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Current management of noninfectious hepatic cystic lesions: A review of the literature

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Abstract

Nonparasitic hepatic cysts consist of a heterogeneous group of disorders, which differ in etiology, prevalence, and manifestations. With improving diagnostic techniques, hepatic cysts are becoming more common. Recent advancements in minimally invasive technology created a new Era in the management of hepatic cystic disease. Herein, the most current recommendations for management of noninfectious hepatic cysts are described, thereby discussing differential diagnosis, new therapeutic modalities and outcomes.

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Key words: Simple hepatic cyst; Noninfectious; Polycystic liver disease; Cystadenoma; Cystadenocarcinoma; Caroli's disease; Sclerotherapy; Fenestration

Core tip: Nonparasitic hepatic cysts consist of a broad spectrum of entities ranging from benign developmental cysts to malignant neoplasms. With recent advancements in diagnostic studies, hepatic cysts are becoming more frequent and better understanding of risk factors, management and long-term outcomes, and further development of current therapeutic modalities are needed.

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INTRODUCTION

Nonparasitic hepatic cysts consist of a heterogeneous group of disorders, which differ in etiology, prevalence, and manifestations, from simple cysts to neoplastic lesions. Differential diagnoses of hepatic cysts include: infectious (hydatid cyst, amebic and pyogenic abscesses), and noninfectious [simple cyst, polycystic liver disease (PCLD), cystadenoma, cystadenocarcinoma and hepatocarcinoma (HCC), cholangiocarcinoma, intrahepatic pseudocysts secondary to pancreatitis, liver hematomas, biliomas, ciliated hepatic foregut cyst] (Table 1). Sometimes, these lesions are not easily differentiated at initial presentation or by imaging studies, and management can become challenging. With improving diagnostic techniques and minimally invasive technology, the management of hepatic cystic disease continues to evolve. We, herein, describe the most current management of noninfectious hepatic cysts, thereby discussing differential diagnosis, treatment options and outcomes.

SIMPLE CYST

Simple hepatic cyst is a biliary malformation, which does not have communication with the intrahepatic biliary tree. Most cysts measure less than 3 cm and are asymptomatic. Microscopically, they are lined by a single layer of cuboid or columnar epithelial cells, resembling biliary epithelial cells. Its origin is derived from aberrant bile ducts that have lost communication with the biliary tree, and continue to secrete intraluminal fluid^[1]. The incidence is larger in adults older than 50 years, with female

Table 1 Differential diagnosis of hepatic cystic lesions

Infectious	
Parasitic	Nonparasitic
Hydatid cyst	Pyogenic liver abscesses
Amebic abscess	
Non-infectious	
Simple cyst	Partially cystic component
PCLD	HCC
Cystadenoma	Cholangiocarcinoma
Cystadenocarcinoma	Intrahepatic pseudocysts (pancreatitis)
Caroli's disease	Bilomas
Peribiliary cyst	Post-traumatic hematoma
Cystic metastases	Giant hemangioma
	Ciliated hepatic foregut cyst
	Congenital (embryonal sarcoma)

PCLD: Polycystic liver disease; HCC: Hepatocellular carcinoma.

to male ratio 1.5:1, and prevalence is around 18% in adult population^[2]. In the majority of patients, liver function tests are within the normal range. Asymptomatic single liver cysts, even when large, do not require treatment or surveillance. Ultrasound (US) is the best imaging modality for recognizing simple cysts, which appear as a circular or oval, anechoic lesion with smooth borders and acoustic posterior enhancement and without septations^[3]. Further imaging studies, including computed tomography (CT), are not routinely required and show non-septated, round and water-dense lesions. In symptomatic patients requiring intervention, either sclerotherapy or surgical fenestration, hydatid cyst should be ruled out in all cases before the operation by serology, and by the patient history of recent travel to endemic areas. In lesions suspicious for cystadenoma, the recommended therapy is surgical resection, as this lesion has malignant potential^[4-8].

Sclerotherapy consists of the destruction of the epithelial lining of the inner surface of the wall to disrupt the intracystic fluid secretion^[9-11]. Under general anesthesia, drainage catheter is introduced by Seldinger technique and under ultrasound guidance, followed by injection of water-soluble contrast to rule out communication with adjacent bile duct or peritoneal cavity. Sclerosing agents include ethanol, minocycline hydrochloride and ethanolamine oleate^[12]. The amount of alcohol injection is limited (100-200 mL) due to the risk of alcohol intoxication, and retention lasts usually between 120-240 min^[13]. The solution is then aspirated before the catheter is removed. The size of the cyst does not play a role in the amount of sclerosing agent given because after cyst collapses, sclerosant will come into contact with the cyst inner wall. Contraindications of sclerotherapy include intracystic bleeding and fistula between the cyst and biliary tree or peritoneum. The optimal efficacy may be seen up to a year after sclerotherapy, and symptomatic recurrence rate is around 20% after 4 mo^[14]. Due to high recurrence rates, management by aspiration followed by sclerotherapy should be reserved for those patients who are not eligible for surgery and general anesthesia^[15].

Surgical fenestration, also known as unroofing, con-

sists of an excision of the roof of the cyst to provide communication between the cyst and the peritoneal cavity. Limitations include cysts involving segments VII or VIII, which have higher recurrence rates due to anatomical position. Hemorrhage and biliary injury are, although rare, possible complications^[16]. There is no associated mortality and morbidity ranges from 0%-15%, with reoperation rates at 9%^[17]. There is no randomized prospective study to date comparing fenestration and sclerotherapy. In most centers, sclerotherapy is attempted first as a noninvasive option, and laparoscopic fenestration is usually indicated in refractory cases. Laparoscopy has become the procedure of choice for deroofting because is associated with significant reduction in hospital stay, postoperative pain and morbidity, and decreased blood loss^[15,18]. To avoid recurrence, it is necessary to resect as much of the wall as possible to prevent closure of the remnant wall and reaccumulation of cyst fluid. However, complete resection of the cyst is not necessary and is associated with higher complication rates. Transposition of omental patch to the cyst bed has been advocated as a means of diminishing recurrence, especially in segments VII and VIII, where early adhesion of the cyst wall to either the diaphragm or abdominal wall may lead to refilling, however this still need to be confirmed by controlled studies^[19].

PCLD

PCLD is a genetic disease responsible for the development of multiple hepatic cysts. It presents in two forms, with or without autosomal dominant polycystic kidney disease (ADPKD)^[20]. Both have an autosomal dominant transmission and similar clinical presentation. PCLD associated with ADPKD is linked with mutations in the *PKD1* (short arm of chromosome 16, encoding polycystin-1) or *PKD2* gene (chromosome 4, encoding polycystin-2), whereas isolated PCLD is associated with heterozygous mutation in *PRKC-SH* or *SEC63* genes^[21-26]. Overall prevalence is the same in gender, but female population is associated with more severe liver disease^[27]. Pregnancy, multiparity, and use of steroids further increase the risk for severe hepatic cystic disease^[28].

In most patients, cysts are small and asymptomatic; when present, symptoms are related mainly to the volume of enlarged liver rather than the volume of a specific cyst, and include abdominal distension, dyspnea, pain and early satiety^[29]. US shows multiple, fluid-filled, round or oval cysts with sharp margins. Cysts do not show contrast enhancement, and it may be extremely difficult to identify vascular and biliary structures adjacent to the cysts. CT scan shows fluid attenuation with no contrast enhancement, and magnetic resonance imaging (MRI) demonstrates hyperintense on T2-weighted and hypointense on T1-weighted images. Gigot's classification^[30] is used for staging based on CT findings: type I, less than 10 large cysts; type II, diffuse involvement of liver parenchyma, but with remaining large areas of noncystic liver parenchyma; and type III, massive, diffuse involvement of

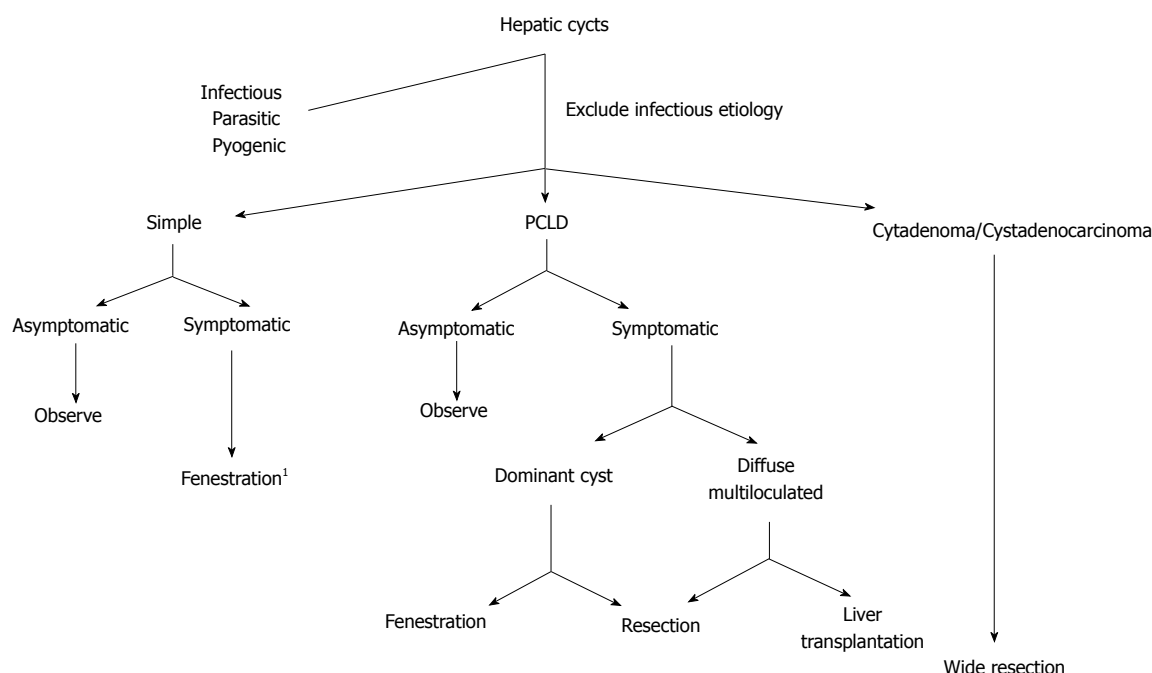


Figure 1 Treatment algorithm for management of noninfectious hepatic cysts (most common etiologies). 1: Although laparoscopic fenestration is considered the best therapeutic approach for management of symptomatic simple cysts, sclerotherapy is still performed as initial management of these lesions in some centers. PCLD: Polycystic liver disease.

liver parenchyma with only a few areas of normal tissue between cysts. Complications are uncommon and include bleeding, rupture and infection of cysts^[31]. The most severe complication is bacterial infection, especially those under dialysis for ADPKD or in immunosuppressed patients after renal transplantation^[32-37]. This is usually managed with aspiration and drainage, and antibiotics. Cholestasis secondary to compression of adjacent biliary duct also may ensue, as well as portal hypertension, resulting from portal or hepatic vein compression^[38]. The incidence of concurrent cerebral aneurysms is 8%, whereas mitral valve prolapse occurs in 25% in those with PCLD associated with ADPKD^[39].

Most current therapies are invasive and consist of surgical removal or emptying of cysts aiming at decompression and reduction of the liver size. Medical management has been proposed in advanced PCLD with diffuse disease^[40,41]. The efficacy of conservative management is still under investigation. Two recent randomized controlled trials have demonstrated that lanreotide, a long-acting somatostatin analogue, was associated with a limited reduction of liver volume in both types of PCLD, measured by CT or MRI as a primary endpoint^[42-46]. Liver volume decreased by 2.9% in the lanreotide group, but increased by 1.6% in the placebo group^[41]. Sclerotherapy and laparoscopic fenestration showed ineffective in the management of PCLD^[47]. Current surgical options include: open fenestration, liver resection, or liver transplantation (Figure 1). Transcatheter embolization has been recently proposed, targeting at decreasing arterial supply to the cyst. Although improvement of symptoms and significant reduction of liver size were observed in

most patients, the limited experience in such technically demanding procedure still pose limitation to widespread use^[48].

Partial liver resection associated with fenestration of the remnant liver has been historically proposed and, although highly successful in some patients, it is associated with high morbidity and mortality rates, and its indications are becoming more selective. Liver transplantation is the only curative modality, and is the only option in patients with anticipated limited efficacy by liver resection^[49-51] (Figure 1).

The appropriate surgical option may be defined based on Gigot's classification; in type I, which corresponds only 10% of cases, laparoscopic fenestration is recommended as first option; in type II, open fenestration is usually implemented; and type III is a contraindication to fenestration and requires resection or liver transplantation in symptomatic cases. If liver transplantation is anticipated, prior fenestration or resection should be avoided to decrease the risk of transplantation^[52-56].

CAROLI'S DISEASE

Caroli's disease is a rare congenital disorder characterized by multiple segmental intrahepatic cystic dilations, firstly described in 1958 by Caroli *et al.*^[57]. It has an autosomal recessive inheritance linked to mutation in *PKHD1* gene, leading to persistent embryonic bile ducts at different levels of the intrahepatic biliary tree. It is also classified as type V choledochal cyst by Todani classification^[58]. As opposed to PCLD, cystic lesions are irregular in shape, fusiform or saccular, and communicate with biliary tree. Patients usu-

ally present with recurrent episodes of cholangitis of unknown source, intrahepatic lithiasis and cholelithiasis^[59-62]. In the presence of congenital hepatic fibrosis and portal hypertension, it is often termed Caroli's syndrome^[63,64]. US shows intrahepatic cystic anechoic areas in which fibrovascular bundles, stones and linear bridging or septum may be present^[65]. The gold standard for diagnosis is direct visualization of the biliary tree, either endoscopic retrograde cholangiopancreatography (ERCP) or percutaneous trans-hepatic cholangiography (PTC)^[66]. Magnetic resonance cholangiopancreatography (MRCP) is an emerging modality for diagnosis of Caroli's disease^[67-69]. It has several advantages: noninvasive, readily available, and ability to visualize the entire biliary tract^[70]. The characteristic appearance on MRCP is the "string of beads" pattern of the ectatic intrahepatic bile ducts. Most common complications of Caroli's disease include cholangitis, sepsis, choledocholithiasis and hepatic abscess. At advanced stages, it may cause hepatic fibrosis with increased risks for cholangiocarcinoma and liver failure^[71].

Management consists of treatment of acute cholangitis and control of sepsis with broad-spectrum antibiotics, ursodeoxycholic acid for hepatolithiasis, and palliative biliary drainage, either *via* PTC or ERCP with or without sphincterotomy. Patients with frequent cholangitis should undergo endoscopic surveillance every 6 to 12 mo^[72]. Surgical options include partial liver resection for segmental or unilobar involvement^[73], or liver transplantation, which is the only curative treatment^[74]. Kassahun *et al.*^[75] described one of the largest experience with patients undergoing liver resection with or without biliodigestive anastomosis for unilobar disease with 84% of patients remained asymptomatic over 4 years follow-up period. Liver transplantation should be offered early in cases of recurrent cholangitis and suspicious for early malignant transformation of the biliary tract. The European transplant registry reported patient survival around 76% at 5 years after transplantation^[76]. In diffuse disease and deemed to liver transplantation, bypass procedures, either choledochojejunostomy or Roux-en-Y hepaticojejunostomy may help palliate symptoms and increase survival.

HEPATIC CYSTADENOMA

Hepatic cystadenoma is a rare benign tumor of unknown etiology, and accounts for 5% of hepatic cystic lesions. Most lesions arise within the intrahepatic bile ducts and occur more commonly in females older than 40 years of age. It may present incidentally or if large, patients may present with jaundice and cholangitis for adjacent biliary compression^[77]. Cystadenoma should be considered in any patient presenting with recurrent liver cysts after fenestration. Diagnostic imaging studies include a multiloculated lesion with internal septations, thickened and irregular wall, mural nodules and papillary projections, calcifications and wall enhancements^[78]. Preoperative planning with direct visualization of biliary anatomy is necessary in most cases with ERCP or PTC, which con-

firm biliary tree communication^[79-81]. The role of MRCP for preoperative imaging still needs to be defined^[81]. It is challenging to distinguish the lesions from hepatic cystadenocarcinomas based on current diagnostic studies^[82]. Irregular wall enhancement and the presence of papillary projections should increase suspicion for biliary cystadenocarcinoma^[83]. Abnormal serum markers, such as CA 19-9 levels and carcinoembryonic antigen, may favor malignant transformation, although this is a variable finding. The current treatment modality is open or laparoscopic liver resection due to the risk of malignancy in all suspected cystadenomas^[84-86] (Figure 1). Frozen sections are not reliable at evaluating these lesions and definitely excluding cystadenocarcinoma. Clear margins are advised due to the risk for synchronous carcinoma or foci of carcinoma *in situ*.

HEPATIC CYSTADENOCARCINOMA

Biliary cystadenocarcinoma is a rare cystic neoplasm of unknown etiology, usually arising intrahepatic or, less frequently, in the extrahepatic bile ducts, and account for 0.41% of hepatic neoplasias^[87]. The majority of these tumors are slow growing, and at the time of presentation, tumor is large with mean diameter at 12 cm, causing abdominal pain, intermittent jaundice, weight loss and ascites^[88]. Current imaging studies are unable to confirm cystadenocarcinomas, however suggestive morphological findings may include: multilocular cyst mass with mural nodules at the periphery, and coarse calcifications. MRI further characterizes the content of the different locules not as serous fluid, but as a complex collection with proteinaceous material and hemorrhagic debris^[89-91]. Preoperative fine-needle aspiration or needle biopsy is contraindicated due to the risk of fluid spilling into abdominal cavity and development of peritoneal carcinomatosis. Overall prognosis is better than HCC or cholangiocarcinoma with 5 years survival rate at 57% after resection, compared with 40% in HCC, and 22% in cholangiocarcinoma^[92]. The only treatment option is formal hepatic resection which has acceptable recurrence rates (10%)^[91,93] (Figure 1). In metastatic disease (20%), chemo- and/or radio-therapy have been advocated with doxorubicin and 5-FU, however results are yet limited^[94].

OTHER CYSTIC LESIONS

Although uncommon, HCC and cholangiocarcinoma may occasionally be cystic, especially in rapidly growing tumors. Giant hemangiomas may rarely present as partly cystic, which corresponds to noncirculating areas, and kinetic of contrast enhancement is typical. Intrahepatic pseudocyst secondary from acute pancreatitis is extremely rare with less than 20 cases reported^[95]. It may be seen in the left lobe along the lesser omentum, or in the right lobe along the portal vein. Pancreatic MRI may be useful to identify rupture of the pancreatic duct^[1]. Liver hematomas may present as cystic lesions in CT scan or US af-

ter liver surgery or trauma, especially after clots liquefy at a later phase. They are spontaneously echogenic on US, hyperdense on CT scan, hyperintense on T1-weighted and hypointense on T2-weighted MRI sequences. Bilomas are cystic lesions surrounded by a fibrous capsule that can occur adjacent to the liver parenchyma secondary from traumatic, iatrogenic or spontaneous lesions.

Cystic hepatic metastases are rare, arising mostly from neuroendocrine, tumors, sarcoma, melanomas or pancreatic cystadenocarcinomas. The presence of increased peripheral vascularization and multiple lesions should rise suspicious for this rare lesion.

Peribiliary cysts arise from cystic enlargement of peribiliary glands, mostly occurring in cirrhotic patients with portal hypertension or after liver transplantation. The cysts are located along common bile duct or within portal tracts, and are usually small and asymptomatic^[96].

Ciliated hepatic foregut cysts are benign lesions that have been described in most gastrointestinal organs, including the liver^[97,98]. They form where the foregut extends during the embryonic period, and are extremely rare. It consists of four-layer border; pseudostratified, ciliated columnar epithelium covering a subepithelial connective tissue, smooth muscle bundles, and an outer fibrous capsule, and usually located in the anterior surface of the liver. Most cases are asymptomatic and incidentally found during abdominal imaging studies, however patients may present with epigastric or right upper quadrant pain^[99]. Imaging characteristics include: predominance in segment IV, small size, subcapsular location. Two thirds are hypoechoic on US, hypodense on CT, and highly hyperintense on T2-weighted images on MRI^[97,100].

Biliary hamartomas (also known as *von Meyenburg* complexes) are asymptomatic lesions, usually discovered incidentally during liver surgery^[101]. They may be associated with Caroli's disease, PCLD and congenital hepatic fibrosis, and identification is clinically important because may be misdiagnosed as liver metastasis^[102].

CONCLUSION

Most hepatic cysts are benign, small, and asymptomatic lesions that are diagnosed incidentally and require no intervention, whereas large, symptomatic or neoplastic cysts need further treatment. Symptomatic simple cyst should be managed with laparoscopic unroofing; PCLD can be managed, in most cases, with partial resection; or liver transplantation for advanced multiloculated disease. Biliary cystadenomas and cystadenocarcinomas require complete resection with clear margins. With recent advancements in diagnostic studies, hepatic cysts are becoming more common and better understanding of risk factors; long-term outcomes and further development of current therapeutic modalities are needed.

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Current role of fenofibrate in the prevention and management of non-alcoholic fatty liver disease

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is a common health problem with a high mortality burden due to its liver- and vascular-specific complications. It is associated with obesity, high-fat diet as well as with type 2 diabetes mellitus (T2DM) and metabolic syndrome (MetS). Impaired hepatic fatty acid (FA) turnover together with insulin resistance are key players in NAFLD pathogenesis. Peroxisome proliferator-activated receptors (PPARs) are involved in lipid and glucose metabolic pathways. The novel concept is that the activation of the PPAR α subunit may protect from liver steatosis. Fenofibrate, by activating PPAR α , effectively improves the atherogenic lipid profile associated with T2DM and MetS. Experimental evidence suggested various protective effects of the drug against liver steatosis. Namely, fenofibrate-related PPAR α activation may enhance the expression of genes promoting hepatic FA β -oxidation. Furthermore, fenofibrate reduces hepatic insulin resistance. It also inhibits the expression of inflammatory mediators involved in non-alcoholic steatohepatitis pathogenesis. These include tumor necrosis factor- α , intercellular cell adhesion molecule-1, vascular cell adhesion molecule-1 and monocyte chemoattractant protein-1. Consequently,

fenofibrate can limit hepatic macrophage infiltration. Other liver-protective effects include decreased oxidative stress and improved liver microvasculature function. Experimental studies showed that fenofibrate can limit liver steatosis associated with high-fat diet, T2DM and obesity-related insulin resistance. Few studies showed that these benefits are also relevant even in the clinical setting. However, these have certain limitations. Namely, these were uncontrolled, their sample size was small, fenofibrate was used as a part of multifactorial approach, while histological data were absent. In this context, there is a need for large prospective studies, including proper control groups and full assessment of liver histology.

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Key words: Fenofibrate; Non-alcoholic fatty liver disease; Steatohepatitis; Peroxisome proliferator-activated receptors

Core tip: Non-alcoholic fatty liver disease (NAFLD) is a common health problem associated with increased liver- and vascular-specific complications. Dyslipidemia, predominantly hypertriglyceridemia, and insulin resistance play a key role in its pathogenesis. Fenofibrate, by activating peroxisome proliferator-activated receptors appears to decrease liver steatosis in experimental animal studies. This benefit can be attributed to its lipid-lowering potency, together with anti-inflammatory and anti-oxidant actions. Also, fenofibrate increases adiponectin levels and the expression of its liver-active receptor. A potential protective role of fenofibrate against NAFLD has also been implied by few small clinical studies. However, this benefit should be further assessed.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a cluster of liver disorders associated with hepatic lipid accumulation (steatosis) in the absence of viral hepatitis or alcohol abuse^[1]. These include a histological spectrum ranging from steatosis alone to non-alcoholic steatohepatitis (NASH)^[1]. In NASH, beyond lipid accumulation, necro-inflammation and fibrosis exist^[2,3]. Approximately 29% of NASH patients will develop cirrhosis within 10 years^[4]. End-stage liver disease and hepatocellular carcinoma are liver-specific endpoints of NAFLD^[3-5].

NAFLD is a common health problem affecting up to 35% of the population in several countries^[5]. It is estimated that 19% of the United States adult population, corresponding to 28.8 million individuals, exhibit ultrasonographic findings of NAFLD^[6]. Increased body weight considerably increases the risk of this abnormality. Among 257 Italian individuals obesity was associated with a 4.6-fold increased risk of hepatic steatosis^[7]. Considering current obesity epidemic it is expected that NAFLD prevalence will rise.

NAFLD is typically asymptomatic^[8]. Non-specific complaints include fatigue, malaise and right upper quadrant discomfort^[8]. Elevation of aminotransferase activities, especially of alanine aminotransferase (ALT) and γ -glutamyltranspeptidase (γ GT), are markers of hepatocellular damage^[9,10]. Lipid accumulation in the liver is identified by non-invasive imaging techniques, including ultrasound and magnetic resonance imaging (MRI)^[9]. However, these techniques cannot discriminate between simple liver steatosis and NASH. In this context, liver biopsy remains the “gold standard”^[11].

From a pathophysiological viewpoint, NAFLD is associated with imbalanced influx *vs* removal of triglycerides (TG) in the liver^[1]. In this context, TG accumulation > 55 mg/g measured by MRI or by histological examination is its key diagnostic feature^[12,13]. Fatty acids (FA) account for approximately 60% of TG in the liver of NAFLD patients, while approximately 15% originate from dietary fat^[14]. *De novo* production is responsible for the rest 25%^[14].

Insulin resistance plays a key role in the pathogenesis of NAFLD^[2,15,16]. This can be attributed to enhanced hepatic FA flux and uptake^[2,15,16]. Insulin resistance results in hyperinsulinemia and increased circulating levels of free FA, which enter hepatocyte cytoplasm to create TG^[2,15,16]. Furthermore, high plasma insulin and glucose levels might stimulate transcription factors associated with enhanced hepatic lipogenesis^[17-19]. In the clinical setting, these abnormalities are mirrored by increased circulating concentration of TG-rich lipoproteins and hypertriglyceridemia. NAFLD is commonly noted in insulin resistant states, including type 2 diabetes (T2DM) and metabolic syndrome (MetS)^[20-27]. Interestingly, it was suggested

that raised activities of ALT or γ GT may predict future development of MetS or T2DM^[20-24]. In this context, NAFLD has been considered as the “hepatic component of MetS”.

It was suggested that NAFLD increases the risk of cardiovascular (CV) events^[20,28,29]. In prospective studies, raised serum activity of liver enzymes independently predicted CV events and/or total and CV mortality^[20]. For example, γ GT activity was raised in 163 patients admitted with an acute ischemic/non-embolic stroke compared with 166 healthy individuals^[30]. Interestingly, patients at the highest quartile for γ GT activity had a 4.7-fold higher risk of ischemic stroke than those at the lowest quartile^[30]. This association was relevant after adjustment for the presence of established CV risk factors^[30]. In accordance with these findings, elevated ALT activity was associated with increased CV- and diabetes-related mortality in 37085 Korean subjects^[31]. Furthermore, NAFLD patients may exhibit enhanced subclinical atherosclerosis compared with non-steatotic individuals^[28]. This may be explained at least in part by the coexistence of NAFLD with an atherogenic risk profile characterized by hyperlipidemia, dysglycemia and hypertension^[32]. Also, in NAFLD the liver overproduces various atherogenic factors, including inflammatory cytokines, coagulation factors and molecules that increase blood pressure^[32].

To date, an established treatment of NAFLD is gradual weight loss. It was shown that dietary intervention or bariatric surgery improved liver function tests and liver histology in patients with NASH^[33-35]. Also, weight reduction by orlistat might be useful^[36,37]. Among 50 overweight subjects dietary intervention together with vitamin E and orlistat achieved significant weight loss^[37]. Reduction ≥ 5 and 9% was associated with improved insulin resistance and hepatic histological findings, respectively^[37]. Also, vitamin E as a potent antioxidant can improve liver histology in non-diabetic NASH. In this context, it is considered a first-line pharmacotherapy for this patient population^[38]. Furthermore, pioglitazone can be useful for patients with biopsy-proven NASH^[38]. However, it should be acknowledged that most patients on pioglitazone in clinical trials were non-diabetic. Also, long-term safety and efficacy has not been evaluated^[38].

NAFLD AND LIPID-LOWERING DRUGS

Interest is increasing regarding the effect of lipid-lowering drugs on NAFLD. Long-term statin treatment has been associated with significant decreases or even normalization of serum aminotransferase activities in patients with NAFLD and dyslipidemia^[39-41]. Liver steatosis assessed by either imaging techniques or biopsy was also diminished^[40,41]. Interestingly, this benefit appears to be associated with greater vascular risk reduction. The Greek Atorvastatin and Coronary Heart Disease Evaluation study included 1600 patients with established coronary heart disease (CHD). These were randomized to atorvastatin or “usual” medical care to achieve low density

lipoprotein cholesterol (LDL-C) goal < 100 mg/dL^[42]. Atorvastatin was more effective in reducing total and CV mortality as well as coronary morbidity compared with “usual” care^[42]. A *post hoc* analysis of this study included 437 patients with moderately abnormal liver function tests at baseline possibly associated with NAFLD^[43]. NAFLD was assumed in patients with moderately elevated aminotransferase activities (< 3x the upper limit of normal) together with relevant ultrasonographic findings, after excluding other causes of abnormal liver function tests^[43]. Statin (mainly atorvastatin) treatment was associated with substantial improvements of aminotransferase activities, whereas non-statin use with further increases^[43]. Interestingly, among patients with abnormal liver function tests those who received a statin experienced a greater reduction of CV events (69% relative risk reduction, $P < 0.0001$) compared with those who did not receive a statin^[43]. Furthermore, among statin-treated patients, those with abnormal liver function tests had fewer CV events compared with those with normal liver function tests (39% relative risk reduction, $P < 0.0001$)^[43]. However, these promising findings have limitations including the *post hoc* analysis and small number of patients. Furthermore, liver biopsy was not performed.

Ezetimibe antagonizes cholesterol absorption by inhibiting Niemann-Pick C1 like-1 protein (NPC1L1)^[44]. This protein is expressed by both enterocytes and hepatocytes. It was suggested that ezetimibe may be useful for the management of NAFLD by inhibiting hepatic cholesterol accumulation^[45]. This can be better achieved by combinations of ezetimibe with drugs facilitating weight loss or enhancing insulin sensitivity^[45].

Fibrates are first-line drugs for reducing TG levels. In this context, they are commonly used for correcting lipid abnormalities in obese patients with MetS and T2DM^[46-48]. Their hypolipidemic action is attributed to activation of the peroxisome proliferator-activated receptors (PPAR), particularly PPAR α ^[49]. PPARs control the transcription of genes regulating lipid and glucose metabolism^[50]. These receptors may also modulate hepatic lipid homeostasis, inflammation and fibrosis, by directing the proliferative and inflammatory response of specific cell types^[50].

PPAR α isotype is highly expressed in metabolically active tissues, including the liver, muscle, intestine and brown adipose tissue^[51]. PPAR α is predominantly expressed by hepatocytes and decreases hepatic lipid accumulation^[50,51]. This is mostly attributed to a regulation of the FA transport and β -oxidative degradation. PPAR α also controls inflammatory responses by inhibiting inflammatory gene expression induced by nuclear factor kappa B (NF- κ B)^[50,51]. Furthermore, it can limit interleukin (IL)-1-associated C-reactive protein expression^[50,52]. In this regard, it has been shown that PPAR α deficient mice are susceptible to hepatic steatosis and NASH^[53-55].

The current concept is that PPAR α activation may prevent these abnormalities^[50,53]. Interestingly, PPAR α agonism reversed steatohepatitis in mice, suggesting a

potential curative role against NASH^[56]. This benefit might be attributed to a downregulated expression of inflammatory genes^[55]. Also, PPAR α activation may reverse fibrosis by reducing the expression of fibrotic markers and the number of stellate cells^[56].

FENOFIBRATE AND NAFLD

Fenofibrate is one of the most used fibrates. This drug alone or in combination with statins improves the atherogenic serum lipid profile, by significantly reducing TG, while raising HDL-C levels^[57-59]. Also, it appears to exert anti-inflammatory and anti-thrombotic actions, while improving endothelial function, particularly in patients with MetS and T2DM^[47,60-63]. In this context, large clinical trials suggest that fenofibrate declines atherosclerosis progression and the risk of vascular events in patients with T2DM and dyslipidemia^[64,65]. However, this benefit should be further assessed.

Interestingly, fenofibrate may improve insulin sensitivity by limiting lipid accumulation in several tissues, including the liver and muscles^[66-69]. This can also be attributed to increased adiponectin together with reduced expression and plasma levels of several other adipokines, including tumor necrosis factor- α (TNF- α), leptin, resistin and plasminogen activator inhibitor (PAI)-1^[66,70,71]. Considering its hypolipidemic and insulin-sensitizing actions it could be assumed that fenofibrate is useful for the prevention and management of NAFLD. Herein, we discuss the role of fenofibrate as a potential treatment option for NAFLD.

Mechanistic implications

Animal studies suggested a protective role of fenofibrate against NAFLD providing explanatory mechanisms. Fenofibrate prevented from high-fat diet-induced hepatic TG accumulation^[72-75]. Consequently, all histological findings of NAFLD, including hepatic steatosis, necroinflammation and collagen deposition, were reversed^[72-74]. These benefits were associated with its lipid-lowering together with anti-inflammatory properties. Namely, fenofibrate prevented from diet-associated weight gain and increases in circulating TG and free FA^[72,73]. It was suggested that PPAR α activation by fenofibrate enhances hepatic FA turnover. Namely, it increased mRNA expression of FA β -oxidation enzymes in obese rats with T2DM^[76]. These include FA transport protein, FA binding protein, carnitine palmitoyltransferase II, as well as medium- and long-chain acyl-CoA dehydrogenase and acyl-CoA oxidase^[76,77].

A high-fat diet-associated increase in the liver inflammatory gene expression may be ameliorated by fenofibrate^[74]. Importantly, this effect was relevant immediately after treatment initiation, before liver steatosis occur. This finding implies a potential protective role of fenofibrate against liver inflammation resulting in NASH^[74]. TNF- α plays a key role in NASH pathogenesis. Its plasma levels can be decreased by fenofibrate^[72]. It appears that TNF- α hepatic expression is reduced by PPAR α activation^[73].

Macrophage infiltration of the liver may be also limited^[72,74]. Inhibition of the liver expression of monocyte chemoattractant protein (MCP)-1, intercellular adhesion molecule (ICAM)-1 and vascular adhesion molecule (VCAM)-1 may help explain this benefit^[75]. This action is PPAR α -dependent^[75]. Anti-inflammatory properties of fenofibrate imply a protective effect against NASH.

Anti-oxidant actions may account for anti-steatotic effects of fenofibrate on the liver^[73,78]. Fenofibrate reduced hepatic steatosis in mice developing hereditary NAFLD without obesity^[78]. Increased expression of genes facilitating FA turnover, while reducing hepatic lipid peroxidation, were mechanisms explaining this benefit^[78].

Reducing hepatic insulin resistance may also account for protective effects of fenofibrate against NAFLD^[72,73,76,79]. This may be mediated by enhanced FA β -oxidation together with eliminated accumulation of diacylglycerols, which have an impact on insulin signaling^[79]. In this context, fenofibrate improved liver steatosis in animal models of obesity-related T2DM and hepatic insulin resistance^[76,79]. Likewise, in a NASH animal model with obesity, dyslipidemia and insulin resistance fenofibrate improved insulin sensitivity and hepatic morphology, while decreasing ALT activity^[77]. In this regard fenofibrate was more effective than rosiglitazone^[77]. Therefore, PPAR α might be preferred over PPAR γ activation for treating insulin resistance-associated NASH.

High-fat diet may adversely affect hepatic microvasculature by narrowing sinusoids and reducing hepatic microcirculatory perfusion^[80]. Consequently, oxygenation of portal venules may be disturbed. It was suggested that these abnormalities promote the development of NAFLD^[80]. It was suggested that PPAR α agonists exert beneficial effects on the microcirculation of several tissues, including the retina, kidney and nerves^[80]. It appears that PPAR α activation inhibits various mediators of vascular damage, including lipotoxicity, inflammation, reactive oxygen species generation, endothelial dysfunction and thrombosis^[80]. Also, it can influence intracellular signalling pathways associated with microvascular complications^[80]. In this context, fenofibrate was associated with a slower progression of retinopathy and albuminuria in the clinical setting of T2DM^[64,80-82]. It was shown that fenofibrate exerts beneficial effects on the liver microvascular environment and oxygen metabolism. Namely, it remarkably improved microvascular patency and tissue oxygenation in high-fat diet-induced NAFLD mice^[83]. These findings imply a potential protective effect of the drug against T2DM-related hepatic steatosis.

Another experimental study investigated a cross-link between anti-steatotic and anti-atherosclerotic effects of fenofibrate^[84]. Microparticles are small membrane vesicles produced by activated and apoptotic cells, being not only biomarkers, but also functional actors in NAFLD and atherosclerosis. In mice with atherosclerosis and NAFLD, fed with Western diet, the expression of microparticles was increased in atherosclerotic lesions and the liver^[84]. Fenofibrate was associated with reduced expression of

microparticles in atherosclerotic lesions, but not in the liver^[84]. Therefore, limiting microparticle expression might not help explain the protective role of fenofibrate against NAFLD.

Adiponectin is an adipokine with various functions associated with insulin sensitivity and inflammation^[85-87]. Reduced adiponectin levels have been associated with increased insulin resistance and risk of vascular events^[85]. Patients with MetS and/or T2DM have low circulating adiponectin levels^[86,87]. These are also decreased in patients with NAFLD, possibly due to enhanced hepatic insulin resistance together with declined FA β -oxidation^[88,89]. In contrast, recombinant adiponectin administration exerted protective effects against NAFLD in mice^[90]. These were attributed to increased β -oxidation and limited hepatic synthesis of FA^[90]. Also, liver production and plasma levels of TNF- α may be reduced^[90]. Adiponectin downregulated aldehyde oxidase 1 *in vivo*^[91]. This enzyme produces reactive oxygen species that promote cell damage and fibrogenesis^[91]. Its activity is high in obesity-related hepatic steatosis^[91]. Adiponectin also inhibited hepatic fibrosis by downregulating connective tissue growth factor *in vitro*^[92]. This molecule promotes liver fibrosis by activating transforming growth factor (TGF) β ^[92]. PPAR α activation appears to play a key role in these benefits of adiponectin; thus fenofibrate exhibited the same properties^[91,92]. Also, clinical and experimental studies showed that fenofibrate increases adiponectin plasma levels^[93,94]. This was associated with vascular benefits of the drug and the rise in HDL-C levels^[93,94].

Except for adiponectin, its liver-specific R2 receptor (AdipoR2) appears to play a role in steatosis and inflammation. At the cellular/molecular level AdipoR2 mRNA was reduced in liver samples of patients with NASH^[95,96]. It was suggested that AdipoR2 may be protective against steatosis-related hepatic insulin resistance^[97]. In contrast, impairment of its expression was associated with decreases PPAR α signaling pathways^[97]. FA load and endoplasmic reticulum stress decreased AdipoR2 levels *in vitro*^[98]. Fenofibrate preserved AdipoR2 levels, while preventing from TG accumulation and endoplasmic reticulum stress^[98].

Clinical evidence

Few clinical studies assessed the effect of fenofibrate on biochemical and imaging surrogates of NAFLD. A study included 186 patients with MetS and both biochemical and ultrasonographic evidence of NAFLD^[99]. These received lifestyle advice and treatment for hypertension, impaired fasting glucose (metformin) and obesity (orlistat)^[99]. For the management of dyslipidemia study participants were randomized to atorvastatin 20 mg/d or micronized fenofibrate 200 mg/d monotherapy or their combination. After 54 wk the percentage of patients having no longer biochemical or ultrasonographic evidence of NAFLD was 67%, 42% and 70% for atorvastatin, fenofibrate and combination, respectively^[99]. Interestingly, each treatment option was independently associated with this benefit.

Other variables independently predicting hepatic steatosis elimination included corrections of low-grade inflammation (high-sensitivity C-reactive protein), anthropometric variables (waist circumference and body weight), the serum lipid profile (TG, LDL-C and total cholesterol levels), systolic blood pressure and glucose levels^[99]. This study highlighted a potential role of multifactorial treatment, including fenofibrate, in reducing hepatic steatosis associated with MetS. However, these results should be considered under certain limitations. For example, no placebo group was included. Furthermore, no biopsy, which is the “gold standard” for NAFLD diagnosis and staging, was performed^[99]. Another small study included 15 patients with T2DM randomized to fenofibrate or pioglitazone^[100]. Pioglitazone significantly improved glucose homeostasis and reduced fasting TG and free fatty acid (FFA) concentrations. These changes were associated with decreased hepatic fat content assessed by MRI^[100]. However, these changes were not relevant in fenofibrate-treated patients. Adding pioglitazone on fenofibrate decreased insulin resistance as well as FFA and TG levels, thereby reducing hepatic fat content^[100]. This finding implies the efficacy of multifactorial treatment in restricting hepatic steatosis in T2DM, as well. In contrast, adding fenofibrate on top of pioglitazone failed to significantly decrease insulin resistance, while lowering circulating TG and FFA levels. These changes were not associated with significantly reduced hepatic steatosis^[100]. Considering these, improving insulin resistance by PPAR γ activation might be preferred over reducing TG levels for the management of T2DM-related NAFLD.

Another study included 16 patients with biopsy-confirmed NAFLD. These were treated with fenofibrate (200 mg/d) for 48 wk^[101]. Fenofibrate was associated with significant improvement of the serum lipid profile and insulin sensitivity. Alkaline phosphatase and γ GT activities were significantly reduced^[101]. The proportion of patients with abnormal aminotransferase activities (> 45 IU/L) was also decreased: 93.7% at baseline *vs* 62.5% following treatment for ALT; 50% at baseline *vs* 18.7% following treatment for AST^[101]. Interestingly, a control liver biopsy at the end of the study showed a significantly decreased grade of hepatocellular ballooning degeneration compared with baseline. However, the grade of steatosis, lobular inflammation, fibrosis and NAFLD activity score did not change significantly^[101].

Data from large clinical trials are lacking. This may be attributed to the exclusion of patients with chronic liver disease due to transient increases in aminotransferase activities associated with fibrate treatment^[102]. Although these elevations occur without any other signal of hepatotoxicity, moderate increases in liver function tests are an indication of adverse effect reaction in large clinical trials. This was also relevant for fenofibrate trials, including the Fenofibrate Intervention and Event Lowering in Diabetes study^[64]. Also, it was suggested that aminotransferases may be direct PPAR α target genes^[103,104]. This implies that fibrate-induced elevations in plasma aminotransferase

activities are a mechanism-related treatment effect. In this context, aminotransferase activities might be problematic as surrogates of NAFLD in studies assessing drug effects on liver steatosis.

CONCLUSION

NAFLD is a common health problem associated with increased liver-specific as well as CV morbidity and mortality. Impaired FA turnover, often associated with insulin resistance, is its pathophysiological hallmark. In the presence of inflammation hepatic steatosis can progress to NASH and eventually cirrhosis. Weight loss, vitamin E and pioglitazone can be useful for the management of this abnormality, while the role of lipid-lowering drugs is being investigated.

PPAR α plays a role in the pathogenesis of NAFLD by regulating lipid and glucose metabolic pathways. The novel concept is that PPAR α activation may be protective and therapeutic against NAFLD. Experimental data suggested such a role of fenofibrate in the setting of high-fat diet, obesity, insulin resistance and T2DM. An improved FA turnover can help explain this benefit. Indeed, fenofibrate appears to enhance the expression of genes promoting FA β -oxidation. Also, its anti-inflammatory together with anti-oxidant actions may prevent from NASH-related necroinflammation, apoptosis and fibrosis. These are attributed to inhibited expression of inflammatory mediators, including TNF- α , MCP-1, VCAM-1 and ICAM-1, together with reduced lipid peroxidation and reactive oxygen species formation. Also, it was suggested that insulin resistance is also improved by fenofibrate. All these effects appear to be PPAR α -dependent. Furthermore, fenofibrate increases the expression and plasma levels of adiponectin, while preserving its liver-active receptor. Beyond insulin-sensitizing effects this adipokine enhances FA hepatic β -oxidation and exerts various anti-inflammatory and anti-fibrotic effects on the liver.

Data are inconclusive regarding the effect of fenofibrate on NAFLD surrogates in the clinical setting. Fenofibrate treatment as a part of multifactorial approach may be useful in MetS and T2DM. In this regard, insulin-sensitizing may be more important than lipid-lowering effects of the drug. However, this benefit should be further established by histological studies. Other limitations of available clinical studies include small sample size and the use of fenofibrate in combination with other strategies. In this context, there is a need for large prospective studies, including proper control groups and full assessment of liver histology.

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Hepatitis C virus infection, microRNA and liver disease progression

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Abstract

Hepatitis C virus (HCV) is a global health problem with an estimated 170-200 million peoples (approximately 3% of world population) are chronically infected worldwide and new infections are predicted to be on rise in coming years. HCV infection remains categorized as a major risk factor for chronic hepatitis, liver cirrhosis and hepatocellular carcinoma worldwide. There has been considerable improvement in our understanding of virus life cycle since, the discovery of HCV two-decades ago. MicroRNAs (miRNAs) are important players in establishment of HCV infection and their propagation in infected hepatocytes. They target crucial host cellular factors needed for productive HCV replication and augmented cell growth. Very first anti-miRNA oligonucleotides, miravirsin has been tested in clinical trial and shown promising results as therapeutic agent in treatment against chronic HCV infection. Deregulated expression of miRNAs has been linked to the pathogenesis associated with HCV infection by controlling signaling pathways such as, proliferation, apoptosis and migration. Circulating miRNAs emerging as growing field in identification of biomarkers in disease progression and their potential as a means of communication between cells inside the liver is an exciting area of research in

future. This review focuses on recent studies enforcing the contribution of miRNAs in HCV life cycle and co-ordinated regulation in HCV mediated liver disease progression.

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Key words: Hepatitis C virus; MicroRNA; Liver disease; Interferon signaling; Circulatory microRNA

Core tip: Hepatitis C virus (HCV) is the major cause of chronic liver disease that gradually progresses from chronic hepatitis to cirrhosis and hepatocellular carcinoma (HCC) during the course of infection. MicroRNAs (miRNAs) are small RNA molecules and have the ability to regulate gene expression by targeting mRNA degradation or translational repression. miRNAs regulate HCV life cycle either by supporting viral replication or by inhibiting interferon signaling pathway. Several miRNAs play important roles in HCV related inflammation, fibrosis and HCC development. This review focuses on the involvement of miRNA in HCV life cycle and virus mediated liver disease progression, emerging role of circulating miRNAs and exploitation of miRNA as alternative therapeutic approach for HCV infection.

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INTRODUCTION

Hepatitis C virus (HCV) was first identified as a non-A, non-B hepatitis more than two decades ago^[1]. It is a single stranded, positive sense RNA virus belongs to family *flaviviridae* and genus *hepacivirus*. The viral genome encodes

for a single precursor polyprotein of approximately 3010 amino acids, which is cleaved by viral and cellular proteases into three structural (core, E1 and E2) and seven non-structural (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) viral proteins. Core protein forms the capsid, which is surrounded by a lipid bilayer containing the glycoproteins, E1 and E2. These viral proteins are responsible for viral replication and various cellular functions^[2]. HCV is a major cause of chronic liver disease, mostly asymptomatic in nature. Majority of the infected patients approximately, 80% develop persistent chronic infection and are at high risk for liver cirrhosis and hepatocellular carcinoma (HCC). An estimated 170-200 million peoples worldwide are infected with hepatitis C^[3] and about 2.7-3.9 million peoples are living with HCV infection in the United States^[4]. In addition, HCC and cirrhosis have been increasing among persons infected with HCV^[5]. Recent approval of HCV NS3/4A protease inhibitors in standard treatment, consisting of pegylated interferon (IFN) alpha, and nucleoside analog, ribavirin (RBV) have shown improved rates of sustained virologic response in HCV infected patients^[6]. Drugs targeted against HCV polymerase, NS5B has also been successfully validated in phase 2 clinical trials for the treatment of HCV infection^[7].

MicroRNAs (miRNAs) were discovered in 1993 during a developmental timing experiment in the nematode *Caenorhabditis elegans*. Till date, human miRNA family has expanded to over 2000 mature miRNAs (miRBase v19.0; <http://www.mirbase.org>) and *in silico* prediction estimates that approximately 60% of human mRNA could be targets of miRNA^[8]. miRNAs constitute a class of non-coding RNAs, about 18-22 nucleotides long and play crucial role in the regulation of gene expression. The production of miRNAs requires several processing steps, first primary miRNAs (pri-miRNAs) are cleaved by the ribonuclease Drosha to produce precursor miRNAs (pre-miRNAs) which in turn, cleaved by the ribonuclease Dicer to produce mature, single stranded miRNAs^[9,10]. Once synthesized, mature miRNA associate with RNA induced silencing complex (RISC) together with Argonaute/EIF2C (AGO) proteins and mediates the target mRNA recognition. miRNA identify target mRNA through specific base-pairing interactions between the 5' end ("seed" region) of miRNA and sites within coding and untranslated regions (UTRs) especially 3' UTR of mRNAs that lead to mRNA destabilization. miRNA inhibits the target gene expression either by mRNA degradation or translational repression. miRNA promotes mRNA cleavage by inducing deadenylation or suppresses protein synthesis by repressing the translation initiation at the cap recognition or inducing ribosomes to drop off prematurely^[11,12]. miRNA biogenesis is beyond the scope of this review and elegant reviews addressing miRNA synthesis and their mechanism of gene regulation is discussed in more detail elsewhere. A combinatorial nature of miRNA regulation, *i.e.*, each miRNA regulates hundreds of different mRNAs and further, a single mRNAs are targeted by multiple

miRNAs, will allow us to focus on regulatory networks that determine the cell fate decisions. Viral infection can elicit changes in cellular miRNA expression profile, and several RNA viruses have been reported to interact directly with cellular miRNAs to facilitate their replication potential^[13].

ROLE OF MIRNAS IN HCV REPLICATION

Recent studies have identified several miRNAs as key players in virus-host interactions, regulating virus replication and pathogenesis during HCV infection. The most abundant miRNA in the liver, miR-122 is regulated by specific, liver-enriched transcription factor, hepatocyte nuclear factor 4 α ^[14] and is responsible for liver homeostasis^[15]. Several studies demonstrated that miR-122 is required for HCV replication in infected cells^[16-18]. miR-122 positively modulates HCV infection through direct interactions with viral RNA and stimulates HCV translation^[19]. It forms an oligomeric complex in which one miR-122 molecule binds to the 5' UTR of HCV RNA with 3' overhanging nucleotides, masking the 5' terminal sequences of HCV genome. Furthermore, specific internal nucleotides as well as 3' terminal nucleotides in miR-122 were absolutely required for maintaining HCV RNA abundance^[20]. miR-122 recruits Argonaute 2 to the 5' end of the viral genome, stabilizing the viral RNA and avoid the degradation in infected cells^[21]. Recent study also demonstrated that miR-122 protects HCV RNA from 5' decay by targeting 5' exonuclease Xrn1^[22]. Exogenous expression of miR-122 allows efficient HCV RNA replication and/or infectious virion production in non-permissive cell line^[23-25]. Apart from regulating viral replication, miR-122 is also involved in cell cycle progression in hepatoma cell line^[26]. miR-122 is known to target cyclin G1 and use of miR-122 inhibitor has been reported to prevent the alcohol-induced increase in HCV RNA and protein levels^[27].

Besides miR-122, other miRNAs have been involved in HCV replication. Overexpression of miR-448 and miR-196 were able to substantially attenuate viral replication by directly targeting CORE and NS5A coding region of the HCV genome, respectively^[28]. Let-7b was also identified as novel cellular miRNAs that directly target HCV genome and elicits anti-HCV activity^[29]. Mutational analysis identified let-7b binding sites at the coding sequences of NS5B and 5'-UTR of HCV genome that were conserved among various HCV genotypes. Overexpression of miR-199a inhibited HCV replication in cells bearing HCV-1b or -2a genome length replicon^[30]. miR-196a inhibits HCV RNA and NS5A protein expression in replicon by regulating HMOX1/Bach1 expression^[31]. In HCV infected patients, lower expression levels of miR-29 was observed in liver and overexpression of miR-29 inhibits viral RNA in HCV infected hepatocytes^[32]. We have demonstrated that miR-130a expression is upregulated in liver biopsy from HCV infected patients as well as in HCV infected hepatocytes

in vitro^[33]. We also observed that knockdown of miR-130a inhibits HCV replication in hepatocytes. Similar observation has been reported on miR-130a mediated regulation of viral replication in HCV infected cells^[34]. Differential upregulation of hsa-miR-130a, hsa-miR-130b, hsa-miR-298, hsa-miR-193a-5p and hsa-miR-371-5p were also observed in HCV Con1 replicon in comparison to control cells. These miRNAs have been associated with cell growth by targeting genes PPAR γ , IRF1 and STAT3 in HCV infected cells^[35]. Differential expression of miRNAs such as, miR-24, miR-149, miR-638 and miR-1181 were also identified following HCV infection and are involved in HCV entry, replication and propagation^[36]. Delivery of miR-17-92 cluster has been reported to inhibit HCV replication by up to 95% *in vitro* cell culture system^[37]. Recently, negative effect of miR-27a has been demonstrated in HCV replication. miR-27a repression increased the cellular lipid content, decreased the buoyant density of HCV particles and increased viral replication and infectivity^[38]. miR-192/miR-215 and miR-491 are capable of enhancing HCV replication in replicon cells^[39]. miR-141 mediated suppression of DLC-1 (a Rho GTPase-activating protein) enhances viral replication in HCV-infected primary human hepatocytes^[40].

ROLE OF MIRNAS IN REGULATION OF INTERFERON RESPONSE IN HCV INFECTION

HCV infection also modulates several miRNAs, which in turn inhibits type 1 IFN signaling pathway. We have demonstrated that HCV inhibits IFITM1, an interferon stimulated gene, by upregulating miR-130a expression in HCV-infected hepatocytes. Introduction of anti-miR-130a in hepatocytes increased IFITM1 expression with concomitantly reduction in HCV replication^[33]. Overexpression of miR-122 has also been associated with inhibition of IFN signaling pathway. Silencing of miR-122 enhances IFN-induced interferon stimulated response element activity, by decreasing expression of SOCS3. This decrease in SOCS3 level was also regulated by enhanced methylation at *SOCS3* gene promoter, implicating additional mechanism of inhibition of HCV replication using antisense oligonucleotides of miR-122^[41]. miRNAs also regulate the expression of target genes involving immune response to viral infections mediated by type I IFN pathway. Upregulated miR-21 suppressed MyD88 and IRAK1 expression in hepatocytes, which subsequently repressed type I IFN effector gene expression and the type I IFN-mediated antiviral response, thereby promoting viral replication^[42]. IFN- α treatment also modulates HCV-specific miRNAs expression in hepatocytes. miR-324-5p and miR-489 shown to be upregulated in the presence of IFN- α while differential expression of miR-30c and miR-130a were observed between HCV-infected Huh7.5 cells treated with or without IFN- α ^[34]. miR-30 cluster targets *SOCS1* and *SOCS3* genes that act as negative regulators of cytokine signaling. Spe-

cifically, SOCS1 and SOCS3 inhibit JAK tyrosine kinase activity and STATs in the JAK-STAT signaling pathway suggesting that IFN- α induced miRNAs modulates gene expression in HCV infected hepatocytes^[34]. IFN- β treatment of Huh-7 cells showed an upregulation of miR-142-3p and miR-128a, and these miRNAs were downregulated in HCV replicon-expressing cells^[43]. IFN- β induced miRNAs, in conjunction with the downregulation of miR-122, was also studied to prevent HCV replication. Introduction of anti-miRs against miR-196, miR-296, miR-351, miR-431 and miR-448, with and without the inclusion of miR-122 mimic, attenuated the IFN- β mediated reduction of viral RNA by approximately 75%^[28]. Treatment with a toll-like receptor-7 (TLR-7) agonist, imiquimod, downregulates miR-146a and miR-155 in PBMCs from HCV infected patients as compared to their expression in PBMCs of healthy individuals^[44].

Increasing evidence also suggests that miRNAs have a profound impact on host defense to HCV infection and clinical outcome of standard HCV therapy. miRNA expression profiles were examined to identify the miRNAs associated with the standard treatment (IFN- α with ribavirin) to CHC patients. Expression levels of 9 miRNAs were significantly different in the sustained virological response (SVR) and non-responder (NR) groups, suggesting that expression pattern of these hepatic miRNA are associated with therapeutic outcome in CHC patients^[45]. The expression level of miR-122 was reportedly associated with early response to IFN treatment. HCV infected patients who did not respond to therapy had significantly lower miR-122 levels as compared to responder^[46].

ROLE OF MIRNAS IN HCV RELATED INFLAMMATION AND FIBROSIS

Many miRNAs have been implicated in various cancers either as oncogenes or tumor suppressor genes. HCV infection induces chronic inflammation and regulation of inflammation related miRNA favors the initiation and progression of HCC. Gene expression analyses identified dysregulation of miR-449a in HCV patients but not in alcoholic and non-alcoholic liver diseases. YKL40 is an inflammatory marker known to be upregulated in patients with chronic liver diseases with fibrosis and miR-449a regulates the expression of YKL40 by targeting NOTCH signaling pathway following HCV infection^[47].

In patients infected with HCV, miR-155 expression levels were markedly increased, and promote hepatocyte proliferation and tumorigenesis by modulating Wnt signaling^[48]. Chronic HCV infection induced liver fibrosis is mediated by upregulation of transforming growth factor (TGF)- β ^[49]. In HCV-infected patient samples and in a mouse carbon tetrachloride fibrosis model, expression levels of miR-21 were positively correlated with fibrotic stage^[50]. miR-21 was shown to target SMAD7, a negative regulator of TGF- β signaling, leading to increased fibrogenesis^[50]. Inhibition of miR-29 was also linked with activation of hepatic stellate cells and collagen

Table 1 Altered expression of microRNAs in association with Hepatitis C virus infection and liver disease progression

miRNA	Expression ¹	Target genes	Function
MiRNAs that facilitates HCV infection			
miR-122	Up	5' UTR in HCV genome <i>Xrn1</i> <i>Cyclin G1</i> <i>SOCS3</i>	Promote HCV replication ^[16-18] and IRES mediated HCV translation ^[21] Inhibit 5' decay of HCV RNA ^[22] Promote Alcohol induced viral replication ^[27] Enhance methylation at SOCS3 gene promoter, inhibits IFN-induced ISRE activity ^[41]
miR-130a	Up	<i>IFITM1</i>	Inhibits type 1 IFN signaling pathway and promotes HCV replication ^[33]
miR-141	Up	<i>DLC-1</i>	Promote viral replication ^[40]
miR-21	Up	<i>MyD88</i> and <i>IRAK1</i>	Negatively regulate IFN signaling ^[42]
MiRNAs that suppresses HCV infection			
miR-448	Unknown	Core region in HCV genome	Inhibits viral replication ^[28]
miR-196/196a	Unknown	NS5A region in HCV genome <i>Bach1</i>	Inhibits viral replication ^[28] Inhibits HCV RNA and NS5A protein expression, relieve oxidative stress by upregulating <i>HMOX1</i> gene expression ^[31]
let-7b	Unknown	<i>NS5B</i> and 5'UTR regions in HCV genome	Reduces HCV infectivity ^[29]
miR-199a	Unknown	5' UTR in HCV genome	Inhibits viral replication ^[30]
miR-27a	Up	<i>RXRα</i> and <i>ABCA1</i>	Regulates lipid metabolism, decrease viral infectivity ^[38]
MiRNAs that promote inflammation and fibrosis upon HCV infection			
miR-449a	Down	<i>NOTCH1</i>	Regulates YKL40 promoter activity and promotes inflammation ^[47]
miR-21	Up	<i>SMAD7</i>	Increase TGF-β signaling and promote fibrosis ^[50]
miR-29	Down	<i>COL1A1</i> , <i>COL3A1</i>	Potentiate fibrosis by activating hepatic stellate cells ^[32]
miR-155	Up	<i>APC</i>	Promote cell proliferation by activating Wnt/β-Catenin signaling pathway ^[48]

¹Denotes endogenous expression in Hepatitis C virus (HCV) infected liver biopsy patients or HCV infected hepatocytes. UTR: Untranslated region; IFN: Interferon; TGF: Transforming growth factor; miR/miRNA: MicroRNA.

synthesis^[32].

ROLE OF MIRNAS IN HCV RELATED HCC

HCC is often considered as a complication of chronic liver disease, comprises of a single group, regardless of the etiology of liver disease. There are several risk factors associated with development of HCC, including chronic hepatitis C infection^[51]. Progression towards HCC involves multiple steps that ultimately lead to deregulation of various signaling pathways and help host cells to acquire metastatic potential in presence of surrounding microenvironment^[52]. Chronic hepatitis C is a major risk factor associated with HCC^[53]. HCV encoded viral proteins both singly or in coordinated manner, interact with host cellular factors and regulate signaling pathways such as, cell proliferation and apoptosis for augmentation of hepatocyte growth that may contribute towards HCC progression^[54]. miRNA dysregulation has been linked with initiation and progression of HCC^[55-57], however, the role of miRNAs in HCV-related HCC is poorly understood. The identification of HCC related miRNA signatures is of great value for the early diagnosis of HCC, before the onset of disease in HCV-positive patients. Limited studies are available addressing the role of miRNA expression in HCV associated HCC. Differential miRNA expression from formalin fixed paraffin embedded HCV infected HCC specimens indicated 10 upregulated and 19 downregulated miRNAs^[58]. Another study in HCV infected patients, 13 miRNAs were shown to be downregulated and were predicted to target genes related to immune response, antigen presentation, cell cycle,

proteasome, and lipid metabolism signaling pathways^[59]. However, validation of these miRNAs and their predicted targets are necessary for conclusive role of particular miRNA in HCV related HCC. A list of altered miRNAs associated with HCV infection and their proposed role in liver disease progression has been summarized in Table 1.

CIRCULATORY MIRNAS IN HCV INFECTION

One of the major challenges in HCV research is early detection of liver disease which allow us for rapid intervention and improved outcome of antiviral treatment. Liver biopsy is often recommended in patients with unexplained elevated serum aminotransferases in order to determine the cause, grade of hepatic inflammation and stage of hepatic fibrosis. Non-invasive or minimally invasive methods need to be developed which can evaluate disease severity and the likelihood of disease progression. Circulating miRNAs have been demonstrated to be very specific and stable in human serum and plasma. In addition, circulating miRNAs display consistent profiles between healthy individuals and significantly altered levels in disease conditions^[60,61]. These characteristics of circulating miRNAs established their potential value as biomarkers for detection and as predictive marker for liver disease progression in HCV infection. We have performed serum/plasma specific miRNA array and observed that several circulating miRNAs are significantly upregulated in sera of HCV infected patients as compared to healthy controls^[62]. We have shown that increased expression of

miR-20a and miR-92a is specific to HCV associated liver disease because we did not observe an upregulation of these miRNA in sera of patients with non-HCV related liver disease. Subsequently, we observed that elevated levels of miR-20a were positively correlated with disease severity in HCV infected patients, however, miR-92a expression is reduced with higher grade of fibrosis in HCV infected patients^[62]. miRNA profiling was also performed to identify the expression of 940 human miRNAs in the serum of HCV infected individuals. Serum levels of miR-134, miR-320c and miR-483-5p were significantly upregulated in HCV infected patients^[63]. Serum levels of miR-122 were correlated with disease parameters in patients with CHC by several groups. The higher levels of miR-122 and miR-192 was observed in sera from patients with CHC and in other etiologies associated with liver injury as compared to sera from healthy controls^[64-68]. The serum level of miR-122 and miR-21 strongly correlates with serum alanine leucine transaminase levels (ALT) and higher necroinflammatory activity in the liver in patients with CHC infection suggesting their potential as a serum biomarker over ALT in predicting the presence of chronic HCV infection^[64,65,68,69], although the specificity of miR-122 for HCV mediated liver disease is questionable. Levels of miR-125b and miR-146a were also increased in the serum of CHC patients compared to healthy controls^[70]. Treatment-naïve patients with chronic HCV infection have been shown to have higher expression of miR-155 in their circulating monocytes as compared to individuals who cleared HCV infection after therapy, suggesting a possible correlation between increased miR-155 and HCV viral presence and/or replication^[70].

Circulating miRNAs in urine were also examined for developing screening methods. Expression of 3 upregulated miRNAs, miR-625, miR-532 and miR-618, were evaluated as non-invasive biomarkers for the early diagnosis of HCC among high-risk HCV positive patients. Elevated expression of miR-625, miR-532 and miR-618 were observed in 56%, 62.5% and 72% of HCC-post HCV positive patients, respectively. In addition, miR-516-5p and miR-650 were found to be down-regulated in 50% and 72% of HCC-post HCV positive patients, respectively. Differential expressions of these miRNAs were predicted to possibly target genes related to HCC development and progression in high risk HCV patients^[71]. The function of these extracellular circulating miRNAs is not well understood. The liver is a complex organ where various cell types reside and interact in close vicinity. During HCV infection, there could be multiple factors that contribute to release of miRNAs in the circulation. Secretion of miRNA from different cell types in a cell-specific manner in response to HCV infection cannot be ruled out. Further studies are warranted to investigate the cellular source of circulating miRNAs.

MIRNA AS THERAPEUTICS IN HCV INFECTION

Therapies that target essential host factors required for

HCV replication could present an effective approach for the development of new HCV antiviral drugs. Therapeutic potential of miR-122 employing antisense oligonucleotide (SPC3649) complementary to the 5'-end of miR-122, has been evaluated in HCV infected chimpanzees^[72]. SPC3649 therapy resulted in a reduction of HCV viral load in the liver and blood of chronically infected chimpanzees. In addition, reduction in viral load was accompanied by normalization of the endogenous interferon pathway, which is maximally induced in chronically infected chimpanzees, suggesting the restoration of host immune response following treatment with SPC3649^[72]. Recently, miravirsin (a locked nucleic acid-modified antisense oligonucleotide for miR-122) showed prolonged dose-dependent reductions in HCV RNA levels without evidence of viral resistance in chronic HCV genotype 1 infected patients in a phase II clinical trial^[73]. No adverse side effects of miR-122 inhibition have been documented in either chimpanzees or chronic HCV infected patients. Additional host cell factors that help HCV for productive replication such as, cyclophilin A and phosphatidylinositol-4-kinase III alpha have emerged as a promising alternative^[74]. The efficacy of anti-miR-122 along with current anti-HCV drugs needs to be evaluated in future trials that could provide better therapy outcome in terms of lesser rate of relapse, interferon free regimens and reduced possibility of drug resistance. miRNAs function either as oncomiRs or tumor suppressors are involved in cell growth regulatory pathways, have rapidly emerged as targets for therapeutics in the pathogenesis of HCV infection. Successful delivery of either miRNA mimics to restore the activity of tumor suppressor miRNAs or anti-miR oligonucleotides for pharmacological inhibition of oncogenic miRNAs, understanding of potential off-target effects and physiologic consequences of long-term miRNA modulation *in vivo* are some of the important factors to keep into consideration in future development of miRNA therapeutics.

CONCLUSION

We are still in the infancy stage of understanding the potential of manipulating miRNAs for the treatment of HCV infection. HCV infection modulates a set of miRNAs that regulate host immune response and cell growth interconnected with multiple signaling pathways. miRNA mediated regulation of gene expression will help us to understand the signaling pathway and disease progression associated with HCV infection. Therapeutic silencing of miR-122 opens a door for novel drugs and therefore, identification of miRNAs with a prominent role in HCV viral life cycle and its implication in liver disease progression is emerging as a therapeutic option against chronic HCV infections. A major drawback of exploiting miRNA as therapy may have adverse side effects because of the biological properties of miRNA where, a single miRNA binds to multiple targets and regulate various signaling pathways simultaneously. On the contrary, recent studies

on circulating miRNAs generates an alternative approach for identification of minimally invasive biomarker for HCV mediated liver disease and as predictive biomarker to categorize patients those may develop end stage liver disease due to viral infection. Indeed, further in-depth studies are needed to identify mechanistic insights behind modulated miRNAs by HCV infection.

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Pure laparoscopic hepatectomy for hepatocellular carcinoma with chronic liver disease

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Abstract

Pure laparoscopic hepatectomy is a less invasive procedure than conventional open hepatectomy for the resection of hepatic lesions. Increases in experiences with the technique, in combination with advances in technology, have promoted the popularity of pure laparoscopic hepatectomy. However, indications for usage and potential contraindications of the procedure remain unresolved. The characteristics and specific advantages of the procedure, especially for hepatocellular carcinoma (HCC) patients with chronic liver diseases, are reviewed and discussed in this paper. For cirrhotic patients with liver tumors, pure laparoscopic hepatectomy minimizes destruction of the collateral blood and lymphatic flow from laparotomy and mobilization, and mesenchymal injury from compression. Therefore, pure laparoscopic hepatectomy has the specific advantage of minimal postoperative ascites production that leads to lowering the risk of disturbance in water or electrolyte balance and hypoproteinemia. It minimizes complications that routinely trigger postoperative serious liver

failure. Under adequate patient positioning and port arrangement, the partial resection of the liver in the area of subphrenic space, peri-inferior vena cava area or next to the attachment of retro-peritoneum is facilitated in pure laparoscopic surgery by providing good vision and manipulation in the small operative field. Furthermore, the features of reduced post-operative adhesion, good vision, and manipulation within the small area between the adhesions make this procedure safer in the context of repeat hepatectomy procedures. These improved features are especially advantageous for patients with liver cirrhosis and multicentric and/or metachronous HCCs.

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Key words: Laparoscopic hepatectomy; Hepatocellular carcinoma; Liver cirrhosis; Chronic liver disease; Liver Tumor; Liver resection; Repeat hepatectomy; Bridging therapy to transplantation; Ascites; Postoperative liver failure

Core tip: For cirrhotic patients with liver tumor, pure laparoscopic hepatectomy minimizes destruction of the collateral blood/lymphatic flow from laparotomy and mobilization, and has advantage of minimal postoperative ascites. It restrains the complications, which trigger the postoperative liver failure. The partial resection in the area of subphrenic space, peri-inferior vena cava area or next to the attachment of retro-peritoneum is facilitated with good vision and manipulation in the small operative field. Furthermore, repeat pure laparoscopic hepatectomy for patients with multicentric/metachronous hepatocellular carcinomas was feasible and safe with the advantages of less post-operative adhesion and good vision and manipulation between the adhesions.

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ma H, Arakawa S, Yoshida R, Isetani M. Pure laparoscopic hepatectomy for hepatocellular carcinoma with chronic liver disease. *World J Hepatol* 2013; 5(9): 487-495 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v5/i9/487.htm> DOI: <http://dx.doi.org/10.4254/wjh.v5.i9.487>

INTRODUCTION

Since the first successful report of laparoscopic liver wedge resection in 1992^[1], pure laparoscopic hepatectomy is thought to be a less invasive procedure than conventional open hepatectomy for the resection of hepatic lesions^[2]. In a comprehensive meta-analysis study, laparoscopic hepatectomy was compared to open hepatic resection in 1678 patients across 26 studies. While it is associated with longer operating times and no differences in oncological outcomes, it is advantageous in several aspects^[3]. Laparoscopic hepatectomy is associated reduced blood loss, decreased portal clamp time, a decrease in overall and liver-specific complications, and shorter post-operative hospital stays^[3]. Recent technological development of devices and accumulation of experiences have facilitated the expansion of the indication of the procedure^[4,5]. Common advantages of laparoscopic surgery, such as early recovery and discharge with smaller post-operative pain and earlier intake, have been reported also for laparoscopic hepatectomy^[6]. However, specific advantages and/or disadvantages of pure laparoscopic hepatectomy for proper indication have not yet been resolved.

Hepatocellular carcinoma (HCC) is the fifth most common primary cancer and the third most common cause of cancer-related deaths worldwide^[7,8]. The treatment options for HCC include surgical resection^[9], liver transplantation^[10], transarterial chemoembolization (TACE), and local ablation therapy^[11]. Most experts think surgical resection and liver transplantation are the best hopes for cure. However, most patients with HCC have underlying chronic liver disease, and hence are at very high risk of developing significant postoperative complications. Although liver transplantation should be considered in patients with deteriorating liver function according to the Milan criteria^[12], hepatic resection should be considered as a primary therapy in patients with well-preserved liver function^[13,14]. When considering the treatment of HCC in patients with liver cirrhosis, the degree of invasive surgical stress, especially to the impaired liver, should be considered in addition to the oncological therapeutic effects. Patients with severe liver cirrhosis have various (overt and preliminary) symptoms, such as: (1) deteriorations of protein synthesis and metabolism; (2) GI tract congestion, ascites, pancytopenia due to portal hypertension and hypersplenism; and (3) susceptibility to infectious diseases and hepatopulmonary syndrome (hypoxaemia) due to increased shunt vessels^[15]. Cirrhotic patients have high morbidity and mortality following anesthesia and surgery^[16] and the risk of abdominal operations increases according to the preoperative Child-Pugh

class of the patients^[17]. Even limited resection following open surgery for severe cirrhotic patients often develops refractory ascites, which leads to fatal complications^[18,19].

In Japan, criteria for selecting patient eligibility for hepatectomy is based on three parameters: (1) the presence or absence of ascites; (2) total serum bilirubin level; and (3) indocyanine green retention rate at 15 min (ICG R15)^[20]. Currently, surgical resection, local ablation therapy, or TACE is adapted to each HCC patient with liver cirrhosis depending on the tumor condition and the liver function. However, a large number of HCC patients with severe liver dysfunction are not able to undergo those treatment modalities due to liver function, tumor size and/or localization. This is especially true after repeat treatments for the disease, including a large number of patients that need repeat treatments for multicentric metachronous lesions occurring in chronic impaired liver. For those patients, "less invasive" pure laparoscopic hepatectomy may provide a good option. The characteristics and advantages of pure laparoscopic hepatectomy for HCC patients with chronic liver diseases are discussed in this paper.

LAPAROSCOPIC HEPATECTOMY FOR HCC PATIENTS: AN OVERVIEW

The benefits of laparoscopic hepatectomy may be particularly advantageous for cirrhotic patients, given the potential for lower levels of parietal and hepatic injury and the preservation of venous and lymphatic collateral circulation. The safety and feasibility of the laparoscopic approach and its short-term benefits for HCC patients with chronic liver dysfunction have been demonstrated by several series^[21-31]. To date, several studies have investigated the major differences between laparoscopic hepatectomy and open hepatectomy (Tables 1 and 2)^[32-39]. Favorable short-term results, including fewer incidences of ascites and liver failure, and shorter postoperative hospital stays, correlate with the laparoscopic procedure. Tranchart *et al.*^[36] reported laparoscopic resection of HCC for selected patients resulted in better postoperative outcomes without long- and short-term oncologic consequences (42 each laparoscopic- and open-hepatectomy patients, with more than 96% Child-Pugh class A patients and mostly anatomical resection). Early postoperative recovery and discharge with less postoperative pain are usual advantages of laparoscopic surgery. Additionally, pure laparoscopic hepatectomy has the advantage of minimal ascites (Table 2), due to preservation of venous and lymphatic collateral circulation, which leads to lower risk of disturbance in water and/or electrolyte balance and hypoproteinemia, disorders that could trigger fatal liver failure. This feature could be the most remarkable specific advantage for postoperative course.

Patients who undergo hepatectomy are exposed three different types of stresses: (1) general, whole-body surgical stress; (2) reduced liver function due to resected liver volume; and (3) surgery-induced injury for liver pa-

Table 1 Recently reported laparoscopic hepatectomy and open hepatectomy comparative studies - general information.

Ref.	No. of cases		Sex (male:female)		Age (yr, mean \pm SD)		Background liver % of LC		Tumor size (cm, mean \pm SD)		Type of resection nonanatomical:LLS: Anatomical	
	LH	OH	LH	OH	LH	OH	LH	OH	LH	OH	LH	OH
Laurent <i>et al</i> ^[32]	13	14	10:3	10:4	62.6 \pm 9.5	65.9 \pm 5.5	NA	NA	3.35 \pm 0.89	3.43 \pm 1.05	0:3:10	0:4:10
Belli <i>et al</i> ^[33]	23	23	10:13	14:9	59.5 \pm 6.84	62.4 \pm 7.7	100.00	100.0	3.10 \pm 0.70	3.24 \pm 0.70	0:5:18	0:6:17
Lai <i>et al</i> ^[34]	25	33	18:7	21:12	59 (35-79) ¹	59.0 (38-77) ¹	92.00	93.9	2.50 (1-7) ¹	2.60 (1-8) ¹	0:6:19	0:2:31
Aldrighetti <i>et al</i> ^[35]	16	16	11:5	12:4	65 \pm 10	71.0 \pm 6.00	56.30	56.3	4.00 \pm 2.20	4.60 \pm 2.50	0:5:11	0:5:11
Tranchart <i>et al</i> ^[36]	42	42	15:27	14:28	63.7 \pm 13.10	65.7 \pm 7.10	73.80	80.9	3.58 \pm 1.75	3.68 \pm 2.09	0:9:33	0:7:35
Kim <i>et al</i> ^[37]	26	29	18:8	20:9	57.84 \pm 9.66	57.08 \pm 9.78	NA	NA	3.15 (1-8) ¹	3.60 (1-19) ¹	0:4:22	0:3:26
Lee <i>et al</i> ^[38]	33	50	24:9	40:10	59 (36-85) ¹	58.50 (32-81) ¹	84.80	64.0	2.50 (1.5-9.0) ¹	2.90 (1.2-9.0) ¹	15:18:0	40:10:0
Truant <i>et al</i> ^[39]	36	53	31:5	47:6	60.6 \pm 10.20	63.30 \pm 7.60	NA	NA	2.90 \pm 1.20	3.1 \pm 1.20	0:22:14	0:26:27

¹Expressed as median (range). LLS: Left lateral sectionectomy; LC: Liver cirrhosis; NA: Not available; LH: Laparoscopic hepatectomy; OH: Open hepatectomy.

Table 2 Recently reported laparoscopic hepatectomy and open hepatectomy comparative studies - operative outcomes

Ref.	Ascites		Liver failure		Hospital stay (d, mean \pm SD)		Mortality	
	LH	OH	LH	OH	LH	OH	LH	OH
Laurent <i>et al</i> ^[32]	1/13	5/14	1/13	5/14	15.3 \pm 8.6	17.3 \pm 18.9	0/13	2/14
Belli <i>et al</i> ^[33]	3/23	8/23	NA	NA	8.2 \pm 2.6	12.04 \pm 3.93	1/23	0/23
Lai <i>et al</i> ^[34]	NA	NA	NA	NA	NA	NA	0/25	1/33
Aldrighetti <i>et al</i> ^[35]	0/16	1/16	NA	NA	6.3 \pm 1.7	9.0 \pm 3.8	0/16	0/16
Tranchart <i>et al</i> ^[36]	3/42	11/42	0/36	4/53	6.7 \pm 5.9	9.6 \pm 3.4	1/42	1/42
Kim <i>et al</i> ^[37]	0/26	1/29	NA	NA	11.08 \pm 4.96	16.07 \pm 10.697	0/26	0/29
Lee <i>et al</i> ^[38]	0/33	2/50	NA	NA	NA	NA	0/33	0/50
Truant <i>et al</i> ^[39]	5/36	12/53	NA	NA	6.5 \pm 2.7	9.50 \pm 4.8	0/36	4/53

LH: Laparoscopic hepatectomy; OH: Open hepatectomy; NA: Not available.

Table 3 Perioperative course after pure laparoscopic hepatectomy

	Cases with severe cirrhosis (<i>n</i> = 7) (ICG R15: \geq 40%, Child-Pugh B/C)	Cases with mild-moderate cirrhosis (<i>n</i> = 30) ¹ [ICG R15: 8.7%-31.1% (median 13.2%), Child-Pugh A]
Operating time (min)	140-341 (232)	130-710 (302)
Intraoperative blood loss (mL)	NC-2750 (100)	NC-2400 (100)
Day of initiation of oral intake in POD	1/3 (2)	1-3 (2)
Drain discharge (as total of 0-3 POD, mL)	279-1990 (919)	60-3350 (416)
Postoperative hospital stay (d)	11/21 (17)	8-254 (18)
Morbidity	14.3% (Cholecystitis)	13.3% (Ileus, refractory ascites, bile leakage)
Mortality	0%	0%

¹Excluded patients with combined surgery. NC: Not countable; POD: Post-operative day; ICG R15: Indocyanine green retention rate at 15 min.

renchyma and environment around the liver caused by destruction of the collateral blood and lymphatic flow by laparotomy and mobilization of the liver and mesenchymal injury caused by compression of the liver. Reduction of surgery-induced injury with pure laparoscopic hepatectomy lowers the risk of refractory ascites, resulting in reduced risk of successive complications and smooth recovery for HCC patients with severe liver cirrhosis.

We also experienced that the perioperative course of HCC patients with severe liver cirrhosis (Child-Pugh class B/C and ICG R15 of \geq 40%) who underwent pure laparoscopic hepatectomy was favorable and comparable to that of the other HCC patients with mild/moderate liver cirrhosis^[40]. As of 2012, 40 patients with HCC and chronic liver disease underwent pure laparoscopic hepatectomy in our hospital. Seven out of 40 patients had severe liver cirrhosis (Child-Pugh class B/C and ICG R15 of \geq 40%). These seven patients and 30 other patients (Child-Pugh class A and ICG R15 of 10.1%-27.4%; three patients were excluded from analysis because of concomitant combined surgery) were compared in perioperative course (Table 3). The perioperative course results, such as intraoperative blood loss, day oral ingestion started, total dose of drain discharge to post operative day 3, morbidity and mortality, were comparable without statistically significant difference in the two groups. Among these seven patients, one underwent living-related liver transplantation 20 mo after hepatectomy.

This extensive review of the literature in combination with our clinical experiences indicate that pure laparo-

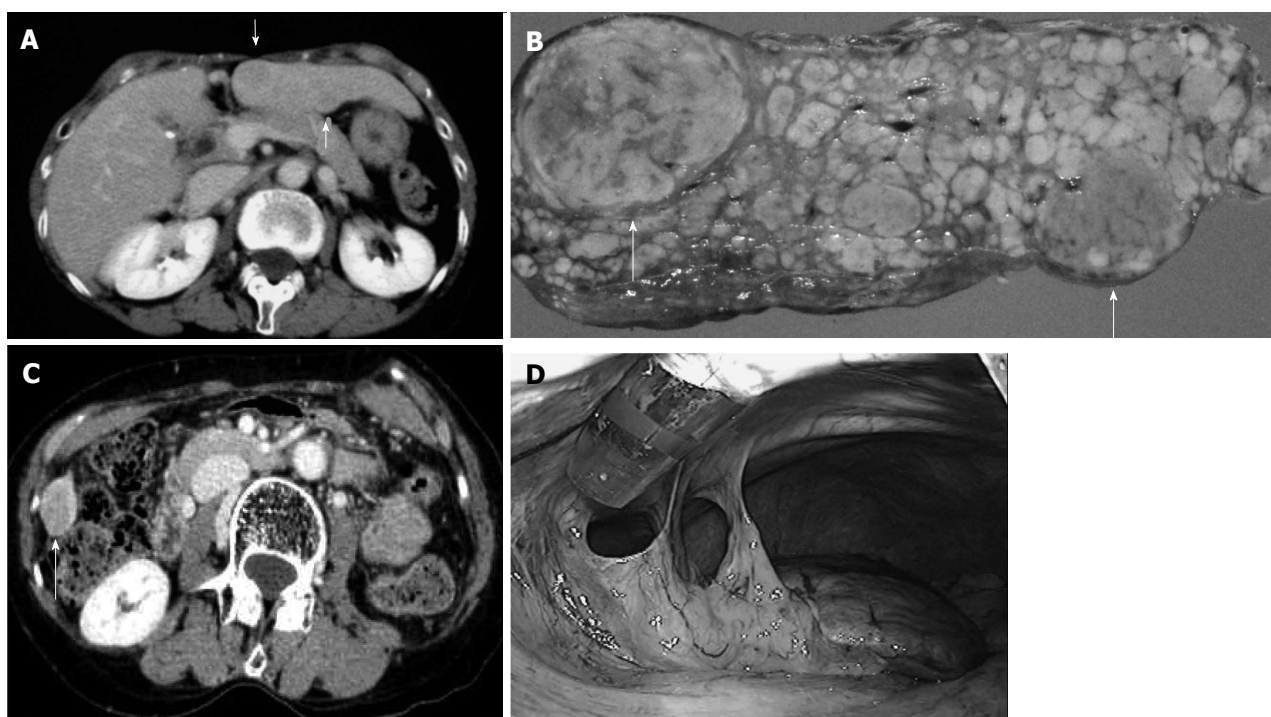


Figure 1 Repeat pure laparoscopic hepatectomy for patients with liver cirrhosis and hepatocellular carcinomas was feasible and safe: case 1. A: Computed tomography scan shows two hepatocellular carcinomas (HCC) in segment 3; B: The tumors (arrows) resected laparoscopically; C: A 69-year-old woman with type-C liver cirrhosis developed a new HCC on the caudal edge of segment 6 of the liver 2 years after the first hepatectomy; D: At the second laparoscopic hepatectomy, there was only mild adhesion around the resected area

scopic hepatectomy is the better therapeutic option for severe cirrhotic patients with tumors on the surface of the liver, in case of difficult adaptation of percutaneous ablation therapy and/or local recurrence after repeat treatments. The procedure may also prove to be an advantageous option in bridging therapy to liver transplantation for HCC patients with severe liver cirrhosis.

LAPAROSCOPIC HEPATECTOMY FOR HCC PATIENTS: ADVANTAGES AND DISADVANTAGES

At the introduction of laparoscopic hepatectomy in 1997, we selected the patients who could undergo adequate oncological pure laparoscopic resection for cancers. The indication of pure laparoscopic hepatectomy had been gradually extended from liver surface partial resection to large anatomical resection (right hepatectomy and posterior/anterior sectionectomy). The inclusion criteria are now a tumor size less than 10 cm without severe adhesion, invasion to major vessels, or a need for reconstruction of vessels or biliary tract.

As of 2012, we have performed forty of pure laparoscopic hepatectomy for HCC with chronic liver diseases, including ten cases of anatomical resections and four cases of repeat hepatectomy. There was no operative-mortality and the rate of morbidity was 12.5%. Tumor numbers are 1-4 and sizes are 1.1-7.8 cm. The median

of their operating time and blood loss was 288 min and 50 mL. From these experiences, we propose the following advantages of laparoscopic hepatectomy for HCC patients: (1) advantageous for repeat procedures: Repeat pure laparoscopic hepatectomy (and combined treatments) for patients with liver cirrhosis and multicentric/metachronous HCCs was feasible and safe. The procedure also resulted in less post-operative adhesion and good vision and manipulation in the small area between the adhesions (case 1, Figures 1 and 2); (2) minimal invasion due to good vision: With adequate port arrangement and positioning of the patients^[41,42], the manipulation in the small operative field is facilitated by good vision of the peri-inferior vena cava (IVC) area, subphrenic space, the area next to the attachment of retro-peritoneum, and the area between the adhesions. Therefore, there is a minimum need for dissection and/or adhesiolysis that could cause destructions of the collateral blood and lymphatic flows (case 2, Figures 3 and 4; case 3, Figures 5 and 6); and (3) better control of bleeding: Instead of compression and elevation of the bleeding field, other techniques are employed. These include meticulous manipulation under magnifying view, pressure control of pneumo-peritoneum and IVC, and various coagulating devices^[43]. The ability of bleeding control is becoming matched to open surgery and anatomical hepatectomies with the exposure of major vessels recently becoming feasible^[41,44-48].

The major disadvantages of laparoscopic surgery are in compression of the bleeding point, palpitation of

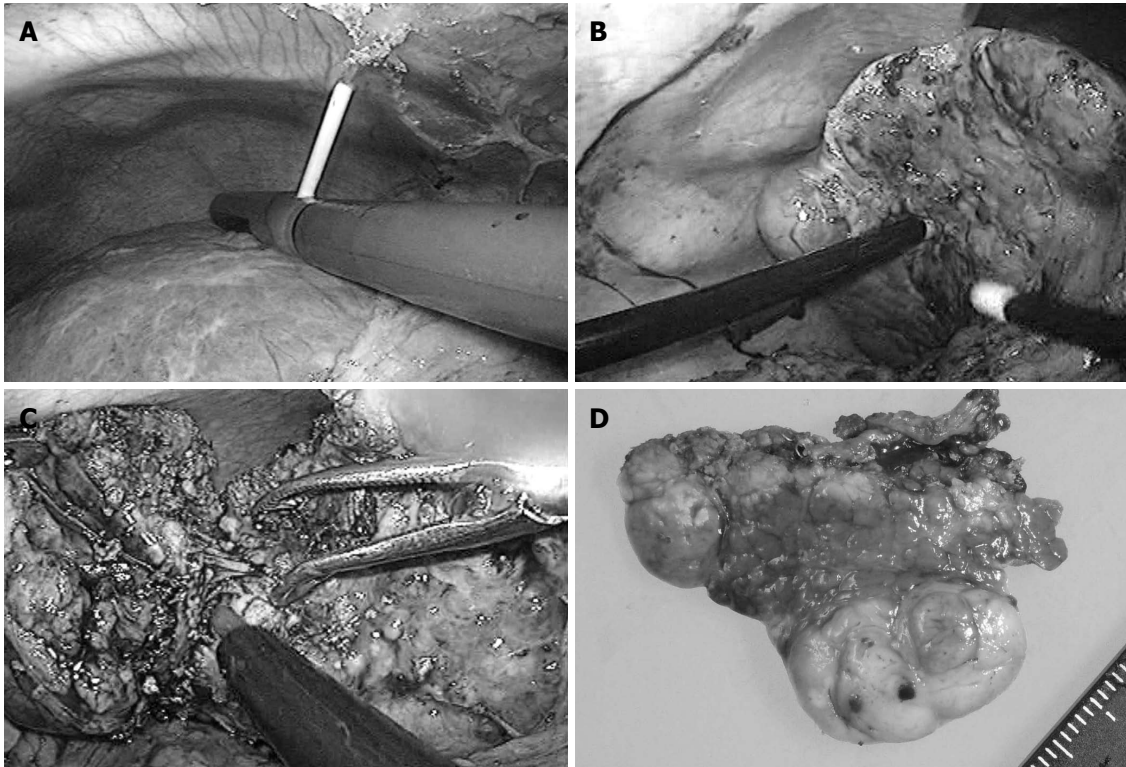


Figure 2 Repeat pure laparoscopic hepatectomy for patients with liver cirrhosis and hepatocellular carcinomas was feasible and safe: case 1. The patient also had two early lesions in segment four, which was treated with laparoscopic microwave coagulation therapy (A). After ablation therapy, the hepatocellular carcinomas (HCC) in segment 6 (B) was resected laparoscopically (C). The resected specimen (D) showed a single nodular HCC. The patient also underwent third hepatectomy for the lesion in segment one next to right adrenal gland two years after this operation.

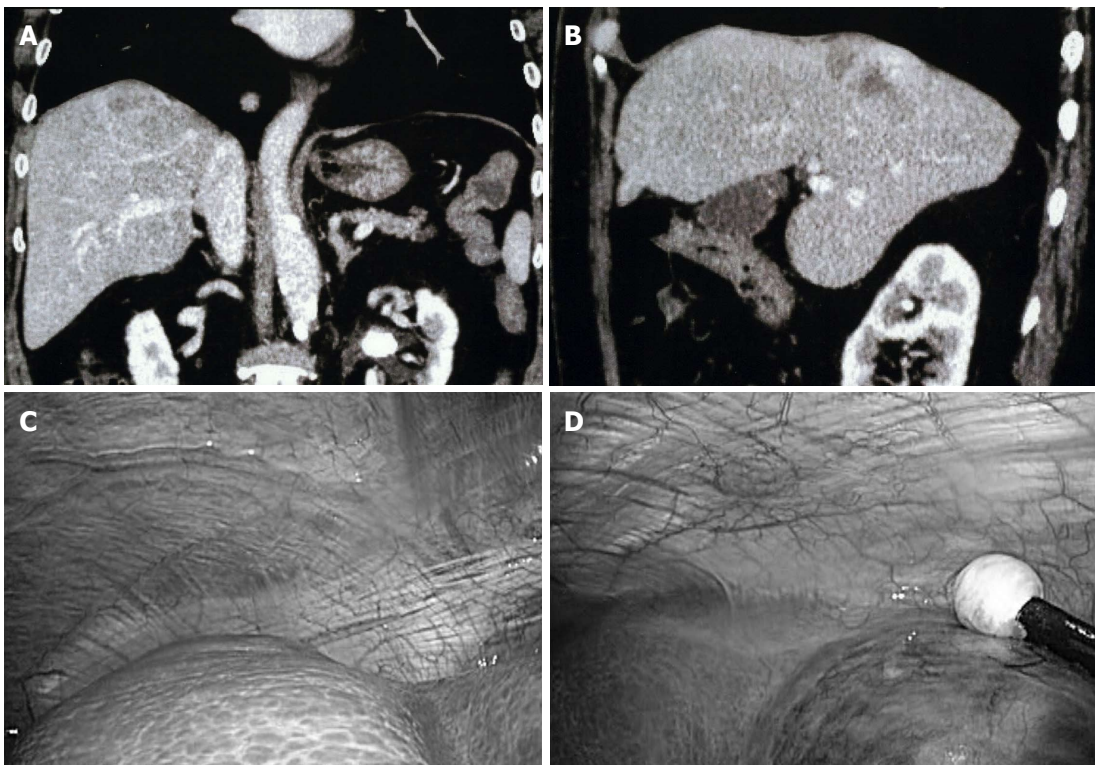


Figure 3 Pure laparoscopic hepatectomy is efficient in the subphrenic space: case 2. An 80-year-old woman with liver cirrhosis developed a hepatocellular carcinomas in the dorsal area of subsegment 8c of the liver revealed in computed tomography examination (A and B). Since the tumor compressed the right hepatic vein and her liver function seemed not to tolerate right hepatectomy or extended anterior sectionectomy, she underwent partial resection of the liver with the dissection and exposure of right hepatic vein and tumor capsule in pure laparoscopic hepatectomy. The tumor was located deeply in the subphrenic space (C) just next to the attachment of retro-peritoneum (D).

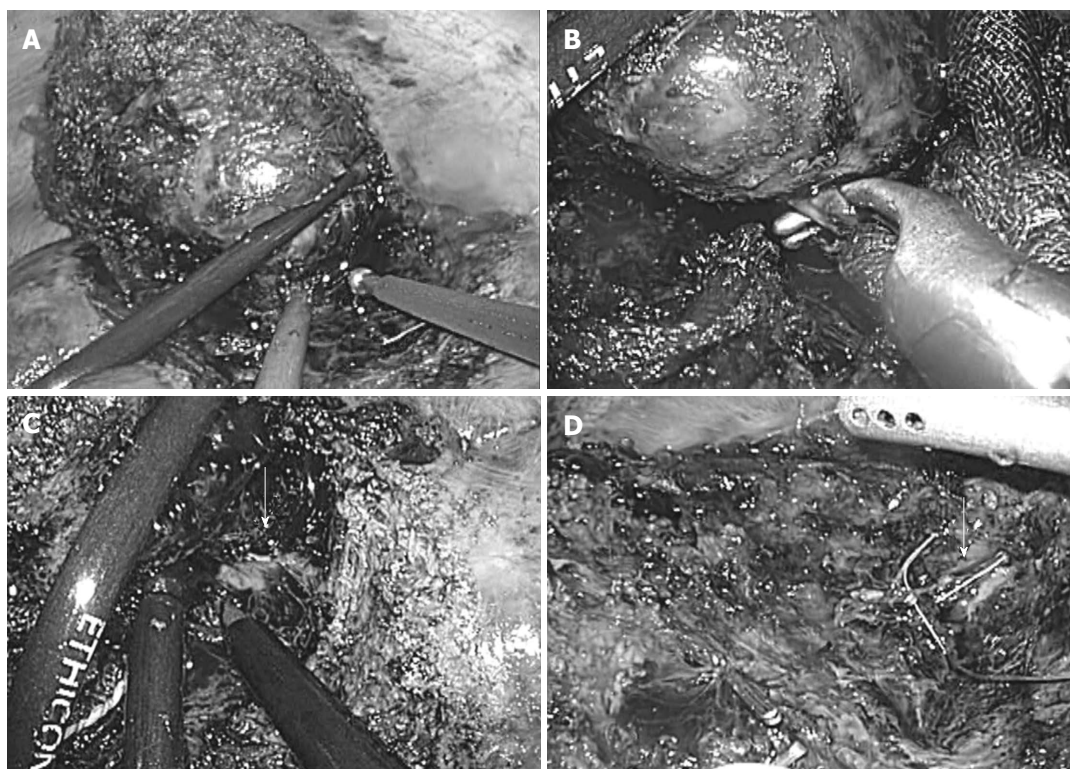


Figure 4 Pure laparoscopic hepatectomy is efficient in the subphrenic space: case 2. A: Resection of the tumor with the exposure of the capsule; B: Encircling and dividing of the direct branch of the right hepatic vein; C: Exposure of right hepatic vein; D: Cutting surface after resection.

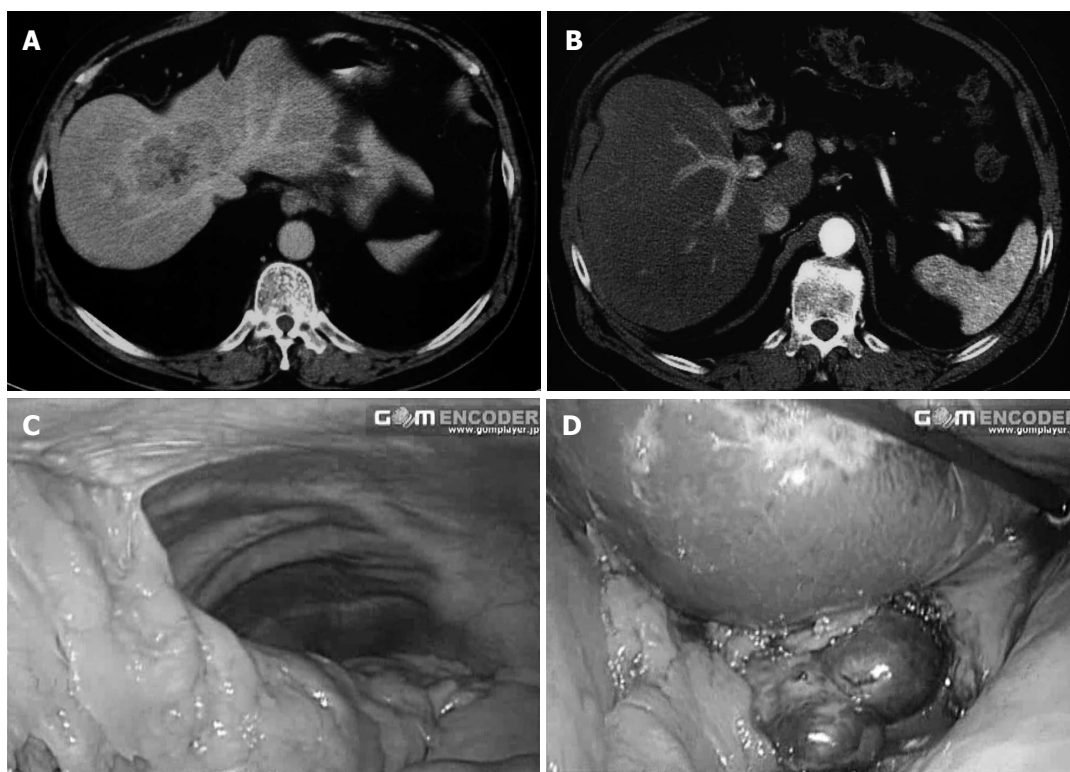


Figure 5 Pure laparoscopic hepatectomy is efficient between the adhesions and the peri-inferior vena cava area: case 3. Two years after a central bisectionectomy for hepatocellular carcinomas (HCC) at the roots of hepatic veins (A), a 66-year-old man developed a new prominent HCC on the left caudate lobe of the liver (B). Following the second pure laparoscopic hepatectomy, there was massive adhesion in the area of right upper abdomen (C). However, good view and access to the tumor were obtained with the dissection of omentum minus (D).



Figure 6 Pure laparoscopic hepatectomy is efficient between the adhesions and the peri-inferior vena cava area: case 3. A and B: Resection of the tumor; C and D: The view after the resection of the tumor.

the tumor, and overview of the whole abdominal fields. However, the advantages we have outlined above indicate that pure laparoscopic surgery should be chosen over open surgery for liver resection under specific conditions.

CONCLUSION

For cirrhotic patients with liver tumors, pure laparoscopic hepatectomy minimizes destruction of the collateral blood and lymphatic flow from laparotomy and mobilization, and mesenchymal injury from compression. Therefore, pure laparoscopic hepatectomy results in minimal postoperative ascites production, which leads to a lower risk of disturbance in water and/or electrolyte balance and hypoproteinemia. It leads to lower complications that could potentially lead to postoperative serious liver failure. These characteristics lead to facilitation of surgical treatments for such patients. Pure laparoscopic hepatectomy also results in improved vision and manipulation in a small operative field under the proper conditions. Further, in cases where it is necessary to perform repeat pure laparoscopic hepatectomy procedures, as well as combined treatments, pure laparoscopic hepatectomy proved to be safer. Finally, there is a minimum need for dissection and/or adhesiolysis which may cause perturbations in the collateral blood and lymphatic flows. These characteristics of pure laparoscopic hepatectomy indicate it is a superior method when compared to open hepatectomy under certain conditions.

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Hepatitis C genotype 6: A concise review and response-guided therapy proposal

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of 60%-90%. Emerging data suggests that a shorter course 24-wk treatment is equally effective as a standard 48-wk treatment, particularly for those patients who attained undetectable HCV RNA at week 4 (RVR). In addition, baseline and on-treatment predictors of response used for other HCV genotypes appear effective with genotype 6. Although some pan-genotypic direct-acting antivirals have completed phase II/III studies (sofosbuvir and simeprevir) with clinical benefit demonstrated in small number of patients with genotype 6, broad availability of these agents in Southeast Asia may not be expected in the near future. While awaiting the newer therapy, response-guided therapy seems appropriate for patients with HCV genotype 6. Patients with RVR (representing > 70% of patients) are suitable for 24-wk treatment with expected SVR rates > 80%. Patients without RVR and/or those with poor response predictors may benefit from 48 wk of therapy, and a detectable HCV RNA at week 12 (with no early virological response) serves as a stopping rule. This treatment scheme is likely to have a major economic impact on HCV therapy, particularly in Southeast Asia, wherein treatment can be truncated securely in the majority of patients with HCV genotype 6.

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Abstract

Hepatitis C genotype 6 is endemic in Southeast Asia [prevalence varies between 10%-60% among all hepatitis C virus (HCV) infection], as well as also sporadically reported outside the area among immigrations. The diagnosis of HCV genotype can be inaccurate with earlier methods of genotyping due to identical 5'-UTR between genotype 6 and 1b, hence the newer genotyping methods with core sequencing are preferred. Risk factors and clinical course of HCV genotype 6 do not differ considerably from other genotypes. Treatment outcome of HCV genotype 6 with a combination of pegylated interferon and ribavirin is superior to genotype 1, and nearly comparable to genotype 3, with expected sustained virological response (SVR) rates

Key words: Hepatitis C; Genotype 6; Epidemiology; Southeast Asia; Treatment; Pegylated interferon; Ribavirin; Response-guided therapy

Core tip: Hepatitis C genotype 6 is endemic in Southeast Asia [prevalence varies between 10%-60% among all hepatitis C virus (HCV) infection], as well as also sporadically reported outside the area among immigrations. The diagnosis of HCV genotype can be inaccurate with earlier methods of genotyping due to identical 5'-UTR between genotype 6 and 1b, hence the newer genotyping methods with core sequencing are preferred. Risk factors and clinical course of HCV genotype 6 do not differ considerably from other geno-

types. Treatment outcome of HCV genotype 6 with a combination of pegylated interferon and ribavirin is superior to genotype 1, and nearly comparable to genotype 3. Emerging data suggests that a shorter course 24-wk treatment is equally effective as a standard 48-wk treatment, particularly for those patients who attained undetectable HCV RNA at week 4.

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INTRODUCTION

Chronic hepatitis C virus (HCV) infection is a worldwide health problem in that it has a global prevalence rate of approximately 3% and affects over 170 million individuals. It is a leading cause of chronic liver disease and hepatocellular carcinoma worldwide in both industrialized and developing countries^[1]. However, geographic differences in the overall prevalence and distribution of HCV genotypes have been well recognized^[1]. The majority (87%) of HCV-infected individuals are from Western Pacific countries (62.2 million), Southeast Asia (32.3 million), Africa (31.9 million), and Eastern Mediterranean countries (21.3 million)^[2,3]. The prevalence of HCV infection is especially higher in Southeast Asia with an estimate prevalence of 2%-12% among general population in some countries^[4], compared to the estimated prevalence of 1.6% in western countries such as the United States^[5]. Hepatitis C genotypes 1, 2, and 3 are widely distributed globally and have been the focus of most experimental and clinical studies. Genotypes 4 and 5 are found mainly in the Africa and Middle East. Genotype 6 and its subtypes are found mainly in Southeast Asia^[2-4,6]. In some countries in Southeast Asia, such as Thailand, Vietnam, and Myanmar, HCV genotype 6 is one of the most common genotype, detected in 10%-60% of all HCV patients^[7-14]. In the past, HCV genotype 6 was believed to be confined to Southeast Asia, but in the changing era of increasing migration of populations, it has been recently reported in nearby areas of Asia, such as China, Taiwan, and Hong Kong (China)^[6,15], and as far as western countries, such as United States, Canada^[16], and Germany^[15]. As globalization (*e.g.*, immigration, travel, and cultural diversity) potentially impacts the epidemiology of HCV, the numbers of patients with HCV genotype 6 seen outside Southeast Asia is expected to increase.

Despite the significant burden of the disease, the creditable data regarding the epidemiology and treatment specifically for HCV genotype 6 are rather limited. This may be largely due to the fact that the majority of the HCV genotype 6-infected population is based in

developing countries with limited research facilities and restricted access to publication. This review is aimed to summarize the current available data regarding the epidemiology and treatment of HCV genotype 6, as well as to propose a response-guided algorithm of treatment.

CLASSIFICATION AND DIAGNOSIS

Substantial genetic diversity led to the identification and classification of various genotypes and subtypes of the HCV among different geographical areas. Currently, 6 major genotypes and more than 80 subtypes have been identified from around the world; the previously reported HCV genotypes of 7, 8, and 9 that are endemic in Southeast Asia have been re-classified as subtypes of genotype 6^[16,17]. Proper classification of HCV genotypes and subtypes is very important clinically and is dependent on nucleotide sequence disparity^[6]. Though the ideal method to accurately identify HCV genotype is by directly sequencing of the entire genome, the current, commercially available methods typically use distinct motifs found within the HCV genome to either indirectly or directly genotype HCV, a more resourceful strategy^[6]. Indirect method of HCV genotyping uses genotype-specific antibodies and competitive enzyme immunoassays (*e.g.*, Murex HCV Serotyping Assays, Murex Diagnostics, Dartford, United Kingdom)^[6]. Direct methods of genotyping include direct sequence analysis of 5'-UTR only (*e.g.*, TruGene HCV 5'NC, Visible Genetics, Toronto, Canada), restriction fragment length polymorphism analysis and reverse hybridization line probe assay for the 5'-UTR only (*e.g.*, INNO-LiPA HCV I, Innogenetics, Zwijnaarde, Belgium) or both 5'-UTR and core regions (INNO-LiPA HCV II, Innogenetics, Ghent, Belgium)^[6]. Selection of genotyping assay is crucial, especially for genotype 6 variants as genotype 6 shares identical 5'-UTR sequences with genotype 1b, thus making earlier genotyping methods based solely on 5'-UTR sequences alone unreliable and those tests with additional HCV core-sequencing preferable^[6,18-21]. Among the newer genotyping methods, INNO-LiPA HCV II assay is one of the most widely used globally. It has been developed on INNO-LiPA HCV I platform with additional sequencing of core regions and demonstrated significant improvement in genotyping accuracy, particularly to differentiate between HCV genotype 1 and genotype 6 variants (about 100% success rate)^[6,18-21].

EPIDEMIOLOGY OF HCV GENOTYPE 6

Epidemiologic studies regarding HCV genotype 6 from different parts of the world are summarized in Table 1. In brief, HCV genotype 6 is particularly common in Southeast Asia (prevalence among all HCV infections are 9%-31% in Thailand^[7-10], 21%-49% in Myanmar^[11,12], 32%-46% in Vietnam^[13,14], > 90% in Lao PDR^[22], and 56% in Cambodia^[23]), and is the most common HCV genotype is some of these countries. In addition, geo-

Table 1 Prevalence of hepatitis C virus genotype 6 in Asia

Country of origin	Population	Genotyping method	Prevalence of HCV genotype 6	Author
Thailand	<i>n</i> = 236; Blood donors throughout the country	Reverse hybridization	18.0%	Kanisanon <i>et al</i> ^[7]
	<i>n</i> = 58; Volunteers from four hospitals located in the North, North-east, South and Center of the country	Core sequencing	8.9%	Sunanchaikarn <i>et al</i> ^[8]
	<i>n</i> = 126; Blood donors in the Northern Thailand	Core sequencing	31.0%	Jutavigittum <i>et al</i> ^[9]
	<i>n</i> = 375; Blood donors in the Central Thailand	Core and NS5B sequencing	18.9%	Akkarathamrongsin <i>et al</i> ^[10]
	<i>n</i> = 40; Immigrant workers from Cambodia (<i>n</i> = 25) and Myanmar (<i>n</i> = 15) in Thailand	Core and NS5B sequencing	56% among Cambodian workers and 26.7% among Myanmar workers	Akkarathamrongsin <i>et al</i> ^[23]
Myanmar	<i>n</i> = 110; Blood donors in Yangon and its suburbs	NS5B sequencing	20.9%	Shinji <i>et al</i> ^[11]
	<i>n</i> = 145; Volunteers from four different border cities of Myanmar	Core sequencing	49% (Genotype 6 was mostly found in the Northern cities)	Lwin <i>et al</i> ^[12]
Vietnam	<i>n</i> = 308; Patients from urban community-based GI practice in Southern Vietnam	Core sequencing	31.5%	Nguyen <i>et al</i> ^[13]
	<i>n</i> = 135; Blood donors in Hanoi (Northern Vietnam)	Core (<i>n</i> = 70) and NS5B (<i>n</i> = 65) sequencing	45.9%	Pham <i>et al</i> ^[14]
Lao PDR	<i>n</i> = 45; Blood donors in Lao PDR	Core and NS5B sequencing	95.6%	Hübschen <i>et al</i> ^[22]
Hong Kong	<i>n</i> = 1055; 949 non-IVDU and 106 IVDU from all over Hong Kong	Core sequencing	27.1% (23.6% among non-IVDU and 58.5% among IVDU)	Zhou <i>et al</i> ^[26]
	<i>n</i> = 212; Blood donors	NA	27%	Prescott <i>et al</i> ^[25]
China	<i>n</i> = 148; Patients from nine regions in China	Core and NS5B sequencing	13% (Genotype 6 was only observed in the South)	Lu <i>et al</i> ^[24]

Source: Ref. [6], with permission. IVDU: Intravenous drug users; NA: Not available; HCV: Hepatitis C virus; GI: Gastrointestinal; PDR: People's Democratic Republic; NA: Not available.

graphical differences of HCV prevalence in each individual country were observed in which genotype 6 appears to be more prevalent in the Northern areas of Thailand, Myanmar, and Vietnam, when compared with the central and southern regions^[9,10,12-14]. It should be noted that the earlier reports of the prevalence of HCV genotype with previous version of HCV genotypic assays may have underestimated the prevalence of HCV genotype 6 (misclassified with genotype 1). Outside Southeast Asia, HCV genotype 6 is also observed in the nearby areas, particularly Hong Kong and the Southern parts of China^[24-26]. Interestingly, HCV genotype 6 is somewhat uncommon in the many countries in Southeast Asia, such as Indonesia, Philippines, and Singapore, as well as in the surrounding countries, such as India, Pakistan, Taiwan, and South Korea^[4,18]. Apart from the aforementioned areas, HCV genotype 6 encountered elsewhere (*e.g.*, United States, Canada, and Germany) were mostly immigrants from Southeast Asia^[15,16].

Nowadays in the Western countries, HCV infections are primarily due to intravenous or nasal drug use and, to a lesser degree, to unsafe medical/surgical procedures, tattooing or acupuncture with unsafe materials, and male homosexual activity^[27]. This contrasts with the principal routes of HCV transmission prior to 1990's of blood transfusion and unsafe injection procedures. Despite conflicting published data, several studies have found that many Asian HCV patients have no identifiable risk factor (up to 50%) of HCV acquisition^[27]. Intravenous

or nasal drug use does not seem to be a major contributing factor to HCV infection. Therefore, inadequately sterilized medical equipment and cultural practices such as acupuncture or cosmetic tattooing are presumably implicated in the transmission of HCV a significant proportion of patients^[18]. A cross-sectional study of 308 Southeast Asian Americans with HCV (41% with genotype 6) reported that risk factors for acquisition for HCV genotype 6 are similar to that of other genotypes, with 41% of patients who could not recall any specific exposure risk^[28]. Nevertheless, higher prevalence of HCV genotype 6 has been described in some certain populations including intravenous drug users and patients with thalassemia major^[10,26,29]. In Hong Kong, HCV genotype 6 was predominantly observed in 58.5%-62.5% among intravenous drug users and 50% among patients with thalassemia major^[26,29]. Correspondingly, Seto *et al*^[30] reported that statistically significant larger proportion of patients with HCV genotype 6 were infected through intravenous drug injections when compare to those with genotype 1 (28.2% *vs* 8.7%, respectively).

CLINICAL FEATURES

There is limited data that specifically addresses the clinical features and natural history of HCV genotype 6. A cross-sectional study performed in 308 Southeast Asians in California found no significant differences in the clinical and virological characteristics (*e.g.*, age, risk factors of

HCV acquisition, alcohol consumption, family history of liver disease, liver functions tests, white blood cell and platelet count, HCV RNA viral load, and liver histology) between HCV genotype 6 and other genotypes. Yet, several studies have suggested that Asian patients tend to be older, have lower body mass index (BMI), consume less alcohol and tobacco, and have more advanced liver histology at presentation than non-Asians^[18,31]. Late presentation in Asian patients may be secondary to the lack of awareness of appropriate screening and the low proportion of patients presenting with identifiable risk factors^[18].

Chronic HCV infection can be associated with various extrahepatic manifestations, including lymphoproliferative (*e.g.*, mixed cryoglobulinemia and lymphoma) and immunological disorders of various organ systems^[32]. The prevalence of lymphoproliferative disorders associated with HCV seems to be geographical heterogeneity^[32,33]. Without clear reasons, it is more prevalent in Southern Europe (with an increased prevalence in patients infected with HCV genotype 2) than in Northern Europe, North America, and Asia^[32,33]. To date, there have been no specific epidemiological and clinical data regarding extrahepatic manifestations of HCV genotype 6. From our experiences, clinically significant extrahepatic manifestations of HCV are rare in Thailand (especially when compared to the relatively high prevalence of HCV in this area).

TREATMENT

Treatment outcomes

A combination of pegylated interferon (PEG-IFN) and ribavirin (RBV) has been the standard treatment for patients with chronic HCV. These drugs are administered for either 48 wk (for HCV genotypes 1, 4, 5, and 6) or for 24 wk (for HCV genotypes 2 and 3), inducing sustained virologic response (SVR) rates of 40%-50% in those with genotype 1, and of > 70-80% in those with genotypes 2 and 3 infections^[27,34]. It should be noted that HCV treatment outcome with PEG-IFN/RBV in Asians seems to be superior to that of non-Asian populations, and this may be due to several factors, such as favorable *IL28B* genotype, low body weight, and HCV genotype misclassification (6 to 1)^[18]. More recently, the treatment durations can be modified according to the virological responses (response-guided therapy)^[27], and in 2011, direct-acting antiviral (DAA)-based triple combination therapies (boceprevir or telaprevir plus PEG-IFN/RBV) have been approved and shown to improve virological outcomes in HCV genotype 1 patients, with an SVR of up to 65%-75% in treatment-naïve patients^[35]. Once achieved, an SVR is associated with long-term clearance of HCV infection, which is regarded as a "cure," as well as with significant improvement of morbidity and mortality of the patient^[27,35]. Among several predictors of SVR to therapy, HCV genotype is considered one of the most robust independent predictors^[27]. Compared

to other genotypes, data regarding the treatment of HCV genotype 6 are scant and mostly generated retrospectively. The available studies suggest that SVR rates in patients infected with HCV genotype 6 (60%-90%) are superior to those in patients with genotype 1 and comparable to patients infected with genotypes 2 and 3^[36-45] (Table 2). The question whether a high treatment response rates in HCV genotype 6 is due to viral factor itself or partially due to host factor, especially favorable *IL28B* genotype among Asians, remains unclear.

Treatment regimens

The optimal dose and treatment duration of HCV genotype 6 have not been well-established. Most of the earlier studies applied PEG-IFN for 48 wk duration with weight-based RBV dose for HCV genotype 6 reported conflicting results with studies comparing 48-wk *vs* 24-wk treatment duration (Table 3). In a retrospective cohort of Nguyen *et al*^[44], SVR was significantly higher in patients treated for 48 wk than in those treated for 24 wk (75% *vs* 39%, respectively; $P = 0.044$). However, a randomized controlled study from Lam *et al*^[45] ($n = 60$) found no significant difference in SVR rates in patients treated with PEG-IFN α -2a/RBV for 48 wk *vs* 24 wk (79% *vs* 70%, respectively; $P = 0.45$). Based on this conflicting evidence, differences in treatment duration recommended by the available guidelines are observed. The 2009 American Association of the Study of Liver Disease^[34] and the 2012 Asian Pacific Association for the Study of the Liver^[46] Practice Guidelines have recommended 48 wk duration of treatment for patients with HCV genotype 6, as for those with genotype 1, whereas the 2011 European Association for the Study of the Liver Practice Guideline has suggested response-guided therapy for HCV genotype 6 with the same algorithm as genotype 2 and 3^[27]. In 2012, the largest randomized controlled trial to date of patients with HCV genotype 6 ($n = 105$) has been published. This study found no statistically significant difference in SVR rates between the genotype 6 patients treated with 24 and 48 wk of PEG-IFN α -2a/RBV (60% *vs* 71%, $P = 0.24$ in the intention-to-treat analysis; 72% *vs* 79%, $P = 0.46$ in the per-protocol analysis)^[47].

Predictors of treatment response and response-guided therapy

For HCV in general, the strongest predictors of SVR are genetic polymorphisms in *IL28B*, genotype, the stage of fibrosis, and undetectable HCV RNA at week 4 of treatment (defined as rapid virological response; RVR)^[27]. Other predictors of response include host factors (*e.g.*, age, BMI, insulin resistance, gender), baseline HCV RNA levels, co-infections, the dose and duration of therapy, virological responses during the treatment, and treatment adherence^[27]. These predictors seem to be valuable for all HCV genotypes and may extrapolate to use for patients with HCV genotype 6 as well. With sparse available data, predictors of response in HCV genotype 6

Table 2 Treatment outcomes of hepatitis C virus genotype 6 (compared to other genotypes)

Ref.	Design/treatment	Genotype	n	SVR	P value ¹
Dev <i>et al</i> ^[36]	Retrospective	6	33	82.5%	NR
	IFN + RBV 52 wk	1	17	61.9%	
Hui <i>et al</i> ^[37]	Prospective	6	16	62.5%	0.04
	IFN + RBV 52 wk	1	24	29.2%	
Cheng <i>et al</i> ^[43]	Retrospective	6	13	69.2%	0.026
	PEG-IFN + RBV (duration not reported)	1	61	32.8%	
		2	18	77.8%	
Fung <i>et al</i> ^[38]	Prospective	6	21	85.7%	0.019
	PEG-IFN + RBV 52 wk	1	21	52.4%	
Nguyen <i>et al</i> ^[40]	Retrospective	6	34	74.0%	0.016
	PEG-IFN + RBV (48 wk for genotype 1 and 6; 24 wk for genotype 2/3)	1	70	49.0%	
		2/3	63	75.0%	
Seto <i>et al</i> ^[30]	Retrospective IFN/PEG-IFN + RBV 52 wk	6	26	92.3%	NR
	IFN/PEG-IFN + RBV 52 wk	1	21	42.9%	
Tsang <i>et al</i> ^[41]	Retrospective	6	70	75.7%	NR
	PEG-IFN + RBV 48 wk	1	70	57.1%	
Zhou <i>et al</i> ^[42]	Retrospective	6	22	81.8%	0.068
	PEG-IFN + RBV (48 wk for genotype 1b; 24 wk for genotype 2/3 and 6)	1b	39	59.0%	
		2/3	42	83.3%	
Tangkijvanich <i>et al</i> ^[48]	Prospective	6	34	76.5%	0.309
	PEG-IFN + RBV (RGT ² for genotype 6; 48 wk for genotype 1; 24 wk for genotype 3)	1	16	62.5%	
		3	16	81.3%	

¹P-value between genotype 6 *vs* genotype 1; ²Response-guided therapy (RGT) define as 24 wk for patients with rapid virological response and 48 wk for those without. PEG-IFN: Pegylated interferon; RBV: Ribavirin; SVR: Sustained virological response.

Table 3 Treatment outcomes of hepatitis C virus genotype 6 by the treatment duration

Ref.	Design/treatment	Duration (wk)	n	SVR	P value
Nguyen <i>et al</i> ^[44]	Retrospective	24	23	39%	0.044
	PEG-IFN 2a/2b + WB-RBV	48	12	75%	
Lam <i>et al</i> ^[45]	Randomized (1:1)	24	27	70%	0.450
	PEG-IFN 2a + WB-RBV	48	33	79%	
Thu-Thuy <i>et al</i> ^[47]	Randomized (1:2)	24	35	60%	0.240
	PEG-IFN 2a + WB-RBV	48	70	71%	
Tangkijvanich <i>et al</i> ^[48]	Prospective	24 if RVR achieved	25	88%	NR
	PEG-IFN 2a + WB-RBV	48 if no RVR	9	44%	

PEG-IFN: Pegylated interferon; RBV: Ribavirin; WB: Weight-based; SVR: Sustained virological response; RVR: Rapid virological response.

have been observed among studies of HCV genotype 6 include younger age (< 40-50 years)^[40,42], low BMI (< 25 kg/m²)^[40], treatment adherence^[40] and RVR^[42,45]. Among these predictors and concordant with observations in other HCV genotypes, RVR was a strong independent predictor of SVR in HCV genotype 6, wherein the positive predictive value (PPV) in achieving SVR in patients with RVR has been 80%-90%^[42,45,47,48]. In Thu thuy *et al*^[47] study, RVR was common (in up to 80% of patients) with a high PPV (75%-86%) and negative predictive value (NPV) for the prediction of SVR (0%-8%), regardless of the treatment duration. Thus, none of the patients who did not have undetectable HCV RNA at week 12 of treatment (defined as early virological response; EVR) subsequently achieved SVR^[47]. Thus, in those who completed treatment protocol, the importance of RVR in the prediction of SVR has been further substantiated; PPV for SVR was 96% with 48-wk treatment group, and was 91% with 24-wk treatment. In addition, a retrospective analysis by Zhou *et al*^[42] demonstrated that the PPV and

NPV of RVR and EVR in patients with HCV genotype 6 are comparable with those in patients with genotype 2/3 infection.

Taken together, it is likely that baseline response predictors together with on-treatment response-guide therapy (RGT) can be utilized for the treatment of HCV genotype 6 in order to optimize treatment outcomes as well as cost-effectiveness (Figure 1). Based on available data, patients with RVR will benefit with 24 wk of therapy, particularly if they are young, with a BMI < 25 kg/m², and have a low viral load, whereas patients with older age, non-CC *IL28B* genotypes, obesity, advanced fibrosis, and high viral loads, would benefit from 48 wk of therapy. The SVR rates among HCV genotype 6 patients with RVR are expected to be at > 80%^[42,45,47], and possibly up to > 90% in those who adhere to therapy^[47]. Alternatively, patients who do not achieve RVR are expected to have low rates of SVR (0%-30%)^[45,47]. If treatment continues, HCV RNA should be checked again at week 12. If HCV RNA is detectable at week 12 then

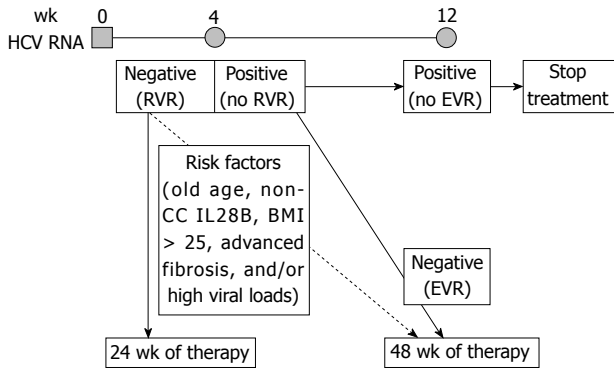


Figure 1 Response-guided therapy in patients with hepatitis C genotype 6. RVR: Rapid virological response; EVR: Early virological response; BMI: Body mass index; HCV: Hepatitis C virus; IL: Interleukin.

treatment should be discontinued, since SVR rates have shown to be near zero in non-EVR patients^[42,45,47]. Correspondingly, a proof-of-concept study ($n = 34$) utilizing RGT for HCV genotype 6 patients with RVR has been firstly reported by Tangkijvanich *et al.*^[48]. In this study, 25 patients who achieved RVR were assigned to receive 24 wk treatment (RGT group) while the remaining 9 patients (no RVR) were assigned for standard 48 wk of PEG-IFN 2a/RBV therapy. SVR rates were significantly higher for RGT group when compared to 48-wk treatment group (88% *vs* 44%, respectively; $P = 0.024$)^[48]. However, the precise role and protocol of RGT for HCV genotype 6 needs a larger prospective study to address.

Treatment adverse events

As previously reported in HCV treatment trials, the common side effects of HCV genotype 6 are of general non-specific symptoms and anemia, which are mild and manageable by supportive measures^[45,47]. Though the incidence and types of side effects caused by therapy with PEG-IFN/RBV seem to be similar among patients of different HCV genotypes, side effect profiles appear to differ among patients of different ethnicities^[6]. Several studies have reported that psychiatric adverse events were less common and ribavirin-induced anemia was more common in Asians than either white or Hispanic patients, and that there were no significant difference between whites and Asians with respect to required ribavirin or PEG-IFN dose reductions^[18]. Notably, the lower rates of psychiatric adverse events in Asians may be partly explained from the potential for underreporting psychiatric problems and/or depression in Asian populations due to associated sociocultural stigma^[49,50], as well as from the absence of confounders such as alcohol use and drug abuse^[51].

Roles of IL28B

Single nucleotide polymorphisms (SNPs) near the *IL28B* gene responsible for encoding IFN-gamma are strongly associated with spontaneous and treatment-induced clearance of HCV^[52,53]. A genome-wide association study of

more than 1600 patients infected with HCV genotype 1 found the rate of SVR following PEG-IFN/RBV treatment to be approximately 80%, 40%, and 25% in *IL28B* genotypes CC, CT, and TT, respectively^[52]. Notably, the favorable C allele is frequently found up to 80% in Asians, which is more common than in Caucasians, Hispanics and African Americans, respectively^[52,54]. This may be part of the reason that SVR rates for HCV genotype 1 among Asian patients are higher (expected 60%-70%) compared to non-Asian populations^[18]. However, the role of *IL28B* for the prediction of HCV clearance in non-1 genotypes is less clear. Studies from HCV genotypes 2, 3, and 4 yielded somewhat conflicting results, though most studies failed to show a significant association of *IL28B* variations with SVR^[53]. Nevertheless, a preliminary study in Chinese genotype 6 HCV patients ($n = 24$) has demonstrated a significant association between *IL28B* polymorphisms (SNPs rs12979860 and rs8099917) and SVR rates^[55].

Roles of viral genome mutations

Studies from Japan and Hong Kong have identified associations between genetic mutations around the interferon sensitivity-determined region (ISDR) of HCV genotype 1b and resistance to IFN-based treatment^[56,57]. Accordingly, sequence diversity of HCV genotype 6a within the extended ISDR (covering 192 base-pairs upstream and 201 base-pairs downstream from the ISDR previously defined in genotype 1b) has shown correlation with antiviral treatment outcomes in a report from China^[58]. However, it should be noted that this observation was not reproducible among HCV genotype 1b patients in Europe and United States^[59,60], which may be partially explained by differences in genetic background, especially the *IL28B* genotypes.

Roles of DAA

At present, there has been no data on the efficacy of current, FDA-approved DAA, boceprevir and telaprevir, on HCV genotype 6. However, some investigational agents with pan-genotypic antiviral activities (*e.g.*, new generation protease inhibitors, NS5B, and cyclophilin inhibitors) have been shown to suppress HCV replication in HCV genotype 6^[61,62]. Recently, sofosbuvir^[63] and simeprevir (TMC435)^[64] have demonstrated clinical benefit in a small number of patients with HCV genotype 6 in the phase II / III studies. However, further studies with larger number of genotype 6 patients are needed in order to establish the regimens and clinical efficacy in this group of patient. While awaiting clinical trials specifically for genotype 6, one would speculate that the use, or off-label use, of pan-genotypic DAA, especially sofosbuvir and simeprevir, for HCV genotype 6 patients may be seen soon, particularly for those who failed standard treatment with PEG-IFN/RBV. It should also be noted that the availability of DAA is currently very limited in most countries in Southeast Asia due to socio-economic

and other barriers.

CONCLUSION

Hepatitis C genotype 6 is endemic in Southeast Asia (prevalence varies between 10%-60% among all HCV infection), as well as also sporadically reported outside the area among immigrations. The diagnosis of HCV genotype can be inaccurate with earlier methods of genotyping due to identical 5'-UTR between genotype 6 and 1b, and the newer genotyping methods with core sequencing are preferable. Risk factors and clinical course of HCV genotype 6 do not considerably differ from the other genotypes. Treatment outcome of HCV genotype 6 with PEG-IFN/RBV is superior to genotype 1, and nearly comparable to genotype 3 (expected SVR rates of 60%-90%). Emerging data suggests that a shorter course 24-wk treatment may be effective as a standard 48-wk-treatment, particularly in those patients who attained RVR. In addition, baseline and on-treatment predictors of response used for other HCV genotypes seem to be useful for genotype 6. Although some pan-genotypic direct acting antivirals have completed phase II / III studies (sofosbuvir and simeprevir) with clinical benefit demonstrated in small number of patients with genotype 6, broad availability of these agents in Southeast Asia may not be expected in the near future. While awaiting the newer therapy, response-guided therapy seems to be appropriate for patients with HCV genotype 6. Patients with RVR (representing > 70% of patients) are suitable for 24 wk treatment with expected SVR rates > 80%. Patients without RVR and/or those with poor response predictors may benefit from 48 wk of therapy, and a detectable HCV RNA at week 12 (no EVR) can be served as stopping rule. This treatment scheme is likely to have a major economic impact on HCV therapy, particularly in Asia, wherein treatment can be truncated securely in the majority of patients with HCV genotype 6.

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Changes of liver fat content and transaminases in obese children after 12-mo nutritional intervention

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Abstract

AIM: To assess a relationship between longitudinal changes in liver fat content and biochemical parameters in obese children after 1-year nutritional intervention.

METHODS: Forty-six obese children, 21 males and 25 females, aged 6-14 years, underwent metabolic measurements, liver ultrasonography (US) and chemical-shift magnetic resonance imaging (MRI) examinations at baseline and after 1-year nutritional intervention. A child was defined obese if her/his body mass index (BMI) was above the age- and sex-adjusted BMI Cole's curve passing through the cut-off of 30 kg/m² at 18 years. BMI Z scores were calculated and adjusted for age and

gender by using the Cole's LMS-method and Italian reference data. Biochemistry included serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Abdominal US and chemical-shift MRI were performed according to a randomized sequence. The same radiologist performed US by a GE Logiq 9 (General Electric Healthcare Medical Systems, Milwaukee, WI, United States) using a 3.5-MHz convex array transducer. Liver echogenicity was evaluated independently on videotape by 3 radiologists unaware of the child and MRI outcomes, and a consensus was established. Another experienced radiologist, unaware of the child and US data, performed the abdominal chemical-shift MRI with a 1-t system NT-Intera (Philips Medical Systems, Best, The Netherlands) and a phased-array coil. Liver fat fraction (FF) on MRI was judged elevated when greater than 9%. A FF > 18% was considered expressing more severe cases of fatty liver according to Fishbein. A nutritional-behavioral intervention was recommended to promote a normocaloric balanced diet and active lifestyle based on the Italian guidelines for treatment of childhood obesity.

RESULTS: Compared to baseline, at the end of intervention children showed lower intakes of energy (mean \pm SD: 2549 \pm 1238 Kcal vs 1770 \pm 622 Kcal, $P < 0.0001$), total fat (90 \pm 47 g vs 52 \pm 23 g, $P < 0.0001$), carbohydrates (356 \pm 174 g vs 241 \pm 111 g, $P = 0.001$), and protein (99 \pm 48 g vs 75 \pm 23 g, $P = 0.006$) intakes. Prevalence of FF \geq 9% declined from 34.8% to 8.7% ($P < 0.01$), with a mean reduction of 7.8% (95%CI: 5.0-10.6). At baseline, FF was associated with liver biochemical parameters (maximum $P < 0.001$). At the end of the intervention association was found with AST ($P = 0.017$). Change of FF was associated with change in AST ($P = 0.027$) and ALT ($P = 0.024$). Rate of increased liver echogenicity declined from 45.6% to 21.7% ($P < 0.0001$). Liver echogenicity was associated with ALT at baseline only ($P < 0.001$). An age- and sex-adjusted multiple regression analysis showed that FF

change was independently associated with change in serum AST (adjusted regression coefficient 0.348, $P = 0.048$).

CONCLUSION: The results suggest that in obese children longitudinal changes in liver fat content based on MRI may be associated with change in serum transaminases suggesting novelty in monitoring nonalcoholic fatty liver disease.

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Key words: Nonalcoholic fatty liver disease; Childhood obesity; Serum transaminases; Magnetic resonance imaging; Nutritional intervention

Core tip: In our study we demonstrate that in obese children longitudinal change in liver fat content evaluated on magnetic resonance imaging is associated with change in serum transaminases and more weakly also with changes in triglyceridemia and apolipoproteins, after a nutritional intervention based on normocaloric balanced diet. Furthermore this is the first study purposely designed to evaluate in obese children whether any relationship may exist of longitudinal changes in liver fat content with changes in liver biochemical parameters. These findings may suggest novelty in clinical monitoring nonalcoholic fatty liver disease severity in childhood obesity.

Verduci E, Pozzato C, Banderali G, Radaelli G, Arrizza C, Rovere A, Riva E, Giovannini M. Changes of liver fat content and transaminases in obese children after 12-mo nutritional intervention. *World J Hepatol* 2013; 5(9): 505-512 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v5/i9/505.htm> DOI: <http://dx.doi.org/10.4254/wjh.v5.i9.505>

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) ranges from fat in the liver to advanced fibrosis and cirrhosis^[1]. Obesity is a condition frequently associated with NAFLD both in children and adults^[1-3]. The overall prevalence of fatty liver based on histological diagnosis in the pediatric population has been estimated to be around 13%^[1]. The highest rate of fatty liver was seen in obese children (32%-50%)^[4,5]. In children, NAFLD is becoming more frequently diagnosed with increasing rates of obesity^[5,6], and might indicate a possible metabolic outcome of obesity^[5]. Therefore, accurate diagnosis of disease as well monitoring of the patients over time remains a major challenge for pediatricians taking care of the growing number of children with NAFLD^[7]. Liver biopsy is the gold standard for establishing diagnosis and severity of NAFLD but it may not be done repeatedly in children. Accordingly, non invasive approaches are important for the clinician. Ultrasonography (US) and chemical-shift

magnetic resonance imaging (MRI), fast to perform, may be useful to detect liver fat content and monitoring NAFLD^[8-14]. On US, fatty liver produces a diffuse increased echogenicity with posterior beam attenuation^[8]. MRI is able to quantify the fat content accurately and identify changes over time in children with NAFLD^[9-15]. Indeed, studies proved that assessment by MRI agrees better than US with the diagnosis of steatosis based on biopsy^[10,12-14]. While, it is still debatable whether in obese children US may be valuable in identifying high hepatic fat accumulation^[8], it may show poor ability in identifying lower fat content compared with MRI^[9,12,13].

Children with NAFLD are usually asymptomatic and come to clinical attention because of elevated liver enzymes or fatty liver seen in incidentally observed elevated serum aminotransferases^[6]. While concordance between serum aminotransferases and US in identifying fatty liver^[12,16,17] may be low, it has been proved for MRI^[12,18]. Despite the potential clinical and practical relevance, there is lack of studies in the current literature assessing the relationship of longitudinal change of liver fat content with liver biochemical parameters in pediatric age.

The aim of the present study was to assess whether any association may exist of longitudinal changes in liver fat content with change in liver biochemical parameters in obese children who underwent a 1-year nutritional intervention.

MATERIALS AND METHODS

Fifty children, aged 6-14 years, were consecutively recruited at the Pediatric Department of the San Paolo Hospital, Milan, Italy, between July 1, 2010 and June 31, 2012 according to the following eligibility criteria. Inclusion criteria were: age > 6 years, obesity, and white parents. Exclusion criteria were: having syndromic, organic and hormonal conditions besides obesity that may predispose to liver disease, including infectious hepatitis B and C, alpha-1-antitrypsin deficiency, medications affecting liver metabolism, diabetes, any alcohol consumption. Children aged < 6 years were not included as it may be difficult to perform accurately MRI for lack of compliance. Obesity was defined according to the International Obesity Task Force^[19].

The parents of eligible children or their legal guardian received detailed explanation about the aim of the study, and signed a consent form. The Hospital Ethics Committee approved the study protocol and gave ethical clearance.

Anthropometric measurements, nutritional and metabolic examinations, US and MRI assessments were performed within 3 ± 1 d before starting intervention (baseline) and one year (± 5 d) after (end of intervention).

Anthropometry and clinical data

Anthropometrical evaluation included measurements of weight and height. The body mass index (BMI) was calculated from the ratio of weight to height² (kg/m²).

A child was defined obese if her/his BMI was above the age- and sex-adjusted BMI Cole's curve passing through the cut-off of 30 kg/m² at 18 years^[19]. BMI Z scores were calculated and adjusted for age and gender by using the Cole's LMS-method^[20] and Italian reference data^[21]. A medical history was additionally collected at baseline from parents by a standardized questionnaire at a personal interview conducted by the same pediatrician.

Biochemistry

Fasting blood samples were taken at 8 h ± 30 min am Flavoured glucose at a dose of 1.75 g/kg body weight (up to a maximum of 75 g) was then given orally, and additional blood samples were taken for measurements of plasma glucose at 120 min. Blood samples were collected and analysed immediately at the Hospital Department of Biochemistry. Total cholesterol (TC), high-density-lipoprotein cholesterol (HDL-C) and triacylglycerol (TG) plasma levels were measured using a dry multiplayer enzymatic method (Ectachem DT-60; Eastman Kodak Co., Rochester, NY). Low-density-lipoprotein cholesterol (LDL-C) serum levels were calculated according to the Friedewald formula [LDL-C = TC - (HDL-C + TG/5)]^[22]. Apolipoprotein A1 and Apolipoprotein B were measured using a Modular analyzer (BNII, Dade Behring; Marburg, Germany). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma glutamyltransferase (γGT) were measured by a Hitachi 917 Analyzer and Roche Diagnostics reagents (both Mannheim, Germany).

Abdominal US and chemical-shift MRI

Abdominal US and chemical-shift MRI were performed according to a randomized sequence. The same radiologist (AR) performed US by a GE Logiq 9 (General Electric Healthcare Medical Systems, Milwaukee, WI, United States) using a 3.5 MHz convex array transducer. Liver echogenicity was evaluated independently on videotape by 3 radiologists unaware of the child and MRI outcomes, and a consensus was established. Absolute agreement among judgments before discussion occurred in 94% of cases at baseline, and in 93.5% of cases at the end of intervention. Echogenicity was graded as follows: grade 0, normal echogenicity; grade 1, slight increase in liver echogenicity, slight exaggeration of liver and kidney echo discrepancy, and relative preservation of echoes from the walls of the portal vein; grade 2, loss of echoes from the walls of the portal veins, particularly from the peripheral branches, greater posterior beam attenuation, and greater discrepancy between hepatic and renal echoes; grade 3, greater reduction in beam penetration, loss of echoes from most of the portal vein wall, including the main branches, and large discrepancy between hepatic and renal echoes^[23].

Another experienced radiologist (CP), unaware of the child and US data, performed the abdominal chemical-shift MRI with a 1-T system NT-Intera (Philips Medical Systems, Best, The Netherlands) and a phased-array coil.

For each child, axial T1-weighted gradient echo images both in-phase [15/6.90 (repetition time msec/echo time msec), flip angle of 25°] and out-of-phase [15/3.45 (repetition time msec/echo time msec), flip angle of 25°] were obtained. The echo time is the time from the application of the radio frequency pulse to peak of the signal. Section thickness was 10 mm, field of view was 375 mm, and matrix was 256 pixels. Scan duration was 1 m/23 s for each in-phase or out-of-phase image. Respiratory compensation was used (scan duration for respiration, 1.6 s). Twenty contiguous transverse images in-phase and out-of-phase were obtained. Two operators not involved in the study and unaware of the patient's disease independently selected the slice showing the best evidence of liver parenchyma to be considered for examination and the region of interest (ROI). Three ROI with the same area (80-100 mm²) were drawn on the corresponding in-phase and out-of-phase images. Two regions were drawn in the right liver, one in the left liver. For each region, the signal intensity was measured on both in-phase and out-of-phase images and the mean pixel signal intensity data were recorded for further calculation. The liver fat fraction (FF) was calculated from the mean pixel signal intensity data using the formula: FF = [SI (in-phase) - SI (out-of-phase)]/2SI (in-phase). The hepatic FF was considered to be normal at a value less than 9% and non-normal as FF ≥ 9%^[10,11]. A FF > 18% was considered expressing more severe cases of fatty liver according to Fishbein^[24]. Additionally the cut off of 5% for FF was used according to studies conducted on obese children, *e.g.*^[14].

Nutritional-behavioral intervention and dietary habits

A nutritional-behavioral intervention was recommended to promote a normocaloric balanced diet and active lifestyle based on the Italian guidelines for treatment of childhood obesity^[25]. Diet was normocaloric (daily caloric intake by sex and age) and consisted of carbohydrate (55%-60%, < 10% high glycemic index carbohydrate), fat (25%-30%, < 10% saturated fatty acids, polyunsaturated up to 10%, monounsaturated up to 15%), protein (12%-15%), fiber [range: age (year) plus 5 - age (year) plus 10, g]^[25]. Children and parents or the legal guardians underwent 1-h nutritional counselling by the same experienced dietician. Written guidelines were given to the parents and children, including general nutritional advices, food choice lists, selected week-menu, recommended average servings for principal food categories according to age and sex. Additionally, recommendations were given to engage in a moderate daily exercise program (30-45 min/d aerobic physical exercise), tailored to individual preferences. Dietary habits of children were assessed at baseline and at the end of intervention by means of an age-adjusted Food Frequency Questionnaire (FFQ) made up of 116 items and designed according to Block^[26]. The same experienced dietician, unaware of the obesity status of children, interviewed mothers for approximately 50 min and each meal was analyzed to find out which food was eaten and how often. Usual portion sizes were esti-

Table 1 Body mass index *-z* score, fat fraction, liver echogenicity, liver biochemical parameters and lipid profile of the studied population at baseline and at the end of intervention, values are mean (SD) + and median or number of observation (%)

Variable	Baseline (<i>n</i> = 46)	End of intervention (<i>n</i> = 46)	¹ <i>P</i> value
BMI <i>Z</i> score	2.3 (0.4); 2.1	1.90 (0.6); 1.9	< 0.001
Fat fraction (%)	7.8 (13.3)	0.01 (8.3)	< 0.001
FF ≥ 5%	21 (45.7)	5 (10.9)	< 0.001
FF ≥ 9%	16 (34.8)	4 (8.7)	< 0.001
Liver echogenicity (%)			
0	25 (54.3)	36 (78.3)	< 0.0001
1	14 (30.4)	9 (19.6)	
2	6 (13.0)	1 (2.2)	
3	1 (2.2)	0 (0.0)	
ALT (U/L)	34.8 (12.0); 29.0	23.8 (8.70); 22.50	< 0.001
AST (U/L)	30.7 (10.1); 29.0	25.9 (7.50); 26.00	< 0.001
γGT (U/L)	15.7 (5.2); 15.0	13.1 (3.80); 12.00	< 0.001
Total cholesterol (mmol/L)	4.39 (0.70); 4.48	4.30 (0.76); 4.23	0.211
HDL cholesterol (mmol/L)	1.22 (0.24); 1.23	1.21 (0.22); 1.24	0.916
LDL cholesterol (mmol/L)	2.64 (0.59); 2.60	2.60 (0.67); 2.56	0.306
Triglycerides (mmol/L)	1.31 (0.62); 1.20	0.99 (0.46); 0.94	< 0.001
Apo A1 (g/L)	1.24 (0.21); 1.21	1.28 (0.21); 1.26	0.122
Apo B (g/L)	0.73 (0.17); 0.70	0.66 (0.15); 0.67	0.022

¹Adjusted for age, sex and duration of obesity; ²Student's *t* test for paired data or Wilcoxon signed-rank test. Normal range^[32]: ALT (5-45 U/L) for age 1-19 years; AST (15-55 U/L) for age 1-9 years and (5-45 U/L) for age 10-19 years; gammaGT (5-32 U/L) for age 4 mo-9 years and (5-24 U/L) for age 10-15 years. SI conversion factors: to convert cholesterol, divide values by 0.0259; to convert triglycerides, divide values by 0.0113; to convert Apo A1 and Apo B values by 0.01. BMI: Body mass index; FF: Fat fraction; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; γGT: Gamma glutamyltransferase; HDL: High-density-lipoprotein; LDL: Low-density-lipoprotein.

mated using household measures, the weight of purchase (e.g., pasta) or unit (e.g., fruit juice). A 24-h recall on the day prior to the hospital admission was further recorded at the end of the interview to standardize the usual serving size.

Medical examinations were scheduled every 3 mo during the intervention period. At each visit, parents of children or their legal guardians filled out a FFQ and physical activity recall to evaluate adherence to lifestyle recommendations. Quantification and analysis of the energy intake and nutrient composition were performed with an ad hoc PC software program developed at our department and based on the Food and Nutrient Data Base issued by the National Institute of Nutrition^[27].

Sample size

The sample size was determined to detect a minimum effect size of 0.5 for a correlation between change in FF and change in liver biochemical parameters. Admitting a type I error level of 0.05 with a power of 95%, and allowing for a drop out up to 10%, 43 children needed to be recruited.

Statistical analysis

Data are expressed as mean (SD) and median or number

of observations (percentage). Intra-subject comparisons were performed by the Student's *t* test for paired data or the Wilcoxon test, as appropriate. The association of liver fat fraction with BMI *Z* score, lipid profile, serum aminotransferases and gamma glutamyltransferase was assessed by Spearman's correlation coefficient. When fat fraction was dichotomized (cut off 9%) Student's *t* test for unpaired data or the Mann-Whitney *U* test were used. A multiple adjusted stepwise regression model was fitted for change of FF, including as covariates change in liver biochemical parameters and lipid profile, and additional potential confounders (age, sex, duration of obesity, change in BMI *Z* score). All values of *P* < 0.05 were considered to indicate statistical significance (two-tailed test). The statistical package For social sciences (SPSS) package version 19.0 for Windows (SPSS, Chicago, IL) was used for the statistical analysis.

RESULTS

Forty-six children (92%), 21 males and 25 females, completed the intervention and had all US, MRI and blood tests performed. No difference was observed among children who completed the study or not, for any variable at baseline (minimum *P* > 0.130). No focal liver lesions were observed in any child. At recruitment, mean (SD; median) of age, duration of obesity was, respectively, 10.1 (2.4; 10.0) years, 4.4 (2.8; 3.4) years. Plasma glucose was 868 (6.3; 86.0) mg/L at fasting and 993 (17.3; 99.5) mg/L at 120 min at baseline, and 856 (6.6; 85.0) mg/L at fasting and 977 (14.2; 98.0) mg/L at 120 min at the end. Compared to baseline at the end of intervention children showed lower intakes of energy (mean ± SD: 2549 ± 1238 Kcal *vs* 1770 ± 622 Kcal, *P* < 0.0001), total fat (90 ± 47 g *vs* 52 ± 23 g, *P* < 0.0001), carbohydrates (356 ± 174 g *vs* 241 ± 111 g, *P* = 0.001), and protein (99 ± 48 g *vs* 75 ± 23 g, *P* = 0.006) intakes. Children spent 2.3 h (1.6 h)/d of physical activity at baseline and 2.8 h (1.8 h)/d at the end of intervention (*P* = 0.174).

Table 1 reports the main characteristics and clinical, liver and lipid profile. After intervention there was a reduction in BMI *Z* score (mean reduction 0.32, 95%CI: 0.21-0.43), triglycerides (0.33, 0.15-0.50) mmol/L, ALT (11.1, 5.6-16.5) mg/dL, AST (4.8, 2.1-7.4) mg/dL, γGT (2.5, 1.5-3.6) U/L and apolipoprotein B (7.2, 1.1-13.2) mg/dL. Rate of increased liver echogenicity declined from 45.6% to 21.7% (*P* < 0.0001). On MRI, rate of liver FF ≥ 9% declined from 34.8% to 8.7% (*P* < 0.001), and from 45.7% to 10.97% (*P* < 0.001) at cut off of 5% for FF, with a mean FF (95%CI) reduction of 7.8 (5.0-10.6)%. In children who exhibited FF ≥ 9% at baseline (*n* = 16) (maximum FF 43%), FF declined from a (mean ± SD: 22.6% ± 11.5% *vs* 6.2% ± 10.5%, *P* < 0.0001), and in 12 was lower than 9%.

At baseline fat fraction was associated with liver biochemical parameters, while at the end of intervention association was found of FF with transaminases. Change of FF was associated with changes in AST and ALT.

Table 2 Spearman correlation coefficient (*P* value) of fat fraction and liver echogenicity with liver function parameters

	AST	ALT	γGT
Baseline			
Fat fraction	0.634 (< 0.0001)	0.456 (< 0.001)	0.469 (< 0.0001)
Liver echogenicity	0.282 (0.0530)	0.437 (< 0.001)	0.232 (0.0750)
End of treatment			
Fat fraction	0.351 (0.0170)	0.297 (0.056)	0.279 (0.0630)
Liver echogenicity	0.260 (0.1030)	0.242 (0.070)	0.116 (0.2540)
Change (end of treatment-baseline)			
Fat fraction	0.326 (0.0270)	0.331 (0.024)	0.271 (0.0720)
Liver echogenicity	0.225 (0.0820)	0.260 (0.063)	0.204 (0.1900)

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γGT: Gamma glutamyltransferase.

Liver echogenicity was associated with ALT at baseline only (Table 2). Fat fraction was also associated with triglycerides (Spearman correlation coefficient, *r* baseline, *r* = 0.516, *P* < 0.0001; end of intervention, *r* = 0.329, *P* = 0.031; change, *r* = 0.517, *P* < 0.0001) and Apo B (baseline, *r* = 0.391, *P* = 0.008; change, *r* = 0.398, *P* = 0.024), but not with cholesterol or Apo A1 (minimum *P* = 0.114). No association of liver echogenicity was found with triglycerides or cholesterol or apolipoproteins (minimum *P* = 0.063). No association of BMI Z score was found with FF or liver echogenicity (minimum *P* = 0.517).

An age and sex adjusted multiple stepwise regression model showed that FF change was independently and positively associated with serum AST level change (adjusted regression coefficient 0.348, *P* = 0.048).

Moreover at baseline acanthosis nigricans, a skin features associated to hyperinsulinemia, was present in 15.4% of the evaluated children and decline to 10.1% after intervention.

Lastly, Table 3 compares liver biochemical and lipid profile when considering the FF dichotomized at 9%. At baseline aminotransferases, γGT and triglycerides were higher in children with liver FF ≥ 9%. At the end of intervention, aminotransferases and total cholesterol levels were higher in children with FF ≥ 9%. Mean difference (95%CI) of BMI Z score and duration of obesity between children with FF ≥ 9% *vs* FF < 9% was respectively 0.15 (-0.12; 0.42) and 1.1 (-0.69; 2.87), at baseline. Out of 16 children with FF ≥ 9%, 8 (50%) showed FF > 18%. Mean (SD) and median of AST, ALT and γGT between children with FF > 18% *vs* 9% < FF < 18% was respectively 44.6 (12.7), 42.0 *vs* 31.9 (8.5), 29.0 (*P* = 0.035); 63.6 (28.5), 55.0 *vs* 37.4 (22.7), 30.5 (*P* = 0.027); 20.2 (5.0), 21.5 *vs* 16.1 (3.7), 16.0 (*P* = 0.063) at baseline. At baseline only ALT values were elevated in children with FF > 18%. After intervention the ALT mean values ranged from 63.6 (28.5), 55 to 32.7 (14.2), 29.0 (*P* < 0.012), value that is in the normal range. In particular out of children with FF > 18% (*n* = 8) 87.5% (*n* = 7) showed elevated ALT values and declined to 25% (*n* = 2) after intervention. Similar results were found when considering a cut off of 5% for FF.

DISCUSSION

This is the first study purposely designed to evaluate in obese children whether any relationship may exist of longitudinal changes in liver fat content with changes in liver biochemical parameters after a nutritional intervention based on normocaloric balanced diet. The study based assessment of liver fat content on MRI, that has been recognized performing better than US for evaluation of liver fat^[13] in the general population, as well in obese adolescents^[16] and children with NAFLD^[14,15].

The overall prevalence of elevated (≥ 9%) liver fat fraction at baseline was 34.8%, that is within the range (32%-40%) estimated in recent studies conducting in obese children and using MRI^[14,15]. At the end of intervention, the liver FF declined in three-quarter of children, and was lower than 9% in 91.3% of children with baseline FF ≥ 9%. Similar findings have been reported in other studies conducted in obese children with NAFLD^[14,15]. In this study, children showed after intervention a decrease in BMI Z score of 13.9%, that may be not unexpected given the decline occurred in dietary intake. Others studies estimated at the end of nutritional interventions a BMI Z score reduction ranging from 12.7%^[15] to about 40%^[14]. It should be noted that the present study may not fully reflect across-country experience. Indeed even in the most successful weight control program for children in the US, the success rate of weight loss may differ^[28]. It cannot be excluded that genetic and environmental factors, different compliance to nutritional intervention may contribute, at least in part, to this difference. Therefore the results of the present study should be contextualized.

Nonalcoholic fatty liver disease, as a component of the metabolic syndrome^[1], is usually suggested by finding elevated serum hepatobiliary enzymes (mostly ALT and GGT)^[12,18,24]. Serum ALT activity is a widely available and inexpensive test for the screening and initial evaluation of NAFLD^[29]. The sensitivity of this biochemical marker, however, remains low because a number pediatric patients may present ALT values in the normal range^[29]. Moreover, given the clinical relevance of identifying children with steatohepatitis (NASH), several studies have described biomarkers associated with pediatric NASH^[29,30]. Patton *et al*^[30], evaluating clinical relation of histopathology with transaminases in pediatric NAFLD, found that AST was superior to ALT in distinguishing NAFLD patterns, while other studies showed ALT performing better^[12,15,24]. Indeed, it is now widely accepted that the degree of ALT elevation does not correlate with the presence or severity of histological findings of NAFLD^[29]. For this reason in the present study we considered not only the transaminases value at baseline and at the end of treatment but also the change.

In the present study obese children with FF ≥ 9% showed higher values of aminotransferases than children with FF < 9% at baseline and at the end of intervention. In particular AST mean values were within the normal range both at baseline and at the end of intervention,

Table 3 Liver biochemical parameters and lipid profile of the studied population at baseline and at the end of intervention in relation to liver FF, dichotomized at 9%, alues are mean (SD) + and median

	Baseline (<i>n</i> = 46)		¹ <i>P</i> value	End of intervention (<i>n</i> = 46)		¹ <i>P</i> value
	FF < 9% (<i>n</i> = 30)	FF ≥ 9% (<i>n</i> = 16)		FF < 9% (<i>n</i> = 42)	FF ≥ 9% (<i>n</i> = 4)	
² AST (UI/L)	26.70 (5.9); 26.00	38.20 (12.3); 40	0.001	29.10 (8.6); 28.50	34.70 (6.4); 33.00	0.015
² ALT (UI/L)	26.50 (7.7); 25.00	50.50 (28.3); 48.50	0.001	30.50 (13.6); 29.00	40.70 (16.1); 40.50	0.015
² γGT (UI/L)	14.40 (5.0); 13.00	18.20 (4.8); 17.50	0.018	15.40 (5.3); 14.50	16.00 (3.7); 15.50	0.092
Triglycerides (mmol/L)	1.14 (0.44); 1.10	1.62 (0.80); 1.38	0.013	0.96 (0.44); 0.89	1.36 (0.45); 1.25	0.090
Total cholesterol (mmol/L)	4.34 (0.69); 4.35	4.48 (0.74); 4.54	0.539	4.22 (0.73); 4.18	5.06 (0.62); 4.98	0.034
HDL cholesterol (mmol/L)	1.23 (0.21); 1.24	1.20 (0.29); 1.17	0.716	1.21 (0.20); 1.24	1.23 (0.36); 1.21	0.950
LDL cholesterol (mmol/L)	2.58 (0.61); 2.58	2.76 (0.56); 2.75	0.348	2.54 (0.63); 2.53	3.21 (0.75); 3.03	0.086
Apo A (g/L)	1.21 (0.01); 1.23	1.29 (0.24); 1.20	0.259	1.29 (0.21); 1.27	1.12 (0.13); 1.12	0.199
Apo B (g/L)	0.71 (0.18); 0.68	0.77 (0.15); 0.70	0.261	0.65 (0.14); 0.62	0.85 (0.13); 0.86	0.064

¹Adjusted for age, sex, BMI Z score and duration of obesity; ²Significance of difference between FF groups (Student's *t* test for unpaired data or the Mann-Whitney *U* test). SI conversion factors: to convert cholesterol, divide values by 0.0259; to convert triglycerides, divide values by 0.0113; to convert Apo A1 and Apo B values by 0.01. Normal range^[32]: ALT [5-45 UI/L] for age 1-19 years; AST [15-55 UI/L] for age 1-9 years and [5-45 UI/L] for age 10-19 years; gammaGT [5-32 U/I] for age 4 mo-9 years and [5-24 UI/L] for age 10-15 years^[32]. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γGT: Gamma glutamyltransferase; HDL: High-density-lipoprotein; LDL: Low-density-lipoprotein.

while ALT mean values was slight superior to the upper normal value at baseline but normal after one year of intervention. Similar results were found when considering a cut off of 5% for FF.

However, considering more severe cases of fatty liver (17.4%) ALT mean values were elevated and normalized after the intervention. In particular the ALT values normalized in 62.5% of children with FF > 18%. The association of liver biochemical parameters with FF was stronger than with liver echogenicity both at baseline and the end of intervention, in accordance with the literature^[8,18]. Change in any liver biochemical parameter was not was associated with change in liver echogenicity, supporting previous findings^[8]. On the contrary, change in AST and ALT was associated with change in liver fat fraction. These results may be expected. Indeed as MRI may agree better than US with the diagnosis of steatosis based on biopsy^[10,12-14], MRI may be a reasonable way to follow liver fat in NAFLD.

Age and sex adjusted regression analysis showed that FF change was independently associated with change in serum AST level. This result could be of clinical and practical relevance when monitoring NAFLD in childhood obesity and suggest that more research is necessary to elucidate the liver serologic biomarkers and their changes through intervention with greatest sensitivity and specificity in predicting steatohepatitis. Additionally, it should be noted that at univariate analysis an association was found between change of liver FF with change of triglyceridemia, and apolipoprotein B. Indeed, while association with change in triglyceridemia may well reflect variation in hepatocyte accumulation of triglycerides^[3], hallmark of NAFLD, association with Apo B, that represents non-high density lipoprotein cholesterol, including very-low density lipoprotein, may suggest the risk to develop non-alcoholic steatohepatitis^[31]. Additionally, longitudinal studies support that NAFLD may be linked with increased risk of cardiovascular disease, independently of classical atherosclerotic risk^[11].

A limitation of the present study is the absence of

a control group of obese children on free diet. Indeed, may be not fully agreement on ethnicity of recruitment of a such control group considering also that the lack of interventions in the treatment of childhood obesity would not met the current requirements of the Italian Society of Pediatrics^[25]. A second limitation is that the gold standard to assess food intake is the 3 d food record, however the Frequency Food Questionnaire is largely used and in the present study was associated with a 24-h recall to standardize the usual serving size. A third limitation is that this study did not plan assessment of fat liver content by liver biopsy, that may be considered unethical in obese “healthy” children, and by proton magnetic resonance spectroscopy, a technique that may show high ability, as well MRI, for the evaluation of hepatic steatosis in general population (Youden's index ranging from 0.647 to 0.842)^[9,13], and additionally filters the signal fat-fraction from potential confounding technical and biological noises.

On the whole, within the limitations of this study, one may conclude that in obese children longitudinal change in liver fat content evaluated on MRI is associated with change in serum transaminases and more weakly also with changes in triglyceridemia and apolipoproteins, suggesting novelty in clinical monitoring NAFLD severity in childhood obesity.

Large longitudinal trials with adequate power are considerable to better investigate the relationship of longitudinal variations of liver fat content with serum liver and lipid profile, to elucidate the liver serologic biomarkers and their changes with greatest sensitivity and specificity in predicting steatohepatitis and their clinical means in NAFLD management.

COMMENTS

Background

Children with nonalcoholic fatty liver disease (NAFLD) are usually asymptomatic and come to clinical attention because of elevated liver enzymes or fatty liver seen in incidentally observed elevated serum aminotransferases. While concordance between serum aminotransferases and ultrasonography (US) in identifying fatty liver may be low, it has been proved for magnetic resonance

imaging (MRI). Despite the potential clinical and practical relevance, there is lack of studies in the current literature assessing the relationship of longitudinal change of liver fat content with liver biochemical parameters in pediatric age.

Research frontiers

Accurate diagnosis of disease as well monitoring of the patients over time remains a major challenge for pediatricians taking care of the growing number of children with NAFLD. The research hotspot is to elucidate the liver sierologic biomarkers and their changes with greatest sensitivity and specificity in predicting steatohepatitis and their clinical means in NAFLD management.

Innovations and breakthroughs

While concordance between serum aminotransferases and US in identifying fatty liver may be low, it has been proved for MRI. However there is lack of studies in the current literature assessing the relationship of longitudinal change of liver fat content with liver biochemical parameters. This is the first study purposely designed to evaluate in obese children whether any relationship may exist of longitudinal changes in liver fat content with changes in liver biochemical parameters after a nutritional intervention based on normocaloric balanced diet.

Applications

These results suggest that in obese children longitudinal change in liver fat content evaluated on MRI is associated with change in serum transaminases and more weakly also with changes in triglyceridemia and apolipoproteins, suggesting novelty in clinical monitoring NAFLD severity in childhood obesity.

Terminology

NAFLD ranges from fat in the liver to advanced fibrosis and cirrhosis. Obesity is a condition frequently associated with NAFLD both in children and adults US and chemical-shift MRI, fast to perform, may be useful to detect liver fat content and monitoring NAFLD. Studies proved that assessment by MRI agrees better than US with the diagnosis of steatosis based on biopsy.

Peer review

This is a very interesting study and a commendable piece of work analyzing the impact of a dietary intervention on liver fat content and biochemical parameters in obese children during a 1-year follow-up. The study provides solid evidence by means of metabolic measurements, liver ultrasonography and magnetic resonance imaging that a nutritional-behavioral intervention based on a normocaloric balanced diet and active lifestyle is useful in reducing fatty liver in obese children. Moreover the correlation between MRI and liver enzyme may be of practical value in NAFLD management in childhood.

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Ciclosporin does not attenuate intracranial hypertension in rats with acute hyperammonaemia

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Abstract

AIM: To investigate the neuroprotective potential of ciclosporin during acute liver failure. We evaluated the effect of intrathecally administered ciclosporin on intracranial pressure, brain water content and aquaporin-4 expression in a rat model with acute hyperammonaemia.

METHODS: Twenty-four male Wistar rats with portacaval anastomosis were randomised into four groups receiving ciclosporin or vehicle and ammonia or saline infusion. Ciclosporin or vehicle was given intrathecally prior to the ammonia or saline infusion. The ammonia or saline infusion was given intravenously for 4 h, while intracranial pressure and arterial pressure was recorded. At the end of the experiment, cerebral cortex and cerebellar brain tissue was analysed for water and aquaporin-4 content.

RESULTS: The following intracranial pressures were found at the end of the experiment: ammonia + ciclosporin: 10.0 ± 1.7 mmHg, ammonia + vehicle: 6.8 ± 1.0 mmHg, saline + ciclosporin: 3.1 ± 0.5 mmHg, saline + vehicle: 3.3 ± 0.6 mmHg. Ammonia infusion had a significant effect on intracranial pressure and brain water content, which both were higher in the groups receiving

ammonia ($P < 0.001$, two-way analysis of variance). Treatment with ciclosporin resulted in relevant tissue concentrations of ciclosporin (> 0.2 micromolar) but did not reduce intracranial pressure after 4 h. Furthermore, ciclosporin did not attenuate the increase in cerebral water content, and did not affect aquaporin-4 expression.

CONCLUSION: Intrathecal administration of ciclosporin does not attenuate intracranial hypertension or brain oedema in rats with portacaval anastomosis and 4 h of ammonia infusion.

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Key words: Brain oedema; Acute liver failure; Neuroprotection; Hyperammonaemia; Ciclosporin

Core tip: Acute liver failure and hyperammonaemia can lead to severe brain oedema. Preserving mitochondrial function by treatment with ciclosporin has shown potential in *in vitro* studies. In this study we evaluated the effect of ciclosporin in a rat model of acute hyperammonaemia on intracranial pressure, brain water content and expression of the water channel aquaporin-4. We did not find a beneficial effect of ciclosporin on intracranial pressure, brain water content or aquaporin-4 expression.

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INTRODUCTION

Development of grade III/IV hepatic encephalopathy in

patients with acute liver failure (ALF) signifies risk of cerebral oedema. Cerebral oedema constitutes a potentially life-threatening complication of ALF if it leads to increased intracranial pressure (ICP). Emergence of intracranial hypertension (ICH) is a poor prognostic sign, and renders the patient at risk of fatal cerebral herniation.

Ammonia is considered to play a pivotal role in the generation of the cerebral oedema, and high arterial ammonia levels have been demonstrated to be predictive of ICH^[1-3].

The cerebral oedema in ALF is characterised by cerebral hyperperfusion and loss of autoregulation of the cerebral blood flow (CBF), and these events are both considered fundamental in the pathogenesis of ICH^[4]. Additional factors have also been implicated in the cerebral oedema associated with ALF, including infection^[5], inflammation^[6] and hyponatraemia^[7].

The cerebral oedema seen during hyperammonaemia is predominantly cytotoxic, and involves astrocyte swelling^[8,9]. The cellular effects of hyperammonaemia are thought to include oxidative stress (OS) and nitrosative stress (NS)^[10-14], induction of the mitochondrial permeability transition (MPT)^[15-17] and activation of mitogen-activated protein kinases (MAPKs)^[18]. Astrocytes are characterised by an abundant presence of water channels, especially aquaporin-4 (AQP4), and ammonia-mediated upregulation of AQP4 has been demonstrated in recent studies of cultured astrocytes and in animal models of ALF^[19-21]. This line of events is thought to lead to mitochondrial dysfunction, reduced oxidative adenosine triphosphate (ATP) production, astrocyte swelling and cerebral dysmetabolism.

Recent studies employing cultured astrocytes have demonstrated suppression of ammonia-induced astrocyte swelling by ciclosporin (Cs) - a widely used immunosuppressant drug belonging to the group of calcineurine inhibitors^[16]. The neuroprotective effects of Cs are most likely attributable to inhibition of the MPT and attenuation of AQP4 upregulation rather than immunosuppression^[16]. The MPT is a calcium-dependent reaction, characterised by a sudden increase in the permeability of the inner mitochondrial membrane mediated by opening of the permeability transition pore (PTP), which causes collapse of the electric potential across the inner mitochondrial membrane. This results in compromised ATP production by oxidative phosphorylation, and subsequently energy failure^[22-25]. Cs blocks the MPT by inhibiting opening of the PTP, thus preserving mitochondrial phosphorylation efficiency and function^[17]. Induction of the MPT in central nervous tissue is strongly implicated in the pathogenesis of astrocyte swelling, although the extent to which the MPT contributes to ICH in ALF is yet to be determined.

Animal models of other disease states such as traumatic brain injury (TBI) have also demonstrated neuroprotective effects of Cs. Findings in such studies show attenuation of axonal damage^[26], and most importantly

inhibition of the MPT securing mitochondrial function^[27]. However, Cs itself also has neurotoxic effects in healthy brain tissue where it reduces mitochondrial function and thereby increases lactate levels and reduces the glutamate and glutamine concentrations in the brains of rats^[28]. This could however be a beneficial feature during hyperammonaemia where both glutamate-induced cytotoxicity and astrocytic glutamine accumulation play pivotal roles^[29]. However, case reports have been published where post-transplant immunosuppression with calcineurine inhibitors has triggered severe hyperammonaemia, mostly in patients with inborn deficiencies of the urea cycle^[30] and we find that the use of Cs under hyperammonaemic conditions needs experimental evaluation before clinical use can be justified.

The use of Cs as a neuroprotectant in animal models of ALF has not yet been evaluated. This might be due in part to the fact that Cs does not cross the blood-brain barrier (BBB) very well^[31] and the BBB is considered to remain largely intact in ALF^[32,33] in contrast to TBI. The challenge of cerebral bioavailability can be overcome by administering Cs intrathecally (*itb*) where the brain tissue concentration of Cs can reach relevant levels for neuroprotection without systemic toxicity^[26]. In the present study, we aimed to examine the effect of Cs administered *itb* on ICP, brain water content and cerebral AQP4 expression in rats with acute hyperammonaemia. We also measured the cortical concentration of glutamate and glutamine. We used a well-established rat model of hepatic encephalopathy, brain oedema, and cerebral hyperperfusion induced by acute hyperammonaemia after construction of a portocaval anastomosis (PCA)^[34].

We hypothesised that Cs would attenuate ICH, preserve normal brain water content and reduce upregulation of AQP4.

MATERIALS AND METHODS

All procedures involving laboratory animals were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and approved by Danish Animal Inspectorate. The experiments were performed in the animal facilities associated with the Hepatology Laboratory, Rigshospitalet, Copenhagen, Denmark.

Animals

Male Wistar rats (Charles River, Sulzfeld, Germany) were housed in plastic cages with free access to water and rodent chow, and kept at constant room temperature and humidity with a 12/12-h light per dark cycle.

The study included 24 anaesthetised animals, and they were randomly assigned to one of the following experimental groups: (1) PCA + ammonia infusion *iv* + Cs *itb* (*n* = 6); (2) PCA + ammonia infusion *iv* + vehicle *itb* (*n* = 6); and (3) PCA + saline *iv* + Cs *itb* (*n* = 6); (4) PCA + saline *iv* + vehicle *itb* (*n* = 6)

Surgical procedure

The PCA was done as an end-to-side anastomosis. In isoflurane anaesthesia, the rats underwent laparotomy. The portal vein and vena cava were isolated, and after the portal vein was ligated and cut, the distal part was sutured onto a hole in the side of the vena cava. The anastomosis was completed in less than 15 min, and the abdomen was sutured in two layers. The animals were returned to their housing, and the experiment started 24 h later.

Experimental procedure

After induction of anaesthesia with isoflurane, 0.2 to 0.3 mL pentobarbital (50 mg/mL) was administered in a tail vein. Every 10 min, the reaction to claw pinching was checked, and supplementary pentobarbital given when necessary. Arterial and venous catheters (PE-50) were inserted into femoral vessels for monitoring blood pressure, *iv* drug administration and blood sampling. The arterial catheters were flushed with 500 IU heparin and one connected to a pressure transducer. The rats were then tracheotomised and mechanically ventilated (Hallowell EMV, E-vet, Haderslev, Denmark) with a respiratory frequency of 65 breaths/min and a tidal volume of 5 to 10 mL with a mixture of atmospheric air and oxygen adjusted to maintain a normal arterial partial pressure of carbon dioxide and avoid hypoxia during the whole experiment. Arterial blood samples were taken every 30 min. for analysis of pO₂ and pCO₂ (ABL 505; Radiometer, Copenhagen, Denmark). The animals were placed in a stereotactic frame and, with the head fixated, a midline scalp incision was made and one small borehole was drilled in the skull. The borehole was used for the placement of a catheter (PE-10) in the cisterna magna, and was used for *itb* administration of Cs/vehicle, and ICP monitoring by connection to a pressure transducer. Continuous measurements of mean arterial pressure (MAP) and ICP were recorded on a computer using the software Perisoft (Perimed, Stockholm, Sweden). During the experiment, body temperature was monitored with an intra-abdominal thermistor and maintained at 37 °C with a ventral heating pad. After an initialisation period, stable baseline values were recorded. Then 150 µL of cerebrospinal fluid was aspirated through the intracranial catheter. In the drug-treated groups, 150 µL of Cs (Sandimmune®, Novartis, Basel, Switzerland) diluted with isotonic saline was infused over 30 min into the cisterna magna using a microdialysis pump (CMA/102) corresponding to a Cs dosage of 10 mg/kg and followed by a flush of saline over 3 min. The vehicle-treated groups were given a vehicle composed of ethanol, ricinus oil and saline [according to the manufacturers information (Novartis)] in an equivalent volume following the same procedure. The catheter was subsequently reconnected to a pressure transducer. In appropriate groups either an *iv* ammonium acetate infusion of 55 µmol/kg per minute or saline infusion 2 mL/h (0.9 mg/mL) was initiated ($t = 0$). The experiment was terminated at $t = 4$ h, and arterial blood samples were taken to determine plasma

levels of alanine aminotransferase, coagulation factors II + VII + X and ammonia. Animals were sacrificed while anaesthetised, and underwent decapitation. The brain was removed from the skull, and dissected into two halves by a sagittal cut. Half of the cerebral cortex and half of the cerebellum were immediately frozen in liquid nitrogen and stored at -80 °C for later analysis of Cs content and AQP4 expression.

Ciclosporin dosage

Based on studies by other groups^[26,27] we chose to administer 10 mg/kg of Cs *itb*, which is sufficient to achieve tissue concentrations able to inhibit MPT (> 0.2 µmol/L) and thus conserve mitochondrial function^[35]. We measured the Cs concentration in the cerebral cortex by liquid chromatography and mass spectrometry (Waters Micromass, Waters Corporation, Milford, MA, United States) after tissue homogenisation and protein precipitation in acetonitrile.

Analysis of brain water content by wet/dry method

The remaining halves of the cerebral cortex and cerebellum were used for determination of brain water content by the wet/dry weight method. The brain tissue was transferred onto pre-weighed glass scales, and weighed on a high-precision scale determining “wet weight”. The specimens were subsequently dried for 24 h in an oven at 110 °C. The dried brain tissue acclimatised in an exicator, and was afterwards weighed determining “dry weight”. The percentage water content of the cortex and the cerebellum was calculated according to the following formula: $[(\text{wetweight} - \text{dryweight}) / \text{wetweight}] \times 100$.

Analysis of AQP4 protein expression by Western blot

Protein analysis was done as previously described^[36]. In brief, samples of frozen cerebral cortex and cerebellar tissue were homogenised and centrifuged. The resultant pellets were resuspended in an acidic buffer and after measuring the protein concentrations, the samples were loaded onto an Invitrogen mini-cell-system (Invitrogen Taastrup, Denmark) with 3 µg protein per lane. Protein was transferred to a polyvinylidene fluoride membrane (Invitrogen) by electroelution and incubated overnight with the primary antibody. The membrane was subsequently washed and then incubated with horseradish-peroxidase-conjugated secondary antibody (SC2020, 1:5000; Santa Cruz Biotechnology). Finally, detection of bound antibody was performed using the enhanced chemiluminescence system (PerkinElmer, Waltham, MA, United States) and camera detecting system LAS 9000 with software ImageGauge 2006 Software (FujiFilm, Stockholm, Sweden).

Analysis of cortical glutamate and glutamine content

The cerebral cortical tissue was weighed and homogenised in a six-fold amount of ice-cold 1 mol/L HClO₄. The homogenate was centrifuged and the supernatant neutralised by ice-cold 1.6 mol/L KOH containing 0.4 mol/L K₂CO₃. The concentrations of glutamate

Table 1 Baseline values

Group	Weight (g)	MAP (mmHg)	ICP (mmHg)
PCA + ammonia infusion + Cs	295.5 ± 11.8	110 ± 4.2	1.0 ± 0.2
PCA + ammonia infusion + vehicle	326.2 ± 15.3	103 ± 4.0	0.8 ± 0.2
PCA + saline infusion + Cs	320.8 ± 12.4	116 ± 2.0	0.6 ± 0.2
PCA + saline infusion + vehicle	308.3 ± 14.7	106 ± 4.2	0.8 ± 0.3

PCA: Portacaval anastomosis; Cs: Ciclosporin; MAP: Mean arterial pressure; ICP: Increased intracranial pressure.

Table 2 Baseline biochemistry

Group	Arterial pH	Arterial pCO ₂ (mmHg)	Arterial pO ₂ (mmHg)	Haemoglobin (mmol/L)	Blood glucose (mmol/L)
PCA + ammonia infusion + Cs	7.46 ± 0.008	37.9 ± 1.2	136.6 ± 7.7	9.0 ± 0.19	6.4 ± 0.29
PCA + ammonia infusion + vehicle	7.47 ± 0.013	38.2 ± 0.4	130.2 ± 3.8	9.4 ± 0.26	5.9 ± 0.52
PCA + saline infusion + Cs	7.49 ± 0.005	35.7 ± 0.5	144.6 ± 1.7	8.6 ± 0.19	6.3 ± 0.21
PCA + saline infusion + vehicle	7.46 ± 0.002	37.9 ± 1.1	129.7 ± 6.7	9.2 ± 0.40	5.3 ± 0.12

PCA: Portacaval anastomosis; Cs: Ciclosporin.

and glutamine were then measured in the supernatant by an enzymatic method using a YSI 2700 (YSI, OH, United States) and the actual cortical concentration in units of mmol/100 g could then be calculated.

Statistical analysis

All results are presented as mean ± SE of the mean. Two-way analysis of variance (ANOVA) was used to evaluate the individual effects of ammonia infusion and Cs on ICP, MAP, brain water and biochemical parameters at the end of the experiment. Normal distribution of data was confirmed by the use of Shapiro-Wilk test of normality. One-way ANOVA was used to evaluate differences in baseline characteristics. An unpaired student's *t* test was used to compare AQP4 levels between groups and correction for multiple comparisons was done by the Holm-Bonferroni method. *P* values below 0.05 were considered significant.

RESULTS

We found no significant differences at baseline between the experimental groups regarding MAP, ICP, body weight, arterial pH, pCO₂, pO₂, blood glucose and Hgb (Tables 1-2). As expected, we found a highly significant effect of ammonia infusion on plasma levels of ammonia at *t* = 4 h (*F* = 201.9, *P* < 0.001). At *t* = 4 h, we found no effect of ammonia or Cs on MAP (data not shown), alanine aminotransferase levels or coagulation factors II, VII and X (Table 3).

At *t* = 4 h, two-way ANOVA demonstrated a highly significant effect of ammonia infusion on ICP (after logarithmic transformation, *F* = 19.41, *P* < 0.001), but

Table 3 Biochemistry at *t* = 4 h

Group	ALT (U/L)	Arterial ammonia (μmol/L)	PP (arb. units)
PCA + ammonia infusion + Cs	525 ± 185	1165.5 ± 87.2	0.32 ± 0.01
PCA + ammonia infusion + vehicle	600 ± 266	900.5 ± 70	0.37 ± 0.02
PCA + saline infusion + Cs	470 ± 221	108.3 ± 5.9	0.40 ± 0.03
PCA + saline infusion + vehicle	830 ± 468	140.3 ± 15	0.33 ± 0.03

PCA: Portacaval anastomosis; Cs: Ciclosporin; ALT: Alanine transferase; PP: Coagulation factor II + VII + X.

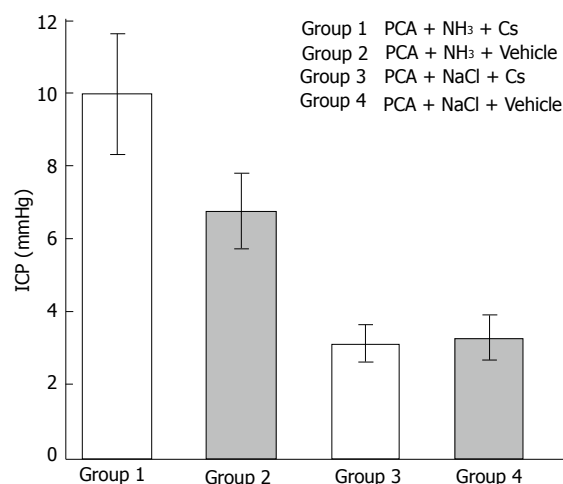


Figure 1 Intracranial pressure. Bar plot of mean ICP in the experimental groups after 4 h of ammonia infusion. Error bars represent SE of the mean. A highly significant effect of ammonia infusion on ICP was found (two-way analysis of variance, *F* = 22.1, *P* < 0.001). We found no effect of ciclosporin. ICP: Intracranial pressure; PCA: Portacaval anastomosis; Cs: Ciclosporin.

no significant effect of Cs (Figure 1). No interaction was found between ammonia and Cs on ICP. Looking at changes in ICP from baseline to *t* = 4 h, we observed a highly significant effect of ammonia (*F* = 20, *P* < 0.001), but not of Cs. In group 1, ICP increased from 1.0 ± 0.2 to 10.0 ± 1.7 mmHg, in group 2 from 0.8 ± 0.2 to 6.8 ± 1.0 mmHg, in group 3 from 0.6 ± 0.2 to 3.1 ± 0.5 and from 0.8 ± 0.3 to 3.3 ± 0.6 mmHg in group 4. Regarding changes in MAP from baseline to *t* = 4 h, we found no effect of ammonia or Cs, but in all groups MAP decreased during the experiment (110 ± 4.2 to 92 ± 10.9 mmHg in group 1, 104 ± 4.0 to 86 ± 5.0 mmHg in group 2, 116 ± 2.0 to 103 ± 4.2 mmHg in group 3 and 106 ± 4.2 to 88 ± 4.5 mmHg in group 4).

Brain water content

Two-way ANOVA demonstrated no significant effect of Cs on cortical or cerebellar water content, but ammonia significantly increased water content in the cerebral cortex (*F* = 7.8, *P* < 0.05) and in the cerebellum (*F* = 16.2, *P* < 0.001) (Figure 2A and B). No interaction was found between ammonia and Cs on cortical or cerebellar water content. There was a significant correlation between ICP and cortical and cerebellar water content (*r*² = 0.20, *P* < 0.05 and *r*² = 0.45, *P* < 0.001 respectively).

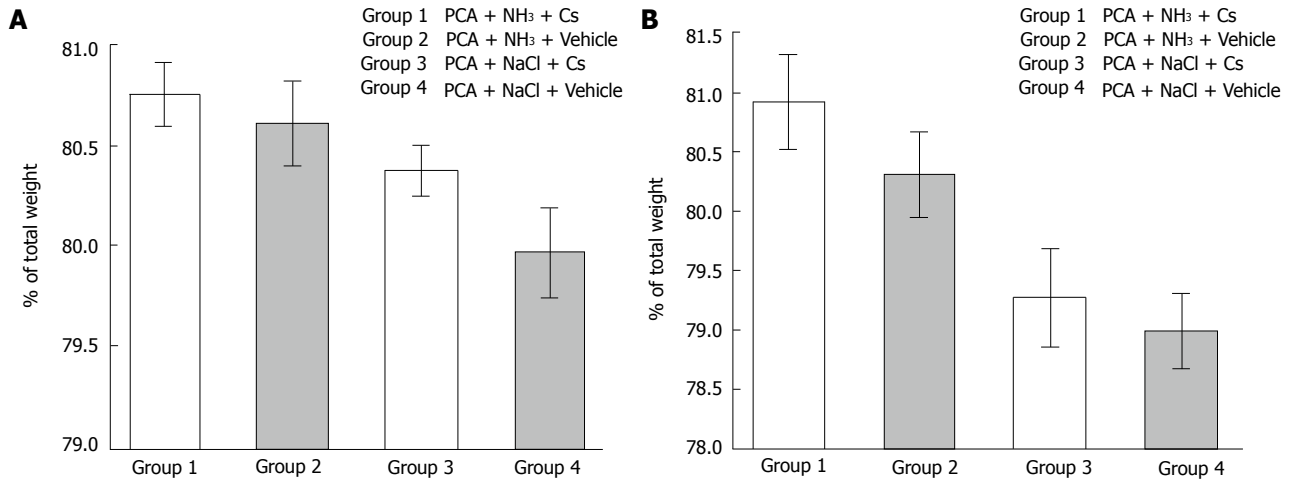


Figure 2 Brain water content. A: Bar plot of mean cortical water content in the experimental groups after 4 h of ammonia infusion. Error bars represent standard error of the mean. A significant effect of ammonia infusion on cortical water content was found (two-way analysis of variance, $F = 7.8$, $P < 0.05$). We found no effect of Cs; B: Bar plot of mean cerebellar water content in the experimental groups after 4 h of ammonia infusion. Error bars represent standard error of the mean. A highly significant effect of ammonia on increased cerebellar water content was found (two-way analysis of variance, $F = 16.2$, $P < 0.001$). We found no effect of Cs. PCA: Portacaval anastomosis; Cs: Ciclosporin.

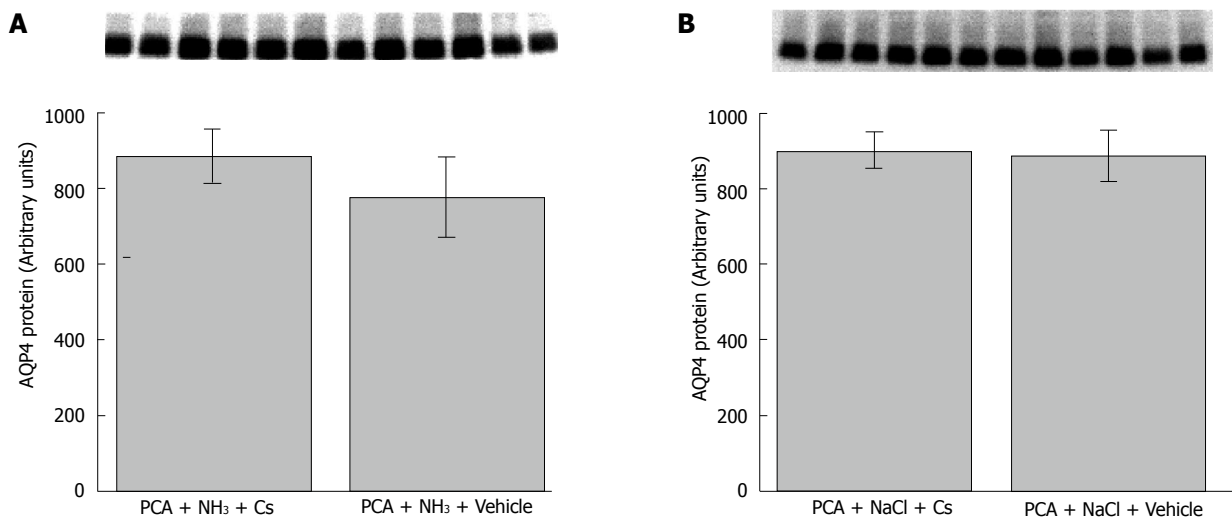


Figure 3 Brain aquaporin-4 expression. A: Bar plot and Western blot of AQP4 content of cerebral cortex in group 1 (six leftmost bands) and group 2 (six rightmost bands). No statistical significant difference was found between groups (unpaired student's t test on densitometric values); B: Bar plot and Western blot of AQP4 content of cerebral cortex in group 3 (six leftmost bands) and group 4 (six rightmost bands). No statistical significant difference was found between groups (unpaired student's t test on densitometric values). AQP: Aquaporin; PCA: Portacaval anastomosis; Cs: Ciclosporin.

Aquaporin-4

We found no significant differences in the mean AQP4 content in the cerebral cortex between group 1 *vs* 2 and group 3 *vs* 4 (Figure 3). This indicates that there was no effect of Cs on AQP4 expression.

Brain tissue concentration of Cs

In the two groups receiving Cs intrathecally, we found a brain cortex concentration of $2.14 \pm 0.74 \mu\text{mol/L}$. The concentrations were not significantly different between the two groups and we did not find a significant correlation between cerebral cortex or cerebellar brain water content and the tissue concentration of Cs (data not shown).

Brain tissue concentration of glutamate and glutamine

In the two groups receiving ammonia, the cortical glutamine concentration was $2.8 \pm 0.09 \text{ mmol/100 g}$ (group 1) and $2.8 \pm 0.14 \text{ mmol/100 g}$ (group 2). In the saline groups the glutamine concentration was $1.2 \pm 0.06 \text{ mmol/100 g}$ (group 3) and $1.0 \pm 0.07 \text{ mmol/100 g}$ (group 4). There was a significant effect of ammonia ($F = 308$, $P < 0.001$) but not of Cs. The glutamate concentration was $1.0 \pm 0.05 \text{ mmol/100 g}$ (group 1), $1.0 \pm 0.07 \text{ mmol/100 g}$ (group 2), $0.9 \pm 0.11 \text{ mmol/100 g}$ (group 3) and $0.9 \pm 0.07 \text{ mmol/100 g}$ (group 4). There was no significant effect of either ammonia or Cs on the glutamate levels.

DISCUSSION

We employed a well-characterised rat model with PCA and acute hyperammonaemia, and found that hyperammonaemia resulted in ICH and increased cortical and cerebellar water content. Furthermore, we found a significant correlation between ICP and brain water. However, our results did not support the hypothesis of a neuroprotective effect of Cs in ALF. Cs did not attenuate ICH and did not preserve normal brain water content. Furthermore, Cs did not affect AQP4 expression or glutamine levels. We also observed an insignificant tendency towards higher ICP in rats treated with ammonia and Cs (group 1) compared with the group receiving ammonia and vehicle (group 2). This speaks against the risk of a type II error causing our negative result and raises the concern that Cs actually might aggravate the cerebral oedema due to intrinsic neurotoxic features. A higher ICP, although within the normal range, has also been observed in TBI patients treated with Cs compared to placebo^[37]. The theoretical background supporting the neuroprotective features of Cs in ALF is expressed in the “Trojan Horse Hypothesis” proposed by Albrecht and Norenberg^[38]. This thesis suggests that astrocytic glutamine is transported into the mitochondria and hydrolysed by phosphate-activated glutaminase. This process yields very high levels of ammonia in this cellular compartment, which are thought to lead to OS and NS formation and induction of the MPT^[39]. MPT is believed to produce additional OS, to activate MAPK and to cause upregulation of AQP4^[18,40]. Precisely how OS and NS induce the MPT and how these factors act in concert to activate MAPK and cause upregulation of AQP4 is not fully understood. Furthermore, clarification of how this line of events mediates astrocyte swelling is yet to be achieved, and it is not clear whether AQP4 facilitates intracellular water accumulation or elimination^[20]. Cs is thought to block the MPT by interacting with Cyclophilin D (CypD)^[41–43]. CypD is a protein endowed with peptidyl-prolyl cis-trans isomerase activity, and constitutes the mitochondrial isoform of cyclophilins^[35,44,45]. Cs binds CypD, and thereby displaces it from the PTP. This displacement is thought to reveal an inhibitory site where the natural inhibitory agent (organic phosphate) is able to bind, thereby lowering the open probability of the PTP^[46,47]. The alleged neuroprotective effects of Cs during hyperammonaemia are thus based on preservation of mitochondrial function by inhibition of the MPT.

Recent studies of cultured astrocytes have demonstrated suppression of ammonia-mediated astrocyte swelling and ammonia-induced AQP4 upregulation by Cs^[21], and thereby supported the pathogenic mechanisms outlined above. While studies of brain metabolism in patients with ALF indirectly support the theory of mitochondrial dysfunction^[48,49], it is important to stress the fact that the occurrence of the MPT and AQP4 upregulation has not been demonstrated in clinical studies. This challenges the potential of this neuroprotective strategy

in clinical practice. Our present study intended to translate the favourable *in vitro* results of Cs as a neuroprotectant to an *in vivo* experiment. The reasons of our conflicting negative results are not easily identified. However, it is likely that astrocytes respond differently to ammonia in an *in vivo* setting compared to cultured astrocytes. Furthermore, additional and mostly undefined factors likely influence brain metabolism, water homeostasis and autoregulation of CBF, which could mask the speculated neuroprotective properties of Cs. The neurotoxic properties of Cs might also have counteracted the beneficial effect observed *in vitro*. On the other hand, animal models of other disease states such as TBI have demonstrated neuroprotective effects of Cs^[26,27] at similar doses and administration time. The divergent results might reflect that the mechanisms involved in brain oedema formation during hyperammonaemia are more complex than we currently understand and certainly belong to a different entity than the brain oedema associated with TBI. Further animal studies of other ALF models and other inhibitors of the MPT might clarify the potential of this neuroprotective strategy.

In conclusion, we found that Cs was unable to reduce ICH in a rat model of brain oedema induced by PCA and acute hyperammonaemia. In addition, we showed that Cs did not prevent the increase in brain water content and did not affect AQP4 expression.

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COMMENTS

Background

Acute liver failure and hyperammonaemia can lead to severe brain oedema. Preserving mitochondrial function by treatment with ciclosporin has shown potential in *in vitro* studies.

Research frontiers

Cerebral oedema constitutes a potentially life-threatening complication of acute liver failure if it leads to increased intracranial pressure. Emergence of intracranial hypertension (ICH) is a poor prognostic sign, and renders the patient at risk of fatal cerebral herniation.

Innovations and breakthroughs

In this study authors evaluated the effect of ciclosporin (Cs) in a rat model of acute hyperammonaemia on intracranial pressure, brain water content and expression of the water channel aquaporin-4. Authors did not find a beneficial effect of ciclosporin on intracranial pressure, brain water content or aquaporin-4 expression.

Applications

Authors found that Cs was unable to reduce ICH in a rat model of brain oedema induced by portocaval anastomosis and acute hyperammonaemia. In addition, authors showed that Cs did not prevent the increase in brain water content and did not affect aquaporin-4 expression.

Peer review

The manuscript written by Larsen *et al* analyzed the effect of ciclosporin on intracranial pressure in rats with hyperammonemia. They also examined cerebral water content and aquaporin-4 expression, and found no favourable effect of ciclosporin on those parameters. The data are inconsistent with *in vitro* analyses, but may give important information on daily practice.

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Format

Journals

English journal article (list all authors and include the PMID where applicable)

- 1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarhoea. *Shijie Huaren Xiaobua Zazhi* 1999; **7**: 285-287

In press

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRS-A Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

- 15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean \pm SD or mean \pm SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as *ν* (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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