ME, Saint S; Healthcare Infection Control Practices Advisory Committee. Guidelines for the prevention of intravascular catheter-related infections. *Am J Infect Control* 2011; **39**: S1-34 [PMID: 21511081 DOI: 10.1016/j.ajic.2011.01.003]

64   **Park CK**, Paes BA, Nagel K, Chan AK, Murthy P; Thrombosis and Hemostasis in Newborns (THiN) Group. Neonatal central venous catheter thrombosis: diagnosis, management and outcome. *Blood Coagul Fibrinolysis* 2014; **25**: 97-106 [PMID: 24477225 DOI: 10.1097/MBC.0b013e328364f9b0]

65   **Aiyagari R**, Song JY, Donohue JE, Yu S, Gaies MG. Central venous catheter-associated complications in infants with single ventricle: comparison of umbilical and femoral venous access routes. *Pediatr Crit Care Med* 2012; **13**: 549-553 [PMID: 22805159 DOI: 10.1097/PCC.0b013e31824fbdb4]

66   **Sethi SK**, Dewan P, Faridi MM, Aggarwal A, Upreti L. Liver abscess, portal vein thrombosis and cavernoma formation following umbilical vein catherisation in two neonates. *Trop Gastroenterol* 2007; **28**: 79-80 [PMID: 18050847]

67   **Shah I**, Bhatnagar S. Liver abscess in a newborn leading to portal vein thrombosis. *Indian J Pediatr* 2009; **76**: 1268-1269 [PMID: 20012786 DOI: 10.1007/s12098-009-0244-5]

68   **Monagle P**, Chan AKC, Goldenberg NA, Ichord RN, Journeycake JM, Nowak-Göttl U, Vesely SK. Antithrombotic therapy in neonates and children: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. *Chest* 2012; **141**: e737S-e801S [PMID: 22315277 DOI: 10.1378/chest.11-2308]

69   **Bhatt MD**, Patel V, Butt ML, Chan AKC, Paes B; Thrombosis and Hemostasis in Newborns (THiN) Group. Outcomes following neonatal portal vein thrombosis: A descriptive, single-center study and review of anticoagulant therapy. *Pediatr Blood Cancer* 2019; **66**: e27572 [PMID: 30520242 DOI: 10.1002/pbc.27572]

70   **Chalmers E**, Ganesen V, Liesner R, Maroo S, Nokes T, Saunders D, Williams M; British Committee for Standards in Haematology. Guideline on the investigation, management and prevention of venous thrombosis in children. *Br J Haematol* 2011; **154**: 196-207 [PMID: 21595646 DOI: 10.1111/j.1365-2141.2010.08543.x]

71   **Demirel N**, Aydin M, Zenciroglu A, Bas AY, Yarali N, Okumus N, Cinar G, Ipek MS. Neonatal thrombo-embolism: risk factors, clinical features and outcome. *Ann Trop Paediatr* 2009; **29**: 271-279 [PMID: 19941750 DOI: 10.1179/027249309X12547917868961]

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