ISSN 1948-5182 (online)

World Journal of *Hepatology*

World J Hepatol 2023 March 27; 15(3): 321-440

Published by Baishideng Publishing Group Inc

World Journal of Hepatology

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INDEXING/ABSTRACTING

The *WJH* is now abstracted and indexed in PubMed, PubMed Central, Emerging Sources Citation Index (Web of Science), Scopus, Reference Citation Analysis, China National Knowledge Infrastructure, China Science and Technology Journal Database, and Superstar Journals Database. The 2022 edition of Journal Citation Reports® cites the 2021 Journal Citation Indicator (JCI) for *WJH* as 0.52. The *WJH*'s CiteScore for 2021 is 3.6 and Scopus CiteScore rank 2021: Hepatology is 42/70.

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: *Yi-Xuan Cai*; **Production Department Director:** *Xiang Li*; **Editorial Office Director:** *Xiang Li*.

NAME OF JOURNAL
World Journal of Hepatology

ISSN
ISSN 1948-5182 (online)

LAUNCH DATE
October 31, 2009

FREQUENCY
Monthly

EDITORS-IN-CHIEF
Nikolaos Pyrsopoulos, Ke-Qin Hu, Koo Jeong Kang

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/1948-5182/editorialboard.htm

PUBLICATION DATE
March 27, 2023

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World J Hepatol 2023 March 27; 15(3): 321-352

DOI: 10.4254/wjh.v15.i3.321

ISSN 1948-5182 (online)

REVIEW

Main factors influencing long-term outcomes of liver transplantation in 2022

Elisa Fuochi, Lorenzo Anastasio, Erica Nicola Lynch, Claudia Campani, Gabriele Dragoni, Stefano Milani, Andrea Galli, Tommaso Innocenti

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Chang A, Thailand; Spataru A, Canada

Received: October 18, 2022
Peer-review started: October 18, 2022
First decision: November 14, 2022
Revised: November 24, 2022
Accepted: February 22, 2023
Article in press: February 22, 2023
Published online: March 27, 2023

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Abstract

Liver transplant (LT) outcomes have markedly improved in the recent decades, even if long-term morbidity and mortality are still considerable. Most of late deaths are independent from graft function and different comorbidities, including complications of metabolic syndrome and *de novo* neoplasms, seem to play a key role in determining long-term outcomes in LT recipients. This review discusses the main factors associated with late mortality and suggests possible strategies to improve long-term management and follow-up after liver transplantation. In particular, the reduction of drug toxicity, the use of tools to identify high-risk patients, and setting up a multidisciplinary team also for long-term management of LT recipients may further improve survival after liver transplantation.

Key Words: Alcohol; Liver transplantation; Long term survival; Metabolic syndrome; Renal dysfunction; Therapy adherence

Core Tip: Survival after liver transplantation has increased in the last decades due to an improvement in early post-transplantation outcomes, underlining the need to shift the focus towards long-term outcomes. We herein discuss the main factors related to long-term morbidity and mortality in liver transplant recipients and outline the main management suggestions and recommendations to improve long-term outcomes.

Citation: Fuochi E, Anastasio L, Lynch EN, Campani C, Dragoni G, Milani S, Galli A, Innocenti T. Main factors influencing long-term outcomes of liver transplantation in 2022. *World J Hepatol* 2023; 15(3): 321-352
URL: https://www.wjgnet.com/1948-5182/full/v15/i3/321.htm
DOI: https://dx.doi.org/10.4254/wjh.v15.i3.321

INTRODUCTION

Liver transplantation (LT) is the only lifesaving treatment option for patients with end-stage liver disease and acute liver failure, and for selected patients with hepatocellular carcinoma in whom other curative treatment options have failed or are not suitable[1]. Over the past decades, early post-transplantation outcomes have significantly improved[2], while the 20-year survival rate still remains only approximately 50%[3]. In fact, long-term survivors have an increased morbidity risk, not only related to "classical" transplant-related complications, such as graft dysfunction, rejection, or liver disease recurrence, but also to factors that are not strictly related to the graft[4]. Metabolic complications, cardiovascular disease, renal dysfunction, and extrahepatic malignancies play a major role in long-term morbidity and mortality of LT patients[1]. Long-term post-transplant management is complex and requires a close follow-up to recognize, manage, and prevent medical complications and comorbidities[1] (Figure 1). The purpose of this review is to discuss the factors associated with long-term morbidity and mortality after LT, describing current recommendations and suggesting possible strategies to improve the management and the follow-up of these patients.

BIBLIOGRAPHIC SEARCH

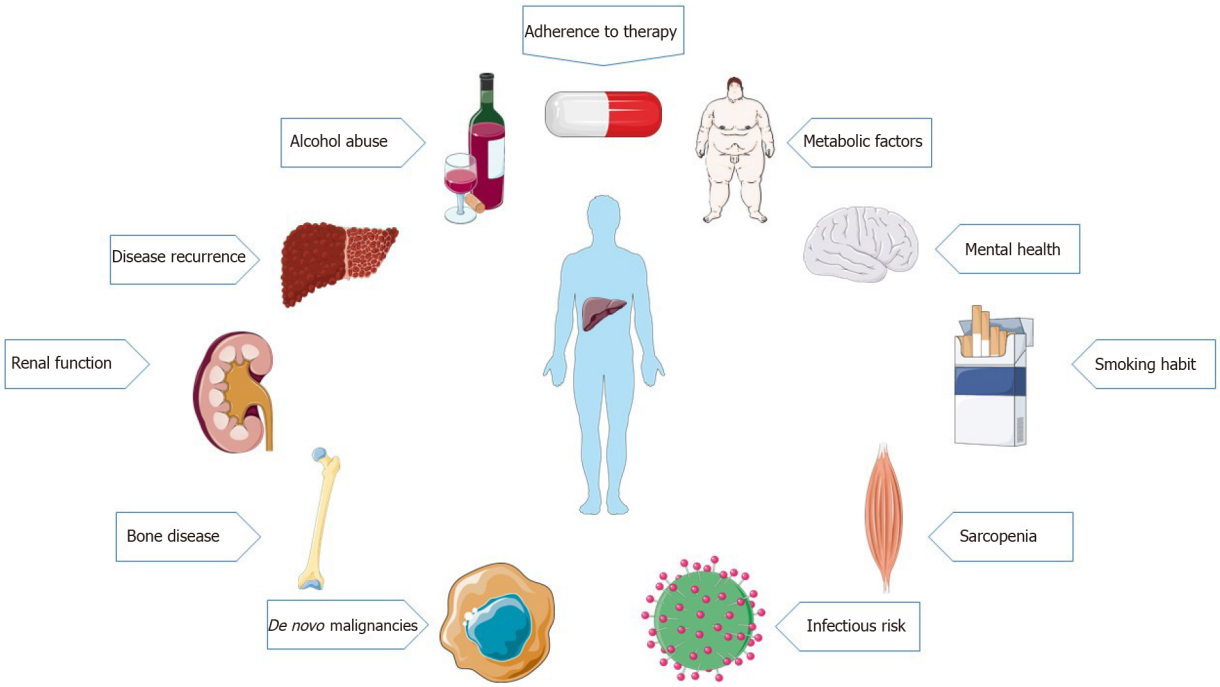
A bibliographic search was conducted by two Authors (Fuochi E and Anastasio L) using PubMed and EMBASE databases, with the following terms: "liver transplant", "liver transplantation", and "orthotopic liver transplantation". Searches of the databases were run on September 13th, 2022. Only papers written in English language were considered. After exclusion of duplicates, the search results were double-blind screened by two reviewers (TI and ENL), and abstracts assessed for eligibility. Reviews, conference abstracts and book chapters were excluded. Articles were declared not relevant by consensus.

METABOLIC FACTORS

Metabolic syndrome (MetS) is generally defined as the presence of three of five risk factors among elevated fasting glucose, reduced high-density lipoprotein cholesterol, elevated triglycerides, obesity, and hypertension[5]. It has been estimated that approximately a quarter of the world population is affected by this condition and its prevalence is still increasing[6].

There is a two-way correlation between metabolic syndrome and LT: On one hand, non-alcoholic steatohepatitis (NASH) is currently the second leading cause for LT waitlist registration/LT in general population and the first leading cause in females, at least in Western countries[7]; on the other hand, the majority of patients who have undergone transplantation develop diabetes mellitus, hyperlipidemia, and arterial hypertension[8]. It has been established that 50%–60% of these patients fulfill the criteria of metabolic syndrome[2]. In the United States, obesity is observed in 30%-40% of LT recipients within the first 5 years after transplantation[9]. Moreover, about 30% of patients suffer from diabetes after transplantation, and pretransplant diabetes is a predisposing factor[10]. Post-transplant development of MetS is due to multiple factors, such as reversal of cirrhosis, increased appetite, use of steroids, and may partly be due to the dysmetabolism of fats and sugars deriving from the use of immunosuppressants [11].

It is widely recognized that there is a strong association between metabolic syndrome and cardiovascular events. Since cardiovascular diseases are listed as the third cause of late mortality in patients who underwent LT[12], estimating cardiovascular risk and managing cardiovascular risk factors are central elements in the management of these patients. In all transplant candidates a cardiac evaluation is mandatory, although there is no ideal way to assess it. Several efforts have been made to



DOI: 10.4254/wjh.v15.i3.321

Figure 1 Main factors affecting long-term morbidity and mortality after liver transplantation.

find a more complete scoring system capable of accurately evaluating cardiovascular risk in these patients. For example, VanWagner has recently proposed the CAR-OLT score, based on age, sex, race, working status, education, liver pathology, and comorbidities to evaluate the coronary risk at one year after transplantation[13]. In 2021, Rachwan *et al*[14] elaborated another score, The coronary artery disease (CAD)-LT score and algorithm, that stratified significant CAD risk as low (≤ 2%), intermediate (3% to 9%), and high ≥ 10%). The score seemed to identify 97% of all significant CAD and potentially avoided unnecessary testing such as cardiac catheterization in low-risk patients[14]. However, none of these scores has been validated yet and it is uncertain whether they are able to predict long-term cardiovascular outcomes after LT.

In the post-transplant setting, European Association for the Study of the Liver guidelines recommend a continuous cardiovascular risk stratification and an aggressive management of metabolic syndrome, with a prompt detection and treatment of modifiable risk factors by means of lifestyle changes, pharmacological therapies, and modifications of the immunosuppression in order to prevent serious cardiovascular complications[1]. American Association for the Study of Liver Diseases (AASLD) Guidelines also recommend dietary counseling for all LT patients to avoid obesity. For patients who fail behavioral weight-loss programs, bariatric surgery may be considered[4]. There is still a debate about the ideal timing of bariatric surgery with respect to transplantation. A recent metanalysis compared the outcomes of bariatric surgery performed before, during, and after LT in a large cohort of obese patients. In all the analyzed groups, the 30-d mortality after surgery was 0%, although patients who underwent bariatric surgery after LT had a higher mortality rate beyond 30 d (7%). The graft survival rate after 1 year was 70% in patients operated before LT, while it rose to 100% for patients who underwent bariatric surgery during LT. Thirty-day minor and major complication rates were 4% and 1%, respectively, if bariatric surgery was performed before transplantation[15]. Further studies are required to define the optimal bariatric procedure and its timing with respect to transplantation[4].

SARCOPENIA

Sarcopenia is defined as the loss of skeletal muscle mass, quality, and function[16].

The overall prevalence of sarcopenia among patients with cirrhosis is 37.5% with an estimated higher prevalence in males, alcohol-related liver disease, and greater severity of cirrhosis[17,18]. Moreover, sarcopenia not only can be present before transplant but may also develop after surgery. This condition can be related to multiple factors such as infections, renal dysfunction, lack of specific nutritional diets, and specific medications[19].

To investigate the correlation between post-transplant sarcopenia and long-term outcome, a study on a population of 382 adult LT recipients has been recently performed in the Netherlands. Stam *et al*[20] measured post-transplant urinary creatinine 24 h excretion rate (24-h CER, a noninvasive marker of total

body muscle mass) one year post-transplantation and found that low CER was associated with increased 10-year mortality and graft failure risk, independently of age, sex, and body surface area. Similarly, patients within the lowest tertile of CER values had worst outcomes in terms of mortality and graft failure, compared to transplant recipients in the highest tertile[20]. It must be noted that, although the 24-h CER index is an established method for assessing skeletal muscle mass, computed tomography (CT) or magnet resonance studies are currently considered the gold standard to assess sarcopenia[21]. However, urinary CER might be an inexpensive and accessible sarcopenia marker, without the need for costly exams or exposure to radiation[22].

In a recent Chinese study, sarcopenia was assessed by measuring psoas muscle index from tomography images obtained within 1 mo after transplantation in 70 male patients. Sarcopenia was identified as being significantly associated with worse post-transplant overall survival (OS) for an average of 63.3 mo of follow-up. Interestingly, sarcopenic patients seem to suffer from higher rates of hepatocarcinoma recurrence, although this difference did not reach statistical significance[23].

Another Asian study also found that sarcopenia, assessed as height-normalized psoas muscle thickness on computed tomography within 2 mo before surgery, was associated with a higher risk of tumor recurrence after transplantation for hepatocellular carcinoma. Authors hypothesized that sarcopenia may promote tumor progression by decreasing levels of certain cytokines (myokines and adipokines) and increasing others, such as tumor necrosis factor (TNF)-α[24]. Further studies are required to confirm these results.

It is important to underline that sarcopenia can also be found in obese people. In a German meta-analysis on 1515 patients, pre-transplant sarcopenic obesity (SO), assessed with different methods, was found to increase overall mortality compared to non-SO at 1, 3 and 5-years follow-up[25]. Unfortunately, using sarcopenia as a predictor of post-transplant survival is still limited by the significant heterogeneity among studies[26]. Many questions remain, including the best modality for assessing muscle mass, the optimal cut-off values for sarcopenia, the ideal timing and frequency of muscle mass assessment, and how to best incorporate the concept of sarcopenia into clinical decision making[27]. In our opinion, sarcopenia should be evaluated before transplantation, for example using CT scan, which is generally easily available as it is required for the global evaluation of the patient before transplantation. Then, the evaluation should be repeated one year after transplantation, using the same method and possibly the same CT machine, in order to compare results.

Treatment of sarcopenia is based on lifestyle modifications. Even if there are no standardized exercise programs, Tandon *et al*[28] recommend 150 min of mild aerobic activity divided in 3-5 d per week and more than two days per week of resistance training in cirrhotic patients. Nutritional intervention prior to transplantation may also play an important role although, to date, studies have been unable to identify strategies that offers convincing benefits. Furthermore, given that sarcopenia can also develop after transplantation, dietary advice by a nutritionist may help to improve patient prognosis. Nutritional supplementations may also play a role in this condition. For example, a recent Italian randomized pilot study reported that a 12-wk supplementation after LT with β-hydroxy-β-methyl-butyrate, an active metabolite of leucine with anabolic effect that inhibits muscle proteolysis, seems to significantly improve muscle mass values in sarcopenic LT patients[29]. However, these supplementations are usually expensive and further studies with larger cohort of patients are needed to confirm these results.

BONE DISEASES

Osteoporosis was defined by the World Health Organization in 1994 as a bone mineral density of less than 2.5 standard deviations below the sex-specific young adult mean[30]. Reduced bone density leads to decreased mechanical strength, thus making the skeleton more prone to fractures[31]. Many studies have reported how fragility fractures cause a significant morbidity and mortality burden in the general population[32], with hip fractures being the most serious, with a 33% cumulative mortality rate in the 12 mo after fracture[33].

One-third of LT recipients have a bone mineral density below the fracture threshold[34] and the fracture rate in these patients has been reported to be as high as 24%–65%[35]. Up to 55% of waitlisted patients might already have osteoporosis, especially women. In this setting osteopenia can be related to different factors such as malnutrition, physical inactivity, malabsorption of vitamin D in cholestatic liver disease, steroid use in patients with autoimmune hepatitis, and direct toxicity in alcohol-related liver damage[36]. Older age, female sex, and low body mass index (BMI) are also risk factors for osteoporosis in the general population. Furthermore, patients with end-stage liver disease present with decreased bone density compared with the age-matched control population[1].

It has been established that low bone mineral density before LT is a risk factor for developing osteoporosis after transplantation[37]. Bone loss often peaks at 6 mo after transplantation, resulting in a high fracture risk[38], even if this trend tends to reverse in the following period, with no deterioration afterward[34]. Multiple factors contribute to increased bone loss after transplantation, including use of corticosteroids, poor nutritional status, vitamin D deficiency, immobility, sarcopenia, hypogonadism, smoking, and alcohol abuse[39].

Current guidelines recommend regular measurement of bone mineral density pre- and post- LT. If osteopenic bone disease is confirmed or if atraumatic fractures are present, patients should be assessed for risk factors for bone loss; in particular, this should include an assessment of calcium intake and 25-hydroxy-vitamin D levels, an evaluation of gonadal and thyroid function, a full medication history, and thoracolumbar radiography[4]. The management of osteopenia and osteoporosis in transplant recipients correlates with recommendations for the general population and involves calcium and vitamin D replacement (if deficient) and weight-bearing exercise (whenever possible). Bisphosphonate therapy must be considered for patients with osteoporosis and/or recurrent fractures[1]. In particular, a recent multicenter randomized double-blind controlled trial evaluated the efficacy of neridronate (an amino-bisphosphonate) in patients with reduced bone mass after transplantation of the heart, liver, or lung. Neridronate, at the dose of 25 mg i.m./mo for 12 mo, significantly increased lumbar bone mineral density in these patients, with a good safety profile, even in case of minor renal impairment[40].

PSYCHOLOGICAL ASPECTS AND QUALITY OF LIFE

The World Health Organization defines quality of life (QoL) as "the individual's perception of their position in life in the context of the culture and value systems in which they live and in relation to their goals, expectations, standards and concerns"[41]. Several studies have highlighted the importance of considering as determinants of successful transplantation not only mere survival rates, but also functional recovery and health-related QoL[42].

Few data are available on long-term QoL perception in transplant recipients. In a single-center cross-sectional study performed in England, QoL perception 10 and 30 years post-transplantation was found to be generally good, being reduced only in older individuals[43]. An American multicenter longitudinal study of 381 patients also reported that the general health perception declined over time in LT patients. There was also a general and progressive worsening of the distress or emotional burden related to physical symptoms such as fatigue, muscle weakness, headaches, and backaches[44]. These results could be explained by the normal age-related general health perception decline. Immunosuppression-related side effects could be at least partly responsible for the worsening of long-term QoL perception[45]. There is no clear association between gender or etiology of liver disease and QoL perception[46,47], although female sex and hepatitis C virus (HCV) related cirrhosis could be associated with worse QoL and higher levels of anxiety[48,49].

Moreover, in the post-transplant setting, patients tend to experience more clinically relevant symptoms of anxiety, depression, and post-traumatic stress compared to the general population[50].

Depressive symptoms appear to determine worse outcomes[51]. DiMartini *et al*[52] divided a cohort of 167 patients transplanted for alcoholic cirrhosis in three groups, according to the evolution of depressive symptoms within the first post-surgery year (consistently low depression levels at all time points; depression levels that rose over time; consistently high depression levels). The Authors found that recipients with increasing depression or persisting depression were more than twice as likely to die for all-cause mortality within the subsequent years. At 10 years follow-up, post-survival rate were significantly lower for the increasing-depression and high-depression groups compared with the low-depression one[52]. In another prospective cohort study, 134 LT patients were assessed for depressive symptoms using a validated questionnaire administrated 3 mo after surgery. Depressive symptoms were significantly associated with a higher 5-year mortality rate. Moreover, the questionnaire score correlated with the mortality rate in this population[53].

Interestingly, some Authors reported how patients receiving appropriate pharmacotherapy for early post-transplant depression had similar long-term survival rates to non-depressed liver-transplant recipients[54]. In addition, a recent Italian study reported how a personalized aerobic and strength training program not only improved metabolic aspects, but also the QoL perception in LT patients, so lifestyle modifications should probably be considered part of mental health management in these patients[55].

European Guidelines suggest that clinical physicians should identify depressive symptoms in the early post-transplantation period and treat them accordingly when present[1]. Unfortunately, the assessment of QoL in LT recipients has not yet been studied thoroughly and is not standardized[56]. More studies are needed to find effective strategies to manage psychological problems in this specific population. In our opinion, studies on larger cohorts of LT patients should be performed to compare different QoL and depressive symptoms questionnaires (for example, questionnaires that are already been validated in other populations such as elderly people or oncologic patients) in order to select the ones that better correlate with long term outcomes after LT.

RENAL DYSFUNCTION

Most of liver-transplanted patients develop impaired kidney function with a variable degree of severity. Within the first 10 years post-surgery, 30%-80% of patients develop chronic kidney disease stages 3-4

[57] and 25–5% of patients require dialysis[58].

Renal function impairment may already be present before LT or develop or worsen after surgery. In LT candidates, renal dysfunction can be related to cirrhosis itself but also to other coexisting conditions such as diabetes, glomerulosclerosis, or IgA nephropathy[59]. Hepatorenal syndrome (HRS)-related kidney injury deriving from intense renal vasoconstriction secondary to complex circulatory changes in cirrhotic patients may not be fully reversible after transplantation[60]. Liver transplantation is considered the definitive treatment for HRS because renal failure is functional and liver disease is the actual cause of the renal impairment[61]. Patients with hepato-renal syndrome seem to have worse survival expectancy than other patients with cirrhosis for any given value of model for end-stage liver disease (MELD) score, which suggests that HRS may be considered a poor-prognosis factor after LT [62]. However, a recent meta-analysis demonstrated that about 83% of HRS patients achieved HRS reversal after LT[63] and HRS-non acute kidney injury seem to have worse outcomes compared to HRS-acute kidney injury[64]. Living-donor LT results in identical long-term outcome when compared with deceased-donor LT in patients with HRS[65]. Many factors may contribute to the development or worsening of kidney failure after transplantation, including perioperative acute kidney injury, hypertension, diabetes mellitus, atherosclerosis and, most importantly, exposure to calcineurin inhibitors (CNI)-based immunosuppressive regimens, especially when it comes to long-term therapies [1]. CNIs might be responsible for more than 70% of chronic kidney injury in post-transplant setting[57].

Many studies have reported an increased risk of death, myocardial infarction, stroke, and major bleeding in chronic kidney disease, especially in its most severe stages[66]; thus, preventing renal deterioration and preserving its function may be a key element in the management of transplanted patients.

Continuous monitoring of renal function is recommended to detect and treat kidney disease at an early stage. Not only serum creatinine, but also an estimating equation to evaluate the glomerular filtration rate should routinary be used. Urinary protein quantification using the concentration ratio of protein to creatinine in a spot urine specimen should be evaluated at least once yearly[4]. Sufficient treatment of potential risk factors such as diabetes and hypertension and avoiding nephrotoxic drugs is recommended and should be started immediately after transplantation[1]. Adjustment of the immunosuppression (IS) (usually on an individual level, especially in patients with impaired kidney function), is mandatory. In particular, reduction or withdrawal of CNI associated-immunosuppression or alternative CNI-free protocols should be considered as soon as possible in patients with impaired renal function[1]. For example, a recent meta-analysis on 769 patients has detected higher estimated glomerular filtration rates (eGFR) at one, 3, and 5 years post transplantation in patients on everolimus therapy (EVR) compared to those receiving CNI standard therapy[67]. In 2019, the observational CERTITUDE study, following patients who had completed the SIMCER trial, found that patients starting EVR therapy at month 1 after transplant with stepwise tacrolimus (TAC) withdrawal had a mean eGFR which was significantly higher compared to patients that were on standard tacrolimus-based regimen at 24 mo after transplant[68]. A phase 2, multicenter, randomized, open-label trial has evaluated the safety and efficacy of EVR initiation even earlier than 1 mo after LT[69]. In this study, patients treated with corticosteroids, TAC, and basiliximab were randomized to receive EVR (1.5 mg twice daily) from the eighth day post-surgery and to gradually minimize or withdraw TAC when EVR was stable at > 5 ng/mL or to continue TAC at 6-12 ng/mL (control group). eGFR was significantly higher in the EVR group, as early as 2 wk after randomization, with similar efficacy rates in the two groups at 3 mo follow up. These studies suggest that EVR based IS, started early after transplantation, might be a valid alternative to CNI-based therapies in patients with renal dysfunction. Despite its positive effect on renal function, early switch to EVR has been associated with higher biopsy-proven acute rejection at 6 mo follow up[70], so that the choice of IS treatment should always be personalized and made weighing up the risks and benefits of the different therapeutic strategies.

Kidney transplantation from deceased or living donors is beneficial in improving survival and should be considered the optimal therapy for LT recipients who develop end-stage renal disease[4].

INFECTION RISK AND VACCINATION

Solid organ transplant recipients are at an increased risk of infection because of the IS required to prevent graft rejection[71]. Vaccination is considered an important strategy to prevent infectious risk not only in the general population but also in transplanted patients[1]. As immunodepression can reduce immune response to vaccines[72] and live attenuated vaccines are not recommended in immunocompromised patient[73], guidelines suggest to perform HAV, HBV, Varicella, *Pneumococcus*, influenza, and tetanus vaccinations prior to transplantation, if possible[1]. Many national guidelines recommend annual influenza vaccination of immunocompromised patients, although the decision to vaccinate is usually at clinical discretion[74]. A meta-analysis conducted on 209 studies has found that transplanted patients, together with HIV and cancer patients, are those who benefit most from the annual boost as it significantly decreases the rate of laboratory-confirmed influenza cases in these patients[75].

Influenza is a considerable public health issue due to its dissemination and contagiousness, causing annually about 4 million severe infection cases and about half of million deaths each year[76]. The

precise epidemiology of influenza in the transplant population is not well known because little data are available describing the incidence of influenza in multi-season and multicenter prospective cohorts, in particular for recipients of allografts other than lung[77]. However, influenza seems to be more common among solid organ transplant recipients compared to the general population, as showed in a 10-year longitudinal study with an incidence of 4.3 cases per 1000 person years[78]. In patients with impaired immunity, influenza is more likely to lead to a lower respiratory infection and can also have unusual manifestations such as rhabdomyolysis and myocarditis[78,79].

A recent multicenter prospective study including 606 transplanted patients from twenty centers in the U.S., Canada, and Spain showed that receiving vaccination for influenza is associated with a decrease in disease severity as determined by the presence of pneumonia and Intensive Care Unit admission[80]. Similar results have been found in another recent Italian study, in which vaccination was associated with fewer hospital admissions for infectious respiratory diseases compared to unvaccinated patients (9.7% *vs* 23.5%). The main reason for vaccination refusal was fear of adverse reaction, impaired health status, or low vaccine efficacy. Interestingly, receiving advice of Reference Center physicians was positively associated with influenza vaccination, highlighting the important role of the transplant hepatologist with regard to vaccine communication and recommendation for high-risk patients[81].

In addition, there is no consistent evidence suggesting an association between influenza vaccine and graft rejection, worsening of allograft function, or other serious adverse events in immunocompromised patients[75].

European and American Guidelines recommend influenza vaccination in transplanted candidates and annual influenza vaccination in liver transplanted patients[1,4]. The Infectious Disease Society of America guidelines suggest administering inactivated influenza vaccine starting from one month after transplantation during community influenza outbreaks[82].

Adherence to seasonal influenza vaccination is still low in immunocompromised patients, reaching a maximum of 50%-60% of patients[83,84]. Accurate counseling by the hepatologist may increase the percentage of vaccinated patients and therefore improve the long-term outcome of these patients[81].

A recent meta-analysis of randomized controlled trial has compared the clinical benefit of high dose trivalent influenza vaccine (TIV) *vs* standard dose in adult patients. One of the 10 analyzed studies also included immunocompromised patients. The meta-analysis found that laboratory-confirmed influenza A (H3N2) was significantly reduced with high-dose TIV, especially in older adults, even if no difference in mortality or hospitalizations was demonstrated[85]. Further studies are needed to compare the efficacy of high-dose *vs* standard-dose TIV in LT patients.

Another strategy to prevent influenza disease is the prophylaxis with neuraminidase inhibitor (Oseltamivir) during periods of local influenza circulation. A randomized controlled trial on 477 immunocompromised subjects, mostly solid organ transplant adult recipients, has found that Oseltamivir, given orally at the dosage of 75 mg daily, significantly reduced laboratory-confirmed influenza incidence in these patients and it was also well tolerated[86]. Also, Oseltamivir does not affect the steady-state pharmacokinetic characteristics of cyclosporine, mycophenolate, or tacrolimus, at least in adult renal transplant patients[87]. In our opinion, Oseltamivir prophylaxis may be considered as a strategy to prevent influenza disease in LT recipients along with vaccination. Oseltamivir is also indicated to prevent serious complications when influenza is established. A recent randomized, placebo-controlled, phase 3 trial found that a single dose of Baloxavir marboxil, a selective inhibitor of influenza cap-dependent endonuclease, has similar efficacy to Oseltamivir in improving influenza symptoms in high-risk healthy individuals[88]. To our knowledge, specific studies on LT population comparing these medications are lacking and further studies are needed to support the use of this drug in transplant patients.

DE NOVO NEOPLASMS

Besides cardiovascular diseases, *de novo* malignancies are the leading cause of mortality after the first post-LT year[1]. LT patients have an 11-fold higher risk of developing cancer compared to the general population[89]. The overall incidence of *de novo* malignancies is considered between 3.1 and 14.4%, with a cumulative risk gradually increasing with posttransplant graft survival, rising to 55% at 15 years[90]. The overall estimated survival rates for all types of neoplasms are reportedly 70, 48, and 39% after 1, 5, and 10 years, respectively[90]. Notably, the probability of survival is generally worse than for a non-transplanted patient with the same tumor at the same stage and location[91].

Recent data suggest that solid organ tumors are becoming the most frequent malignancy in these patients, followed by skin cancers and lymphoproliferative disorders. In particular, Rademacher *et al*[92] have analyzed 1616 LT patients and have found that solid organ tumors were responsible for more than 50% of all the novo malignancies after a mean follow-up of 28 years.

The major causes of *de novo* malignancies in the post-LT course are related not only to the loss of immunovigilance induced by immunosuppressive agents but also to other carcinogenesis risk factors that are shared with the general population[1].

For example, Epstein Barr virus seropositivity before transplantation and aggressive immunosuppressive regimens are considered risk factors for developing lymphoproliferative disorders after transplantation[93]. On the contrary, major risk factors for developing non-melanoma skin cancers in these patients are older age, chronic sun exposure and sunburn, fair skin, and a history of previous skin cancers[94]. Considering solid organ tumors, significantly higher rates of colorectal cancer have been reported in patients with primary sclerosing cholangitis (PSC) and inflammatory bowel disease (IBD), even after LT[95]. Metabolic syndrome, that is common in transplanted patients, as previously discussed[7,8], is associated with a higher risk of endometrial, pancreatic, breast, and colorectal cancer [96]. Human papilloma virus (HPV) infection is associated with various cancers and, especially, with cervical cancer in women[97]. Patients with alcoholic cirrhosis are of particularly have a higher risk of developing upper gastrointestinal, oropharyngeal-laryngeal, and lung cancers, especially if there is also a positive present or past smoking history[98].

Treating modifiable risk factors and thus preventing cancer onset must be part of the clinical management of LT recipients. Smoking cessation and alcohol withdrawal should be promoted to reduce lung and head-neck cancers incidence[99]. Metabolic syndrome, obesity, and diabetes should be managed in order not only to prevent cardiovascular disease, but also to reduce cancer burden in these patients[100]. Sunbed use and sun exposure without adequate protection should be avoided to prevent skin cancers[101]. HPV vaccination is safe in immunosuppressed patients and is indicated to prevent cervical cancer[102]. A recent study has compared the immunological response and tolerability of HPV vaccination in pediatric kidney transplant (KT) recipients *vs* KT non immunosuppressed candidates. The study established that antibody concentration against HPV and seroconversion rates were significantly lower in patients vaccinated after KT compared to those who had been vaccinated before KT. The vaccination was well tolerated in both groups. This study suggests the importance of advocating for HPV vaccination prior to transplantation and acknowledges its safety after transplantation[103]. To our knowledge, there are no specific studies comparing HPV vaccination prior and after LT. Also, future studies are needed to investigate the effect of a supplemental dose of HPV vaccine in transplant recipients who do not seroconvert and to evaluate the long-term persistence of antibodies post-transplantation.

European and American guidelines highlight the importance of cancer screening protocols after LT, especially in high-risk populations, in order to detect *de novo* tumors at an early and potentially curative stage[1,4]. Patients transplanted for alcoholic liver disease should undergo a more intensive surveillance protocol for the detection of upper gastrointestinal, oropharyngeal, laryngeal, and lung cancers[1]. Patients transplanted for PSC with associated IBD should undergo annual colonoscopy to allow early detection of colorectal cancers[1]. American guidelines suggest that all LT recipients should see a dermatologist after transplantation to assess cutaneous lesions. Then, annual evaluation should be performed at least 5 years after transplantation for skin cancer prevention[4]. More data, however, are needed to define the optimal surveillance protocol after LT with individualized emphasis laid on patients' particular risk profiles[1]. A proposal for a possible screening protocol is shown in Table 1.

Immunosuppressants also play a key role in promoting cancer development and progression, not only by inhibiting the body's immune surveillance, but also by several other mechanisms, including the induction of insulin resistance and direct carcinogenic effects[104,105]. A lower incidence of neoplastic disease has been reported in patients treated with mammalian target of rapamycin inhibitors (mTORi) with a gradual tapering of CNI, if compared with patients on standard-dose CNI[106,107]. For this reason many transplant centers frequently add mTORi to CNI or convert to an mTOR inhibitor IS regimen when there are risk factors for malignancy after transplantation, or when a tumor has been diagnosed[1]. Nevertheless, all immunosuppressant regimens could increase *de novo* neoplasms risk, including those based on mTORi[89], so it is advisable to keep IS levels as low as possible, when feasible [108].

SMOKING

The prevalence of patients with a lifetime history of smoking before LT varies between 47% and 60%, while that of active smokers at the time of LT ranges between 10%-12%, with a relapse rate of 7%-12% [109-111]. As expected, patients who quit smoking for a shorter time before LT are those with higher rates of relapse[109]. These figures are subject to variation depending on the considered population, as there are higher rates of smokers among patients who underwent LT for alcohol-associated liver disease (ALD)[112,113]. Cigarette smoke is in fact clearly linked to alcohol consumption[114], which is why smoking habits should be especially investigated in individuals with previous ALD or current known alcohol use.

Other risk factors associated with being an active smoker until and after LT are younger age, higher MELD score, comorbid substance use disorder, and six months or less of alcohol abstinence before LT; alcohol dependence awareness is a protective factor from smoking after LT, underlining the close relationship between the two habits[115].

Table 1 Screening protocols proposal for the surveillance of de novo neoplasms in liver transplantation population

Malignancy	Screening proposal
Skin cancer	Annual dermatological visit[4], shorter follow up interval for high risk patients (*i.e.* every six months)
Lung cancer	Annual thoracic X-Ray; CT-scan in active or past smokers[282]
Colorectal cancer	Perform baseline colonoscopy on patients > 50 years old; annual fecal occult blood test in younger patients or if colonoscopy is negative; annual colonoscopy if patient affected by PSC + inflammatory bowel disease[1]
Ear, nose, and throat cancers	Annual otolaryngological visit in patient with active or past alcohol and/or smoking habit[124]
Renal cancer	Annual abdominal ultrasound
Cervical cancer	Annual papanicolau-test; annual gynecological visit
Breast cancer	Annual mammography, ultrasound evaluation if needed
Prostate cancer	Annual PSA and PSA ratio evaluation

CT: Computed tomography; PSC: Primary sclerosing cholangitis; PSA: Prostate specific antigen.

The majority of studies investigating smoking habits of LT recipients uses self-reported instruments (*i.e.* questionnaires) which appear to be sufficiently reliable in LT candidates, with about 10% not disclosing their smoking habit. In this setting, the use of a biomarker such as serum cotinine can be helpful in detecting deceptive reporting[116].

Tobacco smoking hampers long-term survival[112,117], with a worse prognosis in ALD-transplanted recipients who are active smokers at the time of LT compared to former smokers[115]. Smokers have a 79% higher risk of dying compared to nonsmokers[115]. A history of smoking is not only a well-known risk factor leading to the major causes of death in the long-term post-LT, such as development of cardiovascular complications and *de novo* neoplasms, as showed by a recent meta-analysis[118], but it has also been associated with alcohol relapse in ALD-transplanted patients[119], recurrent viral-hepatitis[120], an augmented risk of IBD flare in PSC-transplanted patients[121], and with an increase in biliary complications[122]. On the other hand, an increased time from smoking cessation to transplantation seems to be a protective factor against developing biliary complications[123].

Undoubtedly, special attention during follow-up is warranted for patients with a history of smoking, by means of screening (annual chest CT and ear-nose-throat evaluation) for early detection of *de novo* malignancies[124] and by actively assessing their smoking status at each visit, focusing on those with particular risk of relapse, implementing tobacco cessation treatments, and, if needed, providing a referral to start behavioral and/or pharmacological treatment[115,116,125,126].

MAINTENANCE IMMUNOSUPPRESSION AND ADHERENCE TO THERAPY

The transplanted liver becomes partially tolerant to immune-mediated injury, so the need for IS declines after the first 90 d[4]. Since the liver is considered a privileged organ in terms of immunological interaction, the clinician's aim has switched from trying to achieve complete suppression of acute rejection to obtaining a reduction of IS-related side effects, as long-term direct and indirect side effects of immunosuppressive therapy are a major cause of morbidity and mortality[1].

Maintenance IS therapy after LT is mainly based on CNI, with TAC being favored over Cyclosporine, with a variable use of other two classes: Antimetabolites like Azathioprine and mycophenolate mofetil, and mTORi, such as sirolimus (SRL) and everolimus. Management of immunosuppressants should take into consideration recipient characteristics, etiology of primary liver disease, and magnitude of alloimmune activation[108].

Each of these drugs has adverse effects (Table 2); for this reason, given the tolerogenic aspect of LT, an immunosuppressant minimization strategy should be considered for each patient (except for those with a history of graft rejection or those transplanted for immune-mediated diseases), while waiting for further development of personalized therapies[108].

Even though IS complete withdrawal should only be limited to clinical trials[108] it always remains an interesting perspective. Recently, Levitsky *et al*[127] conducted a pilot clinical trial of SRL monotherapy withdrawal in 15 selected recipients, who were followed-up for 12 mo after complete IS withdrawal with serial peripheral blood and graft biomarker assessments: 8 (53%) patients were successfully withdrawn from SRL at a median of 18 wk. Interestingly the authors found higher percentages of tolerogenic dendritic cells (HLA-DR +CD11c+ILT3+ILT4+ DC) prior to and after successful SRL withdrawal, compared to those who failed withdrawal. Furthermore, the authors previously identified a real-time PCR based biopsy signature relating to iron metabolism that predicted

Table 2 Maintenance immunosuppressants main adverse effects

Drug class	Adverse effects
CNI	Nephrotoxicity[283], recurrence of HCC[188,284], risk of *de novo* neoplasia[285-287], new onset diabetes mellitus (TAC more than CyA)[288,289], hypertension (CyA more than TAC)[290], dyslipidemia[291] (CyA more than TAC)[292], neurotoxicty[293], weight gain[294,295]
Antimetabolites	Leukopenia, thrombocytopenia, gastrointestinal disturbances (MMF and AZA) diarrhea, CMV reactivation (MMF)[296], pancreatitis, hepatotoxicity, risk of *de novo* neoplasia (AZA)[296,297]
mTORi	Leukopenia, dyslipidemia[298,299], cutaneous and mucosal alterations[300], wound complications, lymphocele[301], hypertension[302]

CNI: Calcineurin inhibitors; HCC: Hepatocellular carcinoma; TAC: Tacrolimus; CyA: Cyclosporine; MMF: Mycophenolate mofetil; AZA: Azathioprine; mTORi: Mammalian target of Rapamicin inhibitors.

tolerance and found that this same signature on pre-weaning biopsy accurately predicted tolerance to withdrawal, with 88% sensitivity, 83% specificity, 88% positive predictive value and 83% negative predictive value.

On the other hand, it must be noted that poor adherence to therapy and/or low blood levels of immunosuppressant are associated with a higher number of acute rejection episodes[128,129], which has been linked to chronic rejection that may lead to re-transplantation or death[130].

Poor-adherence to therapy has been reported in up to 50% of LT recipients[131], even though there is a substantial heterogeneity in the definition of non-compliance[132], often causing difficult comparisons among study results.

Assessing adherence to therapy is also an issue. Electronic monitoring (*e.g.* using pill bottles with a special cap that contains microelectronics to register the time and date of every bottle opening) yields detailed and reliable data but it is time- and cost-consuming[133]. There is also debate for its use as a gold standard, since it may not be feasible in clinical practice[134,135]. Trough levels can be affected by a variety of conditions, such as graft function and the concomitant use of other drugs[136]. Self-reporting could be a reliable method[137], but it lacks of objectivity[138].

A study conducted in kidney transplant recipients showed how a composite score using self-reported non-adherence and/or collateral-reported non-adherence and/or non-therapeutic blood assay variability had the highest sensitivity in assessing non-adherence to therapy[139].

Factors associated with poor compliance to therapy are: Young age[140], divorce, history of substance or alcohol use, mental health disorders, missing clinic appointments[131], belief in alternative medications, high regimen complexity, poor knowledge about medications, and cost issues[137].

As showed by a recent meta-analysis of randomized controlled trials conducted on solid organ recipients (mainly kidney)[134], adherence-enhancing interventions can result in significant increases in total adherence, medication dosing, and timing adherence rates, and even if there is insufficient evidence to assess which type of intervention (mobile health, cognitive, or behavioral) may be maximally effective, probably a combination of multiple interventions led by a multidisciplinary team may improve the immunosuppressive therapy adherence rate for solid organ recipients.

In a review by Burra *et al*[136], the Authors underlined the need to adopt a multidisciplinary approach for LT patient management, where multidisciplinary measures are developed by professional educators, supported by psychologists, and coordinated by physicians.

DISEASE RECURRENCE

ALD

ALD is the main indication for LT in Europe[141] and the United States[142].

There is no standardized definition for relapse thus reported relapse rates vary greatly: from nearly 50% if relapse is intended as alcohol use of any measure[143], to 12% if relapse is intended as harmful alcohol consumption, starting as soon as 1 mo after LT[144]. Interestingly, the study from Faure *et al*[145] reports excessive alcohol consumption post-LT in about 10% of the patients who were not transplanted for ALD as a primary indication, but who reported excessive alcohol consumption before LT, and about 3% of patients who did not report excessive alcohol consumption before LT. For this reason, a thorough history and ongoing monitoring of alcohol consumption in all patients is of great importance during follow-up of LT patients.

Factors associated with alcohol relapse are psychiatric comorbidities, pre-transplant abstinence of less than 6 mo[146], smoking[147], alcohol consumption from an early age[148], noncompliance with appointments or medication[149], and the lack of social support, in particular the absence of a companion in life[150]. Satapathy *et al*[151] have proposed a "Harmful Alcohol Relapse after Liver Transplant" score, that included 4 variables (*i.e.* age at LT, alcohol abstinence measured in months, daily

alcohol use, and history of non-alcohol-related criminal history) to help identify ALD patients at high-risk for harmful alcohol relapse, with an Area Under the Curve (AUC) of 0.79 for predicting relapse after LT.

Lee *et al*[152] developed an artificial intelligence model to predict post-LT harmful alcohol consumption in patients who underwent early liver transplant for alcohol associated hepatitis, using variables generated through content analysis: These variables included the identification of a primary support person, the presence of young children or grandchildren living with the patient, being a home caregiver for children or elderly relatives, opioid abuse, and being religious; this model could predict harmful alcohol consumption in the external validation set with a positive predictive value of 0.82 (95%CI: 0.625–1.000) and a negative predictive value of 0.81 (95%CI: 0.803–0.819), with and AUC of 0.69, indicating potential for AI to assist in the discovery of novel predictors of post-LT hazardous alcohol use, which may be used as a tool to tailor therapies for alcohol use disorder based on a projected likelihood of relapse.

While transplantation for ALD has a favorable outcome even when compared to other etiologies, *de novo* malignancies and cardiovascular events are still more frequent in this category of patients[153]. Excessive alcohol consumption post-LT is associated with a further reduction of long-term survival, with cancer and cardiovascular events as the main causes of death[154,155]. It seems that an average of 5 years follow-up post-LT is needed to observe an increase in liver-related mortality in the excessive alcohol consumption group[156]. It is important to keep in mind that excessive alcohol consumption-related impact on long-term survival can be an issue for every LT recipient, no matter what the primary indication for LT was[145].

Early diagnosis and prevention of relapse is important, given the clinical influence of excessive alcohol consumption post LT. Self-reported alcohol use can lead to deceptive reporting, so that biomarkers can be a supportive tool, in particular liver function tests and metabolites of alcohol, such as urinary ethyl glucuronide[157,158].

A structured management of patients at risk of relapse by a multidisciplinary team, including transplant hepatologist, clinical psychologists, psychiatrists with expertise in alcoholism and social workers is an effective strategy to prevent relapse post-LT[159,160].

In the study by Addolorato *et al*[161] , follow up of LT-recipients by an alcohol addiction unit, formed by internists, physicians in training, and psychologists with expertise in alcoholism, hepatology, and neuroscience, providing multimodal treatment (clinical and medical management, including counseling and pharmacological treatment), proved to be effective in reducing alcohol recidivism and mortality.

To our knowledge, there is still no published RCT evaluating the best intervention to prevent alcohol relapse in LT recipients. However, there is evidence suggesting that a multidisciplinary team approach is an effective way to prevent relapse[159-161]. We still do not have an ideal tool to predict who will relapse after LT, but some risk factors have been identified[147-151], allowing the clinician to focus on specific psychosocial features. Ongoing monitoring for alcohol-relapse is necessary for ALD patients [162], but a thorough history of alcohol consumption and assessment use during follow-up is also important for non-ALD recipients, since excessive alcohol consumption cannot be excluded in this category, also many years after LT[163]. Referral to psychiatric treatment or counseling is recommended in case of relapse, and every patient who underwent LT for ALD should also be encouraged to undertake smoking cessation[1,4].

Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is an indication for LT[1,164] in the first stages of the neoplastic disease, or after downstaging[165]. Recurrence of the disease develops in 8-18% of the recipients after a median time of 12 mo[166,167]. It significantly affects survival, especially if it appears in the first two years after LT, with a median survival after LT of 4 years *vs* 12 years in those without recurrence[168]. Among the many pre- and post-operative risk factors associated with recurrence[169], we can find tumor stage[170,171], vascular invasion[172], high Alpha-fetoprotein (AFP) levels[171] and differentiation grade[173-175], size, and number of nodules[175].

Several prognostic models have been developed using some of these risk factors; these models can be divided in pre- and post-transplant. Among the pre-transplant prognostic models the Milan criteria (solitary HCC with diameter < 5 cm or up to 3 nodules with diameter ≤ 3 cm) represent the benchmark for the selection of HCC patients for LT and the basis for comparison with other proposed criteria[1]. Attempts to expand the Milan criteria usually occur at the expense of HCC recurrence[176]. Other pre-transplant criteria which have demonstrated comparable survival results are those relying on size/number of nodules alone, such as the University of California San Francisco (UCSF) criteria (single nodule ≤ 6.5 cm or 2–3 nodules ≤ 4.5 cm and total tumor diameter ≤ 8 cm)[177] and the Up-to-7 criteria (sum of the largest tumor size and number of lesions < 7)[178] and those including AFP beside the size/number of nodules, such as Metroticket 2.0 (AFP levels + tumor number and size of the largest tumor) which showed good results compared to the above-mentioned models[179]. Every nodule with an intermediate-to-high probability of harboring HCC according to LI-RADS protocol seems to contribute to tumor burden and should be entered in the Metroticket 2.0 calculator in order to grant appropriate performance[180]. It has also been demonstrated how incorporating the modified RECIST criteria in response to neoadjuvant therapies into the Metroticket 2.0 framework can improve its predictive ability

[181]. In any case, it is mandatory to evaluate not only preoperative but also postoperative predictors of recurrence as there may be a mismatch between radiological findings before surgery and postoperative pathological assessments[182].

Among the post-transplant criteria, Parfitt *et al*[183] developed a risk score to predict HCC recurrence, based on microvascular invasion, tumor size, satellitosis and giant/bizarre cells visible at low power which was subsequently externally validated[184], showing a sensitivity of 80%, specificity of 79%, and an area under the ROC curve (AUROC) of 0.80.

Mehta *et al*[185] developed a simple prognostic score (RETREAT score) involving patients transplanted within the Milan Criteria, using 3 variables: AFP levels, the presence of microvascular invasion, and the sum of the diameter of the largest viable tumor plus the number of viable tumors. The score was able to stratify 5-year HCC recurrence risks ranging from less than 3% in those with a risk score of 0 to higher than 75% with a risk score of 5 or higher.

In this setting, the choice of the IS regimen is extremely important since mTORi, primary SRL, seem to have a protective effect against HCC recurrence[186,187] while CNI therapy is associated with an increased risk of tumor recurrence[188], even though the current recommendation is to minimize IS [108], with no mention to a specific IS regimen. AASLD guidelines also suggest considering an IS regimen including SRL, started several weeks after transplantation, for patients undergoing transplantation for HCC[4].

Strategies for preventing HCC recurrence mainly rely on an adequate pre-transplant selection of candidates[176] and on optimizing IS regimen[108] since there currently is no approved adjuvant therapy that has demonstrated prolonged disease-free survival[189-191]. On this basis, early diagnosis of HCC recurrence gains a central role in the post-LT care but there is no consensus about screening for recurrence, translating in a significant variability in center practices. In fact, Aggarwal *et al*[192] recently conducted a survey among 48 American adult liver transplant centers: There was considerable variation in the duration of surveillance, with 48% of the reporting centers maintaining surveillance for 5 years, while 18% discontinued surveillance after 2 years; 38 out of 48 centers used a risk stratification method for disease recurrence post-LT, categorizing patients into high and low risk groups, mostly based on the presence of microvascular invasion, tumor differentiation grade, discrepancy between pretransplant radiologic tumor size or number and explant pathology, and serum AFP measured before LT or at the time of LT. As expected, AFP was the most commonly used biomarker for detecting recurrence and 13 centers used specific cutoff values for serum AFP (between 100 and 500 ng/mL). On the other hand, 21% of the reporting centers employed solely abdominal/pelvic imaging and only 5% including bone imaging. The most frequently used imaging monitoring routine was every 3–4 mo in the first year, followed by every 6 mo in the second year, and every 6–12 mo at 3 years or beyond. For patients who were thought to be at a higher risk for HCC recurrence, 21 of the 38 facilities that stratified HCC recurrence risk had a more stringent "high risk" surveillance protocol, with a significant variability among the centers: In the first five years after liver transplantation, imaging was most frequently reported every 3 to 6 mo. Only in a few centers surveillance was interrupted after two years of follow-up (14%).

A reasonable surveillance strategy should therefore include chest and abdomen imaging and serum AFP monitoring, and should be more rigorous for those patients with high risk features for recurrence, especially during the first year after LT[193]. Most authors report monitoring for HCC recurrence post-LT with thoracic CT, abdominal CT or MRI, and AFP levels with 3- to 6-mo intervals in the first 2 or 3 years, increasing the interval between exams after the 2 or 3-year timepoint[194].

Increased surveillance may improve post-recurrence survival, though optimal surveillance strategies have yet to be proven; currently, no surveillance guidelines exist in this setting[195,196].

In fact, it is not clear whether screening for recurrence is worthwhile at all, due to poor results of systemic treatment for recurrence after LT[197]. Thus, surveillance should be customized according to a known recurrence pattern (*i.e.* frequent time and space frame) in order to be more cost-effective[198], considering that recurrence beyond 5 year is less common and associated with better prognosis[199]. Recurrence of HCC can be intrahepatic and/or extrahepatic, with the lung and bones as the most common extrahepatic sites of recurrence[199,200].

The study from Ladabaum *et al*[201] suggests that the relatively small gains in life-expectancy that may be achieved by screening for recurrence after LT are likely to be associated with relatively high incremental costs per life-year gained, and that the greatest benefit of screening is more likely to be derived by screening patients whose explant pathology exceeded the Milan criteria and by limiting screening to the first two years after LT.

Lee *et al*[195] showed how increased surveillance, measured by cumulative exposure to surveillance (CETS-*i.e.* the cumulative sum of all the protected intervals that each surveillance test provides) is associated with improved post-recurrence survival and a higher probability of aggressive treatment: In particular 252 d of CETS in the first 24 mo after LT would yield the best sensitivity and specificity for identifying disease which can be treated with either resection or ablation.

The above mentioned RETREAT score underwent a subsequent validation by Mehta *et al*[202] who found that a higher score is associated with a shorter time to HCC recurrence; the authors proposed a cost-saving surveillance strategy in which no surveillance is needed for patients with a score of 0, surveillance every 6 mo for 2 years is warranted for those with a score of 1-3 and for 5 years for those

with a score of 4, while for those with a score > 5 surveillance is warranted every 3– 4 mo for 2 years then every 6 mo for 2-5 years.

Further studies addressing the optimal strategy, the survival benefit, and the cost-effectiveness of surveillance for HCC recurrence should be undertaken, given that not being amenable to a curative-intent treatment has been found to be a poor prognosis factor in recurrent HCC post-LT[203].

Regarding treatment options, European guidelines suggest that treatment of HCC recurrence should probably follow the same algorithms used for immunocompetent patients, and also considering re-transplantation in selected cases[1], while American guidelines state that resection or ablation is usually the treatment of choice for a solitary extrahepatic metastasis or intrahepatic recurrence of HCC and that ablation with radiofrequency (RFA) is the best treatment for small solitary recurrences[4]. It seems that patients undergoing surgical treatment have a better post-recurrence survival, for both in intra-[204] and extrahepatic[205] recurrence, compared to those not undergoing resection, with a reported OS of 20-27 mo after HCC recurrence, in those who underwent surgery, significantly superior to patients who received only nonsurgical therapy (9-10 mo) or best supportive care (2-4 mo)[204,206]. There is still a dearth of information regarding locoregional treatments for the management of HCC recurrence following LT, so further data is needed. Among the few studies evaluating locoregional treatment we can find a study by Huang *et al*[207], which was conducted on 78 patients who had recurrence of HCC post-LT and found no significant difference in terms of OS or recurrence-free survival between the group undergoing surgical resection and the group undergoing RFA.

In a retrospective study conducted on 28 patients with HCC recurrence, Zhou *et al*[208] compared the outcomes of 14 patients receiving chemoembolization to 14 matched control subjects not receiving chemoembolization: Patients who underwent chemoembolization had significantly longer OS after LT and after the diagnosis of HCC recurrence (median OS after LT 865 d, median OS after HCC recurrence 286 d) compared to those who did not (median OS after LT 228 days, median OS after HCC recurrence 85 d), respectively, with no severe complications, and 57% in the treatment group showing partial response. The development of new recurrence, both intra- and extrahepatic, was still high in both groups (86% in those receiving chemoembolization *vs* 93% in those who did not), implying that the improved survival in patients receiving chemoembolization is likely attributed to the control of established tumors instead of the prevention of new lesions.

Regarding systemic therapies, Mancuso *et al*[209] conducted a meta-analysis of studies on survival and safety of sorafenib for HCC recurrence after LT with the aim of estimating the 1-year rates of survival: Overall the median survival was 10.5 mo (range 5 to 21.3). The pooled estimate of the 1-year survival rate was 63% (range, 18%–90%) with a significant heterogeneity among studies ($P < 0.0001$). Studies on sorafenib have shown that systemic therapy improves survival when compared to optimal supportive care alone. Patients experienced considerable medication toxicity, along with poorly tolerated side effects[210,211]. Close monitoring is necessary and should be even closer if IS regimen includes mTOR inhibitors as well, as the association between Sorafenib and mTORi showed an increased frequency of dose reduction and discontinuation due to adverse events[212,213]. Regorafenib could be an option in patients progressing while on treatment with sorafenib[214].

Recently Iavarone *et al*[215] conducted an observational multicenter retrospective study on 81 LT patients with HCC recurrence who discontinued first-line sorafenib (36 treated subsequently with regorafenib and 45 undergoing BSC at sorafenib discontinuation): The median OS was significantly longer in the group treated with regorafenib than in the group undergoing BSC (13.1 mo *vs* 5.5 mo, $P < 0.01$); treatment with regorafenib was an independent predictor of reduced mortality (hazard ratio, 0.37, $P = 0.02$).

Scarce data is currently available for other tyrosine kinase inhibitors (lenvatinib and cabozantinib), which would allow to propose new treatment sequences for patients with HCC recurrence after LT[198].

Another promising option for post-transplant HCC recurrence includes immunotherapy: The combination of atezolizumab (an anti-PD-L1) and bevacizumab (anti-vascular endothelial growth factor monoclonal antibody) proved to be superior to sorafenib in non-LT setting[216].

It must be noted that there is evidence that immunotherapy can interfere with post-transplant immunological tolerance and lead to allograft rejection that is resistant to treatment[217].

Luo *et al*[218] recently conducted a pooled analysis of the published cases of post-LT immunotherapy-treated HCC, including 29 patients: The overall response rate (complete response and partial response) to immunotherapy was 31.3% , including 18.8% with complete response and 12.5% with partial response

In the immune checkpoint inhibitors subgroups, including 19 patients, rejection was experienced by 6 out of 19 patients (32%), including 5 receiving nivolumab and 1 receiving pembrolizumab; allograft rejection exhibited a tendency to occur shortly after immunotherapy initiation, at a median time of 12 d and patients who started immunotherapy shortly after LT seemed to be at a higher risk of rejection than those starting after a longer interval of time.

After a median follow-up of 3 mo 68% of patients died (13/19), but only 23% (3/13) of those deaths were due to early rejection; the authors concluded that allograft rejection can be lethal, but the possibility of rejection-related death justifies considering immunotherapy as a backup plan because disease progression invariably results in death.

Post-transplant HCC recurrence currently represents a diagnostic and therapeutic challenge, and as such, patients transplanted for HCC need a close surveillance and an individualized management discussed in a multidisciplinary team in case of recurrence.

Autoimmune diseases

Reported recurrence rates vary among the different autoimmune diseases: The prevalence of recurrent primary biliary cholangitis (PBC) ranges between 9% and 35% with mean time to recurrence between 1.6 and 6.5 years[219]. Autoimmune hepatitis (AIH) recurs in 8%-12% of patients within the first year after LT and 36%-68% after 5 years[220].

PSC recurs in about 20% of patients after a median time of 4.6 years[221] with a cumulative incidence up to 45% at 6 years[222].

Diagnosing recurrence of autoimmune disease can be challenging, leading to a substantial variation of the data reported in the literature[219], since there are many conditions in the transplanted liver that can mimic autoimmune diseases (*e.g.* ischemia related biliary insults, hepatic artery thrombosis and/or chronic ductopenic rejection, infectious cholangitis can mimic PSC; acute cellular rejection can mimic PBC and AIH)[223].

Recurrence of PBC has little impact on patient and graft survival, with a reported patient survival at 5 and 10 year of 96% and 83% in those with disease recurrence[224], with a proportion of graft lost to disease recurrence of about 5%[225].

Reported patient survival for AIH is approximately 79% and 70% at 5 and 10 years, while graft survival is 73% and 63% after 5 and 10 years of follow up. Compared to recurrence of PBC and PSC, AIH recurrence leads to an increased risk of death due to infection or graft rejection[226].

Patient survival for PSC recurrence is approximately 86% and 70% at 5 and 10 years whereas reported graft survival at 5 and 10 years is 79% and 60%, respectively[227]. There is some controversy about whether recurrence of the disease affects survival[219,221,227,228]. A recent study conducted analyzing the European Liver Transplant Registry[229] shows that PSC recurrence has a negative impact on both graft and patient survival, leading to higher number of re-transplantations and a 33% decrease in 10-year graft survival.

As showed by a recent meta-analysis, the only identified risk factor for PBC recurrence[230] is the use of tacrolimus while the use of preventive UDCA was a protective factor. UDCA is also an effective treatment for disease recurrence[231,232], while there is a lack of data on the use of Obeticholic Acid and Fibrates for the treatment of recurrent PBC.

Risk factors associated with recurrent AIH are younger age at LT, use of mycophenolate mofetil post-LT, sex mismatch and high IgG pre-LT[233], suboptimal IS, disease type and severity[234], histological findings of severe disease in native liver[235]; of note, long term use of low-dose corticosteroid after LT seems to reduce the incidence of recurrent disease with a good safety profile[233,236].

The choice of the best treatment for recurrent AIH depends on the severity of presentation: For mild recurrence, such as asymptomatic disease with minimal changes in liver biochemistry and histology, an adjustment of the IS regimen may be sufficient, while severe disease recurrence may require re-introducing or increasing the dose of corticosteroids, or adding another immunosuppressive drugs [237]. Although this strategy remains controversial[233]. Re-transplantation may be required for patients with recurrent AIH who present with liver failure and graft loss[237].

Another meta-analysis showed that identified risk factors for PSC recurrence[238] are intact colon before LT and IBD presence, cholangiocarcinoma, advanced donor age, higher MELD score, acute cellular rejection (ACR) and multiple episodes of ACR.

Autoimmune etiology is a risk factor for Late T-Cell mediated rejection, which is associated with reduced graft survival, that is why particular attention is warranted in the IS protocol, even though the optimal IS regimen has not been defined[108].

In this scenario, early diagnosis of recurrence gains particular importance, even though diagnosis could be challenging[223] and liver function tests alteration should be considered highly suspicious for disease recurrence[239], while also considering other risk factors[240].

Montano-Loza *et al*[240] proposed strategies to reduce the risk of autoimmune liver disease recurrence after LT, acting on the main risk factors for recurrence: Treatment of active cirrhosis and normalization of transaminases and IgG for AIH in the pre-transplantation period plus long term corticosteroid use after LT; use of preventive Ursodeoxycholic Acid for PBC; control of IBD and considering pre- or post-transplant colectomy in patients with PSC.

In summary, prevention of recurrence in the future will probably rely on identification of risk profiles starting from the pre-transplantation phase and on tailored IS protocols.

Non-alcoholic fatty liver disease /NASH

The proportion of transplants performed for NASH has increased significantly over time[241], outpacing HCV[142], and metabolic liver disease has become a top indication for LT worldwide[242]. Post-transplant outcomes of NASH patients are generally good, even though there is some controversy about the overall survival rates for patients transplanted for NASH cirrhosis or HCC compared to other etiologies; graft survival rates are comparable[241,243,244].

Pre-LT screening for MetS is mandatory given the high prevalence in this population[245].

The distinction between recurrent and *de novo* non-alcoholic fatty liver disease (NAFLD) after LT is made clinically by accurately identifying the preexisting liver disease. There are few data on NAFLD after LT and a broad range in the recurrence or *de novo* rate depending on which diagnostic criteria is used[246]. There are no histologic characteristics to distinguish recurrent from *de novo* NAFLD after LT and histological findings in recurrent or *de novo* NAFLD in the allograft are considered to be the same as in immune-competent native livers[246].

Recurrence of NAFLD is up to 100% in patients who were transplanted for NASH after 1 to 5 years of follow-up, while reported NASH recurrence rates over a comparable follow-up period are between 4%–57%, with 2%-5% demonstrating compensated cirrhosis. Over a similar time period, the incidence of *de novo* NAFLD ranged from 18%- 78%, whereas *de novo* NASH ranged from 13% to 17%, showing lower rates of *de novo* disease compared to recurrence[247-249]. A meta-analysis on the incidence and risk of NAFLD/NASH post-LT reported the rate of cirrhosis in recurrent NAFLD recipients to range from 1 to 11%, with one study reporting the rate as high as 29%, whereas the rate of cirrhosis in *de novo* NAFLD LT recipients was 14% at five years after LT[250]. Despite the lack of data, NAFLD/NASH cirrhosis post-LT is probably a rare cause of death or graft loss in the first years post-LT given the good 5-year graft survival rate[246], but further data is needed on the long term. A recent study has pointed at recent pre-LT cardiovascular history and a combined donor-recipient age of 135 as major prognostic factors [251].

Management recommendations for LT recipients are the same as those for other NAFLD/NASH patients[252].

Diet and lifestyle changes have in fact a main role in the treatment of fatty liver disease[253]. NASH can be resolved with a weight loss of at least 7% of total body weight, and fibrosis can be stabilized or can regress with a weight loss of at least 10% of total body weight; a lower target of weight loss of 3%-5% is advised for patients with lean NAFLD[254]. Patients with fatty liver disease should follow the Mediterranean diet, mainly constituted by fresh fruit, vegetables, legumes, whole grains, fish, olive oil, nuts, and seeds while limiting the consumption of red and processed meat as well as commercially produced fructose[254]. This type of diet has been showed to reduce liver steatosis[255] and improve liver stiffness[256]. Patients with NAFLD should also consider engaging in regular physical activity, aiming for 150–300 min of moderate–intensity aerobic exercise per week[254], since it has been found that exercise alone, even without dietary intervention, can significantly decrease liver fat. Both European[257] and American[252] guidelines recommend that pharmacological treatments, aimed primarily at improving liver disease, should be limited to those with biopsy-proven NASH and fibrosis. It is also recommended to consider pharmacological treatment for patients with less severe disease who are at high risk of disease progression (*i.e.* with diabetes, MetS, persistently increased ALT, high necroinflammation). As of today, no NASH drug has been approved by Food and Drugs Administration, European Medicines Agency, or any other leading regulatory agencies[258]. Bariatric surgery should be proposed in case of non-response to lifestyle changes and pharmacotherapy[257].

Regarding modification to the IS regimen, ILTS advises corticosteroids minimization where possible, since they carry a significant risk for all components of MetS, and CNI minimization to mitigate post-transplant weight gain and hypertension[108].

There are no societal or professional guidelines for post-transplant surveillance in NASH LT patients at the moment, nor a frequency of post-LT monitoring for recurrent or *de novo* NASH has been defined, given the low likelihood of clinically significant recurrence of NASH. Conversely, high-risk individuals identified during the pretransplant work-up, such as those with PNPLA3 polymorphism or hypopituitarism, definitely require closer surveillance[246].

Suggested strategies for NAFLD/NASH screening in LT patients include annual ultrasound and liver enzymes monitoring: If fatty liver disease is identified or suspected then the patient should undergo noninvasive tests for fibrosis assessment[259]. A metanalysis by Bhat *et al*[260] showed how transient elastography (TE) performed better that APRI and FIB-4 at diagnosing recurrent fibrosis in LT recipients, but none of the studies included in the analysis was specific for NAFLD/NASH. A recent study from Siddiqui *et al*[261] conducted on 99 patients who underwent LT, showed how TE can detect advanced fibrosis with an AUROC of 0.94 and exclude advanced fibrosis with a negative predictive value of 0.99 when a liver stiffness cutoff value of 10.5 kPa is used; furthermore a controlled attenuation parameter cutoff value of 270 dB/m can identify any hepatic steatosis with an AUROC of 0.88. Along with TE, also Magnetic Resonance Elastography is an accurate method for assessing liver fibrosis in LT recipients[262]. Singh *et al*[263] conducted a pooled analysis including 6 cohorts where the mean AUROC values for diagnosis of advanced fibrosis and cirrhosis were respectively 0.83 (0.61-0.88) and 0.96 (0-93-0.98), with a good diagnostic performance even after stratification based on sex, BMI and degree of inflammation.

Beyond being a way to perform a differential diagnosis with other potential causes for elevated liver enzymes, liver biopsy remains the gold standard for the diagnosis of post-LT NAFLD/NASH; patients who have an established diagnosis of recurrent or *de novo* NAFLD can probably be followed with serial noninvasive testing to diagnose advanced fibrotic disease, but given the lack of data regarding fibrosis monitoring and the presence of factors that can influence these tests, suspected fibrotic disease at noninvasive testing still needs confirmation with biopsy[259].

While *de novo* or recurrent NASH appears to have little effect on prognosis, infections and cardiovascular diseases are among the leading causes of mortality[241,264].

A multidisciplinary approach in the management of NAFLD/NASH transplanted patients, promoting increased physical activity, diet modifications, behavioral therapy, and pharmacological treatment, when necessary, should be explored[265,266].

Unfortunately, in many cases a full multidisciplinary team is not available for the patient due to limited resources; either way the active assistance of the physicians is essential since their advice to lose weight has favorable impacts on the likelihood that patients will adhere to the suggested lifestyle changes[253].

Viral hepatitis

HCV recurrence post-LT was an issue with a major impact on the prognosis in the pre-direct acting antiviral (DAA) era, given its shortened natural history in the LT setting (development of cirrhosis in 10-30% of patients after a median of 5 years)[267-269].

The advent of DAA therapy was a "game changer", with HCV recurrence as cause of death or re-transplantation decreasing from 5.89% in the Interferon era to 0.60% in DAA era over a three-year period[270].

LT may not remove HBV from a persistently infected host because HBV may reside in extra-hepatic sites and serve as a source of reactivation. As a result, in a chronically infected patient, after LT, prophylaxis is used to avoid reactivation rather than re-infection or recurrence of HBV. Because of this, lifetime antiviral prophylaxis is required[271]. Treatment with hepatitis B immunoglobulin (HBIG) and Nucleos(t)ide Analogues is an effective strategy to prevent HBV recurrence in most HBV-infected patients undergoing LT, showing very low recurrence rates[272]; monotherapy with entecavir or tenofovir is probably not sufficient to prevent graft reinfection but is considered sufficient to prevent disease recurrence[1].

Risk factors for HBV recurrence include a high HBV-DNA level at LT, presence of HBeAg positivity, HCC, anti-viral drug resistance, and HBIG monoprophylaxis[1,273,274].

Recurrence is defined by the presence of HBsAg in the serum and detectable quantities of DNA, and it is typically linked to clinical evidence of recurrent disease. The goal of treatment is to keep HBV replication under control throughout time to prevent graft loss, even though there is no standard follow-up protocol for early diagnosis[1,275]. Therapy with ETV and/or TDF seems to be efficient and safe when used for treatment of HBV recurrence after LT[276]. Treated recurrence is associated with good prognosis[277-279] but care should be taken in patients transplanted for HCC since HBV recurrence could be a signal of HCC recurrence[277,278].

CONCLUSION

With the dramatic improvement in short-term survival of LT recipients that occurred in recent years, the focus of the physician is shifting to the improvement of long-term outcomes[280]. The main causes of late mortality in this category are not liver related[2]. In this review, we described the main comorbidities and risk factors affecting LT recipients, which in most cases are preventable, can be treated, or are amenable of screening measures, even though there is a lack of consensus to define the best strategy for the follow-up and management of part of these factors, for which we reported some of the suggested approaches (Table 3).

The hepatologist's role in long-term management of LT recipients is becoming more complex with the increase of comorbidities/risk factors that can affect long-term outcomes. A multidisciplinary approach could help overcome this complexity. The importance of a multidisciplinary team is underlined by current guidelines[1,164] with regard to pre-LT evaluation, and its value is recognized in some particular settings such as the prevention and management of alcohol relapse or to improve adherence to therapy[1,161].

The availability of a "long-term management multidisciplinary team" dedicated to LT recipients, could handle or prevent the onset of the aforementioned comorbidities/risk factors and should be composed by psychiatrists, psychologists, cardiologists, general practitioners, nutritionists, dieticians, social workers, and should be coordinated by the transplant hepatologist. We believe this approach could improve the long-term outcomes of the LT recipients.

A body of the evidence is already available for the identification of high-risk patients[14,151,185] and such prognostic ability could allow healthcare providers to focus on those who could benefit the most from preventive measures in a cost-effective manner.

Furthermore, a special mention should go to maintenance immunosuppression, which has a strong impact on patient long-term survival: Although further studies are needed to propose IS withdrawal [108], which should therefore be limited to clinical trials, early minimization of IS (when feasible) seems a rational strategy to limit adverse events and improve long-term outcomes. A deeper understanding of the immunological pathways of rejection would allow to design more specific and safer drugs, in order to tailor therapy[281].

Table 3 Comorbidity/risk factors impairing long-term survival with suggested follow-up and management

Risk Factor/Comorbidity	Follow-up	Management
Metabolic syndrome	(1) Electrocardiogram and transthoracic echocardiography before transplantation. If patient older than 50 and has multiple cardiovascular risk factors, perform a cardiopulmonary exercise test. If coronary disease is suspected, coronary angiography should be executed[1]; and (2) Repeatedly perform a cardiovascular risk stratification after transplantation (for example, every 6 mo)[1].	(1) Aggressive and rapid management of metabolic risk factors in the form of lifestyle changes, pharmacological therapies and modifications of the IS[1]; (2) Dietary counseling for all LT patients[4]; and (3) Consider bariatric surgery in patients who failed behavioral weight-loss programs[4].
Sarcopenia	(1) Sarcopenia evaluation before LT (for example, measuring skeletal mass from CT scan, even required for other reasons)[23]; and (2) New assessment at 1 year after transplantation (possibly using the same method and the same CT machine, in order to compare results. Consider also 24-CER evaluation[20]).	(1) Manage sarcopenia with lifestyle modification and nutritional interventions both pre and post transplantation[1, 28]; (2) Consider nutritional counseling after transplantation; and (3) Consider nutritional supplementations (*e.g.* with HMB)[29].
Osteoporosis	(1) Regular measurement of bone mineral density pre- and post-LT, with a follow up timing dependent on the severity of the disease; and (2) If osteopenic bone disease is confirmed or if atraumatic fractures are present, assess calcium intake, vitamin D levels, gonadal and thyroid function, a full medication history, and thoracolumbar radiography[4].	(1) Manage osteoporosis with calcium and vitamin D replacement (if deficient), consider a weight-bearing exercise pre-operative program[1]; and (2) Consider bisphosphonate therapy (for example, Neridronate, at the dose of 25 mg i.m./mo for 12 mo)[40].
Psychological health and QoL	Actively look for depressive symptoms since the early post-transplantation period[1].	(1) Treat promptly depressive symptoms with adequate pharmacotherapy[54]; (2) Consider psychological support by a specialist if needed; and (3) Propose lifestyle modifications (in particular, personalized aerobic and strength training programs)[55].
Renal dysfunction	(1) Continuous monitoring of renal function with serum creatinine and glomerular filtration rate measurements[1]; and (2) Urinary protein quantification at least once yearly[4].	(1) Treat potential risk factors (diabetes, hypertension); (2) Avoid nephrotoxic drugs[1]; (3) Adjustment of the IS; reduction or withdrawal of CNI or use alternative CNI-free protocols as soon as possible in impaired renal function (for example, EVR combination regimens starting 1 mo after transplantation[67,68]; and (4) Consider kidney transplantation in end-stage renal disease[4].
Infectious risk	Hepatologist accurate counseling to increase the percentage of vaccinated patients[81].	(1) Perform HAV, HBV, varicella, Pneumococcus, influenza and tetanus vaccinations prior to transplantation; (2) Administer inactivated influenza vaccine starting one month after transplantation during community influenza outbreak; (3) Annual influenza vaccination in liver transplanted patients[4]; and (4) Consider Oseltamivir prophylaxis during periods of local influenza circulation[86].
De novo malignancies	Define and follow a surveillance protocol with individualized emphasis laid on patients' particular risk profiles[1].	(1) Treating modifiable risk factors: stop smoking, alcohol withdrawal[99], metabolic syndrome management[100], avoid sunbed use and sun exposure[101], promote HPV vaccination[102]; and (2) Use mTOR- based therapy if possible[1] or a CNI-mTOR combined therapy always at the lowest effective dose.
Smoking	(1) Assess smoking status at each visit, focusing on those with particular risk of relapse (ALD); and (2) Use of biomarkers (serum Cotinine)[116].	(1) Encourage to undertake smoking cessation[4]; and (2) Referral for behavioral/pharmacological therapy[125,126].
Adherence to therapy	Self- and collateral-reported non-adherence, trough levels (considering graft-function and concomitant use of other drugs)[136,139] with particular attention to younger patients and those missing clinic appointments, with a history of substance or alcohol use, mental health needs, divorced or high regimen complexity[131,137,140].	Multidisciplinary measures developed by professional educators, supported by psychologists, and coordinated by physicians[1,136].
Alcohol abuse relapse	(1) Assessing alcohol use at each clinic visit, with particular attention to those transplanted for ALD with pre-transplant abstinence of less than 6 mo, with psychiatric comorbidities, smoking, noncompliant with clinic appointment or medication and lacking social support[147,149,150]; and (2) Assessment by self-reported alcohol use, liver function tests and metabolites of alcohol, such as urinary ethyl glucuronide[157,158].	(1) Preventive structured management by a multidisciplinary team including transplant hepatologist, clinical psychologists and psychiatrists with expertise in alcoholism and social workers[159,160]; (2) Encouraging smoking cessation; and (3) Referral to psychiatric treatment or counselling in case of relapse[1,4].
HCC recurrence	(1) Thoracic CT – abdominal CT or MRI and AFP levels with 3- to 6-mo intervals in the first 2 or 3 years, increasing the interval between exams from that date[194]; (2) Selection of patients who needs a stricter follow-up using prognostic criteria such as: AFP levels, the presence of microvascular invasion, the diameter of the largest viable tumor and the number of viable tumors[202]; and (3) Particular attention to patients transplanted outside of Milan Criteria[176].	(1) Minimizing overall IS; consider adding mTORi[108]; (2) Individualized management of HCC discussed in a multidisciplinary team[303]; and (3) Surgical treatment when feasible[204,205].

Autoimmune disease recurrence	(1) Monitoring liver function tests[223,229] and performing liver biopsy and/or cholangiography when deemed necessary[1]; and (2) Exclude mimicking conditions (ischemia related biliary insults, hepatic artery thrombosis and/or chronic ductopenic rejection, infectious cholangitis for PSC, rejection histological mimicking for PBC and AIH)[223].	(1) AIH: Treatment of active cirrhosis and normalization of transaminases and IgG in the pre-transplantation period plus long-term corticosteroid use after LT; (2) PBC: Use of preventive Ursodeoxycholic Acid; and (3) PSC: Control of IBD and considering pre- or post-transplant colectomy in patients with difficult to control PSC[240].
NASH recurrence	(1) Early identification of Metabolic Syndrome components pre- and post-transplant[304]; (2) Annual screening with US and liver function tests; (3) Noninvasive testing and liver stiffness measurement in case of alterations at the annual screening; and (4) Biopsy in case of suspected fibrotic disease[259].	(1) Management as for other NAFLD/NASH patients (multidisciplinary approach with diet and lifestyle modification, pharmacological treatment and bariatric surgery when necessary[252,257]; (2) Treating metabolic syndrome components)[265,266]; and (3) Corticosteroids and CNI minimization when possible[108].
Viral hepatitis recurrence	(1) Liver function test monitoring; (2) HCV-RNA titres[111]; (3) HBV DNA and HBsAg monitoring[1,275]; (4) Regular assessment of graft damage[1]; and (5) Particular attention to patients transplanted for HCC with HBV recurrence/reactivation[277,278].	(1) Treating HCV before LT when possible; use of DAA[1, 164]; and (2) HBIG and NUCs to prevent HBV recurrence/reactivation[1,272].

LT: Liver transplantation; CT: Computed tomography; CNI: Calcineurin inhibitors; EVR: Everolimus; HAV: Hepatitis A virus; HBV: Hepatitis B virus; HPV: Human papilloma virus; mTORi: Mammalian target of Rapamicin inhibitors; ALD: Alcohol-related liver disease; HCC: Hepatocellular carcinoma; AFP: Alpha fetoprotein; PSC: Primary sclerosing cholangitis; PBC: Primary biliary cholangitis; AIH: Autoimmune hepatitis; NASH: Non-alcoholic steatohepatitis; HCV: Hepatitis C virus; HBIG: Hepatitis B immunoglobulin; NUCs: Nucleos(t)ide analogues.

The question "How can we improve long-term outcomes after liver transplantation?" has no clear and simple answer. The combination of reduction of drugs toxicity, the use of precise instruments that allow to detect high-risk patients and the presence of a multidisciplinary team coordinated by an hepatologist could probably be the key for the improvement of long-term outcomes after LT.

ACKNOWLEDGEMENTS

The figures were drawn by using pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License.

FOOTNOTES

Author contributions: Fuochi E and Anastasio L performed the bibliographic search and drafted the initial manuscript; Innocenti T and Lynch EN re-screened the search results; Lynch EN provided English language revision as a native speaker; Lynch EN, Campani C, Dragoni G, Milani S, Galli A, and Innocenti T revised the article critically for important intellectual content; and all Authors approved the final version of the manuscript.

Conflict-of-interest statement: Authors declare no conflict of interests for this article.

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S-Editor: Wang JL
L-Editor: A
P-Editor: Wang JL

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World Journal of Hepatology

Submit a Manuscript: https://www.f6publishing.com

World J Hepatol 2023 March 27; 15(3): 353-363

DOI: 10.4254/wjh.v15.i3.353

ISSN 1948-5182 (online)

MINIREVIEWS

COVID-19 and liver dysfunction in children: Current views and new hypotheses

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Specialty type: Gastroenterology and hepatology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Ulasoglu C, Turkey; Wishahi M

Received: October 28, 2022
Peer-review started: October 28, 2022
First decision: January 3, 2023
Revised: January 14, 2023
Accepted: March 17, 2023
Article in press: March 17, 2023
Published online: March 27, 2023

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Abstract

Coronavirus disease 2019 (COVID-19) poses an extremely serious global impact on public healthcare for individuals of all ages, including children. Increasing evidence has shown that liver abnormalities are commonly found in children with COVID-19, and age-related features in innate and adaptive response have been demonstrated. However, there are few reports and studies on COVID-19 related liver injury in children, and the data are scattered. So that many contradictions have arose. This situation is not only due to the serious ethical issues in studying pediatric patients with COVID-19, but also because of the short duration and wide coverage of the COVID-19 epidemic, the severity and complexity of clinical cases varied, as did the inclusion criteria for case reporting and patient outcomes. Therefore, we totaled the incidences, characteristics and pathomechanism of liver injury in children since the COVID-19 outbreak. The etiology of COVID-19-related liver injury is divided into three categories: (1) The direct mechanism involves severe acute respiratory syndrome coronavirus 2 binding to angiotensin-converting enzyme 2 in the liver or bile duct to exert direct toxicity; (2) the indirect mechanisms include an inflammatory immune response and hypoxia; and (3) COVID-19-related treatments, such as mechanical ventilation and antiviral drugs, may cause liver injury. In summary, this minireview provides fundamental insights into COVID-19 and liver dysfunction in children.

Key Words: COVID-19; SARS-CoV-2; Children; Liver injury; Inflammatory immune response; Cytokine storm

Core Tip: There are few cases of liver injury in children with coronavirus disease 2019 (COVID-19) and clinical reports are scarce. We collected reports on COVID-19-related liver injury (CRLI) in children over the last two years and divided the etiology of CRLI into three categories: (1) The direct mechanism involves severe acute respiratory syndrome coronavirus 2 binding to angiotensin-converting enzyme 2 in the liver or bile duct to exert direct toxicity; (2) the indirect mechanisms include an inflammatory immune response and hypoxia; and (3) COVID-19-related treatments, such as mechanical ventilation and antiviral drugs, may cause liver injury. We also discuss the current controversies regarding the pathophysiology of CRLI.

Citation: Yun YF, Feng ZY, Zhang JJ. COVID-19 and liver dysfunction in children: Current views and new hypotheses. *World J Hepatol* 2023; 15(3): 353-363
URL: https://www.wjgnet.com/1948-5182/full/v15/i3/353.htm
DOI: https://dx.doi.org/10.4254/wjh.v15.i3.353

INTRODUCTION

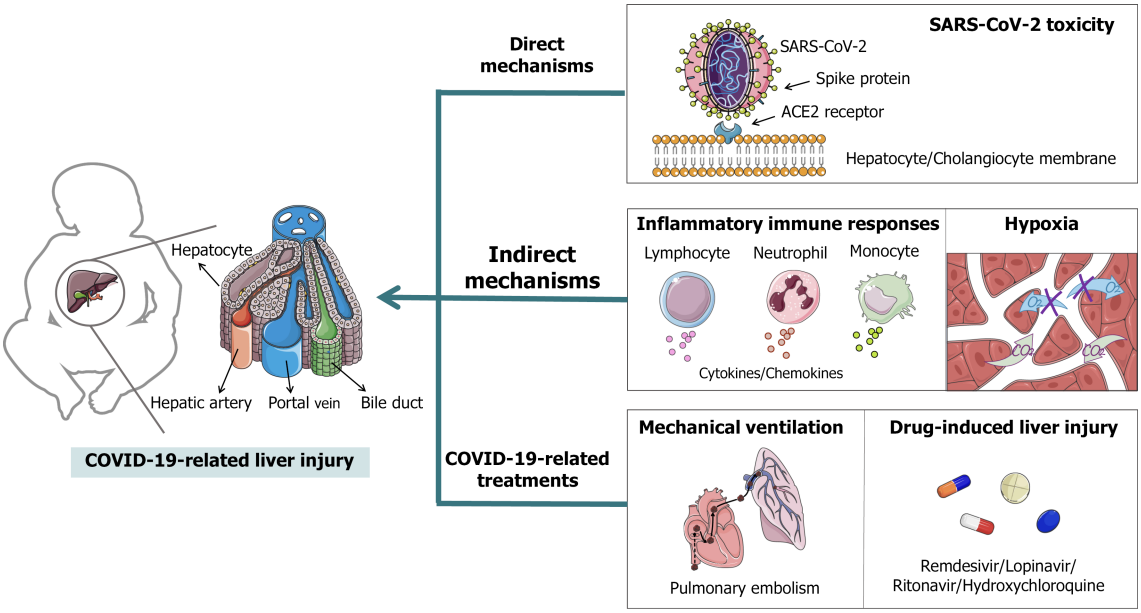
Coronavirus disease 2019 (COVID-19) is an infectious respiratory disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). In response to this global health crisis, governments and medical institutions have been actively working to improve epidemic prevention measures, and diagnostic and treatment methods, all of which have significantly reduced the transmission rate and mortality rate[1-3]. However, this crisis is not yet over, and the physical damage caused by COVID-19 is gradually expanding from respiratory to systemic diseases. In addition to inducing acute respiratory distress syndrome (ARDS)[4,5], COVID-19 also causes damage to organs such as the liver, gastrointestinal tract, kidney, heart and nervous system[6-9]. The liver is an important center for regulating physiological processes such as nutrient and exogenous drug metabolism, immunity, endocrinology and blood volume[10]. Liver injury due to any cause (*e.g.*, viral infection, nutritional overload or tumor burden) is a global health problem. COVID-19-related liver injury (CRLI) is defined as any liver injury that occurs during the disease course and treatment in COVID-19 patients, regardless of the presence of prior liver disease[11]. A study showed that approximately 2–11% of patients had underlying chronic liver disease and 14%-53% developed liver injury during the course of COVID-19[12].

COVID-19 mainly occurs in the elderly and people with potential complications[13]. The lethality of infection increases logarithmically and linearly with age in those over 30 years, but children have a lower prevalence and tend to be asymptomatic or have mild to moderate disease[14]. Therefore, currently published case reports mainly describe adult patients, resulting in a lack of details in pediatric cases. However, most infections in children originate from family contacts, they play an important role in disease transmission and have become a key target population for epidemic prevention and control measures[15]. Meta-analyses have shown that liver injury is common in children, but is often overlooked[16]. Therefore, we focused on pediatric patients with CRLI and divided the pathogenesis of CRLI in children into three categories: direct, indirect and treatment-related pathogenesis (Figure 1).

FEATURES OF CRLI IN CHILDREN

COVID-19 can cause varying severity of liver injury, as evidenced by abnormal elevations in alanine aminotransferase (ALT) and aspartate aminotransferase (AST), accompanied by mild elevations in alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), total bilirubin (TBIL) and a reduction in albumin[17]. The abnormal liver enzyme levels in serum include: ALT > 40 U/L, AST > 40 U/L, GGT > 49 U/L, ALP > 135 U/L, TBIL > 17.1 μmol/L and albumin < 3 g/dL[18]. Recently, several studies have provided the results of abnormal liver tests in pediatric COVID-19. Alkan *et al*[19] found that 130 (44.2%) of 294 patients (age range: 14 d-18 years) with COVID-19 had abnormal liver function and most patients (33.3%) were characterized by elevated ALT, and other patients had elevated ALT (5.1%), ALP (6.6%), GGT (8.9%) and TBIL (3.8%). In addition, decreased albumin was also observed by Esmaeili *et al*[20] and Liu *et al*[21]. In their studies, the proportion of decreased albumin in pediatric patients was 16.7%[20] and 18.2%[21], respectively.

In general, the main manifestations of CRLI in children were mildly elevated ALT/AST and most research has confirmed this, for instance, Parri *et al*[22] reported on 130 children (age range: 0-17 years) with COVID-19 in Italy, and 8/68 (11.8%) children had elevated ALT and 11/60 (18.3%) had elevated AST. The analysis by Du *et al*[23] showed that ALT and AST increased in 9 (5.0%) and 24 (13.3%) of 180 subjects (age range: 0-15 years), respectively, and 11 (6.3%) of 174 subjects showed increased ALP levels. Thus, the elevation of liver enzymes in pediatric patients is not significant, which may be due to the fact that COVID-19 is mainly mild in children.



DOI: 10.4254/wjh.v15.i3.353

Figure 1 The possible pathophysiological mechanisms of Coronavirus disease 2019-related liver injury in children. SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; ACE2: Angiotensin-converting enzyme 2; COVID-19: Coronavirus disease 2019.

In addition, Sun *et al*[24] conducted a single center observational study of 8 children (age range: 2 mo-15 years) with severe COVID-19 and the results showed that ALT was increased in 4 (50.0%) cases but increased AST was not observed. It is possible that sometimes abnormally elevated ALT/AST is not a sufficient indicator of liver injury. The related studies on the features of CRLI in children are summarized in Table 1.

DIRECT PATHOPHYSIOLOGICAL MECHANISMS

Toxicity of SARS-CoV-2 to hepatocytes and cholangiocytes

Genome sequencing, and phylogenetic and structural analyses have confirmed that SARS-CoV-2 can bind to angiotensin-converting enzyme 2 (ACE2) of host cells depending on its spike protein, and this binding can mediate membrane fusion and viral invasion[25]. ACE2 is not only highly expressed in alveolar cells, but also distributed in various organs throughout the body, including the liver[26]. Thus, the direct pathological basis of CRLI is the viral virulence of SARS-CoV-2, and it can bind to ACE2 on liver endothelial cells and exert toxicity causing hepatocyte damage[27,28]. Unlike adults, children have milder symptoms of CRLI, possibly due to lower ACE2 expression, lower maturity, and weaker function (*e.g.*, binding to SARS-CoV-2). However, in contrast, ACE2 expression decreases with age; thus, ACE2 Levels are higher in children than in adults[29]. Moreover, one of the functions of ACE2 is to convert angiotensin (Ang) II to Ang(1-7), which has anti-inflammatory and anti-liver fibrosis effects[30,31]. Therefore, besides the ability to mediate viral infections, the distribution of ACE2 in different age groups and the "dual action" of it on organ damage require further investigation.

Interestingly, Chai *et al*[32] indicated that ACE2 is highly expressed on cholangiocytes compared to hepatocytes and that SARS-CoV-2 may prefer to bind directly to ACE2 on cholangiocytes. Cholangiocytes are epithelial cells that line the intrahepatic and extrahepatic bile ducts and play an important role in liver regeneration and immune response[33]. This suggests that the liver abnormalities in COVID-19 patients may not be directly caused by hepatocyte injury and that the potential damage to cholangiocytes by SARS-CoV-2 may have a profound effect on the liver.

In addition, CRLI can be classified into three categories according to the degree of liver enzymes exceeding the upper limit of normal (ULN) (Table 2). Patients were classified as hepatocyte injury type when they had raised ALT and/or AST more than 3×ULN; patients were classified as cholangiocyte injury type when they had raised ALP and/or GGT more than 2×ULN; when the first two requirements were met simultaneously, patients were considered to have mixed injury type[18,34]. There are obvious differences in CRLI types between adults and children. Cai *et al*[18] found that the number of liver injuries in 318 adult COVID-19 patients with abnormal liver test results was as follows: mixed type (43.4%) > cholestatic type (29.2%) > hepatocellular type (20.8%). Furthermore, elevation of ALP, a marker of bile duct injury, is less common than abnormal liver enzymes in adults[35]. With regard to children, Alkan *et al*[19] found that the number of liver injuries in 130 pediatric patients was as follows:

Table 1 Laboratory features of coronavirus disease 2019-related liver injury in children

Ref.	Number of patients	Age range	Abnormal liver function	E-ALT	E-AST	E-ALP	E-GGT	E-TBIL	D-albumi, *n* (%)
Alkan *et al*[19]	294	14 d-18 years	130 (44.2%)	15 (5.1%)	98 (33.3%)	19 (6.6%)	26 (8.9%)	11 (3.8%)	NA
Esmaeili *et al* [20]	18	3-10 years	6 (33.3%)	5 (27.8%)	7 (38.9%)	0	NA	3 (16.7%)	3 (16.7%)
Liu *et al*[21]	46	0-1 year	20 (43.5%)	11 (25.0%)	20 (45.5%)	NA	NA	6 (13.6%)	8 (18.2%)
Parri *et al*[22]	130	0-17 years	NA	8/68 (11.8%)	11/60 (18.3%)	NA	NA	NA	NA
Du *et al*[23]	182	0-15 years	NA	9/180 (5.0%)	24/180 (13.3%)	11/174 (6.3%)	NA	NA	NA
Sun *et al*[24]	8	2 mo-15 years	4 (50.0%)	4 (50.0%)	0	NA	NA	0	NA

E-ALT: Elevated alanine aminotransferase; E-AST: Elevated aspartate aminotransferase; E-ALP: Elevated alkaline phosphatase; E-GGT: Elevated gamma-glutamyl transferase; E-TBIL: Elevated total bilirubin; D-albumin: Decreased albumin; NA: Not available.

Table 2 The frequencies of different coronavirus disease 2019-related liver injury types in adults and children

CRLI types	Liver test parameters[18]	Adults[18]	Children[19]
Hepatocyte type	ALT/AST ≥ 3×ULN	66 (20.8%)	24 (18.5%)
Cholangiocyte type	ALP/GGT ≥ 2×ULN	93 (29.2%)	93 (71.5%)
Mixed type	ALT/AST ≥ 3×ULN and ALP/GGT ≥ 2×ULN	138 (43.4%)	13 (10.0%)

CRLI: Coronavirus disease 2019-related liver injury; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyl transferase; ULN: Upper limit of normal.

cholestatic type (71.5%) > hepatocellular type (18.5%) > mixed type (10.0%). The rate of ALP elevation in pediatric patients was slightly more than ALT (Table 1). Cholestatic liver injury is especially common in children under 5 years of age, and this may be related to ACE2 being less distributed in hepatocytes and more distributed in cholangiocytes in younger children[19]. Therefore, we should be more concerned about cholestatic liver injury in pediatric patients with COVID-19.

INDIRECT PATHOPHYSIOLOGICAL MECHANISMS

Inflammatory immune response-mediated liver injury

Inflammatory response and immune response are inseparable systemic responses at the organ, tissue, cellular and molecular levels. A moderate inflammatory immune response (IIR) plays a crucial part in protecting the body from pathological damage in the internal and external environment. However, an excessive IIR is the pathological basis for the development of multiple systemic diseases. An increasing number of studies have shown that the systemic IIR induced by SARS-CoV-2 has an intricate pathophysiological link to liver injury.

Dysfunction of innate and adaptive immune responses: Natural killer (NK) cells and natural antibodies are key components of the innate immune system and can be the first to respond to new viruses[36,37]. The adaptive immune response then comes into play and produces highly specific memory T and B cells to clear the virus and prevent reinfection[38]. However, dysfunctional innate and adaptive immune responses mediate the host damage caused by SARS-CoV-2[39].

The innate immune system detects SARS-CoV-2 mainly by two strategies: first, the presence of SARS-CoV-2 can be detected using various pattern recognition receptors, such as plasmacytoid dendritic cells detecting incoming viral genomic RNA in the intranuclear body *via* toll-like receptor (TLR); second, during viral replication, double-stranded RNA intermediates can be recognized by the RIG-I like receptor (RLR), a cytoplasmic RNA sensor. Following the engagement of the TLR and RLR, downstream signaling activates the transcription of interferons (IFNs) and pro-inflammatory cytokines and chemokines[40]. SARS-CoV-2 can block innate immune recognition and signal transduction using the

expression of viral proteins[41]. Uncontrolled innate immune signaling may produce excess cytokines, which can trigger inflammation and worsen the condition.

With regard to the adaptive immune system, its three basic components are B cells, CD4+ T cells and CD8+ T cells. B cells can rapidly produce neutralizing antibodies after infection with SARS-CoV-2, and the antibody target is the spike protein of the virus[42]. T-cell responses were detected in almost all SARS-CoV-2 infections, and virus-specific CD4+ T cells can differentiate into Th1 cells and T follicular helper cells, which have antiviral activity through the production of IFN-γ and related cytokines[43]. The CD8+ T cells are essential for clearing virus by killing infected cells. In milder symptoms, SARS-CoV-2 specific CD4+ and CD8+ T cells can respond rapidly in the acute phase of COVID-19 and thus exert antiviral effects[44].

Recently, studies have revealed changes in the immune responses of COVID-19 pediatric patients (Table 3). Diao *et al*[45] retrospectively reviewed 522 patients (age range: 5 d-97 years) and demonstrated that more than 70% of the patients had a significant reduction in total T cells, CD4+ and CD8+ T cells, but the reduction was age-dependent as the younger patients had the least reduction in T cells. In addition, unlike in adults, CD4+ T cells were even increased in moderate pediatric cases[46]. Also, compared with mild cases, moderate cases had higher levels of IL-10, complement (C) 4 and NK cells, while neutrophils were significantly lower[46]. These findings suggested that the innate cells such as NK cells and neutrophils play a crucial role in the initial phase and the CD4+ T cells perform a function in the later phase of COVID-19. Studies have shown that CD4+ T cells and IL-10 Levels are positively correlated with CRLI biomarkers in pediatric patients[46], and although the IL-10 derived from CD4+ T cells plays an important anti-inflammatory role in mild patients, excess IL-10 may cause liver injury with COVID-19 progression.

Furthermore, Li *et al*[47] showed that in children with severe COVID-19, CD3+, CD3+CD4+ (helper T cells), and CD3+CD8+ (memory T cells) counts were decreased, and the pro-inflammatory cytokine IL-6 and immune regulatory cytokine IL-10 were increased. Other inflammatory cytokines such as IL-2, IL-4, IL-10, TNF-α and IFN-γ were also detected[47]. However, the inflammatory cytokines IL-2, IL-4, IL-6, TNF-α and IFN-γ were rarely increased in mild and moderate pediatric patients[46]. These results suggested that although the adaptive immunity of children is relatively weak, their innate immunity is less likely to be disordered after SARS-CoV-2 infection and the cytokine storm associated with inflammation is not severe in most pediatric patients. Therefore, the direct liver injury caused by cytokine storms in children may not be as severe as in adults. However, recent research found that CRLI is most common in children under the age of five years[19]. This illustrated that SARS-CoV-2 infection can still trigger a series of damages once it exceeds the threshold due to the weak adaptive immunity of younger children.

Chemokines are small molecule proteins that have the ability to induce targeted chemotaxis of immune cells during inflammation and they also play an important role in dealing with viral infections [48]. Several studies have shown that the main pro-inflammatory chemokines such as CXCL [chemokine (C-X-C motif) ligand] 8, CXCL9, CXCL10, CCL (CC chemokine ligand) 2, CCL3 and CCL5 were increased in patients with aggravation of SARS-CoV-2 infection[48]. However, the levels of CXCL8, CXCL10, and CCL2 were unchanged in pediatric COVID-19 according to Warner *et al*[49]. These findings also suggested that the IIR in pediatric patients with COVID-19 is less severe than in adults.

B cells play a key role in immune regulation and antibody secretion. Previous studies have indicated that total B cells in COVID-19 patients were induced[23,48]. Du *et al*[23] conducted an analysis of 182 pediatric COVID-19 patients with different severity and showed that the levels of immunoglobulin (Ig) E, IgG and IgA were generally in the normal range or were elevated in isolated cases among mild or moderate patients. However, IgG and IgM counts were induced in severe COVID-19 patients[23]. Wu *et al*[46] noted that Igs including IgG, IgA and IgM were negatively correlated with biomarkers in the liver of pediatric patients. This may be the reason why CRLI is more common in severe patients and AST is only slightly elevated in mild patients.

Multisystem inflammatory syndrome in children-mediated liver injury: Recently, clinical reports have shown that children infected with SARS-CoV-2 for several weeks may develop a characteristic complication: multisystem inflammatory syndrome in children (MIS-C). A national study initiated in 2020 at Boston Children's Hospital, Boston, MA, United States, with real-time monitoring of approximately 35 United States children's hospitals, reported that of 186 MIS-C cases, 131 were positive for SARS-CoV-2[50]. MIS-C presents with persistent acute fever, abdominal pain, diarrhea, rash, lymphadenopathy, and in severe cases appendicitis and peritonitis, which may progress to multiorgan dysfunction[51,52]. Different types of liver injury mediated by MIS-C are being reported successively. Giannattasio *et al*[53] reviewed 55 pediatric patients (mean age 6.5 ± 3.7 years) with MIS-C and showed that 16 patients had acute liver injury (ALI) at admission and 10 more patients developed ALI during observation, ALI was defined by the presence of ALT elevation > 40 U/L. Furthermore, a 14-year-old boy developed MIS-C after SARS-COV-2 infection which was followed by hepatic steatosis, and the researchers also found elevated levels of ALT, AST and indices of cholestasis[54]. Another 10-month-old boy developed fulminant acute liver failure due to MIS-C[55]. The pathophysiology of MIS-C-mediated liver injury may also be related to the IIR as described previously.

Table 3 Immune features in pediatric patients with varying degrees of coronavirus disease 2019

Biomarker	Severe	Moderate	Mild/asymptomatic
T lymphocyte[45-47]	CD3+↓; CD4+ ↓; CD8+ ↓; CD3+CD4+ ↓; CD3+CD8+ ↓	CD3+ →; CD4+ →/↑; CD8+ →	CD3+ →; CD4+ →; CD8+ →
B lymphocyte[23,46]	↓	→/↑	→/↑
Innate cell[23,46]	Monocytes ↑; Neutrophils ↑; NK cells ↓	Monocytes ↑; NK cells ↑; Neutrophils ↓	Monocytes →; Neutrophils →; NK cells →
Immunological parameters [23,46]	IgE ↑; IgG ↓; IgA ↑; IgM ↓; C3 ↑/↓; C4 ↑/↓	IgE →/↑; IgG →/↑; IgA →/↑; IgM →; C3 ↑/↓; C4 ↑	IgE →/↑; IgG →/↑; IgA →/↑; IgM →; C3 ↑/↓; C4 →
Inflammatory cytokine[23, 46,47]	IL-2 ↑; IL-4 ↑; IL-6 ↑; IL-10 ↑; IFN-γ ↑; TNF-α ↑	IL-2 →; IL-4 →; IL-6 →; IL-10 ↑; IFN-γ →; TNF-α →	IL-2 →; IL-4 →; IL-6 →; IL-10 →; IFN-γ →; TNF-α →
Chemokines[49]	CXCL10 →; CXCL8 →; CCL2 →	CXCL10 →; CXCL8 →; CCL2 →	CXCL10 →; CXCL8 →; CCL2 →

↑: Increased; ↓: Decreased; →: Unchanged.
NK: Nature killer; Ig: Immunoglobulin; C: Complement; IL: Interleukin; IFN: Interferon; TNF: Tumor necrosis factor; CXCL: Chemokine (C-X-C motif) ligand; CCL: CC chemokine ligand.

Complement dysfunction-mediated liver injury: A new pathological mechanism of CRLI is dysfunction of the complement system. Complement is also part of the immune system which provides innate defense against pathogens and mediates inflammatory reactions. However, during SARS-CoV-2 infection, an overactive complement response leads to systemic inflammation, and a negative complement response promotes viral replication and infection, thereby exacerbating disease and inducing damage to other organs[56]. Du *et al*[23] published a report on 183 pediatric patients with COVID-19, C3 was elevated and decreased in 12.4% and 18.6% of severe patients, respectively, and C4 was elevated and decreased in 3.7% and 4.3% of severe patients, respectively (Table 3). In addition, it has been shown that complement correlates with the coagulation cascade and dysregulated complement activation may also contribute to the hypercoagulable state in severe COVID-19 patients[57]. For example, a report by Antala *et al*[58] showed that of four children with CRLI, two had complement dysfunction and resulted in microangiopathy, one of which showed rapid improvement in liver function after treatment with eculizumab. All of these findings demonstrate that severe CRLI may be associated with complement dysfunction and microangiopathy features.

Hypoxia-mediated liver injury

The liver normally consumes 20% of whole body oxygen due to its dual blood flow system in the hepatic artery and portal system. In addition, the liver is able to extract 95% of blood-oxygen in order to maintain oxygen uptake[59]. It is well-known that ARDS is the most significant complication of COVID-19, which usually presents with respiratory distress, hypoxemia and acute respiratory failure[4,5]. All of these are important risk factors contributing to hypoxic hepatitis (HH). HH is characterized by a large and rapid increase in serum transaminases due to a decrease in oxygen delivery to the liver[60]. Furthermore, inflammatory cytokines may reduce the ability of hepatocytes to extract oxygen from the blood leading to hepatocyte death[61]. Thus, IIR caused by SARS-CoV-2 infection may promote the development of HH. Current studies suggest that HH is uncommon in patients with COVID-19, but has a very high mortality rate. For instance, Wu *et al*[62] identified 8 adult cases with HH among 3041 COVID-19 patients, and only 1 (12.5%) patient was discharged, and 7 (87.5%) died. Despite the lack of related reports on HH in pediatric patients, it is also a warning signal that we should be more concerned about the possibility of HH in children.

COVID-19-RELATED TREATMENT CAUSES LIVER INJURY

Mechanical ventilation-mediated liver injury

Approximately 23% of patients with SARS-CoV-2 infection developed pulmonary embolism[63]; therefore, some form of ventilation support, such as a high-flow nasal cannula, non-invasive and invasive mechanical ventilation, is required to prevent hemodynamic instability[64]. Woodruff *et al*[65] investigated COVID-19 -associated hospitalization surveillance network of 14 states in United States, they found that 691 (30.1%) patients required ICU admission and 122 (5.3%) patients needed invasive mechanical ventilation among 2293 hospitalized children (aged < 18 years). Moreover, other several researches also have showed 6% to 18% pediatric patients of COVID-19 required mechanical ventilation and 3% have died[66-71]. Current pediatric ventilation strategies are usually based on adult reports,

which may lead to increased pulmonary vascular resistance and thus reduced right ventricular (RV) activity[72]. RV dysfunction is a good predictor of heart failure[73]. As the liver is the largest visceral organ in the human body and receives up to 25% of the entire cardiac output, RV failure can not only aggravate liver injury by liver congestion attributed to elevated central venous pressure, but also ischemic hepatitis[59]. Additionally, a multivariate regression analysis showed a significant increase in the severity of COVID-19 among pediatric patients receiving mechanical ventilation[74]. Therefore, physicians should pay attention to the changes in cardiac function and the possibility of subsequent liver injury when mechanical ventilation is given to pediatric patients.

Drug-induced liver injury

Drugs are mainly metabolized by the liver. Drug-related liver injury (DRLI) remains an important focus in the monitoring of new drugs and drug repurposing. At present, the use of anti-SARS-CoV-2 drugs in pediatric patients is dependent on the evidence from adult clinical cases due to the emergency of COVID-19. The Italian Society of Infectious Pediatric Diseases recommends the use of remdesivir in pediatric patients with severe COVID-19 in whom renal and liver functions are normal, lopinavir/ritonavir should only be considered if remdesivir is incompatible or unavailable, dexamethasone and tocilizumab can be administered in patients with ARDS or MIS-C[75]. A medication guidance from a North American institution suggested using hydroxychloroquine as first-line treatment in children under 12 years and as second-line treatment in children above 12 years[76]. DRLI in pediatric cases is predominantly characterized by elevated liver enzymes as described by Goldman *et al*[77] in 77 children with severe COVID-19 treated with remdesivir, where 3 patients discontinued remdesivir due to elevated liver enzyme levels. The evaluation of other antiviral drugs in the pediatric population is uncommon. Although there are fewer pediatric patients with severe COVID-19, the use of antiviral drugs still deserves a separate discussion to develop a more appropriate therapy for children.

POINTS OF CONTENTION ON CRLI

The above conclusions are drawn from a limited number of pediatric cases and there are serious ethical questions about research on children with COVID-19. Therefore, many conflicting views remain to be further explored, for example: (1) "SARS-CoV-2 binds to ACE2 and exerts direct liver injury" *vs* "Ang(1-7) produced by ACE2 hydrolysis of Ang II has anti-inflammatory and anti-fibrotic effects on the liver"; (2) "The expression and function of ACE2 are weaker in children" *vs* "The expression of ACE2 decreases with age"; (3) "Cholestatic liver injury is more common in children" *vs* "The elevation of biliary injury marker ALP was not significant"; (4) "Cytokine storm can lead to inflammation and liver injury" *vs* "Cytokine storm is mild in pediatric patients"; and (5) "Inhibition of the complement system may aggravate viral infection and cause liver injury" *vs* "Excessive activation of the complement system may induce inflammation and cause liver injury".

It is normal for these contradictions to emerge. As the short duration and wide coverage of the COVID-19 epidemic, the severity and complexity of clinical cases vary, and the criteria for inclusion and the outcome of patients are also different among case reports. For the longer term future, we should continue to focus on CRLI to address these issues.

CONCLUSION

With the continuous progress of COVID-19, liver injury is becoming a research focus. We have divided the etiology of CRLI in children into three categories, and the possible pathophysiological mechanisms are discussed separately. Of these, the direct mechanism involves SARS-CoV-2 binding to ACE2 in the liver or bile duct to exert direct toxicity, the indirect mechanism includes IIR and hypoxia, and COVID-19-related treatments may also cause liver injury under some circumstances, such as the use of mechanical ventilation and antiviral drugs. In summary, children are characterized by strong innate immunity but weak adaptive immunity, and the IIR resulting from SARS-CoV-2 is still the main cause of liver injury. The evaluation of liver injury in pediatric patients with severe COVID-19, especially those with MIS-C, should be a focus. Another focus is the toxicity of SARS-CoV-2 to cholangiocytes, as children more commonly have cholestatic liver injury. In addition, hypoxia may promote liver injury due to the high incidence of ARDS complications. Finally, liver injury induced during COVID-19 treatment is often overlooked. Mechanical ventilation in children with respiratory distress can lead to the risk of RV dysfunction and subsequent liver injury, and the use of antiviral drugs in children may also lead to DRLI. In order to reach a consensus on the etiology of CRLI, more pediatric case reports, more detailed classifications and more in-depth studies are pending.

FOOTNOTES

Author contributions: Yun YF and Zhang JJ conceived of the contents of the manuscript; Yun YF wrote the manuscript and prepared the figures and tables; Zhang JJ and Feng ZY revised the manuscript; all the authors read and approved the final manuscript.

Supported by the National Natural Science Foundation of China, No. 22004063; the Natural Science Foundation of Jiangsu Province, No. 20200303; Program for Innovative Talents and Entrepreneur in Jiangsu, No. 021413006001; the Fundamental Research Funds for the Central Universities, No. 021414380504; and State Key Laboratory of Analytical Chemistry for Life Science, No. 5431ZZXM2206.

Conflict-of-interest statement: The authors have no conflict of interest to disclose.

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S-Editor: Chang KL
L-Editor: A
P-Editor: Chang KL

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World Journal of Hepatology

Submit a Manuscript: https://www.f6publishing.com

World J Hepatol 2023 March 27; 15(3): 364-376

DOI: 10.4254/wjh.v15.i3.364

ISSN 1948-5182 (online)

MINIREVIEWS

May 2022 acute hepatitis outbreak, is there a role for COVID-19 and other viruses?

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Specialty type: Gastroenterology and hepatology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Bernabe-Ortiz JC, Peru; Naderi D, Iran

Received: October 21, 2022
Peer-review started: October 21, 2022
First decision: January 3, 2023
Revised: January 6, 2023
Accepted: March 14, 2023
Article in press: March 14, 2023
Published online: March 27, 2023

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Abstract

There has been an increasing number of reported cases of acute hepatitis of unknown origin in previously healthy children since first reported on March 31, 2022. This clinical syndrome is identified by jaundice and markedly elevated liver enzymes with increased aspartate transaminase and/or alanine aminotransaminase (greater than 500 IU/L). We conducted an inclusive literature review with respect to acute hepatitis outbreaks in children using the search terms acute hepatitis, outbreak, children, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), coronavirus disease 2019 (COVID-19), and adenovirus. According to the cumulative data presented in four main studies, the median age is 4 years, with a male predominance (1.3:1). Jaundice was the most common clinical mani-

festation (69%), followed by vomiting (63%), anorexia (52.9%), diarrhea (47.2%), abdominal pain (39%), pyrexia (33.3%), pale stool (30%), and dark urine (30%). Coryza and lethargy were reported in 16.6%, while pruritus was reported in 2% of cases. Acute liver failure was observed in 25% of cases. The exact mechanism of this acute hepatitis outbreak is still not entirely clear. Adenoviruses and SARS-CoV-2 were detected in a significant number of patients. Coinfection with adenovirus and SARS-CoV-2 could be a possible underlying mechanism. However, other possible infections and mechanisms must be considered in the pathogenesis of this condition. Acute hepatitis of unknown origin in children has been a serious problem since the start of the COVID-19 pandemic but has not yet been sufficiently addressed. Many questions remain regarding the underlying mechanisms leading to acute liver failure in children, and it is likely that extensive future research is needed.

Key Words: Acute hepatitis of unknown origin; Children; Adenovirus; SARS-CoV-2; COVID-19; Hepatic failure

Core Tip: There has been an increasing number of acute hepatitis of unknown origin in children since first reported on March 31, 2022. The exact mechanism of this acute hepatitis outbreak is still unclear. Still, the increased detection rate of adenoviruses and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) may imply a key role for these viruses in the pathogenesis of this childhood condition. Coinfection with adenovirus and SARS-CoV-2 could also play a role, but comprehensive research is still needed to reach an exact mechanism. Until an aetiology is uncovered, the focus should be placed on the prevention of this syndrome in children *via* the use of proper hygiene.

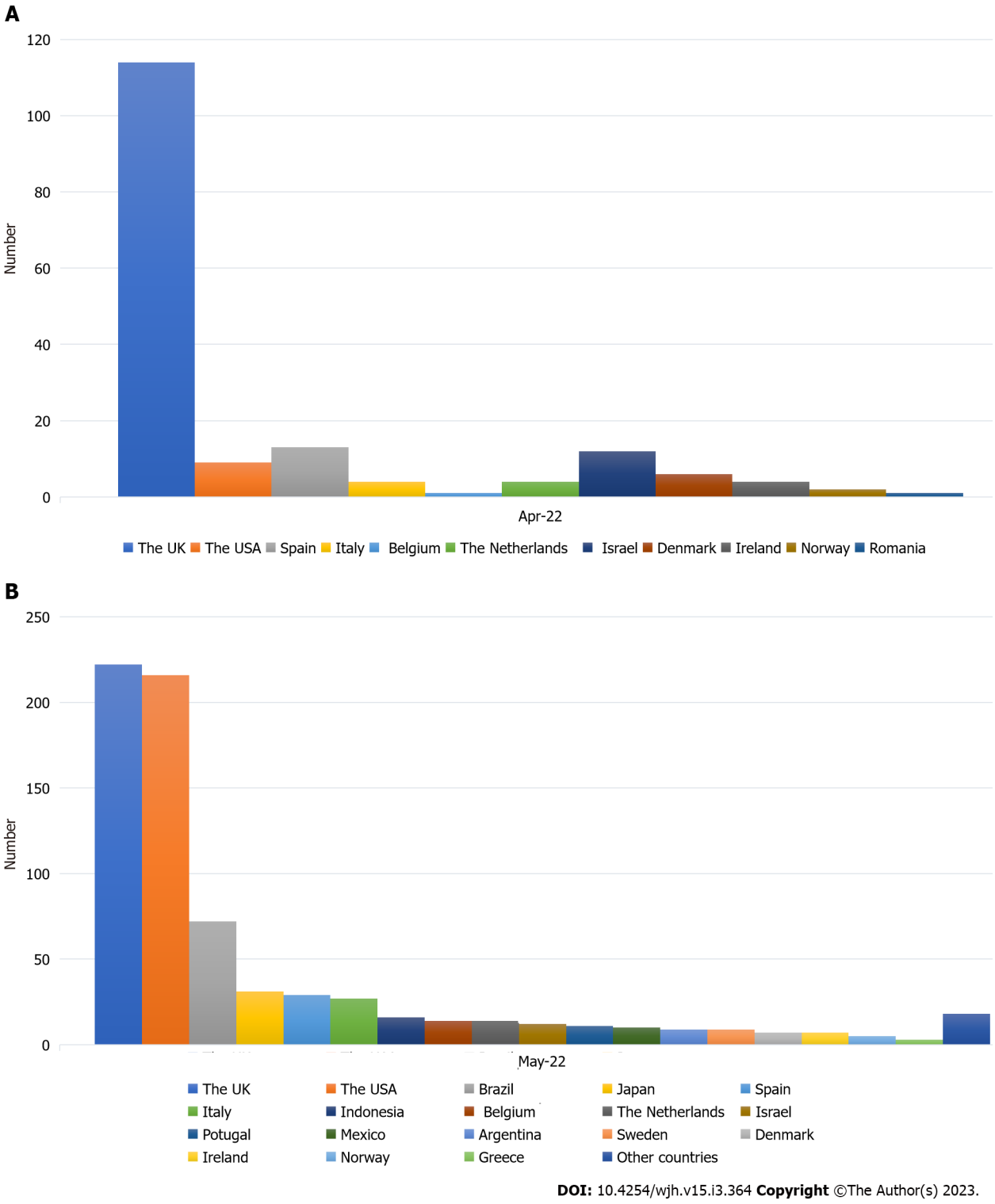
Citation: Elbeltagi R, Al-Beltagi M, Saeed NK, Bediwy AS, Toema O. May 2022 acute hepatitis outbreak, is there a role for COVID-19 and other viruses? *World J Hepatol* 2023; 15(3): 364-376
URL: https://www.wjgnet.com/1948-5182/full/v15/i3/364.htm
DOI: https://dx.doi.org/10.4254/wjh.v15.i3.364

INTRODUCTION

Since it was first reported on March 31, 2022, in Scotland, and with the recent increase in the reported cases since April 15, 2022, in the United Kingdom, a reason for acute hepatitis of unknown origin in previously healthy children has not been clearly defined. There is speculation as to whether this phenomenon represents a true increase in the number of cases or if it is an inflated statistic due to increased awareness and reporting. By the last week of April 2022, there were 169 cases of acute hepatitis of unknown origin in children aged 1 mo to 16 years reported from 11 countries, including The United Kingdom, Spain, Israel, The United States, Denmark, Ireland, Netherlands, Italy, Norway, France, Romania, and Belgium[1]. By the end of May 2022, the number of reported cases increased to 746, reported from 36 countries in 4 continents, mainly Europe and America (Figure 1)[2].

The clinical syndrome caused by acute hepatitis is identified by jaundice and markedly elevated liver enzymes, with increases in aspartate transaminase (AST) and/or alanine aminotransaminase (ALT) to greater than 500 IU/L. These findings may be preceded by gastrointestinal symptoms such as abdominal pain, vomiting, and diarrhoea. Fever is also reported in a few cases, but most affected children are afebrile. Furthermore, many patients experience more severe complications, such as liver failure[3]. Most hepatotropic viruses that cause acute hepatitis, such as hepatitis A, B, C, D, and E viruses, are not detected in acute hepatitis of unknown origin. About 10% of cases require liver transplantation, with 1 fatal case being reported. Despite most cases being reported from Europe, Israel, and The United States, there is no link between traveling to any specific country and developing the syndrome[4].

The exact mechanism of this type of acute hepatitis outbreak is still not known. However, adenovirus is reported in 74 cases, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in 20 cases, and coinfection in 19 cases. Meanwhile, data from The United Kingdom and the Netherlands show an increase in adenovirus infection in the community concurrently with the rise in the number of cases of acute hepatitis of unknown origin[5]. Although there is some evidence for the role of adenovirus with or without SARS-CoV-2 coinfection in the aetiology of this syndrome, other factors, such as immunopathogenesis and non-infection-related factors, could play a role. This review aims to shed light on the understanding of this syndrome[6].

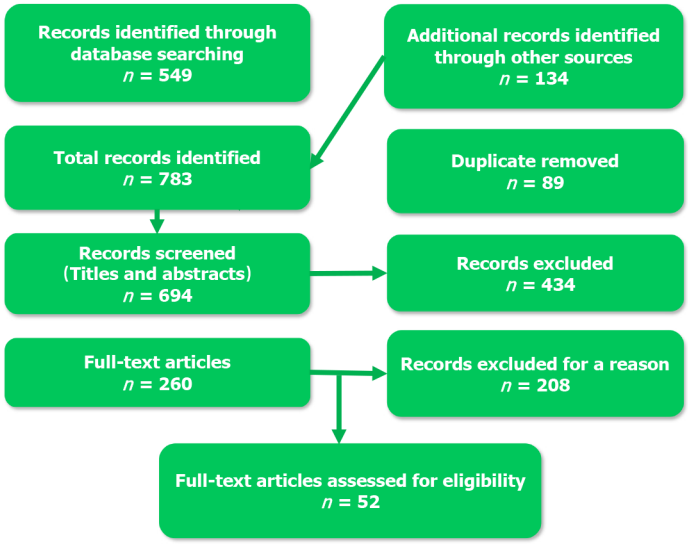


DOI: 10.4254/wjh.v15.i3.364

Figure 1 Reported cases of acute hepatitis syndrome of unknown aetiology in children. A: April 2022; B: May 2022. Apr: April; The UK: The United Kingdom; The USA: The United States of America.

METHODOLOGY

We conducted an inclusive literature review by searching various electronic databases for reports on acute hepatitis outbreaks in children. Databases searched included PubMed, Embase, Cumulative Index to Nursing and Allied Health Literature, Web of Science, Cochrane Library, Scopus, Library and Information Science Abstracts, Google search, and the National Library of Medicine catalogue. The search included reports published before August 31, 2022. Search terms utilized included acute hepatitis, outbreak, children, SARS-CoV-2, coronavirus disease 2019 (COVID-19), and adenovirus. Reference lists were inspected, and citation searches were also done on the included studies. We included open access papers published in English. Figure 2 shows a flow chart of the reviewed articles.



DOI: 10.4254/wjh.v15.i3.364

Figure 2 Flow chart of studies included in this review.

We reviewed 260 articles concerned with acute hepatitis of unknown aetiology outbreaks in children; information from 52 was included in this review.

CLINICAL SPECTRUM OF ACUTE HEPATITIS OF UNKNOWN CAUSE IN CHILDREN

The World Health Organization (WHO) has classified cases of severe acute hepatitis of unknown origin in children occurring during the current outbreak into three categories: (1) Confirmed; (2) Probable; and (3) Epi-linked. As there are no well-defined diagnostic criteria for this clinical entity, the probable case definition is the most widely used. Probable cases are those which present with manifestations of acute hepatitis with elevated serum aminotransferase levels of greater than 500 IU/L and without evidence of infection with hepatitis A-E viruses. The patient population is defined as children aged 16 years or younger as of October 1, 2021, with the youngest reported patient being 1 mo old. An epi-linked case is defined as a patient of any age presenting with acute hepatitis who has had a history of close contact with a probable case since October 1, 2021, and has no evidence of hepatitis A-E virus infection[7]. Three-quarters of reported cases in European countries are younger than age 5, as many studies have been focused on children younger than 10 years[8]. According to cumulative data presented in 4 main studies (2 from The United Kingdom and 2 from The United States), the median age of presentation is 4 years, with a male predominance (1.3:1). Jaundice is the commonlyommon reported clinical manifestation (69%), followed by vomiting (63%), anorexia (52.9%), diarrhoea (47.2%), abdominal pain (39%), pyrexia (33.3%), pale stool (30%), and dark urine (30%). Coryza and lethargy are reported in 16.6%, while pruritus is reported in 2% of cases. Acute liver failure is reported in 25% of these cases. See Table 1 for an overview of these data[9-12].

Kelgeri *et al*[9] found that in 44 cases of childhood acute hepatitis of unknown origin, hepatitis most commonly resolved. However, progression to fulminant liver failure requiring liver transplantation is reported in 14% of these cases. This finding underscores the severity of this condition and emphasizes the importance of recognizing its potential outcomes. In affected children, a prodromal phase is typically indicated by biochemical testing. Ultrasound findings of gallbladder wall thickening, pericholecystic fluid, mesenteric lymphadenopathy, and mild hepatosplenomegaly indicate a potential viral aetiology. If these findings are present, an extensive viral workup is required, especially if infection with an adenovirus is suspected. Laboratory tests essential to the diagnosis of suspected acute hepatitis of unknown origin are summarized in Table 2.

GUT-LIVER AXIS AND HEPATIC DISEASES

The gut microbiome affects various essential processes, including immunological, structural, metabolic, and neurological functions. For this reason, gut microbiome status can considerably impact physical and mental health. The gut is colonized by over 1000 microbial species, a process that starts *in utero* and continues after birth in an ongoing, complex, dynamic manner to promote gut maturation and development[13]. Although the number of microbial species in the gut microbiota of children and

Table 1 Clinical data from various studies, *n* (%)

Ref.	Kelgeri *et al*[9], 2022	Cates *et al*[10], 2022	Marsh *et al*[11], 2022	Baker *et al*[12], 2022	Cumulative data
Demographic data					
Country	UK	USA	Scotland, UK	Alabama, USA	
Number of patients	44	296	13	9	362
Age (yr), median (range)	4 (1-7)	2.2 (0–9.7)	3.9 (3-5)	2 (1.66-5.7)	3 (0-9.7)
Male/female ratio	0.83:1	1.42:1	1.2:1	0.28:1	1.3:1
Clinical findings					
Jaundice	41 (93)	71/123 (57.7)	8/9 (88.9)	8/9 (88.9)	128/185 (69)
Vomiting	24 (54)	76/123 (61.8)	4/4 (100)	7/9 (77.8)	113/180 (63)
Diarrhoea	14 (32)	61/123 (49.6)	4/4 (100)	6/9 (66.7)	85/180 (47.2)
Pale stools	13 (30)	/	/	/	13/44 (30)
Abdominal pain	12 (27)	48/123 (39.0)	7/9 (77.8)	/	69/176 (39)
Lethargy	10 (23)	15/123 (12.2)	4/4 (100)	1/9 (11.1)	30/180 (16.6)
Dark urine	6 (14)	44/123 (35.8)	/	/	50/167 (30.0)
Coryza	6 (14)	20/123 (16.3)	/	3/9 (33.3)	29/176 (16.5)
Pyrexia	4 (9)	51/123 (41.5)	0/4 (0)	5/9 (55.6)	60/180 (33.3)
Pruritus	1 (2)	/	/	/	1/44 (2.0)
Anorexia		65/123 (52.9)	/	/	65/123 (52.9)
Acute live failure	6 (14)	37/123 (30.1)	/	1/9 (11.1)	44/176 (25)

UK: The United Kingdom; USA: The United States of America.

adolescents mirrors that of adult, the relative abundance of species varies. In children and adolescents, there are more abundant *Faecalibacterium spp.*, *Bifidobacterium spp.*, and subspecies of *Lachnospiraceae*[14]. In addition, fungi and viruses are also present in the gut. Virobiota of the gut include bacteriophages that can infect prokaryotic cells, viruses that can infect eukaryotic host cells, and virus-derived genetic particles embedded in host chromosomes; the term "virome" refers to the entire complement of viral genetic elements found in the human genome[15].

In the oro- and nasopharyngeal areas, bacteriophages, coronaviruses, herpes viruses, adenoviruses, respiratory syncytial viruses, picornaviruses, influenza A viruses, and other uncharacterized eukaryotic viruses are frequently encountered. Common gastrointestinal viruses include bacteriophages, adenoviruses, caliciviruses, parvoviruses, picornaviruses, papillomaviruses, astroviruses, plant viruses, and other uncharacterized eukaryotic viruses[16]. Some eukaryotic DNA viruses, such as anelloviruses, herpesviruses, human bocavirus, and adenoviruses, and some RNA viruses, such as picobirnaviruses and parechoviruses can continue shedding for months. For this reason, these viruses form a significant fraction of the typical human virome due to their capacity for persistent infection[17]. Unfortunately, human adenoviruses in the gut can be reactivated and cause persistent infection, leading to serious morbidity and mortality, especially in immunosuppressed patients (*e.g.*, children with hematopoietic disorders)[18].

The liver-gut microbiome axis, which also includes virobiota, is a bidirectional pathway in which portal veins transport gut-derived products directly from the gut to the liver, and bile and antibodies produced in the liver are transported back to the gut (Figure 3). Gut microbiome products preserve the immune homeostasis of the intestine and liver. Conversely, some microbial-derived metabolites such as ethanol, trimethylamine, short-chain fatty acids, and secondary bile acids may play a role in liver disease. Meanwhile, liver diseases such as cirrhosis can induce significant changes to the gut microbiome due to impairment of the vascular, epithelial, and immune barriers of the intestine[19]. Accordingly, gut dysbiosis can induce an abnormal mucosal immune response and lead to homeostatic imbalance. This resulting imbalance causes microbes and immune cells to migrate to the liver, provoking inflammation and associated hepatic injury, and may also influence neoplastic processes[20, 21].

Table 2 Suggested workup in the diagnosis of acute hepatitis of unknown aetiology

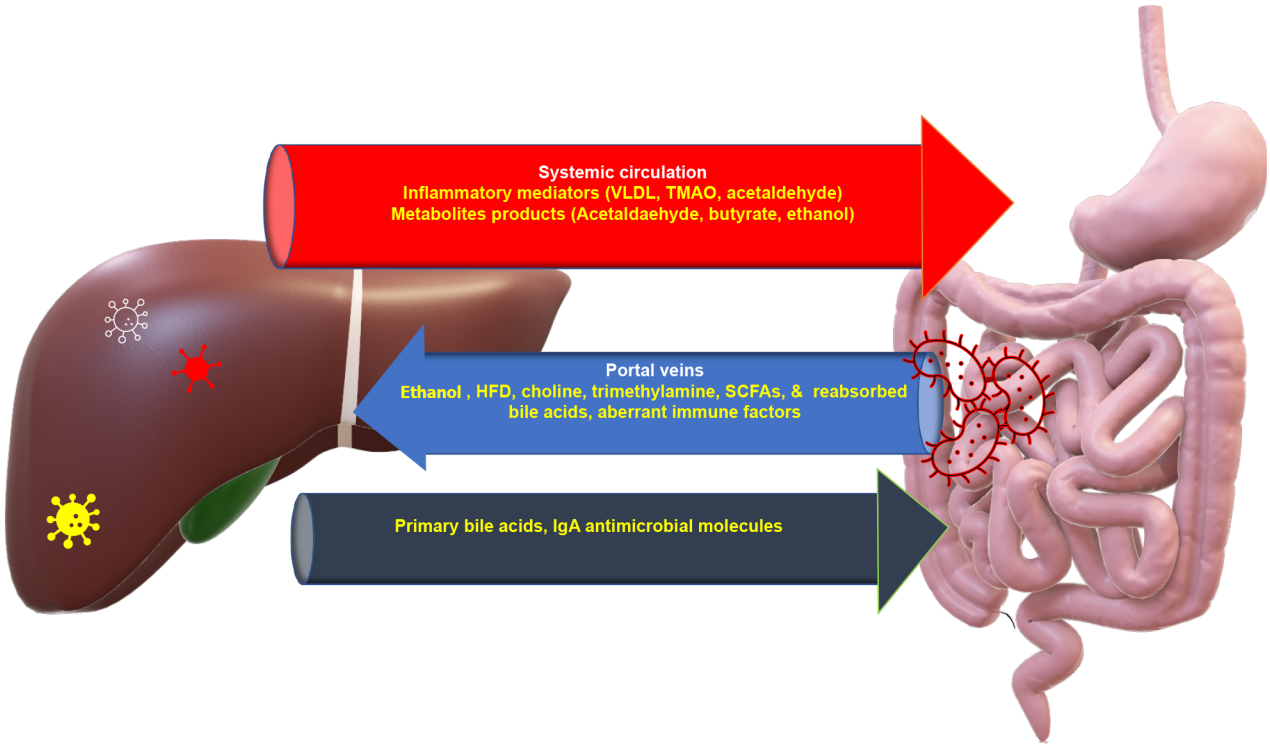
Item		Sample test
History		To be taken according to WHO for case definition, probable or confirmed case. History of traveling to high-risk areas endemic to hepatitis viruses, exposure to a local outbreak, household contact, sharing personal items with an infected person, attendance at day-cares, history of transfusion-dependent illness, or exposure to tattoos and/or body piercing using nonsterile techniques
Clinical examination		Low-grade fever, fatigue, anorexia, nausea, vomiting, enlarged and tender liver with/without splenomegaly, jaundice, abdominal pain, dark urine, pale or clay-coloured stool
Liver Functions		Total bilirubin, conjugated bilirubin, liver enzymes (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and gamma-glutamyl transferase), albumin, and prothrombin time
Liver biopsy		Staining with haematoxylin and eosin in selected cases
Imaging studies		Usually not required; may be needed to rule out biliary obstruction and other aetiologies for elevated liver enzymes and to exclude complications such as cirrhosis and hepatocellular carcinoma. Abdominal ultrasound: Shows enlarged liver with decreased (acute) or increased (chronic) echogenicity, brighter portal vein, periportal oedema, gallbladder wall thickening, and ascites. CT findings of acute hepatitis are nonspecific: Hepatomegaly, gallbladder wall thickening, periportal oedema, and ascites
Tests for autoimmune hepatitis		Autoantibodies such as ANAs and anti-SMAs
Detecting viral causes of hepatitis	Serology	Antibodies against Hepatitis A-E, Epstein-Barr virus, cytomegalovirus, HIV, varicella, adenovirus, SARS-CoV-2 (anti-S and anti-N antibodies)
	Culture	Blood: Adenovirus, cytomegalovirus, Epstein-Barr virus, herpes simplex virus, influenza viruses. Rectal Swab: Adenovirus, rotavirus, enteroviruses
	PCR	Blood: For hepatitis A, C, E, adenovirus, cytomegalovirus, enteroviruses, Epstein-Bar virus, Parechovirus, Herpes simplex virus, HHV 6 and 7. Throat Swab: Respiratory virus screening by multiplex assay (including Adenovirus, Influenza, Parainfluenza, Respiratory Syncytial Virus, Rhinovirus, Human bocavirus 1-3, Human metapneumovirus, Enteroviruses, SARS-CoV-2, *etc.*). Stool: For enteric viruses screening by multiplex assay (including Norovirus, Enteroviruses, Rotavirus, Astrovirus, Sapovirus)
Detecting bacterial causes of hepatitis	Serology	Antibodies against: *Brucella spp., Bartonella henselae, Borrelia burgdorferi* (when epidemiologically appropriate)
	Culture	Blood: Routine procedures for bacterial pathogens, when clinically applicable. Throat Swab: *Streptococcus* group A. Stool: *Salmonella, Shigella, Campylobacter, E. coli* 0157. Urine: Routine procedures for bacterial pathogens, when clinically applicable
	PCR	Stool or rectal swab: Enteric bacterial pathogens. Urine: *Leptospira spp*
Toxicological screening		Blood and urine by different methods, including mass spectrometry: Drugs (*e.g.*, acetaminophen, antibiotics, antiepileptics, herbal medicines) or toxins (*e.g.*, carbon tetrachloride)
Metabolic work-up		Ceruloplasmin; 24 h of urinary copper excretion; Celiac disease screening; Urine organic acid profile; Plasma amino acids; Plasma acylcarnitine; Whole exome and mitochondrial gene examination to rule out other inborn metabolic disorders that can cause liver injury; Other metabolic work-up according to the clinical scenario

ANA: Antinuclear antibody; CT: Computerized tomography; HIV: Human immunodeficiency virus; HHV: Human herpesvirus; PCR: Polymerase chain reaction; SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2; SMA: Smooth muscle antibody; WHO: World Health Organization.

ADENOVIRUS HEPATOTROPIC EFFECTS

Adenoviruses are medium-sized, nonenveloped, double-stranded DNA viruses. They are named for their first isolation from the adenoid in 1953. Adenoviruses are widespread viruses that classically trigger mild cold- or flu-like disease, pneumonia, conjunctivitis, and acute gastroenteritis in all age groups throughout the year[22]. Adenoviruses are not typically hepatotropic in immunocompetent children. However, they can still cause hepatitis in children with liver stem cell transplantation, immunosuppressed children (*e.g.*, with severe combined immune deficiency), and children receiving chemotherapy for solid malignant neoplasms.

The species C adenoviruses are the most commonly implicated in adenovirus-associated hepatitis, with type 5 being the most frequently encountered[23]. Furthermore, human species F adenoviruses (*e.g.*, types 40 and 41) are well-known causes of paediatric gastroenteritis. Chhabra *et al*[24] showed that F adenovirus type 41 is more widespread than type 40 in the setting of viral gastroenteritis in children younger than 5 years. Despite mainly causing respiratory infections, adenoviruses can produce transient nonspecific "reactive hepatitis" findings in children during an active infection, with AST and ALT levels used as markers of hepatitis severity[25]. Adenovirus infection can be diagnosed by direct antigen detection (in blood, stool, or respiratory samples), polymerase chain reaction (PCR) amplification, virus culture and isolation, and serology. Specimens are ideally collected within 1 week of symptom onset. Positive serology is expected in most children by the age of 4 years, but a 4-fold or more increase in the titre of adenovirus-specific antibodies is considered evidence of a recent infection. Adenovirus typing



DOI: 10.4254/wjh.v15.i3.364

Figure 3 Gut-liver axis. Mutual effects of the gut and liver through systemic and portal circulation and biliary enterohepatic circulation. HFD: High-fat diet; IgA: Immunoglobulin A; SCFAs: Short-chain fatty acids; TMAO: Trimethylamine N-oxide; VLDL: Very-low-density lipoprotein.

can be performed with molecular tests, and is essential from an epidemiological point of view[26]. When performing adenovirus molecular testing in suspected cases of acute hepatitis of unknown cause, whole blood samples instead of serum or plasma should be used as it has a higher viral yield[12].

Out of 74 cases of acute hepatitis of unknown aetiology with detected adenovirus, 18 patients were identified by molecular tests as serotype F41, and a few others were serotype F40[27]. This finding may indicate that enteric adenoviral infection may be related to hepatic infection by means of the gut-liver axis, a topic that warrants further research[28]. The low viral load in the clinical samples necessitated PCR amplification of part of the viral hexon gene followed by Sanger sequencing for the detection of adenoviruses. However, notably, children presenting with acute hepatic failure had a high viral load. Importantly, there are also intratypic genetic variations in adenoviruses of serotype F type 41[29]. A study at an Alabama hospital in August 2022 showed three different strains of adenovirus serotype F41, observed in 5 patients with acute hepatitis of unknown aetiology. This finding may indicate a low probability with regard to an outbreak being caused by a specific adenovirus serotype[30].

Meanwhile, serotyping data obtained from 4 adenovirus-positive patients in the European cohort showed 2 with serotype F41, 1 with serotype F40, and 1 with a serotype of "other." This supports the potential of adenovirus to negatively affect the liver after reaching it through the gut-liver axis. This adenovirus pathogenicity could be attributed to the development of mutations that promote hepatotropism, but this hypothesis needs to be confirmed by whole genome sequencing to detect any such mutation[28]. Despite being isolated from a significant number of children with acute hepatitis of unknown origin, the exact role of adenoviruses in the pathogenicity of this condition still needs to be confirmed. Infection with adenoviruses is usually mild and resolves spontaneously. However, the infection can be associated with high morbidity and mortality in immunocompromised children, particularly those with allogeneic stem cell transplants. Although adenoviruses have been widely studied, there is currently no anti-adenoviral treatment approved by the United States Food and Drug Administration. As of this review, cidofovir and ribavirin are the only antiviral drugs used as first-line therapy to treat adenoviral infections. Brincidofovir has no nephrotoxicity and has better bioavailability than cidofovir, but this drug is no longer manufactured[31,32].

CORONAVIRUSES HEPATOTROPIC EFFECTS

Despite pulmonary symptoms being the dominant finding in the clinical presentation of COVID-19, SARS-CoV-2 may also affect other organs such as the liver[33]. The liver is affected by 14%–53% of SARS-CoV-2 infections, regardless of preexisting liver disease[34]. SARS-CoV-2 accesses the liver *via*

binding angiotensin-converting enzyme-2 receptors, which are strongly expressed on cholangiocytes, minimally expressed on hepatocytes, and absent on Kupffer cells[35]. COVID-19-associated liver injury could be related to immune-mediated damage with a severe inflammatory response to SARS-CoV-2 infection, direct cytotoxicity due to active viral replication inside the liver cells (Figure 4), COVID-19-associated anoxic liver damage, drug-associated liver injury, or reactivation of preexisting liver infections (*e.g.*, Hepatitis B)[30]. SARS-CoV-2 infection can also activate autoimmune hepatitis *via* systemic immune hyperstimulation, molecular mimicry, or both[31].

Meanwhile, few cases of autoimmune hepatitis have been reported after SARS-CoV-2 vaccination, and those that have been reported all showed complete remission with steroid therapy[36]. Crisan *et al* [37] showed that patients who presented with elevated liver enzymes and abnormal chemistries on arrival were more likely to have worse disease and poorer outcome. The presence of fibrosis in hospitalized patients with COVID-19 is associated with increased mortality. Therefore, regular monitoring of liver function should be standard for all COVID-19 patients, and serological testing for specific hepatotropic viruses (*e.g.*, Hepatitis B or C according to the local epidemiological status) should be strongly considered[38].

Figure 4 shows the effects of COVID-19 infection on the liver, which is first evidenced by increased liver enzymes. The virus reaches the liver from the gut-liver-lung axis, which may be re-shed back to the gut through the bile. These effects are mediated through the impact of hypoxia, systemic venous congestion, immune-mediated hepatic damage by inflammatory mediators induced by SARS-CoV-2 infection, the direct hepatic cytopathic effect of SARS-CoV-2, and the hepatotoxic effects of some medications used to treat SARS-CoV-2 infection such as azithromycin, chloroquine, lopinavir, ritonavir, and tocilizumab. Hepatic damage can also result from SARS-CoV-2 reactivation of pre-existing liver diseases such as hepatitis B or C[39,40].

SYNERGISM BETWEEN SARS-COV-2 AND ADENOVIRUS

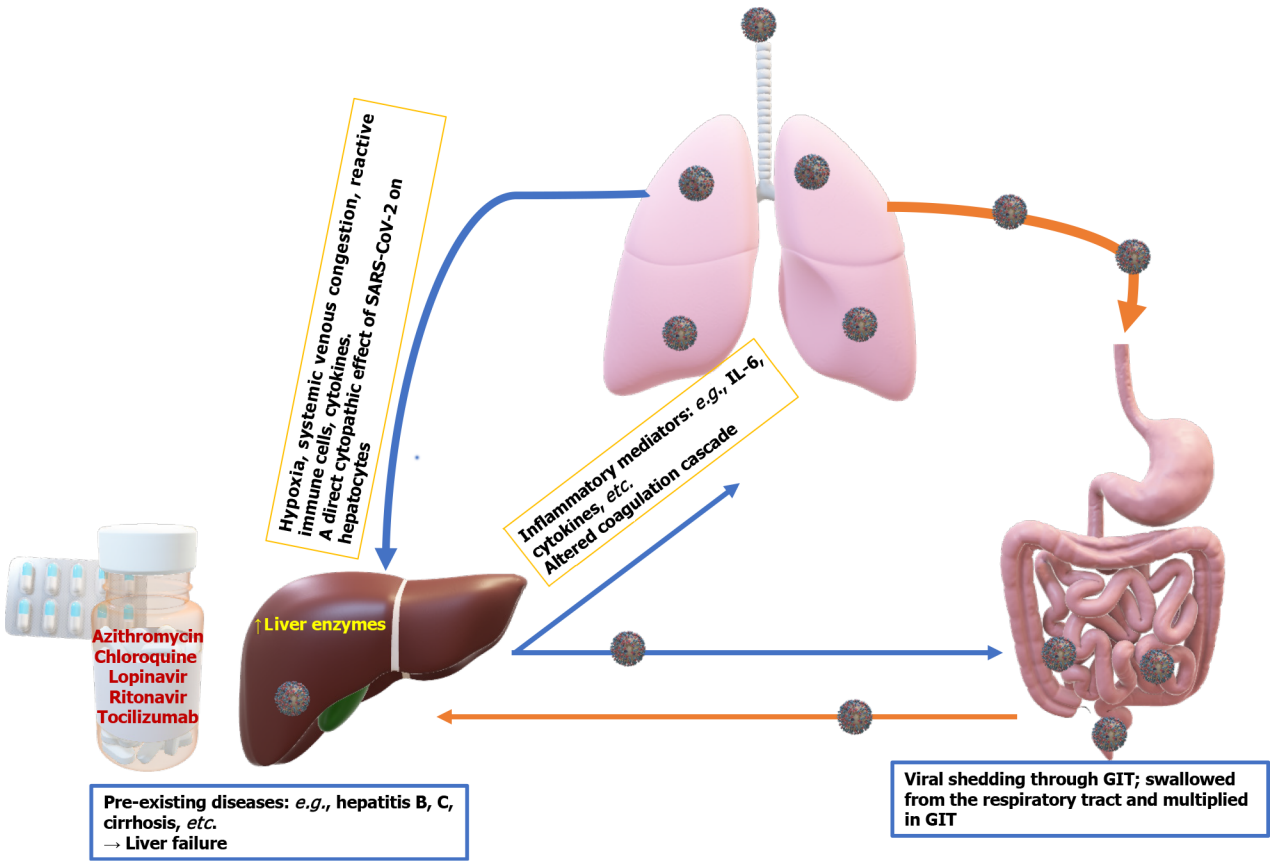
Many studies have shown an increased incidence of adenovirus among children infected with SARS-CoV-2. Coinfection with SARS-CoV-2 and adenovirus may also occur. Mohammadi *et al*[41] showed that the rate of SARS-CoV-2 and adenovirus coinfection is 1.1%, and all cases had mild respiratory disease. Another study from The United States showed a 0.4% rate of SARS-CoV-2 and adenovirus coinfection, being the third most common coinfection after rhinovirus/enterovirus and influenza A viruses[42]. Another study from the United Kingdom showed a 2% rate of coinfection with SARS-CoV-2 and adenovirus which increased the odds of death by 1.22[43]. Finally, a study from China found a slightly higher coinfection rate of 2.8%, associated with a worse diagnosis than bacterial coinfection[44].

EPSTEIN-BARR VIRUS AS A POSSIBLE CAUSE

Epstein-Barr virus (EBV) is a member of the herpesvirus family. It causes a heterogeneous group of infections in children and adults with a classic presentation (infectious mononucleosis) or other atypical presentations. Baker *et al*[12] showed that EBV was identified in 6 out of 9 children with acute hepatitis of unknown cause, verified using molecular methods. However, due to the absence of IgM, these cases could represent the reactivation of an old EBV infection and not a primary infection. However, EBV was reported to cause acute hepatitis in adults as well. García-Martínez *et al*[45] reported coinfection of SARS-CoV-2 and EBV in a 19-year-old woman who presented with pyrexia and bilateral eyelid and hemifacial swelling and was found to have splenomegaly, cervical lymphadenopathy, and elevated AST and ALT. In addition, Nadeem *et al*[46] described the reactivation of an EBV infection in a 62-year-old man attributed to coinfection with SARS-CoV-2. This patient was also found to have elevated AST and ALT. Despite these cases, the role of EBV in the pathogenesis of hepatitis is unclear, as many other confounding factors were present.

QUESTIONS NEED TO BE ANSWERED

Although both adenoviruses and SARS-CoV-2 are not typically hepatotropic viruses and rarely cause acute hepatitis in immunocompetent patients, coinfection with both viruses may produce significant effects on the liver and induce an acute hepatitis-like syndrome. Many questions remain, and further research may lead to key information regarding acute hepatitis of unknown origin. As there is an increased rate of autoimmune diseases after COVID-19 and its vaccines[47], could acute hepatitis of unknown cause be a COVID-19 immune-triggered reaction? Could this syndrome be caused by new variants of either adenovirus or SARS-CoV-2? Could coinfection with SARS-CoV-2 and adenoviruses trigger aggravated inflammatory responses affecting a sensitized liver and consequently induce acute hepatitis? Could acute hepatitis be a local form of the multisystem inflammatory syndrome, as



DOI: 10.4254/wjh.v15.i3.364

Figure 4 Effect of coronavirus disease 2019 infection on the liver as indicated by increased liver enzymes. The virus reaches the liver from the gut-liver-lung axis and may be re-shed back to the gut through the bile. These effects are mediated through the impact of hypoxia, systemic venous congestion, immune-mediated hepatic damage by inflammatory mediators induced by severe acute respiratory syndrome coronavirus-2 infection (SARS-CoV-2), the direct hepatic cytopathic effect of SARS-CoV-2, and the hepatotoxic effects of some medications used to treat SARS-CoV-2 infection such as azithromycin, chloroquine, lopinavir, ritonavir, and tocilizumab. GIT: Gastrointestinal tract; IL-6: Interleukin 6.

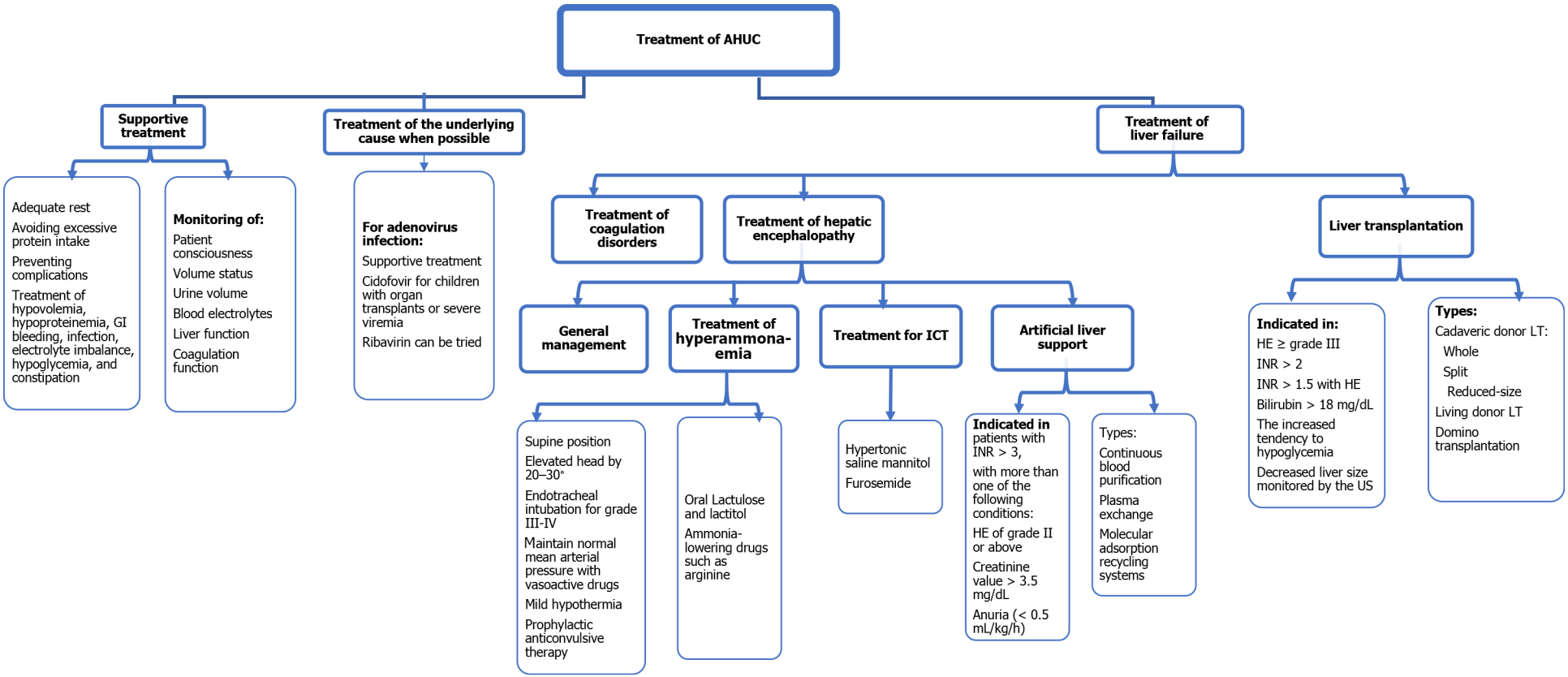
described by Cantor *et al*[47]? Could adenoviruses serve as a vector for SARS-CoV-2, easing the entry of SARS-CoV-2 in hepatocytes? Could this syndrome of acute hepatitis be related to other undiscovered microbial or non-microbial agents? Other aetiologies cannot be ignored. Despite being isolated from cases with acute hepatitis of unknown cause, the role of adenoviruses in the pathogenesis of this syndrome is not yet proven. We must strive to answer these questions and better define the correlation between SARS-CoV-2 and adenovirus infection and the development of this syndrome.

TREATMENT

Treatment of acute hepatitis of unknown cause in children is mainly symptom-based, supporting the recovery of liver function and treating complications as they arise. Cidofovir can be used when adenovirus infection is suspected, particularly in children with an organ transplant or severe viremia[48,49]. When multisystem inflammatory syndrome in children (MIS-C) is suspected to cause acute hepatitis, the treatment protocol is the same as the management of MIS-C[47]. Liver transplantation is indicated in children with acute fulminant hepatic failure refractory to aggressive therapy[50]. A summary of the treatment of acute hepatitis of unknown cause is illustrated in Figure 5.

PREVENTION

As SARS-CoV-2, adenoviruses, EBV, and other viruses are strongly suspected as potential mediators of acute hepatitis of unknown cause, appropriate hand hygiene and regular surface disinfection are essential to reduce viral spread. Hand and respiratory hygiene manoeuvres can reduce the spread of nonenveloped viruses such as adenoviruses[51]. Moreover, it is key that healthcare professionals know the signs and symptoms of hepatitis in children. In suspected cases, clinicians should order serum ALT



DOI: 10.4254/wjh.v15.i3.364

Figure 5 Summary of the treatment of acute hepatitis of unknown cause. AHUC: Acute hepatitis of unknown cause; GI: Gastrointestinal; HE: Hepatic encephalopathy; ICT: Increased intracranial tension.

and AST transaminase testing to ensure efficient detection of cases as early as possible.

CONCLUSION

There has been an increasing number of acute hepatitis of unknown origin in children since first reported on March 31, 2022. The exact aetiology of this condition in children, which was observed to increase in prevalence during the COVID-19 pandemic, is still unclear. Despite adenoviruses and SARS-CoV-2 being isolated from some patients with acute hepatitis of unknown cause, the role of these viruses in the pathogenesis of this syndrome is not yet proven. Coinfection with SARS-CoV-2 and other

viruses may relate to the pathogenesis of this condition. However, many questions remain and will require comprehensive research to better understand this correlation. Until a better understanding is reached, emphasis must be placed on preventing the development of acute hepatitis in children by using proper hygiene (*e.g.* hand washing, frequent surface disinfection) to reduce viral spread. Treatment of acute hepatitis of unknown cause in children is mainly symptom-based, supporting liver recovery and treating complications as they arise.

ACKNOWLEDGEMENTS

We thank the anonymous referees and editors for their valuable suggestions.

FOOTNOTES

Author contributions: Elbeltagi R, Al-Beltagi M, Saeed NK, Bediwy AS, Toema O collected the data and wrote and revised the manuscript.

Conflict-of-interest statement: All authors declare that they have no competing interests.

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S-Editor: Liu GL
L-Editor: Filipodia
P-Editor: Liu GL

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World Journal of Hepatology

Submit a Manuscript: https://www.f6publishing.com

World J Hepatol 2023 March 27; 15(3): 377-385

DOI: 10.4254/wjh.v15.i3.377

ISSN 1948-5182 (online)

MINIREVIEWS

Challenges and recommendations when selecting empirical antibiotics in patients with cirrhosis

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Specialty type: Gastroenterology and hepatology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): D, D, D
Grade E (Poor): 0

P-Reviewer: Ferrarese A, Italy; Kumar R, India; Rabago LR, Spain

Received: November 12, 2022
Peer-review started: November 12, 2022
First decision: November 23, 2022
Revised: December 28, 2022
Accepted: March 10, 2023
Article in press: March 10, 2023
Published online: March 27, 2023

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Abstract

There is abundant evidence that bacterial infections are severe complications in patients with cirrhosis, being the most frequent trigger of acute-on-chronic liver failure and causing death in one of every four patients during hospitalization. For these reasons, early diagnosis and effective treatment of infections are mandatory to improve patient outcomes. However, treating physicians are challenged in daily practice since diagnosing bacterial infections is not always straightforward. This situation might lead to delayed antibiotic initiation or prescription of ineffective regimens, which are associated with poor outcomes. On the other hand, prescribing broad-spectrum antibiotics to all patients suspected of bacterial infections might favor bacterial resistance development. This is a significant concern given the alarming number of infections caused by multidrug-resistant microorganisms worldwide. Therefore, it is paramount to know the local epidemiology to propose tailored guidelines for empirical antibiotic selection in patients with cirrhosis in whom bacterial infections are suspected or confirmed. In this article, we will revise current knowledge in this area and highlight the importance of surveillance programs.

Key Words: Bacterial infections; Cirrhosis; Multidrug resistance; Antibiotic prophylaxis; Antibiotic stewardship

Core Tip: Practitioners who participate in caring for patients with cirrhosis are challenged when using antibiotics rationally. On one side, bacterial infections are frequent, severe, and not always straightforward to diagnose; on the other, scant granular data is publicly available about the causal microorganisms and their susceptibility patterns. According to experts, empiric antibiotic treatments should cover 80% of the common pathogens in stable patients and 90% in critically ill patients with suspected infections. Therefore, it is necessary to know the microorganisms expected to be involved in the most frequent bacterial infections and their susceptibility patterns to develop evidence-based guidelines. This opens a window of opportunity for research because bacterial infections and multidrug resistance are global health issues expected to grow over the following decades.

Citation: Dirchwolf M, Gomez Perdiguero G, Grech IM, Marciano S. Challenges and recommendations when selecting empirical antibiotics in patients with cirrhosis. *World J Hepatol* 2023; 15(3): 377-385
URL: https://www.wjgnet.com/1948-5182/full/v15/i3/377.htm
DOI: https://dx.doi.org/10.4254/wjh.v15.i3.377

INTRODUCTION

Impact of bacterial infections in patients with cirrhosis

Bacterial infections are extremely frequent in patients with cirrhosis, present in about 25%-46% of those hospitalized for an acute decompensating event. In two third of cases, infections are diagnosed at admission, whereas the remaining patients develop nosocomial infections[1,2]. The commonest infections in patients with cirrhosis include spontaneous bacterial peritonitis (SBP), urinary tract infection, pneumonia, spontaneous bacteremia, and skin and soft tissue infections[3]. Although gram-negative enteric organisms were the primary pathogens involved, gram-positive infections are increasing in prevalence. This situation might be favored by antibiotic prophylaxis, medical procedures, and prior hospitalizations, among other risk factors[2,4].

Bacterial infections are currently recognized as a surrogate for the final stage of chronic liver disease [5,6]. Even though any type of decompensation in patients with cirrhosis is associated with worsening survival, not all decompensating events carry the same weight in patients' prognosis. The relevance of bacterial infections as a prognostic factor has been clearly stated in a meta-analysis that found that they increase mortality four-fold in this population, considering 30% of patients die within one month and another 30% die one year after these infections are diagnosed[7].

Factors associated with an increased risk of infection are poor liver function, variceal bleeding, low ascitic fluid protein levels, prior SBP, and hospitalization[8]. In addition, bacterial infections have also been defined in the large prospective cohort study CANONIC as the most frequent trigger of acute-on-chronic liver failure (ACLF), negatively impacting patients' prognosis irrespective of the resolution of the infection[5]. In fact, infections as precipitant or complications arise in 50% of patients with ACLF and 70% of patients with three or more organ failures[9].

Challenges in timely diagnosis and treatment of bacterial infections

Early diagnosis of bacterial infections is crucial for the rapid initiation of antibiotic treatment[8]. However, this poses a challenge since they are often oligo-symptomatic. For example, only one-half of patients with cirrhosis and bacterial infections develop fever, and most do not present leukocytosis or systemic inflammatory response criteria[10]. This is why high clinical suspicion is critical; in fact, the European Association for the Study of the Liver (EASL) position paper on bacterial infections recommends that all patients with cirrhosis admitted to the hospital should be considered infected until proven otherwise[8]. Furthermore, it should also be considered in patients with cirrhosis that deteriorate their clinical status while admitted to the hospital[10].

A rapid evaluation, including physical examination, ascitic and/or hydrothorax evaluation, and a chest X-ray, might rule in or out some of the most frequent infections in patients with cirrhosis, such as SBP, spontaneous bacterial empyema, pneumonia, and skin and soft tissue infections. However, urinary tract infection and spontaneous bacteriemia, representing more than 40% of the infections[3], are not easy to approach because their diagnosis is mainly based on cultures, which are usually available 24 to 48 h after the initial evaluation. In practice, the difficulty of ruling out these two infections might lead to unnecessary empiric antibiotic prescriptions.

Several biomarkers have been assessed to aid in promptly diagnosing bacterial infections. C-reactive protein, ferritin, or leukocyte count lack specificity for bacterial infections[11]. Furthermore, they can be influenced by immune dysfunction and hypersplenism, presenting lower values than expected[10,11]. Procalcitonin has been proposed as a more specific marker for bacterial infection. Nearly all tissues produce this biomarker in response to endotoxin or mediators released during bacterial infections, such as interleukin (IL)-1β, tumor necrosis factor-alpha, and IL-6. It has been proposed that it highly

correlates with the severity of bacterial infections and may help distinguish bacterial from viral infections or non-infectious inflammatory syndromes[8,11]. In a meta-analysis of more than 1000 patients with infections and cirrhosis, procalcitonin and C-reactive protein had acceptable accuracy for diagnosing bacterial infection among patients with cirrhosis compared with patients with normal liver function; however, their suggested applications differ. Procalcitonin was suggested as a rule-in tool [positive likelihood ratio = 7.38, 95% confidence interval (CI): 4.70-11.58], whereas C-reactive protein was suggested as a rule-out tool (negative likelihood ratio = 0.23, 95%CI: 0.13-0.41)[12]. Ultra-sensitive procalcitonin has been suggested more recently as a valuable tool for bacterial infection diagnosis, with a sensitivity of 97% and a negative predictive value of 98%, considering a cutoff value of 0.098 ng/mL [13]. Despite these promising data, these tools have yet to be integrated into everyday clinical practice.

Due to all these limitations, other auxiliary tools have been proposed and validated in this population to diagnose sepsis. One of these is the Sepsis-3 score, which defines sepsis as a Sequential/Sepsis-related Organ Failure Assessment (SOFA) score of at least two points at intensive care unit (ICU) admission or an increase in the SOFA score during ICU hospitalization and suspected infection[14,15]. This updated clinical score aims to achieve greater consistency for future trials and ease earlier diagnosis and management of patients with sepsis or at its risk[15]. Similarly, the qSOFA score considers a surrogate of poor prognosis the presence of at least two of the following: Respiratory rate of 22 breaths per minute or greater, altered mental status, or systolic blood pressure of 100 mmHg or lower[16]. This simplified score had a greater predictive validity for in-hospital mortality than SOFA and systemic inflammatory response syndrome when used outside of an ICU setting[17]. However, these scores must be broadly validated to be used as the standard of care.

When a bacterial infection is suspected in patients with cirrhosis, the immediate initiation of antibiotics is crucial in improving the prognosis. Similarly, to the scores mentioned above, the recommendation derives from studies and guidelines considering the general population. In the Surviving Sepsis Campaign 2021, the initiation of antimicrobials is considered an emergency in patients with sepsis or septic shock. In this latter group, for each hour of delay upon administration of antimicrobials, there is a 4%-13% increase in the odds of in-hospital mortality[14]. Similar findings have been reported in patients with cirrhosis and septic shock, where each hour of delay in using appropriate antimicrobials was associated with higher mortality[18,19].

Challenges in the selection of antibiotic prophylaxis or empiric treatment in the multidrug-resistant era

It has been stated in a consensus conference regarding infections in patients with cirrhosis that randomized clinical trials on antibiotic prophylaxis are affected by several methodological pitfalls: The majority of them were under-powered, considered short follow-up periods, had methodological flaws, and were conducted more than two decades ago, in a completely different epidemiological context than the one faced today[20]. Current recommendations are based on the results of these studies, which were performed in an epidemiological setting where microorganisms responsible for infections were rarely multidrug-resistant and when gram-negative bacilli predominated over gram-positive cocci. This has changed radically in the last 20 years, with an increasing prevalence of multidrug-resistant microorganisms (MDRO), especially in patients with decompensated cirrhosis prone to hospitalizations, prolonged antibiotic prophylaxis, and invasive procedures[21]. In fact, in a recent worldwide prospective multicenter study performed by Piano *et al*[3], the global prevalence of MDRO reached 34%. These findings differed significantly by country, with a prevalence higher than 70% in India, between 20%-30% in Argentina, Canada, and several western European countries, and lower than 20% in the United States and Russia. The consequences are not trivial: Infections caused by MDRO were associated with a lower efficacy of empirical antibiotic treatment, a longer duration of antibiotic therapy, a lower rate of resolution of the infection, and a higher incidence of septic shock than those with non-MDRO infections. Most importantly, mortality was significantly higher in patients with MDRO infections[3].

Rectal colonization by MDRO may guide empirical antibiotic therapy. A recently published study showed that MDRO rectal colonization is prevalent in critically ill patients with cirrhosis (up to 47% at admission) and is associated with an increased risk of infections caused by the MDRO colonizing strains [22]. Furthermore, colonization by MDRO has also been associated with higher mortality in the liver transplant waiting list[23] and higher mortality in patients with cirrhosis and hepatocellular carcinoma [24]. All in all, the frequency of rectal colonization surveillance and its interpretation when selecting empirical therapy is yet to be defined[25].

According to experts, empiric antibiotic treatment should effectively cover approximately 80% of expected bacteria in non-critically ill patients and 90% in critically ill patients[26]. However, in the scenario mentioned above in which infections by gram-positive bacteria and multidrug organisms are increasing, prior recommendations may need to be revised. Thus, the current challenge is whether we can still safely choose antibiotic prophylaxis and treatment based on the current practice guidelines or whether these general recommendations should be regularly updated and tailored according to local epidemiological information.

Antibiotic prophylaxis in patients with cirrhosis

Antibiotic prophylaxis should be prescribed in specific clinical situations where there is a high risk for bacterial infections and when the benefit of their use outweighs the risk for adverse events and the development of antibiotic resistance[10].

Antibiotic prophylaxis in patients with acute gastrointestinal bleeding: There is broad consensus regarding prescribing antibiotic prophylaxis in acute gastrointestinal bleeding in patients with cirrhosis. This is mainly based on their high rate of bacterial infections without antibiotic use (up to 50% during the first days of hospitalization) and on the efficacy of prophylaxis in preventing infections, re-bleeding, and death[27]. Furthermore, the proposed duration of treatment is of only seven days. Thus, the risk of inducing multidrug resistance is lower than in more extended prophylaxis strategies. Regarding the choice of antimicrobial agent, a meta-analysis reports several antibiotics regimens that have a beneficial effect, with cephalosporins, quinolones, and quinolones plus beta-lactams having a more substantial protective effect than other antibiotics. Notably, no significant difference between quinolones and cephalosporins was observed[28]. However, due to the emergence of quinolone-resistant organisms, most international guidelines recommend ceftriaxone as the antibiotic of choice[27,29-31]. In countries such as the United States, where norfloxacin has been discontinued, ceftriaxone is the only recommended option[32]. The EASL 2013 position paper suggests oral norfloxacin twice daily in patients with preserved liver function as the regimen of choice, endorsing ceftriaxone in patients with decompensated cirrhosis (those with at least two of the following findings: Ascites, severe malnutrition, encephalopathy, or jaundice). Additionally, oral nitrofurantoin or ertapenem is recommended in patients with infections caused by extended-spectrum b-lactamase-producing *Enterobacteriaceae* in the last three to six months[8]. However, in a more recent publication, this scientific society endorses the use of ceftriaxone 1 g/24 h for up to seven days not only in patients with advanced cirrhosis but also in those on quinolone prophylaxis and hospital settings with a high prevalence of quinolone-resistant bacterial infections, recommending oral quinolones only for the remaining patients. They stress these recommendations should be evaluated and cross-checked from the perspective of local resistance patterns[33].

When assessing the effectiveness of current antibiotic prophylaxis strategies, a recent large multicenter study of patients with cirrhosis and variceal bleeding found that almost 20% of patients developed a bacterial infection despite using the recommendations mentioned above[34]. On the other hand, the need for routine antibiotic prophylaxis has been questioned in less severely ill patients (Child-Pugh A) due to their lower risk of infections and death[35].

Despite an acceptable consensus regarding the use of ceftriaxone as the prophylaxis of choice, this should be adapted considering the growing worldwide prevalence of MDRO, the severity of the underlying liver disease, and/or the setting of the bleeding episode (community-onset *vs* nosocomial). For example, antibiotic prophylaxis should not be the same in a patient admitted for variceal bleeding as in a patient who bleeds while in the ICU receiving antibiotics for a prior bacterial infection.

Long-term primary and secondary prophylaxis of SBP: Primary prophylaxis is proposed for patients with ascites and severe impairment of liver function, without a prior episode of SBP. The criteria used differs slightly according to different guidelines. The EASL guidelines recommend primary prophylaxis should be started on patients with low protein concentration in ascites (< 1.5 g/L), liver failure (Child-Turcotte-Pugh score > 9 and bilirubin > 3 mg/dL), and either renal dysfunction or hyponatremia[33]. In contrast, the American Association for the Study of Liver Diseases (AASLD) 2021 practice guidelines suggest primary prophylaxis could be considered in patients with the same threshold of ascitic protein accompanied by liver failure (Child-Turcotte-Pugh score > 9 and bilirubin > 3 mg/dL), renal dysfunction or hyponatremia[31]. In the latter guideline, primary prophylaxis is left to each physician's discretion since available studies are considered of variable quality and thus insufficient to support a consensus guidance recommendation. The impact of primary prophylaxis on overall survival, and not only on SBP occurrence, is a topic of ongoing research. Recently, the effect of long-term (six months) primary prophylaxis with norfloxacin has been evaluated in a randomized controlled trial that included 291 Child C patients. The risk of death at six months was significantly lower in patients with ascites fluid protein concentrations < 1.5 g/L, whereas it had no effect in patients with higher ascites protein count. Interestingly, norfloxacin significantly decreased any gram-negative bacterial infection without increasing infections caused by Clostridium difficile or MDROs[36]. Further data regarding the efficacy and safety of primary prophylaxis of SBP is expected from the ASEPTIC trial, which aims to evaluate the impact of cotrimoxazole treatment *vs* placebo during 18 mo of therapy in overall survival SBP incidence, and antimicrobial resistance, among other objectives[37].

Secondary prophylaxis (*i.e.*, in patients with at least one prior episode of SBP) rationale is based on the high risk of SBP recurrence, and the significant impact antibiotic prophylaxis has on reducing its incidence. In a trial performed more than 30 years ago, secondary prophylaxis with norfloxacin significantly reduced the probability of SBP recurrence compared to placebo (20% *vs* 68%, respectively)[38]. However, the current benefit of secondary prophylaxis with norfloxacin has recently been challenged due to the growing prevalence of quinolone-resistant bacteria and heterogeneous results in observational studies[39,40]. Several alternative strategies have been proposed to norfloxacin, using

other antimicrobials such as ciprofloxacin, rifaximin, ceftriaxone, or cotrimoxazole with different frequencies of administration (daily, five days a week, weekly). Interestingly, in a recently published meta-analysis, only daily rifaximin significantly reduced SBP recurrence compared to other antibiotics or placebo[41]. However, due to methodological concerns affecting available trials, rifaximin is not considered the standard of care for prophylaxis of SBP[42]. This poses a challenge for the treating physician when facing a patients who are under rifaximin treatment for hepatic encephalopathy that need to start prophylaxis for SBP: The aforementioned EASL guidelines state that no recommendation can be provided to guide the choice of antimicrobial among patients already on rifaximin[33]: Choosing either antibiotic or both becomes a personalized choice.

Rational selection of empiric antibiotics: Easier said than done

In daily practice, various forces drive the decision to start empiric antibiotic treatment. Given the high incidence and severe impact of bacterial infections in patients with cirrhosis, it is likely that antibiotics are overused in this population. In fact, a recent sub-analysis of the ATTIRE clinical trial suggested that half of the antibiotics prescribed to hospitalized patients with decompensated cirrhosis might not be necessary[43].

That said, the next step after confirmation or suspicion of sepsis is to start an empiric antibiotic treatment, which will be selected taking into account the site of the infection (SBP, urinary tract infection, *etc.*), the type of infection (community-acquired, health-care-associated, or nosocomial), and the pattern of resistance according to the local epidemiology. However, it is also important to consider the degree of liver failure, renal function, and potential allergies, among other variables. Another critical factor that has to be taken into account is the severity of the infection, which might be explored by evaluating the presence and number of organ failures or by calculating scores like CLIF-C AD, CLIF-C ACLF, and quick SOFA, among others[33], as was previously discussed.

Several models to predict the risk of infection by multidrug-resistance organisms were published to refine the selection of the empirical antibiotic treatment. Unfortunately, none were developed or validated in patients with cirrhosis, and their performance was moderate[44,45]. The most desirable tool to guide the selection of antibiotics would be real-time techniques that inform on the involved microorganisms and their antibiotic susceptibility pattern. Gram stain preparation is the only widely available and straightforward approach, but it provides limited information. However, in the future, other rapid molecular tests still under development or validation could give this information in minutes or hours and might help select empirical treatments in patients with cirrhosis[46].

Guidelines for antibiotic selection and protocols for rapid evaluation of patients with suspicion of sepsis are very helpful[47]. However, the need for knowledge about the expected local microorganisms and their susceptibility patterns are some of the barriers to developing these guidelines. Therefore, the World Health Assembly proposed a plan for antimicrobial resistance in 2015, which enhances surveillance of antimicrobial susceptibility patterns to generate evidence-based empiric antibiotic recommendations. Surveillance can be performed at different levels, from single institutions to states or countries. But ideally, each institution should count on sufficient granular data to generate its recommendations which would guide the treating physician to select the shortest treatment duration with the lowest-spectrum antibiotic, which will cover 80%-90% of the anticipated microorganisms using an adequate dose and route of administration[3,48].

It is known that keeping an active surveillance program that performs periodic reports and recommendations requires a multidisciplinary expert team, is time-consuming, and is costly[49]. Therefore, scientific societies or governmental organizations should implement and lead these programs and report their results at different levels. For example, Argentina and Uruguay launched a surveillance program for bacterial infections in patients with cirrhosis in October 2018, which hepatologists, infectious diseases, and epidemiologists lead and aims to serve as a platform to perform evidence-based recommendations regarding empirical antibiotic selection in this population[50].

The most recently published recommendations for empiric antibiotic treatment in patients with cirrhosis can be found in the AASLD and EASL guidelines for managing patients with decompensated cirrhosis (Table 1)[31,33]. These recommendations should be adopted with caution after revisiting the epidemiological particularities that a given center or region might have and discussing them with infectious disease specialists and microbiologists.

For example, for the case of empirical treatment of SBP, guidelines suggest using a third-generation cephalosporin or piperacillin-tazobactam. However, it should be noted that there are essential differences among third-generation cephalosporins. Ceftazidime, ceftriaxone, and cefepime are mainly used to treat community-acquired SBP, but their spectrum varies. Generally speaking, cefepime and ceftriaxone cover most gram-negative and gram-positive bacteria, which are expected to cause community-acquired SBP. However, ceftazidime does not cover gram-positive bacteria, like *Streptococcus spp*, which are known to be highly prevalent in some regions in patients with community-acquired infections, like SBP and spontaneous bacteremia[39,51]. Similarly, these guidelines recommend using fluoroquinolones (ciprofloxacin or levofloxacin) in patients with community-acquired urinary tract infection, which might offer inadequate coverage in regions where the prevalence of resistance of community uropathogens to fluoroquinolones is known or expected to be high.

Table 1 Empiric antibiotic recommendations in patients with cirrhosis, according to source, severity and type of infection

Infection	AASLD	EASL
Spontaneous infections (peritonitis, bacteremia[1], empyema)	Community acquired: Third-generation cephalosporins	Community acquired: Third-generation cephalosporins or piperacillin/tazobactam Healthcare-associated: Area dependent: Like nosocomial infections if high prevalence of MDRO or sepsis
	Nosocomial: Piperacillin/tazobactam and daptomycin (if known VRE in past or evidence of GI colonization) or meropenem if known to harbor MDR gram-negative organisms	Nosocomial: Carbapenems alone or carbapenems and daptomycin, vancomycin or linezolid if high prevalence of MDR gram-positive bacteria or sepsis
Pyelonephritis/urinary tract infection	Uncomplicated pyelonephritis: Fluoroquinolones (ciprofloxacin or levofloxacin). Severe pyelonephritis: Third-generation cephalosporins (*e.g.*, ceftriaxone). If recent antibiotic exposure: Piperacilin/tazobactam or carbapenem	Community acquired: Uncomplicated: Ciprofloxacin or cotrimoxazole. If sepsis: Third-generation cephalosporins or piperacillin/tazobactam. Healthcare-associated: Area dependent: Like nosocomial infections if high prevalence of MDROs or if sepsis. Nosocomial: Uncomplicated: Fosfomycin or nitrofurantoin. If sepsis: Meropenem and teicoplanin or vancomycin
Pneumonia	Community acquired: (1) Non-severe: B-lactam and macrolide or respiratory fluoroquinolones; and (2) Severe: B-lactam and macrolide or B-lactam and fluoroquinolones. Vancomycin can be added if patient has prior respiratory isolation of MRSA. Hospital acquired (not ventilator associated): (1) Non-severe (not septic, not intubated): One of the following: Piperacillin/tazobactam or cefepime or levofloxacin. Vancomycin can be added if MRSA was isolated in the last 90 d or if antibiotics were used in the last 90 d; and (2) Severe (presence of sepsis or requiring intubation). One of the following: Piperacilin tazobactam or cefepime or meropenem and levofloxacin. Vancomycin can be added if MRSA was isolated in the last 90 d or if antibiotics were used in the last 90 d. Pseudomonas coverage: If there is prior respiratory isolation of pseudomonas of recent use of parenteral antibiotics or hospitalization	Community acquired: Piperacillin/tazobactam or ceftriaxone and macrolide or levofloxacin or moxifloxacin. Healthcare-associated: Area dependent: Like nosocomial infections if high prevalence of MDROs or if sepsis. Nosocomial: Ceftazidime or meropenem and levofloxacin ± glycopeptides or linezolid
Cellulitis	Moderate (with systemic signs of infection): Penicillin or ceftriaxone or cefazolin or clindamycin. Severe (failed antibiotics, presence of sepsis): Vancomycin and piperacillin/tazobactam	Community acquired: Piperacillin/tazobactam or third-generation cephalosporins and oxacillin. Healthcare-associated: Area dependent: Like nosocomial infections if high prevalence of MDROs or if sepsis. Nosocomial: Third-generation cephalosporin or meropenem and oxacillin or glycopeptides or daptomycin or linezolid

[1]European Association for the Study of the Liver refers only to spontaneous bacterial peritonitis and spontaneous bacterial empyema.
AASLD: American Association for the Study of the Liver; EASL: European Association for the Study of the Liver; GI: Gastrointestinal; MDR: Multidrug-resistant; MDROs: Multidrug-resistant microorganisms; MRSA: Methicillin-resistant *Staphylococcus aureus*; VRE: Vancomycin-resistant enterococcus.

Final thoughts

There is an evident conflict between ensuring adequate antibiotic prophylaxis or empiric treatment and rationalizing broad-spectrum antibiotics in patients with cirrhosis. After reviewing the literature in search of information that may be useful to guide the rational use of antibiotics in this population, several shortcomings emerge. There is insufficient granular data on the susceptibility patterns of the microorganisms involved in bacterial infections. This should stimulate research and publications of descriptive studies that serve as a platform for developing evidence-based guidelines. Many centers worldwide likely have valuable information that needs to be published. Part of the complexity of this type of research is that the microorganisms involved and their susceptibility patterns change over time. Therefore, it is necessary to have sustained surveillance programs and not just short-term studies.

CONCLUSION

Since the World Health Organization anticipates that drug resistance will have a catastrophic impact on health systems and the global economy by 2050, all healthcare professionals that participate at different levels in the care of patients with cirrhosis should advocate for the rational use of antibiotics.

ACKNOWLEDGEMENTS

To Astrid Smud and Laura Barcan from the Infectious Disease Unit of the Hospital Italiano de Buenos Aires for their continuous support.

FOOTNOTES

Author contributions: All authors performed the literature research, wrote and revised the manuscript.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

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S-Editor: Wang JJ
L-Editor: A
P-Editor: Wang JJ

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World J Hepatol 2023 March 27; 15(3): 386-392

DOI: 10.4254/wjh.v15.i3.386

ISSN 1948-5182 (online)

MINIREVIEWS

Emerging role of engineered exosomes in nonalcoholic fatty liver disease

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Specialty type: Gastroenterology and hepatology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Forlano R, United Kingdom; Thandassery RB, United States

Received: December 26, 2022
Peer-review started: December 26, 2022
First decision: February 1, 2023
Revised: February 20, 2023
Accepted: March 15, 2023
Article in press: March 15, 2023
Published online: March 27, 2023

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide. NAFLD comprises a continuum of liver abnormalities from nonalcoholic fatty liver to nonalcoholic steatohepatitis, and can even lead to cirrhosis and liver cancer. However, a well-established treatment for NAFLD has yet to be identified. Exosomes have become an ideal drug delivery tool because of their high transmissibility, low immunogenicity, easy accessibility and targeting. Exosomes with specific modifications, known as engineered exosomes, have the potential to treat a variety of diseases. Here, we review the treatment of NAFLD with engineered exosomes and the potential use of exosomes as biomarkers and therapeutic targets for NAFLD.

Key Words: Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Exosome; Engineered exosome; Targeted therapy

Core Tip: Nonalcoholic fatty liver disease (NAFLD) is the fastest growing chronic disease in the world. As the disease progresses, NAFLD can lead to liver fibrosis, cirrhosis and even liver cancer. However, a well-established treatment for NAFLD has yet to be identified. Exosomes are small extracellular vesicles secreted by cells. Owing to their high delivery efficiency and biocompatibility, exosomes are expected to become a new means of drug delivery and precise treatment for a variety of diseases, including NAFLD.

Citation: Ding J, Xu C, Xu M, He XY, Li WN, He F. Emerging role of engineered exosomes in nonalcoholic fatty liver disease. *World J Hepatol* 2023; 15(3): 386-392
URL: https://www.wjgnet.com/1948-5182/full/v15/i3/386.htm
DOI: https://dx.doi.org/10.4254/wjh.v15.i3.386

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a metabolic disease that is prevalent worldwide affecting at least a quarter of the population[1]. NAFLD is a continuum of liver abnormalities from nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH) that can even lead to cirrhosis and liver cancer. NAFL is reversible, whereas NASH with cirrhosis is difficult to reverse[2]. Therefore, it is critical to explore the pathogenesis of NAFLD and identify therapeutic targets to treat or prevent its development. Exosomes are extracellular vesicles with a particle size of 30-150 nm that play a crucial role in communication between cells[3]. Some macromolecules such as RNA or proteins in exosomes are associated with the occurrence and development of liver-related diseases and can be used as potential molecular markers in the diagnosis of NAFLD[4]. Processed and modified exosomes (known as engineered exosomes) may also facilitate the study of NAFLD and the development of new therapeutic strategies[5]. In this review, the mechanism and function of engineered exosomes in the development of NAFLD are reviewed (Figure 1).

ENGINEERED EXOSOMES AND LIPID METABOLISM

The liver is the largest metabolic organ and a hub of lipid metabolism. Abnormal changes in lipid metabolism in the liver lead to the development of metabolic diseases[6]. A research team found that the release of exosomes in cultured astrocytes from apolipoprotein E knockout mice was significantly reduced compared to wild-type controls, and a PI3K inhibitor (LY294002) rescued the release of exosomes. They confirmed that the release of exosomes was regulated by cellular cholesterol through stimulation of the PI3K/Akt signalling pathway[7].

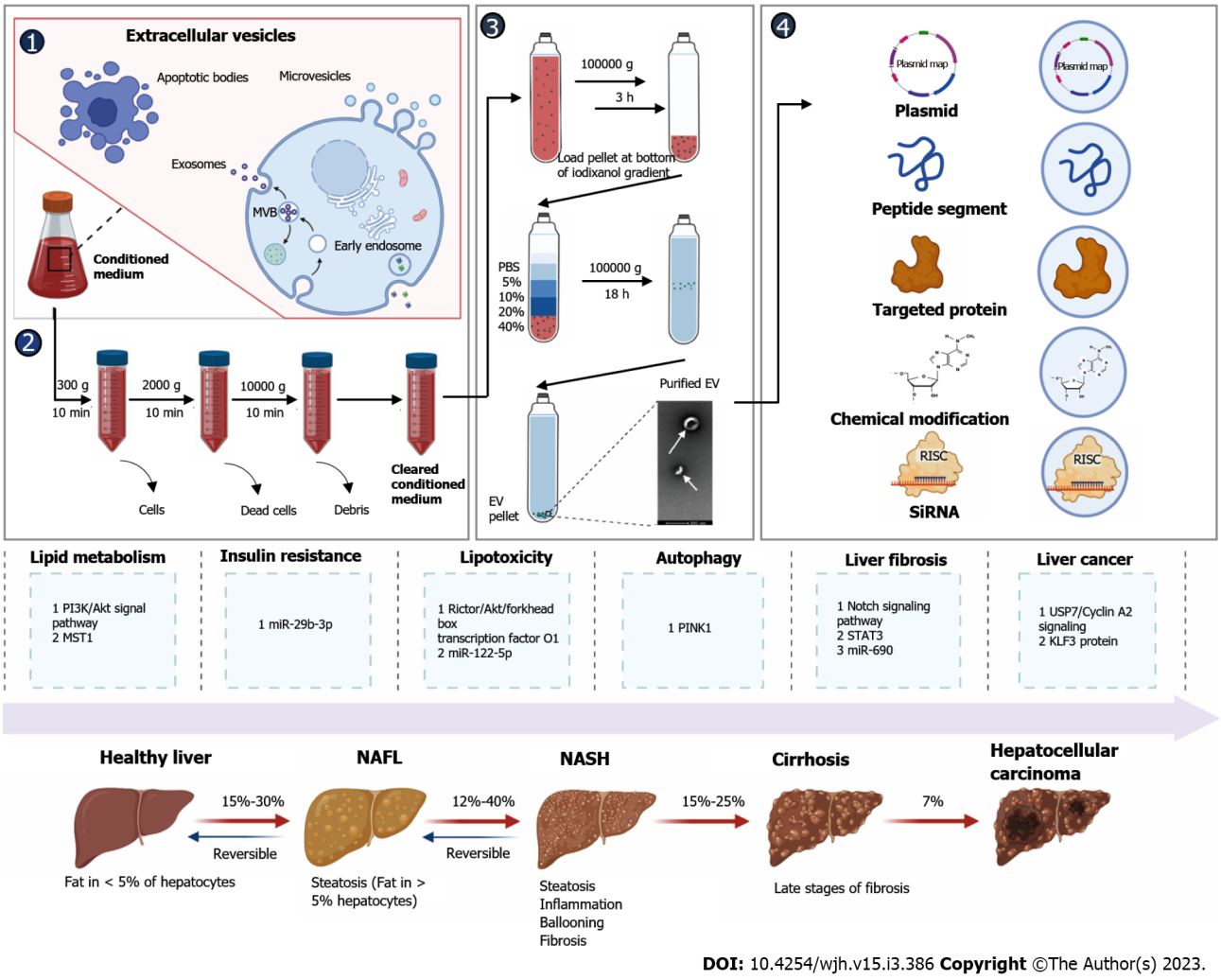
Li *et al*[8] systematically screened for microRNA expression using high-throughput small RNA sequencing and found that miR-199a-5p was significantly upregulated in adipose tissue in a mouse model of high-fat diet (HFD). Further studies confirmed that exosomal miR-199a-5p promoted lipid accumulation in the liver through induction of macrophage stimulating 1 (MST1) expression and fatty acid metabolism. Cheng *et al*[9] found that exosomal miR-627-5p reversed insulin resistance, prevented liver injury, normalized glucose and lipid metabolism and reduced lipid deposition in a rat model of NAFLD.

Brown adipose tissue (BAT) strongly promotes energy expenditure and shows good potential in the treatment of obesity. Zhou *et al*[10] treated HFD-fed mice with engineered exosomes derived from the serum of young healthy mice or from BAT. They found that treatment with BAT exosomes significantly promoted oxygen consumption in recipient cells, thus alleviating metabolic syndrome in HFD-fed mice.

Li *et al*[11] used a low-density lipoprotein receptor-deficient mouse (Ldlr mouse) as a model for hypercholesterolemia. Ldlr mRNA was encapsulated into exosomes by overexpression of Ldlr in donor AML12 mouse hepatocytes. The authors found that engineered exosomes loaded with Ldlr mRNA could restore the expression of Ldlr in the livers of Ldlr-deficient mice and rescue hypercholesterolemia. This study suggests that engineered exosomes may be an effective therapy for patients with hypercholesterolemia.

ENGINEERED EXOSOMES AND INSULIN RESISTANCE

Insulin resistance is now believed to play a key role in the onset and progression of NAFLD[12]. A HFD reduces insulin sensitivity. Kumar *et al*[13] found that feeding a HFD changed the lipid composition of intestinal exosomes. These exosomes were found to be absorbed by macrophages and hepatocytes, resulting in inhibition of the insulin signalling pathway. Castaño *et al*[14] found that obesity can alter the



DOI: 10.4254/wjh.v15.i3.386

Figure 1 Diagram shows the correlation between nonalcoholic fatty liver disease and engineered exosome. MVB: Multivesicular body; EV: Extracellular vesicles; NASH: Nonalcoholic steatohepatitis; NAFL: Nonalcoholic fatty liver; STAT3: Signal transducer and activator of transcription 3; RISC: RNA-induced silencing complex; MST1: Mammalian STE20-like kinase 1; USP7: Ubiquitin specific peptidase 7; KLF3: Kruppel-like factor 3; PINK: PETN induced kinase 1; PI3K: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase; Akt: Protein kinase B.

expression and composition of miRNAs in mouse plasma exosomes. Ying *et al*[15] found that miR-690, an exosome-derived miRNA from M2-polarized macrophages, improved insulin sensitivity in obese mice. Su *et al*[16] found that exosomes derived from the bone marrow mesenchymal stem cells (BM-MSCs) of aged mice could be ingested by fat, muscle and liver cells, leading to insulin resistance *in vivo* and *in vitro*. The authors found that the amount of miR-29b-3p in exosomes released by BM-MSCs was significantly increased in aged mice. Furthermore, they found that inhibition of miR-29b-3p with an aptamer-mediated nanocomposite delivery system improved insulin resistance in aged mice.

ENGINEERED EXOSOMES AND LIPOTOXICITY

Lipotoxicity promotes proinflammatory M1 polarization of liver macrophages during the development of NAFLD[17,18]. Liu *et al*[19] found that miR-192-5p-rich hepatocyte-exosomes induced by lipotoxic injury promoted macrophage M1 polarization and liver inflammation through Rictor/Akt/forkhead box transcription factor O1 signalling. Zhao *et al*[20] found that cholesterol-induced lysosomal dysfunction increased exosome release from hepatocytes, leading to M1 polarization and macrophage-induced inflammation in a miR-122-5p-dependent manner. Human umbilical cord mesenchymal stem cells (HUC-MSCs) are increasingly being studied in clinical trials of end-stage liver disease due to their excellent tissue repair and anti-inflammatory effects. Shi *et al*[21] found that HUC-MSC-derived exosomes could protect against methionine- and choline-deficient L-amino acid diet (MCD)-induced NASH.

Lipotoxicity can damage mitochondria and induce oxidative stress during the progression of NAFLD [22,23]. Studies have shown that adipocytes respond to mitochondrial stress by rapidly and vigorously

releasing exosomes[24]. Similarly, exosomes derived from chemically induced human hepatic progenitors inhibit cell death induced by oxidative stress[25].

ENGINEERED EXOSOMES AND AUTOPHAGY

Autophagy is a process in which cells degrade and metabolize their own damaged organelles or protein aggregates that plays a key role in maintaining liver homeostasis[26]. Increasing evidence suggests that autophagy plays a very important role in lipid metabolism. Autophagy mainly protects cells and regulates inflammation in NAFLD[26]. Because autophagy and exosomal biogenesis share common elements, some studies have found that plasma exosomal levels are higher in NAFLD patients than in healthy controls[27]. Luo *et al*[28] found that miR-27a inhibited mitochondrial autophagy and promoted NAFLD-associated liver fibrosis by negatively regulating PINK1 expression *via* lipotoxic hepatocyte exosomes. A research team established a model of hepatocyte injury and apoptosis induced by D-galactosamine and lipopolysaccharide (D-GalN/LPS) to study the protective effect of bone marrow mesenchymal stem cell (BMSC)-derived exosomes on liver injury. They found that BMSC-derived exosomes attenuated D-GaIN/LPS-induced hepatocyte apoptosis by activating autophagy *in vitro*[29]. Similar studies have shown that upregulation of miR-96-5p in BMSCs and their exosomes ameliorated NASH *via* caspase-2[30].

ENGINEERED EXOSOMES AND LIVER FIBROSIS

It is generally believed that during the development of NAFLD, liver-related cells are replaced by fibrotic scar tissue, giving rise to liver fibrosis or cirrhosis, which are associated with poor prognosis and mortality in patients with NASH[2]. The Notch signalling pathway is a key mediator of cellular differentiation, proliferation and apoptosis[31]. We designed hairpin-type decoy oligodeoxynucleotides (ODNs) for RBP-J to inhibit the activation of Notch signalling. ODNs were loaded into HEK293T-derived exosomes by electroporation. Furthermore, we observed that tail vein-injected exosomes were mainly taken up by hepatic macrophages in mice with hepatic fibrosis. RBP-J decoy ODNs delivered by exosomes efficiently inhibited Notch signalling in macrophages and ameliorated liver fibrosis in mice [32].

Hou *et al*[33] found that myeloid cell-specific IL-6 signalling promoted miR-223-enriched exosome production and attenuated NAFLD-associated fibrosis. Tang *et al*[34] found that exosomes embedded with siRNAs or antisense oligonucleotides targeting signal transducer and activator of transcription 3 (STAT3) could attenuate liver fibrosis. Gao *et al*[35] showed that Kupffer cells produced endogenous miR-690 and shuttled this miRNA to other hepatocytes through exosomal secretion. Treatment with miR-690 inhibitors reduced fibrosis and steatosis in a NASH model. Wang *et al*[36] found that miR-6766-3p-rich 3D human embryonic stem cell (hESC) exosomes could ameliorate liver fibrosis by targeting the TGFβ RII-SMADS pathway in hepatic stellate cells. Ji *et al*[37] developed an exosome-liposome hybrid loaded with clodronate-nintedanib that impaired hepatic fibrosis by reducing the activation of Kupffer cells.

CRISPR-Cas9 gene editing has become a powerful therapeutic technology. However, there is a lack of safe and effective *in vivo* delivery systems for CRISPR-Cas9, especially for tissue-specific vectors[38]. Luo *et al*[39] used exosome-mediated CRISPR/dCas9-VP64 delivery to reprogram hepatic stellate cells to construct engineered exosomes for the treatment of liver fibrosis. Similarly, Wan *et al*[40] delivered exosome-mediated Cas9 ribonucleoprotein complexes for tissue-specific gene therapy in liver disease.

ENGINEERED EXOSOMES AND LIVER CANCER

Without timely intervention, NAFLD inevitably results in liver cancer[41]. Liver cancer is the fourth leading cause of cancer-related death worldwide and occurs in patients with various chronic liver diseases[42]. To date, the exact pathogenesis of NAFLD-induced liver cancer is not fully understood, but may involve DNA damage responses, inflammation, autophagy, and disruption of the gut microbiota [41].

Adipose tissue is known to play a role in energy storage and metabolic regulation by secreting adipokines[43]. Studies have demonstrated that exosomal circRNA secreted by adipocytes promotes tumour growth by inhibiting miR-34a and activating the USP7/Cyclin A2 signalling pathway[44].

An acidic microenvironment has been shown to promote the release of exosomes, which are considered to be cell-to-cell communication agents involved in cancer progression and metastasis[45]. Tian *et al*[46] found that exosomal miR-21 and miR-10b induced by the acidic microenvironment in liver cancer could promote cancer cell proliferation and metastasis and be used as prognostic molecular markers and therapeutic targets for liver cancer.

Macrophage-derived exosomes play multiple roles in cancer initiation and progression[47]. Zhang *et al*[48] found that exosomes derived from RBP-J overexpressing macrophages inhibited the progression of liver cancer by miR-499b-5p/JAM3. M2 macrophages can influence tumour development by secreting various cytokines, including exosomes. Some studies suggest that M2 macrophage-derived exosomes modified by miR-660-5p-related oligonucleotides enhanced the development of hepatocellular carcinoma by regulating KLF3[49].

ENGINEERED EXOSOMES INVOLVED IN THE DIAGNOSIS OF NAFLD

Exosomes can be derived from healthy and stressed cells to provide a snapshot of the cell of origin under physiological and pathological conditions. Hepatocyte-derived exosomes released from stressed/injured hepatocytes have been identified as a partial cause of liver disease progression and liver injury, so circulating exosomes may serve as biomarkers of NAFLD. Nanopasmon-enhanced scattering of gold nanoparticles coupled with hepatocyte-specific antibodies was used to identify hepatocyte-derived exosomes[50]. Furthermore, microarray analysis of exosomal miRNAs isolated from the serum of 41 patients with NAFLD (diagnosed using liver biopsy) suggested that serum exosomal miRNAs could be used to assess the severity of NAFLD and identify potential targets for NAFLD treatment[33]. One of the determinants of liver degeneration in the progression of NAFLD is Wnt/frizzled (FZD) signalling; for example, FZD7 delivered by plasma-derived exosomes is a good candidate for a novel and effective biomarker for the diagnosis and prognosis of NAFLD[51].

CONCLUSION

The incidence of NAFLD is rapidly increasing with changes in lifestyle and dietary habits[1]. Exosomes not only mediate communication between cells but can also be engineered to deliver specific substances. Engineered exosomes have shown some effects on NAFLD in animal experiments. Owing to their low immunogenicity and liver targeting[52,53], engineered exosomes have great potential to treat NAFLD.

FOOTNOTES

Author contributions: Ding J contributed to writing the original draft; Xu C contributed to picture making; Xu M, He XY, and Li WN contributed to data collection; He F contributed to designed the review and revised the final version.

Supported by National Natural Science Foundation of China, No. 81970535.

Conflict-of-interest statement: All the authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Country/Territory of origin: China

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S-Editor: Liu JH
L-Editor: A
P-Editor: Liu JH

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World Journal of Hepatology

Submit a Manuscript: https://www.f6publishing.com

World J Hepatol 2023 March 27; 15(3): 393-409

DOI: 10.4254/wjh.v15.i3.393

ISSN 1948-5182 (online)

ORIGINAL ARTICLE

Basic Study

mRNA transcriptome profiling of human hepatocellular carcinoma cells HepG2 treated with *Catharanthus roseus*-silver nanoparticles

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Specialty type: Nanoscience and nanotechnology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Liu W, China; Rajeshwari K, India

Received: October 27, 2022
Peer-review started: October 27, 2022
First decision: December 31, 2022
Revised: January 17, 2023
Accepted: March 3, 2023
Article in press: March 3, 2023
Published online: March 27, 2023

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Abstract

BACKGROUND

The demand for the development of cancer nanomedicine has increased due to its great therapeutic value that can overcome the limitations of conventional cancer therapy. However, the presence of various bioactive compounds in crude plant extracts used for the synthesis of silver nanoparticles (AgNPs) makes its precise mechanisms of action unclear.

AIM

To assessed the mRNA transcriptome profiling of human HepG2 cells exposed to *Catharanthus roseus* G. Don (*C. roseus*)-AgNPs.

METHODS

The proliferative activity of hepatocellular carcinoma (HepG2) and normal human liver (THLE3) cells treated with *C. roseus*AgNPs were measured using MTT assay. The RNA samples were extracted and sequenced using BGIseq500 platform. This is followed by data filtering, mapping, gene expression analysis, differentially expression genes analysis, Gene Ontology analysis, and pathway analysis.

RESULTS

The mean IC_{50} values of *C. roseus*AgNPs on HepG2 was 4.38 ± 1.59 μg/mL while on THLE3 cells was 800 ± 1.55 μg/mL. Transcriptome profiling revealed an alteration of 296 genes. *C. roseus*AgNPs induced the expression of stress-associated genes such as *MT*, *HSP* and *HMOX-1*. Cellular signalling pathways

were potentially activated through MAPK, TNF and TGF pathways that are responsible for apoptosis and cell cycle arrest. The alteration of *ARF6*, *EHD2, FGFR3, RhoA, EEA1, VPS28, VPS25, and TSG101* indicated the uptake of *C. roseus*-AgNPs *via* both clathrin-dependent and clathrin-independent endocytosis.

CONCLUSION

This study provides new insights into gene expression study of biosynthesised AgNPs on cancer cells. The cytotoxicity effect is mediated by the aberrant gene alteration, and more interestingly the unique selective antiproliferative properties indicate the *C. roseus*AgNPs as an ideal anticancer candidate.

Key Words: *Catharanthus roseus*; HepG2; Silver nanoparticles; Transcriptome; oxidative stress; Apoptosis; Cell cycle

Core Tip: Despite the increased attention on cancer nanomedicine which is advantageous to overcome the limitations of conventional cancer treatment, the information on the selectivity and detailed mechanisms at the cellular and molecular level remain unclear. To evaluate its selectivity effects, the proliferative activity of both liver cancer cells HepG2 and normal liver cells THLE-3 in response to *Catharanthus roseus*-silver nanoparticles (*C. roseus*-AgNPs) was assessed. To determine the possible signalling pathways induced by the *C. roseus*-AgNPs, the mRNA transcriptome profiling of hepatocellular carcinoma cell line HepG2 was performed, highlighting the expression of genes associated with oxidative stress, apoptosis, and cell cycle arrest. The elucidation of its selectivity effects and detailed wide genome screening would enlighten the cellular and molecular signalling pathways and provide a strong basis towards the development of *C. roseus*-AgNPs as an anticancer drug for liver cancer.

Citation: Azhar NA, Abu Bakar SA, Citartan M, Ahmad NH. mRNA transcriptome profiling of human hepatocellular carcinoma cells HepG2 treated with *Catharanthus roseus*-silver nanoparticles. *World J Hepatol* 2023; 15(3): 393-409

URL: https://www.wjgnet.com/1948-5182/full/v15/i3/393.htm

DOI: https://dx.doi.org/10.4254/wjh.v15.i3.393

INTRODUCTION

Nanoparticles are materials or discrete clusters of atoms having dimensions within 1-100 nm[1]. Having a large surface area-to-volume ratio with unique biological properties, nanoparticles have gained immense usage in the early diagnosis and treatment of cancer, the application of which is termed nano oncology[2,3]. Nanoparticles can offer an alternative to the current conventional chemotherapeutic agents which although exhibit high efficacy in killing cancer cells, still suffer from significant drawbacks due to the poor specificity in causing severe damage to healthy cells[4]. Amongst various nanoparticles, silver nanoparticles (AgNPs) have been reported to demonstrate a significant biological effect, particularly in the healthcare industry[5,6]. Concurrently, the market demand for eco-friendly, hazard-free, and cost-effective synthesis of AgNPs was higher as many of the common nanoparticle production methods involved hazardous chemicals and high energy- consumption[7]. One of the most effective biogenic approaches is to use plant extract that contains metabolites, which can enhance the reduction of silver ions. Plant extract-mediated silver nanoparticle synthesis is found to have a promising anticancer property. Plant extract-based synthesis is largely favoured due to the lower degree of adverse effect as well as the low cost of synthesis that enables large-scale production. Moreover, biologically active ingredients or phytomolecules in the plant extract act as reducing agents to promote the synthesis of AgNPs[8]. A previous study has corroborated the anticancer property of AgNPs, for example, biosynthesised AgNPs using *Acalypha Indica*, which exhibited anti-cancer activity against human breast cancer cell line MDA-MB-231[9]. In another study, AgNPs synthesised using leaf extract of *Tropaeolum majus* L. also demonstrated anti-cancer properties on the MCF7 cell line[10]. These findings cumulatively proved the anti-cancer property of the biogenic AgNPs.

Previously, an herbal plant *Catharanthus roseus* (*C. roseus*) G. Don has demonstrated its ability as a reducing agent to synthesise AgNPs. This plant is commonly known as periwinkle which belongs to the Apocynaceae family[11]. This plant is very synonymous with its content, indolomonoterpenic alkaloids vincristine and vinblastine[12]. These compounds are commonly used in the treatment of several

malignant conditions, such as Hodgkin's and non-Hodgkin's lymphomas, acute lymphoblastic leukaemia, neuroblastoma and breast carcinoma[13]. These alkaloids may be responsible for the reduction of the silver ions to AgNPs and at the same time exert their function by disrupting the mitotic spindle apparatus of microtubules through tubulin interaction, thus blocking the mitosis process, and arresting the cancer cells during metaphase[14].

An understanding of the anti-cancer mechanisms of AgNPs at the molecular level would provide detailed insight into various physiological processes involved. This is achievable *via* transcriptome analysis, a holistic view of gene expression. An overview or snapshot of the gene expression landscape could reveal the intricate molecular network that underlies the myriad of biological processes in a cell. As compared to hybridisation-based RNA quantification methods such as microarray analysis, this sequencing-based transcriptome detection can perform well within a wide range of circumstances, where this method could quantify gene expression with low background, high accuracy, and high reproducibility levels with significant dynamic range transcriptome analysis can detect subtle changes in gene expression, mutations, splice variants and fusion genes that cannot be identified by microarrays [15].

Fuelled by the intriguing capacity of the transcriptome analysis, in this study, we endeavoured to carry out an mRNA transcriptome profiling of the human hepatocellular carcinoma cell (HepG2) treated with AgNPs synthesised using an aqueous extract of *C. roseus* G. Don. The human hepatocellular carcinoma cell (HepG2) was used as a representative *in vitro* cancer cell line model, due to its known well-characterised property of cell line and its wide usage in many toxicity studies for screening hepatotoxic compounds[16]. To the best of our knowledge, there was no study reported on the transcriptome profiling of cancer cells treated with plant extract-mediated synthesised AgNPs. As such, this study is the first study that focuses on the transcriptome profiling of cancer cells treated with AgNPs synthesised using plant extract. This study can be a significant step in identifying potential genes that are regulated by the treatment of *C. roseus*-AgNPs on HepG2 cells, which will lead to the establishment of the underlying molecular network of the mechanistic actions of the AgNPs.

MATERIALS AND METHODS

Preparation of cell line

The hepatocellular carcinoma cell line HepG2 used in this study was purchased from American Type Culture Collection (ATCC, Cat. HB-8065™, Rockville, MD, United States). Complete RPMI-1640 medium supplemented with 10% heat-inactivated foetal bovine serum, 1% penicillin-streptomycin (v/v) and 1% L-glutamine (v/v) was used to culture and maintain the cells. All the reagents were purchased from Nacalai Tesque (Kyoto, Japan). Meanwhile, a normal liver cell line (THLE-3) (ATCC) was cultured in Bronchial Epithelial Cell Growth Basal Medium (Lonza, Basel, Switzerland) supplemented with frozen additives without gentamycin/Amphotericin and Epinephrine, 5 ng/mL EGF, 70 ng/mL Phosphoethanolamine and 10% fetal bovine serum. The incubator used for the cell culture work was set at 37 °C with 5% CO_2 (Shellab, Cornelius, OR, United States). Upon reaching 80% confluency, the cells were subcultured and transferred into new cell culture flasks. The cells were seeded at a concentration of 1×10^5 cells/mL.

Preparation of C. roseus G. Don aqueous extract

The *C. roseus* aqueous extract was prepared according to our previous study[17]. A voucher specimen of *C. roseus* plant was deposited at the Herbarium of Universiti Sains Malaysia with reference number 10933. The leaves were washed using free-flowing clean water and left dried in an oven at 40 °C. The leaves were first ground before mixing with double distilled water with a ratio of 50 g: 1 L in a conical flask. Following overnight incubation in a water bath at 40 °C, the mixture was centrifuged at 2000 rpm for 15 min. The filtered supernatant was freeze-dried and ready to be used for the preparation of *C. roseus*-AgNPs.

Preparation of C. roseus G. Don-AgNPs

The *C. roseus*-AgNPs used in this study have been successfully synthesised, optimised, and characterised in our previous study[5]. The optimised *C. roseus*-AgNPs consist of 10% of *C. roseus* aqueous extract and 5 mmol/L of silver nitrate ($AgNO_3$) solution. The mixture was allowed to react in a dark environment at room temperature for 24 h until the colour changes from light yellowish to dark brownish. The mixture was then collected and centrifuged for 15 min at 10000 rpm. The supernatant was discarded while the pellet was collected and freeze-dried.

Cell viability

The proliferative activity of HepG2 and THLE-3 cells was assessed using Cell Titer 96® AQueous Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, WI, United States) which consists of (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) dye solution or also known as MTT and solubilisation solution. The method was performed according to the manufacturer's protocol. HepG2

cells and THLE-3 cells were seeded in a 96-well plate (Eppendorf, Hamburg, Germany) at a concentration of 1 × 10^5 cells/mL. Cells were treated with *C. roseus*-AgNPs (Merck, Billerica, MA, United States) in serial dilution manner which was 1.96 µg/mL, 3.91 µg/mL, 7.82 µg/mL, 15.63 µg/mL, 31.25 µg/mL, 62.5 µg/mL, 125 µg/mL, 250 µg/mL, 500 µg/mL, and 1000 µg/mL. The cells were incubated for 24, 48, and 72 h at 37 °C, 5% incubator. Untreated cells were used as a control. Each sample size was prepared in triplicate. Following the indicated incubation time, each well was added with 20 µL of MTT reagent and further incubated for 4 h in a humidified 5% CO_2 incubator at 37 °C. After 4 h of incubation, 100 µL of stop solution was added to each well and incubated for 1 h to solubilise the formazan. The absorbance at 570 nm was recorded using a microplate reader (Bio Tek, Winooski, VT, United States). The half-maximal inhibitory concentration (IC_{50}) values were calculated based on the following formula:

% Cell Viability = [Mean OD_{sample} - OD_{blank}]/[Mean $OD_{control}$ - OD_{blank}] × 100

OD = Optical Density

Treatment of HepG2 cells with C. roseus-AgNPs and total RNA extraction

The HepG2 cells were seeded approximately at 1 × 10^5 cells/mL. The seeded cells were treated with *C. roseus*-AgNPs at a concentration of 4.95 µg/mL, which is the IC_{50} value used in our previous study[5] and incubated for 72 h at 37 °C in a humidified atmosphere of 5% CO_2. Untreated HepG2 cells were used as a control. After 72 h of exposure, the cells were washed with PBS and immediately lysed and homogenised in TRIzol™ Reagent (Thermo Fisher, Waltham, MA, United States). Total RNA extraction was carried out using the manufacturer's protocol. The resulting pellet was solubilised in RNAse-free water and was kept at -80 °C until further processing. The purity and concentration of RNA (260/280 ratio) were determined using Nanodrop 2000 spectrophotometer (NanoDrop Products, Wilmington, DE, United States). The integrity of the total isolated RNA was assessed by Agilent 2100 Bioanalyser (Agilent RNA 6000 Nano Kit, Santa Clara, CA, United States).

Beijing Genomics Institute sequencing

All RNA samples were sent to Beijing Genomics Institute (BGI, Shenzen, China) for sequencing. The total RNA extracted was pre-processed for transcriptome sequencing. The poly-A-containing mRNA molecules were captured and purified using a technique probe. The purified RNA molecules were reverse-transcribed into the first-strand cDNA, subsequently followed by the second-strand cDNA synthesis using Polymerase I and treatment with RNase H. The resulting product was purified and enriched with PCR amplification. The PCR amplicon was quantified by Invitrogen Qubit 2.0 Fluorometer (Thermo Fisher). The amplicon libraries were pooled together to make a single-strand DNA circle (ssDNA circle). DNA nanoballs (DNBs) were generated from the ssDNA circle by rolling circle amplification and loaded into a flow cell in which DNB binding sites are patterned nano-arrays. Sequencing was carried out using a paired-end 100 bp sequencing strategy on the BGIseq500 platform.

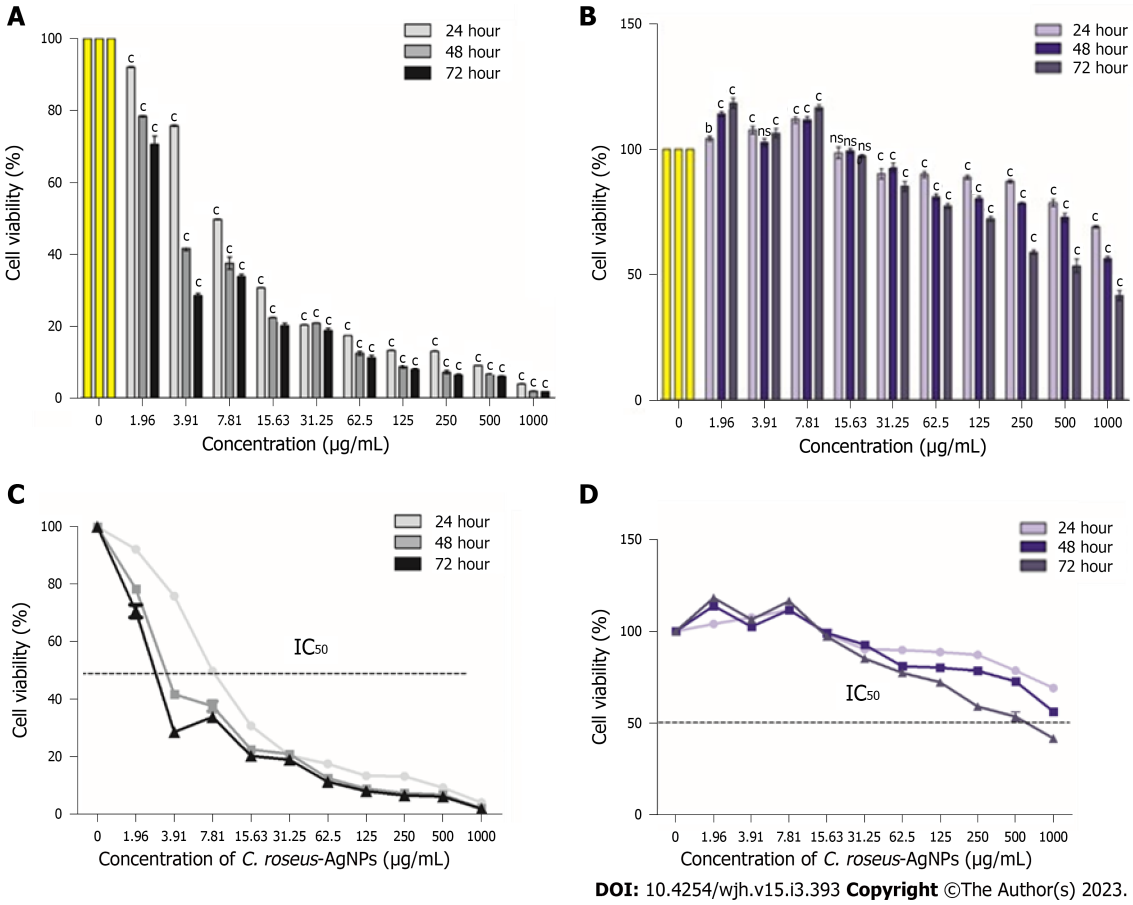
Bioinformatics analysis

High-quality genome sequencing data was developed by removing the adapter, poor quality and low complexity reads. The cleaned sequences were mapped onto the reference genome (hg19), subsequently followed by the identification of the novel genes, SNP (single nucleotide polymorphism), InDels (insertions and deletions) and the detection of gene splicing. Differential Gene Expressions were obtained by applying a paired, two-tailed t-test to the calculated expression data of the treated and untreated samples. Gene Ontology (GO) analysis was used to analyse the enrichment of gene sets associated with biological processes, molecular functions, and cellular components. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was carried out to permit the pathway annotation to the differentially expression genes (DEGs). A *P* value less than 0.1 is considered a statistically significant difference.

RESULTS

Cytotoxicity of C. roseus-AgNPs on HepG2 Cells

In this study, the cytotoxic effects of *C. roseus*-AgNPs were assessed on HepG2 cells and normal liver epithelial cells, THLE-3 cells. Figure 1A shows the cytotoxicity effects of HepG2 cells after treatment with *C. roseus*-AgNPs. In comparison to the untreated cells, *C. roseus*-AgNPs significantly ($P < 0.001$) inhibited the proliferation of HepG2 cells at all concentrations and incubation periods in time- and dose-dependent manner, indicating the cytotoxic effect of *C. roseus*-AgNPs towards HepG2, with 7.79 %, 21.59%, and 30.15% of cells were inhibited at the lowest concentration of *C. roseus*-AgNPs at 24, 48 and 72 h, respectively. HepG2 cells showed a consistent percentage decrement of cell viability upon the treatment, and only 1.78 % average of the cells survived between 24 to 72 h of incubation at the highest concentration of *C. roseus*-AgNPs. The percentage of *C. roseus*-AgNPs cytotoxicity compared to the untreated cells was used to determine the IC_{50} values as illustrated in Figure 1B where the IC_{50} were 7.81 ± 0.02 µg/mL, 3.87 ± 0.02 µg/mL, and 3.20 ± 0.04 µg/mL at 24, 48, and 72 h of incubation, respectively.



DOI: 10.4254/wjh.v15.i3.393

Figure 1 Cytotoxicity evaluation of *Catharanthus roseus*-silver nanoparticles on HepG2 and THLE-3 cells. A: The cytotoxicity of HepG2 cell lines treated with different concentrations of *Catharanthus roseus*-silver nanoparticles (*C. roseus*-AgNPs); B: The IC_{50} of *C. roseus*-AgNPs on HepG2 cells; C: The cytotoxicity of THLE3 cell lines treated with different concentrations of *C. roseus*-AgNPs; D: The IC_{50} of *C. roseus*-AgNPs on THLE-3 cells. All experiments were done in triplicate, and the data represent means ± standard deviations. The comparison between each concentration with untreated cells was done using two-way ANOVA with Dunnet post-test to detect any significant differences ($^bP < 0.01$; $^cP < 0.001$; ns not significant). *C. roseus*-AgNPs: *Catharanthus roseus*-silver nanoparticles.

Figure 1C shows the effect of THLE-3 treated with *C. roseus*-AgNPs. The results demonstrate an intriguing finding, where the *C. roseus*-AgNPs demonstrated a substantial ($P < 0.001$) increment in THLE3 proliferation at concentrations of 1.96 µg/mL and 7.81 µg/mL for all incubation times. On the contrary, during all incubation times, there was no significant difference at concentrations of 15.63 µg/mL. However, at concentrations 31.25 µg/mL to 1000 µg/mL, *C. roseus*-AgNPs significantly ($P < 0.001$) inhibited the proliferation of THLE3 cells as compared to untreated THLE3 cells. After 72 h, approximately 55.78% of cells survived at the highest concentration of *C. roseus*-AgNPs. There were no IC_{50} values at concentrations for 24 and 48 h, but at 72 h, the IC_{50} was recorded at 615 ± 0.05µg/mL µg/mL, as depicted in Figure 1D. Based on the results, we observed that *C. roseus*-AgNPs was found to inhibit the growth of the HepG2 cell line with a mean IC_{50} value of 4.95 ± 0.03 µg/mL. Contrarily, *C. roseus*-AgNPs showed very weak inhibition activity toward THLE3 cells with IC_{50} value of 615 ± 0.05 µg/mL.

Quantitative and qualitative measurement of total RNA

Total isolated RNA was quantified using Bioanalyser. As depicted in Supplementary Figure 1, the representative electropherogram indicated two intact bands that are visible in each sample. These two bands represent 28s and 18s ribosomal RNA, respectively. RNA integrity number (RIN) was then determined, which is the value of the RNA integrity. The value that falls within a range between 8 to 10 showed an acceptable value of RIN[18]. The RIN and ribosomal ratio values acquired for both untreated and *C. roseus*-AgNPs treated HepG2 cells were 9.6 and 9.4, respectively. Both RIN values were within the acceptable range.

mRNA Transcriptome sequencing

Sequencing data filtering: Two samples were sequenced using the DNBseq platform and the result was about 6.98 Gb bases per sample. The distribution of the base quality was shown in Supplementary Figure 2. As observed in both Supplementary Figure 2A and B, the percentage of clean reads was 93.69% and 94.03%, respectively.

Genome mapping: After read filtering, the clean reads were mapped to the reference genome using *HISAT2*[19]. On average, 95.88 % of reads were found to be mapped to the population of human genomes and the consistency of the mapping result for each sample suggests that the samples were comparable. The mapping details are shown in Table 1.

Gene expression analysis: To get a complete reference for the gene mapping and expression, novel coding transcripts were merged with the reference transcripts, and clean reads were mapped to them using Bowtie2[20]. The gene expression level for each sample was calculated with RSEM[21]. The result of this analysis is summarised in Table 2.

The sufficiency of sequencing data for bioinformatics analysis was approached using sequencing data saturation analysis. As the number of sequenced reads increased, the number of identified genes also increased. On the other hand, when the number of sequenced reads reached a certain amount, the determining gene growth curve flattens, indicating the identified gene reached saturation. Supplementary Figure 3 displays the saturation analysis for each sample.

Reads coverage and distribution of each detected transcript are shown in Supplementary Figure 4 and Supplementary Figure 5, respectively. This approach allows access to the excellent quality of the samples and sequencing data sufficiency by showing the completely covered transcripts and evenly distributed reads throughout the transcript. These results suggest that both untreated and treated HepG2 had excellent sample quality and sufficient sequencing. Correlation between samples was assessed by Pearson correlation coefficient calculations for all gene expressions between the samples, as shown in Figure 2.

The identification of differentially expressed genes: DEGs were determined by using DEseq2 and passion Dis algorithms. The distribution of DEGs is summarised using the volcano plot as shown in Figure 3. The treatment of HepG2 cells with *C. roseus*-AgNPs revealed 296 DEGs, with 182 genes were upregulated while 114 genes were downregulated (Figure 3A).

Gene ontology analysis of DEGs: The identified DEGs were subjected to Gene ontology analysis. GO unveiled three ontologies which are related to molecular biological function, cellular components, and biological processes. The classification result is depicted in Figure 4.

Pathway analysis of DEGs: KEGG pathway classification and functional enrichment were generated based on DEGs. Pathway enrichment result is shown in Table 3 and the network enrichment is depicted in Figure 5.

DISCUSSION

C. roseus-AgNPs exhibited anti-cancer properties with negligible effect on normal cells

Our group has previously demonstrated the anti-cancer properties of *C. roseus*-AgNPs on cancer cells [22]. The anti-cancer properties of the *C. roseus*-AgNPs were estimated by IC_{50}, which represents the concentration of *C. roseus*-AgNPs required to inhibit 50% of the total cells[23]. According to the IC_{50} value (800 ± 1.55 μg/mL) observed at 72 h, the THLE3 cells substantially ($P < 0.001$) inhibited only at very high concentrations of *C. roseus*-AgNPs. On the other hand, the IC_{50} of the *C. roseus*-AgNPs on the HepG2 cells was 4.38 ± 1.59 μg/mL. This study revealed that *C. roseus*-AgNPs showed a significant ($P < 0.001$) cytotoxicity towards HepG2 cells as compared to THLE3 cells. *C. roseus*-AgNPs can inhibit the progressive development of HepG2 while causing very insignificant toxicity to normal cells at low concentrations. Several studies have also shown that biosynthesised AgNPs show no toxicity against normal cells while demonstrating cytotoxic effects against cancer cells[24-29]. For example, a study by Halkai *et al*[24] showed that fungal-derived AgNPs exerted minimal cytotoxicity against human gingival fibroblast cell line. Additionally, Sriram *et al*[25] also reported similar observations in their experiments, where AgNPs acted as an anti-proliferative agent by effectively inhibiting the development of Dalton's lymphoma ascites cell lines without causing toxicity on normal cell lines. The findings from our study agreed with the previous reports, corroborating the potentiality of *C. roseus*-AgNPs as an anti-cancer agent.

mRNA transcriptome analysis identified 296 protein-coding genes

An in-depth understanding of the anti-cancer properties of the *C. roseus*-AgNPs entails the identification of the genes that act in concert in orchestrating the effect. As transcriptome analysis can provide an overarching view of the gene expression profile under a certain condition or state, it was adopted in our effort to comprehend the underlying mechanisms of the anti-cancer activity of *C. roseus*-AgNPs against HepG2 cells. In the present study, the untreated HepG2 cells and *C. roseus*-AgNPs treated HepG2 cells were subjected to mRNA transcriptome analysis using the BGI DNBseq Platform. As revealed by the mRNA transcriptome analysis, it was found that the treatment of HepG2 cells with *C. roseus*-AgNPs has resulted in the regulation of 296 protein-coding genes, of which 182 genes were upregulated while 114

Table 1 Summary of genome mapping

Sample	Total clean reads	Total mapping ratio	Uniquely mapping ratio
Untreated HepG2	70025052	95.94%	76.60%
C. roseus-AgNPs treated HepG2	72598578	95.82%	75.94%

C. roseus-AgNPs: *Catharanthus roseus*-silver nanoparticles.

Table 2 Summary of gene mapping ratio

Sample	Total clean reads	Total mapping ratio	Uniquely mapping ratio
Untreated HepG2	70025052	66.79%	63.92%
C. roseus-AgNPs treated HepG2	72598578	62.94%	60.15%

C. roseus-AgNPs: *Catharanthus roseus*-silver nanoparticles.

Table 3 Pathway functional enrichment results

Pathway ID	Pathway	Genes	Hits	Adj *P* value
K05200	Pathway in cancer	*FAS, GADD45A, BAX, PMAIP, BID, JUN, CXCL8, HMOX1, STAT1, FOS, CEBPA, VEGFA, FGF5, EGF, RHOA, FADD, FH, SMAD, MTOR, NFκBIA, CDKN1A, WNT4*[a], *WNT7A*[a], *FGFR3*[a], *BMP4*[a], *CDK4*[a], *CDK2*[a], *MDM*[a]	29/530	3.28E-12
K04115	p53 signaling pathway	*FAS, GADD45A, SERPINE, THBS1, CDK4, BAX, CDK2, PERP, SESN1, SESN2, PMAIP1, BID, IGFBP3*[a], *MDM2*[a]	14/72	1.28E-13
K04210	Apoptosis	*FAS, GADD45A, BAX, PMAIP1, BID, JUN, FOS, FADD, NFKBIA, BCL2A1, ATF4, MCL1, TNFSF10*[a], *RIPK1*[a]	14/136	9.61E-10
K04144	Endocytosis	*ARF6, RHOA, EHD2, FGFR3, HSPA6, HSPA1L, VPS28, EEA1, VPS25, TSG101, STAM, SMAD, EHD4, LDLR, TFRC*[a], *MDM2*[a]	16/244	4.08E-08
K04010	MAPK signaling pathway	*FAS, GADD45A, JUN, FOS, VEGFA, FGF5, CDKN1A, EGF, ATF4, HSPA6, HSPA1L, CDKN1A, HSPB1, DUSP1, NR4A1, EFNA4*[a], *FGFR3*[a], *ERBB3*[a], *SKP2*[a]	16/295	5.58E-07
K04668	TNF signalling pathway	*FAS, JUN, FOS, FADD, NFKBIA, ATF4, RIPK1*[a], *CCL2, NOD2, CCL20*[a]	10/110	9.07E-07
K04350	TGF beta signaling pathway	*THBS1, RHOA, SMAD2, SMAD4, BMP4, SMAD7, BAMBI, BMP6*	8/90	1.66E-05
K02010	Cell cycle	*GADD45A, SOX15, CDK4*[a], *CDK2*[a], *MDM2, SMAD2, SMAD4, CDKN1A, PCNA*[a], *MCM3*[a], *SKP2*[a]	9/124	2.09E-05
K04978	Mineral absorption	*HMOX1, MT1F, MT1X, MT1H, MT1B, FTH1*	6/51	3.58E-05

[a]Downregulated genes.

genes were downregulated, as shown in Figure 3.

GO analysis exhibited that the highest fraction of the regulated genes were involved in "cellular and signalling response" followed by "biological regulation", "regulation of biological process", "metabolic process" and "response to stimulus" (Figure 4). The underlying pathways regulated by the genes are the p53 signalling pathway, pathway in cancer, apoptosis pathway, endocytic pathway, MAPK signalling pathway, TNF signallingg pathway, TGF signallingg pathway, cell cycle pathway and mineral absorption pathway.

C. roseus-AgNPs induced the expression of stress-associated genes such as MT, HSP and HMOX-1

C. roseus-AgNPs treatment of the HepG2 cells was found to upregulate several members of the gene isoforms that encode metallothionein (MT), such as *MT1F, MT1X, MT1H, and MT1B*. MTs are intracellular proteins that contain approximately 30% thiol-containing cysteine residues, which can bind several cytotoxic agents, including platinum compounds, alkylating agents, and metal ions such as zinc and copper[30]. MTs also regulate various pathophysiological processes such as apoptosis, and angiogenesis and could also act as radical scavengers by protecting the cells from free radicals[31]. As such, an increased level of MT is an indicator that the cells were undergoing 'stress' and the cells are

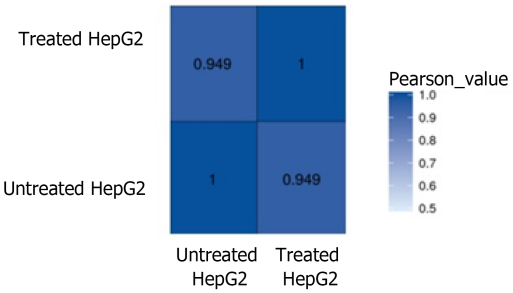


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Figure 2 The correlation analysis between samples. The colour represents the correlation coefficient.

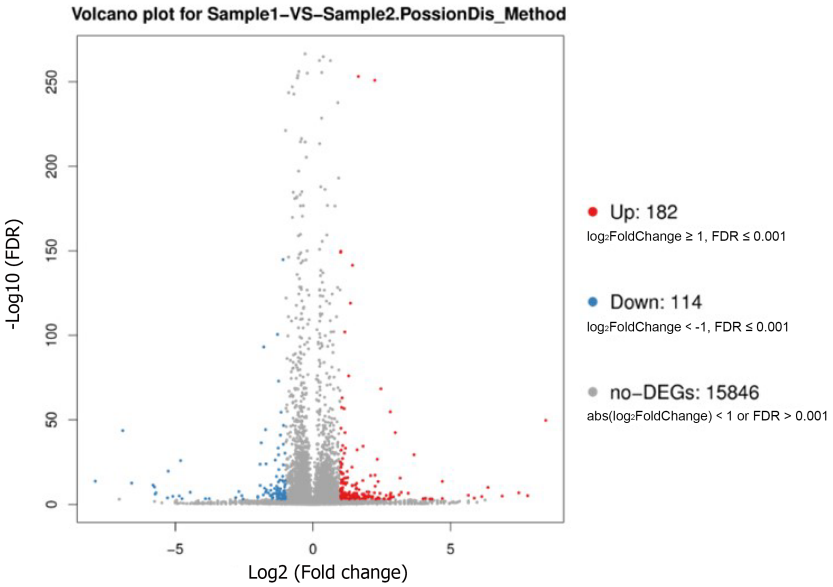

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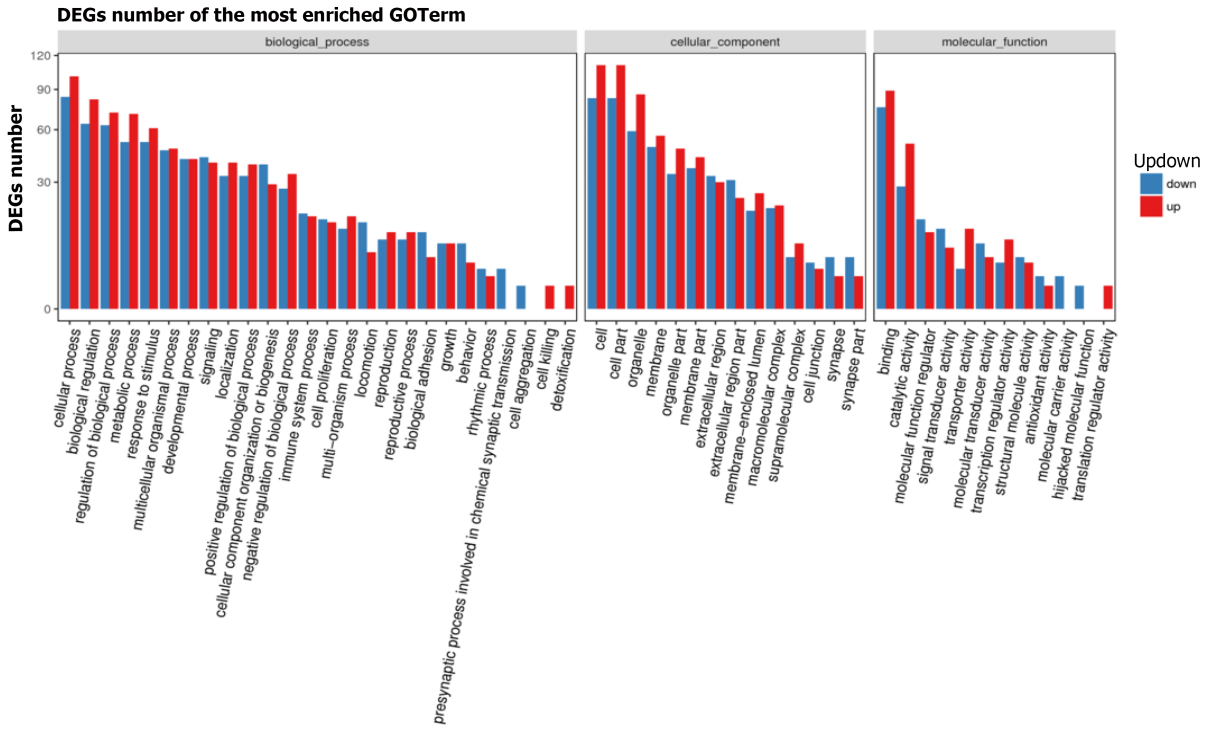
Figure 3 Volcano plot of differentially expression genes. Red points represent upregulated differentially expression genes (DEGs). Blue points represent down-regulated DEGs. Grey points represent non-DEGs. DEGs: Differentially expression genes.

striving to mitigate the cytotoxic effect of the anticancer drug, in this case, *C. roseus*-AgNPs[32]. This finding is also in agreement with the findings by Woo *et al*[33], who reported that *Javanese medaka*, a type of seawater organism showed MT upregulation upon exposure to AgNPs. On the other hand, heat-shock genes such as *HSPA1L*, *HSPB1*, and *HSPA6* were also found to be upregulated in HepG2 cells exposed to *C. roseus*-AgNPs. *HSPs* are upregulated by stress signals such as high temperature, decreased availability of oxygen, infectious agents, and inflammatory mediators[34]. The increased expression level of HSPs is needed to counteract the stress, which is induced by *C. roseus*-AgNPs in this study. Furthermore, the up-regulation of oxidative stress-related genes *HMOX-1* was also documented in our experiment. *HMOX-1* is a reactive oxygen species (ROS) sensor that has antioxidant and anti-inflammatory properties[35]. During stress conditions, HMOX-1 catalyse the degradation of the Heme group into biliverdin, carbon monoxide, and iron[36]. Similar increased expression of *HMOX-1* was also observed by Gurunathan *et al*[37], in mouse embryonic fibroblast cells upon treatment with AgNPs. Collectively, the upregulation of stress-response genes such as *MTs, HSPs*, and *HMOX-1* in this study indicates that *C. roseus*-AgNPs exposure invokes the cell's defensive response in negating effects of cellular stresses caused by *C. roseus*-AgNPs. The increased expression of stress-response genes indirectly reflects the cytotoxic effect of *C. roseus*-AgNPs. We have also observed significant production of NO and ROS in our previous study upon treatment of HepG2 cells with *C. roseus*-AgNPs[38]. These findings are substantial and in agreement with the previous findings, whereby upregulation of *MTs, HSPs,* and *HMOX-1* was observed in cells exposed to AgNPs[39-41].

C. roseus-AgNPs increased expression of tumour suppressor genes and apoptotic genes

The most intriguing finding in our study is that *C. roseus*-AgNps treatment on HepG2 cells induces the expression of growth arrest and DNA damage-inducible alpha (*GADD45A*) gene, which is a type of



DOI: 10.4254/wjh.v15.i3.393

Figure 4 Gene Ontology classification of upregulated and downregulated differentially expression genes.

tumour suppressor gene that regulates processes such as DNA repair, cell cycle control, senescence, and genotoxic stress[42]. The expression of the *GADD45A* gene in cell cycle inhibition is also regulated by p53. p53 protein is involved in maintaining genetic integrity and regulating the cellular response towards genotoxic stress by inducing cell cycle arrest or apoptosis to prevent tumorigenesis[43]. *p53* is negatively regulated by *MDM2*. Interestingly, our experimental findings demonstrated that *MDM2* was downregulated in *C. roseus*-AgNPs treated HepG2 cells, suggesting that its inhibitory effect against p53 was ameliorated, causing the upregulation of the *p53* gene. As such, the expression of the *p53* gene elevates, causing the suppression of the proliferation of cancer cells. Sahu *et al*[44] reported similar observations in their study where under normal conditions, *p53* was constitutively expressed, but inactivated by its negative regulator, *MDM2*. However, during cellular stresses, *MDM2* was downregulated which in turn caused the upregulation of *p53* gene. The upregulation of the *p53* gene is indicative of the anticancer effect of *C. roseus*-AgNPs in amplifying the tumour suppressor activity of the cancer cells. Besides the upregulation of the tumour-suppressor genes, apoptotic-related genes *BAX* and *FAS* were also found to be upregulated, suggesting the anticancer efficacy of the *C. roseus*-AgNPs in promoting apoptosis in cancer cells.

C. roseus-AgNPs activated signal transduction pathways such as MAPK signalling pathway

The MAPK pathway is a series of protein kinase cascade essential in regulating numerous physiological functions including inflammation, cell stress response, cell differentiation, cell division, cell proliferation, metabolism, motility, and apoptosis[45]. Treatment with *C. roseus*-AgNPs activated MAPK signalling pathway in HepG2 cells. In this study, several genes that are involved in MAPK pathways were found to be regulated such as *FAS, GADD45A, P53, JUN*, and *FOS*. As indicated previously, *GADD45A*, a tumour suppressor gene which could also be involved in the MAPK signalling pathway was found to be upregulated upon treatment of the HepG2 cells with *C. roseus*-AgNPs. Increased expression of *GADD45A* conduces to baicalein-induced apoptosis and activation of MAPK signalling pathway[46]. In this study, activation of MAPK signalling pathway also upregulates the *p53* gene as mentioned previously, as MAP kinase phosphorylates and activates the p53 protein in response to stressful stimuli induced by *C. roseus*-AgNPs[47]. Taken together, activation of MAPK pathway prepares the cell for counteracting actions such as inflammation, cell stress response, and apoptosis upon treatment with *C. roseus*-AgNPs, which indirectly implies the anticancer properties harboured by these nanoparticles.

C. roseus-AgNPs activated TNF signalling pathway

TNF alpha is a pro-inflammatory cytokine that acts by binding to TNF-R1 and TNF-R2 receptors, resulting in the recruitment of signal transducers that activate the effector, leading to the activation of caspases and two transcription factors, NF-κB, as well as MAPKs such as ERK, p38, and JNK, which will

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Figure 5 Network enrichment result. The darker the colour indicates the highest enrichment pathways. The larger the area, the higher the degree of enrichment.

induce apoptosis and necrosis[48]. In this study, the treatment of *C. roseus*-AgNPs caused the upregulation of several genes related to TNF signalling pathway such as FADD, NF-κbia), ATF4, CCL2, NOD2. FADD protein interacts directly with TRADD, which are signal transducers that activate NF-κB and trigger apoptosis[49]. The overexpression of the *FADD* genes in our study suggests that treatment with *C. roseus*-AgNPs eventually promotes apoptosis. Similar overexpression was also reported in the previous study, whereby AgNPs treated MDA-MB-436 cells showed an increase in the level of *FADD* gene[50]. In this study, the upregulation of *ATF4* was also found. The overexpression of *ATF4* was reported by Iwasaki *et al*[51], which happens in response to metabolic stresses caused by SFAs and ER stressors. *RIPK1* gene is involved in the system that controls cell survival, signalling nodes in cell death and inflammation and cytokine production. The downregulation of the RIPK1 gene in this study upon treatment with *C. roseus*-AgNPs can induce apoptosis *via* the cleavage activity of the caspase 3 associated pathway[52]. Qiu *et al*[53] reported similar observations in their experiments. CCL20 is known to enhance cancer cell progression[54]. The downregulation of the *CCL20* gene in this study suggests that *C. roseus*-AgNPs are able to induce inflammation through TRAIL as reported by a previous study[55].

C. roseus-AgNPs elicited the activation of TGF-β signalling pathway

TGF-β signalling pathway plays a crucial role in controlling various fundamental aspects of cellular activities such as cellular growth, development, differentiation, and apoptosis[56]. As a secreted polypeptide, TGF-β functions *via* receptor serine/threonine kinases and intracellular SMAD effectors [57]. TGF-β acts as a tumour suppressor at the early stage of cancer while it also acts as a pro-metastatic factor in the later stages of cancer[58]. Exposure of HepG2 cells to *C. roseus*-AgNPs activates TGF-β signalling pathway. The effect of *C. roseus*-AgNPs is analogous to a previous study, whereby ellagic acid was found to exert anti-proliferation effects by activating TGF-β/Smad3 signalling pathway[59]. Transcriptome analysis also showed that isoforms of SMAD, which are part of TGF-β pathway were also upregulated. Moreover, BMPs such as *BMP4* and *BAMP6*, which are extracellular signalling molecules that belong to the TGF-β pathway, were also upregulated. The tumour suppressor effect mediated by TGF-β pathway was imparted upon treatment with *C. roseus*-AgNPs, which corroborates its anticancer property.

The uptake of C. roseus-AgNPs occurred via endocytosis

Endocytosis involves the formation of small membrane vesicles (60-120 nm) that transports various

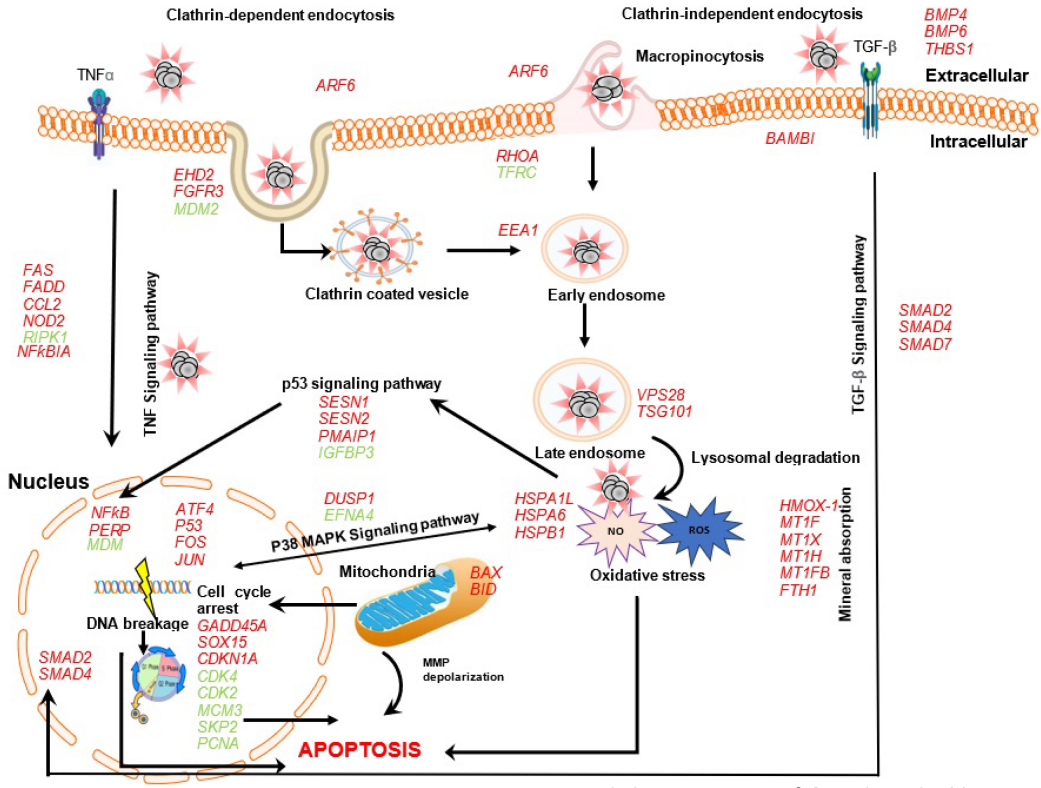
molecules or cargo from the plasma membrane to the cytoplasm. Though there are several types of endocytosis, previous studies have shown that clathrin-dependent endocytosis and macropinocytosis are the major routes of transportation of AgNPs into the cells[60]. The observations also agree with the results reported by Treuel *et al*[61] that endocytosis has been demonstrated to be a key mechanism in driving the cellular uptake of AgNPs, with NPs entering cells *via* early endosomes, late endosomes, and lysosomes. In this study, a few genes such as *ARF6, EHD2, FGFR3, RhoA, EEA1, VPS28, VPS25, and TSG101,* were upregulated, suggesting that the uptake of *C. roseus*-AgNPs can occur *via* the clathrin-dependent or clathrin-independent endocytosis pathway. *ARF6* gene, also known as ADP-ribosylation factor 6, is a small GTPase that regulates endocytic membrane trafficking and actin remodelling[62]. The upregulation of *ARF6* gene in this study is consistent with the findings of Tanabe *et al*[62], which suggest that *ARF6* gene regulates the membrane trafficking between the plasma membrane and endosome *via* clathrin-dependent or clathrin-independent endocytosis[63]. A previous study by Morén *et al*[63] showed that the overexpression of *EHD2* gene inhibited the formation of caveolae. Interestingly, our study demonstrated an upregulation of *EHD2* gene, which encodes a member of the EH domain-containing protein family. EHD2 protein has an N-terminal domain that interacts with the actin cytoskeleton and a C-terminal EH domain that binds to an EH domain-binding protein[64]. This interaction appears to link clathrin-dependent endocytosis and actin, implying that this gene is involved in the endocytic pathway, particularly clathrin-dependent endocytosis[65]. These findings suggest that clathrin-dependent endocytosis was one of the major uptake mechanisms of *C. roseus*-AgNPs while ruling out the involvement of possible involvement of caveolin-dependant endocytosis.

Another interesting finding in this study is the potential involvement of macropinocytosis, attributable to the upregulation of *RhoA* gene macropinocytosis. This gene encodes a member of the Rho family of small GTPases, which regulates macropinocytosis *via* active and inactive GTP-binding while simultaneously playing an important role in the remodelling of the actin skeleton during macropinocytosis[66,67]. According to Patel *et al*[67], after the macropinocytic cups closed to form macropinosomes, the expression of another Rho subtype, *RhoA*, increased significantly. This corroborates our findings in this study on the *RhoA* gene upregulation, which suggests that *C. roseus*-AgNPs uptake also could occur *via* macropinocytosis. The overexpression of *EEA1* in this study indicated that the formation of early endosomes occurs during the uptake of *C. roseus*-AgNPs. This finding is consistent with prior work, which demonstrated the high frequency of the *EEA1* gene in early endosomes that are the primary sorting station in the endocytic pathway[68]. On the other hand, *TSG101* and *VPS28* genes are involved in late endosomal trafficking[69]; the upregulation of *TSG101* and *VPS28* genes in this study suggests that late endosome was formed during the *C. roseus*-AgNPs uptake. This finding was in line with a previous study, where the expression of TSG101 and VPS28 was found to be increased[70]. The upregulation of the stress-responsive genes as mentioned previously in our study is also indicative of the successful uptake of *C. roseus*-AgNPs into the HepG2 cells, as the generation of free radicals that induced stress in the cells can be caused by the leaching of Ag^+ from AgNPs into the cytosol, because of high acidic lysosome rupture. *TFRC* gene encodes a cell surface receptor necessary for cellular uptake by the process of receptor-mediated endocytosis[71]. The downregulation of the *TFRC* gene in this study suggests that the expression of the gene was induced to reduce endocytosis *via* negative feedback regulation as a response to cellular homeostasis. Our finding also is in tandem with the findings of Wang *et al*[72], who have also noticed a drop in the expression of the *TFRC* gene, which could be due to negative feedback for defensive actions.

The uptake of C. roseus-AgNPs arrested cell cycle

Cancer progression is associated with aberrancy in the cell cycle, such as the anomalous expression of CDKs[73]. CDKs are usually highly expressed, causing the uncontrolled proliferation of cancer cells [74]. Upon the treatment of the HepG2 cells with *C. roseus*-AgNPs, *CDK4, and CDK2* were found to be downregulated, implying the antagonistic effect of the nanoparticles against the cell cycle protein. A previous study reported that the blockage of Go/G1 was accompanied by the downregulation of the cell cycle regulators *CDK4* and *CDK2*[75]. In this study, the downregulation of *CDK4* and *CDK2* suggested that *C. roseus*-AgNPs was arrested at Go/G1. Another important observation is the upregulation of *GADD45A*, which caused a decrease in the *SKP2* expression. Overexpression of *SKP2* is associated with the cell cycle progression and as the *SKP2* expression was found to be reduced in the present study, it is surmised that the cell proliferation is being forestalled. Moreover, the reduced expression of *SKP2* is also associated with the increased expression of *CDKN1A*, which is an inhibitor of cell cycle progression by inhibiting the activity of cyclin-dependent kinase expression[76]. The upregulation of *CDKN1A* corroborates the anticancer activities of *C. roseus*-AgNPs that can induce cell cycle arrest. *MCM3* is a member of minichromosome maintenance family that is associated with tumour invasiveness[77]. The treatment of the *C. roseus*-AgNPs caused the downregulation of *MCM3,* in HepG2 cells, ratifying the anticancer activities of *C. roseus*-AgNPs in alleviating tumour aggressiveness.

The overall proposed mechanism as depicted in Figure 6, consists of clathrin-dependent and clathrin-independent endocytosis. The signalling pathways indicate the involvement of the up and down-regulated genes in various cellular organelles. The understanding of cellular and molecular mechanisms would provide a strong justification of the rationale of *C. roseus* G. Don-AgNPs as anticancer compounds for liver cancer therapy.



DOI: 10.4254/wjh.v15.i3.393

Figure 6 Model of cytotoxicity mechanism in HepG2 cell treated with *C. roseus*-AgNPs that involves the upregulated genes (in red) and downregulated genes (in green).

CONCLUSION

In this study, the treatment of HepG2 cells with *C. roseus*-AgNPs has resulted in the increase of the expression of tumour suppressor genes, apoptotic genes, and activation of signal transduction pathway such as mitogen-activated protein kinase (MAPK) signalling pathway, endocytosis signalling pathway, TNF signalling pathway, TGF-Beta signalling pathway as well as cell cycle arrest. Collectively, the findings from our study have demonstrated the anti-cancer properties of *C. roseus*-AgNPs, with insignificant effects on normal cells. The therapeutic property of the *C. roseus* G. Don-AgNPs should be further explored in the future as part of the endeavours to surrogate or complement the current conventional chemotherapeutic-based intervention.

ARTICLE HIGHLIGHTS

Research background

Conventional chemotherapy and radiotherapies based on x-ray and gamma-ray radiations are the most widespread techniques in the world for the treatment of malignant diseases due to their ability to penetrate tissues and thus allow them to reach deep sites. The only limitation of these treatments is the lack of selectivity between the tumour and the healthy surrounding tissues. Interestingly, previous studies have shown that silver nanoparticles (AgNPs) have the ability to selectively induce cytotoxic effects on cancer cells, as compared to normal cells. Therefore, the present study aims to evaluate the cytotoxic effects of AgNPs synthesised by *C. roseus* aqueous extract against liver carcinoma cells HepG2 and normal liver cells THLE-3, by assessing the proliferative activity followed by the mRNA transcriptome profiling analysis.

Research motivation

Due to the limitations of the conventional treatment like non-specificity and less effectiveness, novel strategies are in demand to solve these issues. Amongst all, the use of plant-synthesised silver nanoparticles has gained attention as they are known for non-toxic properties, are cost-effective, are easily assessable and environmentally friendly. The unique properties of nano-sized nanoparticles have been reported can penetrate cancer cells effectively. In this study, the anticancer activity was evaluated

at both cellular and molecular levels to gain insight into its mechanisms.

Research objectives

To evaluate the proliferative activity of the human hepatocellular carcinoma cells HepG2 in response to the *Catharanthus roseus*-silver nanoparticles (*C. roseus*-AgNPs), in comparison to the normal liver cells THLE-3 cells.

Research methods

To evaluate the proliferative activity, the hepatocellular carcinoma cells HepG2 and normal human liver cells THLE3 were treated with standardised *Catharanthus roseus*-silver nanoparticles (*C. roseus*AgNPs) in a double dilution manner and analysed using MTT assay. To elucidate the gene expression study, the RNA samples were extracted and sequenced using BGIseq500 platform. This is followed by data filtering, mapping, gene expression analysis, DEGs analysis, GO analysis, and pathway analysis.

Research results

The proliferative activity revealed selective effects, indicating that the *Catharanthus roseus*-silver nanoparticles were cytotoxic on hepatocellular carcinoma cells HepG2 cells but not on the normal liver cells THLE3 cells. The transcriptome analysis has resulted in the regulation of 296 protein-coding genes, of which 182 genes were upregulated while 114 genes were downregulated. The most intriguing finding is the expression of tumour suppressor gene GADD45A, responsible for the regulation of DNA repair, cell cycle control and genotoxic stress. The expression of this gene is regulated by p53. The upregulated GADD45A was supported by the downregulated MDM2, which is the negative regulator for p53. Our findings revealed the activation of several signalling pathways including the mitogen-activated protein kinase signalling pathway, TNF signalling pathway and TGF-β signalling pathway. These pathways are the main regulator in fundamental intracellular activities such as apoptosis, cell cycle and cellular growth. The upregulation of *ARF6, EHD2, FGFR3, RhoA, EEA1, VPS28, VPS25* and *TSG101* indicated that the *C. roseus*-AgNPs were taken up by HepG2 cells *via* both clathrin-dependent and clathrin-independent.

Research conclusions

The selective proliferative activity between cancerous and normal liver cells indicates a promising potential of *Catharanthus roseus*-silver nanoparticles (*C. roseus*-AgNPs) as an effective anticancer agent. The understanding of the molecular signalling pathways induced by the genes associated with oxidative stress, apoptosis and cell cycle arrest provides the novelty towards the development and establishment of *C. roseus*-AgNPs as an anticancer drug for hepatocellular carcinoma. Moreover, we propose that the uptake was *via* both clathrin-dependent and clathrin-independent endocytosis. These findings would explain the cytotoxicity mechanisms of the *C. roseus*-AgNPs at cellular and molecular level towards hepatocellular carcinoma cells HepG2.

Research perspectives

While the endocytic pathways emphasise the action of the selectively permeable plasma membrane on the nanomaterials, cytotoxicity of silver nanoparticles (AgNPs) generally involves the cells' downstream activity, including reactive oxygen species (ROS)- dependent pathway, cell cycle arrest and genotoxicity. Moreover, the small-sized AgNPs can easily penetrate the cells and bind to macromolecules including proteins and DNA, either directly or indirectly although the exact mechanism for this interaction has not been clarified. The physicochemical characteristics that make AgNPs so useful can be the main reason they might be dangerous to cells, and at a higher level to human health. Therefore, to avoid these problems, the AgNPs must be engineered from either biocompatible, nontoxic, biodegradable material or materials have with minimal toxic effects.

ACKNOWLEDGEMENTS

We would like to offer special appreciation to all laboratory staff and postgraduate students under the laboratory of the Advanced Medical and Dental Institute for their guidance, assistance, and support. A preprint has previously been published by Azhar *et al*, 2018 (Available from: Preprints:2021090431, [DOI: 10.20944/preprints202109.0431.v1]).

FOOTNOTES

Author contributions: Ahmad NH contributed to the methodology; Ahmad NH contributed to the validation; Azhar NA contributed to the conceptualisation; Azhar NA and Abu Bakar SA contributed to the formal analysis; Azhar NA contributed to the investigation; Ahmad NH contributed to the resources; Azhar NA, Abu Bakar SA, Citartan M and

Ahmad NH contributed to the data curation; Azhar NA contributed to original draft preparation; Ahmad NH and Citartan M contributed to the review and editing; Ahmad NH and Citartan M contributed to the supervision; Ahmad NH contributed to project administration; Ahmad NH contributed to funding acquisition; all authors have read and agreed to the published version of the manuscript.

Supported by Fundamental Research Grant Scheme from the Malaysian Ministry of Higher Education, No. FRGS/1/2015/SG03/USM/03/1.

Conflict-of-interest statement: The authors declare no conflict of interest.

Data sharing statement: No additional data are available.

Country/Territory of origin: Malaysia

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S-Editor: Chang KL
L-Editor: A
P-Editor: Chang KL

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World Journal of Hepatology

Submit a Manuscript: https://www.f6publishing.com

World J Hepatol 2023 March 27; 15(3): 410-418

DOI: 10.4254/wjh.v15.i3.410

ISSN 1948-5182 (online)

ORIGINAL ARTICLE

Retrospective Cohort Study

Adherence to guideline-directed hepatocellular carcinoma screening: A single-center US experience

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Specialty type: Gastroenterology and hepatology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Gatselis NK, Greece; Jin Y, China; Kumar SKY, India

Received: November 11, 2022
Peer-review started: November 12, 2022
First decision: January 5, 2023
Revised: January 16, 2023
Accepted: February 21, 2023
Article in press: February 21, 2023
Published online: March 27, 2023

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Abstract

BACKGROUND

The American Association for the Study of Liver Disease recommends screening patients with cirrhosis for hepatocellular carcinoma (HCC) using imaging with or without alpha-fetoprotein every six months. Unfortunately, screening rates remain inadequate.

AIM

To assess root causes of screening failure in a subspecialty hepatology clinic.

METHODS

The authors identified patients with cirrhosis seen in a subspecialty hepatology clinic and determined whether they underwent appropriate screening, defined as two cross-sectional images between five and seven months apart. The authors characterized the primary driver of screening failure. Finally, other hepatologists were surveyed to determine provider perceptions of screening failure causes.

RESULTS

1034 patients were identified with an average age of 61 years and a mean MELD of 8.1 ± 3.8. Hepatitis C virus was the most common cirrhosis etiology. 489 (47%) underwent appropriate screening. No demographic or clinical differences were detected between those who underwent appropriate screening and those who did

not. The most common etiologies of screening failure, in descending order, were: radiology unable to schedule timely imaging, provider did not order imaging, patient canceled follow up appointment, appointments scheduled too far apart, lost to follow up, no-show to radiology appointment, and provider canceled appointment. Hepatologists surveyed believed the most common cause of screening failure was no-show to radiology.

CONCLUSION
Rates of screening were poor even in a subspecialty hepatology clinic. Screening failure was mostly due to systemic factors such as radiology availability and time between hepatology appointments rather than individual error.

Key Words: Hepatocellular carcinoma; Cirrhosis; Health maintenance; Quality improvement; Screening; Hepatology

Core Tip: This study reinforces existing knowledge that screening rates for Hepatocellular carcinoma are woefully inadequate, even in a subspecialty hepatology clinic. Unlike previous studies, ours identifies specific failure points, showing that screening failures are driven more by systemic issues than by physician or patient error.

Citation: King WW, Richhart R, Culpepper T, Mota M, Banerjee D, Ismael M, Chakraborty J, Ladna M, Khan W, Ruiz N, Wilson J, Altshuler E, Clark V, Cabrera R. Adherence to guideline-directed hepatocellular carcinoma screening: A single-center US experience. *World J Hepatol* 2023; 15(3): 410-418
URL: https://www.wjgnet.com/1948-5182/full/v15/i3/410.htm
DOI: https://dx.doi.org/10.4254/wjh.v15.i3.410

INTRODUCTION

Hepatocellular carcinoma (HCC) represents the sixth leading cause of cancer and the third leading cause of cancer death worldwide[1]. The most common and important risk factor for HCC is cirrhosis [2]. Estimates of the annual incidence of HCC among patients with cirrhosis range from 1 to 8%[3,4]. The lifetime incidence in patients with cirrhosis may be as high as 32% and is increasing in the United States[3,5-7].

The American Association for the Study of Liver Disease (AASLD) recommends screening for HCC with abdominal ultrasound, computed tomography, or magnetic resonance imaging, with or without alpha-fetoprotein, every six months[8-10]. Adherence to AASLD guidelines correlates with improved survival, as demonstrated in a French cohort study of 1671 patients at 35 centers. Patients who adhered to semi-annual screening protocols had increased lead-time adjusted survival[11]. A theoretical model by Sarasin *et al*[12] predicted an increase in life expectancy among patients with Child-Turcot-Pugh A cirrhosis with HCC screening if the expected incidence of HCC is at least 1.5% per year. Unfortunately, predictive algorithms to stratify patients by HCC risk have failed external clinical validation[13]. Much research now focuses on blood-based biomarkers for simple and accessible point of care screening, but these strategies are not yet ready for clinical practice[14].

Unfortunately, adherence to screening guidelines remains poor[15-17]. A 2011 retrospective cohort study of 13002 patients with cirrhosis across 128 Veterans Affairs medical centers showed that only 12% had received appropriate screening[18]. A 2012 systematic review by Singal *et al*[15] found the surveillance rate among all patients with cirrhosis to be only 18.4%, although it was higher (51.7% *vs* 16.9%) among patients followed in subspecialty gastroenterology clinics. A subsequent retrospective cohort study performed by the same group found that only 2% of patients received consistent surveillance; 33% had inconsistent surveillance, and 65% had no surveillance over 3 years[19]. A qualitative study within the Veterans Health Administration similarly found that following with a subspecialist, whether gastroenterology or infectious disease, significantly increased HCC screening rates[20]. Poor knowledge and vigilance of screening protocols among primary care providers has been well-documented[21,22]. Other factors included distance to a screening site and lead time between screening order and screening date[20]. Socioeconomic factors also contribute to screening utilization [23,24]. Primary care-based clinical reminders have also been shown to improve screening rates[25]. Singal *et al*[26] showed that a mailed outreach program increases HCC screening rates.

Many previous studies examined patients diagnosed with HCC to identify factors related to lack of screening[18,27,28]. Our group sought to collect data on all patients at a subspecialty hepatology clinic to retrospectively identify risk factors for screening failure among all patients with cirrhosis, not just those with HCC. We hypothesized that there may be additional factors not previously identified that contribute to screening failure.

The purpose of this study was two-fold: (1) To determine the rate of appropriate HCC screening in patients with cirrhosis in a subspecialty practice in which screening guidelines are well known; and (2) to identify barriers at an institutional and provider level as well as the patient-related factors. The data will be used to improve adherence to guideline-directed screening protocols *via* future quality improvement initiatives.

MATERIALS AND METHODS

The electronic medical record was queried for billing codes from the 9th revision of the International Classification of Diseases (ICD-9) or ICD-10 to identify patients. Demographic, disease etiology, and laboratory data were collected. Inclusion criteria included patients with cirrhosis who were seen at least twice in the subspecialty hepatology clinic between August 2015 and August 2017. The charts were then manually reviewed to confirm that each patient was appropriate for screening based on AASLD guidelines. Exclusion criteria included prior liver transplantation and prior HCC.

Next, the authors determined whether the patients had been appropriately screened, defined as having undergone two imaging studies (abdominal ultrasonography, contrasted computed tomography, or magnetic resonance imaging) within 150 to 210 days of each other during the study period. Because the AASLD guidelines suggested an optional role for -fetoprotein, the authors did not look for -fetoprotein measurement. The charts of these patients were reviewed to determine the primary cause of screening failure. The reason for failure was categorized based on the screening barriers listed below. For patients with multifactorial screening failure, the first failed step in the screening process was counted as the primary reason for failure. For example, if a patient canceled a hepatology appointment and subsequently did not receive orders for imaging, the reason for screening failure was attributed to the clinic cancellation. The hierarchy of steps, in order, were: loss to follow-up, patient clinic appointment cancellation, physician clinic appointment cancellation, appointments more than 7 mo apart, failure to order imaging, failure to schedule imaging, or failure to present to radiology.

Finally, eight hepatologists in the clinic who were not involved in this study were anonymously surveyed on their perceptions of risk factors for screening failure.

Statistical significance was defined using $\alpha < 0.05$. Continuous variables were abnormally distributed according to Shapiro-Wilk testing. Therefore, comparisons were made using the Mann-Whitney *U* test. Categorical variables were compared using chi-square or Fisher's exact testing. The study protocol was reviewed and approved by our institutional IRB prior to any data collection and study procedures.

RESULTS

The authors identified 1276 patients who met the inclusion criteria. 242 were removed due to meeting exclusion criteria. Therefore, a total of 1034 patients were analyzed. The study population had an average age of 61 years, was 55% male, and was 83% White. Hepatitis C virus was the most common cirrhosis etiology, accounting for 51% of participants. The mean MELD score was 8.1 (SD 3.8). No statistically significant differences were detected in baseline characteristics between patients who underwent appropriate screening and those who did not (Table 1).

489 (47%) patients underwent appropriate screening during the study period. 410 (40%) underwent two imaging studies that were outside the time range criterion. Six percent of patients had only one imaging study, and 7% had none (Figure 1). The most common cause of HCC screening failure was delays in scheduling of imaging studies (Figure 2). Patient-centered factors, including appointment cancellations, no-shows, and loss to follow up accounted for 36% of screening failures. System failures were classified as delays in radiology and hepatology scheduling as well as physician cancellation of follow-up appointments. These accounted for 40% of screening failures. Lack of physician order accounted for 21%.

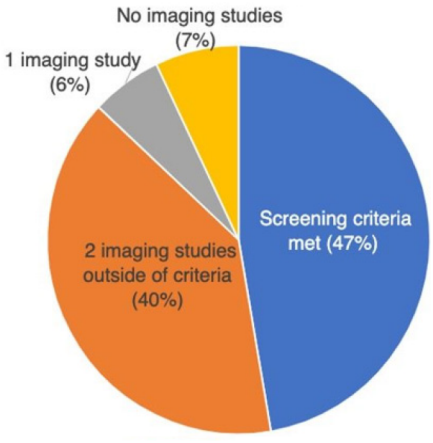
All of those who received their care exclusively within the public university medical system were referred to the radiology department within the institution. 35 patients who followed with community-based gastroenterologists and came to the institution for periodic subspecialty consultation elected to undergo HCC screening with local private radiologists.

All patients diagnosed with HCC experienced delays in screening. One was diagnosed at stage IVb and passed away due to HCC. One was lost to follow-up following discovery of a 3.1 cm nodule on an magnetic resonance imaging protocoled for liver masses. Two underwent Y-90 transarterial radioembolization and partial surgical hepatectomy. One of these patients ultimately elected to transition to hospice and passed away due to worsening hepatic decompensation; the other is still alive.

Table 1 Baseline patient characteristics, *n* (%)

Baseline patient characteristic	Met screening criteria (*n* = 463)	Failed screening criteria (*n* = 545)	*P* value
Age	61.4 ± 10.7	60.2 ± 10.5	0.06
Gender			0.37
Male	261 (56)	292 (54)	
Female	202 (44)	253 (46)	
Race			0.85
White	385 (83)	452 (83)	
African-American	48 (10)	55 (10)	
Other	20 (4)	30 (6)	
Unknown	5 (1)	5 (1)	
County of residence			0.61
Same county as institution	115 (25)	143 (26)	
Different county than institution	348 (75)	402 (74)	
Etiology			0.64
NASH	144 (31)	157 (29)	
AIH	21 (5)	25 (5)	
PBC	34 (7)	37 (7)	
PSC	19 (4)	13 (2)	
HCV	229 (49)	283 (52)	
HBV	29 (6)	37 (7)	
AALD	44 (10)	63 (12)	
MELD	8.2 ± 3.8	8.1 ± 3.8	0.65

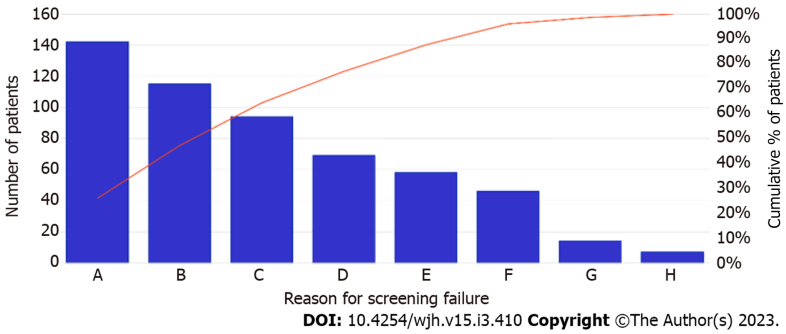
NASH: Non-alcoholic steatohepatitis; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; PSC: Primary sclerosing cholangitis; HCV: Hepatitis C virus; HBV: Hepatitis B virus; AALD: Alcohol-associated liver disease.



DOI: 10.4254/wjh.v15.i3.410

Figure 1 Rates of appropriate hepatocellular carcinoma screening in a sub-specialty hepatology clinic. HCC: Hepatocellular carcinoma.

In a poll, other hepatologists at the same institution believed the most common causes of screening failure, in order, to be: failure to present to radiology, patient clinic appointment cancellation, loss to follow up, and failure to order imaging. Human error and deferral to primary care provider (PCP) were the most cited reasons for failure to order screening.



DOI: 10.4254/wjh.v15.i3.410

Figure 2 Pareto chart of root cause of screening failures. A: Radiology unable to schedule timely imaging; B: Physician/Provider did not order imaging; C: Patient canceled hepatology follow up appointment; D: Hepatology follow up appointments scheduled too far apart; E: Patient lost to follow up; F: Patient no show to radiology appointment; G-H: Physician/Provider canceled hepatology follow up appointment.

DISCUSSION

Despite guidelines that were well-known to the providers in the subspecialty hepatology practice, fewer than half of the patients in our cohort underwent appropriate screening during the study period. The findings are consistent with previous studies and add to the growing evidence that HCC screening rates are grossly insufficient.

However, our study illuminates some nuances in the reasons for screening failure. Most screening failures in our cohort were institutional rather than patient-driven or secondary to physician oversight. We were able to investigate what happened after the order was placed for screening to evaluate the system factors that contribute. Radiology scheduling failure, whether from inability to contact the patient or unavailability of timely imaging appointments, was the primary reason for lack of adherence. The multiple failure points both highlight the complexity of care coordination for cirrhosis patients in a subspecialty clinic and offer targets for intervention and improvement.

Failure to order screening was the second leading risk factor among subspecialty hepatologists in this cohort. Other investigators have demonstrated poor knowledge of screening protocols among primary care providers (PCPs), which can explain lack of adherence to guidelines. However, we do not believe a knowledge deficit was a major contributing factor in a subspecialty clinic. Many hepatologists cited deferral to PCP as a reason for not ordering screening, even though knowledge among PCPs remains poor. The authors also speculate that a busy, often overbooked clinic with competing priorities makes even the most diligent hepatologists forget to order screening. It is difficult to order abdominal imaging while counseling a patient that they will die from cirrhosis unless they overcome innumerable psychosocial barriers to abstain from alcohol for long enough to become a liver transplant candidate.

This study has several important limitations. Firstly, the window for "appropriate screening" in this study was 5 to 7 mo, which is narrower than the 4–8-mo window suggested by the AASLD, resulting in a positive bias toward ineffective screening. Secondly, patients who had two imaging studies 6 mo apart were considered "appropriately screened," regardless of whether a third imaging study was completed on time. This data simplification may have resulted in an overestimation of the screening rate. Thirdly, the attribution of screening failure to a single step fails to capture the multifactorial nature of screening failure. For example, a patient for whom radiology did not schedule an imaging study because the physician did not order one because they missed their clinic appointment would be classified as "no show," even though the provider could have ordered the screening even without the patient there. Finally, the logistical complexity of the screening process leaves room for interpretation variation between multiple investigators, even with rigorous standardization.

The debate over the proper length of screening is likely to continue, with many authors pointing out that longer intervals have not been studied. Some experts, including the National Cancer Institute, have opined that hepatologists ought to abandon screening protocols entirely due to a lack of survival benefit [29-32]. Furthermore, the World Gastroenterology Organization suggests that screening in low and middle-resource settings is appropriate only if the patient would have access to HCC treatments[33]. However, we contend that every effort be made to adhere to current practice guidelines when resources are available. Our findings demonstrate the need for future measures to address system and provider level improvements. We have implemented an automatic reminder in the electronic medical record for physicians and other healthcare professionals and targeted reminders *via* main or electronic media for patients. In addition, our findings highlight the need for serum biomarkers for HCC screening, which would eliminate the logistical delays with radiology[34].

CONCLUSION

In conclusion, the rate of appropriate HCC screening, though above the estimated national average, was inadequate in this patient population. The reasons for failure were multifactorial, but the primary driver was delays in radiology scheduling. These data immediately identify targets for future quality improvement initiatives.

ARTICLE HIGHLIGHTS

Research background

The American Association for the Study of Liver Disease recommends that patients with cirrhosis be screened for hepatocellular carcinoma (HCC) every six months. Other researchers have shown that adherence to these guidelines is poor, but little is known about the causes of this failure.

Research motivation

The authors noted that many patients in their own subspecialty hepatology practice did not undergo appropriate screening. They studied factors contributing to screening failure in order to develop a possible quality improvement initiative.

Research objectives

The authors sought to identify root causes of HCC screening failure among patients with cirrhosis in their subspecialty heaptology clinic.

Research methods

The authors identified patients with cirrhosis in their subspecialty hepatology clinic and determined whether they underwent appropriate screening. The authors reviewed the medical records of patients who did not undergo appropriate screening to identify the root causes of screening failure.

Research results

Among 1034 patients, only 489 underwent appropriate screening. The most common causes of screening failure, in descending order, were: radiology unable to schedule timely imaging, provider did not order imaging, patient canceled follow up appointment, appointments scheduled too far apart, lost to follow up, no-show to radiology appointment, and provider canceled appointment.

Research conclusions

Even in a subspecialty hepatology clinic in which providers strive to follow guideline-based HCC screening, rates of screening were still poor. Most of the barriers to appropriate screening were due to systemic factors such as radiology availability, rather than to individual error.

Research perspectives

HCC screening is vital to the comprehensive care of patients with cirrhosis, yet systemic and institutional barriers often prevent patients from receiving adequate care. The root causes identified in this article immediately suggest areas for possible quality improvement and provide guidance to those at other institutions.

ACKNOWLEDGEMENTS

The authors would like to thank Hanzhi Gao for her statistical expertise.

FOOTNOTES

Author contributions: King WW conducted a plurality of this work; Mota M, Clark V, and Cabrera R developed the study concept and design; King W, Richhart R, Culpepper T, Mota M, Banerjee D, Ismael M, Chakraborty J, Ladna M, Khan W, Ruiz N, and Wilson J performed data acquisition; King W and Culpepper T performed statistical analysis; King W wrote the manuscript; Richhart R, Culpepper T, Altshuler E, Clark V, and Cabrera R assisted with revision and editing; Clark V and Cabrera R supervised this work.

Institutional review board statement: This research was approved by the Institutional Review Board of the University of Florida.

Informed consent statement: Informed consent was therefore not obtained from each individual patient. This process was approved by the institutional review board at the authors' home institution, the University of Florida. Please contact the corresponding author with any questions or concerns.

Conflict-of-interest statement: The authors have no conflicts of interest.

Data sharing statement: De-identified patient data, study protocols, and statistical analysis protocols are available to be shared.

STROBE statement: STROBE statement: The authors have read the STROBE Statement – checklist of items, and the manuscript was prepared and revised according to the STROBE Statement – checklist of items.

Country/Territory of origin: United States

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Corresponding Author's Membership in Professional Societies: American College of Gastroenterology.

S-Editor: Ma YJ
L-Editor: A
P-Editor: Ma YJ

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World Journal of Hepatology

Submit a Manuscript: https://www.f6publishing.com

World J Hepatol 2023 March 27; 15(3): 419-430

DOI: 10.4254/wjh.v15.i3.419

ISSN 1948-5182 (online)

ORIGINAL ARTICLE

Retrospective Study

To scan or not to scan: Use of transient elastography in an integrated health system

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Specialty type: Gastroenterology and hepatology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Pham TTT, Viet Nam; Taura K, Japan; Tolunay HE, Turkey

Received: October 21, 2022
Peer-review started: October 21, 2022
First decision: December 23, 2022
Revised: January 6, 2023
Accepted: March 1, 2023
Article in press: March 1, 2023
Published online: March 27, 2023

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Abstract

BACKGROUND

Non-invasive tests, such as Fibrosis-4 index and transient elastography (commonly FibroScan), are utilized in clinical pathways to risk stratify and diagnose non-alcoholic fatty liver disease (NAFLD). In 2018, a clinical decision support tool (CDST) was implemented to guide primary care providers (PCPs) on use of FibroScan for NAFLD.

AIM

To analyze how this CDST impacted health care utilization and patient outcomes.

METHODS

We performed a retrospective review of adults who had FibroScan for NAFLD indication from January 2015 to December 2017 (pre-CDST) or January 2018 to December 2020 (post-CDST). Outcomes included FibroScan result, laboratory tests, imaging studies, specialty referral, patient morbidity and mortality.

RESULTS

We identified 958 patients who had FibroScan, 115 before and 843 after the CDST was implemented. The percentage of FibroScans ordered by PCPs increased from 33% to 67.1%. The percentage of patients diagnosed with early F1 fibrosis, on a scale from F0 to F4, increased from 7.8% to 14.2%. Those diagnosed with advanced F4 fibrosis decreased from 28.7% to 16.5%. There were fewer laboratory tests, imaging studies and biopsy after the CDST was implemented. Though there were more specialty referrals placed after the CDST was implemented, multi-

variate analysis revealed that healthcare utilization aligned with fibrosis score, whereby patients with more advanced disease had more referrals. Very few patients were hospitalized or died.

CONCLUSION
This CDST empowered PCPs to diagnose and manage patients with NAFLD with appropriate allocation of care towards patients with more advanced disease.

Key Words: Non-alcoholic fatty liver disease; Transient elastography; FibroScan; Clinical decision support tool; Health care utilization; Primary care

Core Tip: This was a retrospective study of nearly 1000 patients with non-alcoholic fatty liver disease who underwent FibroScan. The purpose of this study was to compare patients before and after a clinical decision support tool was implemented. This tool was designed to guide primary care providers on the management of non-alcoholic fatty liver disease. After the tool was released, we saw higher rates of early-stage fibrosis diagnosed by FibroScan. We saw appropriate allocation of care, whereby patients with advanced fibrosis had more labs, imaging studies and specialty referrals. These results suggest non-alcoholic fatty liver disease can feasibly be diagnosed and managed in the primary care setting.

Citation: Stein L, Mittal R, Song H, Chung J, Sahota A. To scan or not to scan: Use of transient elastography in an integrated health system. *World J Hepatol* 2023; 15(3): 419-430
URL: https://www.wjgnet.com/1948-5182/full/v15/i3/419.htm
DOI: https://dx.doi.org/10.4254/wjh.v15.i3.419

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of liver disease worldwide, affecting over 25% of the population[1]. In the United States alone, this translates to over 80 million individuals[2]. In its early stage, NAFLD. is reversible. However, disease progression results in irreversible fibrosis and cirrhosis and portends significant risk of hepatocellular carcinoma.

Historically, liver biopsy was the gold standard for diagnosis of NAFLD[3]. However, advancements in non-invasive testing are beginning to change the standard, with safer, cost-effective[4,5], accurate[6] and readily accessible modalities[7,8] that can be utilized in the primary care setting[9]. In the United States, the most common modality is transient elastography, often delivered by the FibroScan device (Echosens, Paris, France).

The evolution of non-invasive tests, like FibroScan, has enabled clinical pathways by which primary care physicians (PCPs) can identify patients with liver disease prior to utilization of specialty services [10,11]. Recently, professional societies[12,13] have started to embrace these diagnostic tools and clinical pathways in their recommendations. However, very few retrospective[14] and prospective[15,16] studies have assessed the effectiveness of these pathways in clinical practice. To date, no singular study has assessed management, appropriateness of care and patient outcomes for NAFLD patients who have undergone FibroScan.

In 2018, a clinical decision support tool (CDST) for NAFLD was implemented in Kaiser Permanente Los Angeles Medical Center (KPLAMC), a tertiary care center in Southern California. The goals of this CDST were to: (1) Educate and guide PCPs in identifying patients with NAFLD; (2) Risk-stratify patients *via* non-invasive tests; and (3) Triage patients based on risk, whereby lower risk patients were educated about lifestyle modification and higher risk patients were offered specialty referral for advanced care. We sought to determine the impact of this CDST on health care utilization, practice patterns and patient outcomes.

MATERIALS AND METHODS

Clinical pathway

This study was centered around a CDST, part of a user-facing app, called Aura, on the electronic health record (EHR). Aura-based CDSTs populate patient clinical data to allow clinicians to calculate scores and receive recommendations. This CDST was based on the Fib-4 index, a validated calculator to predict liver fibrosis and cirrhosis[17]. If the score was below 1.3, the recommendation included lifestyle

counseling and repeating the score in 3 years. If the score met a threshold of 1.3, a FibroScan was recommended. If the score was above 3.25, FibroScan and specialty referral to gastroenterology and hepatology was recommended (Figure 1).

Study population

The primary population included persons ≥ 18 years who underwent FibroScan for NAFLD indication at KPLAMC from January 1, 2015 to December 31, 2020. KPLAMC is the tertiary referral center for Kaiser Permanente Southern California (KPSC), the largest integrated health system in the state of California. KPLAMC cares for over 275000 adult members, representing about 16% of the population [18].

Study design and data source

A retrospective study. The cohort was identified using an internal database of patients for whom FibroScan was performed. The population was stratified by time of FibroScan, either before (pre-CDST) or after (post-CDST) introduction of the CDST to clinical workflow. Patients were excluded from analysis if pregnant within 1 year of FibroScan. Patients with incomplete data were also excluded (Figure 2).

Data were gathered and extracted from this cohort *via* the KPSC Health Connect Database using International Classification of Diseases and Current Procedural Terminology codes (Supplementary Tables 1 and 2). Certain variables were confirmed by manual chart review. The KPSC Institutional Review Board approved the study.

Outcomes

The primary outcome was health care utilization - who underwent FibroScan and what was the result of the scan. Variables included age, sex, body mass index (BMI), race, insurance type and medical comorbidities such as concomitant chronic liver diseases and risk factors for metabolic syndrome. FibroScan results included fibrosis score, steatosis score, probe type used and category of physician who ordered the scan, either primary or specialty care.

The secondary outcomes included clinical management, hospitalization rate and mortality within one year of FibroScan. Clinical management was subdivided into three categories - laboratory tests, imaging studies, biopsy and specialty referral. Laboratory tests included liver function test, international normalized ratio, creatinine and complete blood count. Imaging studies included computerized tomography (CT)-4 phase liver, magnetic resonance imaging (MRI) liver, right upper quadrant ultrasound and repeat FibroScan. Specialty referral included gastroenterology, hepatology and health education, for services like diet and weight loss. Primary hospital admission diagnoses included hepatic encephalopathy, variceal bleeding, spontaneous bacterial peritonitis and liver cancer (Supplementary Table 2). Deceased patients who died within the first year after FibroScan were captured and cause of death was identified.

Statistical analysis

Statistical significance was calculated by chi-square and Kruskal-Wallis for categorical and continuous variables, respectively. All *P*-values were determined to be significant if they were below the 0.05 threshold.

Subgroup analysis included a multivariable logistic regression to quantify the relationship between clinical management - laboratory tests, imaging studies and specialty referrals - and fibrosis score. The multicollinearity and variance inflation factor were checked and determined to be negligible. For multivariate logistic regression, the p-value was calculated by the Wald Test, with multicollinearity between variables checked with high correlation of 0.8, tolerance below 0.1 and variance inflation factor of above 10. All analyses were done using SAS 9.4 and SAS Enterprise Guide 7.15 (SAS Institute, Cary, NC, United States).

RESULTS

Patient characteristics

We identified 958 patients who underwent FibroScan from January 1, 2015 to December 31, 2020. Of these, 115 patients had FibroScan from January 1, 2015 to December 31, 2017 (pre-CDST) and 843 patients had FibroScan from January 1, 2018 to December 31, 2020 (post-CDST). Patient demographics and clinical characteristics are represented in Tables 1 and 2.

In the pre-CDST cohort, mean age was 58.3 ± 13.78 years with over half (53.9%) being female. Mean BMI was 31.6 ± 6.13. The majority racial group was Hispanic (47.8%), followed by non-Hispanic White (25.2%) and Asian (19.1%). Most patients had commercial health insurance (64.3%) while many others had Medicare (24.3%). Patients carried comorbid diagnoses of diabetes mellitus (45.2%), hyperlipidemia (52.2%) and obstructive sleep apnea (14.7%). Very few patients had comorbid liver diseases. The post-CDST cohort had statistically similar data to the pre-CDST cohort with one exception. Mean BMI in the

Table 1 Patient demographics

Characteristic	Pre-clinical decision support tool (*n* = 115)	Post-clinical decision support tool (*n* = 843)	*P* value
Age, year	58.3 ± 13.78	57.1 ± 14.02	0.3777
Female (%)	53.9	53.3	0.8956
Body mass index	31.6 ± 6.13	33.1 ± 7.10	0.0358
Race (%)			0.4486
African American	4.3	3.3	
Asian	19.1	17.4	
Hispanic	47.8	56.8	
Non-Hispanic White	25.2	19.9	
Other, unknown	3.5	2.5	
Insurance plan type (%)			0.1312
Commercial, private pay	64.3	64.7	
Dual	3.5	5.9	
Medicaid	6.1	4.5	
Medicare	24.3	24.7	
Other, unknown	1.7	0.2	
Medical Comorbidities (%)			
Chronic hepatitis B	2.6	1.8	0.5415
Chronic hepatitis C	4.3	1.3	0.0172
Diabetes mellitus	45.2	42.4	0.5572
Hepatocellular carcinoma	0	0	
Hyperlipidemia	52.2	58.6	0.1891
Liver transplant	0	0.4	0.5212
Obstructive sleep apnea	14.7	13.3	0.6662
Polycystic ovarian syndrome	1.7	0.5	0.1076
Primary biliary cholangitis	0.9	0.4	0.4242
Primary sclerosing cholangitis	0	0	

Data are expressed as mean ± SD or *n* (%).

post-CDST cohort was 33.1 ± 7.1 (P = 0.0358).

FibroScan data

In the pre-CDST cohort, 33% of FibroScans were ordered by PCPs. In the post-CDST cohort, 67.1% of FibroScans were ordered by PCPs. In both cohorts, a little over half (55.7%-56%) of probes used during FibroScan were XL.

Regarding FibroScan results, 9 patients, representing 7.8% of the pre-CDST cohort, had low grade F1 fibrosis. In the post-CDST cohort, this increased to 120 patients with F1 fibrosis, representing 14.2% (P = 0.0142). Additionally, 33 patients in the pre-CDST cohort had advanced F4 fibrosis, representing 28.7%. This decreased to 16.5%, a total of 139 patients, in the post-CDST cohort with F4 fibrosis (P = 0.0142). The percentage of patients with advanced steatosis S3 increased from 43.5% in the pre-CDST cohort to 67.3% in the post-CDST cohort ($P \leq 0.0001$).

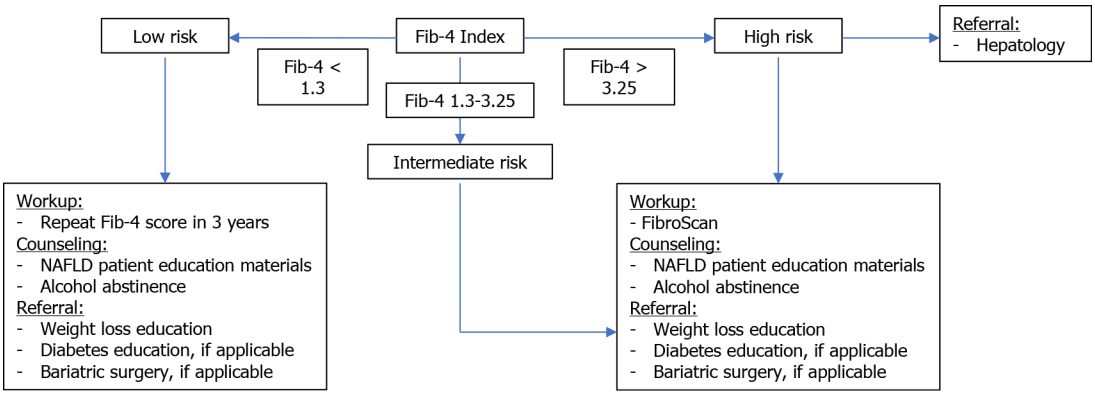
Clinical management

Laboratory tests: In the pre-CDST cohort, an average of 7.2 tests were performed *per* patient in the first year after FibroScan. This significantly decreased to 5.3 Laboratory tests in the post-CDST cohort ($P < 0.0001$). When subdivided by type of test, this significant decrease remained true (Figure 3A).

Table 2 FibroScan data

Parameter	Pre-clinical decision support tool (*n* = 115)	Post-clinical decision support tool (*n* = 843)	*P* value
Physician ordering FibroScan (%)			< 0.0001
Primary care	33	67.1	
Specialty care	67	32.9	
Exam probe used (%)			0.9453
Medium	44.3	44	
Extra large (XL)	55.7	56	
FibroScan result (%)			
Fibrosis score			0.0142
F0	32.2	38.1	
F1	7.8	14.2	
F2	17.4	17.9	
F3	13.9	13.3	
F4	28.7	16.5	
Steatosis score			< 0.0001
S0	43.5	8.1	
S1	4.3	10	
S2	8.7	14.7	
S3	43.5	67.3	

Data are expressed as *n* (%).

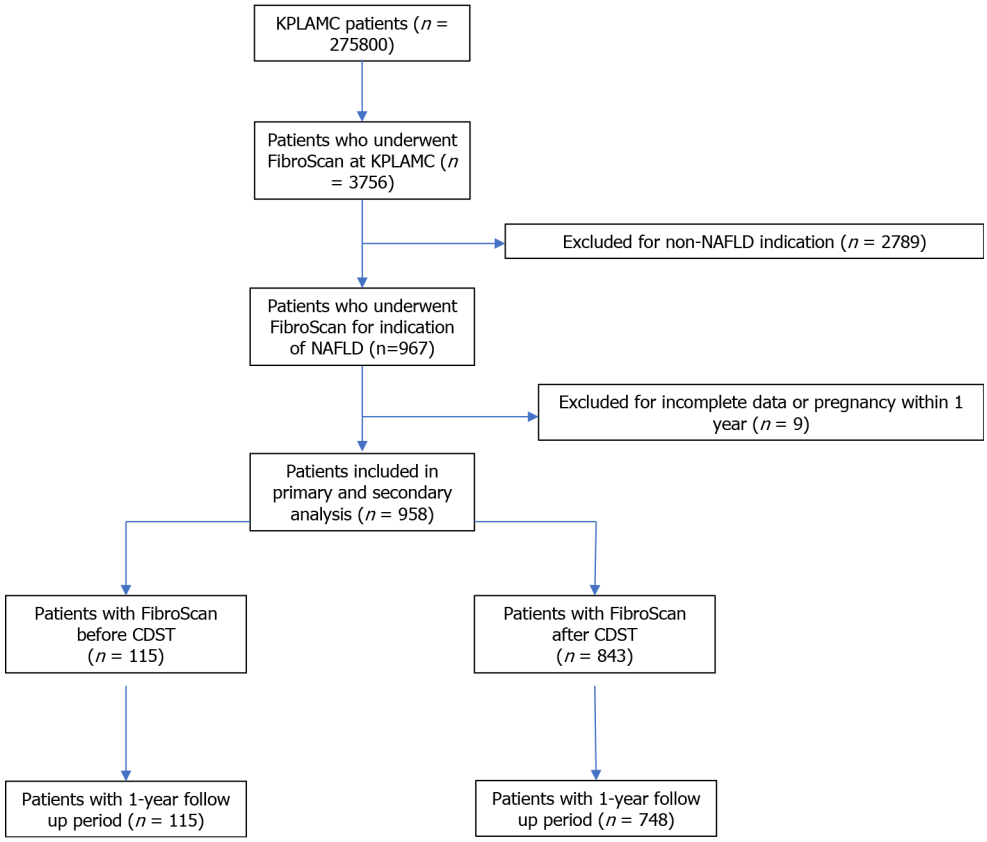


DOI: 10.4254/wjh.v15.i3.419

Figure 1 Clinical decision support tool workflow. Fib-4: Fibrosis-4; NAFLD: Non-alcoholic fatty liver disease.

Imaging studies: The percentage of patients who had an MRI 4-phase liver decreased from 13.9% in the pre-CDST cohort to 12.8% in the post-CDST cohort (P = 0.7486). The percentage of patients who had an MRI liver decreased from 16.5% in the pre-CDST cohort to 10.6% in the post-CDST cohort (P = 0.0607). The percentage of patients who had a right upper quadrant ultrasound decreased significantly from 30.4% in the pre-CDST cohort to 20.7% in the post-CDST cohort (P = 0.0193). The percentage of patients who had a repeat FibroScan increased from 1.7% in the pre-CDST cohort to 2.7% in the post-CDST cohort (P = 0.5538, Figure 3B).

Biopsy: In the pre-CDST cohort, 8.7% of patients had liver biopsy within the first year. This decreased significantly to 2.7% in the post-CDST cohort (P = 0.001). The average number of months to biopsy was similar in both cohorts, 3.9 mo in the pre-CDST cohort *vs* 3.5 mo in the post-CDST cohort (P = 0.9822, Figure 3B).



DOI: 10.4254/wjh.v15.i3.419

Figure 2 Cohort flow chart. KPLAMC: Kaiser Permanente Los Angeles Medical Center; NAFLD: Non-alcoholic fatty liver disease; CDST: Clinical decision support tool.

Of those who were referred for liver biopsy, 82.8% of patients had fibrosis scores of F3 or F4 from FibroScan. Of the biopsies done, 76.9% resulted in fibrosis scores that agreed with the patient's FibroScan result. The remaining 23.1% were discordant to the FibroScan result. In all the discordant biopsy results, Fibroscan overestimated the fibrosis score from the biopsy pathology.

Specialty referral: The percentage of patients for whom gastroenterology referral was placed increased from 5.2% in the pre-CDST cohort to 7.5% in the post-CDST cohort (P = 0.3803). The percentage of patients for whom hepatology referral was placed increased significantly from 2.6% in the pre-CDST cohort to 12.8% in the post-CDST cohort (P = 0.0014). The percentage of patients for whom health education referrals were placed increased significantly from 21.7% in the pre-CDST cohort to 35.2% in the post-CDST cohort (P = 0.0045, Figure 3B).

Patient outcomes

Morbidity: In the pre-CDST cohort, no patients were hospitalized for complications of liver disease in the first year. In the pre-CDST cohort at any time in the study time frame, 4 patients were hospitalized for hepatic encephalopathy, 1 patient was hospitalized for variceal bleeding and 1 patient was hospitalized for spontaneous bacterial peritonitis.

In the post-CDST cohort, 1 patient was hospitalized for hepatic encephalopathy and 2 patients were hospitalized for liver cancer in the first year. In the post-CDST cohort at any time in the study time frame, 4 patients were hospitalized for hepatic encephalopathy and 5 patients were hospitalized for liver cancer (Table 3).

Mortality: In the pre-CDST cohort, 1 patient died in the first year. In the pre-CDST cohort at any time, 9 patients died. In the post-CDST cohort, 7 patients died in the first year. In the post-CDST cohort at any time, 17 patients died. No patients died of complications of liver disease. Cause of death was primarily cardiovascular or complications of coronavirus disease 19 (COVID-19) (Table 3).

Multivariable analysis: The likelihood of healthcare utilization across all categories - laboratory tests, imaging studies and specialty referrals - increased with advancing fibrosis, most prominent in F4 fibrosis (Table 4). The reference group for this analysis was F0 fibrosis patients, unless otherwise specified.

Table 3 Patient morbidity and mortality

Variable	Pre-clinical decision support tool	Post-clinical decision support tool
Patients hospitalized in first year for:		
Hepatic encephalopathy	0	1
Variceal bleeding	0	0
Spontaneous bacterial peritonitis	0	0
Liver cancer	0	2
Patients hospitalized at anytime for:		
Hepatic encephalopathy	4	4
Variceal bleeding	1	0
Spontaneous bacterial peritonitis	1	0
Liver cancer	0	5
Patients deceased in first year	1	7
Patients deceased at any time	9	17

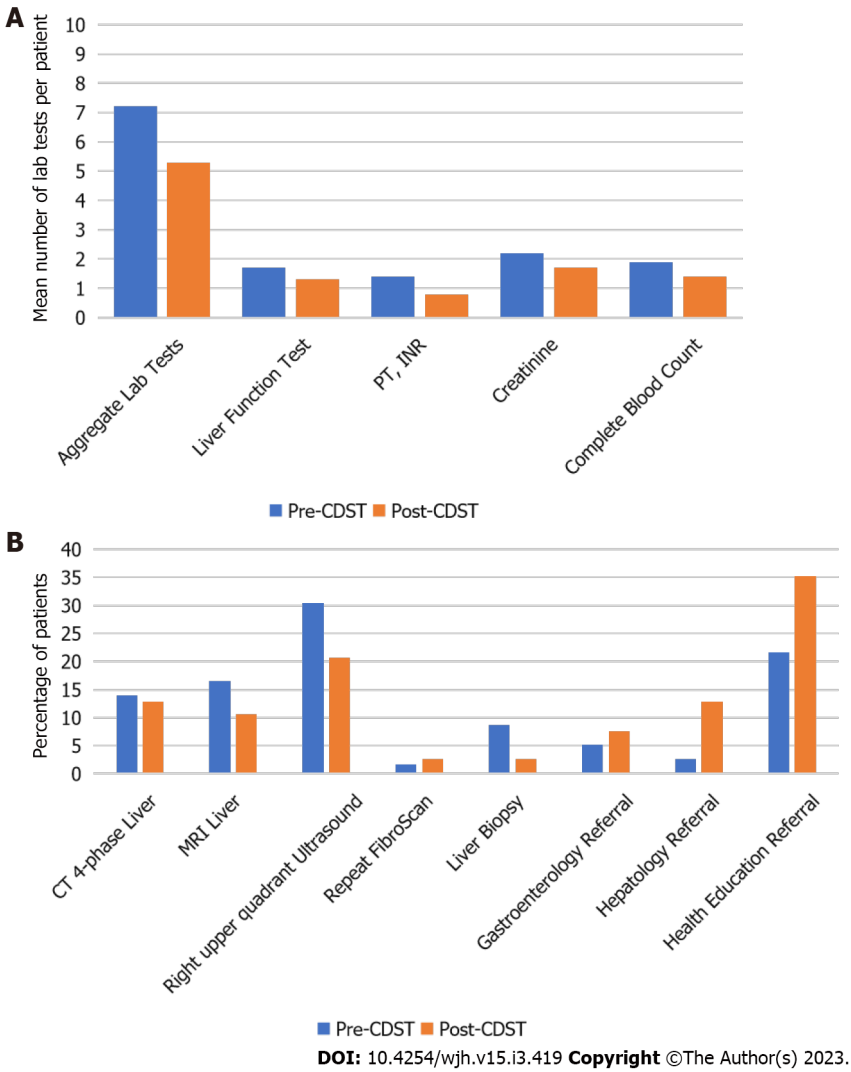
Data are expressed as raw numbers.

Table 4 Multivariable analysis

Variable	Fibrosis score	Odds ratio	95% confidence interval	*P* value
Lab tests	F1 *vs* F0	0.955	0.621-1.469	0.8354
	F2 *vs* F0	1.055	0.711-1.566	0.7886
	F3 *vs* F0	1.507	0.946-2.4	0.0845
	F4 *vs* F0	2.477	1.522-3.953	0.0001
Imaging study	F1 *vs* F0	0.825	0.506-1.343	0.4386
	F2 *vs* F0	1.287	0.855-1.937	0.2259
	F3 *vs* F0	4.703	3.064-7.218	< 0.0001
	F4 *vs* F0	7.188	4.793-10.78	< 0.0001
Gastroenterology referral	F1 *vs* F0	2.362	0.909-6.141	0.0778
	F2 *vs* F0	1.47	0.549-3.939	0.4431
	F3 *vs* F0	6.195	2.786-13.775	< 0.0001
	F4 *vs* F0	4.122	1.85-9.14	0.0005
Hepatology referral	F1 *vs* F2	0.181	0.04-0.813	0.0258
	F3 *vs* F2	4.438	2.253-8.739	< 0.0001
	F4 *vs* F2	4.55	2.385-8.681	< 0.0001
Health education referral	F1 *vs* F0	1.415	0.882-2.272	0.1501
	F2 *vs* F0	1.463	0.957-2.236	0.0786
	F3 *vs* F0	2.054	1.305-3.233	0.0019
	F4 *vs* F0	3.589	2.391-5.387	< 0.0001

Those with F3 fibrosis were 1.507 times as likely to have a laboratory test than those with F0 fibrosis ($P = 0.0845$). Those with F4 fibrosis were 2.477 times as likely to have a laboratory test than those with F0 fibrosis ($P = 0.0001$).

Those with F3 fibrosis were 4.703 times as likely to have an imaging study than those with F0 fibrosis ($P < 0.0001$). Those with F4 fibrosis were 7.188 times as likely to have an imaging study than those with F0 fibrosis ($P < 0.0001$).



DOI: 10.4254/wjh.v15.i3.419

Figure 3 Clinical management in first year following FibroScan. A: Laboratory tests; B: Imaging studies and specialty referral. CDST: Clinical decision support tool; CT: Computerized tomography; MRI: Magnetic resonance imaging.

Those with F3 fibrosis were 6.195 times as likely to have a gastroenterology referral than those with F0 fibrosis ($P < 0.0001$). Those with F4 fibrosis were 4.122 times as likely to have a gastroenterology referral than those with F0 fibrosis ($P = 0.0005$).

Due to low numbers, comparisons for hepatology referrals were made with F2 fibrosis patients rather than F0 or F1 fibrosis patients. Those with F3 fibrosis were 4.438 times as likely to have a hepatology referral than those with F2 fibrosis ($P < 0.0001$). Those with F4 fibrosis were 4.55 times as likely to have a hepatology referral than those with F2 fibrosis ($P < 0.0001$).

Those with F3 fibrosis were 2.054 times as likely to have a health education referral than those with F0 fibrosis ($P = 0.0019$). Those with F4 fibrosis were 3.589 times as likely to have a health education referral than those with F0 fibrosis ($P < 0.0001$).

DISCUSSION

This study examined the demographics, clinical management, morbidity and mortality of a cohort of patients with NAFLD who underwent FibroScan, a non-invasive test to diagnose liver fibrosis. This study was centered around a CDST designed to guide PCPs in the care of patients with NAFLD. We compared patients before and after the CDST was implemented to determine its impact on health care utilization, practice patterns and patient outcomes.

The CDST pathway, combining Fib-4 and FibroScan, was chosen in particular because of robust clinical data supporting its use in the NAFLD population. When compared head-to-head with other scoring systems, Fib-4 has a high negative predictive value[19], making it an ideal rule out test in detecting advanced fibrosis and cirrhosis[20,21]. Furthermore, FibroScan has a high positive predictive value for the measurement of liver stiffness[22], to rule in advanced fibrosis and cirrhosis, and thus risk-

stratify patients. When Fib-4 and FibroScan are used in tandem, it is predicted that 87% of unnecessary further assessments may be avoided[23].

Our data revealed three important findings. First is regarding FibroScan orders. Prior to the CDST, about two-thirds of all FibroScans were ordered by specialty providers. Additionally, the overall number of scans ordered by any provider during that time was low. This indicates either poor understanding of the test's presence or low level of confidence in the test itself. After the CDST, not only did the overall number of scans increase 7-fold, but also, the majority of scans - about two-thirds - were ordered by PCPs. This drastic shift shows that the CDST achieved its goal of educating PCPS on the utility of FibroScan and fostered a new confidence in the test, leading to higher rates of utilization.

As such, the average BMI of patients in the post-CDST cohort was statistically significantly higher than those in the pre-CDST cohort. We attribute this difference to provider education regarding risk factors for NAFLD. When the CDST was implemented, PCPs were alerted of its presence and provided educational materials in the form of EHR alerts, informational emails and formal lectures. Since obesity is a known risk factor for NAFLD, it is likely that PCPs thought to screen patients with higher BMIs.

The second important finding is regarding fibrosis score. In the pre-CDST cohort, fibrosis scores had a bi-modal distribution. About half of the patients either had no fibrosis (F0) or had advanced fibrosis (F4). Conversely, the post-CDST cohort contained almost half the number of patients with advanced fibrosis (F4) and also twice the number of patients with early fibrosis (F1). This change shows that the CDST captured patients earlier in the disease process. As we know, while early fibrosis is reversible, advanced fibrosis and cirrhosis is not. Early recognition and diagnosis are crucial.

The third important finding is regarding care utilization. In aggregate, the utilization rates of laboratory tests, imaging studies and biopsy decreased with the introduction of the CDST. In particular, there was no significant difference in gastroenterology referral for patients with early fibrosis (F0-F1). Furthermore, patients with advanced fibrosis (F3-F4) had more tests and studies done and more referrals placed. This not only represents appropriate allocation and utilization of care, but also may serve to quell providers' worries that identification of NAFLD patients may lead to unnecessary testing, in particular endoscopies for variceal surveillance[24].

Regarding strengths and weaknesses, this study cohort is robust and diverse and can reasonably be extrapolated to the national population. To date, no singular study of a clinical pathway has assessed management, appropriateness of care and patient outcomes in the NAFLD population. Unfortunately, the study period included the COVID-19 pandemic, which is known to have resulted in decreased rates of care utilization and delivery[25].

CONCLUSION

This study is of particular importance. PCPs see more than 300 cases of NAFLD for every 1000 patient encounters[26]. The average annual cost of care *per* NAFLD patient with private health insurance in the United States is $7804 for a new diagnosis and $3789 for long-term management[27]. Not only is NAFLD independently associated with 17% higher annual attributable healthcare costs, but also more advanced disease, F3 and above, is associated with a 40% increase in median annual healthcare cost when compared to F2 and below[28]. The lion share of this increase in cost can be attributed to liver biopsy, imaging and hospitalizations[27].

Not only is the prevalence of NAFLD and NASH projected to increase by up to 56% in the next 10 years[29], but also high primary care workload and physician burnout[30] necessitates action and education. Early and accurate diagnosis of fibrosis in NAFLD patients, particularly those with advanced disease, is necessary to determine the patient's prognosis and guide clinical decision making.

Workflows such as this CDST can not only help patients attain adequate, appropriate, preventative care, but also can help streamline primary care clinical practice and empower physicians beyond the liver clinic to appropriately recognize and manage high risk NAFLD. Future directions for this work include longitudinal study of this population and clinical workflow in multiple centers on a national and international scale.

ARTICLE HIGHLIGHTS

Research background

Non-alcoholic fatty liver disease (NAFLD) is a growing problem, affecting over 25% of the global population. Non-invasive tests are being used more and more to risk stratify and diagnose patients with NAFLD. However, there is a paucity of data for how these tests are being used for clinical decision making in real-world practice.

Research motivation

We examined a clinical decision support tool (CDST) designed to guide primary care providers (PCPs)

in the care of patients with NAFLD.

Research objectives

To evaluate health care utilization, practice patterns and patient outcomes of patients who underwent Fib-roScan for NAFLD indication.

Research methods

A retrospective review of 958 adult patients who underwent FibroScan. Patients were compared before and after introduction of the CDST. Univariate and multivariate logistic regression models were performed in statistical analyses.

Research results

Introduction of the CDST allowed for more patients with early fibrosis and fewer patients with advanced fibrosis to be identified. Overall, fewer labs, imaging studies and biopsies were ordered after the CDST. Providers appropriately ordered more specialty referrals for patients with more advanced fibrosis.

Research conclusions

This CDST empowered PCPs to diagnose and manage patients with NAFLD with appropriate allocation of care towards patients with more advanced disease.

Research perspectives

Non-alcoholic fatty liver disease can feasibly be diagnosed and managed in the primary care setting. Future research is required to streamline and refine care of this patient population.

FOOTNOTES

Author contributions: Stein L, Mittal R and Sahota A designed the study; Song H and Chung J gathered the data and performed the statistical analysis; Stein L wrote the manuscript; all authors read, edited and approved the final manuscript.

Institutional review board statement: The study was reviewed and approved by the Kaiser Permanente Southern California Institutional Review Board (Approval No. #12674).

Informed consent statement: Because of the nature of a retrospective study, signed informed consent form is not needed. However, Kaiser Permanente Los Angeles Medical Center has given permission to conduct this study.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: No additional data are available.

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S-Editor: Liu GL
L-Editor: A
P-Editor: Liu GL

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World Journal of Hepatology

Submit a Manuscript: https://www.f6publishing.com

World J Hepatol 2023 March 27; 15(3): 431-440

DOI: 10.4254/wjh.v15.i3.431

ISSN 1948-5182 (online)

ORIGINAL ARTICLE

Retrospective Study

Coexistent alcohol-related cirrhosis and chronic pancreatitis have a comparable phenotype to either disease alone: A comparative retrospective analysis

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Specialty type: Gastroenterology and hepatology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Maslennikov R, Russia; Tantau AI, Romania

Received: December 7, 2022
Peer-review started: December 7, 2022
First decision: December 19, 2022
Revised: February 3, 2023
Accepted: March 9, 2023
Article in press: March 9, 2023
Published online: March 27, 2023

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Abstract

BACKGROUND

Alcohol use disorder is a prevalent disease in the United States. It is a well-demonstrated cause of recurrent and long-standing liver and pancreatic injury which can lead to alcohol-related liver cirrhosis (ALC) and chronic pancreatitis (ACP). ALC and ACP are associated with significant healthcare utilization, cost burden, and mortality. The prevalence of coexistent disease (CD) ranges widely in the literature and the intersection between ALC and ACP is inconsistently characterized. As such, the clinical profile of coexistent ALC and ACP remains poorly understood. We hypothesized that patients with CD have a worse phenotype when compared to single organ disease.

AIM

To compare the clinical profile and outcomes of patients with CD from those with ALC or ACP Only.

METHODS

In this retrospective comparative analysis, we reviewed international classification of disease 9/10 codes and electronic health records of adult patients with verified ALC Only (n = 135), ACP Only (n = 87), and CD (n = 133) who received

care at UPMC Presbyterian-Shadyside Hospital. ALC was defined by histology, imaging or clinical evidence of cirrhosis or hepatic decompensation. ACP was defined by imaging findings of pancreatic calcifications, moderate-severe pancreatic duct dilatation, irregularity or atrophy. We compared demographics, pertinent clinical variables, healthcare utilization, and mortality for patients with CD with those who had single organ disease.

RESULTS

Compared to CD or ACP Only, patients with ALC Only were more likely to be older, Caucasian, have higher body mass index, and Hepatitis B or C infection. CD patients (*vs* ALC Only) were less likely to have imaging evidence of cirrhosis and portal hypertension despite possessing similar MELD-Na and Child C scores at the most recent contact. CD patients (*vs* ACP Only) were less likely to have acute or recurrent acute pancreatitis, diabetes mellitus, insulin use, oral pancreatic enzyme therapy, and need for endoscopic therapy or pancreatic surgery. The number of hospitalizations in patients with CD were similar to ACP Only but significantly higher than ALC Only. The overall mortality in patients with CD was similar to ALC Only but trended to be higher than ACP Only ($P = 0.10$).

CONCLUSION

CD does not have a worse phenotype compared with single organ disease. The dominant phenotype in CD is similar to ALC Only which should be the focus in longitudinal follow-up.

Key Words: Alcohol; Cirrhosis; Chronic pancreatitis; Overlap; Phenotype

Core Tip: Patients with coexistent alcohol-related cirrhosis and alcohol-related chronic pancreatitis do not have a worse phenotype when compared with single organ disease patients. The dominant phenotype in patients with coexistent disease (CD) in terms of overall survival and markers of advanced liver disease was similar to patients with Alcohol-related Cirrhosis Only. Coexistent disease patients also had lower prevalence of disease-related manifestations when compared with those who had single organ disease. Patients with CD may not need to be monitored at a higher degree, but the primary focus for longitudinal follow-up should be on alcohol-related cirrhosis.

Citation: Lu M, Sun Y, Feldman R, Saul M, Althouse A, Arteel G, Yadav D. Coexistent alcohol-related cirrhosis and chronic pancreatitis have a comparable phenotype to either disease alone: A comparative retrospective analysis. *World J Hepatol* 2023; 15(3): 431-440

URL: https://www.wjgnet.com/1948-5182/full/v15/i3/431.htm

DOI: https://dx.doi.org/10.4254/wjh.v15.i3.431

INTRODUCTION

Alcohol use disorder (AUD) is a disease affecting over 14 million adults in the United States[1]. Long-standing alcohol use is a well-established cause of liver and pancreatic injury that can culminate in alcohol-related liver cirrhosis (ALC) and alcohol-related chronic pancreatitis (ACP)[2,3]. The complications of ALC and ACP are major causes of morbidity and mortality associated with alcohol misuse[4-6].

The liver and pancreas are developmentally related and share a number of functional similarities; they also exhibit common features of alcohol-induced injury. The quantity of alcohol misuse is the primary risk factor for developing both diseases and leads to the metabolic stress and low-grade inflammation that stimulates maladaptive fibrotic changes[7]. Susceptibility for developing ALC and/or ACP also relates to non-modifiable risk factors such as race, genetics, and environment[8-11]. ALC-related complications range from ascites and portosystemic encephalopathy to hepatorenal syndrome and hepatocellular carcinoma, and it is estimated that alcohol use accounts for 20%-36% of cirrhosis cases [12-14]. The rate of cirrhosis-related hospitalizations and annual costs have been increasing[15,16]. Comparably, the long-standing inflammatory state in chronic pancreatitis (CP) results in irreversible parenchymal destruction and dysfunction. ACP often begins with an index acute pancreatitis event that progresses to CP as dictated by the severity and number of recurrent episodes of acute pancreatitis[17]. Commonly attributed to alcohol consumption in the North American population, complications from CP include chronic pain, exocrine/endocrine insufficiency, and pancreatic adenocarcinoma[18-20] and poor quality of life[21].

Although ALC and ACP have been well-studied in isolation, patients with overlap of ALC and ACP (*i.e.*, Coexistent Disease) is inconsistently characterized in the literature. Some studies have failed to demonstrate any association between ALC and ACP[22,23] while others suggest interconnectivity between alcohol-related liver and pancreas disease. For instance, alcohol-related liver disease can lead to pancreatic exocrine insufficiency and accumulation of fatty acid ethyl esters which contributes to further progression of alcohol-related liver[24] and pancreas disease[25], while ACP can cause and exacerbate portal hypertension which worsens the complications of liver disease[26]. Furthermore, emerging data from the United States in recent years suggests that coexistent disease (CD) represent only a small fraction of patients with AUD. Although estimates of prevalence of CD in the literature range widely from 0%-75%, a meta-analysis performed by our group revealed a pooled prevalence of ACP in ALC and ALC in ACP to be 16.2% and 21.5% respectively[27].

To date, published studies have yet to define the clinical profile of patients with CD and its differences from single-organ disease. We hypothesized that patients with CD will have a more advanced phenotype and worse outcomes when compared with patients who have single organ (ALC Only or ACP Only) disease. To test this hypothesis, we performed a detailed comparative analysis of well-characterized patients with ACP Only, ALC Only, and CD who received care in a large healthcare system cohort.

MATERIALS AND METHODS

Study population

The study was approved by the University of Pittsburgh's Institutional Review Board. The patient pool consisted of those who were aged ≥ 18 years, had one or more inpatient, emergency room, and outpatient encounters at any UPMC facility from January 1, 2006 to December 31, 2017 with international classifications of diseases (ICD) versions 9 and/or 10 codes for AUD, alcohol-related liver disease or pancreatitis (Supplementary material), had 12 or more months of contact with the UPMC system, and received care at UPMC Presbyterian-Shadyside campus at some time during their care at UPMC[28]. Among these patients, we randomly identified a subset who received a diagnosis of ALC Only (n = 202), ACP Only (n = 200) and both ALC and ACP (n = 200). Unlike ALC for which etiology-specific codes are routinely used in clinical practice, ICD-9 classification for pancreatitis did not include etiology-specific codes, which became available with the ICD-10 coding system. In our dataset, as only a small portion of patients received an ICD-10 diagnosis of ACP, we identified patients as ACP by the diagnosis of AUD at any time in addition to CP, as was described previously[28].

Analysis and review of the Electronic Health Records of the 602 randomly identified patients was performed by 2 authors (ML, YS) under the supervision of the senior author using pre-defined criteria to verify the diagnosis of cirrhosis and CP. Cirrhosis was defined by histologic findings, imaging evidence of cirrhosis or portal hypertension, or clinical signs of hepatic decompensation. CP was defined by imaging findings of pancreatic calcifications, moderate-severe pancreatic ductal dilation, pancreatic ductal stricture or gland atrophy. To ensure that patients with ALC Only did not have any clinical pancreatic disease, we excluded patients with a verified diagnosis of ALC who had prior acute or recurrent acute pancreatitis. Similarly, among patients with verified ACP Only, we excluded those who had prior alcohol-related hepatitis. Patients with a verified diagnosis of ALC Only, ACP Only and both ALC and ACP (CD) formed the study population.

Data collection

For each patient with a verified diagnosis, we reviewed the Electronic Health Records to retrieve detailed information on demographics, alcohol and tobacco use, pertinent clinical information for ALC and ACP, healthcare utilization and overall survival until March 3, 2021. Information relevant to liver disease included details of verification criteria fulfilled, clinical features of portal hypertension, hepatic decompensation, history of alcohol-related hepatitis, Child-Pugh and MELD scores, need for liver transplantation, and treatments received. For CP, in addition to the verification criteria fulfilled, information was collected on clinical features of CP, laboratory tests, dual-energy X-ray absorptiometry (DEXA) scan results, and treatments for CP or its complications.

Analytic approach and statistical analyses

We report demographic and disease-specific information for each of the three groups. Continuous variables are presented as mean ± standard deviation or median (interquartile range), and categorical variables were reported as n (%). Statistical comparisons were made using t-test and Kruskal-Wallis test for continuous variables and chi-square tests for categorical variables. Survival from time of first diagnosis is reported using the Kaplan-Meier method. Cox proportional-hazards models are used to report the hazard ratio (HR) and 95% confidence intervals (CI) for patients with ALC Only *vs* ACP Only and CD *vs* ACP Only while adjusting for age at diagnosis, sex, and race. All statistical analyses were performed using R, version 4.1.3 by biomedical statisticians (RF, AA).

RESULTS

Study population and demographics

The final study population consisted of 355 patients with verified diagnosis - 135 with ALC Only, 87 with ACP Only, and 133 with CD. Select characteristics of these patients are presented in Table 1. When compared with CD, patients with ALC Only were older at the time of study entry, had higher body mass index, were more likely to be Caucasian and more likely to have Hepatitis B and C infections. While roughly one-thirds of patients with CD or ALC Only were female, only 23% of ACP patients were female. The median duration of contact was greater than 10 years and was comparable between groups. The median number of non-elective hospital admissions for CD and ACP Only were comparable and significantly greater than patients with ALC Only. During follow-up, the number of patients who died in the CD, ALC Only, and ACP Only group was 80 (60%), 82 (61%), and 36 (41%), respectively. Survival analysis using Cox-regression after controlling for age, sex and race (Figure 1) demonstrated that the survival between ALC Only and ACP Only was similar (HR 1.22, 95%CI 0.82-1.82, $P = 0.32$), while there is a trend towards lower survival in patients with CD when compared to ACP Only (HR 1.40, 95%CI 0.94-2.09, $P = 0.10$).

Comparisons between CD vs ALC Only

Select disease-specific characteristics of patients with CD and ALC Only are shown in Table 2. Patients with ALC Only underwent liver biopsy more often than those with Coexistent disease (33.3% *vs* 16.5%, $P = 0.002$). Patients with ALC Only were more likely to have radiographic evidence of cirrhosis (93% *vs* 76%, $P \leq 0.001$) and portal hypertension (74% *vs* 59%, $P = 0.006$) on imaging. Although MELD and Child-Pugh scores at most recent contact were similar among patients with CD and ALC Only, some specific clinical features differed between the two groups. Specifically, while patients with CD were more likely to have a history of spontaneous bacterial peritonitis, those with ALC Only were more likely to have esophageal varices, need for variceal banding, treatment with beta blockers, and hepatocellular carcinoma. Other features of decompensated liver disease (*e.g.*, ascites) or treatments (*e.g.*, TIPS) were similar between the two groups.

Comparisons between CD and ACP Only

Morphologic appearance of the pancreas was generally similar among patients with CD and ACP Only (Table 3). In regards to the clinical manifestations, patients with ACP Only were more likely to have a history of acute or recurrent acute pancreatitis, receive pancreatic enzyme replacement therapy, ERCP, and pancreatic surgery than patients with CD. Patients with ACP Only were also more likely to have endocrine dysfunction, as characterized by a higher prevalence of diabetes, need for insulin therapy, and poor glycemic control as reflected by a higher hemoglobin A1c level at the time of last contact. Other clinical features or therapies were similar between the two groups.

DISCUSSION

As the largest study of its kind, this work endeavors to further characterize patients at the intersection of ALC and ACP. Our retrospective analysis of patients with a verified diagnosis of ALC Only, ACP Only or CD reveals that during a similar period of observation, although patients with CD had differences in some disease-related manifestations, they did not have worse phenotype than counterparts with single organ disease. Furthermore, our findings suggest that patients with CD potentially need not be monitored at a higher degree, but the primary focus should be on the management of ALC.

Patients included in this study represent the most severe phenotypes of alcohol-related liver or pancreas disease who received care at a tertiary care center during the course of their illness. Among them, we observed that the dominant phenotype in patients with CD to be similar to that of ALC, specifically the two most important indicators of outcomes (*i.e.* overall survival and MELD-Na and Child C scores in patients with CD were similar to patients with ALC Only). This suggests that patients with alcohol-related pancreatic disease who are identified to have alcohol-related liver disease need to be assessed and monitored for early identification of cirrhosis or cirrhosis-related complications so they can be managed in a timely manner.

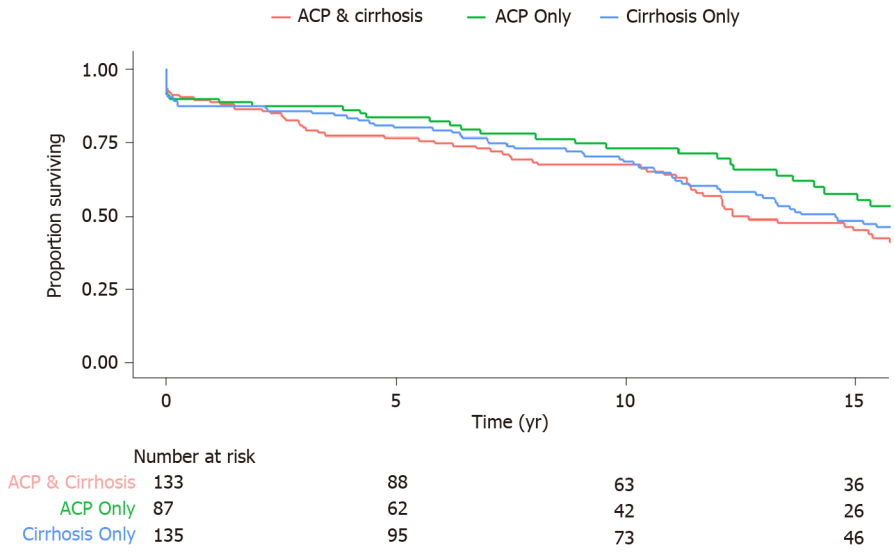
Patients with CD shared similar demographic attributes with those of single organ disease such as the sex distribution of ALC Only patients as well as age, racial distribution and body mass index (BMI) of ACP Only patients. Of note, although our prior study showed that the prevalence of alcohol-related pancreatic disease in those with alcohol-related liver disease was 2-4 folds higher in blacks compared to other races[28], the racial difference was not present in this study. This may be related to the inclusion of patients with the most severe phenotypes in this study as noted above, which may not be representative of the full spectrum of alcohol-related liver and pancreas disease.

When comparing patients with CD with those who had single organ disease, we observed some demographic differences. For instance, patients with CD were younger than those with ALC Only but similar to patients with ACP Only. Although our retrospective study was not designed to evaluate this

Table 1 Select demographics and characteristics in the study population, *n* (%)

	CD (*n* = 133)	ALC only (*n* = 135)	ACP only (*n* = 87)	*P* value (CD *vs* ALC only)	*P* valve (CD *vs* ACP only)
Age (at study entry), yr – mean ± SD	51.7 ± 12.0	54.6 ± 9.8	51.0 ± 12.3	**0.029**	0.684
Female	49 (38)	42 (31)	20 (23)	0.322	**0.03**
Race				**0.015**	0.52
Caucasian	97 (73)	113 (84)	61 (70)		
Black	30 (23)	22 (16)	24 (28)		
Other	6 (5)	0 (0)	2 (2)		
Body mass index[a] – mean ± SD	24.2 ± 7.0	27.8 ± 6.5	23.3 ± 5.4	**< 0.001**	0.281
Tobacco use	117 (88)	109 (81)	81 (93)	0.104	0.127
Smoking (one or more packs per day	31 (23)	23 (17)	24 (28)	0.29	0.392
Alcohol use (duration), yr – mean ± SD	26.7 ± 16.0	29.4 ± 13.8	23 ± 18.4	0.595	0.762
Hepatitis B Infection	7 (5)	17 (13)	2 (2)	**0.036**	0.278
Hepatitis C Infection	31 (23)	58 (43)	14 (16)	**0.001**	0.194
Non-Elective Hospital Admissions[b] – median (IQR)	4 (1 - 12)	3 (0 - 7)	4 (1 - 8)	**0.007**	0.57
Duration of observation, yr – mean ± SD	10.8 ± 7.9	12.4 ± 7.6	11.8 ± 7.6	0.107	0.36

[a]At most recent contact.
[b]Compared using Kruskal-Wallis test. ACP: Alcohol-related chronic pancreatitis; ALC: alcohol-related liver cirrhosis; CD: Coexistent disease; IQR: Interquartile range.



DOI: 10.4254/wjh.v15.i3.431

Figure 1 Survival analysis for the three clinical groups from time of first diagnosis to the last contact. ACP: Alcohol-related chronic pancreatitis.

systematically, a potential explanation is an earlier identification of CP based on clinical symptoms and/or imaging studies in patients with alcohol-related liver disease. Similarly, patients with CD had BMI similar to ACP but lower than patients with ALC likely related to malabsorption. The alternative explanation in a subset of patients with ALC may be fluid retention related to portal hypertension.

Other than spontaneous bacterial peritonitis, patients with CD in general had a lower burden of disease-related manifestations when compared with patients who had ALC Only and ACP Only. The reason for this is unclear but a possible explanation may be the recognition of disease overlap at an earlier stage, *e.g.* alcohol-related liver disease in patients with ACP or alcohol-related pancreatitis in patients with ALC. In terms of healthcare utilization, the burden of non-elective admissions in patients

Table 2 Select disease-specific characteristics in patients with coexistent disease *vs* alcohol-related liver cirrhosis only, *n* (%)

Characteristics present	CD (*n* = 133)	ALC only (*n* = 135)	*P* value
Verification criteria fulfilled			
Liver biopsy performed	22 (17)	45 (33)	**0.002**
Cirrhosis on biopsy	13 (59)	22 (49)	0.349
Cirrhosis on imaging[b]	101 (76)	126 (93)	**< 0.001**
Portal hypertension features on imaging	78 (59)	100 (74)	**0.006**
Alcohol-Related hepatitis	48 (36)	34 (25)	0.052
MELD score[a]	19.3 ± 8.98	18.7 ± 8.89	0.614
Child-Pugh score[a]			0.69
A	41 (31)	48 (36)	
B	49 (39)	48 (36)	
C	43 (32)	39 (29)	
Complications of portal hypertension			
Esophageal varices on EGD	46 (35)	68 (50)	**< 0.001**
Esophageal variceal hemorrhage	11 (8)	17 (13)	0.247
Ascites	96 (72)	83 (62)	0.063
Spontaneous bacterial peritonitis	22 (17)	9 (7)	**0.011**
Portosystemic encephalopathy	62 (47)	68 (50)	0.539
Hepatocellular carcinoma	6 (5)	23 (17)	**0.001**
End-stage renal disease requiring CRRT/HD	15 (11)	13 (10)	0.644
Treatment of portal hypertension/complications			
Esophageal variceal banding	11 (8)	22 (16)	**0.046**
TIPS	3 (2)	8 (6)	0.13
Beta-blocker use[a]	18 (14)	38 (28)	**0.004**
Diuretic use[a]	57 (43)	65 (48)	0.415
Large volume paracentesis	29 (22)	39 (29)	0.183
Antibiotics for SBP prophylaxis[a]	9 (7)	11 (8)	0.68
Lactulose and/or rifaximin use[a]	50 (38)	55 (41)	0.632
Transplant evaluation	14 (11)	19 (14)	0.402
Liver transplantation	3 (2)	10 (7)	**0.05**

[a]At most recent contact.

[b]Ultrasound, computed tomography scan magnetic resonance imaging, elastography. ALC: Alcohol-related liver cirrhosis; CD: Coexistent disease; CRRT: Continuous renal replacement therapy; HD: Homeodomain; EGD: Esophagogastroduodenoscopy; SBP: Spontaneous bacterial peritonitis.

with CD mirrored those of ACP Only patients.

Strengths of our study include the largest sample size to evaluate the phenotype of patients with CD, rigorous review of medical records to verify diagnosis and data collection by review of medical records and a long observation period which ensures capture of clinical events. Our study also has limitations. Being a retrospective study from a single-center tertiary academic medical center may have resulted in our study population to be of higher complexity and limit generalizability of our findings. Our study population includes patients with concomitant Hepatitis B and C infections. While the prevalence of these infections rates represent the traits of our underlying clinical population, hepatitis B and C infections may attribute to or confound the severity of hepatic disease. Although our review of records within the UPMC system was complimented by availability of medical records from other institutions whenever possible through Care Everywhere, there is a possibility of underestimation of clinical events. Finally, clinical events and demographics have the potential to be misclassified in the dataset due to missing or incomplete information.

Table 3 Select disease-specific characteristics in patients with coexistent disease *vs* alcohol-related chronic pancreatitis only, *n* (%)

Characteristics present	CD (*n* = 133)	Only ACP (*n* = 87)	*P* value
Verification criteria fulfilled on imaging			
Pancreatic calcifications	88 (66)	64 (74)	0.246
Moderate-severe ductal dilatation	38 (29)	30 (35)	0.354
Moderate-severe ductal structure	18 (14)	14 (16)	0.599
Any gland atrophy	77 (58)	46 (53)	0.463
Moderate-severe gland atrophy	10 (13)	7 (15)	0.88
Gland atrophy not reported	67 (87)	37 (80)	0.25
Diagnosis based on EUS alone	6 (5)	5 (6)	0.681
Chronic pancreatitis features			
Acute pancreatitis	101 (76)	74 (85)	**0.009**
Age at first pancreatitis, yr – mean ± SD	48.1 ± 15.2	41.5 ± 10.6	0.112
Recurrent acute pancreatitis	61 (46)	53 (61)	**0.023**
Chronic abdominal pain[a]	56 (42)	44 (51)	0.189
Pancreatic pseudocyst	29 (22)	22 (25)	0.549
Diabetes mellitus	54 (41)	50 (58)	**0.011**
Exocrine pancreatic insufficiency (Fecal elastase < 100 and/or steatorrhea)	24 (18)	14 (16)	0.708
Pancreatic adenocarcinoma	2 (2)	4 (5)	0.163
Treatment of chronic pancreatitis/complications			
Oral anti-diabetic therapy[a]	11 (20)	13 (26)	0.113
Insulin therapy[a]	46 (85)	37 (74)	0.209
Pancreatic enzymatic replacement therapy[a]	35 (26)	39 (45)	**0.004**
Chronic opiate therapy[a]	59 (44)	33 (38)	0.381
Treatment by chronic pain specialist	20 (15)	20 (23)	0.124
Celiac plexus block	0 (0)	2 (2)	0.077
ERCP	41 (31)	43 (49)	**0.004**
Pseudocyst drainage (endoscopic/surgical)	18 (14)	13 (15)	0.743
Pancreatic surgery	13 (10)	19 (22)	**0.012**
Pertinent test results			
Hemoglobin A1C[a] – mean ± SD	6.4 ± 2.3	7.3 ± 2.1	**0.01**
Vitamin D deficiency	48 (36)	25 (29)	0.104
DEXA scan performed	28 (21)	19 (22)	0.855
Osteopenia on DEXA scan	12 (43)	10 (53)	0.51
Osteopenia on DEXA scan	8 (29)	5 (26)	0.865

[a]At most recent contact. ACP: Alcohol-related chronic pancreatitis; CD: Coexistent disease; ERCP: Endoscopic retrograde cholangiopancreatography; DEXA: Dual-energy-xray-absorptiometry.

CONCLUSION

Contrary to our working hypothesis, patients with Coexistent ALC and ACP did not have a worse phenotype when compared with single organ disease patients. The dominant phenotype in patients with CD in terms of overall survival and markers of advanced liver disease was similar to patients with ALC Only. CD patients also had lower prevalence of disease-related manifestations when compared with those who had single organ disease. Our findings suggest that patients with CD may not need to be monitored at a higher degree, but the primary focus for longitudinal follow-up should be on ALC.

ARTICLE HIGHLIGHTS

Research background

Heavy alcohol use is a known cause of liver and pancreatic injury that can lead to alcohol-related liver cirrhosis (ALC) and alcohol-related chronic pancreatitis (ACP). These diseases are associated with significant morbidity, mortality, and healthcare utilization and spending.

Research motivation

While both ALC and ACP are well-characterized, there is a subset of patient with both ALC and ACP (coexistent disease) that is poorly understood.

Research objectives

We aim to characterize the clinical profile of patients with coexistent disease (CD) and its differences from those with ALC Only or ACP Only.

Research methods

The study population consisted of adult patient encounters at UPMC facilities from 2006 to 2017 with more than 12 mo of contact. We identified subsets of patients with ACP Only, ALC Only, and CD based on international classifications of diseases codes and reviewed the Electronic Health Record to verify diagnoses and abstract clinical information. Statistical comparisons were made using t-test and Kruskal-Wallis test for continuous variables and chi-square tests for categorical variables. Survival from time of first diagnosis is reported using the Kaplan-Meier method. Cox proportional-hazards models are used to report the hazard ratio and 95% confidence intervals while adjusting for age at diagnosis, sex, and race.

Research results

The median duration of contact was greater than 10 years and was comparable between groups. The median number of non-elective hospital admissions for CD and ACP Only were comparable and significantly greater than patients with ALC Only. The number of patients who died in follow-up in CD, ALC Only, and ACP Only groups was 80 (60%), 82 (61%), and 36 (41%). Using Cox regression, survival was similar between ALC Only *vs* ACP Only and CD *vs* ACP Only. Despite comparable MELD-Na and Child-Pugh scores between CD and ALC Only patients, those with ALC Only were more likely to have esophageal varices, need for variceal banding, treatment with beta blockers, and hepatocellular carcinoma. Patients with ACP Only were more likely to have acute pancreatitis, need for endoscopic or surgical intervention, and endocrine dysfunction.

Research conclusions

Patients with CD did not have a worse phenotype compared to patients with ACP Only or ALC Only.

Research perspectives

As the largest study of its kind, this work hopes to characterize patients at the intersection of ALC and ACP. Given our findings, we observed that the dominant phenotype in CD is similar to that of ALC Only, suggesting that patients with alcohol-related pancreatic disease who are newly identified to have alcohol-related liver disease should be closely monitored for liver cirrhosis and its complications.

ACKNOWLEDGEMENTS

The authors thank the Enhancing MEntoring to Improve Research in GastroEnterology (EMERGE) Program of the Division of Gastroenterology, Hepatology and Nutrition, University of Pittsburgh for supporting this project.

FOOTNOTES

Author contributions: Yadav D designed the research, contributed to the analysis and wrote the paper; Lu M, Sun Y performed the research, contributed to the analysis and wrote the paper; Arteel G contributed to the design of the study and provided clinical advice; Saul M retrieved patient information from UPMC databases, Feldman R, Althouse A performed the research and statistical analysis. All authors reviewed and approved the final version of the manuscript.

Institutional review board statement: This study was reviewed and approved by the Institutional Review Board of the University of Pittsburgh (STUDY 20100015).

Informed consent statement: We obtained a waiver of informed consent since the research represents no more than minimal risk of harm to subjects and involves no procedures for which written consent is normally required outside of the research context.

Conflict-of-interest statement: All the authors declare that they have no conflict of interest.

Data sharing statement: The dataset for this study is available from the corresponding author on reasonable request and fulfilment of regulatory requirements.

Country/Territory of origin: United States

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S-Editor: Liu JH
L-Editor: A
P-Editor: Liu JH

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