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REVIEW

Rodent models and metabolomics in non-alcoholic fatty liver disease: What can we learn?

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Abstract

Non-alcoholic fatty liver disease (NAFLD) prevalence has increased drastically in recent decades, affecting up to 25% of the world's population. NAFLD is a spectrum of different diseases that starts with asymptomatic steatosis and continues with development of an inflammatory response called steatohepatitis, which can progress to fibrosis. Several molecular and metabolic changes are required for the hepatocyte to finally vary its function; hence a "multiple hit" hypothesis seems a more accurate proposal. Previous studies and current knowledge suggest that in most cases, NAFLD initiates and progresses through most of nine hallmarks of the disease, although the triggers and mechanisms for these can vary widely. The use of animal models remains crucial for understanding the disease and for developing tools based on biological knowledge. Among certain requirements to be met, a good model must imitate certain aspects of the human NAFLD disorder, be reliable and reproducible, have low mortality, and be compatible with a simple and feasible method. Metabolism studies in these models provides a direct reflection of the workings of the cell and may be a useful approach to better understand the initiation and progression of the disease. Metabolomics seems a valid tool for studying metabolic pathways and crosstalk between organs affected in animal models of NAFLD and for the discovery and validation of relevant biomarkers with biological understanding. In this review, we provide a brief introduction to NAFLD hallmarks, the five groups of animal models available for studying NAFLD and the potential role of metabolomics in the study of experimental NAFLD.

Key Words: Non-alcoholic fatty liver disease; Liver disease; Rodent models; Metabolic profiling; Metabolomics; Biomarkers

Core Tip: Non-alcoholic fatty liver disease (NAFLD) is a spectrum of different diseases that starts with asymptomatic steatosis, continues with steatohepatitis, and can progress to fibrosis. Current knowledge suggests that NAFLD initiates and progresses through most of nine hallmarks. Animal models remain crucial for understanding the disease and for developing tools based on biological knowledge. Metabolomics seems a valid tool for studying metabolic pathways and organ crosstalk in NAFLD. In this review, we provide a brief introduction to NAFLD hallmarks, the five groups of animal models available for studying NAFLD and the potential role of metabolomics in the study of experimental NAFLD.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) prevalence has increased drastically in the last decades, affecting up to 25% of the world's population[1]. The rise of disorders such as obesity and type 2 diabetes mellitus, as well as changes in lifestyle and diet composition, have led to a worldwide increase in the incidence of NAFLD[2-5]. Given that NAFLD reduces life expectancy by four years and triggers the appearance of different comorbidities such as cardiovascular disease, kidney damage or osteoporosis[3-5], it seems vital for specialists to establish accurate and precise guidelines or strategies to address the disease[6]. Assuming that the first stages of NAFLD are reversible[2] and to control the disease worldwide, there is a need for new non-invasive methods based on diagnostic and predictive biomarkers to help diagnose NAFLD in these early stages and avoid of the biopsy, which remains the gold standard diagnostic method[7,8]. The use of animal models remains crucial for understanding the disease[9] and for developing tools based on biological knowledge. In this review, we will provide an updated summary on NAFLD development, the importance of experimental animals uses, the rodent models currently applied, and use of metabolomics as a new methodology for improving understanding and management of NAFLD.

NAFLD DISEASE

NAFLD is a spectrum of different diseases that starts with asymptomatic steatosis (NAFL) and continues with onset of an inflammatory response called steatohepatitis (NASH), which can progress to fibrosis. This hepatic fibrosis may produce cirrhosis and eventually, hepatocellular carcinoma (HCC)[2]. The first theory to explain NASH development, proposed in 1998, was known as the "two hits" hypothesis[10]. The first hit was fat storage in the hepatocytes, which would induce steatosis, the second hit being increased oxidative stress in the hepatocytes which would stimulate lipid peroxidation. It was believed this double hit was necessary to induce disease onset[10]. Currently, the "two hits" concept is considered old-fashioned by many experts. The hepatocyte needs several molecular and metabolic changes for its function to finally vary. Instead, it seems more precise to propose a "multiple hit" hypothesis[11]. This premise is intended to provide greater insight into NAFLD pathology and considers the different events that can take place in predisposed subjects during development of the disorder. Fat accumulation and synthesis of reactive oxygen species are essential events, yet other phenomena are also important and can be considered hallmarks of NAFLD initiation and progression (Figure 1).

Environmental factors

Among environmental factors, the most prominent are dietary habits, physical activity, and socio-economic aspects. Increased calories intake, and consumption of high-sugar and high-fat diets increases the risk of developing not only NAFLD but also conditions such as obesity and type 2 diabetes mellitus [4,8,12]. Hallsworth *et al*[13] was the first to show an association between sedentary behavior and physical activity levels in patients with NAFLD, finding that these patients were on average more

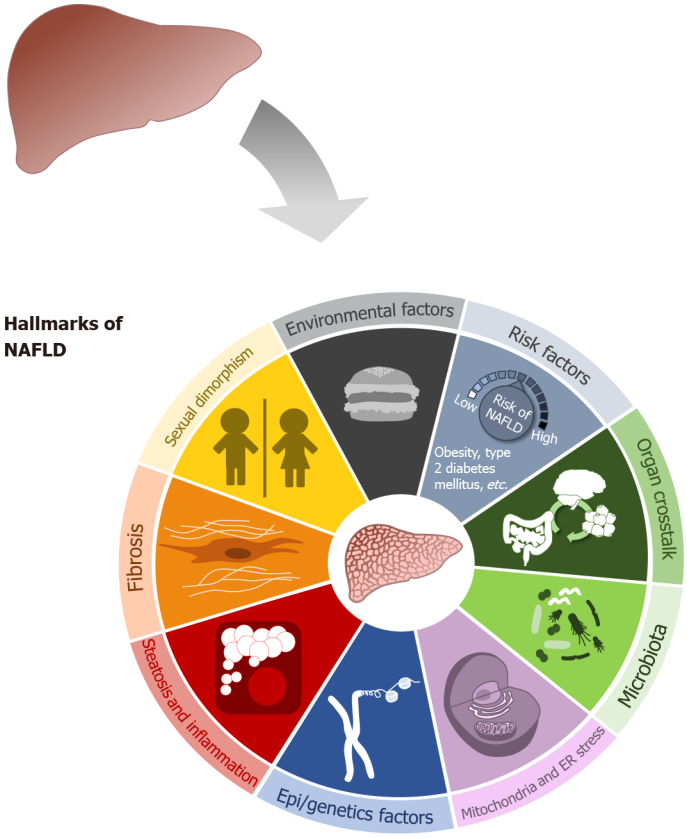

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Figure 1 Hallmarks of non-alcoholic fatty liver disease. Previous studies and current knowledge suggest that in most cases, non-alcoholic fatty liver disease initiates and progresses through most of these nine hallmarks, although the triggers and mechanisms for them can be diverse. NAFLD: Non-alcoholic fatty liver disease.

sedentary, walked less and spent less time on physical activity. Furthermore, it has been demonstrated that lifestyle interventions in diet and physical activity could improve the disease prognosis[12]. Finally, regarding socio-economic aspects, the role of educational level and family economic status in development of NAFLD is still under debate[4].

Intracellular factors

At the cellular level, important events such as mitochondrial dysfunction[14], endoplasmic reticulum (ER) stress[15,16], and activation of the inflammasome[17] contribute to fat accumulation in cells (steatosis) and inflammation. Genetic variants and epigenetic factors must also be taken into account in NAFLD progression[11,18]. A decade ago, PNPLA3 I148M was the first genetic variant reported to be associated with NAFLD. Currently, 13 genetic variants have been linked to increased risk of NAFLD or NASH, with the exception of the variant UCP2 866, which reduces the risk of NASH[18]. Some of these variants, such as TM6SF2, PNPLA3, NCAN, and PPP1R3B, have been linked to inherited NAFLD[8].

Extracellular factors

As a complete organ, the liver includes many non-parenchymal cells besides hepatocytes which contribute to the proper functioning of the organ. Among these are liver sinusoidal endothelial cells (LSECs), hepatic stellate cells (HSCs) and several immune cells, such as Kupffer cells[19,20]. Most of these cell types are essential to maintain homeostasis in the liver at the extracellular level, and alteration in their function has been associated with the NAFLD progression. LSECs maintain portal pressure and inhibit HSCs and Kupffer cells activation. During the first reversible stage of NAFLD, LSECs lose their functions, and, in turn, induce inflammation and fibrosis[21,22]. HSCs contribute to initiation and progression of liver fibrosis[19,23,24], one of the hallmarks of NAFLD evolution. Immune cells can be activated during liver disease creating a pro-inflammatory environment in the organ which contributes to NASH, fibrosis, cirrhosis, and HCC progression[19,25].

Organ crosstalk

NAFLD illness is not limited to hepatic disease: the NAFLD liver interacts with other organs, creating an organ crosstalk[26], which provides further support for the "multiple hits" hypothesis. As a first example, adipose tissue (AT) dysfunction is related to NAFLD disease[11,26]. Excess fat consumption

produces hypertrophy in adipocytes. AT can release several hormones or cytokines called adipokines which generate a pro-inflammatory environment[27]. This inflammatory state occurs first in the AT, then in the liver[28]. Furthermore, noncoding RNA[29] and extracellular vesicles[26,30] from the AT are linked to development of NAFLD and cell-to-cell communication. In the context of NAFLD, the gut-liver axis refers to the relationship between gut integrity, gut microbiota, and the liver[11,26,31]. Both organs are directly connected by the portal vein. In general, the gut presents different kinds of barriers and mechanisms to maintain its integrity. One function of these barriers is to control the passage of substances into the portal vein and the liver[31,32]. Further evidence suggests that the intestinal barriers are altered, and intestinal permeability is increased in NAFLD disease. Taking advantage of this altered permeability, bacteria can translocate more easily into the blood, enter the portal vein and finally reach the liver[32]. Increased gut permeability and bacterial translocation are associated with liver cirrhosis [31]. The gut microbiota is also altered in NAFLD due to intestinal microbial dysbiosis[33]. It has been shown that bacteria phyla are modified under high-fat diet-induced liver steatosis in rodent models[34] and human studies of NAFLD, NASH, and cirrhosis[35]. Variations in bacteria composition lead to altered concentration of some metabolites. This phenomenon, added to reduced permeability, triggers the arrival of molecules such as lipopolysaccharides in the systemic circulation and activation of Toll-like receptor in cells. Moreover, metabolism of trimethylamine which can be oxidized in the liver ultimately forming trimethylamine N-oxide, has been linked to NAFLD progression and cardiovascular disease[33]. Additionally, the liver has been associated with the brain[26]. The arcuate nucleus of the hypothalamus regulates satiety. In 2005, De Souza *et al*[36] proved that a high-fat diet caused several proinflammatory-related changes in mRNA expression in the hypothalamus of Wistar. Furthermore, cirrhotic patients can develop hepatic encephalopathy, a neurological comorbidity associated with NAFLD disease[37,38]. Finally, the kidney and the liver have also been linked. The study of Musso *et al* [39] in 2014 revealed that NAFLD severity was correlated with severity of chronic kidney disease (CKD). Many pathways are shared between NAFLD and CKD, so progression of NAFLD will contribute to CKD progression and vice versa[40].

Sexual dimorphism

NAFLD affects more men than women[41,42], due to the protective role of estrogens against disease development[43-46]. Nonetheless, in women of a certain age and under certain risk factors, incidence is higher than in men and they experience a more aggressive disease course. These risk factors are: (1) Earlier age of menarche; (2) Polycystic ovary syndrome; (3) Gestational diabetes; and (4) Menopause[46-49]. Interestingly, sex differences extend beyond incidence rates: NAFLD appears to develop in distinct ways in males and females[50-54]. However, further studies on about molecular processes are needed for enhanced insight into sexual dimorphism in NAFLD[55].

RODENT MODELS IN NAFLD

NAFLD is a complex disease which affects many hepatic parameters, as well as functions of other organs. With current methodologies, it is virtually impossible to study the "multiple hits" hypothesis of NAFLD as a whole in humans, because this requires access to multiple tissues, biofluids, and controlled environments. Animal models therefore remain essential for studying initiation and progression of NAFLD, and present various advantages over clinical research: (1) The possibility to obtain multiple samples and carry out longitudinal studies; (2) Shorter time to disease onset; (3) The possibility of controlling the variables of our model; and (4) Use of genetically modified animals to study a specific gene or metabolic pathway alteration. Compared to *in vitro* studies, animal models can be used to study the whole liver and organ crosstalk between the liver and other organs[56].

Nevertheless, a perfect animal model[9,57] providing information on all potential triggers and causes of NAFLD is elusive. Therefore, it is vital to know the stage of the disease to be studied and which model reproduces the physiopathological characteristics we want to study. Focusing on model selection, among key common characteristics, a good model must imitate certain aspects of the human NAFLD disorder, be reliable and reproducible, have low mortality, and be compatible with simple and viable methods[9]. Development of obesity or insulin resistance, AT inflammation, alterations of intestinal physiology, and a specific liver phenotype (Table 1) are traits that mimic human NAFLD[58,59]. Several animal models can be used to study metabolic diseases, including NAFLD, but rodents are the most commonly used. Rodent models are preferred because they easily develop obesity, type 2 diabetes mellitus, and NAFLD[60]. In mice, the ideal model genetic background is the strain C57BL/6, and specifically the substrain C57BL/6J, as C57BL/6J mice are more insulin resistant than C57BL/6N mice [61], which allows for better isolation of the NAFLD process from other metabolic alterations. For rat models, Wistar or Sprague Dawley rats are usually chosen, although other models besides rats and mice, such as New Zeland white rabbits, Guinea pigs, or Tree shrews, have also been used[60]. Rabbits, and many non-rodent models like pigs, have the important advantage of longer pre-pubertal stages, which allow them to mimic the subclinical NAFLD situation in children with greater precision than would be possible with mice or rats[59,62]. Also, pigs are anatomically and metabolically more similar

Table 1 Summary of existing rodent models of non-alcoholic fatty liver disease

Rodent models	Obesity	Insulin resistance	Steatosis	NASH	Fibrosis	HCC
Dietary						
Deficient diet						
MCD	No	Hepatic IR	Yes	Yes	Yes	No
CDAA	No	No	Yes	Yes	Yes	Yes
High-amount diet						
HFD	Yes	Yes	Yes	Yes	Yes	No
HFHS	Yes	Yes	Yes	Yes	Yes	No
High fructose diet	No	Yes	Yes	No	No	No
HFHC	Yes	Yes	Yes	Yes	Yes	No
Atherogenic diet (cholesterol + cholate)	No	Hepatic IR	Yes	Yes	Yes	No
Cafeteria diet or Western diet	Yes	Yes	Yes	Yes	No	-
ALIOS	Yes	Yes	Yes	Yes	Yes	Yes
AMLN	Yes	Yes	Yes	Yes	Yes	No
DIAMOND	Yes	Yes	Yes	Yes	Yes	Yes
Genetic						
ob/ob	Yes	Yes	Yes	No	No	No
db/db	Yes	Yes	Yes	No	No	No
KK-Ay	Yes	Yes	Yes	No	No	No
foz/foz	Yes	Yes	Yes	No	No	No
fa/fa	Yes	Yes	Yes	No	No	No
PTEN knockout	No	No	Yes	Yes	Yes	Yes
PPAR-α knockout	No	No	Yes	No	No	No
SREBP-1c transgenic	No	Yes	Yes	No	No	No
Chemicals						
Tetracycline	No	No	Yes	Yes	Yes	-
CCl_4	No	No	Yes	Yes	Yes	Yes
TAA	-	-	Yes	Yes	Yes	Yes
STZ	-	-	-	Yes	-	-
DMN	-	-	No	Yes	Yes	Yes
DEN	No	-	Yes	Yes	Yes	Yes
Porphyrinogenic agents (DDC or GF)	-	-	Yes	Yes	-	-
MSG	Yes	Yes	Yes	Yes	No	Yes
Tunicamycin	-	-	Yes	Yes	-	-
Surgical						
CBDL	-	-	Yes	Yes	Yes	-
Combined models						
ob/ob + MCD diet	Yes	-	Yes	Yes	No	No
db/db + MCD diet	Yes	Yes	Yes	Yes	Yes	No
HFD + thermoneutral housing at 30 ºC	-	-	-	Yes	Yes	-

HFD + CCl_4	No	-	Yes	Yes	Yes	Yes
HFD + DEN	Yes	-	Yes	Yes	Yes	Yes
CDAA + CCl_4	No	-	Yes	Yes	Yes	Yes
STAM model	No	-	Yes	Yes	Yes	Yes

ALIOS: American lifestyle-induced obesity syndrome model (high-fat + trans-fat + fructose); AMLN: Amylin liver NASH model; CBDL: Common bile duct ligation; CCl_4: Carbon tetrachloride; CDAA: Choline-deficient, L-amino defined diet; DDC: 3,5-diethoxycarbonly-1,4-dihydrocollidine; DEN: Diethylnitrosamine; DIAMOND: Diet-induced animal model of non-alcoholic fatty liver disease mice; DMN: Dimethylnitrosamine; GF: Griseofulvin; HCC: Hepatocellular carcinoma; HFD: High-fat diet; HFHC: High-fat high-cholesterol diet; HFHS: High-fat high-sugars diet (mainly fructose or sucrose); MCD: Methionine and choline deficient diet; MSG: Monosodium glutamate; NASH: Non-alcoholic steatohepatitis; STZ: Streptozotocin; STAM: Stelic animal model of NASH (STZ + HFD); TAA: Thioacetamide.

to humans than rodent models. Nonetheless, these non-murine species have some drawbacks, such as they involve more complicated and less generally established genetic approaches, and housing larger animals can be more difficult from a logistic and economic point of view[59]. Models of smaller size and shorter lifetimes than mice and rats have also been explored. For example, use of zebrafish as a NAFLD model is recently increasing an inexpensive model in which NAFLD develops quickly[63].

Despite the wide variety of models, rodents are still the preferred species for experimental NAFLD research because of their small size, ease of maintenance, short life span, and available genetic resources. The current rodent models used in NAFLD can be stratified into five main groups, depending on the disease inducer: dietary, genetic, chemical, surgical, and combined (mix of different models). Pathological characteristics of these rodent models are summarized in (Table 1).

Dietary models

Dietary models, which can be classified as deficient or high amount diets, are an excellent option for studying NAFLD disease. Deficient diets are not generally found in humans, as they are based on absence of essential elements. However, in animals, methionine and choline-deficient diet (MCD) or choline-deficient, L-amino defined diet are effective in generating liver damage[64,65]. The diets most closely resembling humans experience are the high amount calorie diets with an excessively high amount of specific nutrients, mainly fats and sugars[9,57,59,66]. Different diets can be defined by the high concentration of nutrients or how they are combined. Among these are high-fat diets, high-cholesterol and cholate diets (atherogenic diet), high-fat high-cholesterol diets, high-sugar diets based on fructose or sucrose, and high-fat high-sugar diets. Their effects on NAFLD development are shown in (Table 1). Lastly, there are different animal models of NAFLD based on diets that promote NASH in a short period: (1) American lifestyle-induced obesity syndrome model (ALIOS model); (2) Amylin liver NASH model (AMLN model); and (3) Diet-induced animal model of NAFLD mice (DIAMOND model) [57]. The ALIOS model is based on a high-fat diet (45% fats, 2% trans fats), drinking water with fructose and glucose, and a sedentary behavior (cages without wire racks), promoting obesity[67]. The AMLN model is based on a high-fat (40% fats, 18% trans fats), high-fructose (22%) and cholesterol (2%) diet[68, 69]. ALIOS and AMLN are very similar, but with different fat percentages, and in the AMLN model fructose is given in pellet form rather than in drinking water[68]. A variant of the AMLN model called the Gubra amylin NASH (GAN) diet is currently used, with the same composition, but trans-fat-free diet and with increased saturated fatty acids[70]. The DIAMOND model is based on a high-fat (42%), high-carbohydrate and cholesterol (0.1%) diet but with an added high-fructose and glucose solution [71]. All these models are modified Western or Cafeteria diets (combination of fat and sugars) presenting more or less the same composition but in different proportions[63,72].

Genetic models

Genetic models allow us to study genetic and pathophysiological consequences of alterations in certain genes potentially involved in NAFLD development. These models are based on mechanistic hypotheses and have the main limitation that every specific mutation in a single gene is not usually found in humans[9]. Nevertheless, they provide two major advantages over other models: first, the means to study disease mechanisms in NAFLD, and second, the opportunity to improve our knowledge of a specific mechanism in the disease models[73]. Nowadays, genetic engineering tools have facilitated generation of transgenic animals and knockouts, either by commercial houses or in academic laboratories[62,73-75]. There are many genetic models for NAFLD, each one based on different pathways affected in the disease[76]. The genetic models most commonly used in the study of NAFLD are reported in (Table 1).

Chemical models

The most widespread chemical models for studying NAFLD are those based on liver damage through tetracycline, carbon tetrachloride (CCl_4), thioacetamide (TAA), and streptozotocin[9,65]. These models

can produce significant liver damage depending on the experimental exposure time (days, weeks, or months) and the dose delivered, but in the focus is generally, on producing liver steatosis and fibrosis [63,65,74,77]. Treatment with the chemicals diethylnitrosamine (DEN) or dimethylnitrosamine (DMN) is typically used to induce HCC and the approach may be too aggressive for studying NAFLD alone[56,60,74]. The porphyrinogenic agents (3,5-diethoxycarbonyly-1,4-dihydrocollidine (DDC) and griseofulvin (GF) and the chemical monosodium glutamate (MSG) are less often used but can also induce steatosis and NASH[78]. The chemical Tunicamycin produces ER stress in the hepatocytes which can in turn induce steatosis[79,80]. Overall, chemical models represent a faster and more dramatic way to study liver damage, but the disease initiation and progression bears less resemblance to human NAFLD than diet or genetic models.

Surgical models

Hepatobiliary system surgery can induce NAFLD in experimental models. The most common surgical model is Bile Duct Ligation (BDL), which is used to produce fibrosis, cirrhosis and as a consequence, liver failure in rodents[65,74,81]. BDL can be performed in mice and rats[65], but this model is difficult to implement in mice, as several surgical complications can arise[56]. Surgical models are the least used models of NAFLD because of their complexity and lack of similarity to human NAFLD.

Combined models

Genetic models do not usually develop NASH, fibrosis, or HCC spontaneously, so they are often supplemented with diet to achieve worse liver damage[9,57,62]. This is also the case with chemical models, in which the dose for inducing liver damage is often too aggressive but combining a low dose with some NAFLD-inducing diet modifications can help producing a model that progresses at a slower pace, which allows detection of the different stages of NAFLD progression[60,65,66,77]. These combined models genetic plus diet or chemical plus diet, are also a common option for studying NAFLD[76].

METABOLOMICS IN NAFLD RODENT MODELS

Currently, liver function is routinely controlled by blood analysis in which clinicians test for transaminases, albumin, platelets, bilirubin and clotting factors. Patients presenting abnormal levels of these parameters, especially transaminases, and whose medical history reveals risk factors for diabetes, obesity or metabolic syndrome, undergoes a non-invasive imaging method, mainly ultrasonography and elastography, to confirm the presence of steatosis and fibrosis in the liver. If the result is positive, the NAFLD fibrosis score and FIB-4 index scores can be applied. Depending on the score, patients are classified as at low, medium or high risk of fibrosis. The goal of these imaging methods is to detect whether fibrosis is present, due to the different follow-up required in patients with fibrosis. An invasive imaging method, biopsy, is performed on those with a high risk of fibrosis or with an unclear diagnosis under non-invasive imaging methods[8,82-84]. Nowadays, biopsy remains the gold-standard for diagnosis of hepatic steatosis, NASH and fibrosis, as histology confirms tissue damage[7,8]. Biopsy has a relatively high incidence of false negatives, since the fragment finally analyzed only represents about 1/50000 of the organ and analysis may vary between pathologists[7]. Moreover, non-invasive imaging methods also present disadvantages. Steatosis can only be detected at over 30% and these methods cannot determine whether NASH is present[85,86]. We are still far from achieving the main objective: NAFLD prevention and a rapid diagnosis. New non-invasive diagnostic methods are needed, and one alternative could be use of metabolomics in the search for new biomarkers.

Personalized medicine has become a fundamental strategy in the future of healthcare. The possibility of tailor-made treatments for patient groups will help streamline healthcare costs and enhance efficacy and safety of interventions. The transition to a personalized medicine model has been facilitated by recent advances in "omics" technologies that are allowing the degree of personalization in the diagnosis and treatment of different diseases to be increased to levels unimaginable just a few years ago[87]. Metabolomics is an emerging research area and can be considered, at a biochemical level, as the end of the “omic” cascade since changes in the metabolome constitute the organism's last response to genetic, chemical and environmental alterations[63].

Small biochemicals are the end products of all the regulatory processes present in a cell, tissue, or organism, including transcriptional and translational regulation and posttranslational modifications. Consequently, metabolic changes are among the best reporters of the organism's response to a disease process. The application of metabolomics to the study of metabolic diseases may increase our understanding of the pathophysiological processes involved, and thus help us to identify potential biomarkers. The identification and quantification of these low molecular weight molecules define the metabolic phenotype of these diseases and studying the metabolic changes that occur in response to different pathophysiological processes may help establish the mechanisms underlying the disease.

Metabolites can be measured in several body fluids or tissues, although plasma and urine are the most frequently used samples in metabolic research, they are readily available and have clinical relevance as a source of potential biomarkers. Almost all cells in the body communicate with plasma,

either directly or through different tissues and biological fluids, releasing at least part of their intracellular content. By contrast, urine is produced by renal filtration of plasma and is widely considered to be among the most important samples for diagnosis as it contains not only many plasma components but also the catabolic products of different metabolic pathways.

Metabolic fingerprinting and metabolic profiling are two different approaches to the study of metabolites in biological samples. Metabolic fingerprinting does not aim to identify the entire set of metabolites but rather to compare patterns or fingerprints of metabolites that change in response to a disease state, pharmacological therapies, or environmental alterations. This approach can be used as a diagnostic tool to evaluate the disease state by comparing healthy controls and disease subjects. Nonetheless, qualitative and quantitative analyses are required to understand the mechanisms underlying a disease. Metabolite profiling focuses on the analysis of a group of metabolites related to a specific metabolic pathway. In this approach, target metabolites are selected beforehand and are assessed using specific analytical methods.

The analytic techniques used to study the metabolome are mass spectroscopy (MS), nuclear magnetic resonance (NMR), or a combination of both[88,89]. Each technique has its own strengths and weaknesses[88,90]. An advantage of NMR technique, is that it can be used to study tissues, including liver, without destroying the sample with the proton high-resolution magic-angle spinning probe (HR-MAS)[90,91].

Metabolomics is a very powerful tool for the study of metabolic diseases[90,92], yet applications of metabolomics to NAFLD is an understudied area. Nonetheless, some studies demonstrate the importance of measuring metabolites for better characterization of the disease. NAFLD is a metabolic illness, hence metabolomics as a technique offers the opportunity to better understand the metabolic alterations in NAFLD progression[87,92,93] and patient stratification[89]. MS and NMR have been used to study NAFLD progression in rodent models. Articles yielded from the keyword search using the term "metabolomics" and "rodent models" are shown in (Table 2). Metabolomics studies have been carried out in dietary, chemical, genetic and combined models of NAFLD. Including metabolic alterations could broaden the search for specific metabolomics biomarkers which would help in disease diagnosis.

Despite the diversity of models used in previous metabolomics studies on NAFLD rodent models (Table 2), some common findings can be extracted. Fatty acids are stored as triacylglycerols in the liver when not catabolized by β-oxidation. Consequently, fatty liver seems to be a rearrangement of lipids in the liver and not just fat storage. Most studies in liver tissue of rodent models have revealed massive accumulation of triacylglycerols (see liver extract studies in Table 2). The well-known adipocyte origin of some of these triacylglycerols suggests AT as a potential source of triacylglycerols deposited in the liver in NAFLD. Furthermore, almost all studies in NAFLD rodent models report alterations in other metabolites like glucose, lactate, pyruvate, and alanine, suggesting that NAFLD is involved in cytosolic glycolysis and oxidative stress[97,112,119]. Metabolism of branched-chain amino acids also seems to be altered in NAFLD. A previous study including human subjects and animal models in the context of hepatic insulin resistance demonstrated a link between BCAA and the tri-carboxylic acid cycle[106,108]. The integration of findings in human and rodent model studies seems very complex. In a translational human-animal study, Han *et al*[123] studied the progression of fatty liver and liver steatosis, finding changes in metabolic networks related to amino acids and bile acids. However, these results were significantly different between animals and humans. Among others, taurine, a well-known amino acid with protective and antioxidant properties, was increased in humans but not in rat models. Finally, consistent finding in different rodent and human studies on NAFLD is an increased level in serum of bile acids, important molecules which signal many processes in the liver and are involved in lipid and glucose homeostasis.

CONCLUSION

NAFLD is the most prevalent liver disease worldwide. Approaches from different perspectives have led to increased insight into many aspects of the disease. Knowledge of the disease has increased with the use of animal models, especially those in rodents. Although, the perfect animal model does not exist, some models perfectly mimic several aspects of NAFLD development and have become very useful tools to address the disease in the search for biomarkers of the early reversible stages. Studying metabolism in these models provides a direct reflection of what happens inside the cell. Metabolomics seems an important tool for studying metabolic pathways and crosstalk between organs affected in animal models of NAFLD, and for identifying and validating relevant biomarkers with biological understanding.

Table 2 Studies related to "non-alcoholic steatohepatitis", "rodent models" and "metabolomics"

Rodent model	Produced by	Animals used	Biological sample	Platform used	Ref.
Dietary	HFD	C57BL/6 mice. 6-wk-old	Liver extract and serum	UPLC-QTOF-MS and GC-MS	Kim *et al*[94]
	HFD and Paigen diet	BALB/c mice. 6-wk-old	Liver extract and urine	^{1}H-NMR	Klein *et al*[95]
	HFD and HCD	C57BL/6N mice. 6-wk-old	Urine	^{1}H-NMR	Jung *et al*[96]
	HFD and HCD	Wistar rats. 6-wk-old	Liver extract	^{1}H-NMR	Bertram *et al*[97]
	HFD	C57BL/6S1ac mice. 4-wk-old	Urine	^{1}H-NMR and UPLC-QTOF-MS	Li *et al*[98]
	HFD	C57BL/6J mice. 6-wk-old	Serum	UHPLC-QTOF-MS and GC-MS	Lai *et al*[99]
	High-fructose and saturated fatty acid diet	Sprague-Dawley rats	Liver extract	HR-MAS and ^{1}H-NMR	Tranchida *et al*[100]
	HFHCC diet	C57BL/6J mice. 8-wk-old	Liver extract and plasma	GC-TOF MS and CSH-QTOF MS	Tu *et al*[101]
	HFD	Sprague-Dawley rats. 4-6-wk-old	Liver extract	LC-MS	Wan *et al*[102]
	HFD	Sprague-Dawley. 6-wk-old	Urine and feces	^{1}H-NMR	Chen *et al*[103]
	MCD	C57BL/6J mice. 8-wk-old	Feces	GC-MS	Ye *et al*[104]
	HFD	Swiss albino mice	Serum and feces	^{1}H-NMR	Carvalho *et al*[105]
	High fat-sucrose diet	Sprague-Dawley rats. 6-wk-old	Serum	HPLC-QTOF-MS	Xu *et al*[106]
	MCD and atherogenic diet	C57BL/6J mice. 10-wk-old	Liver extract	MS	Montandon *et al*[107]
	HFD	Sprague-Dawley rats. 6-8-wk-old	Serum	LC-MS	Cui *et al*[108]
	HFD	Sprague-Dawley, Fisher 344 and Brown-Norway rats. 5-wk-old	Liver extract	LC-MS	Boyce *et al*[109]
Genetic	Db/db mice	C57BL/6J mice. 10-wk-old	Liver extract	^{1}H-NMR and UPLC-QTOF-MS	Kim *et al*[110]
	Ob/ob mice	B6.Cg-*Lepob*/J mice. 8-wk-old	Liver extract	HR-MAS and ^{1}H-NMR	Gogiashvili *et al*[111]
Chemical	DEN	Sprague-Dawley rats. 4-wk-old	Liver extract	^{1}H-NMR	Wang *et al*[112]
	CCl_4	Wistar rats	Plasma	UPLC-QTOF-MS	Li *et al*[113]
	CCl_4	Sprague-Dawley rats. 4-wk-old	Urine	GC-TOF MS	Jiang *et al*[114]
	CCl_4	Wistar rats	Liver extract	GC-MS	Song *et al*[115]
	CCl_4	Sprague-Dawley rats	Urine	^{1}H-NMR	Wu *et al*[116]
	CCl_4	Wistar rats	Urine	GC-MS	Fang *et al*[117]
	CCl_4	Sprague-Dawley rats. 1-yr-old	Serum and urine	UPLC-QTOF-MS	Chang *et al*[118]
	CCl_4	Sprague-Dawley rats. 7-wk-old	Serum	^{1}H-NMR	Li *et al*[119]
	CCl_4	Sprague-Dawley rats	Serum	^{1}H-NMR	Liu *et al*[120]
	DEN	Sprague-Dawley rats. 6-wk-old	Serum	^{1}H-NMR	Yang *et al*[121]
Combined model	Combined (genetic + dietary) with HCD	Acyl knockouts mice on a C57BL6/J background. 4-wk-old	Serum	LC-MS	Zhao *et al*[122]

CCl_4: Carbon tetrachloride; CSH-QTOF MS: Reverse-phase lipid chromatography-quadrupole/time-of-flight mass spectrometry; DEN: Diethylnitrosamine; GC-MS: Gas chromatography-mass spectrometry; GC-TOF MS: Gas chromatography-time-of-flight mass spectrometry; HCD: High-carbohydrate diet; HFD: High-fat diet; HFHCC: High-fat, high cholesterol, cholate diet; HPLC-QTOF-MS: High-performance liquid chromatography quadrupole time of flight mass spectrometry; ^{1}H-NMR: Proton nuclear magnetic resonance; LC-MS: Liquid chromatography-mass spectrometry; MCD: Methionine, and choline-deficient diet; MS: Mass spectroscopy; UPLC-QTOF-MS: Ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry.

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REVIEW

Hepatobiliary manifestations in inflammatory bowel disease: A practical approach

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Abstract

Inflammatory bowel diseases (IBD) are associated with various hepatobiliary disorders. They can occur at any moment in the course of the disease or associated with the treatment. The prevalence of liver dysfunction can reach up to 50% in different studies. Nonalcoholic fatty liver disease is considered the most common hepatobiliary complication in IBD, while primary sclerosing cholangitis is the most specific. Management of hepatic manifestations in IBD involves a multidisciplinary approach that includes a high index of suspicion and joint management with hepatologists. The medical confrontation with abnormal liver tests must include an exhaustive study to determine if these patterns can be related to IBD, associated diseases or to the therapies used.

Key Words: Extraintestinal manifestations; Inflammatory bowel disease; Sclerosing primary cholangitis; Hepatic steatosis; Liver toxicity

Core Tip: Inflammatory bowel diseases are associated with various hepatobiliary disorders. They can occur at any moment in the course of the disease or associated with the treatment. Although hepatic manifestations are known, they are not always searched for in a directed manner. This review article presents the main hepatobiliary manifestations, including those caused by new therapies (biologics and small molecules). Finally, we propose a management algorithm.

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INTRODUCTION

Inflammatory bowel diseases (IBD) are associated with various hepatobiliary disorders. They can occur at any moment in the course of the disease or associated with the treatment. The prevalence of liver dysfunction can reach up to 50% in different studies. Non-alcoholic fatty liver disease (NAFLD) is considered the most common hepatobiliary complication in IBD, while primary sclerosing cholangitis (PSC) is the most specific. Management of hepatic manifestations in IBD involves a multidisciplinary approach that includes a high index of suspicion and joint management with hepatologists. The medical confrontation with abnormal liver tests must include an exhaustive study to determine if these patterns can be related to IBD, associated diseases or to the therapies used (Figure 1).

AUTOIMMUNE DISEASES ASSOCIATED WITH IBD

Primary sclerosing cholangitis

It is defined as a chronic cholestatic liver disease characterized by intra and/or extra-hepatic bile duct lesions[1]. The incidence in the adult population is 1.1 per 100 thousand inhabitants and the prevalence is between 8.5-13.6 per 100 thousand patients/year[2-4]. PSC is more common in men with a 2:1 ratio and its age of manifestation is usually in the 4th to 5th decade of life. The prevalence of IBD in patients with PSC is 70%-80%, the most common being ulcerative colitis (UC), while only 2%-7% of patients with IBD will develop PSC[5,6]. The prevalence of PSC may increase when using techniques with greater diagnostic sensitivity, improving the survival rate[7]. Lunder *et al*[8] found 3 times more PSC performing magnetic resonance cholangiopancreatography (MRCP) as screening in all patients with IBD (7.5% *vs* 2.2%) when comparing only based on the clinical picture or altered liver function tests. The prevalence of PSC among relatives is 100 times higher than that of the general population, this has made it possible to establish, through genomic association studies, the presence of HLA-B* 08 and DR*03 as a risk locus[9]. The pathophysiology of PSC is unknown, nonetheless it is suggested that there is an autoimmune component given its association with the presence of autoantibodies such as: Anti-neutrophil cytoplasmic antibodies (ANCA), anti-nuclear antibodies (ANA) and anti-smooth muscle antibodies (ASMA)[10]. However, this factor has been questioned given that there is a higher prevalence of PSC in men (contrary to most immune-mediated diseases), the limited effectiveness of immunosuppressive drugs, and the lack of a specific autoantibody[10]. One theory is that PSC is a consequence of the sustained inflammatory response as a product of bacterial and viral translocation typical of IBD[8]. These bacterial/viral products would go to the portal system contributing to the exaggerated inflammatory response of the cholangiocyte, which evolves into fibrosis[11,12]. Diagnosis: The most frequent symptoms are abdominal pain, jaundice, itching, and metabolic bone disease, cholangitis and decompensated cirrhosis; however, between 21%-44% of patients may be asymptomatic at the time of diagnosis. Within the laboratory test results, the persistent elevation of alkaline phosphatase (ALP) and gamma glutamyl transpeptidase (GGT) stand out, oftentimes being an incidental finding[13]. The non-invasive evaluation with MRCP allows to optimally evaluate the presence of multifocal segmental stenosis of the intra- and extra-hepatic biliary tree that give the typical beaded appearance; this technique has a sensitivity of 86% and a specificity of 77%, including a sensitivity of 98.9% to detect cholangiocarcinoma (CCA) in this group of patients[14]. Currently, endoscopic retrograde cholangiography has a therapeutic role in cases of suspected dominant biliary tree stenosis. Liver biopsy is used in the event of diagnostic doubts or suspicion of small duct involvement. The classic finding is the presence of "onion skin" periductal fibrosis which leads to ductopenia and cholestasia that can be present in 50% of biopsies as well as the presence of obliterative fibrosing cholangitis, which is found only in 5% of patients[15]. Recently, it was observed that the senescent cholangiocyte (with p16 + immunohistochemical marker) is associated with both clinical and histological severity, so it could

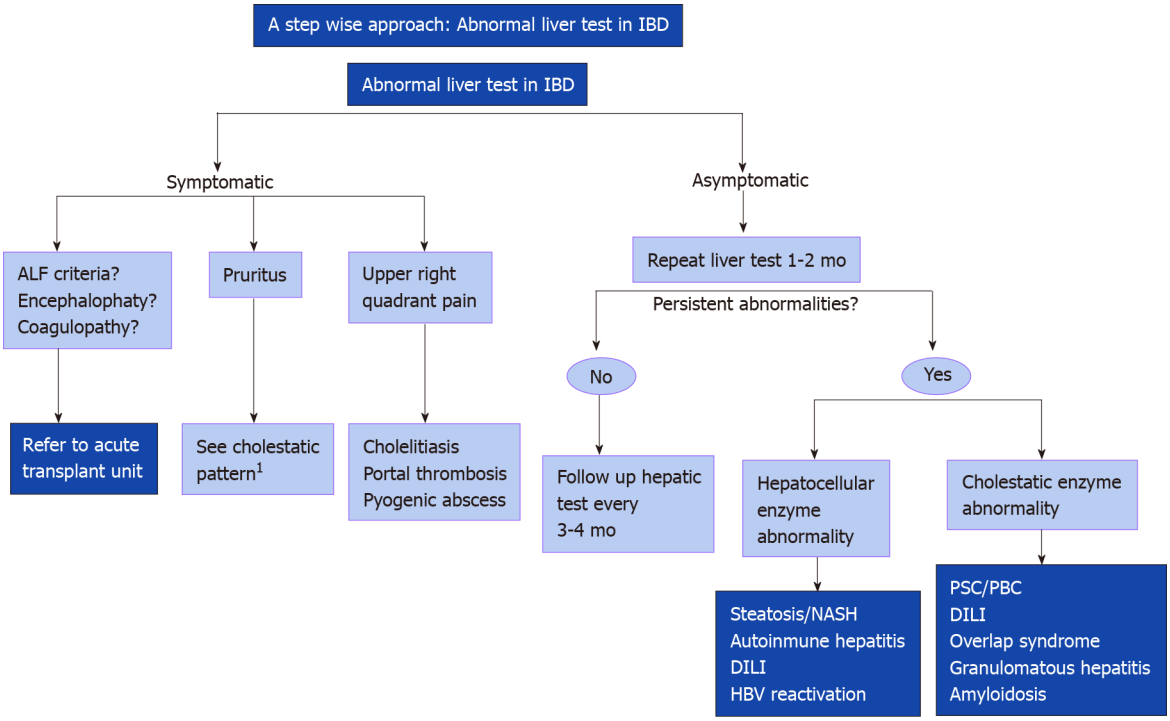

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Figure 1 A stepwise approach: Abnormal liver test in inflammatory bowel disease.[1]If the study is negative consider liver biopsy. Created with Biorender. IBD: Inflammatory bowel disease; ALF: Acute liver failure; NASH: Nonalcoholic steatohepatitis; HBV: Hepatitis B virus; PSC: Primary sclerosing cholangitis; PBC: Primary biliar cholangitis; DILI: Drug induced liver injury.

represent a new target for prognosis and therapy[16].

UC-PSC: UC represents 80% of IBD cases in patients with PSC, while indeterminate colitis and Crohn disease (CD) constitute the remaining 20%[2]. The clinical course tends to be milder, and the colonic involvement is generally extensive; mild, with greater inflammatory involvement of the ascending colon, mucosa rectal sparing and associated with backwash ileitis[17,18]. Despite having an asymptomatic clinical course, the risk of CRC is significantly higher[19,20].

CD-PSC: Similarly, to UC, the clinical course of CD tends to be more benign, with the predominant phenotype being inflammatory[17]. Rectal involvement is lower compared to UC (20% *vs* 68%, $P = 0.07$), with right colonic involvement being more common (50% *vs* 29% in UC, $P = 0.3$)[18]. From a prognostic point of view, in an IBD-PSC cohort in England, these diseases association increased risk of death [hazard ratio (HR): 3.2] and CRC (2.4). In that group the CRC was diagnosed a lower median age (59 years *vs* 69 years without PSC). In patients younger than 40 years at the PSC diagnosis the liver transplantation and PSC-related events were more frequently than in people more than 60 years (75% *vs* 31%)[21]. Patients with UC had an increased risk of liver disease progression compared with patients with CD (HR: 1.56; $P < 0.001$) or no IBD (HR: 1.15; $P = 0.002$)[22].

Small duct disease: Approximately 20%-30% of PSC correspond to small duct involvement. Defined as liver histopathology periductal, concentric fibrosis; fibro-obliterative cholangitis or primary ductular involvement with normal MRCP. Progression towards classic disease is 3%-7% per year[23]. This subtype of the disease is associated with both types of IBD, however there is a higher proportion of CD *vs* UC (22% *vs* 6%[24,25]). On the other hand, the prognosis of this group of patients is considerably better than classic PSC, with a greater average liver transplant-free survival. In general, it has a lower risk of developing CCA in comparison with main duct involvement[9].

Immunoglobulin G 4-related sclerosing cholangitis: IBD has been associated with autoimmune pancreatitis (AIP) and immunoglobulin G (IgG)4-related sclerosing cholangitis. This is the biliary manifestation of IgG4-related disease, a systemic fibroinflammatory condition that is characterized by mass lesions and/or strictures with classical histopathological findings in involved organ (salivary glands, retroperitoneum, kidneys, and lymph nodes)[26,27]. It is the most common extrapancreatic manifestation in patients with AIP type 1. Diagnosis is based in histopathological appearances, radiological features, and serological abnormalities. Typically have elevated serum IgG4 levels (> 135 mg/dL) and histopathologic findings (> 10 IgG4-positive plasma cells per high-power field. Diffuse or segmental narrowing of the intrahepatic and/or extrahepatic bile duct associated with thickening of the

bile duct wall. Usually presents with obstructive jaundice (70% to 80%), weight loss, and abdominal pain. Age of onset is (50-60)'s, predominately men. 75% PSC-patients have underlying IBD compare to IgG4 cholangitis in which case only 5% develops IBD[28]. IgG4+ plasma cells have been identified in colon tissue from patients with refractory forms of IBD patients. There appears to be association between disease activity and reduced fecal elastase or elevated serum IgG4, although the latter finding was more prevalent in patients with UC[29]. Differential diagnosis with pancreatic or biliary cancers, PSC, secondary sclerosing cholangitis is a challenge. The mainstay of treatment is systemic corticosteroids and most of the time a steroid trail is used to confirm the diagnosis. The response is good in two-thirds of strictures cases, different than PSC patients[30].

Post orthotopic liver transplantation IBD: There are discrepancies regarding the clinical course of post-liver transplant in IBD patients. About 30% may have a more severe course that includes clinical, endoscopic and histological deterioration within 10 years[31]. *De novo* IBD (that which develops after transplantation) is 10 times more frequent in patients transplanted due to PSC *vs* the general population, with a risk of 10%-11% at the 5 years mark and 14%-30% at the 10 years mark[31,32]. The use of cyclosporine and azathioprine (AZA) is preferred over tacrolimus given the protective factor during the clinical course[31,32].

Primary biliary cholangitis

It corresponds to a chronic autoimmune cholestatic disease of unknown cause that is histologically presented as a chronic non-suppurative destructive cholangitis[33]. Like all cholestatic diseases, its clinical manifestations range from asymptomatic to itching and fatigue. It is characterized by the presence of anti-mitochondrial antibodies and alteration of GGT and ALP levels[33]. Although its association with several autoimmune diseases such as: Sicca, Sjögren, among others, is known, its association with IBD is anecdotal. Liberal *et al*[34], described a series of 151 patients with primary biliary cirrhosis (PBC), of which only 6 had concomitant IBD. In all cases, PBC was diagnosed delayed from the IBD. Its association was similar with both UC and CD and its average age of diagnosis was the 5th decade of life. Although it is an infrequent association, it is important to keep it in mind since the use of ursodeoxycholic acid (UDCA) allows normalization of liver function tests and impacts the prognosis of the liver disease.

Autoimmune hepatitis unlike PSC

Autoimmune hepatitis (AIH) has the classic autoimmune disease behavior, being more frequent in women, with positive antibodies and responding to immunosuppressive therapy[35]. The clinical spectrum of autoimmune hepatitis ranges from asymptomatic elevation of liver function tests, passing through acute on chronic liver failure to cirrhosis. There are two types of AIH based in serological autoantibodies. Type 1 is characterized by ANA and/or SMA/anti-actin antibodies. Atypical pANCA-positive is more frequent in type 1 rather than type 2. Type 1 AIH affect mostly adults, have chronic presentation usually, hypergammaglobulinemia and other concomitant disease are autoimmune thyroiditis, rheumatological diseases and inflammatory back pain (IBP). It could present as autoinmmune sclerosing cholangitis (ASC) in children and PSC overlap in adults and remission after drug withdrawal is possible. Type 2 AIH is characterized by antibodies to liver kidney microsome type 1 (anti-LKM1), usually in the absence of ANA and SMA. Affect frequently children under 14 years, with acute onset at presentation. The most common concomitant diseases are autoimmune thyroiditis, diabetes mellitus and vitiligo and rarely presents as ASC or PSC in children and adults respectively. Usually need long term immunosuppressive therapy[36]. The diagnosis consists of the combination of epidemiological factors, serology with antinuclear and ASMA and liver biopsy, which is mandatory for diagnostic and prognostic purposes. The most frequent histological findings are the presence of lobular involvement, plasma cell infiltrate, involvement of the limiting lamina and pseudo rosette formation [37]. Immunosuppressive therapy is the mainstay of treatment, being corticosteroid and thiopurine association the first line of treatment. In resistant cases, drugs such as mycophenolate, calcineurin inhibitors and even anti-tumor necrosis factor (TNF)-α can be used[37]. Its association with IBD is of low prevalence, the most frequent being AIH with UC and to a lesser extent with CD or non-classifiable IBD [38,39]. In the most significant study, out of 105 patients with AIH, only 17 had findings suggestive of UC. Clinically, patients with AIH and IBD, mostly UC, developed AIH at younger age, had a lower remission rate, a higher rate of treatment failure, and progressed more to cirrhosis[40,41].

Overlap syndrome

It seems logical to raise overlaps when clinical, analytical, and imaging findings are shared or overlapped. This way, the presence of PSC/AIH overlap can be considered when there is a MRCP that shows typical findings of PSC but with AIH compatible antibodies with an elevated IgG and a histological finding of interface hepatitis. In the same way, AIH/PSC overlap can be considered when the diagnosis of AIH coincides with pruritus, elevated ALP and GGT, and alterations in the MRCP. The overlap between AIH and PBC is the most frequent in the general population, the Paris criteria are used to guide the diagnosis[42]. There is also an overlap between AIH and PSC and less frequently PBC and

PSC[43]. In IBD, there are no established diagnostic criteria, which makes it even more difficult to establish the real incidence of this entity; on the other hand, its appearance is usually sequential over the years[44,9]. The prevalence of this entity is lower than PSC alone and it is more commonly diagnosed in the pediatric and adolescent population[9,45]. Given the autoimmune nature of the disease associated with cholestasia, the concomitant use of immunosuppressive therapy (corticosteroids and thiopurines) and UDCA is recommended, however, there are no randomized studies that support this strategy. The clinical evolution of these patients seems to be similar to those of PSC/IBD without significant differences in its behavior in the few published reports[46].

ASC

This is a form of SC with strong autoimmune features with overlap with AIH, virtually all patients have raised IgG levels and autoimmune liver serology identical to AIH type 1, being ANA and/or SMA positive[47]. It was originally described in pediatric patients. In the initial report the patients were mostly men with concomitant IBD[48]. The largest prospective study was done in King's College Hospital with 55 pediatric patients with definite or probable AIH diagnosis accord to International Autoimmune Hepatitis Group. In that cohort, half of patients with anormal MRCP were classified as ASC, of those 44% had IBD compared to 18% of patients with HAI. In the retrospective largest cohort including 718 patients with PSC, 33% had concomitant AIH[49]. ASC disease in equally frequently in women and men and possession of HLA DRB1*1301 is associated to ASC, while possession of DRB1*0301 confers susceptibility to AIH type 1 and of DRB1*0701 to AIH type 2[50]. Histological differences between AIH and ASC patients included a higher median inflammatory activity index in AIH compared to ASC, and a higher frequency of cholangitis in ASC, but they are quite similar, and the final diagnosis is based in MRCP anormal findings. A clear diagnosis criterion for ASC is lacking. There are no randomized controlled treatment trials for ASC and these patients are treated with therapy based in UDCA and prednisolone ± AZA. In follow up MRCP, disease showed progression in half of ASC with an estimated 10 years transplant-free survival of 65%[51]. In a prospective study in ASC and AIH disease biochemical remission is similar, but the liver transplant rate was higher in ASC than in AIH in a 13 years period[52]. Seems like AIH and ASC are different disease based in different gender distribution, HLA association, IBD association and outcomes. There is some concern whether adult PSC is a late-stage phenotype of ASC and it is necessary long term follow up period to clarify this[47]. There are data suggesting progression of liver disease and post-liver transplantation recurrence of both AIH and ASC are associated with poorly controlled IBD[52,53]. Some studies demonstrated lower PSC recurrence post liver transplantation in patients with colectomy after surgery[54].

NAFLD

It is characterized by fat storage in > 5% of hepatocytes. Its development is directly related to obesity, insulin resistance and metabolic syndrome (MS), being currently considered the hepatic manifestation of MS. The clinical and histological spectrum is broad, from simple steatosis to steatohepatitis with inflammation nonalcoholic steatohepatitis (NASH), progression into fibrosis, liver cirrhosis, and hepatocellular carcinoma. The diagnosis requires the exclusion of secondary causes, such as daily alcohol intake (> 30 g/men and > 20 g/women) and the use of steatogenic drugs[54]. Demonstration of fat infiltration, either through histology (biopsy) or imaging, is required for diagnosis. Ultrasonography (US) is the most widely used technique, with a sensitivity of 85% [95% confidence interval (CI): 79.5%-88.9%] and a specificity of 94% (95%CI: 87.2%-97%) for the diagnosis of NAFLD[55]. Current data suggests an increase in the prevalence of NAFLD, currently estimated to be 25% globally (95%CI: 10-22-28). It is the leading cause of chronic liver disease in the western world and a growing cause of liver transplantation worldwide[54,56,57]. In IBD patients, it appears to be at least similar to or greater than in the general population and is currently considered the most frequent hepatobiliary manifestation in these patients[54]. The reports are varied with a prevalence ranging between 8%-71%[1]. This heterogeneity depends on the sample size, diagnostic criteria, and design used in the various studies, but also on the origin of the population studied, the year of study, and probably the change in pharmacological therapies in recent decades. A recent meta-analysis that included 19 studies (n = 5620 subjects), in which the diagnosis of NAFLD was based on imaging techniques, liver biopsy or transient liver elastography, reported a prevalence of NAFLD of 27.5% in patients with IBD (95%CI: 20.7%-34.2%)[56], quite similar to that of the general population[2]. The cumulative prevalence was higher in recent studies (2016 to 2018) compared to the cumulative prevalence of previous studies [(33.0%, 95%CI: 22.0%-44.1%) *vs* (21.3%, 95%CI: 13.1%-29.5%); P = 0.09], which may be related to the increase in obesity and MS in recent years. In turn, in studies of IBD patients with an 18-year follow-up, it showed that 48% of patients with CD and 44% of patients with UC presented NAFLD diagnosis by US. The presence of NAFLD was associated with older age, hypertension, and higher body mass index (BMI) in both groups[58]. Using transient liver elastography, it was observed that 32.8% of patients with IBD met NAFLD criteria and even 12.2% of patients already had liver fibrosis at the time of the study[59]. In patients with IBD, a higher prevalence of NAFLD has been observed in older patients [mean difference (MD) = 8.22; 95%CI:

6.22-10.22], with history of type 2 diabetes mellitus [odd ratio (OR) = 3.85; 95%CI: 2.49-5.95], hypertension (OR = 3.18; 95%CI: 2.36-4.28), obesity (OR = 2.79; 95%CI: 1.73-4.50), insulin resistance (OR = 6.66; 95%CI: 1.28-34.77) , MS (OR = 4.96; 95%CI: 3.05-8.05), chronic kidney disease (OR = 4.83; 95%CI: 1.79-13.04), methotrexate (MTX) treatment (OR = 1.76; 95%CI: 1.02-3.06), history of intestinal surgery (OR = 1.28; 95%CI: 1.02-1.62) and duration of IBD (MD = 5.60; 95%CI: 2.24-8.97)[60]. A recent retrospective study showed that the presence of clinical activity (Harvey Bradshaw Index > 4), history of intestinal resection, endoscopic activity, and the use of AZA would be risk factors with statistical association for NAFLD in patients with CD. In the case of UC, there was an association between NAFLD and endoscopic activity[61]. MS appears not to be the only triggering factor for NAFLD in patients with IBD. The prevalence of MS in patients with coexisting IBD-NAFLD could be lower than in NAFLD patients without IBD[62,63], thus the prevalence of these risk factors such as obesity, hypertension, dyslipidemia and type 2 diabetes was significantly lower in those patients with coexistence of IBD-NAFLD compared with the group of patients who only had NAFLD[64]. Another study that included 232 patients (78 patients with NAFLD-IBD, 154 patients with NAFLD only) showed that the patients with NAFLD-IBD were younger compared to the group of NAFLD patients without IBD, had lower body weight, smaller abdominal circumference and lower prevalence of MS (23.1% *vs* 56.6% respectively, $P < 0.001$)[65]. In patients with IBD-NAFLD, the severity of IBD was associated with greater severity of hepatic steatosis as measured by abdominal US[65]. These findings suggest that patients with IBD develop fatty liver disease with fewer metabolic risk factors than the population without IBD and that the severity of IBD could influence the degree of steatosis. This raises the existence of other factors, outside of metabolic ones, that could play a role in the coexistence of both diseases. The degree of chronic inflammation, the role of intestinal barrier disruption, increased intestinal permeability, microbial dysbiosis, immune activation, and drug-induced hepatotoxicity are factors that should be evaluated in directed studies[66]. Thus, the risk factors for NAFLD in IBD could be divided into those related to MS-obesity and those related to IBD itself in Figure 2. The actual prognostic impact of the NAFLD-IBD association is unclear. Steroids, especially higher doses, and longer duration, and immunomodulators used to treat IBD may increase the risk of progression to NAFLD. They increased weight gain and metabolic parameters, although there is no evidence that medications alone are responsible. TNF-α inhibitors could have a protecting roll in IBD patients from developing NAFLD. A systematic review was carried out through October 2017, this did not demonstrate a significant association between medication treatment in IBD and the risk of developed NAFLD. This suggests a complex, multifactorial relationship between IBD and NAFLD[67]. The coexistence of NAFLD-IBD poses a challenge in the management strategies of these patients. The presence of NAFLD and mainly the presence of NASH, can increase the risk drug induced liver injury (DILI), limiting the use of certain immunosuppressive therapies. It has been observed that in patients with IBD on immunosuppressive therapy, those who displayed an elevation of aminotransferases levels had a higher prevalence of NAFLD[68]. Thus, NAFLD could represent a risk factor in patients who require immunosuppressive drugs with hepatotoxic potential. A two-fold increase in mortality was reported in hospitalized patients with IBD and concomitant chronic liver disease (mainly cirrhosis due to NAFLD) compared to those without liver disease (2.7% *vs* 1.3%, $P < 0.01$)[69], which suggests that in patients with IBD and risk factors, the existence of NAFLD should be actively sought in order to plan a therapeutic strategy and rule out other differential diagnosis. Treatment of NAFLD should be based on managing metabolic risk factors and lifestyle changes. The objective is to achieve a weight reduction of at least 7%, which has been associated with biochemical and histological improvement[19,69]. Currently there are no specific recommendations for the management of NAFLD in patients with IBD. Control of metabolic risk factors and maintenance of IBD remission should be emphasized. Screening, prevention, and early treatment of NAFLD should be part of the comprehensive management of patients with IBD, especially those with risk factors.

DILI IN THE MANAGEMENT OF IBD

Given the new therapeutic options in IBD, there is a risk that these patients will develop DILI during the management of their disease, requiring a timely evaluation by an hepatologist in case of suspected hepatotoxicity[70]. DILI in a patient with IBD may manifest with elevated alanine aminotransferase (ALT) and aspartate aminotransferase (hepatocellular pattern); ALP and GGT (cholestasis pattern), jaundice (hyperbilirubinemia) or a mixed pattern. This complication can occur acutely, with the development of acute liver failure, autoimmune hepatitis, and reactivation of hepatitis B, and a percentage of these patients may develop chronic damage[71]. Therapies with 5-aminosalicylates, as well as immunomodulators, can cause alterations in liver function tests, these have been widely described in the literature, they are summarized in Table 1[72-78].

Salicylates

Sulfasalazine is an anti-inflammatory medication consisting of both 5-aminosalycilic acid and sulfapyridine. The last molecule causes more frequently toxicity, with a characteristic hypersensitivity

Table 1 Possible, drug induced liver injury, prevention and what to do

Drug	Prevention	Hepatic injury	Treatment
5-Amino salicilyc acid	Check before start treatment annual check	Drug induced hepatitis; Drug induced cholestasic; Granuloma formation (sulfa)	Stop drug; Follow-up
Tiopurines (azathioprine/6MP)	Check before treatment: TPMT and liver test; Check every week in first month, withing 2 wk in 2nd mo, every 3 mo	Drug induced hepatitis; Drug induced cholestasic; Sinusoidal obstruction syndrome; Nodular regenerative hyperplasia; Peliosis hepatitis	Drug induced hepatitis and cholestasic are idyosincratic reactions; More cases in the first three months of treatment; Stop drug
Methotrexate	Check before start treatment; Check every 2 wk until 2nd mo; Check every 3 mo; Add folic acid	Fibrosis/cirrhosis; Steatohepatitis	Stop MTX if ALT > 3 times; Despite alcohol; Comsume; Fibroscan

6MP: 6-mercapthopurine; MTX: Methotrexate; TPMT: Thiopurine methyltransferase; ALT: Alanino transferase.

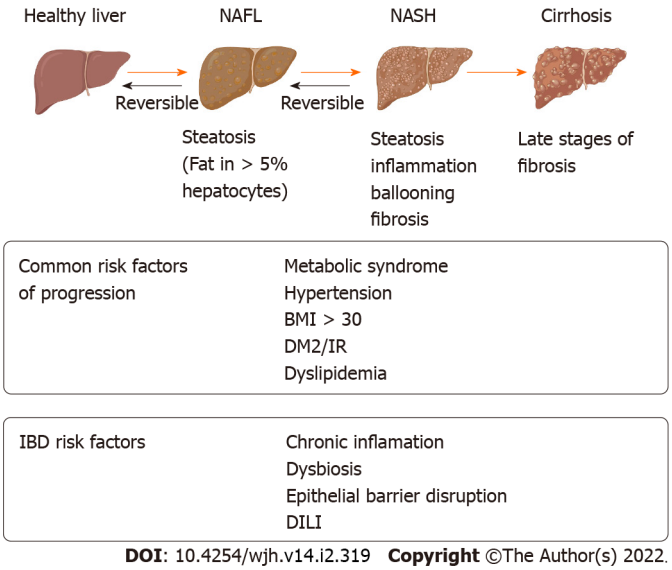

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Figure 2 Non-alcoholic fatty liver disease risk factor in inflammatory bowel disease. Created with Biorender. IBD: Inflammatory bowel disease; NAFL: Nonalcoholic fatty liver; NASH: Nonalcoholic steatohepatitis; BMI: Body mass index; DM2: Diabetes mellitus 2; IR: Insulin resistance; DILI: Drug induced liver injury.

reaction with hepatitis and withdrawal and steroids therapy could be required[79]. Other clinical manifestations are granulomatous hepatitis, cholestatic liver disease, and rarely acute liver failure. In a study included 4.7 million prescription, acute hepatitis occurred in only 9 patients[80]. Mesalamine is more commonly used in IBD patients and DILI associated is very low, 0%-4% of patients on this drug [81].

Thiopurines

Thiopurines, AZA and mercaptopurine (MP), are commonly used in IBD patients and have proven efficacy in maintenance remission. They are used as monotherapy or associated to biological therapy. AZA is transformed into 6MP *via* glutathione depending process. 6MP undergoes complex three enzymatic transformations to 6-thioguanine nucleotide (6-TGN), the active metabolite of thiopurine drugs. This can take alternative two other pathways, being converted to 6 thiouric acid or being metabolized to 6-methyl mercaptopurine (6MMP) by thiopurine methyltransferase (TPMT). This enzyme has an important role in the balance between 6-TGN and 6MMP. In IBD patients data support 6-TGN and 6MMP to improve clinical response and safety profile of thiopurines respectively. 6MMP is an inactive and potentially hepatotoxic metabolite. The therapeutic use of these drugs may be limited by dose-related or idiosyncratic adverse effects[72]. A subgroup of patients, "hyperme-thylators", 30%, preferentially produced 6MMP instead of 6-TGN producing treatment resistance and risk of hepatotoxicity. In this group, hepatotoxicity can be reversed by reducing the AZA dose by 75 % and adding xanthine oxidase inhibitors (allopurinol). The steady-state thiopurine metabolite concentrations are generally reached after approximately 4-8 wk of therapy to be measured. Blood levels of 6MMP > 5700 pmol/(8 × 10 red blood cell) are associated with a threefold hepatotoxicity risk[82] but toxicity has also been observed in patients with low 6MMP concentrations. Several factors, such as smoking, obesity,

ethnicity, and genetic factors, may influence the response to thiopurine therapy. NAFLD is a risk factor for the development of hepatotoxicity in IBD patients on thiopurines[83]. A multivariable analysis determinate early predictor for the development of hepatotoxicity in patients on stable thiopurine dose demonstrated increased risk with older people (> 50 years), BMI (> 25), gender (male) and 6MMP level > 3615 pmol 1 wk after treatment initiation[84]. These drugs have been reported to induce liver injury in up to 17% of patients[72]. Although the incidence varies depending of the hepatotoxicity definition in different studies. In a study hepatoxicity was define as ALT twice the upper limit normal and the incidence was 2.6% per patient-year[85]. There are three types of liver injury: (1) Hypersensitivity reaction; (2) Acute cholestatic or hepatitis pattern mostly idiosyncratic; and (3) Long term dose dependent endothelial injury involving sinusoidal dilatation, peliosis of the liver, sinusoidal obstruction syndrome and regenerative nodular hyperplasia with portal hypertension as manifestation[86]. Mostly cases the severity is mild and return to normal values even without drug dose modifications. Thiopurine withdrawal could be necessary in less than 4%, when severe cholestasis jaundice, moderate/severe injury without improvement after 50% dose reduction or development of endothelial chronic injuries[85].

MTX

MTX is an antagonist of the dihydrofolate reductase enzyme. The initial studies determinate hepatotoxicity risk was accumulative doses dependent but lately a meta-analysis showed that there is no association between the cumulative dose of methotrexate and the development of liver damage[86]. In a study about MTX for IBD, there was a 14.3% incidence of hepatotoxicity (defined as ALT or GGT > 1.5 ULN) that occurred after a median latency of 26 mo. Adverse events were more frequently seen in patients who were not taking concomitant folic acid supplementation, so it is recommended[87]. In a study with 518 patients treated with MTX, 44 patients (8.5%) had FibroScan and/or FibroTest results suggesting severe liver fibrosis. In a multivariate analysis, factors associated with this were the BMI > 28 kg/m^2 and high alcohol consumption[79]. So, it is a very important practice to modified these factors in this population of IBD with MTX treatment. Every time an increased in liver labs occurs in patients been treated with MTX, is necessary to rule out other diagnosis. Today we have tools for assessment in a non-invasive way liver fibrosis. Transient elastography allows to evaluate with a good accuracy[88].

Glucocorticoids

Corticosteroids are considered safe drugs regarding to hepatotoxicity. Only one case of effervescent prednisolone form induced hepatitis has been reported associated with IBD[89]. Another new case was reported with use of prednisolone recently. It contains sodic metasulfobenzoate and there is some concern whether itself is the cause of hepatotoxicity[90]. Generally, we assumed steroids are rarely cause of DILI in this setting of patients. We don´t have to forget that steroids could induce or deteriorate NAFLD[91] and could also reactivate viral hepatitis with prolonged use[40].

Biological therapies

Anti-TNF: Anti-TNF [infliximab (IFX), adalimumab (ADA), golimumab and certolizumab], these agents have been associated with various adverse events, including alterations in liver function tests[91], with a prevalence of 2.5% (free ammonia > 2.5 times the upper limit of normal) and 16% (ALT > 3 times the upper normal limit) respectively[2]. The latter, generally mild and transient, occurs more frequently after the 2nd to 5th infusion of IFX. Long-standing IBD, use of IFX as monotherapy, increased BMI, and hepatic steatosis have been some of the observed risk factors[92]. Less frequently, cases of autoimmune hepatitis have been described, with the switch to ADA being possible since it is not a class effect[93]. The effects on liver function tests with ADA, golimumab and certolizumab are less frequent[94]. Its discontinuation has been suggested in case of transaminase elevations > 3 times the normal value[95]. Liver function tests should be evaluated at the start of any anti-TNF agent therapy and then routinely monitored every 4 mo[95].

Anti-integrins: Anti-integrins are humanized monoclonal antibodies that block the adhesion and migration of leukocytes from blood vessels to inflamed tissue. DILI due to vedolizumab is uncommon and subsides after the suspension of the biological drug[96,97]. Varies from asymptomatic elevation of transaminase levels to symptomatic hepatocellular or cholestatic involvement[98].

Anti-interleukin 12/23: Ustekinumab is a fully humanized G1 immunoglobulin that binds to the p40 subunit of interleukin (IL)-12/23, which has been shown to be effective in inducing and maintaining remission in patients with CD and moderate to severe UC[99]. The pivotal studies in CD (UNITI-1, UNITI-2 and IM-UNITI) and UC (UNIFI) did not demonstrate an increased risk of hepatotoxicity[100, 101], which has been confirmed in subsequent studies[102]. Even though there is no formal recommendation for follow-up, monitoring of liver function tests every 6 mo is suggested[103].

Small molecules Janus-Kinases: Tofacitinib, an inhibitor of type 3, 1 and, to a lesser extent, type 2 Janus-kinases, and tyrosine kinase, has been shown to be effective in inducing and maintaining remission in patients with moderate to severe UC[99]. Subsequent studies have shown no risk of liver

damage in patients treated with tofacitinib when compared with placebo[104]. It also seems prudent to monitor liver function tests every 6 mo.

Sphingosine 1-phosphate receptors: Ozanimod a small molecule selective agonist against phosphate-1-sphingosine type 1 and 5 receptors that prevents lymphocyte trafficking to the intestine has recently been approved for moderate-to-severe active UC[105]. The increase in GGT was seen in 5.3% of the patients[106]. Studies carried out on the real world should confirm the safety of this drug.

The combination of two biological agents or one biological with a small molecule aims to produce a synergic effect, thus increasing the probability of achieving remission of intestinal inflammatory activity and of extraintestinal manifestations. Recently, a study with high-risk IBD, refractory to biological therapy and small molecules, demonstrated that a combined strategy does not increase the risk of adverse events (including liver damage)[107]. Will be necessary new studies to confirm it.

VIRAL HEPATITIS IN IBD

Hepatitis B virus reactivation

Like other immunosuppressive therapies (including the use of corticosteroids in high doses or for prolonged periods of time), anti-TNF, anti-integrin therapy has been associated with hepatitis B virus (HBV) reactivation, especially in patients with hepatitis B surface antigen (HBsAg) positive[107,108]. It is for this reason that every patient must be tested with HBsAg, hepatitis B surface antibody (HBsAb) and the total HBcAb before starting the biological agent, being necessary prophylactic therapy in case of presenting a positive surface antigen[109]. To date, there is no information on cases of HBV reactivation in IBD patients treated with ustekinumab. In the case of tofacitinib, reactivations of the HBV have been observed in patients with rheumatoid arthritis[110], so it is prudent to carry out a control before starting therapy.

Hepatitis C virus

The prevalence of hepatitis C virus (HCV) infection in IBD patients seems to be lower than expected, similar to the general population. These results indicate that IBD patients in western European countries should no longer be considered as a risk group for HBV or HCV infection. Numerous case reports indicate that anti-TNF-α therapy in the setting of HCV appears to be safe. However, the long-term effect of anti-TNF-α agents on HCV is not. In particular, while the use of anti-TNFα in non-cirrhotic patients appears safe, it is contraindicated in patients with decompensated cirrhosis[111]. On the other hand, anti-TNF-α drugs seem to reduce inflammation through TNF-α inhibition, playing a role in the pathogenesis of HCV[112]. There are few and small HCV reactivation studies, the HCV reactivation was 8/51 (15.7%) and in 1/10 (10%) HCV RNA positive patients, respectively[113,114]. All cases of reactivation had a very mild course, except for one patient, who died. No recommendations have been proposed for HCV screening prior to starting immunomodulators. However, HCV antibody screening should be routinely performed upon the completion of liver function tests before starting immunosuppressive therapy[115].

CCA

Development of PSC in IBD patients had increased risks of CCA (HR, 28.46), hepatocellular carcinoma (HR, 21.00), pancreatic cancer (HR, 5.26), and gallbladder cancer (HR, 9.19)[2]. Patients with PSC with or without IBD are also at increased risk of primary hepatobiliary neoplasia and CCA. Although IBD may be a risk factor for CCA, likely *via* PSC, it is not clear that IBD confers any added risk for CCA in PSC patients[116]. The lifetime CCA incidence in PSC patients is between 5%-10%, affecting people in the fourth decade of life. It is usually a perihiliar neoplasia (75%) and fibrosis is not necessary for its development. Most of the tumors are diagnosis in the first four years after PSC diagnosis, being more than 50% in the first year since the diagnosis. These are frequently detected in its advanced stages when prognosis is poor[117,118]. The mortality in IBD-PSC patients is higher than IBD patients without PSC, being malignance the main factor. CCA have reduced survival compared to CRC. The survival curve in patients with CRC was similar to the PSC-IBD without CRC or CCA probably related to periodic colonoscopy surveillance that allow early CRC diagnosis[117]. Well known CRC surveillance annually recommendations are established but in the field of CCA there is lack of robust evidence in PSC population surveillance. Although most of clinicians ask for MRCP and CA19-9 marker annually it's well known the limited specificity in the setting of PSC and the difficulty in image diagnosis in early stages. Risk factors associated with CCA among patients with PSC-IBD patients are not well known and are traying to be find to stratify patients. One recently study of the large cohort showed, in a multivariable model, that duration of IBD was the only independent predictor of increase risk of CCA, with a 33% increase risk per 10 years of IBD. And in the subset patients with colectomy when the

indication of surgery was CRC or dysplasia the risk was increased compare those with colectomy for refractory disease (HR, 2.68, 95%CI: 1.01-7.07) after adjustment for disease duration[118]. The pathobiological mechanisms underlying are not clear but the altered bile acids and microbiome environment that exists in IBD may persist after colectomy[119,120]. It seems as colectomy does not modify the increased risk of CCA associated with prolonged IBD, persisting the risk after colectomy. So, in these setting of patients, specific surveillance may be appropriate.

OTHER HEPATO-BILIARY MANIFESTATIONS

Cholelithiasis

It is a frequent pathology in patients with IBD. In the study by Fousekis *et al*[58], cholelithiasis was the second hepato-biliary manifestation. Patients with CD have double the risk of developing cholelithiasis in relation to control subjects, while UC show no differences with the control population[121,122]. A prevalence of 11%-34% has been estimated in CD[28]. The underlying pathophysiological mechanism appears to be multifactorial. Patients with CD present gallbladder hypomotility with decreased emptying and gallbladder ectasia[123]. The involvement of the ileum would lead to a reduction in the reabsorption of bile salts, with the consequent alteration in the enterohepatic circulation and secondarily an increase in biliary cholesterol saturation[9]. The risk would also be related to the degree and extent of ileo-caecal involvement and the number of intestinal resections[121,124]. Ileocolonic involvement, with more than 15 years of disease, with frequent outbreaks (> 3), prolonged hospital stays or a number greater than 3 hospitalizations, according to the number of intestinal surgeries or ileal resection (> 30 cm) and total parenteral nutrition requirements have been reported as a risk in CD[121].

Portal venous thrombosis

Thromboembolic events are more frequent than in the general population[125]. Prevalence studies indicate that 1.3% of patients can present it, with a mortality of 50%[126]. Porto-mesenteric axis thrombosis is a rare form of venous thrombosis, reporting a prevalence of 0.1%-1.7% in patients with IBD, being found in the postoperative period of digestive surgery or in an imaging study[127-129]. A retrospective multicenter study reported that up to 40% of patients with IBD and porto-mesenteric thrombosis may present an associated prothrombotic factor, the most frequent being hyperhomocysteinemia due to folate and vitamin B12 deficiency[130]. Additionally, patients with IBD may have an imbalance between coagulation factors, with a decrease in the level of antithrombin III and an elevation of factors V-VIII, of the platelet count and of the fibrinogen level, which can finally lead to a prothrombotic state. IBD may itself be a risk factor for thromboembolism[131]. The clinical manifestations and risk factors are summarized in Figure 3[28,130]. The European Crohn's and Colitis Organisation guidelines recommend measures to prevent thromboembolic events during hospitalization or during exacerbation of IBD[132]. In the presence of porto-mesenteric thrombosis, evaluation of acquired and hereditary prothrombotic conditions and early anticoagulant treatment are recommended[132]. Portal hypertension non-cirrhotic intrahepatic portal hypertension (NCIPH) is associated to Schistosomiasis; toxins/drugs (arsenic, vitamin A, AZA, 6-thioguanine), immune disorders (Felty' syndrome, common variable immunodeficiency disorder) and myeloproliferative syndromes. More recent evidence of associated gut disorders has been described. In a retrospective cohort of NCIPH, three (9%) patients had UC while five of 31 (16%) tested had celiac disease[133]. Microvascular damage described induced by thiopurines are *veno*-occlusive disease, peliosis hepatis, perisinusoidal fibrosis and nodular regenerative hyperplasia (NRH). This is an uncommon condition characterized by the diffuse transformation of normal hepatic parenchyma into small, regenerative nodules with little to no fibrosis. Vascular flow impairment induces diffuse hepatocyte hyperplasia and nodule formation[134]. The mechanisms of NRH in patients with IBD include immunological and thrombotic factors in addition to external factors such as AZA. The uncontrolled inflammatory response itself in patients with IBD stimulates these factors, which also could cause NRH[135]. The largest case series describing NRH in IBD patients reported a total of 37 cases, between 1994 and 2005, in 11 hospitals. The cumulative risk of NRH could be estimated from the experience in one of center as 0.50% at five years and 1.25% at 10 years and in the multivariate analysis was associated to male sex, stricturing behavior IBD patients treated with AZA[136]. NRH may be detected using biopsies or magnetic resonance imaging (MRI). Recently MRI was proponed as an alternative diagnostic test but the sensitivity and specificity were relatively low[136]. NRH is most often diagnosed after there is evidence of portal hypertension. A low platelet count may be the earliest manifestation of NRH to consider in long-term thiopurine therapy. Generally, the prognosis of NRH is better than that of chronic liver disease and is related to the complications of portal hypertension and the severity of the underlying disease. NRH is probably not reversible, even after stopping the treatment with AZA[134].

Pyogenic liver abscess

They are a rare complication of IBD, with a reported inci-dence greater than the general population (6.72 *vs* 4.06 per 10000 person-years; spontaneously hypertensive rats = 1.46 (95%CI: 1.01-2.12)[137]. Most of

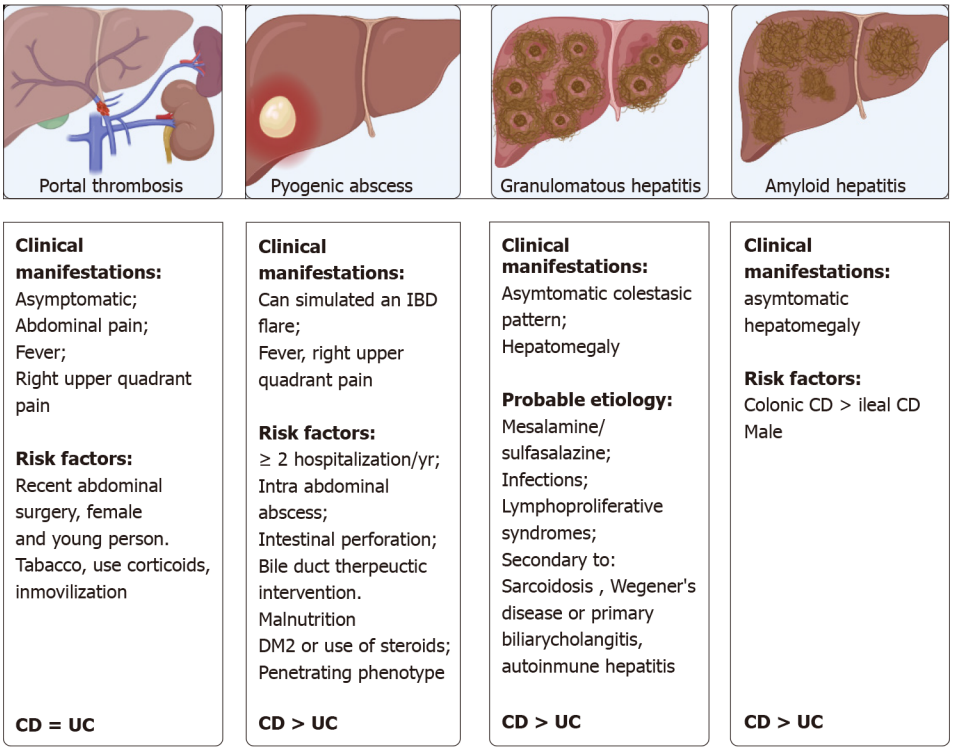

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Figure 3 Others hepatic manifestations. Created with Biorender. DM2: Diabetes mellitus 2; IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis.

the cases described are in patients with CD[138]. The clinical manifestations and risk factors are summarized in Figure 3[139,140]. The most frequent etiological agents are *Streptococcus milleri* and Gram-negative anaerobic bacilli[138,141], with a mortality rate close to 38%, with worse results when the diagnosis is late or when there are multiple abscesses of biliary origin[142]. Management does not differ from management in the general population and involves broad spectrum intravenous antibiotics for prolonged periods of time and when necessary, percutaneous, or surgical drainage.

Granulomatous hepatitis

Granulomatous hepatitis is also an infrequent manifestation in patients with IBD, being mainly described in CD. A prevalence of less than 1% has been reported and is characterized by the presence of non-calcified hepatic granulomas demonstrated in the histological study (liver biopsy)[41]. The clinical manifestations and probable etiologies are summarized in Figure 3[143,144]. With a good prognosis, it would improve with the control of the causative agent, such as mesalamine suspension[145].

Hepatic amyloidosis

Secondary amyloidosis consists in the storage of insoluble protein fragments, called amyloid, in various organs. This pathology is infrequent in patients with IBD, with a prevalence of 0.5%, being more frequent in CD with a prevalence that varies between 0.9% and 3%[133]. The clinical manifestations and risk factors are summarized in Figure 3[146]. Treatment is based on controlling intestinal inflammation, thus reducing the release of acute phase reactants, such as amyloid A. Resolution of amyloidosis has been reported after resection of the compromised intestine[147].

CONCLUSION

Undoubtedly, a multidisciplinary management allows a timely diagnosis of hepatobiliary manifestations that are frequent in both CD and UC (summarized in Table 2). Its diagnosis has prognostic implications, given the risk of progressing to chronic liver disease and its possible association with neoplastic diseases. In regard to new therapies, although they have been classified as safe, there is a risk of alterations in liver function tests, being more frequent with anti-TNF biological agents. However, in all these, either small molecule or biological therapies, it is advisable to carry out a control of liver function tests prior to starting treatment and sequentially according to the type of therapy used, and a HBV screen to avoid risks of reactivation.

Table 2 Summary of different diseases according to liver disease pattern

Type of manifestation	Diagnostic	Treatment
	Cholestasis	
PSC	MRCP	UDCA
SDD	Liver biopsy	UDCA
IgG4 cholangitis	MRCP + liver biopsy + Ig4 subclass in blood	Glucocorticoids
PBC	AMA serology or liver biopsy in some cases	UDCA
DILI	Approach based in ruling out others diagnosis and likelihood depending the drug and latency	Withdrawal the drug and steroids in some hypersensibility cases
Cholangiocarcinoma	MRCP + CA199 markers	Surgery, chemotherapy, liver transplantation (special cases)
	Hepatocelular pattern	
NAFLD	Abdominal US, fatty liver indexs, ruling out other diagnosis, liver biopsy in some cases	Change style of life with loose weight, calories restricted diet, exercise and control IBD inflammatory activity
AIH	Serology (ANA, ASM, LKM1, IgG, liver biopsy). Simplified autoimmune hepatitis score	Azathioprine ± steroids
Chronic viral hepatitis	Serology, non-invasive fibrosis tests	DDA in HCV and long-term antiviral in HBV
DILI		Withdrawal the drug and steroids in some hypersensibility cases
	Mix pattern	
Overlap/AIC	MRCP + liver biopsy	Azathioprine ± steroids + UDCA
DILI		Withdrawal the drug and steroids in some hypersensibility cases
Chronic viral hepatitis	Serological markers	DDA in HCV and long term antiviralin HBV

MCRP: Magnetic resonance cholangiopancreatography; UDCA: Ursodeoxycholic acid; ANA: Anti-nuclear antibodies; SMA: Anti-smooth muscle antibodies; LKM1: Liver kidney microsome type 1; DDA: Direct-acting antiviral; HCV: Hepatitis C virus; HBV: Hepatitis B virus; AIC: Anterior insular cortex; DILI: Drug induced liver injury; PSC: Primary sclerosing cholangitis; AIH: Autoimmune hepatitis; NAFLD: Non-alcoholic fatty liver disease; US: Ultrasonography; ASM: Anti-smooth muscle; IBD: Inflammatory bowel disease; IgG: Immunoglobulin G; SDD: Small duct disease.

FOOTNOTES

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MINIREVIEWS

Cytomegalovirus infection in liver-transplanted children

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Abstract

Cytomegalovirus (CMV) infection is a common complication of liver transplantation in children. The CMV serostatus of recipients and donors is the primary risk factor, and prophylaxis or pre-emptive strategies are recommended for high-risk patients. Graft rejection, coinfection and Epstein-Bar virus reactivation, which can lead to post-transplant lymphoproliferative disease, are indirect effects of CMV infection. Assessment of CMV infection viral load should be routinely performed upon clinical suspicion. However, tissue-invasive CMV disease is not associated with CMV viraemia and requires confirmation by tissue pathology. Oral valganciclovir and intravenous ganciclovir are equivalent treatments, and the duration of treatment depends on factors including CMV viral load, tissue pathology, and clinical response. Risk stratification by donor and recipient status prior to transplantation and post-transplantation antiviral prophylaxis or pre-emptive therapy are recommended. Adult guidelines have been established but additional study of the effectiveness of the preventive guidelines in children is needed. This review summarizes the burden, risk factors, clinical manifestations, laboratory evaluation, treatment, and prevention of CMV infection in children after liver transplantation.

Key Words: Cytomegalovirus; Children; Liver transplantation; Pediatric; Infection

Core Tip: Cytomegalovirus (CMV) infection after liver transplantation in children is a serious complication, with high morbidity resulting from direct and indirect effects. Despite risk stratification, pre-emptive therapy and antiviral prophylaxis, late CMV infection frequently occurs in transplant recipients. If CMV infection is suspected during outpatient visits, then prompt detection is key. If CMV infection is detected, then decreasing immunosuppressants should be prioritized before initiation of antiviral therapy. Oral valganciclovir and intravenous ganciclovir are the mainstays of treatment, with variable duration depending on CMV manifestations, viral load, histopathology, and clinical response.

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INTRODUCTION

Cytomegalovirus (CMV) infection is common in both immunocompetent and immunocompromised hosts, and the manifestations of primary infection in adolescents and young adults can be serious. In immunocompromised hosts, both primary and latent CMV infection can cause serious disease. The indirect effects of mixed infection, post-transplant lymphoproliferative disease, and graft rejection, are all of great concern. Hence, prevention and prompt management of incident CMV infections are necessary and rapid access to measures to predict and detect CMV infections with high accuracy are required.

TERMINOLOGY

CMV infection is defined as evidence of CMV replication regardless of symptoms. The evidence may involve isolation and identification of the CMV virus or detection of viral proteins or nucleic acids in any specimen. The detection of CMV in the blood may be by standard or shell techniques, CMV pp65 antigen, CMV DNA, or CMV RNA, with CMV viraemia, antigenaemia, DNAemia, or RNAemia[1-3].

CMV reinfection is CMV infection by a different strain from an exogenous source documented by molecular techniques or sequencing of specific regions. Patients with CMV reinfection develop new immune responses to the viral epitopes that are different from the previous primary CMV infection, known as a polymorphic gene[3].

CMV reactivation is a CMV infection that results from reactivation of latent endogenous CMV.

CMV disease includes evidence of CMV infection in combination with attributable symptoms that can be classified as CMV syndrome and tissue-invasive CMV disease or compartmentalized CMV disease. CMV syndrome, which includes fever, malaise, and/or myelosuppression[3], has no organ- or tissue-specific manifestations. Tissue-invasive CMV disease has primary organ-specific pathology and organ-specific manifestations.

PREVALENCE

CMV seroprevalence, or evidence of infection, varies worldwide (from 45% to 100%) in reproductive-age women[4]. Seroprevalence is highest in South America, Africa, and Asia and lowest in Western Europe and the United States. Factors related to high seroprevalence are older age, low socioeconomic status, crowded or unsanitary living conditions, and low education level[5,6]. The age-adjusted seroprevalence of CMV infection was reported as 50.4% in the United States[5] and as 20.7%-28.2% in children aged 1-5 years and as 36.3%-37.5% in those aged 6-11 years[7].

CMV infection can be serious in recipients who were seronegative prior to liver transplantation. Consequently, the risk of infection is stratified by recipient and donor serostatus as seropositive donors with seronegative recipients (D^+/R^-), seropositive donors with seropositive recipients (D^+/R^+), seronegative donors with seropositive recipients (D^-/R^+), and seronegative donors with seronegative recipients (D^-/R^-). A study of a series of 146 liver transplant patients reported a higher incidence of post-transplant CMV infection in the 14 children (71.4%) than in the 132 adults (33.4%)[8] because of the high proportion of CMV-naïve recipients. The children also developed CMV infection significantly sooner than the adults, with a mean time to viraemia of 11.5 *vs* 30 d[8].

Antiviral prophylaxis and pre-emptive therapy are intended to decrease CMV infections and disease in liver transplant patients. Without prevention therapy, 18%-85% of adults develop CMV infection and 15%-40% develop CMV disease[9-11], ranging from 1%-2% in D^-/R^- procedures and 44%-65% in D^+/R^- procedures[12]. In young children, the incidence of CMV infection ranged from 44% to 65% within 6 mo and up to 2 years in follow-up[13-15]. A study by Saitoh *et al*[13] in Japan reported that in children with pre-emptive therapy, CMV antigenaemia occurred following 63% of the D^+/R^- procedures, 38% of the D^+/R^+ procedures 11% of the D^-/R^+ procedures, and 6% of the D^-/R^- procedures. CMV disease occurred with 11% of the D^+/R^- procedures, 2% of the D^+/R^+ procedures, 0% of the D^-/R^+ procedures, and 6% of the D^-/R^- procedures. A study by Verma *et al*[14] in the United Kingdom, reported late CMV infection in 10.5% and disease in 4.4% of children following liver transplant. None of the D^-/R^- children had late CMV infection or disease 2 years post-liver transplant.

PATHOGENESIS

CMV infection in liver recipients can manifest as a primary infection, reinfection by exogenous virus, or reactivation of endogenous virus in the host cells. After the virus infects the host cells, it replicates slowly, leading to persistent, latent viral infection in recipient cells. Systemic inflammation can cause reactivation of the latent viral state and development of CMV infection. Viral latency at cellular sites may also serve as a route for transmitting the virus to susceptible recipients[12]. The main targets of CMV are epithelial cells[16], with transmission of the virus occurring from host to host *via* mucosal epithelium, as in gastrointestinal CMV infection. Immature dendritic cells underlying the mucosa are also sites of viral replication and shedding, leading to viral spread by lymphatic circulation[17]. In solid organs, the main targets of CMV are mesenchymal and endothelial cells[16,18]. Viral spread within the organ results from infection of connective tissue cells. Infection of endothelial cells contributes to haematogenous spread into organ tissues.

While CMV infection manifests directly as a clinical disease, it can also modulate the host's immune system to lead to indirect effects that cause acute early allograft rejection or late allograft dysfunction. Moreover, immune system dysregulation and immunosuppression associated with impairment of CD4+ T cells and macrophages may increase the susceptibility to opportunistic bacterial, fungal, or viral infections including Epstein-Bar virus (EBV) and human herpes virus (HHV)-6[12]. CMV can also infect host vascular endothelial cells and cause the downregulation of genes responsible for the production of extracellular matrix components such as collagen type I and fibronectin, resulting in the development of vascular thrombosis[19].

Protective responses against CMV infection include both innate and cell-mediated immunity. Innate immunity involves Toll-like receptor 2 (TLR2), a pattern recognition receptor that recognizes CMV antigen and consequently promotes antiviral peptide and cytokine production[12]. Tissue dendritic cells are a frontline target of the virus. Cell-mediated immunity is the primary immune response against CMV infection in liver transplant recipients. Interferon-gamma (IFN-γ) produced by CD8+ T cells is associated with a decreased risk of CMV disease, and cytokine production is stimulated by recognition of the CMV pp65 antigen by CD8+ T cells[20,21]. An enzyme-linked immunosorbent spot assay is available to confirm CD4+ and CD8+ cell-mediated immune function and quantify IFN-γ produced in response to CMV[22-24]. In addition, humoural immunity against CMV infection develops through production of neutralizing antibodies that target CMV glycoprotein B, which has contributed to the development of a CMV vaccine[25]. Neutralizing antibodies can also be generated against other CMV envelope glycoproteins.

RISK FACTORS OF CMV INFECTION AND DISEASE AFTER LIVER TRANSPLANTATION

CMV serostatus of the recipient and donor

The incidence of CMV infection is generally highest in D^+/R^- liver transplant recipients, and recent studies have reported up to 95% of all recipients with CMV antigenaemia were in either D^+/R^- or D^+/R^+ groups[13,14]. The time from transplantation to the onset of CMV viraemia was also shown to be significantly shorter for D^+/R^- patients than for those in the other groups[26]. The evidence supports stratification of liver transplant candidates by the recipient and donor CMV serostatus[27]. D^-/R^- or D^+/R^+ patients are considered at low risk, while those who are D^-/R^+ are considered intermediate risk and those who are D^+/R^- are considered at high risk[27] (Table 1).

Viral burden

It has been documented that patients with a high initial or an increasing viral load tend to have an increased risk of developing CMV disease after liver transplant[28-30], and early detection is important for clinical management. The viral load cut-off for predicting CMV disease varies with the method of detection. Gerna *et al*[31] found that CMV disease developed in patients with a mean blood CMV viral

Table 1 Risk of cytomegalovirus disease after liver transplantation

Risk factors	
CMV serostatus of recipient and donor	D^+/R^-
	D^+/R^+ and D^-/R^+
Viral burden (initial CMV viral load)	High CMV viral load
	Rate of viral load increasing
Immunosuppressive agents	Antibody to CD3-receptor: OKT3 or muromonab
	Basiliximab
	Corticosteroids
	Mycophenolate mofetil
	Calcineurin inhibitors: Tacrolimus, sirolimus, and cyclosporine
Recipient immunity	TLR2 gene mutation, mutation of mannose-binding lectin
	Upregulation of programmed death-1 receptors
Recipient underlying liver disease	Hepatoblastoma with pre-transplant chemotherapy
Other risk factors	Virus-to-virus interaction (HHV6, HCV, fungal infection), transfusion of non-leucocyte-depleted blood products, volume of blood loss, liver transplantation because of fulminant liver failure, older age, non-white race, female sex, CVVH after liver transplant, septic shock, renal insufficiency

CMV: Cytomegalovirus; CVVH: Continuous venovenous haemofiltration; D: Donor; HCV: Hepatitis C virus; HHV-6: Human herpes virus-6; R: Recipient; TLR2: Toll-like receptor 2.

load of 1740 copies/mL. Assay of CMV DNA by real-time quantitative polymerase chain reaction (PCR) [32-34] showed a cut-off value of 180 copies/mL (164 IU/mL) is associated with an increased incidence of severe CMV disease in adult liver transplant recipients[35]. The lack of an international reference standard limits the generalization of study cut-off values for worldwide use. The World Health Organization (WHO) has a reference standard for plasma quantitative nucleic acid testing (QNAT) that transplantation centres can use for calibration[36,37]. International references are needed for other assay methods (Table 1).

Immunosuppressive agents

Drugs that interfere with host immune function also influence the risk of CMV disease. Generally, immunosuppressive agents involving the cytotoxic immune response cause a loss of CMV infection control. They include lymphocyte-depleting drugs used in the induction and rejection phases. OKT3, or muromonab, a murine monoclonal antibody against the CD3 receptor found in mature T lymphocytes, has been correlated with an increased risk of CMV infection[15]. Other drugs that increase the risk of CMV infection include corticosteroids[38], mycophenolate mofetil[39,40], and basiliximab[41]. Calcineurin inhibitors, such as tacrolimus, sirolimus, and cyclosporine, which are commonly used in paediatric patients, have also been associated with an increased risk of CMV disease[41,42]. Tacrolimus and sirolimus concentrations have been correlated with increased viral load in whole blood and plasma from paediatric liver recipients[42]. Monitoring drug levels in patients receiving tacrolimus or sirolimus was recommended, as the correlation between circulating levels and the administered dose was not strong. The assay may be performed with either whole blood or plasma, as the viral load results obtained with each type of sample were highly correlated[42]. Newer drugs, such as mechanistic target of rapamycin inhibitors, have a weaker association with the risk of CMV infection[12] (Table 1).

Recipient immunity

The immune status of liver transplant recipients also contributes to the risk of CMV infection[12]. Defects in innate immunity, such as TLR2 gene mutations, are correlated with an increased risk of tissue-invasive disease[43]. Other defects in innate immunity associated with the risk of CMV infection include mutation of mannose-binding lectin and upregulation of programmed death-1 receptors[44,45] (Table 1).

Underlying liver disease in the recipient

Some underlying liver diseases in recipients before liver transplantation have been associated with the risk of CMV infection. Acute liver failure and hepatoblastoma patients receiving post-transplant chemotherapy had significantly increased risk of CMV infection[13,46]. Recipients with cholestatic liver

disease before transplantation had a decreased risk of CMV infection and those with biliary atresia were reported to have a lower risk of CMV infection[13], with a reported odds ratio of 0.288[15] (Table 1).

Other risk factors

Other risk factors include virus-to-virus interaction [HHV-6, hepatitis C virus (HCV)], fungal infection, transfusion of non-leucocyte-depleted blood products, blood loss volume, liver transplantation because of fulminant liver failure, older age, non-white race, female sex, haemodialysis, septic shock, and renal insufficiency[47,48] (Table 1).

CMV MANIFESTATIONS

Primary infection, reinfection, and reactivation can occur in liver transplant recipients. Primary infection is the development of CMV viraemia in a previously unexposed seronegative recipient, excluding cases with the passive acquisition of CMV antibodies from blood products or immunoglobulin (Ig). The manifestations of primary CMV infection are more severe than CMV reinfection or reactivation from latent endogenous virus[49]. Current guidelines consider D^+/R^- children to be the most prone to developing severe CMV disease from primary infection[50].

Direct effect of CMV or CMV disease

Patients can manifest CMV syndrome or CMV tissue-invasive disease.

CMV syndrome: Systemic manifestations include the detection of CMV in the blood, together with at least two of the following: Fever; new-onset malaise or fatigue; leukopenia or neutropenia in two separate measurements; 5% atypical lymphocytes; thrombocytopenia; and transaminitis three-times the upper normal limit. Fever is defined by a body temperature > 38 °C for at least 2 d within a period of 4 d. Some patients develop lymphadenopathies, hepatosplenomegaly, pharyngitis, and a mononucleosis-like syndrome consisting of a rubelliform rash associated with febrile illness. Less common manifestations include migratory polyarthritis, mainly involving the fingers, knees, and toes[51-53].

CMV tissue-invasive disease: The most common organ involvement in post-liver transplant includes the gastrointestinal tract, liver, and lungs[12]. Gastrointestinal CMV disease may manifest with clinical features such as odynophagia, dysphagia, abdominal pain, diarrhoea, haematochezia, and severe iron deficiency anaemia that could imply gastritis, oesophagitis, enteritis, or colitis (Figure 1)[12]. CMV may also infect the liver allograft, causing CMV hepatitis in which an abnormal liver function test may not clearly distinguish it from allograft rejection. Other less common CMV manifestations include central nervous system (CNS) disease, retinitis, nephritis, cystitis, myocarditis, pancreatitis and cholangitis[54]. However, the diagnosis of tissue-invasive disease is challenging and often requires invasive investigations. Confirmation of CMV CNS disease requires the presentation of CNS symptoms and evidence of CMV infection in cerebrospinal fluid or brain biopsy. CMV retinitis is diagnosed by fundoscopic examination. The diagnosis of CMV nephritis, cystitis, myocarditis, or pancreatitis requires the detection of CMV together with cytopathological evidence in biopsies of the involved organ.

Indirect effects of CMV

Apart from CMV disease, indirect effects such as CMV-associated graft failure, vanishing bile duct syndrome, allograft fibrosis, chronic ductopenic rejection, vascular thrombosis, and new-onset diabetes mellitus may occur[12]. CMV-associated graft failure may be difficult to distinguish from graft failure from other causes, including immune-mediated graft rejection, haematologic disease, drug toxicity, or other infections, such as HHV-6, EBV, and adenovirus. A diagnosis of exclusion is required[3]. Some patients may manifest with coinfection reactivation or opportunistic HCV, HHV-6, HHV-7, fungal, nocardial, or bacterial infections, or EBV-associated post-transplant lymphoproliferative disease, infections.

INVESTIGATION

A definitive diagnosis of invasive tissue disease requires the detection of CMV in a tissue specimen from the affected organ[55]. The gold standard for testing is the detection of either CMV cytopathology or CMV antigen by immunohistochemistry. Other methods of detecting CMV infection and disease are described below (Tables 2 and 3).

Cell culture

In conventional cultures, human fibroblast cells are inoculated with a clinical specimen and have an incubation period of 2 d to 21 d. In shell vial assays, the incubation time is shortened to approximately

Table 2 Cytomegalovirus assays and clinical use

Investigation	Sample	Uses	Properties
Cell culture			
Traditional cell culture (human fibroblast cells)	Tissue or non-tissue (blood, urine, oral secretion) sample	Not widely available	Highly specific
Shell vial assay (centrifugation-amplification technique)			Can be tested for phenotypic susceptibility; Takes a long time (2 to 21 d), more rapid with the shell vial assay (16 h)
Histopathology of organ-specific tissues			
Plain histological microscopy	Tissue sample	Gold standard for diagnosis of tissue-invasive CMV disease	Low sensitivity but very high specificity
Immunohistochemistry		Used for reference of endpoint of treatment of tissue-invasive CMV disease	
Molecular diagnosis (detection of viral genome)			
Plasma quantitative nucleic acid testing (plasma QNAT)	Blood (plasma or whole blood)	Used to detect CMV DNAemia with high sensitivity; used in diagnosis, surveillance to guide pre-emptive antiviral treatment, and therapeutic monitoring	Generally high sensitivity but less sensitivity in R^+ patients
Tissue QNAT	Tissue sample	Need more clinical trial studies	Better specificity but a lack of studies
Real-time PCR	Blood	Alternative to conventional plasma QNAT	More rapid and precise
NASBA assay	Blood	Under study as an alternative to conventional quantitative antigenaemia as a guide for starting pre-emptive therapy	Increased sensitivity for detection of CMV viraemia
Direct viral pp65 antigen detection	Whole blood or plasma	Diagnosis of CMV infection by detecting antigenaemia; Quantitative result, can guide initiation of pre-emptive therapy	After the blood collection, the sample must be processed within 6 h; False-negatives in patients with neutropenia
Serological analysis (viral antibody detection)			
CMV IgG antibody testing	Plasma	Diagnosis of CMV infection	Better sensitivity and specificity; also positive in past infection
CMV IgM antibody testing		Pre-transplant assessment for serostatus of the donor and the recipient	Low sensitivity and specificity for diagnosis
Viral cellular response detection			
QuantiFERON-CMV assay: IFN-γ released measurement	Plasma	Prognostic marker for risk of developing CMV disease: a positive result is associated with a lower incidenceMonitoring during prophylaxis or pre-emptive therapy	High positive predictive value but low negative value

CMV: Cytomegalovirus; D: Donor; IFN-γ; Interferon-gamma; NASBA: Nucleic acid sequence-based amplification; QNAT: Quantitative nucleic acid testing; R: Recipient; PCR: Polymerase chain reaction; Ig: Immunoglobulin.

16 h by a centrifugation-amplification technique. CMV can be cultured from any type of sample, but non-tissue samples have low sensitivity. The current guidelines do not recommend viral culture of blood, urine, or oral secretions for diagnosing active CMV infection[45]. Viral culturing of tissue samples has high sensitivity but is not widely available[1] (Tables 2 and 3).

Histopathology

Histopathological diagnosis of CMV infection requires the finding of typical cytopathic changes including foci of flat and swollen cells. Immunohistochemistry of tissue biopsies has high specificity but low sensitivity depending on the distribution of infected tissues. Frozen sections of biopsy samples or preparations made by centrifuging cells onto a slide can be stained with fluorescently-labelled antibodies to early CMV antigens. CMV infection is confirmed by the CMV antigen-positive inclusion bodies (Figure 2) (Tables 2 and 3).

Molecular diagnosis (detection of viral genome)

QNAT of CMV viral load in blood plasma samples has high sensitivity for detection of CMV DNAemia,

Table 3 Uses of available cytomegalovirus assays

Use	Assay
Diagnosis	CMV viral load by plasma QNAT; CMV viral load by real-time PCR assay; pp65 antigen testing; CMV IgG/IgM antibodies
Diagnosis of tissue-invasive CMV disease	Histopathology
Pre-transplant risk stratification	CMV IgG/IgM antibodies
Threshold for initiation of pre-emptive therapy	CMV viral load by plasma QNAT; Quantitative pp65 antigen measurement; NASBA assay
Monitoring or endpoint (prophylaxis, pre-emptive or treatment)	CMV viral load by plasma QNAT; QuantiFERON-CMV assay
Endpoint of treatment of tissue-invasive CMV disease	Histopathology
Prediction of developing CMV disease	QuantiFERON-CMV assay

CMV: Cytomegalovirus; Ig: Immunoglobulin; PCR: Polymerase chain reaction; QNAT: Quantitative nucleic acid testing.

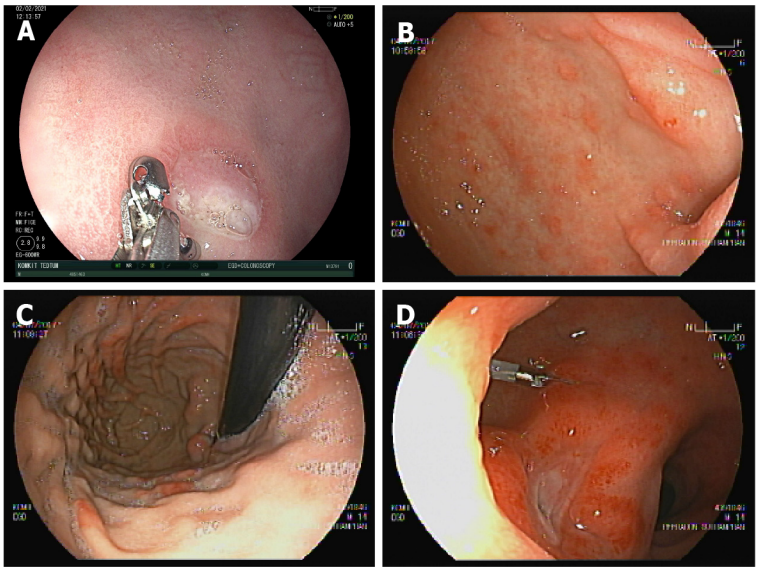

Figure 1 Cytomegalovirus tissue infection of the stomach and duodenum in a 13-mo-old boy and a 14-year-old boy with D^+/R^- serostatus at transplant. Neither patient received antiviral prophylaxis. A and B: The 13-mo-old boy with D^+/R^- serostatus at transplant presented with severe anaemia at 3 mo; C and D: The 14-year-old boy presented with haematemesis at 2 mo after liver transplantation.

especially in D^+/R^- patients, but the sensitivity may be lower in R^+ patients[56,57]. Current guidelines recommend using plasma QNAT for diagnosis, surveillance to guide pre-emptive antiviral treatment, and therapeutic monitoring. The assay must be calibrated according to WHO standards and reported as IU/mL. The absolute value and rate of increase indicated by plasma QNAT are both correlated with the risk of progression to CMV disease and are predictive of CMV disease[28,51]. QNAT may be performed in either plasma or whole blood specimens, but it is recommended to use the same type of specimen and the same type of assay during monitoring of a patient[55]. Tissue QNAT has greater specificity than plasma QNAT, but the available evidence is not adequate to identify a recommended threshold for routine diagnosis[55]. Other specimens, including urine and oral secretions are not recommended for the surveillance and diagnosis of CMV disease by QNAT[55]. In addition to its usefulness in diagnosis, CMV viral load correlates with the duration of treatment and risk of relapse[58].

Other diagnostic assays are real-time PCR and nucleic acid sequence-based amplification (NASBA)[59]. Real-time PCR targets the conserved region of the CMV DNA polymerase gene, regardless of the presence of any viral mutation, allows the quantitative measurement of viral nucleic acids, and is more rapid and precise than conventional quantitative PCR[60]. NASBA detects unspliced viral mRNAs located in a background of DNA and has been studied as an alternative to quantitative antigenaemia as a guide for starting pre-emptive therapy and as a more sensitive assay for the detection of CMV isolation in blood (Tables 2 and 3).

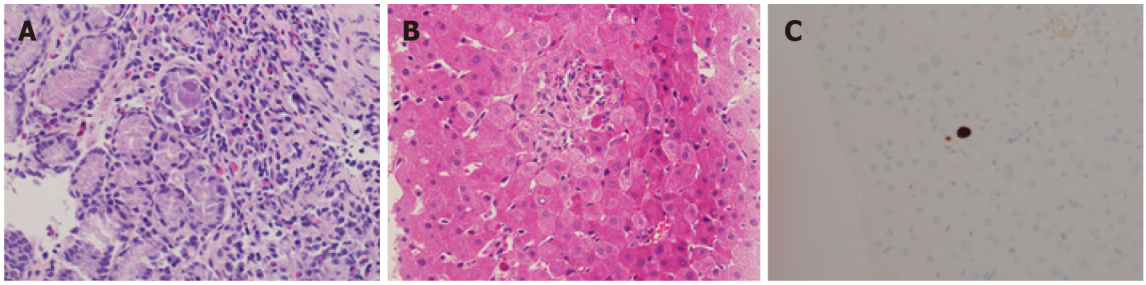

Figure 2 Biopsies showing chronic active gastritis. A: Cytomegalovirus inclusion bodies are seen within mucous cells. The gastric biopsy is characterized by enlarged cells with basophilic nuclear and cytoplasmic inclusions; B: Liver biopsy shows a neutrophilic microabscess surrounding a hepatocyte with granular basophilic cytoplasmic cytomegalovirus inclusions; C: Positive cytomegalovirus immunohistochemistry in liver tissue.

Direct assay of viral antigen

Direct assay of CMV antigen in whole blood or plasma can detect antigenaemia. The pp65 protein antigen is synthesized by the virus in infected host cells, and the sample should be processed within 6 h after collection, as the number of antigen-positive cells significantly decreases with time[61]. Fluorescence-labelled anti-pp65 antibody binds to the pp65 antigen in peripheral blood leucocytes, and the quantitative results are reported as the number of positive cells in 2 × 10^5 peripheral blood leucocytes. False-negative results are usually obtained in patients with neutropenia[62]. In clinical practice, the detection of CMV antigenaemia can diagnose CMV infection and guide the initiation of pre-emptive therapy (Tables 2 and 3).

Serological assay of viral antibodies

CMV infection can also be detected by serological assay of viral antibodies. CMV IgG antibody testing is recommended. Tests for IgG combined with IgM and for IgM exclusively are not recommended because of their low specificity[55]. IgM antibodies can persist for months in patients with a previous primary CMV infection, and even though IgG has better sensitivity and specificity than IgM, the results must be interpreted with caution in patients with past CMV infection. The techniques available currently are complement fixation, enzyme-linked immunosorbent assay (ELISA), anti-complement immunofluorescence, radioimmunoassay, and indirect haemagglutination. The primary clinical use of serologic assays is in the pre-transplant assessment of donor and recipient CMV serostatus (Tables 2 and 3).

Viral cellular response detection

The QuantiFERON-CMV assay is an ELISA that detects of IFN-γ production following stimulation by CMV antigen. The assay reflects cell-mediated immunity by measuring IFN-γ levels following *in vitro* stimulation of CD8+ T cells by CMV peptides. The subsequent incidence of CMV disease in immunocompromised patients is significantly lower among those with a positive result than those with a negative result[21,50,63]. A multicentre cohort study showed that the positive and negative predictive values of the assay were 0.90 and 0.27, respectively[50]. Many assays are in use in some centres for monitoring during prophylaxis or pre-emptive therapy[55] (Tables 2 and 3).

TREATMENT

Early detection of CMV infection is necessary for the management of transplant patients, and reflects the index of suspicion from clinical features of tissue-invasive CMV disease or CMV syndrome and the results of monitoring blood for CMV DNA in asymptomatic CMV infections. A lower total intensity of calcineurin inhibitors is associated with better early CMV DNAemia eradication[64]. Consequently, if significant CMV viraemia or tissue-invasive CMV disease is diagnosed, then reducing current immunosuppressive therapy, especially in those with severe CMV disease or a high viral load, is the priority.

Medication

Specific antiviral drugs against CMV infection are intravenous ganciclovir and oral valganciclovir. If tolerated, oral drugs are preferred for mild to moderate CMV disease and asymptomatic CMV DNAemia because they are associated with shorter hospital stays and fewer complications than intravenous drugs. Oral valganciclovir is preferred to oral ganciclovir because of its better bioavailability[65]. A study found that oral valganciclovir is safe and noninferior compared with intravenous ganciclovir[66], but in life-threatening CMV disease, intravenous ganciclovir is preferred to reach the optimal drug level rapidly. The current guidelines recommend the administration of 5 mg/kg intravenous ganciclovir every 12 h as initial therapy, with dosage adjustments in patients with renal

insufficiency. After the desired clinical response has been achieved, switching to oral therapy may be considered if it is well tolerated[55]. In cases of asymptomatic CMV infection and CMV syndrome, after a duration of treatment of a minimum of 2 wk with clinical resolution and no evidence of CMV DNAemia, eradication is defined as a CMV viral load of < 200 IU/mL in one or two consecutive weekly samples[55]. Patients with tissue-invasive CMV disease usually have minimally detectable or undetectable viraemia; it is not recommended to use CMV PCR to assay serum viral load as a guide for antiviral discontinuation. The decision to discontinue antiviral medication should be based on the clinical response, including the histopathology of the involved tissue. In patients with gastrointestinal CMV disease, clinicians should consider colonoscopy or upper endoscopy with histologic evidence of invasive CMV infection to indicate disease eradication instead of using serum CMV viral load[65].

Monitoring and alternative regimens

During treatment, patient surveillance includes complete blood count for leucopenic side effects, renal function monitoring to guide antiviral dosage adjustment, and weekly quantitative CMV nucleic acid testing to assess medication response. Apart from renal adjustment, lowering the antiviral dosage is not recommended because of concern of treatment failure. Antiviral switching because of leucopenia is considered after discontinuation of other myelosuppressive agents or the addition of granulocyte colony-stimulating factor. The use of foscarnet or cidofovir as an alternative antiviral medication can then be considered[1,55].

Ganciclovir-resistant CMV disease

If the patient's CMV DNAemia remains persistently positive or is recurrently positive despite prolonged antiviral therapy for more than 6 wk of cumulative exposure to ganciclovir or more than 2 wk of ongoing full-dose therapy[55], then antiviral drug resistance testing should be considered. Factors that increase the risk of developing resistant strains include prolonged use of ganciclovir, typically for more than 5 mo, high-risk pairs, especially D^+/R^-, a history of exposure to strongly immunosuppressive agents, or inadequate drug delivery. Paediatric cohort studies have reported an incidence of ganciclovir resistance of approximately 2%-4%, which might have been under-reported[67,68].

Current guidelines recommend medications much like those used in adults[55], but because of a lack of controlled trials, the drug of choice has not yet been identified. Current guidelines include an algorithm to select appropriate medications[55]. The regimen includes the addition or switching of antiviral medication to intravenous foscarnet or a dosage escalation of intravenous ganciclovir. The regimen is then adjusted after genetic testing for antiviral drug resistance. Cidofovir is considered if genetic testing shows resistance to foscarnet. In the case of multidrug resistance, a combination of intravenous antiviral drugs is recommended[55]. The guidelines suggest a combination of intravenous foscarnet and high-dose ganciclovir[55]. Other medications, including brincidofovir, letermovir, and maribavir, are still under clinical study[1,55].

PREVENTION

Pre-organ transplant screening

Pre-organ transplant screening helps to detect patients at risk of CMV disease and who require prophylaxis and patients with clinically significant occult CMV infection requiring pre-emptive therapy. Pre-transplant serostatus screening is thus necessary for risk stratification. The modalities rely on recipient age. Either urine/saliva for CMV shell culture or serum/whole blood for CMV QNAT combined with CMV IgG antibody testing are recommended for recipients younger than 18 mo of age [26]. Single CMV IgG antibody testing is not recommended because maternal CMV IgG antibody can be found in some patients younger than 18 mo of age who acquire passive immunization during the perinatal period. In recipients are older than 18 mo of age, CMV IgG testing alone can be used[27]. If either CMV culture or CMV QNAT is positive, the patient is considered seropositive. However, donors younger than 18 mo of age who are seropositive for CMV IgM are also assumed to be seropositive[51]. As the peak incidence of CMV disease occurs during the first 3 mo after transplantation[8], CMV surveillance with weekly QNAT for the first 12 wk is recommended[55,69].

Pre-emptive therapy

Viral threshold: In pre-emptive therapy, antiviral drugs are provided to asymptomatic patients with evidence of CMV infection. QNAT is the preferred test because of the rapid results with high sensitivity. Patients with a test showing a positive viral load above a clinically significant threshold are given pre-emptive treatment, but there is no universally recommended viral load threshold for management initiation because of a lack of standardized assays[55]. The thresholds are assay- and centre-specific, and it is recommended that each centre establish its own threshold[55]. Paediatric studies at a centre in India used QNAT assays and a cut-off value of 500 copies/mL[8], and a study in Italy used real-time PCR assay of CMV DNA in blood and a cut-off value of 650 copies/mL[70]. pp65 antigenemia has also been

used as a threshold for pre-emptive therapy at many centres. A centre in Japan used a cut-off of 5 pp65-positive cells per 50000 leucocytes to indicate CMV antigenaemia[13].

Medications: Intravenous ganciclovir and oral valganciclovir are recommended for pre-emptive therapy. Oral ganciclovir is less effective than oral valganciclovir. A study reported that despite administration of oral ganciclovir, breakthrough CMV syndrome was observed[71]. In some centres, intravenous ganciclovir is initially given, and switched to oral valganciclovir until the course of the pre-emptive therapy is completed. Intravenous ganciclovir is generally given at 5 mg/kg every 24 h. The recommended valganciclovir dosage is 15 mg/kg once daily for patients who weigh less than 15 kg or 500 mg/m^2 once daily for patients who weight more than 15 kg. The maximum dose is 900 mg/dose once daily[27]. The dosage of valganciclovir is adjusted to both body surface area and kidney function assessed by creatinine clearance.

The optimal duration of intravenous ganciclovir prophylaxis has not been determined, and varies from 14 d to 3 mo and is extended to 6 mo at some centres[51]. The time from transplantation to onset of CMV viraemia or disease was not significantly different in those who received ≤ 14 d or > 14 d of postoperative ganciclovir prophylaxis[26]. The treatment duration for low-risk D^-/R^- patients should be assessed by clinical follow-up. The intermediate-risk group should be treated for 3 mo, and the high-risk group should be treated for 6 mo. Because of the lack of a recommended optimal cut-off duration, the treatment duration can be adjusted according to the physician's judgment. A negative blood CMV viral load in two samples taken 2 wk apart can also be considered a guide for discontinuation of therapy[8]. The efficacy of the pre-emptive protocol has been studied in some trials. In the study by Pappo *et al*[72], liver-transplanted children were given oral valganciclovir 17 mg/kg/d for 3-6 mo, leading to a decrease in the incidence of CMV infection. A study by Ueno *et al*[73], reported that the incidence of CMV infection in patients with 1 year prophylaxis decreased by more than 80.5% compared with a regimen of less than 1 year. The pre-emptive regimen decreased the cost of treating CMV infection and disease[70].

Monitoring

Drug toxicity should be monitored by complete blood counts, kidney function tests, such as blood urea nitrogen and creatinine, and hepatic transaminase enzymes every 1-2 wk in the first month post-transplant and then monthly until completion of prophylaxis.

A study on post-prophylactic delayed-onset CMV disease found that the peak incidence in paediatric patients occurred at about 3 mo after cessation of antiviral prophylaxis following liver transplantation [51]. This finding led to the recommenda-tion of post-prophylaxis surveillance of CMV for at least the first 3 mo of treatment in high- and moderate-risk recipients[27]. The surveillance can be by either quantitative CMV PCR or QNAT monthly for 12 mo post-prophylaxis. Low-risk recipients may not need surveillance; however, if any febrile illnesses occur, quantitative PCR is required regardless of the recipient risk status.

Systemic antiviral prophylaxis: Patients selected for systemic antiviral prophylaxis include those at high risk as D^+/R^- serostatus. Patients with D^-/R^- serostatus may not require prophylaxis, but universal systemic antiviral prophylaxis is given to all patients at some transplant centres regardless of their serostatus.

Medication

The antiviral medications used for prophylaxis include acyclovir, valacyclovir, intravenous or oral ganciclovir, and valganciclovir. Valganciclovir is the most frequently used agent and ganciclovir is more effective than acyclovir in reducing the incidence of CMV disease[74]. Because of the clinical trials with high power, the effectiveness of oral valganciclovir and oral ganciclovir remain controversial. Some studies found that oral valganciclovir contributed to a lower incidence of early-onset CMV disease than oral ganciclovir[75], but valganciclovir has a higher incidence of tissue-invasive and late-onset CMV disease than oral ganciclovir[76]. The duration of systemic prophylaxis in clinical practice is typically 3-6 mo after transplantation. Current guidelines recommend at least 3-6 mo of treatment in children with a serostatus of D^+/R^- and 3-4 mo or 2-4 wk in other groups, with CMV surveillance at the end of therapy [55]. The summary of management for CMV disease was described in Table 4.

CMV vaccination

Several CMV vaccines have been evaluated in clinical trials, but the results were not promising. Poor protection against infection may be a result of the nature of the virus, which can evade and modulate the immune system. The most promising vaccines are derived from viral glycoprotein B, and are progressing to phase II clinical trials. An initial study in children found that the vaccine was safe and effective in developing immunity, with an efficacy of 43%. The vaccine also reduce the duration of treatment in post-solid organ transplant recipients[80]. Virus-like particles consisting of a fusion product of extracellular domain glycoprotein B and vesicular stomatitis virus G-protein induced high titres of neutralizing antibodies[81]. Live-attenuated vaccine has shown a good safety profile, inducing both humoural and cell-mediated immunity, and reducing the incidence of severe infection[82,83]. However, vaccines still fail to prevent infection in seronegative solid organ transplant recipients. A disabled

Table 4 Summary of pre-emptive, prophylaxis and treatment of cytomegalovirus disease in post-liver transplant patients

Condition	Pre-emptive		Prophylaxis	Treatment
Monitoring and endpoint	Monitoring: Weekly or every 2 wk CBC, BUN, Cr, AST, and ALT for first month and then monthly; Monthly CMV QNAT for 12 mo. Endpoint: CMV QNAT for VL negative for two samples 2 wk apart		Monitoring: Weekly CMV QNAT. Endpoint: CMV QNAT for VL negative for two samples 2 wk apart	Monitoring: Weekly CBC, BUN, Cr; Weekly CMV QNAT. Endpoint: CMV syndrome: Clinical resolution; VL less than 200 IU/mL on 1-2 consecutive weeks; Tissue-invasive CMV disease: Clinical resolution; Histologic evidence
Cut-off for start medication	Reference	Verma *et al*[8,14]; Saitoh *et al*[13]; Martín-Gandul *et al*[77]; Atabani *et al*[58]; Griffiths *et al*[78]	-	Kotton *et al*[55]
	Values	Non-specific: VL 500 copies/mL; VL 650 copies/mL; pp65 Ag 5 per 50000 leucocytes. D^+/R^-: Plasma VL 1500 IU/mL. D^+/R^- and R^+: Plasma VL 2275 IU/mL or 2500 copies/mL; Whole blood VL 2520 or 3000 copies/mL. R^+: VL 3983 IU/mL	None (risk donor/recipient pair-based)	VL > 200 IU/mL for 2 consecutive weeks
Duration	Reference	Razonable *et al*[32,38;71]; Razonable[39]; Razonable and Humar[51]; Razonable and Hayden[56]; Razonable[79]; Pappo *et al*[72]; Ueno *et al*[73]; Kotton *et al*[55]	Kotton *et al*[55]	Kotton *et al*[55]
	Values	Non-specific: 14 d to 3 mo; Extended to 6 mo; Extended to 12 mo. High risk: 6 mo. Intermediate risk: 3 mo. Low risk (D^-/R^-): Clinical follow-up	D^+/R^-: 3-6 mo. Others: 3-4 mo or 2-4 wk with CMV surveillance	At least 2 wk
Drug/dose/route	First-line: Ganciclovir (5 mg/kg IV q 24 h); Valganciclovir (< 15 kg: 15 mg/kg/dose po once daily; > 15 kg: 500 mg/m^2/dose po once daily); Maximum dose: 900 mg/dose once daily; Combined ganciclovir then valganciclovir		First-line: Ganciclovir (same dose as pre-emptive); Valganciclovir (same dose as pre-emptive)	First-line: Ganciclovir [5 mg/kg IV q 12 h (+/- with dose adjustment for renal function)]. Second-line (ganciclovir-induced leucopenia): Foscarnet [60 mg/kg IV q 8 h or 90 mg/kg IV q 12 h (+/- with dose adjustment for renal function)]; Cidofovir [5 mg/kg once weekly × 2 doses then every 2 wk (+/- with dose adjustment for renal function)]. For ganciclovir-resistant [Ganciclovir: 7.5-10 mg/kg IV q 12 h (+/- with dose adjustment for renal function). Add or switch to Foscarnet. Switch to Cidofovir

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BUN: Blood urea nitrogen; CBC: Complete blood count; CMV: Cytomegalovirus; Cr: Creatinine; Ig: Immunoglobulin; QNAT: Quantitative nucleic acid testing; VL: Viral load; D: Donor; R: Recipient.

infectious single cycle vaccine induced neutralizing antibodies and cell-mediated immunity against CMV infection in non-human primates and had an acceptable safety profile[83]. Peptide-based, DNA-based, and vector vaccines are currently under investigation in phase I clinical trials[84,85].

New strategies

Currently, a hybrid strategy of systemic antiviral prophylaxis followed by pre-emptive medication is being used at some centres. Universal prophylaxis with intravenous ganciclovir for at least 2 wk followed by intravenous ganciclovir for at least an additional 2 wk as universal pre-emptive therapy or pre-emptive therapy has been used for patients with detectable CMV DNA[26,69]. The regimen is effective for the prevention of tissue-invasive CMV disease[69], and the effectiveness is similar to that of pre-emptive therapy alone. However, the duration of antiviral treatment was significantly shorter with pre-emptive therapy alone[31]. More studies of the effectiveness of hybrid strategy are needed.

CONCLUSION

Infection after liver transplantation is a common, frequently serious complication. CMV infection that increases the mortality of children with liver transplants because of its direct and indirect effects. Preventive interventions include risk stratification prior to liver transplantation and regular monitoring for prompt diagnosis of CMV infection. If CMV infection is detected, prompt treatment can lead to favourable outcomes.

FOOTNOTES

Author contributions: Onpoaree N and Sintusek P reviewed the literature and wrote the manuscript; Sunpavat A interpreted the histopathological data and provided the histopathology images; Sintusek P provided the endoscopic images; and all authors approved the final manuscript.

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MINIREVIEWS

Hepatocellular carcinoma in patients with metabolic dysfunction-associated fatty liver disease: Can we stratify at-risk populations?

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Abstract

Metabolic dysfunction-associated fatty liver disease (MAFLD) is a new nomenclature recently proposed by a panel of international experts so that the entity is defined based on positive criteria and linked to pathogenesis, replacing the traditional non-alcoholic fatty liver disease (NAFLD), a definition based on exclusion criteria. NAFLD/MAFLD is currently the most common form of chronic liver disease worldwide and is a growing risk factor for development of hepatocellular carcinoma (HCC). It is estimated than 25% of the global population have NAFLD and is projected to increase in the next years. Major Scientific Societies agree that surveillance for HCC should be indicated in patients with NAFLD/MAFLD and cirrhosis but differ in non-cirrhotic patients (including those with advanced fibrosis). Several studies have shown that the annual incidence rate of HCC in NAFLD-cirrhosis is greater than 1%, thus surveillance for HCC is cost-effective. Risk factors that increase HCC incidence in these patients are male gender, older age, presence of diabetes and any degree of alcohol consumption. In non-cirrhotic patients, the incidence of HCC is much lower and variable, being a great challenge to stratify the risk of HCC in this group. Furthermore, large epidemiological studies based on the general population have shown that diabetes and obesity significantly increase risk of HCC. Some genetic variants may also play a role modifying the HCC occurrence among patients with NAFLD. The purpose of this review is to discuss the epidemiology, clinical and genetic risk factors that may influence the risk of HCC in NAFLD/MAFLD patients and propose screening strategy to translate into better patient care.

Key Words: Hepatocellular carcinoma; Metabolic dysfunction-associated fatty liver

disease; Nonalcoholic fatty liver disease; Surveillance for hepatocellular carcinoma; Incidence of hepatocellular carcinoma; Nonalcoholic steatohepatitis

Core Tip: Metabolic dysfunction-associated fatty liver disease (MAFLD) affects 25% of general population worldwide. Within that huge number of patients, a minority will progress to cirrhosis, with an annual incidence rate of hepatocellular carcinoma (HCC) > 1%. In them, surveillance for HCC by means of ultrasound with or without alpha-fetoprotein measurement is cost-effective. In patients with MAFLD cirrhosis who are men, older and diabetic, risk is even higher and magnetic resonance imaging might be a better screening test. However, the great challenge is stratifying the HCC risk in patients with MAFLD without cirrhosis. Factors that can help to stratify their risk (genetic, demographic, metabolic, non-invasive fibrosis tests) will be reviewed.

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INTRODUCTION

Metabolic dysfunction-associated fatty liver disease (MAFLD) previously known as non-alcoholic fatty liver disease (NAFLD) represents a condition of excessive accumulation of fat in the liver of people with features of metabolic syndrome regardless of alcohol consumption. Recently, a panel of international experts from 22 countries proposed this new nomenclature assigning the disease a name linked with its pathogenesis to overcome the negative definition originally attributed to NAFLD[1,2]. Definition of NAFLD was based on the presence of steatosis in > 5% of hepatocytes and the exclusion of secondary causes of hepatic fat accumulation such as significant alcohol consumption, long-term use of steatogenic medications, and other known causes of liver disease[3]. On the contrary, definition of MAFLD is based on positive criteria, independently of the presence of other liver diseases. The diagnosis of MAFLD is based on the evidence of liver steatosis, in addition to one of the following three criteria: Overweight/obesity, presence of type 2 diabetes mellitus, or evidence of metabolic dysregulation[1,2]. Fat accumulation in the liver may be shown by histology, imaging (ultrasonography, controlled attenuation parameter by FibroScan®, magnetic resonance imaging–derived proton density fat fraction, computed tomography) or blood biomarkers (fatty liver index).

The Consensus panel further recognized that the exclusion of alcohol intake or hepatitis B virus (HBV) or hepatitis C virus (HCV) infections is no longer a prerequisite for the diagnosis of MAFLD. Patients who meet the diagnostic criteria for MAFLD and have in addition one of these concomitant diseases should be defined as having a dual etiology fatty liver disease[1]. Subsequently, expert panels of the Latin American Association for the Study of the Liver[4] and also from Middle East and north Africa[5] reached consensus to endorse the proposal on the redefinition of fatty liver disease and the new nomenclature (MAFLD).

MAFLD encompass a spectrum of conditions that may be limited to excessive liver fat (simple steatosis) or progress to non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis and to hepatocellular carcinoma (HCC)[3]. MAFLD is mainly driven by hyperalimentation, unhealthy diets, sedentary behavior, leading to central and visceral adiposity, insulin resistance, overweight/obesity and metabolic syndrome. A recent meta-analysis, based on 86 studies from 22 countries estimated that global prevalence of NAFLD is 25.24% (95%CI: 22.10-28.65), peaking at 31.79%% in the Middle East, 30.45% in South America and as low as 13.48% in Africa[6]. The prevalence of NASH in the general population is not well known, since a liver biopsy is required to confirm this condition. It has been estimated that it can range from 1.5% to 6.45%[6]. One of the most worrying aspects of NASH is that modeling-projected prevalence (estimated in eight countries) is to increase by 15%-56% between 2016 and 2030[7]. Other Markov model-based study, but limited to United States, concluded that prevalent NAFLD and NASH cases are forecasted to increase 21% and 63%, respectively between 2015 and 2030, while incidence of decompensated cirrhosis and HCC will increase 168% and 137%, respectively, by 2030[8]. Results of both modeling studies suggest increasing cases of advanced liver disease in the coming years; taken together with an unmet of effective therapeutic approach means that MAFLD will be the leading cause of cirrhosis, liver transplantation and HCC in the next decades[7,8].

Primary liver cancer (PLC) is the sixth more frequent cancer in general population, with more than 900.000 new cases every year worldwide[9], but it ranks as the second one among cancer deaths, because of its bad prognosis[9,10]. Overall survival of patients is very low and the incident cases/deaths ratio is 0.9. HCC accounts more than 90% of all PLCs. The poor prognosis of HCC is mostly due to the fact that it usually emerges in patients with chronic liver disease and advanced fibrosis or cirrhosis. When patients are diagnosed by symptoms, they usually have large or multiple tumors. Then, the impaired liver function reserve due to cirrhosis prevents curative treatment by surgical resection; or the tumor extension is beyond the Milan criteria, generally accepted limit for liver transplant treatment. The only way to improve survival in patients with HCC is to make diagnosis in an asymptomatic stage, through a surveillance program. This is possible because we know who are the patients at risk of developing HCC. Main risk factors for HCC include chronic infections for HBV and HCV, alcoholic liver disease (ALD) and MAFLD, with some geographic differences. In East Asia and Africa, hepatitis B is the first etiology being aflatoxin contamination a cofactor in some regions whereas in western countries, hepatitis C and alcoholic cirrhosis are the main causes of HCC. NASH and cryptogenic cirrhosis (probably "burnt-out" NASH) have historically ranked third in series from the United States [11] and Latin America[12]. However, changes are currently taking place and 2 recent studies agreed that NASH is the most rapidly growing indication for liver transplant among patients with HCC in United States[13,14]. According to the most recently published study, NASH now accounts for 18% of all HCC cases who are listed, meaning an 8-fold growth since 2002, and being the second most frequent cause, after hepatitis C[14].

Regarding which MAFLD patients to include in surveillance protocols for HCC, there are some discrepancies between the recommendations of the main Scientific Societies. Based on historical modeling studies performed in hypothetical patients with chronic hepatitis C, surveillance for HCC is assumed to be cost-effective when the annual incidence rate is equal to or greater than 1.5%. Therefore, all the recommendations agree that patients with MAFLD and cirrhosis should be included in that protocols; but they differ in respect to patients with advanced fibrosis (bridging fibrosis or F3 fibrosis). The last "Practice Guidance on Management of HCC" by the American Association for the Study of Liver Diseases states that "the risk of HCC is significantly lower in those with HCV or NAFLD without cirrhosis compared to those with cirrhosis, surveillance not being recommended for the former"[15]. The last Guidelines on "Management of hepatocellular carcinoma" by the European Association for the Study of the Liver affirms that "the role of surveillance for patients with NAFLD without cirrhosis is unclear (evidence low)"[10]; and in a table that lists "Categories of adult patients in whom surveillance is recommended", it states "Non-cirrhotic F3 patients, regardless of etiology may be considered for surveillance based on an individual risk assessment (evidence low; recommendation weak)[10]. On the contrary, the "American Gastroenterological Association Clinical Practice Update on Screening and Surveillance for HCC in Patients with Nonalcoholic Fatty Liver Disease", in its second Best Practice Advice, states that "patients with NAFLD with non-invasive markers showing evidence of advanced liver fibrosis or cirrhosis should be considered for HCC screening"[16].

It is worth mentioning that, unlike what happens with patients with hepatitis C or ALD, where HCC arises in cirrhotic liver in approximately 90% of cases; several case-control studies assessing characteristics of HCC in NAFLD patients have shown a significantly lower percentage of cirrhosis in NAFLD cases than in other etiology controls. The prevalence of cirrhosis among MAFLD cases with HCC has ranged from as low as 51%[17], 52.8%[18], 53.8%[19] to 77.2%[20]. Although fibrosis, cirrhosis and HCC appear to be the generic responses to any kind of chronic liver injury, the time-course and sequence of events appear to be even less predictable in MAFLD than other liver diseases. Nevertheless, in large population-based studies performed in non-selected NAFLD patients, the incidence rate of HCC is extremely low[21]. Although occasionally some case of HCC may appear in a patient with NAFLD and non-advanced fibrosis, it is generally considered that the risk is too low to justify the use of surveillance in all comers.

In this review, we will update the knowledge on epidemiological aspects of HCC in patients with MAFLD and analyze which are the factors that increase the risk between patients with and without cirrhosis. To address this, we will answer the following questions: What is the incidence of HCC in MAFLD, with and without cirrhosis? What are the clinical risk factors of HCC in MAFLD? What are the genetic phenotypes associated with HCC in MAFLD? Which are the differences between HCC diagnosed in patients with MAFLD compared to other etiologies? How can we "best screen" HCC in MAFLD at 2021?

It is important to recognize that, although the acronyms NAFLD and MAFLD refer to the same disease and will be used interchangeably in this review, all available information comes from studies previously conducted in patients with NAFLD.

INCIDENCE RATE OF HCC AND FACTORS THAT INCREASE THE RISK IN PATIENTS WITH MAFLD AND CIRRHOSIS

Several studies have compared the incidence rate of HCC between cohorts of patients with advanced

fibrosis/cirrhosis due to NAFLD or cryptogenic cirrhosis with HCV-related advanced fibrosis/cirrhosis cohort[22-27]. The Table 1 shows the main results of them.

One of the most representative studies within this group is that of Ascha and his colleagues at Cleveland Clinic[26]. Their primary objectives were to estimate the incidence of HCC between patients with NASH-cirrhosis and HCV-cirrhosis; and secondary, to identify risk factors for the occurrence of HCC. They reviewed data from 195 patients with NASH-cirrhosis and 315 patients with HCV-cirrhosis who had been referred for liver transplantation. The median age was significantly higher in patients with NASH than HCV (56.6 and 48.2 years, respectively, $P < 0.001$); but significantly fewer NASH patients were men compared with HCV patients (44.1% *vs* 76.5%, respectively; $P < 0.001$]. During a median follow-up of 3.2 years, HCC was diagnosed in 25 of 195 (12.8%) NASH-cirrhotic patients compared with 64/315 (20.3%) HCV-cirrhotic patients ($P = 0.03$). The annual cumulative incidence of HCC in patients with NASH cirrhosis was 2.6% compared with 4.0% for patients with HCV cirrhosis ($P = 0.09$)[26]. In multivariate analysis, they observed that older age [hazard ratio (HR), 1.08 (95%CI: 1.02-1.1); $P = 0.006$] and any alcohol consumption [HR, 3.8 (95%CI: 1.6-8.9); $P = 0.002$] were the only factors independently associated with development of HCC in the population with NASH-cirrhosis[26].

In other study, Sanyal *et al*[23] prospectively compared outcomes between 152 patients with NASH-related cirrhosis matched with 150 patients with HCV-cirrhosis[23]. Baseline characteristics of both groups were comparable in respect to liver function tests, Child-Pugh and MELD scores though patients with NASH had a higher frequency of metabolic syndrome features, such as diabetes or arterial hypertension. Over a 10-year follow-up, patients with NASH-cirrhosis had a significantly higher cardiac mortality than HCV cirrhotic patients ($P = 0.03$). By contrast, patients with HCV-cirrhosis had a significantly higher rate of general mortality ($P = 0.04$), development of ascites ($P < 0.006$) or progression to liver decompensation than patients with NASH-cirrhosis. In addition, patients with HCV had a significantly higher risk of developing HCC than NASH patients [17% (25/147) *vs* 6.7% (10/149), respectively, $P < 0.01$] (subtracting 3 patients from each group who had HCC at baseline). In Sanyal study, however, no HCC related risk factors were identified[23].

A large prospective, multicenter, international study compared the course of 247 patients with NAFLD and biopsy-proven advanced fibrosis or cirrhosis with that of 264 patients with hepatitis C and similar fibrosis stages (F3-F4)[27]. Patients with NAFLD were older (54.7 years *vs* 48.3 years, respectively, $P < 0.001$) with a higher percentage of females than HCV patients (60.3% *vs* 35.2%, respectively, $P < 0.001$). Mean follow-up were 85.6 and 74.9 mo in NAFLD group compared to HCV, respectively. After adjusting for baseline differences in age and gender, the cumulative incidence of liver-related complications was lower in the NAFLD than in the HCV cohort ($P = 0.03$), including incident HCC (6 *vs* 18 cases; $P = 0.03$). Among the 247 patients with NAFLD, 118 (47.8%) had F3 fibrosis and 129 (52.2%), Child A cirrhosis at the baseline. All cases of HCC in NAFLD group occurred among patients with cirrhosis (6 out of 129, 4.6%). In this study, no predictive factors for the development of HCC were identified[27].

A study from Japan retrospectively compared outcomes between 68 patients with NASH-cirrhosis and 69 matched HCV-cirrhosis patients[25]. The 5-year occurrence rate of HCC was 11.3% in the NASH group *vs* 30.5% in the HCV group. HCC incidence showed a slightly higher rate in the HCV group, but the difference was not significant ($P = 0.185$). An important finding of this study was that HCC was the leading cause of death in both groups (9 deaths in the NASH group and 19 in the HCV group)[25]. Moreover, in multivariate analysis, risk factors for the HCC occurrence were not identified.

Another study from France, retrospectively analyzed survival and cirrhosis complications in 27 overweight patients with cryptogenic cirrhosis and 391 patients with HCV-cirrhosis[22]. Patients with cryptogenic cirrhosis plus obesity were older than HCV cirrhotic patients (62.1 and 53.7 years, respectively; $P < 0.001$) but the sex ratio (male/female) was not significantly different between both groups. To avoid bias in the results based by the older age of cryptogenic cirrhosis plus obesity patients, a further analysis matched by age and low or no alcohol consumption compared outcomes between them and 85 HCV-cirrhotic patients[22]. The French study showed a slightly higher occurrence of HCC in the group with cryptogenic cirrhosis plus obesity than HCV cirrhosis group (30% *vs* 21%, respectively) though this tendency was not statistically significant[22].

In summary, this set of studies comparing outcomes between patients with NAFLD and hepatitis C and advanced fibrosis/cirrhosis mostly showed a slightly lower incidence of HCC in patients with NAFLD than in patients with HCV[23-27]. Only one of them[26] had the primary objective of investigating the incidence of HCC in NASH cirrhosis and found an annual incidence rate of 2.6%; while the others analyzed the appearance of cirrhosis complications in general. The yield of HCC incident cases in the individual studies was not as high and this may have prevented for identification of independent risk factors for HCC development at the multivariate analysis. Ascha *et al*[26] found that older age and any alcohol consumption were independent predictors of HCC occurrence[26]. To be noted, excessive alcohol intake is excluded (by former definition) in NAFLD patient groups but alcohol consumption may have played a role in HCV patient cohorts. This issue may not have been analyzed in detail in retrospective studies.

A large retrospective study by Kanwal *et al*[28] was conducted analyzing the Veterans Health Administration (VHA) database in United States, with the objective of estimating the risk of incident HCC among patients with MAFLD[28]. They compared 296.707 NAFLD patients with 296.707 matched

Table 1 Studies that evaluated hepatocellular carcinoma risk in a cohort with cirrhosis or advanced fibrosis due to nonalcoholic steatohepatitis or cryptogenic cirrhosis (presumptively nonalcoholic steatohepatitis-related) and in a comparison cohort with hepatitis C virus-related cirrhosis or advanced fibrosis

Ref.	*n*	Age (yr)	Male gender (%)	HCC incidence	*P* value
Ratziu *et al*[22]	27 CC-O	62.1 ± 10.6	(M/F) 1.7	8/27 (29.6%)	NS
	85 HCV	62.1 ± 10.6	(M/F) 1.7	18/85 (21%)	
Sanyal *et al*[23]	152 NASH	54.7 ± 11.6	39.7	10/149 (6.7%)	NS
	150 HCV	48.3 ± 11.3	64.8	25/147 (17%)	
Kojima *et al*[24]	24 CC	58.2 ± 10.6	NA	9/24 (37.5%)	$P < 0.01$
	48 HCV	58.7 ± 8.1	NA	36/48 (75%)	
Yatsuji *et al*[25]	68 NASH	62.7 ± 13.2	43	5-yr 11.3%	NS
	69 HCV	61.3 ± 5.8	43	5-yr 30.5%	
Ascha *et al*[26]	195 NASH	56.6	44.1	Annual cumulative 2.6%	$P = 0.09$
	315 HCV	48.2	76.5	Annual cumulative 4.0%	
Bhala *et al*[27]	247 NAFLD	54.7	39.5	6/247 (2.4%)	$P = 0.03$
	264 HCV	48.3	67.5	18/264 (6.8%)	

NAFLD: Nonalcoholic fatty liver disease; HCC: Hepatocellular carcinoma; NASH: Nonalcoholic steatohepatitis; HCV: Hepatitis C virus; CC-O: Overweight patients with cryptogenic cirrhosis; (M/F): Male/female ratio; NS: Non-significant; CC: Cryptogenic cirrhosis.

controls. Patients with NAFLD-cirrhosis had an annual incidence of HCC of 10.6/1000 persons-year (PYs). Among patients with cirrhosis, HCC incidence ranged from 1.6 to 23.7/1000 PYs, depending on other demographic characteristics like male gender, age older than 65 year and Hispanic race. The annual incidence *per* 1000 PYs (95%CI) was 11.05 (9.83–12.39) in men *vs* 1.62 (0.20–5.85) in women with cirrhosis; or 13.43 (10.82–16.49) in older than 65 years *vs* 9.74 (8.46–11.17) in younger than 65 years; or 23.76 (12.27–41.50) in Hispanics *vs* 11.94 (9.11–15.37) in Whites. The risk of HCC was the highest in older Hispanics with cirrhosis[28].

In a recently published paper, investigators from Mayo Clinic at Rochester, United States, assessed, as primary aim, the association of diabetes and HCC in patients with NASH and cirrhosis. Secondary aim was to analyze the association between other metabolic risk factors and HCC[29]. The retrospective cohort included 354 patients who did not have HCC at baseline. Mean age was 61.5 years and 41% were male. Diabetes was present in 253 (71%) patients at baseline. Follow-up duration was a median of 46 and 47 mo for diabetics and nondiabetics, respectively. HCC was diagnosed in 30 cases, 27 out of 253 patients with diabetes and 3 out of 101 patients without diabetes. The 5-year cumulative incidence rate of HCC was 7.8% (95%CI: 5.1-11.8) in the total population: 10.2% (95%CI: 6.6-15.5) for diabetics *vs* 1.7% (95%CI: 2.4-11.5) for nondiabetics[29]. In multivariable analysis, 3 factors were identified as independent predictors of HCC development: Older age, serum albumin levels, and diabetes[29]. In addition, authors revised the United Network for Organ Sharing (UNOS)/Organ Procurement and Transplantation Network (OPTN) registry data to identify all adult patients who were registered on the waitlist for liver transplant in United States with diagnosis of NASH [or cryptogenic cirrhosis plus body mass index (BMI) ≥ 30] between 2003 and 2016. There were 6.630 patients with diagnosis of cirrhosis due to NAFLD, of whom 58% had diabetes. The 5-year cumulative incidence rate of HCC was 5.6% (95%CI: 4.9-6.3). Multivariate analysis showed that age, male sex, diabetes and low serum albumin level were independent risk factors for developing HCC[29].

Corey *et al*[30] performed a retrospective case-control study of patients with cirrhosis due to NAFLD followed-up in 5 academic centers from United States[30]. They evaluated 244 patients with NAFLD cirrhosis: 94 cases with HCC and 150 controls without HCC. Mean age was 59 years, male sex in 54.7%. On multivariate analysis, the strongest association with presence of HCC was male gender (OR = 4.3, 95%CI: 1.83-10.3, $P = 0.001$). Age was associated with HCC as well (OR = 1.082, 95%CI: 1.03-1.13, $P = 0.001$); and Hispanic ethnicity (contrary to what was described in the VHA study[28]) was associated with a decreased prevalence of HCC (OR = 0.3, 95%CI: 0.09-0.994, $P = 0.048$)[30].

CHARACTERISTICS OF PATIENTS WITH MAFLD WHO DEVELOP HCC

Several studies have shown that patients with HCC emerging in MAFLD have some significant

differences from patients with HCC arising in other chronic liver diseases. Firstly, patients with NAFLD plus HCC use to be older than other etiologies HCC[11,20,22,31]. Secondly, tumors tend to be larger as a result of a higher percentage of NAFLD patients being diagnosed outside of a surveillance program[19,20,32]. As a consequence, some studies showed a shorter survival in patients with NAFLD and HCC compared to controls with other etiologies of cirrhosis and HCC[11,22]. However, other studies have found a similar[19,20] or longer[31] overall survival in NAFLD patients with HCC even though they had a more advanced stage. This could be due to the third and very important difference: A lower percentage of cirrhosis in MAFLD patients who develop HCC compared to their controls with hepatitis B or C or alcoholic liver disease and HCC[17-20,31,33]; giving them greater access to surgical resections of tumors. In fact, a recently published review evaluated the outcomes of patients with NAFLD and HCC who underwent surgical resection, finding that HCC occurs frequently in non-cirrhotic livers. Authors stated that all the papers showed a better overall survival after surgery in patients with NAFLD compared to other etiologies[34].

The Table 2 shows studies assessing presence of cirrhosis in patients with HCC due to NAFLD *vs* other etiologies.

In summary, the incidence of HCC in patients with MAFLD related cirrhosis show a rate that is above the accepted threshold as cost-effective to indicate surveillance protocols. Among the predictors of increased risk of developing HCC, several studies coincided in favor of the presence of male gender, older age and type 2 diabetes as independently significant risk factors. Also, we would like to point out the study by Ascha *et al*[26] (vide supra) where any degree of alcohol consumption (in patients who by the former definition do not have a significant intake) may increase the risk of HCC occurrence.

INCIDENCE RATE OF HCC AND FACTORS THAT INCREASE THE RISK IN PATIENTS WITH MAFLD WITHOUT CIRRHOSIS

In the previously mentioned retrospective study based on the VHA database (vide supra), analyzing more than 290.000 NAFLD patients and more than 290.000 matched controls, only 0.4% of patients had a diagnosis of cirrhosis at baseline and other 1.4% were confirmed as having cirrhosis later during the study. Mean follow-up was approximately 9 years in both groups. The annual HCC incidence rate was estimated in 0.21/1000 PYs (95%CI: 0.19-0.22) for NAFLD patients; significantly higher than that found in controls, 0.02/1000 PYs (95%CI: 0.02-0.03). NAFLD was associated with a 7.6-fold higher risk of HCC, after adjusting for race and metabolic syndrome features. Multivariate analysis showed that factors that significantly increase the risk of HCC among NAFLD patients are presence of diabetes [adj. HR 3.03 (95%CI: 2.52–3.64), $P < 0.0001$], age ≥ 65 years [adj. HR 1.83 (95%CI: 1.53–2.18), $P < 0.0001$] and Hispanic ethnicity [adj. HR 1.59 (95%CI: 1.14–2.20), $P = 0.005$][28].

Studies based on the general population that evaluate incidence rate of HCC in patients with NAFLD usually show a fairly low risk. In an elegant study from Taiwan, Lee *et al*[35] using Taiwan's National Health Insurance Research Database, evaluated the HCC incidence rate of NAFLD cohort comparing with general population. They recruited 18.080 patients with NAFLD, with a median follow-up of 6.32 years. The 10-year cumulative incidence of HCC was 2.73% (95%CI: 1.69–3.76%) in the total cohort[35]. Multivariate analysis verified that elevated alanine aminotransferase (ALT) was independently associated with an increased HCC risk [HR 6.80, (95%CI: 3.0-15.42), $P < 0.001$]. Another independent risk factor identified was age [HR 1.08 *per* year, (95%CI: 1.05–1.11)]; and statin use was independently associated with a reduction in HCC risk [HR 0.29, (95%CI: 0.12–0.68)]. By combining 2 independent risk factors, the risk of HCC can be better stratified: 10-year cumulative HCC incidence was highest in older (age > 55 years) patients with elevated ALT (12.41%, 95%CI: 5.99–18.83%), but lowest in younger patients without ALT elevation (0.36%, 95%CI: 0–1.08%)[35].

In another large study based on the general population, data were extracted from four European primary care databases (United Kingdom, Netherlands, Italy and Spain)[36]. Subjects who had a recorded diagnosis of NAFLD or NASH were analyzed up for incident cirrhosis and HCC diagnoses and each NAFLD patient was matched up to 100 controls by practice site, gender and age. Among 18.782.281 adults, 136.703 patients with coded NAFLD/NASH were identified. Hazard ratio for HCC in patients compared to controls was 3.51 (95%CI: 1.72–7.16). The strongest independent predictor of a diagnosis of HCC or cirrhosis was baseline presence of diabetes, which doubled the risk of developing these outcomes (HR 2.3, 95%CI: 1.9–2.78)[36].

The study by Adams and colleagues at Mayo Clinic, based on the general population using the Rochester Epidemiology Project, showed that patients with NAFLD have a lower survival than the control population[37]. They identified 420 patients diagnosed with NAFLD (mostly by imaging methods) and compared their overall survival and liver morbidity with the general Minnesota population of the same age and sex. In a mean follow-up of 7.6 years, 53 of 420 (12.6%) patients died. Standardized mortality ratio of NAFLD cohort was 1.34 (95%CI: 1.003–1.76; $P < 0.03$). Independent predictors of mortality were age, impaired fasting glucose and cirrhosis (HR, 3.1, 95%CI: 1.2–7.8)[37]. Only 21 (5%) patients were diagnosed with cirrhosis during this relatively short period of time, and 2 developed HCC. Even so, liver disease was the third leading cause of death among NAFLD patients as

Table 2 Studies that analyzed the prevalence of cirrhosis among patients with nonalcoholic fatty liver disease-related hepatocellular carcinoma and in controls with other etiologies-related hepatocellular carcinoma

Ref.	*n*	Prevalence of cirrhosis (in percentage)
Ertle *et al*[18]	36 NAFLD	52.8
	35 HCV	94.3 in HCV
	29 HBV	93.1 in HBV
	19 ALD	94.7 in ALD
Reddy *et al*[31]	52 NAFLD	73.1
	162 HCV/ALD	93.8
Dyson *et al*[20]	136 NAFLD	77.2
	178 ALD	100 in ALD
	65 HCV	96.9 in HCV
	29 HBV	82.7 in HBV
Mittal *et al*[33]	107 NAFLD	65.4
	1133 ALD	88.9 in ALD
	952 HCV	91.1 in HCV
	65 HBV	92.3 in HBV
Piscaglia *et al*[19]	145 NAFLD	53.8
	611 HCV	97.2 in HCV
Yasui *et al*[17]	87	51
	No control group	NA

NAFLD: Nonalcoholic fatty liver disease; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HBV: Hepatitis B virus; ALD: Alcoholic liver disease; NA: Not available.

compared with the 13rd leading cause of death in control population[37].

Studies conducted in hospitals (clinic-based studies) presumably include "sicker" patients, in whom the risk of outcomes such as cirrhosis or HCC might be increased. However, in the follow-up of unselected NAFLD patients enrolled from an ultrasound (US) diagnosis, the incidence of HCC may continue to be extremely low. In a Japanese retrospective cohort study, 6.508 patients with NAFLD diagnosed by abdominal US were followed-up for 5.6 years. The cumulative rates of HCC were 0.02% at year 4, 0.19% at year 8, and 0.51% at year 12[38]. The annual rate of incident HCC was 0.043%. In this study, multivariate analysis identified 4 independent risk factors for developing HCC: Serum aspartate aminotransferase (AST) level ≥ 40 IU/L [HR 8.20; (95%CI: 2.56– 26.26) $P < 0.001$]; platelet count < 150 × 10^3/μL [HR 7.19; (95%CI: 2.26–23.26) $P = 0.001$]; age ≥ 60 years [HR 4.27; (95%CI: 1.30–14.01) $P = 0.017$] and diabetes [HR 3.21; (95%CI: 1.09–9.50) $P = 0.035$][38].

In another large retrospective study based on the VHA database, Kanwal *et al*[39] evaluated the independent and joint effects of different metabolic traits (diabetes, hypertension, dyslipidemia and obesity) on the risk of developing cirrhosis and HCC[39]. The cohort consisted of 271.906 patients with NAFLD who did not have cirrhosis or HCC at baseline, mean age was 55.5 ± 12.8 years, 94.3% were men, 28.7% had diabetes, 70.3% had arterial hypertension, mean BMI was 31.6 ± 5.6. During a mean follow-up of 9.3 years, 22.794 patients (8.4%) progressed to cirrhosis whereas HCC was diagnosed in 253 patients. Diabetes was the only factor independently associated with the risk of HCC by the multivariate analysis, the risk of HCC was nearly 2.8-fold higher than those without diabetes (adjusted HR = 2.77, 95%CI: 2.03-3.77). Obesity and dyslipidemia were associated with 31% increase in HCC risk. However, these associations, like hypertension, did not reach statistical significance[39].

In another clinic-based Japanese, retrospective study, 1600 older than 60 years NAFLD patients (diagnosed by US) and 1600 older than 60 years matched HCV patients were enrolled with the aim of investigating the cumulative incidence of malignant diseases, including HCC[40]. At the baseline, there were several significant differences between both groups. Metabolic parameters such as triglycerides, total cholesterol, fasting plasma glucose were more elevated in NAFLD patients, but AST, ALT, alpha-fetoprotein (AFP) were more elevated and platelet count more decreased in hepatitis C patients[40]. No data was described on the liver fibrosis stages of the patients. Mean observation period was 8.2 years in both groups. Cumulative development rate of malignant diseases at the 10th year was 13.9% in the

NAFLD group and 28.2% in the HCV group by Kaplan–Meier method (risk ratio 2.27; $P < 0.001$). However, the incidence rate of HCC was clearly pronounced in the HCV cohort, where 267 HCC cases were diagnosed (20.86/1000 PY) compared to 10 HCC cases diagnosed in the NAFLD group (0.78/1000 PY) ($P < 0.001$)[40]. In the NAFLD group, multivariate analysis showed that independent predictors of malignancies were age of ≥ 70 years (HR 2.10; 95%CI: 1.38–3.17; $P < 0.001$), current smoking (HR 1.64; 95%CI: 1.18–2.27; $P = 0.003$), and elevated glucose level (HR 1.32; 95%CI: 1.08–1.61; $P = 0.007$)[40].

Dam-Larsen *et al*[41,42] published 2 papers examining the long-term prognosis of 2 cohorts of patients, one with nonalcoholic fatty liver (NAFL) and the other, with alcoholic fatty liver (AFL)[41,42]. In the first study, they evaluated the risk of development of cirrhosis and death in 215 patients (109 with NAFL and 106 with AFL) who had underwent a liver biopsy. All the patients had biopsy-proven single steatosis, without NASH. During a median follow up time of 16.7 years in the NAFL and 9.2 years in the AFL group, only one NAFL patient developed cirrhosis compared with 22 patients in the alcoholic group[41]. Survival estimates in NAFL group were not different from the Danish population. In the last study, the aim was to conduct an extended, clinical follow-up in both NAFL and AFL patients, to analyze for potential risk factors for survival and development of cirrhosis, and to describe the causes of death[42]. This second analysis enrolled 170 patients with NAFL and 247 patients with AFL whose liver biopsies had been taken between the years 1976 and 1987. All surviving patients were contacted in 2003 and invited to attend a clinical follow up visit. Median follow-up times in the whole cohort were 20.7 years and 12.8 years in the NAFL and AFL groups, respectively. Two (1.2%) patients with NAFL and 54 (22%) with AFL, respectively, were diagnosed as having cirrhosis during follow-up. Forty-eight NAFL patients died during the study period and one of them died from cirrhosis. Within the AFL group, 188 patients died, 32 of them (17%) from cirrhosis. Regarding HCC as a cause of death, there was no cases in NAFLD group and one in AFL group[42].

Many studies have been published attempting to assess the risk of HCC or other liver complications in patients with non-cirrhotic NAFLD, but they have many limitations and weaknesses. Most of them were retrospective and heterogeneous in terms of the inclusion criteria; did not have data on liver fibrosis stages; or they had too short a follow-up to assess hard outcomes such as HCC or complications of cirrhosis. In addition, most of them had relatively few cases of HCC diagnosed and multivariate analysis trying to identify risk factors were powerless. Therefore, it is difficult to draw conclusions or make recommendations on in whom to indicate surveillance for HCC in patients with MAFLD, especially when there is no information on liver fibrosis. Table 3 summarizes some studies that analyzed the incidence rate of HCC in patients with NAFLD without cirrhosis and which were the independent risk factors found in the multivariate analysis.

At the same time, it is important to note that large epidemiological studies carried out in the general population have shown a significant association between the presence of diabetes, or obesity and even metabolic syndrome and PLC.

DIABETES MELLITUS, OBESITY, METABOLIC SYNDROME AND RISK OF PLC

The association of diabetes and PLC has been established for many years. A Swedish population-based cohort study analyzed the risk of developing PLC and biliary tract cancers among 153.852 patients with diabetes, identifying incident cases of cancer during follow-up through the Swedish Cancer Registry [43]. The incidence of PLC was increased fourfold (standardized incidence ratio = 4.1; 95%CI: 3.8-4.5). Even after excluding diabetic patients with concomitant conditions that predispose to HCC, such as alcoholism, cirrhosis, and hepatitis, it was observed an excess risk of approximately threefold[43].

El–Serag *et al*[44] identified all patients with a hospital discharge diagnosed of diabetes between 1985 and 1990 using the records of VHA and assigned randomly 3 controls for every patient, excluding those with concomitant liver disease[44]. The study cohort included 173.643 patients with diabetes and 650.620 controls without diabetes, followed through 2000 for the occurrence of NAFLD related cirrhosis or HCC. Diabetes was associated with 2-fold increase for HCC (HR 2.16, 95%CI: 1.86-2.52, $P < 0.0001$), independently of alcoholic liver disease, viral hepatitis, or demographic features[44].

A multicenter Italian hospital-based study also found that body mass index ≥ 30 kg/m^2 and diabetes mellitus (OR 3.7, 95%CI: 1.7–8.4) were associated to HCC risk[45]; and these associations persisted among subjects without HBV and/or HCV infection[45].

After many cohort studies suggested a strong association between type 2 diabetes mellitus and HCC, a systematic review and meta-analysis was performed, including 25 cohort studies[46]. Diabetes mellitus was associated with an increased incidence of HCC [summary relative risks (SRR) = 2.01, 95%CI: 1.61–2.51]. Increased incidence of HCC in patients with diabetes was independent of geographic location, alcohol consumption, history of cirrhosis, or infections with HBV or HCV[46].

Multiple studies have suggested that metformin, a first-line diabetes medication, may reduce the incidence of HCC and other cancers. Although the mechanism is not well understood, this was initially shown in animal models of HCC and then assessed in many human studies. A recent meta-analysis of 19 studies involving 550.882 diabetic patients concluded that metformin use reduced the liver cancer incidence by 48% (OR 0.52; 95%CI: 0.40–0.68) compared with nonusers[47]. The association remained

Table 3 Incidence rate of hepatocellular carcinoma and independent risk factors among patients with non-alcoholic fatty liver disease/metabolic-dysfunction associated fatty liver disease without cirrhosis

Ref.	Population studied	*n*	Mean follow-up (yr)	Incidence rate of HCC	Independent risk factors
Kanwal *et al* [28]	VHA database (United States)	295.623	9.0 ± 2.2	0.08/1000 person-years	Male gender; Age > 65 yr; hispanics
Lee *et al*[35]	General population-based study (Taiwan of China)	18.081	Median 6.32	10-yr cumulative incidence 2.73% (95%CI: 1.69–3.76)	Age > 55 yr; elevated ALT
Alexander *et al* [36]	General population-based study (Europe)	136.703	Median 3.3	0.3/1000 person-years	Diabetes
Kawamura *et al* [38]	Clinic-based study (Japan)	6.508	Median 5.6	Annual incidence 0.043%	AST ≥ 40 IU/L; platelet count < 150 × 10^3 /μL; age > 60 yr; diabetes
Arase *et al*[40]	Clinic-based study (Japan)	1.600	8.2	0.78/1000 person-years	Age > 70 yr; smoking; elevated glucose level
Kanwal *et al* [39]	VHA database (United States)	271.906	9.3 ± 2.7	253 cases[1]	Diabetes

[1]Incidence rate of hepatocellular carcinoma was not calculated.

HCC: Hepatocellular carcinoma; VHA: Veterans Health Administration; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

after adjusting for hepatitis B/C virus infection, cirrhosis, obesity, behavioral factors, and time-related bias. Sensitivity analysis showed that the beneficial effect of metformin was observed in 10 cohort studies and in 9 case-control studies but not when 2 randomized controlled trials were considered separately (they were probably underpowered or with short period of follow-up)[47]. To avoid time-related biases, a propensity score-matched retrospective cohort was constructed enrolling 84.434 veterans newly prescribed metformin or a sulfonylurea as monotherapy (42.217 new metformin users and 42.217 matched-new sulfonylurea users. Metformin treatment was associated with a reduction in liver cancer [adj. HR 0.44, (95%CI: 0.31-0.64)] compared to sulfonylurea therapy[48]. In subgroup analysis, metformin compared to sulfonylurea was also associated with lower liver cancer incidence in both patients with and without baseline cirrhosis and when the cohort was stratified by statin use at baseline[48].

At the same time, many cohort studies have shown association between overweight, obesity and risk of liver cancer. Already in 2007, a meta-analysis including 10 studies with 6.042 cases, concluded that, compared with normal weight individuals, the SRR of liver cancer was 1.89 (95%CI: 1.51–2.36) for those with obesity[49]. Subsequently, more case-control studies were published and the meta-analyzes were updated. One of them, published in 2012, included 26 prospective studies and more than 25.000 PLC cases. Obesity was associated with an increased risk of PLC (SRRs 1.83, 95%CI: 1.59–2.11)[50], and this association was even further in obese males than obese females (P = 0.027). Subgroup analyses revealed that positive associations were independent of geographic locations, alcohol consumption, history of diabetes or infections with HBV and/or HCV[50]. Therefore, body of evidence suggests that obesity increases the risk of HCC, that is approximately twice that of normal weight individuals. However, it is still uncertain whether there is a gender difference in the association between obesity and PLC. A new meta-analysis was conducted to quantitatively and precisely evaluate the gender difference in that association[51]. The results showed increased relative risks (RR) of HCC incidence for obese men than women (RR 2.04, 95%CI: 1.70–2.44 *vs* RR 1.56, 95%CI: 1.37–1.78, respectively, P = 0.02)[51]. Furthermore, the RR's of HCC incidence for men and women were compared between non-Asian and Asian countries. The RR's of HCC incidence were significantly higher in obese men than obese women in non-Asian studies (RR 2.31, 95%CI: 1.85–2.91, *vs* RR 1.56 (95%CI: 1.31–1.86, respectively, P = 0.01) but not in Asian countries[51].

More importantly, there is a linear relationship between increasing BMI and death from various types of cancer, including PLC[52]. A prospective investigation was conducted in United States in a large cohort of men and women with the aim to determine the relations between BMI and the risk of death from cancer at specific sites. More than 900.000 adults (free of cancer at baseline) were enrolled in 1982. During 16 years of follow-up there were 57.145 deaths from cancer. As compared with men of normal weight, men with a BMI ≥ 35.0 had significantly elevated RRs of death from cancer, which ranged from 1.23 (95%CI: 1.11 to 1.36) for death from any cancer to 4.52 (95%CI: 2.94 to 6.94) for death from liver cancer. There was a significant positive linear trend in death rates with increasing BMI for several types of cancers (esophageal, stomach, colorectal, pancreatic, gallbladder cancers, *etc.*) but the one with the highest risk was the PLC, which was increased by 4-5 times in men with BMI > 35[52].

Furthermore, metabolic syndrome (as defined by the United States National Cholesterol Education Program Adult Treatment Panel III criteria) has also been shown to be a significant risk factor for

development of HCC in the general population. Cases of HCC (n = 3.649) were identified in the Surveillance, Epidemiology, and End Results (SEER)-Medicare database, in United States. Control group was composed by 195.953 persons residing in the same regions. By adjusted multiple logistic regression analyses, metabolic syndrome was significantly associated with increased risk of HCC (OR 2.13; 95%CI: 1.96-2.31, P < 0.0001)[53].

GENETIC VARIANTS ASSOCIATED WITH HCC IN MAFLD

Recent advances in the field of Genetics allow obtaining comprehensive data on the genetic alterations associated with MAFLD-related HCC. Genome wide association studies (GWAS) look for links between single nucleotide polymorphism (SNP) and disease phenotype. Differential gene expression results from gene mutations in regulatory elements or epigenetic changes, which plays an important role in susceptibility to the development of HCC. Of over 100 Loci examined in GWAS and candidate gene studies, genetic variations in 5 genes have emerged as reproducibly and robustly predisposing individuals to development of MAFLD (*PNPLA3*, *TM6SF2*, *GCKR*, *MBOAT7* and *HSD17B13*)[54]. While unexplained variance remains despite these discoveries, indicating that future GWAS may reveal additional associations[54].

The patatin-like phospholipase domain-containing protein 3 (PNPLA3) genetic mutation on rs738409 c.444C>G SNP is a well-known risk factor for hepatic steatosis, disease severity, fibrosis stage and progression to NAFLD-related HCC[55-58]. This variant was most common in Hispanics, who are more susceptible to MAFLD[59]. Singal *et al*[60] performed a systematic review and meta-analysis of 24 studies with 9.915 patients and found that PNPLA3 was associated with an increased risk of HCC in patients with cirrhosis (OR 1.40, 95%CI: 1.12–1.75)[60]. Upon subgroup analysis, PNPLA3 was found to be an independent risk factor for HCC in patients with NAFLD or alcoholic liver disease-related cirrhosis (OR 1.67, 95%CI: 1.27-2.21), but not among other etiologies[60]. Liu *et al*[57] in a case-control study of 100 NAFLD related HCC and 275 controls with histologically characterized NAFLD, reported that bearing the PNPLA3 rs738409 c.444C>G was associated with GG homozygotes exhibiting a 5-fold increased risk of HCC in patients with NAFLD and when compared with United Kingdom general population the risk-effect was even more pronounced[57]. This association persisted following multivariate adjustment for age, gender, diabetes, BMI and presence of cirrhosis[57]. Interestingly, its effects to promote the full spectrum of NAFLD are modulated by interactions with environmental factors[61] and other gene variants[58,62].

A rs58542926 c.449C>T SNP in transmembrane-6-superfamily member 2 (TM6SF2) gene is associated with increased liver fat content, NASH and fibrosis progression[58,63-65]. Noteworthy, the TM6SF2 rs58542926 c.449C>T variant is associated with lower levels of serum cholesterol, LDL-cholesterol and triglycerides, and is protective against cardiovascular disease[65]. To evaluate the association between NAFLD-related HCC risk and TM6SF2 rs58542926 c.449C>T variant, Liu and coworkers reported that the TM6SF2 variant confers increased predisposition to NAFLD-related HCC (OR 1.922, 95%CI: 1.31–2.81)[64]. However, this association was not significant when risk factors including gender, age, diabetes and cirrhosis were considered by multivariate analysis[64].

A SNP in the glucokinase regulator (GCKR), rs1260326 and rs780094 variants are associated with increased susceptibility to NAFLD and fibrosis progression[66-69]. However, only GCKR rs1260326 variant predispose to NASH-related HCC (OR 1.84 95%CI: 1.23-2.75)[69]. Both GCKR variants interact with PNPLA3 rs738409 c.444C>G in elevating susceptibility to NASH in people with both risk alleles[66,68].

A SNP rs641738 g.54173068 C>T variant of the gene encoding membrane bound O-acyltransferase domain-containing 7 (MBOAT7) has been linked with an increased risk of hepatic steatosis, inflammation and fibrosis[70,71]. To ascertain the NAFLD-related HCC risk with MBOAT7 rs641738 variant, Donati and coworkers evaluated an Italian cohort of 765 NAFLD patients where MBOAT7 rs641738 variant was associated with NAFLD-HCC independently of clinical factors or fibrosis stage (OR 2.18, 95%CI: 1.30–3.63)[72].

The rs72613567 T>TA in the hydroxysteroid 17-β dehydrogenase 13 gene (HSD17B13) have recently been linked with a reduced risk of chronic liver disease[73]. The HSD17B13 rs72613567 variant in NAFLD-cohort patients is associated with decreased steatohepatitis and severe fibrosis[73,74]. Associations between the HSD17B13 rs72613567 variant and reduced odds of HCC in a variety of etiologies including NAFD and ALD have been reported[75,76].

Since MAFLD is a complex disease, therefore, it is logical that combining genetic variants into a risk score will improve prognostic accuracy over a singular genetic variant. Based on this concept, Donati and coworkers observed a significant association between the number of risk alleles variants (PNPLA3, TM6SF2 and MBOAT7) and HCC (OR *per* allele 1.56, 95%CI: 1.31–1.86; OR complete positive alleles 9.25, 95%CI: 3.83–22.8) that was not affected after adjusted for clinical factors and fibrosis stage[72]. In this cohort, HCC risk was 9% in the population with 0–1 risk allele, 19% in the population with 2–3 risk alleles, and 31% in the population with ≥ 4 risk alleles[72]. In the same work, Donati *et al*[72] developed a combined clinical and polygenic risk score (PRS) to predict HCC, the model had a 0.96 ± 0.4 area

under the receiving operating characteristic curve (AUROC) for detecting HCC cases, with optimal cutoff of 96% sensitivity and 89% specificity for HCC risk in the Italian NAFLD cohort. Recently, Gellert-Kristensen and coworkers demonstrated that a PRS, combining the 3 genetic variants in *PNPLA3*, *TM6SF2* and *HSD17B13*, was associated with risk of cirrhosis and HCC in fatty liver disease (both NAFLD and alcohol-related) from Denmark and United Kingdom general population[77]. The score ranged from 0 to 6 depending on the number of risk alleles, a score of ≥ 5 was associated with a 12-fold increased risk of cirrhosis and a 29-fold increased risk of HCC[77]. Bianco and colleagues, evaluated a PRS to improve HCC risk stratification in NAFLD (n = 1699) and general population cohort (United Kingdom BioBank), combining PNPLA3-TM6SF2-GCKR-MBOAT7 (PRS) and then adjusted for HSD17B13 (PRS-5). In the NAFLD cohort, PRS were associated with an approximately 12-fold increased odds of severe fibrosis and an approximately 9-fold increased odds of HCC (OR 9.2, 95%CI: 5.2–16.3 for PRS; and OR 9.1, 95%CI: 5.2–16.0 PRS-5)[78]. The association was independent of age, gender, diabetes and BMI but not of severe fibrosis. In the NAFLD cohort, the AUROC for HCC was 0.64 for PRS and 0.65 for PRS-5, the best single cut-off for PRS with 43% sensitivity and 80% specificity and for PRS-5 with 43% sensitivity and 79% specificity[78]. These promising polygenic risk prediction scores adjusted for conventional risk factors may, in the future, have the potential to guide care of patients with MAFLD. It is likely that genetic risk variants will need to be combined with other variables, such as clinical parameters, to improve score performance[72].

SCREENING TESTS FOR HCC SURVEILLANCE IN PATIENTS WITH MAFLD

In addition to deciding which patients with MAFLD should be involved in surveillance protocols for HCC, it would be necessary to address which are the best screening tests. For many years now, Scientific Societies have recommended the use of hepatic US with or without serum AFP measurement every 6 mo, based on its cost-effectiveness, acceptability for patients, easy accessibility and HCC doubling time. However, the sensitivity of this strategy to detect tumors eligible for curative treatment is not ideal. The ultimate goal of HCC surveillance is to increase patient survival, and for this, early stage tumors (within Milan criteria) must be diagnosed. A recent meta-analysis showed that sensitivities of liver US alone or with AFP measurement to detect early-stage HCC were 45% and 63%, respectively [79]. Furthermore, inadequate liver ultrasound quality may be more common in overweight or obese patients.

A retrospective cohort study was conducted to determine factors associated with inadequate US quality in HCC surveillance. Among 941 US examinations performed in cirrhotic patients, 191 (20.3%) studies were considered as inadequate for excluding HCC[80]. By multivariate analysis, inadequate quality was associated with male gender, BMI category, Child–Pugh B or C, and alcoholic or NASH related cirrhosis. In NASH-cirrhosis, the risk of having an inadequate US quality increased almost 3 times (OR 2.87, 95%CI: 1.71–4.80); and hepatic US was inadequate in over one-third of patients with BMI > 35[80].

The adequacy of ultrasound in assessing the cirrhotic liver to exclude nodular lesions depends not only on the patient factors but also of the operator. However, there are no regulations worldwide on the expertise that a radiologist must have to perform US examinations in the heterogeneous cirrhotic liver for HCC surveillance. The LI-RADS group (endorsed by the American College of Radiology) has proposed a US visualization score[81], where A category means “No or minimal limitations”; B, “Moderate limitations”. In B category, the concept is “limitations may obscure small masses”. Examples are moderate beam attenuation or some portions of the liver were not visualized. Finally, score C means “Severe limitations”, the concept is “Limitations significantly lower sensitivity for focal liver lesions” and examples are majority (> 50%) of liver or diaphragm were not visualized[81]. When the US visualization score is B or particularly when is C, an additional imaging method should be indicated as screening test (computed tomography or magnetic resonance imaging). Furthermore, patients at higher risk for HCC (*e.g.*, NASH cirrhosis plus diabetes), even with adequate US, should perhaps be screened with a more sensitive test.

A prospective Korean study by Kim *et al*[82] was performed to compare the HCC detection rate of hepatic US and liver-specific contrast enhanced magnetic resonance imaging (MRI) in patients with cirrhosis who were at high risk for HCC[82]. They enrolled 407 patients with cirrhosis and an estimated annual risk of HCC greater than 5% who underwent 1 to 3 biannual screening examinations with paired US and liver-specific contrast-enhanced MRI. HCC were diagnosed in 43 patients. They found that the HCC detection rate of MRI was 86.0% (37/43), significantly higher than the 27.9% (12/43) of US (P < 0.001) and 74.4% of tumors were in a very-early stage (a single nodule < 2 cm), with 67.4% of patients receiving curative treatments[82].

MRI is not used routinely in surveillance protocols for HCC because it would not be cost-effective in patients with low or intermediate risk. MRI is less affordable, more expensive and much more time consuming than US. However, in patients with very high HCC risk or in whom US is suboptimal, MRI could become the primary screening test. To minimize the costs and scanning time, protocols of abbreviated MRI (AMRI) are being tested[83,84] showing high percentages of sensitivity and specificity

in the preliminary results. AMRI protocols will play a role in the future in surveillance for HCC in patients at high risk or in whom the quality of US is inadequate.

CONCLUSION

The increasing prevalence of overweight, obesity, insulin resistance and type 2 diabetes have made MAFLD the most common chronic liver disease and a real challenge for physicians and health systems worldwide. Patients with NASH and advanced fibrosis or cirrhosis are the most prone to developing complications of cirrhosis including HCC. The diagnosis of NASH can only be confirmed by liver biopsy. However, given the huge number of patients with MAFLD and the invasiveness of the method, most patients will not undergo a liver biopsy. Nevertheless, the stage of liver fibrosis (and not the condition NASH/no NASH) is the main driver of liver-related morbidity and mortality. Non-invasive tests (NITs) to assess liver fibrosis stage, especially the non-proprietary ones such as FIB4[85] and the NAFLD fibrosis score[86], and different types of elastography[87] are being used with increasing frequency in the management of patients with NAFLD. The most appropriate algorithm to determine liver fibrosis in patients with MAFLD is beyond the scope of this review. Briefly, when a combination of 2 NITs (*e.g.*, FIB4 plus elastography or FIB4 plus NAFLD fibrosis score) yields a result below the low cut-off value, this has a high negative predictive value to rule out advanced fibrosis/cirrhosis in patients with NAFLD. It is assumed that the patient will not present liver-related morbidity in the short time and it is not necessary to include this patient in a surveillance protocol for HCC.

On the other end, patients with an unequivocal diagnosis of cirrhosis should be involved in surveillance for HCC. In most of them, conventional screening tests will be used, liver US plus serum AFP measurement every 6 mo; following the recommended recall procedures when any of the tests yield a positive result[10,15]. As mentioned, in a percentage of patients with NASH cirrhosis and/or with obesity, the quality of the US will not be adequate to confidently rule out nodular liver lesions. In addition, it may happen that patients with NAFLD and cirrhosis have factors that significantly increase the risk of HCC. In these 2 situations, the primary screening test could be MRI or an AMRI protocol. According to our review, factors that have been repeatedly found to increase the risk of HCC in cirrhosis due to MAFLD are male gender, older age, the presence of diabetes and, in some studies, decreased levels of serum albumin and any degree of alcohol intake. Table 4 suggests different risk categories for HCC in patients with NAFLD/MAFLD.

The risk of HCC occurrence is much more difficult to stratify in patients with MAFLD who do not have a diagnosis of cirrhosis. As mentioned, the "AGA Clinical Practice Update on Screening and Surveillance for Hepatocellular Carcinoma in Patients with NAFLD" states that patients with NITs showing evidence of advanced liver fibrosis or cirrhosis should be considered for HCC screening[16]. That expert panel recommends to combine at least 2 NITs and to better stratify risk and maximize specificity, they propose using higher than usual cut-off values for noninvasive detection of cirrhosis: 16.1 kPa for transient elastography; and 5 kPa for magnetic resonance elastography[16].

In the majority of patients with NAFLD (approximately 55%-60%) the NITs exclude advanced fibrosis and in a minority of them (approximately 10%-15%), they confirm severity of liver damage. However, there are a large number of patients in whom NITs show indeterminate results, which do not allow to rule out or confirm advanced fibrosis/cirrhosis. This gray area is where it is most challenging to stratify the risk of HCC or other complications of cirrhosis. According to findings of population and clinic-based studies in patients with diagnosis of NAFLD by US that we have revised, elevated AST levels[38] and decreased platelet counts[38] (both could be surrogates of severe fibrosis) and also elevated ALT levels [35] increase independently the risk of HCC. Furthermore, in many of these studies older age[28,35,38,40] and diabetes[28,36,38,39] were shown to be independent risk factors for HCC occurrence as well. In addition, multiple large, population-based studies have shown that individuals with diabetes[43-47], obesity[45,49-52], or metabolic syndrome[53] have a 2-3 times higher risk of HCC occurrence than their controls in the population analyzed. All of these epidemiological, cross-sectional studies demonstrate the association between diabetes, obesity, metabolic syndrome and HCC, without being able to analyze the causal relationship and the mechanisms involved. It is assumed that the link between these metabolic traits and the appearance of HCC is through the hepatic manifestation of metabolic syndrome, NASH, advanced fibrosis and cirrhosis. Finally, the study of genetic alterations that predispose to MAFLD, advanced fibrosis and increased risk of HCC will be an important tool in the near future, when PRSs become easily available. Only 2 factors have been associated with a significant decrease in the risk of HCC occurrence: Metformin use in diabetic patients[47,48] and statin use in NAFLD patients[35].

Figure 1 shows the main factors that can increase the risk of HCC in patients with MAFLD. It is worth to emphasize that the most influential risk is the presence of cirrhosis. In patients with cirrhosis, there is no doubt that they should be included in surveillance for HCC, with conventional tests or occasionally, with MRI.

In patients without cirrhosis but in whom NITs suggest the presence of advanced fibrosis, it seems reasonable to indicate surveillance for HCC with conventional screening tests, US plus AFP, twice a

Table 4 Different risk categories for hepatocellular carcinoma in patients with non-alcoholic fatty liver disease/metabolic-dysfunction associated fatty liver disease[1]

Risk for HCC	Patients	
Very high	Cirrhosis plus	Male gender
		Older age
		Diabetes
		Low serum albumin
High	Cirrhosis	
	F3 fibrosis plus	Elevated AST[2]
		Low platelets[2]
		Older age
		Diabetes
Low	F3 fibrosis	
Very low	Mild or no liver fibrosis	

[1]Study of polygenic risk score will be important for better stratification of hepatocellular carcinoma risk when clinically available.
[2]Probably understaged liver fibrosis.
HCC: Hepatocellular carcinoma.

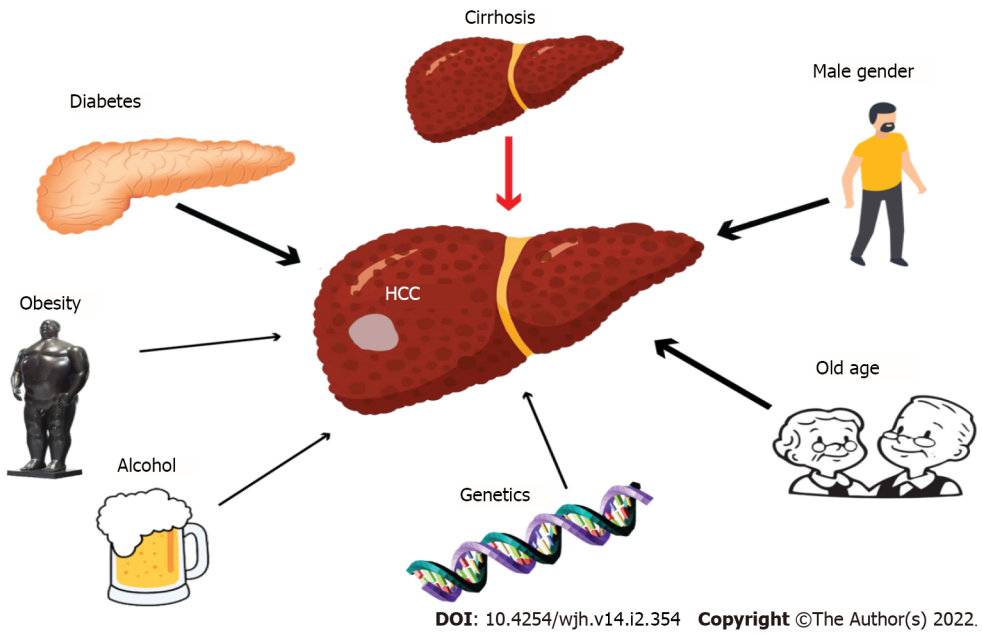

Figure 1 Factors that can increase the risk of hepatocellular carcinoma in patients with non-alcoholic fatty liver disease/metabolic-dysfunction associated fatty liver disease. Presence of cirrhosis is the factor that has the greatest influence on the incidence of hepatocellular carcinoma. HCC: Hepatocellular carcinoma.

year. In patients in whom NITs show intermediate results, there is, to date, no recommendation that can be made based on scientific evidence. The treating physician should consider the presence of additional risk factors that were described in this review and decide accordingly. As an example, a male patient, older than 65 years and with diabetes or elevated AST has a higher risk of HCC than a female patient, 45 years old, without diabetes or with normal AST. Further prospective, longitudinal, cooperative studies have to be carried out in this group of patients to better understand the risk of HCC and which factors may modify its incidence. This will allow better risk stratification, optimize surveillance and improve tests adequacy.

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MINIREVIEWS

Immunomodulation by probiotics and prebiotics in hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the most prevalent primary malignancy in patients suffering from chronic liver diseases and cirrhosis. Recent attention has been paid to the involvement of the gut-liver axis (GLA) in HCC pathogenesis. This axis results from a bidirectional, anatomical and functional relationship between the gastrointestinal system and the liver. Moreover, the complex network of interactions between the intestinal microbiome and the liver plays a crucial role in modulation of the HCC-tumor microenvironment, contributing to the pathogenesis of HCC by exposing the liver to pathogen-associated molecular patterns, such as bacterial lipopolysaccharides, DNA, peptidoglycans and flagellin. Indeed, the alteration of gut microflora may disturb the intestinal barrier, bringing several toll-like receptor ligands to the liver thus activating the inflammatory response. This review explores the new therapeutic opportunities that may arise from novel insights into the mechanisms by which microbiota immunomodulation, represented by probiotics, and prebiotics, affects HCC through the GLA.

Key Words: Hepatocellular carcinoma; Gut microbiota; Probiotics; Prebiotics; Gut-liver axis; Immunomodulation

Core Tip: In patients with chronic liver disease and cirrhosis, hepatocellular carcinoma (HCC) is the most common primary malignancy. Recent attention has been paid to the involvement of the gut-liver axis (GLA) in HCC pathogenesis. This review explores the potential for new treatment options as a result of novel insights into the processes by which microbiota immunomodulation, represented by probiotics and prebiotics, affects HCC through the GLA.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the leading cause of cancer-related death worldwide and it is the most common primary tumor in people with cirrhosis and chronic liver disease[1]. Males are diagnosed with HCC at a greater rate than females (2.4:1) in Eastern and Southern Asia, Middle and Western Africa, Micronesia/Poly-nesia and Melanesia[2]. Today, infection with hepatitis B virus (HBV) and hepatitis C virus (HCV), as well as dietary aflatoxin and alcohol abuse, are all significant risk factors for HCC occurrence. Despite the fact that HBV and HCV account for 80%-90% of total HCC cases, the obesity epidemic, the development of effective direct acting antivirals for HCV, and the availability of a universal HBV vaccination may alter HCC epidemiology in the future[3].

Recently, an increase in the incidence of non-alcoholic steatohepatitis (NASH) and non-alcoholic fatty liver disease (NAFLD) has been accompanied by an increase in the incidence of NASH-related HCC[4]. Aristolochic acid and tobacco have also been identified as probable pathogenetic cofactors in HCC, according to several findings on mutational signatures[5].

Specific advances in our understanding of the processes related to NASH-associated HCC have offered new insights into the tumor microenvironment contributions, generated by a mutual interplay between the immune system and gut microbiota (GM), defined as the assemblage of microorganisms such as bacteria, eukaryotes, archaea, and viruses inhabiting the intestine[6-9]. Indeed, important factors linked to the immune-microbiome interplay such as leaky gut, endotoxemia, toll-like receptors (TLR), dysbiosis, and immunomodulation have been associated with HCC develop-ment[10]. Although the liver does not come into direct contact with bacteria, it is anatomically connected to the gut[11]. Of note, the gastrointestinal tract influences homeostasis, preserving an intact barrier against bacterial lipopoly-saccharides (LPS) and intestinal bacteria. The most often utilized marker for the translocation of inflammatory bacterial microbiota-associated molecular patterns (MAMPs) is LPS.

LPS is an element found in gram-negative bacteria cell walls that triggers inflammation *via* TLR4. Low-grade exposure to GM-derived metabolites and MAMPs occurs during the physiologic transit of nutrient-rich blood from the colon to the liver, despite the extremely effective multi-level intestinal barrier[11]. Bacterial translocation and LPS build up cause intestinal bacterial overgrowth and changes in GM composition when intestinal permeability is enhanced. Furthermore, a leaky gut allows dysbiotic microbiota-associated bacterial metabolites and MAMPs to more easily translocate and reach the liver. Degradation, detoxification, and clearance of LPS and other microbial products are all hampered in patients with chronic liver diseases or cirrhosis, since the damaged liver is exposed to a wider spectrum of TLR ligands, as well as other bacterial products and metabolites[12]. Indeed, it has been noted that altered microbiota is generally present in HCC patients[13]. In addition, unlike bacterial species, it was discovered that the iron transport, microbial metabolism, and energy-producing system of HCC patients and healthy controls varied considerably[13].

TLR4 produced by activated stellate cells reacts to low LPS concentrations, causing fibrosis and cirrhosis development. An animal model of hepatocarcinogenesis showed that the GM and TLR4 activation have been shown to enhance HCC development by increasing cell proliferation and suppressing apoptosis[14].

As the gut-liver axis (GLA) is involved in HCC pathogenesis, the study of the mutual interplay between the microbiota and immune response and their cross-talk with the tumor microenvironment are an important focus of current clinical research. Our review investigates and analyzes the potential therapeutic benefits of emerging insights into the mechanisms by which microbiota immunomodulation, as represented by probiotics and prebiotics, impacts HCC *via* the GLA.

GLA

The GM as a "virtual metabolic organ" establishes an axis with several extraintestinal organs, such as the brain, kidneys, bone and cardiovascular system. However, in recent years, the GLA has received considerable interest[15]. The GLA is the result of a bidirectional, anatomical and functional relationship

between the gastrointestinal system and the liver, largely *via* the portal circulation (Figure 1). A complex network of interactions between the enteric microbiome and the liver regulates and stabilizes their symbiotic connection, which includes metabolic and immunological crosstalk[16,17]. Antigens (from harmful microorganisms or food) enter through these linkages and are identified by dendritic cells, which then activate the adaptive immune system by regulating T cell responses. Pathogen-associated molecular patterns (PAMPs) (*e.g.,* LPS, DNA and flagellin activate nuclear factor kappa B and peptidoglycans) *via* nod-like receptors and TLRs, as a result, inflammatory cytokines and chemokines are secreted and reach the portal circulation. PAMPs can activate stellate cells implicated in fibrosis development and progression, in addition to hepatocyte injury, and Kupffer cells are much more susceptible to LPS than hepatocytes[18].

Involvement of the microbiome in HCC

As previously reported, the GM contribution to HCC etiopathogenesis is complex and elaborate. A disturbed intestinal barrier brings a series of TLR ligands to the liver and activates the inflammatory response in different ways: (1) *Via* upregulation of hepatic stellate cell proliferation and downregulation of hepatocyte apoptosis, the TLR signaling pathways induce liver tumorigenesis[14]; and (2) Lastly, in HCC, inadequate immunosurveillance is linked to an aberrant intestinal microbiota. Furthermore, *via* increasing oxidative stress, inflammatory response and steatosis, the microbiota dysbiosis might be linked to HCC development[19].

In general, changes in the makeup of microbial profiles are thought to have a role in tumorigenesis [9]. In fact, recent research has revealed a link between certain bacterial profiles and HCC patients[20], showing high amounts of *Escherichia coli* and other gram-negative bacteria in the intestinal bacterial flora, which are linked to elevated LPS levels in serum[21]. Moreover, *Fusobacterium* and *Oribacterium* are the bacteria most often identified from a tongue swab of HCC affected subjects. On the other hand, the intestinal HCC microbiome showed reduced amounts of *Lactobacillus spp*., *Bifidobacterium spp*., and *Enterococcus spp*.[22]. A more recent report examined bacterial diversity in cirrhosis and HCC patients [20]. A decrease in the fecal microbial diversity from healthy controls to cirrhosis and an increase from cirrhosis to early HCC with cirrhosis (both induced by chronic HBV infection) was observed. In addition, different microbiota markers were detected[20]. Moreover, the authors observed a decrease in *Verrucomicrobia* with a simultaneous increase in *Actinobacteria*.

Furthermore, augmented levels of *Bacteroides* and *Ruminococcaceae* and increased levels of *Akkermansia* and *Bifidobacterium* were detected in patients with NASH-induced cirrhosis and HCC, in comparison to NASH-induced cirrhosis without HCC[23]. In the same study, the authors discovered a link between enteric microbiota patterns and calprotectin levels as well as systemic inflammation. The GM of patients with HBV-associated HCC and non-HBV non-HCV (NBNC) associated HCC was compared in further research. In comparison to healthy controls or to patients with NBNC-related HCC, those with HBV-related HCC had a substantially richer fecal microbiota. Patients with NBNC-related HCC had higher amounts of pro-inflammatory bacteria (*Escherichia coli*, *Enterococcus*) and lower amounts of anti-inflammatory bacteria (*Faecalibacterium*, *Ruminococcus*, *Ruminoclostridium*), leading to lower quantities of anti-inflammatory short-chain fatty acids (SCFA) in their feces[24]. The GM of HCC patients receiving liver transplantation was also compared to the intestinal microbiota of individuals who did not have HCC but had a similar etiology of cirrhosis. An augmented abundance of fecal *Escherichia coli* was linked with HCC[25]. Additionally, *Helicobacter spp*., was found in liver HCC suggesting that intestine translocation might be a possible cause of carcinogenesis[26]. To this end, the enteric microflora profile might potentially indicate reaction rates in HCC patients undergoing treatment with immune checkpoint inhibitors[27], suggesting that the microbiome could be used for liver cancer immunotherapy[28].

Overall, alterations in microbial profiles in HCC did not appear to be consistent among investigations, presumably due to differences in etiologies, geographical locations, and dietary intakes. Differences between patients with cirrhosis and HCC, as well as cirrhosis alone, appear to be lower than those between healthy individuals and those with cirrhosis. As a result, microbiome-based diagnostic tools are anticipated to be more potent for cirrhosis identification than for HCC diagnosis. However, rather than particular HCC-associated abnormalities, the microbiome's functional effect on HCC development is more likely to be linked to cirrhosis-related changes, which may increase HCC advancement.

Therapeutic impact of probiotics in HCC

A recent study has shown that antibiotics can help with small-intestinal bacterial overgrowth-related liver damage, demonstrating the connection between intestinal microbiota and liver disorders[29]. Probiotics are microorganisms that have a beneficial effect on humans and are presently being studied as a potential therapy for chronic liver disease[16,29]. Indeed, they can support the growth of enteric microbes producing anti-inflammatory metabolites, which exert an immune suppressive effect.

Probiotics can help microbiota generate anti-inflammatory compounds with tumor-suppressing properties. Probiotics have a significant influence on the GLA, with anti-inflammatory and immunomodulatory effects on GM and gut barrier function, as well as a metabolic influence on organs beyond the gastrointestinal tract. They can affect the generation of immunomodulating GM compounds that show anti-tumor properties (Figure 2). Furthermore, supplementation with probiotics increased the

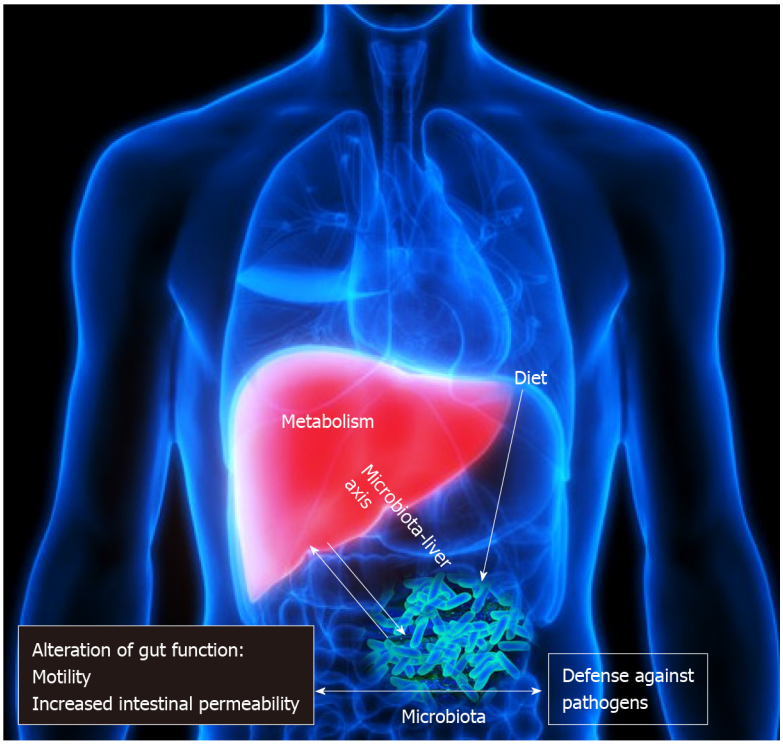

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Figure 1 The gut liver axis and microbiota.

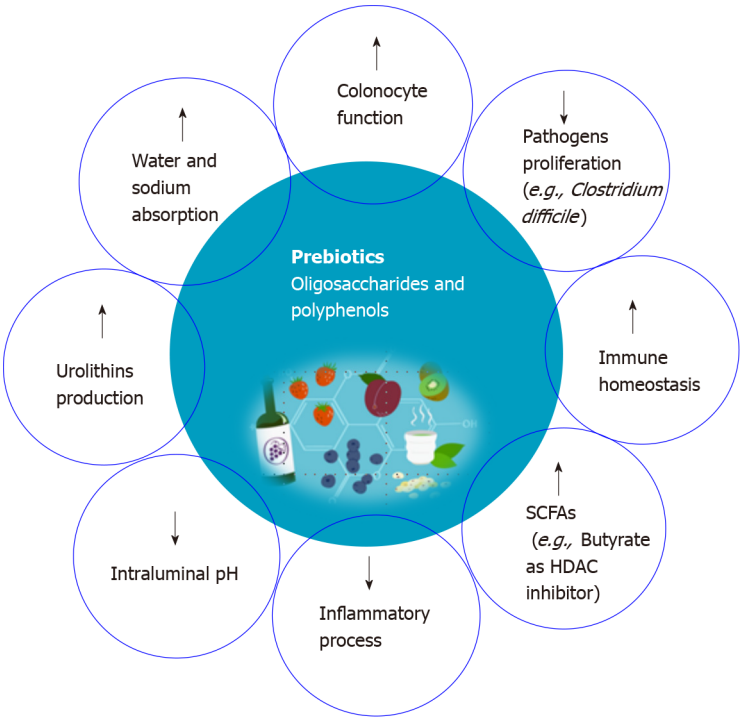

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Figure 2 Proposed mechanism of action of probiotics against hepatocellular carcinoma progression. HDAC: Histone deacetylase; SCFAs: Short-chain fatty acids.

expression of some anti-inflammatory cytokines, such as interleukin (IL)-13, IL-10, and IL-27.

Downregulation of angiogenic factors and receptors, VEGFA, Fms related receptor tyrosine kinase 1, ANGPT2, and kinase insert domain receptor, were seen in mice fed a particular probiotic combination [30]. However, not all probiotic species have the same immunomodulatory effect on the gut microflora. Different strains of *Lactobacillus spp*., for example, are linked to both pro-obesity and anti-obesity effects

[31]. Research on mice with diet-induced obesity found that distinct targeted enteric bacteria modification using vancomycin *vs* probiotic strain *Lactobacillus salivarius* resulted in divergent metabolic effects, although microbiota modifications were identical. By contrast, a recent human investigation in obese adolescents focussing on the impact of *Lactobacillus salivarius Ls*-33 on a number of inflammatory biomarkers, found no evidence of an effect on the metabolic syndrome[32,33]. These findings indicate that the role of the same probiotics could be different due to various conditions; therefore, attention should be paid to evaluating the different impacting factors.

The use of antimicrobials and probiotics in chronic liver illnesses should be based on the GLA pathophysiology. Despite a consistent number of findings from animal and human research, further case-control prospective studies with a large number of patients are needed to fully understand this issue. Obesity-induced intestinal microbial dysbiosis can lead to HCC, according to a mouse model[34]. Aflatoxin-induced HCC is the subject of research in relation to therapeutic HCC prevention with probiotics. In fact, aflatoxin contamination of foods is an etiological risk factor for HCC in underdeveloped nations. Probiotics may help to suppress aflatoxin B-induced hepatocarcinogenesis, correct gut dysbiosis, lower LPS levels, and shrink tumors, according to findings from human and animal model studies[14,35]. Moreover, in a mouse model, treatment with *Lactobacillus plantarum* (*L plantarum*)C88 (isolated from Chinese traditional fermented meals) boosted fecal aflatoxin B1 excretion and controlled the defence system's antioxidant deficiency[35]. Additionally, a probiotic yogurt containing *Lactobacillus rhamnosus*, *Streptococcus thermophilus*, and *Wiessella cibaria* also reduced aflatoxin metabolites in the urine of children who ate aflatoxin B1-infected maize.

The binding of aflatoxin metabolites to probiotic bacteria may have resulted in lower intestinal absorption, inducing a reduction in aflatoxin metabolites in urine[36].

In addition, the ability of probiotic bacteria to facilitate epigenetic modification of host gene expression is advantageous in reducing HCC development[37]. The crosstalk between host and enteric microflora, where gene expression is controlled by several methods such as DNA methylation and histone modification, demonstrates bacterial control of host gene expression[38]. In mice treated with a colon carcinogen, it has been observed that *Lactobacillus acidophilus* (*L. acidophilus*) and *Bifidobacterium bifidum* can decrease the expression of Kristen rat sarcoma viral oncogene homolog, oncomirs (microRNA-221and microRNA 155) and the oncogenes BCL2-like 2 (Bcl-w) in the liver. Moreover, mice treated with these probiotic bacteria had higher levels of the tumor suppressor microRNA-122 and the tumor suppressor gene transcription factor PU.1.[37]. Probiotic supplementation may minimize the incidence of HCC by safeguarding the hepatocyte genome, which is important in the pathophysiology of HCC. Probiotic fermented milk and chlorophyllin were shown to reduce the expression of rasp-21, c-myc, cyclin D1, and Bcl-2 in an HCC rat model, slowing tumor development and volume by 40%. Mice treated with probiotics have significant amounts of fecal *Oscillibacter* and *Prevotella*[39].

Microbial PAMPs can induce liver cancer progression *via* TLR-mediated inflammatory responses, as previously described. The treatment with bacteria having probiotic properties has been demonstrated to decrease the development of HCC in the liver by reducing the expression of TLR-induced inflammation. When rats with induced liver cirrhosis were treated with *L plantarum* probiotic bacteria, they showed lower TLR4 expression and less liver damage[40]. In addition, gut sterilization and TLR4 inactivation reduced HCC by 80% to 90%, indicating that they might be used as HCC preventive methods[14].

Furthermore, the antiviral action of probiotic bacteria may slow HCC progression by avoiding persistent HBV infection. The release of HBV surface antigen (HBsAg) was diminished by a cell extract of *Bifidobacterium adolescentis* by reducing the transcription of HBsAg gene, which contrasted the infection. Despite the fact that the amount of HBV DNA in the cells did not change considerably, probiotic therapy drastically reduced the amount of extracellular HBV DNA available[41]. Furthermore, treatment with *Lactobacillus bulgaricus* induced lowered viral load and cellular deterioration[42]. On the other hand, probiotic bacteria supplements can also help liver function during HCV infection. In addition, HCV-positive patients, treated with *Enterococcus faecalis* lowered blood levels of the aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (liver damage indicators)[43].

Furthermore, NAFLD is also a key etiological risk factor in HCC development. Supplementation with the probiotic bacteria *L. acidophilus* and *Bifidobacterium lactis* can help NAFLD patients with liver damage, as seen by lower ALT, AST, and total cholesterol blood levels[44]. Probiotic therapy lowered body weight and total body fat content in obese NAFLD patients. By suppressing the pro-inflammatory cytokine tumor necrosis factor-α, probiotics also reduced hepatic inflammation in obese NAFLD patients[45]. Additionally, GM changes seem to be the cause of the hepatoprotective and anti-inflammatory actions of probiotic bacteria in NAFLD patients. In rats fed with a high-sucrose and a high-fat diet, probiotic meals improved diet-induced loss of intestinal microbiota diversity, intestinal epithelial barrier function and colonization resistance. Restoration of enteric microbiota and gut epithelial barrier function reduced NAFLD progression by lowering serum LPS levels and reducing TLR4-mediated hepatic inflammation[36].

Finally, probiotic cell components and metabolites increase the production of tight junction proteins, which helps to maintain gut epithelial integrity. Tight junction integrity is critical for preventing translocation of pathogens or exogenous substances across intestinal epithelia and consequently intestinal inflammation. Constituents of tight junctions comprise integral membrane proteins, such as occludin, claudin (CLDN) family members, JAMs 1-3, cingulin, and cytoplasmic plaque proteins, such as zonular

occludins-1 (ZO-1) and ZO-2[46,47]. Different components of the CLDN family have been reported to be affected during hepatocarcinogenesis[48-51]. However, in HCC patients undergoing hepatectomy, downregulation of ZO-1 is linked to a poor prognosis[52]. Probiotic supplementation can elicit considerable upregulation and relocalization of interepithelial tight junction proteins by activating numerous TLR produced by the intestinal epithelium. In terms of the effect of probiotic therapy on tight junctions, it was discovered that a VSL#3 combination maintained the epithelial barrier in acute colitis by avoiding reduced tight junction protein expression and an increased apoptotic ratio[53]. Moreover, in a mouse model of high-fat diet or obesity-induced liver steatosis, supplementation with a multispecies probiotic (including *Bifidobacteria*, *Lactobacilli* and *Streptococcus*) formulation helped to maintain tight-junction proteins ZO-1 and ZO-2, and reduced hepatic triglyceride concentration compared with a high-fat diet alone[54]. In older rats, a probiotic cocktail including *Lactobacillus* and *Enterococcus* strains reduced microbiota dysbiosis, leaky gut, inflammation, metabolic dysfunctions, and physical function loss caused by a high-fat diet. The GM regulated by probiotics decreased leaky gut by strengthening tight junctions, which lowered inflammation[55].

Prebiotics as a novel therapeutic approach

Prebiotics are substrates that are used selectively by host bacteria to provide a health benefit[56]. Health effects are mostly related to the production of SCFA through prebiotics fermentation by a limited number of genera/species of the intestinal microbiota such as *Lactobacilli* and *Bifidobacteria*[57]. *Bifidobacteria* are the most common bacterial species in the human GM, and they are thought to benefit human health by preserving the resident microbiota's balance. SCFAs such as acetate, propionate, and butyrate exert several biologic activities that positively influence the structure and function of the microbial community. Some of these beneficial effects include controlling colonocyte function, promoting water and electrolyte absorption, decreasing the intraluminal pH, inhibiting pathogen proliferation, modifying the immune homeostasis of the gut and modulating the inflammatory processes[56].

The improvement of mucosal barrier functions exerted by the modulation of GM composition improves conditions such as cirrhosis and consequently may prevent HCC. Prebiotics such as butyrate are not metabolized in the mitochondria of tumor cells but enter the cell nucleus to regulate gene expression. In fact, the functions of butyrate including inhibition of histone deacetylase, decrease tumorigenesis (Figure 3)[58].

The most studied prebiotic classes are galactans (galacto-oligosaccharides) and inulin-type fructans [*e.g.*, fructo-oligosaccharides (FOS), inulin, oligofructose]. There are other oligosaccharides such as isomalto-oligosaccharides and soybean oligosaccharides that have recently gained interest. Dietary sources of prebiotic oligosaccharides are various types of vegetables, fruits, grains and legumes. Depending on their water solubility, dietary fibers are classed as insoluble (*e.g.*, hemicellulose, cellulose, and lignin) and soluble (*e.g.*, fructans, gums, and pectins). Only soluble fibers can be metabolized by the GM and have "prebiotic" functions which can affect host health[59].

In particular, the two prebiotics most researched are FOS and inulin, which have been shown to stimulate the growth of beneficial *Bifidobacteria* that are implicated in cancer prevention (Figure 3)[60].

Inulin is a non-digestible functional polysaccharide, present in plants such as onions, chicory, Jerusalem artichoke, leeks, garlic, bananas, rye, barley and wheat[61]. A recent study showed that inulin modulates GM and hepatic fatty acid composition limiting dysbiosis and its negative effect on host health[62].

FOS are mostly present in plants such as garlic, onions, leeks, chicory root, Jerusalem artichokes, asparagus, and bananas. New findings suggest that FOS can help to achieve colonization resistance against *Clostridium difficile*. Several studies have reported the association between liver cancer and intestinal microflora and indicate that *Clostridium* cluster XIVa, *Clostridium* cluster XI, or other strains catalyzing the transformation from primary to secondary bile acids could be triggers of hepatic cancerogenesis[34,63,64]. Moreover, research has shown that *Clostridia* species are enriched in the guts of mice with liver cancer, documenting the association between cancer and dysbiosis[65].

A study evaluating the relationship between food groups and liver cancer risk reported that specific subgroups of vegetables, rich in inulin and FOS, such as celery, mushrooms, Chinese chives, onions, garlic, garlic shoots, asparagus, lettuce, garland chrysanthemum, legumes, squash and carrots, had a strong inverse relationship with liver cancer, indicating their protective effects against HCC[66] (Figure 4). A cohort study involving 125455 individuals from the Nurses' Health Study and the Health Professionals Follow-up Study found that wholegrain, bran, and cereal fiber consump-tion may help reduce HCC[67].

Moreover, eating whole grains and dietary fiber, particularly cereal fiber, has been linked to a decreased risk of obesity and NAFLD, both of which are known predisposing factors for HCC, as previously documented[68]. In fact, long-term consumption of whole grains has been suggested to reduce the risk of HCC by improving gut integrity and changing GM composition[69]. Moreover, a meta-analysis of 19 studies involving 1290045 participants (3912 with HCC) showed that every 100 g/d increase in vegetable intake decreases the risk of HCC by 8%[70].

A potential role of diet in HCC development has recently been clarified by a systematic review (30 studies involving 5222534 participants) that investigated the association between diet and HCC. In particular, certain dietary patterns, such as the Mediterranean diet, the Urban Prudent Dietary Pattern,

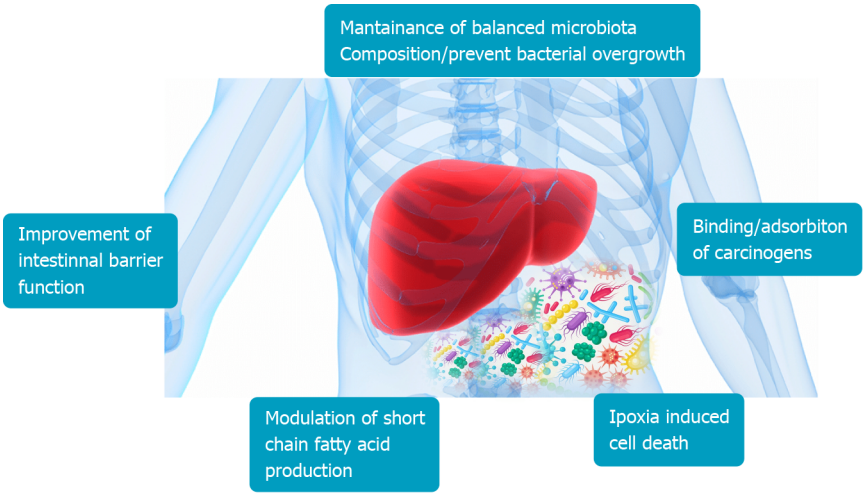

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Figure 3 Role of prebiotics on immunomodulation in hepatocellular carcinoma. Prebiotics contribute to short-chain fatty acids such as acetate, propionate, and butyrate production, which positively influence the structure and function of the microbial community. Butyrate functions include inhibition of histone deacetylase, and decreased tumorigenesis. In addition, inulin improves gut barrier integrity, thereby reducing pro-inflammatory lipopolysaccharides-toll-like receptors 4 signaling in macrophages during alcoholic liver disease. Fructo-oligosaccharides and inulin stimulates the growth of beneficial *Bifidobacteria* that are implicated in cancer prevention.

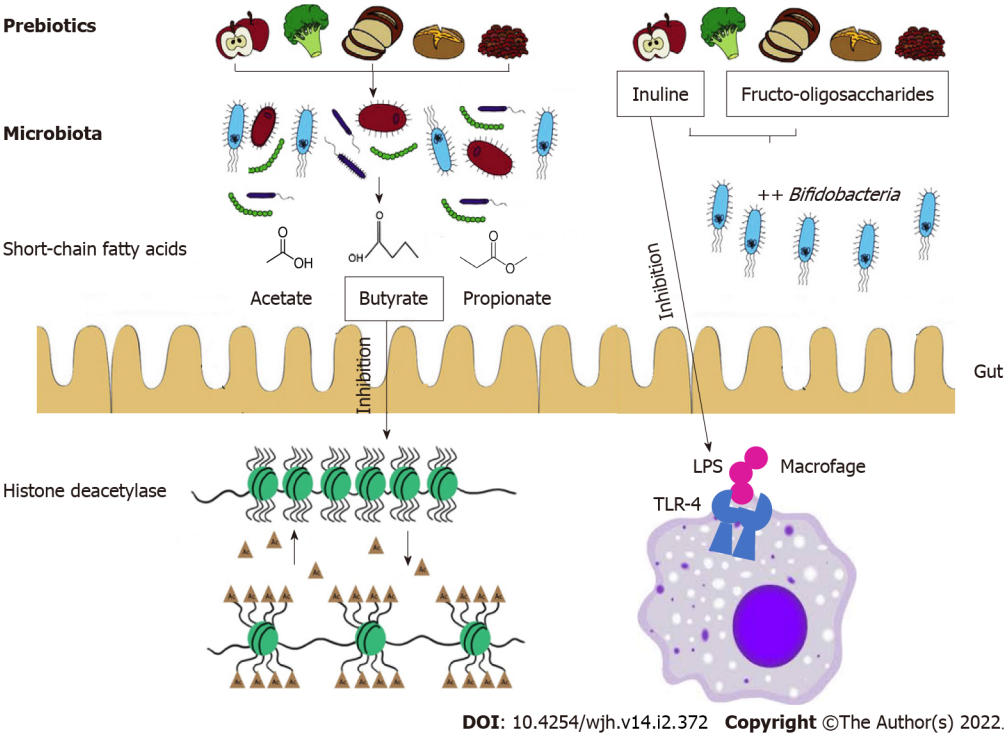

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Figure 4 Effect of prebiotics in cancer prevention. LPS: Lipopolysaccharides; TLR: Toll-like receptors.

the Alternative Healthy Eating Index-2010, and the Traditional Cantonese Dietary Pattern, were found to lower the incidence of HCC[71].

Recently, other substances besides non-digestible oligosaccharides, such as plant polyphenols are considered to have prebiotic effects. These compounds are not absorbed in the small intestine and, therefore, reach the colon where they are processed by the colonic microbiota conferring benefit to the host health[71]. They comprise flavonoids, phenolic acids and lignins found in nuts, tea, wine, vegetables and fruits. Polyphenols, due their immunomodulation activity, can have chemopre-ventive effects in HCC progression[72]. In particular, polyphenols contained in tea have been reported to have positive effects on GM homeostasis by decreasing the growth of pathogenic bacteria[73]. One of them is the antioxidant ellagic acid, present especially in nuts and berries, which is metabolized by microbiota in the colon into urolithins that have cancer preventive properties[74].

A prospective cohort study found that a higher consumption of tree nuts (hazelnuts, almonds, macadamias, pecans, cashews, and pistachios) (mean 1.25 serving per week) was associated with a reduced risk of HCC[75,76].

Coffee contains phenolic agents which have anticarcinogenic properties and its consumption has been demonstrated by various studies to reduce HCC risk[77].

Moreover, the cancer preventive effect of the association of oligosaccharides with polyphenols was also recently investigated. A study examined the effect of the combination of FOS and pectins with raspberry polyphenols on cecal microbial fermentation and regulation of liver lipid metabolism and inflammation, and concluded that FOS and pectins enhanced the effects of the raspberry polyphenolic extract against disorders related to NAFLD[78].

Furthermore, prebiotics use has been explored as an approach to modulate intestinal barrier function through maintaining tight junction (TJ) integrity. Prebiotics have demonstrated effects on GM composition that could lead to beneficial changes in TJ protein expression and distribution. However, the effect of prebiotics on TJ by microbiota composition modulation is less recognized than those produced by probiotic supplementation.

The prebiotics which showed beneficial effects on intestinal TJs in various studies are FOS, inulin, and galacto-oligosaccharide. A recent study investigated the effect of FOS on AMP-activated protein kinase (AMPK) activity and TJ assembly under non-inflammatory and inflammatory conditions using T84 cells as an intestinal epithelial cell model, and showed that FOS promoted intestinal epithelial TJ assembly through AMPK activation[79]. In addition, other studies demonstrated that prebiotic supplementation results in robust activation of AMPK[80,81].

A study showed that inulin fermentation increased occludin, CLDN-3 and ZO-1 RNA expression that could reinforce the barrier function[82]. Even galacto-oligosaccharide supplementation increased ZO-1, occludin and CLDN-1 protein expression[83].

CONCLUSION

HCC development is a complicated process influenced by a number of etiological risk factors. However, several studies have shown that probiotics and prebiotics have a beneficial effect on GM regulation and decrease procarcinogenic factors in the liver. It is noteworthy that the most remarkable aspect is the importance of a balanced diet with proper nutrition (which includes higher intake of vegetables, fish, white meat, and coffee, and reduced intake of fat, red meat, and alcohol), especially for those patients suffering liver, and especially chronic, diseases[84]. A Mediterranean diet has recently been shown to reduce the incidence of HCC, providing a new paradigm for future research[85]. Moreover, scientific investigations have revealed the possibility of developing cancer-preventive symbiotic (combination of probiotic and prebiotic) functional foods[86]. Of course, further research is needed to fully identify the bioactive probiotic metabolites (post-biotics) of certain dietary phytochemicals and to understand the possible mechanism(s) by which these post-biotics interact with the host[87]. In the future, the development of dietary methods and adjunct treatments to prevent the development of HCC may be supported by the creation of combinations of symbiotics or post-biotics with improved anticancer potential.

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FOOTNOTES

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Development of the nervous system in mouse liver

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Abstract

BACKGROUND

The role of the hepatic nervous system in liver development remains unclear. We previously created functional human micro-hepatic tissue in mice by co-culturing human hepatic endodermal cells with endothelial and mesenchymal cells. However, they lacked Glisson's sheath [the portal tract (PT)]. The PT consists of branches of the hepatic artery (HA), portal vein, and intrahepatic bile duct (IHBD), collectively called the portal triad, together with autonomic nerves.

AIM

To evaluate the development of the mouse hepatic nervous network in the PT using immunohistochemistry.

METHODS

Liver samples from C57BL/6J mice were harvested at different developmental time periods, from embryonic day (E) 10.5 to postnatal day (P) 56. Thin sections of the surface cut through the hepatic hilus were examined using protein gene product 9.5 (PGP9.5) and cytokeratin 19 (CK19) antibodies, markers of nerve fibers (NFs), and biliary epithelial cells (BECs), respectively. The numbers of NFs and IHBDs were separately counted in a PT around the hepatic hilus (center) and the peripheral area (periphery) of the liver, comparing the average values between the center and the periphery at each developmental stage. NF-IHBD and NF-HA contacts in a PT were counted, and their relationship was quantified. SRY-related high mobility group-box gene 9 (SOX9), another BEC marker; hepatocyte

nuclear factor 4α (HNF4α), a marker of hepatocytes; and Jagged-1, a Notch ligand, were also immunostained to observe the PT development.

RESULTS

HNF4α was expressed in the nucleus, and Jagged-1 was diffusely positive in the primitive liver at E10.5; however, the PGP9.5 and CK19 were negative in the fetal liver. SOX9-positive cells were scattered in the periportal area in the liver at E12.5. The Jagged-1 was mainly expressed in the periportal tissue, and the number of SOX9-positive cells increased at E16.5. SOX9-positive cells constructed the ductal plate and primitive IHBDs mainly at the center, and SOX-9-positive IHBDs partly acquired CK19 positivity at the same period. PGP9.5-positive bodies were first found at E16.5 and HAs were first found at P0 in the periportal tissue of the center. Therefore, primitive PT structures were first constructed at P0 in the center. Along with remodeling of the periportal tissue, the number of CK19-positive IHBDs and PGP9.5-positive NFs gradually increased, and PTs were also formed in the periphery until P5. The numbers of NFs and IHBDs were significantly higher in the center than in the periphery from E16.5 to P5. The numbers of NFs and IHBDs reached the adult level at P28, with decreased differences between the center and periphery. NFs associated more frequently with HAs than IHBDs in PTs at the early phase after birth, after which the number of NF-IHBD contacts gradually increased.

CONCLUSION

Mouse hepatic NFs first emerge at the center just before birth and extend toward the periphery. The interaction between NFs and IHBDs or HAs plays important roles in the morphogenesis of PT structure.

Key Words: Nervous system; Liver; Portal tract; Hepatic artery; Immunohistochemistry; Tissue engineering

Core Tip: The portal tract (PT) consists of branches of the hepatic artery (HA), portal vein, intrahepatic bile ducts (IHBD), and autonomic nerves. This study evaluated the mouse hepatic nervous system development using immunohistochemistry. Hepatic nerve fibers (NFs) first emerge at the hepatic hilus just before birth and extend toward the periphery with IHBD in the PT after birth. The hepatic NFs associated more frequently with the HA than the IHBD in the PT after birth. The hepatic NFs may play important roles in the morphogenesis and stabilization of the PT during development of the liver.

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INTRODUCTION

Recently, structures resembling whole organs, termed organoids, have been generated from stem cells through the development of three-dimensional culture systems[1]. The liver is the largest organ in the abdomen and is a pivotal center of metabolism, detoxification, and digestion. The mammalian liver has a structural and functional unit called the liver lobule, and in the periphery of the lobule, Glisson's sheath, also known as the portal tract (PT) consisting of the portal vein (PV), bile duct (BD), and hepatic artery (HA), develops[2]. We previously created the three-dimensional vascularized functional human micro-hepatic tissue in mice by co-culturing human hepatic endodermal cells with endothelial and mesenchymal cells derived from human-induced pluripotent stem cells (iPSCs)[3]; however, they lacked PTs. The construction of stable blood vessels is a fundamental challenge for tissue engineering in regenerative medicine[4]. During evolution, organs have come to perform complex functions, requiring an increased degree of information processing by neurons and a supply of nutrients by blood vessels [5]. Therefore, neurons, which arose earlier in evolution than vessel, may also should be important to construct functional artificial organs. No current organoid systems contain an integrated peripheral nervous system; however, Workman *et al*[6] successfully generated human-iPSC-derived intestinal tissue with a functional enteric nervous system[6]. The hepatic nervous system exerts important roles in the overall regulation of the organism, for example, in the glucose and lipid metabolism, circadian rhythm, regeneration, hepatic blood flow, food intake and obesity[7-9]. Development of vessels and BDs

in the liver have been thoroughly explored[10]. It has been known for many years that intrahepatic bile duct (IHBD) development is initiated near the hilum of the liver before progressing toward the periphery of the lobes[10]. At the late phase of mouse embryonic development, differentiated cholangiocytes [biliary epithelial cells (BECs)] are discontinuously scattered in the periportal mesenchyme and form ductal plate (DP) and biliary cysts followed by the development of ductal structure and incorporation into the continuous network structure after birth[11,12]. In addition, the angiogenic growth factors produced by periportal mesenchymal cells and BECs seem to provide a molecular link between the developing biliary and arterial structures[13]. However, the role of the hepatic nervous system has not been elucidated in liver development[14]. Recently, Tanimizu *et al*[15] reported that IHBDs guide the extension of nerve fibers (NFs) by secreting nerve growth factor (NGF) during their development. NFs with IHBDs extend through the PT from the hepatic hilus to the periphery[15]. But it has not been clarified if the hepatic nerve network plays important roles in the morphogenesis and stabilization of the PTs after birth.

In this work, we histologically observed the mouse liver and evaluated the developmental process of PTs, with a special focus on the hepatic nervous system in PTs and the correlation between NFs and BDs or vessels using immunohistochemistry. This study aims to explore the role of nerve cells in the development of the liver and whether the addition of nerve cells is useful for constructing liver organoids with PT structures.

MATERIALS AND METHODS

Animals

Pregnant female C57BL/6J mice were purchased from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan) to acquire embryos and newborn mice for the experiments. Adult male mice (8-wk-old) were also purchased from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan). The mice were bred and maintained according to the Yokohama City University institutional guidelines for the use of laboratory animals. All experimental procedures were approved by the institutional review board of the Animal Research Center, Yokohama City University School of Medicine (No. 075). Liver samples of C57BL/6J mice were collected at different developmental periods, beginning at embryonic day (E) 10.5 until 8-week-old [adult animals; postnatal day (P) 56]. Specifically, samples were collected at E10.5, E11.5, E12.5, E13.5, E15.5, E16.5, E17.5, P0, P3, P5, P7, P28, and P56 (n = 4 for each stage).

Hematoxylin-eosin staining and immunostaining

Neonatal and adult liver tissues were fixed in 4% paraformaldehyde and were paraffin-embedded. Then, 3-μm thin sections of cut surface through the hepatic hilus were examined for hematoxylin-eosin (HE) and immunostaining. The primary antibodies used for immunostaining are listed in Table 1. We used protein gene product 9.5 (PGP9.5) as a marker of neurons and cytokeratin 19 (CK19) as a marker of BEC. A preliminary immunohistochemical study was performed to select antibodies appropriate for identifying NFs. Control fetal liver specimens were immunohistochemically stained for a panel of antibodies for PGP9.5 (monoclonal, Abcam, Cambridge, MA, United States), S100 protein (polyclonal, Agilent, Santa Clara, CA, United States), and β tubulin III (monoclonal, Merk. Darmstadt, Germany). As a result, immunostaining of PGP9.5 most clearly and specifically identified NFs. Therefore, we used PGP9.5 for the study. SRY-related high mobility group-box gene 9 (SOX9), another BEC marker; hepatocyte nuclear factor 4α (HNF4α), a marker of hepatocytes; and Jagged-1, a Notch ligand, were also immunostained to observe the PT ontogeny.

Immunohistochemistry was performed using the Envision method according to the manufacturer's instructions (Agilent). Each thin section was deparaffinized, and antigens were retrieved for immunohistochemical reactions (pH 9.0, 20 min) by PT Link (Agilent). After blocking the endogenous peroxidase with hydrogen peroxide, slides were incubated with primary antibodies overnight at 4 °C. The sections were then washed, and the antigen was visualized using the DAKO Autostainer using the Envision flex kit (Agilent). The slides were counterstained with hematoxylin for 30 s and dehydrated and sealed with coverslips. The slides were then examined microscopically. Positive and negative control tissues were stained in each run. Images were acquired using a light microscope (Olympus Corporation, Tokyo, Japan).

The HAs in the PT were identified under HE staining as vessels with thick walls composed by a tunica media layer that contains concentric rings of smooth muscle.

Observations and quantitative analysis

It has been known for many years that IHBD development is initiated near the hilum of the liver before progressing toward the periphery of the lobes[10]. The development of IHBDs and NFs were examined near the hepatic hilus (center) and in the peripheral region (periphery) separately. Figure 1 indicates the center and the periphery. Inside and outside of the red line were defined as the center (around the hepatic hilus) and periphery, respectively (Figure 1). In addition, thin sections of the surface cut through the hepatic hilus (center) were examined using PGP9.5 and CK19 antibodies, which are markers of NFs

Table 1 Antibodies used for immunohistochemistry

Antibody	Origin	Dilution	Source
PGP9.5 (13C4/I3C4) monoclonal	Mouse	× 500	Abcam
CK-19 (EP1580Y) monoclonal	Rabbit	× 200	Abcam
SOX9 polyclonal	Rabbit	× 1000	Chemicon International
HNF4α (K9218) monoclonal	Mouse	× 100	Thermo Fisher Scientific
Jagged-1 (EPR4290) monoclonal	Rabbit	× 300	Abcam

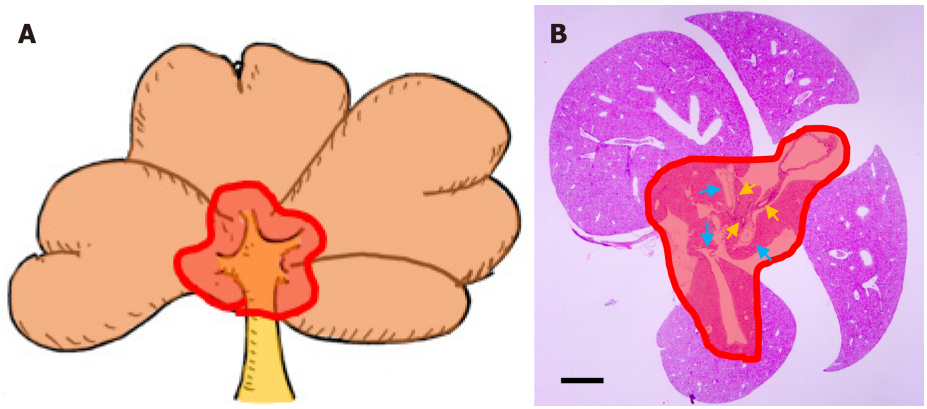

Figure 1 Anatomy of the mouse liver. Schema of the mouse liver and microphotograph of the mouse liver at postnatal day 7. The inside and outside of the red line were defined as the center (around the hepatic hilus) and periphery, respectively. Histologically, cytokeratin 19-positive hilar bile duct (yellow arrows) and portal vein (blue arrows) were found in the center. Scale bar = 1 mm. A: Schema of the mouse liver; B: Microphotograph of the mouse liver at postnatal day 7.

and BDs, respectively. Primitive BDs and NFs in the liver have been reported to emerge in the periportal mesenchyme around the newborn period. Then, PVs and periportal mesenchyme evolve into PTs. The numbers of NFs and BDs were separately counted in three randomly selected PVs with periportal tissue or a PT in the center and the periphery of the liver, with the average values being compared between the center and periphery at each developmental time point.

Quantification of the relationship between NFs and IHBDs or NFs and HAs in PTs was made by manually counting the numbers of NFs that contacted IHBDs or HAs in the same three selected PTs in which the number of NFs was counted using a light microscope.

Statistical analyses

means ± SD are typically reported. Statistical analyses were performed using the an unpaired-t test using GraphPad Prism v9.02 (GraphPad, La Jolla, CA, United States). Differences at $P < 0.05$ were considered to be statistically significant.

RESULTS

Histology of mouse PTs

PTs of adult mice (P56) consist of PV, HA, BD, lymphatic vessels, and autonomic nerve branches. These components are also found in human PTs. Many small NFs were observed (as PGP9.5-positive staining) around the PV, HA, and BD, and they produced plexuses (Figure 2).

Development of hepatic NFs and IHBD in the hepatogenesis

The hepatocyte maker HNF4α is already expressed in the nuclei of many cells in the primitive liver at E10.5; however, PGP9.5 expression was mainly found in the neural tube at the dorsal area of the embryo. PGP9.5 and CK19 were not found in the liver from E10.5 to E11.5, indicating that there is no evidence for the formation of NFs and BDs at this stage (Figure 3).

At E12.5, the Notch ligand Jagged-1 was diffusely positive in the liver, and SOX9-positive cells (BECs) were scattered in the periportal area; however, CK19- and PGP9.5-positive cells were not found in the fetal liver from E12.5 to E15.5 (Figure 4). Hilar BD (extrahepatic BD) strongly expressed SOX9 and weakly expressed CK19. PGP9.5-positive bodies were found in the mesenchyme around the hilar BD (Figure 4B).

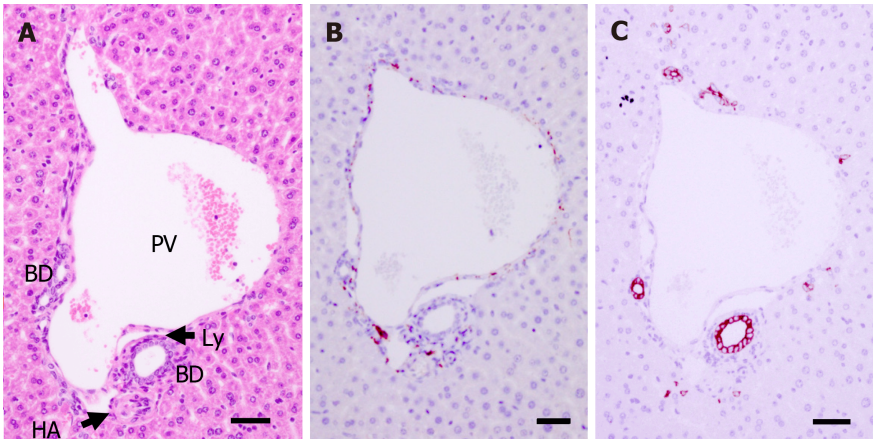

Figure 2 Histology of the portal tract of a postnatal day 56 mouse. A: Morphological analysis of a portal tract *via* hematoxylin-eosin staining; B and C: Neurons and bile ducts formed around the portal tract are shown by protein gene product 9.5 staining (B) and cytokeratin 19 staining (C), respectively. PV: Portal vein; HA: Hepatic artery; BD: Bile duct; Ly: Lymphatic vessel. Scale bar = 50 μm.

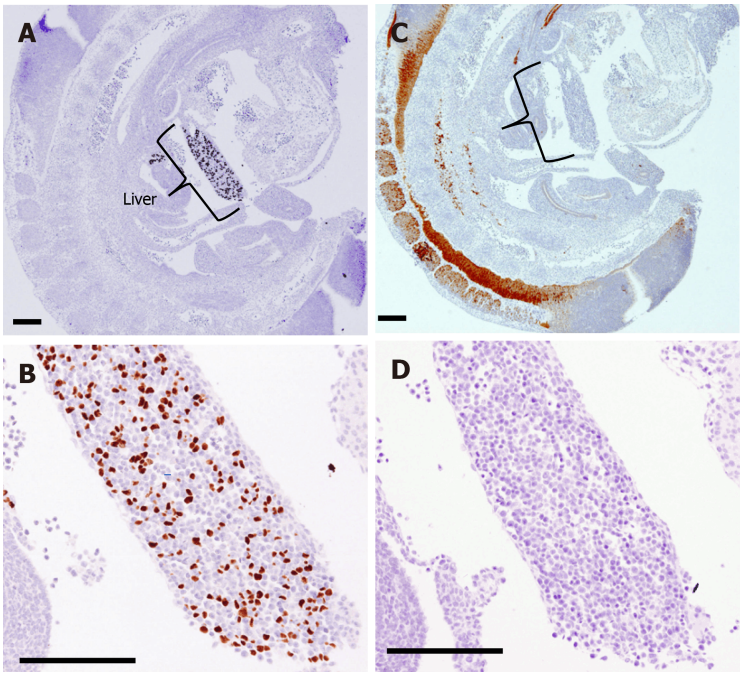

Figure 3 Immunohistochemical analysis of the liver at embryonic day 11.5 by hepatocyte nuclear factor 4α and protein gene product 9.5. A and B: Hepatic progenitors were observed by hepatocyte nuclear factor 4α (HNF4α) immunostaining; B: Liver tissue at a higher magnification than shown in A; C and D: Neurons or neural progenitors were observed by protein gene product 9.5 (PGP9.5) immunostaining; D: Liver tissue at a higher magnification than shown in C. HNF4α was expressed in the nuclei of many cells in the primitive liver; however, PGP9.5 expression was mainly found in the neural tube at the dorsal area of the embryo and but not found in the liver. Scale bar = 200 μm.

Subsequently, DPs and primitive IHBDs were formed around PVs from E16.5 to P3 (Figures 5 and 6). Jagged-1 was mainly expressed in the periportal tissue, and no cells in the periportal tissue expressed HNF4α in this phase. SOX9-positive cells formed DPs and primitive IHBDs, and CK19-positive cells were first expressed at E16.5 in several SOX9-positive ductal structures, especially around large PVs in the center. PGP9.5-positive bodies were also first found in the periportal tissue in the center at E16.5 (Figure 5). HA emerged at P0, and thus primitive PTs were constructed at first in the center. The numbers of NFs and CK19-positive IHBDs initially increased in the center and then increased in the periphery as well. Jagged-1-positive periportal tissue was thinner in the periphery than in the center, and the number of NFs and IHBDs was smaller in the periphery than in the center in this phase (Figure 6).

Along with periportal tissue remodeling, excess periportal cells underwent regression, and CK19-positive cells constructed IHBDs not only in the center but also in the periphery from P5 to P7. The distribution of PGP9.5-positive NFs was primarily found in contact with the HA and PV branches and less in contact with IHBD (Figure 7).

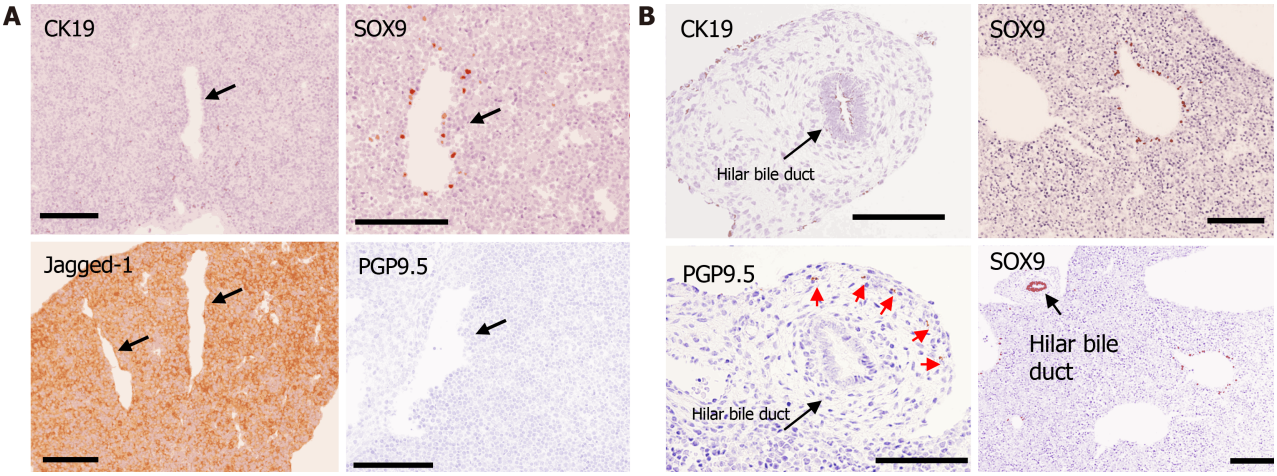

Figure 4 Immunohistochemical analysis. A: Immunohistochemical analysis of the liver at embryonic day 12.5. Jagged-1 was diffusely positive in the liver (lower left panel), and SRY-related high mobility group-box gene 9 (SOX9)-positive bile duct progenitor cells were found around the large vessels presumably defined as portal veins (upper right panel); however, cytokeratin 19 (CK19) (another marker for bile duct)- and protein gene product 9.5 (PGP9.5) (a marker for neurons)-positive cells were not found (shown in the upper left panel and lower right panels, respectively). Arrows indicate portal veins; B: Immunohistochemical analysis of the center of the liver at embryonic day 15.5. SOX9-positive bile duct progenitor cells were found around the large vessel presumably defined as portal veins. The hilar bile duct strongly expressed SOX9 and weakly expressed CK19. PGP9.5-positive bodies were only found in the parenchyma around the hilar bile duct (red arrow). Scale bars = 200 μm. CK19: Cytokeratin 19; SOX9: SRY-related high mobility group-box gene 9; PGP9.5: Protein gene product 9.5.

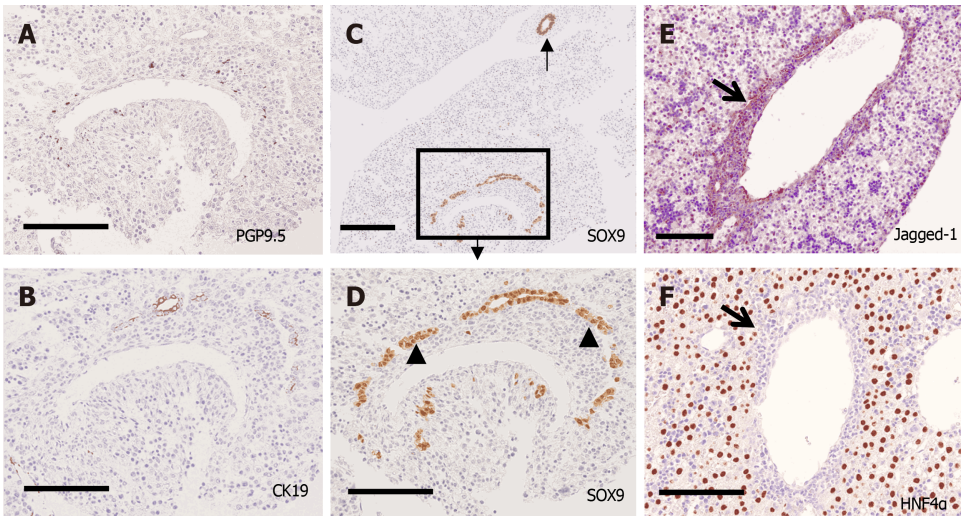

Figure 5 Immunohistochemical analysis of the center of the liver at embryonic day 16.5 and embryonic day 17.5. A-D: Embryonic day 16.5; E and F: E17.5; A: Protein gene product 9.5 (PGP9.5)-positive bodies were found in the periportal tissue; B: Cytokeratin 19 (CK19) was partly positive in SRY-related high mobility group-box gene 9 (SOX9)-positive structures; C and D: SOX9-positive cells partly from double-layered cylindrical structures called ductal plates (arrowhead) and partly form ductal structures; C: The arrow indicates the hilar bile duct; E: Jagged-1 was mainly expressed in the periportal tissue (thick arrow); F: Hepatocyte nuclear factor 4α was expressed in the liver parenchyma but not in the Jagged-1-positive periportal tissue (thick arrow). Scale bars = 200 μm. CK19: Cytokeratin 19; SOX9: SRY-related high mobility group-box gene 9; PGP9.5: Protein gene product 9.5; HNF4α: Hepatocyte nuclear factor 4α.

PT formation reached the adult level at P28. PV, BD, NF, and HA were found in PTs both in the center and periphery. According to PGP9.5-positive staining, many small NFs were found around the PVs, and they seemed to produce nerve plexuses surrounding the PVs. However, NFs were found only in the PTs and not in the liver parenchyma. Jagged-1 expression was mainly found in the BD and PV (Figure 8). HNF4α was clearly expressed in the nuclei of hepatocytes, and SOX9 was expressed in the nuclei of BEC during the entire period of this experiment.

Quantitative analysis of the NF and IHBD

NFs and BDs were first found in the periportal tissue of the center at E16.5. Figure 9 shows quantification of the NF and IHBD in a PT during mouse liver development. In our results, the entire process of PGP9.5-positive NFs and CK19-positive IHBD development progressed from around the hepatic hilus to the peripheral PTs. The numbers of NFs and IHBDs in the PT were larger in the center than in the periphery at the early phase of NF and IHBD development, and the differences were statistically

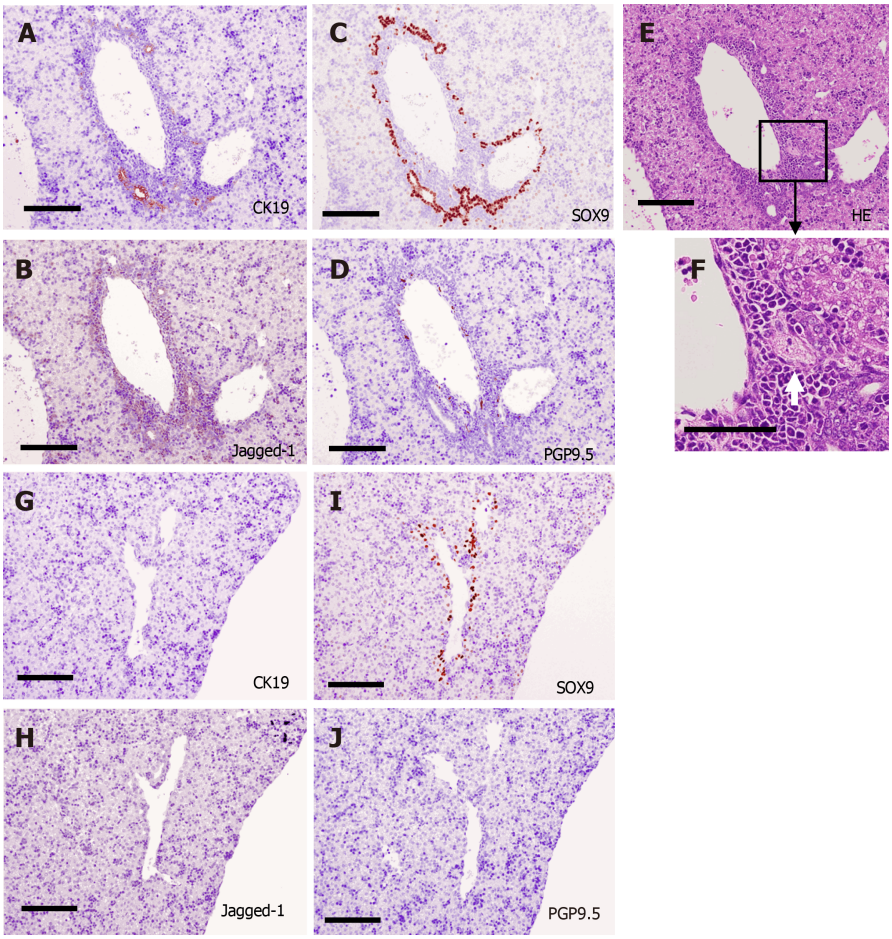

Figure 6 Immunohistochemical analysis of the area around the portal vein at postnatal day 0. A-F: Center of the liver; A-C: Many SRY-related high mobility group-box gene 9 (SOX9)-positive cells were found in Jagged-1-positive periportal tissue, and they formed cytokeratin 19 (CK19)-positive bile ducts in the center; D: Protein gene product 9.5 (PGP9.5)-positive nerve fibers were found in the periportal tissue in the center; E and F: Branching of the hepatic artery was found in the periportal tissue (white arrow); G-J: Periphery of the liver. The Jagged-1-positive periportal tissue was thinner in the periphery than in the center, and the number of biliary structures was smaller in the periphery. Neither CK19-positive bile ducts nor PGP9.5-positive nerve fibers were found in the periphery. Scale bars = 200 μm. HE: Hematoxylin-eosin; CK19: Cytokeratin 19; SOX9: SRY-related high mobility group-box gene 9; PGP9.5: Protein gene product 9.5.

significant at E17.5, P0, and P5 for NFs and at E17.5 for IHBDs. Their numbers gradually increased in the PTs until P7 and then plateaued in the center. On the other hand, their numbers increased even after P7 in the periphery. These results indicate that NFs first emerge at the center just before birth, extend toward the periphery with BDs after birth.

Quantification of NF-IHBD and NF-HA interactions

Quantification of NF-IHBD and NF-HA interactions in a PT was made by counting the numbers of NFs that contacted with IHBDs or HAs after birth (Figure 10). NF contacts were observed earlier with HA than with IHBD. The number of NF-HA contacts was larger than the number of NF-IHBD contacts in the center, and the difference was statistically significant from P0 to P7. NF contacts also emerged in the periphery after P3. The number of NF-HA contacts was larger than the number of NF-IHBD contacts in the periphery as well. These results indicate that NF-HA interactions may begin earlier than NF-IHBD interactions.

DISCUSSION

In-depth studies of both the differentiation and morphogenesis of the liver are prerequisites for *in vitro* and *in vivo* reconstitution of hepatic tissue for regenerative medicine. Our study describes morphological and immunohistochemical analyses mainly focused on the development of mouse intrahepatic nerve networks. Organogenesis of the liver and biliary tract occurs from the ventral posterior foregut endoderm near the cardiac mesenchyme as the hepatic diverticulum. The liver is largely composed of hepatocytes and cholangiocytes, which are differentiated from bipotent liver progenitors, the hepatoblasts[10]. It has been known for many years that BD development is initiated near the hepatic hilum

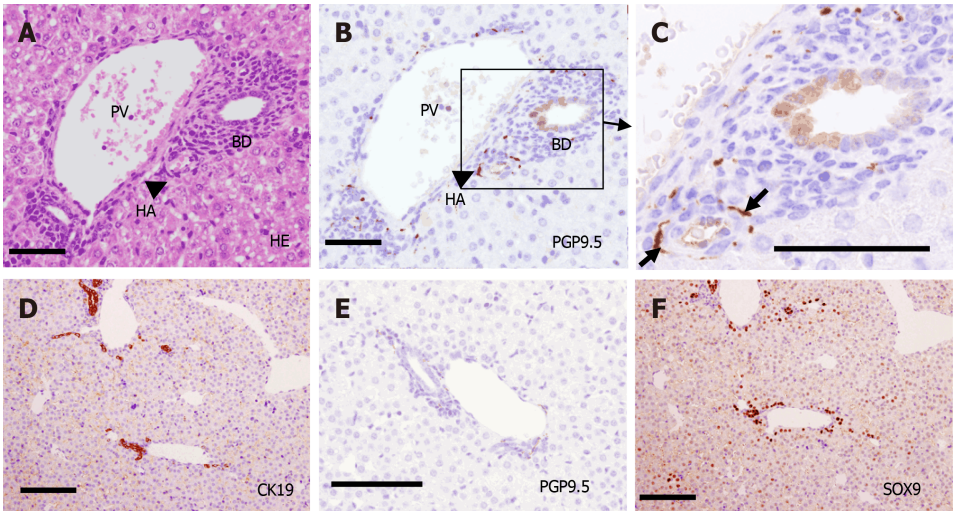

Figure 7 Immunohistochemical analysis of the portal tract at postnatal day 7. A-C: Center of the liver. Distribution of protein gene product 9.5 (PGP9.5)-positive nerve fibers were mainly observed around the hepatic artery (C, thick arrow) and portal vein branches, with less association with bile ducts (B and C); D-F: Periphery of the liver. SRY-related high mobility group-box gene 9-positive cells constructed cytokeratin 19 (CK19)-positive bile ducts not only in the center but also in the periphery. The number of PGP9.5-positive nerve fibers increased in the portal tracts even in the periphery. Scale bar = 200 μm. PV: Portal vein; HA: Hepatic artery; BD: Bile duct; HE: Hematoxylin-eosin; CK19: Cytokeratin 19; SOX9: SRY-related high mobility group-box gene 9; PGP9.5: Protein gene product 9.5.

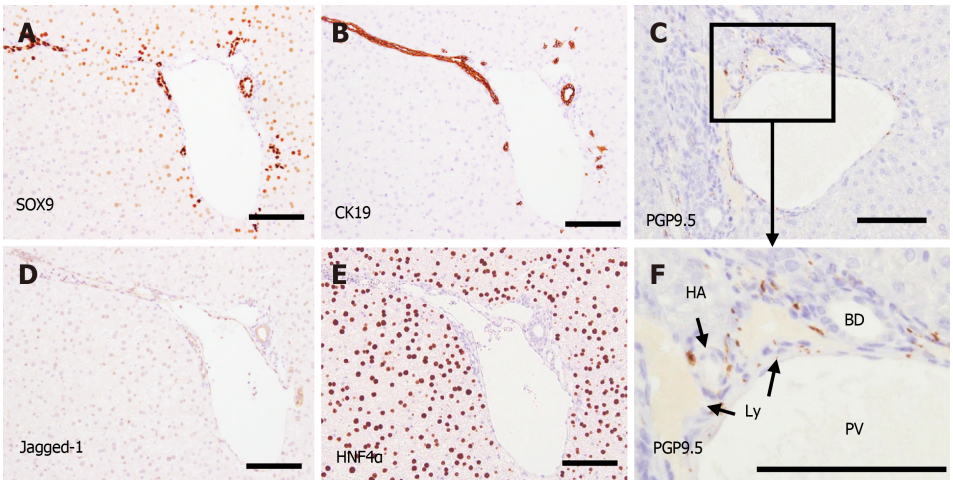

Figure 8 Immunohistochemical analysis of the portal tract in the periphery of the liver at postnatal day 28. A: SRY-related high mobility group-box gene 9 (SOX9) was expressed in the nuclei of biliary cells; B, C and F: Many cytokeratin 19-positive biliary cells (B) and protein gene product 9.5-positive nerve fibers (C and F) were found in the portal tracts even in the periphery. No nerve fibers were found in the liver parenchyma; D: Jagged-1 expression was mainly found in the bile ducts and vessels; E: Hepatocyte nuclear factor 4α was expressed in the entire liver parenchyma. Scale bars = 200 μm. PV: Portal vein; HA: Hepatic artery; BD: Bile duct; Ly: Lymphatic vessel; CK19: Cytokeratin 19; SOX9: SRY-related high mobility group-box gene 9; PGP9.5: Protein gene product 9.5.

before progressing toward the periphery of the lobes. Our understanding of the BD morphogenesis has recently improved with advanced three-dimensional imaging and computer-assisted analysis and with retrograde ink injection enabling visualization of the BD lumina in the whole liver[11,12,16,17]. However, the studies have not sufficiently investigated the embryological development of liver innervation.

Tanimizu *et al*[15] demonstrated that morphogenesis of IHBDs in the mouse liver gradually spread along the periportal tissue from the hepatic hilus toward the periphery, and the formation of nerve networks followed IHBD development after birth[15]. In addition, they suspected that IHBDs mainly guide the extension of NFs by secreting NGF during NF development in the mouse liver. We previously prepared total RNA from CD45- and Ter119-negative murine liver cells at various developmental stages (E10.5, E11.5, E13.5, E15.5, E17.5, E19.5, P0, P3, and postnatal week 8) using an RNeasy Mini Kit (Qiagen, Venlo, Netherlands). RNA for gene expression profiling was hybridized to the Whole Mouse Genome Agilent 4 × 44K v2 Oligonucleotide Microarray (Agilent) according to the manufacturer's instructions [3]. To analyze gene expression changes associated with liver development based on microarray data, the processed raw signal intensity of each probe was subjected to 75th percentile normalization. The microarray data also showed an elevated *Ngf* RNA level in the liver just before birth (Supplemen-

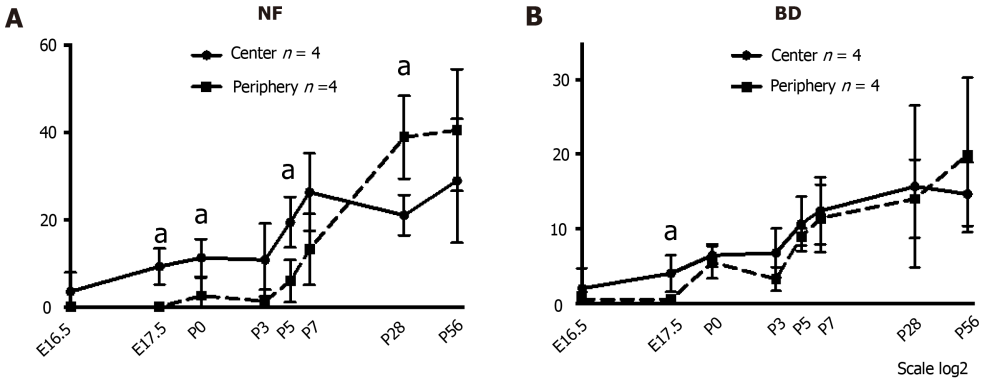

Figure 9 The number of the nerve fibers and intrahepatic bile ducts during murine liver development. A: NF; B: BD. Nerve fibers and bile ducts were first found in the periportal area of the center at embryonic day 16.5. The numbers of nerve fibers and bile ducts were significantly larger, especially at the early phase of development, in the center than in the periphery. Their numbers gradually increased in the portal tracts until postnatal day 7 in the center and increased thereafter in the periphery. NF: Nerve fiber; BD: Bile duct; E: Embryonic day; P: Postnatal day. $^{a}P < 0.05$.

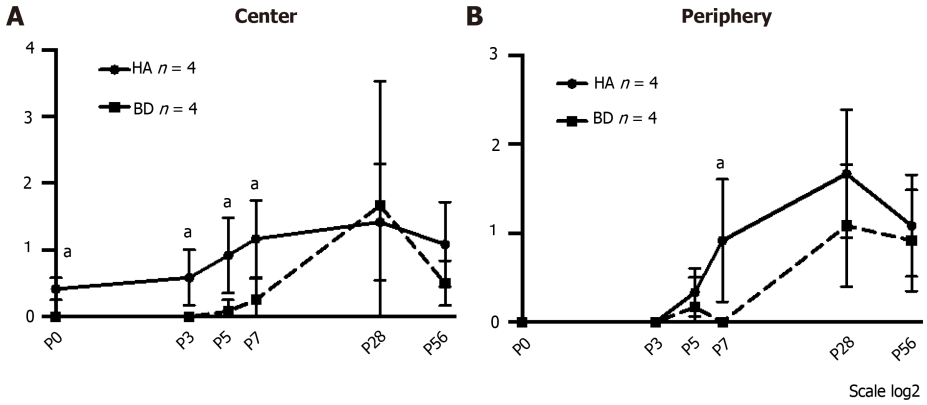

Figure 10 Number of contacts between nerve fibers and hepatic arteries or intrahepatic bile ducts. A: Center; B: Periphery. Nerve fiber (NF) contacts were observed at an earlier phase in the hepatic artery (HA) than in the intrahepatic bile duct (IHBD). The number of NF-HA contacts was significantly larger than that of NF-IHBD contacts from P0 to P7 in the center. NF contacts emerged also in the periphery after P3. The number of NF-HA contacts was larger than that of NF-IHBD contacts in the periphery as well. NF: Nerve fiber; BD: Bile duct; HA: Hepatic artery; E: Embryonic day; P: Postnatal day. $^{a}P < 0.05$.

tary Figure 1). However, our mouse histological and quantitative studies demonstrated that IHBDs and NFs simultaneously appeared in the periportal tissue around the hepatic hilus just before birth and thereafter gradually spread toward the periphery. This difference was thought to occur because we counted the number of IHBD using the BEC marker CK19, which is expressed at a later phase than SOX9.

The primary function of the fetal liver is hematopoiesis, and the liver receives little innervation during early development. After birth, the role of the liver changes to bile production, metabolism, and protein synthesis, and many different nerve types modulate these functions[18]. The innervation of the liver is different from that of the gastrointestinal tract, which consists of intrinsic ganglionated plexuses situated between the muscle layers of the gut wall. The liver is thought not to contain neural crest-derived intrinsic neurons[18]. For example, experimental studies using a quail/ chick interspecies grafting technique in the chicken embryos supported evidence for this apparent lack of intrinsic neurons in the liver[19]. An ontogenetic study of neuropeptide Y (Npy), a marker of sympathetic nerves showed that sympathetic NFs were not apparent for most of the embryonic period of development until E19. After birth, the density of sympathetic NFs increases to reach a maximum level at 1 wk. In conclusion, the authors suggested that sympathetic NFs derive from extrinsic sources because no neuronal somata were positive for Npy in the fetal liver. In addition, they suspected that sympathetic NFs might enter the liver *via* the hepatic hilus with HA and PV. Intriguingly, the density of Npy-positive NFs decreased after 1 wk and reached adult level at 2 wk postnatally. Therefore, they thought that transient Npy expression might play an important role in the developing liver[20,21]. In our experiment, neuronal marker-positive bodies were not found during most of the embryonic period in the liver and were only found in the parenchyma of the hilar BD until E15.5. Neuronal marker-positive bodies first emerged around the hepatic hilus at E16.5 and spread toward the periphery thereafter. These results are consistent with results of previous studies.

The NFs are classified by neurotransmitters into aminergic, cholinergic, peptidergic, and nitrergic NFs[22]. The distribution and role of each type of NF have been extensively studied. In addition, in the normal human adult liver, many types of immunoreactive peptidergic nerves have been detected[23-26]. Tiniakos *et al*[14] reported that Npy-positive NFs, which were examined closely in mouse liver by Ding *et al*[20,21], were the most abundant peptidergic NFs in the human adult liver and were distributed in PTs and in the acinus along sinusoids. However, no Npy-positive NFs were found in the human fetal liver[14]. On the other hand, during the third trimester, other peptidergic NFs were identified within PTs with a transient expression of galanin, somatostatin and calcitonin-gene-related peptide (CGRP) in the human fetal liver, but these NFs were not observed in the human adult liver. Therefore, the authors suspected that the intrahepatic peptidergic network might play an important role in liver morphogenesis, as Ding *et al*[20,21] suggested. Our previous microarray data in mice also showed transient elevation of *Npy* and *Cgrp* RNA levels around birth; however, galanin and somatostatin RNA was kept at a high level even after birth, different from human studies (Supplementary Figure 1).

The innervation of the mammalian liver is largely classified as either PT innervation or parenchymal innervation. Parenchymal innervation is composed of NFs present in the hepatic parenchyma. There are no significant differences in PT innervation among species. In contrast, parenchymal innervation is found in humans and guinea pigs but not found in mice and rats[27-30]. Our observations in mice also showed no NFs in the liver parenchyma even in the adult stage. Similarly, the timing of hepatic innervation is different among species. In some human histological studies, NFs emerge before birth in the human liver, much earlier than in the mouse liver. IHBD morphogenesis is thought to start in the fetal liver with the alignment of BEC, which constitute the double-layered cylindrical DP in the PT[17,31]. The DP and primitive IHBD in humans also emerge earlier than in the mouse, and the DP is first found at weeks of gestation (GW) 7 in the hepatic hilus[32,33]. However, many NFs were already found in the hepatic hilus at GW 7, and direct innervation into the DP has also been reported[22]. Therefore, the process of development of the intrahepatic nerve network in humans may be different from in the mouse liver. As already mentioned, many studies have reported that the liver does not appear to be colonized by intrinsic neurons[14,18-20]. To the contrary, Terada reported in a human study that a few neural marker-positive bodies emerged in large PTs at GW 8 and small PTs in the periphery at GW 11; then, the number increased thereafter. Therefore, the neural marker-positive bodies were thought to arise from the intrinsic portal mesenchyme to develop the nerve network in the human liver. However, how intrahepatic NFs connect with the extrinsic nervous system remains unclear[22].

In our study, during PT development, the PV was initially found as a large vessel and constituted a periportal tissue for the development of the IHBDs, HA branching, and NFs. Fabris *et al*[13] reported that the periportal mesenchyme instructs IHBD development, and VEGF secreted from BECs promotes HA morphogenesis. The interaction between developing BDs and HAs may slightly differ between humans and mice, since arterial morphogenesis in humans occurs along the DP in the fetal liver[34], whereas in mice, HAs emerged around birth in the periportal tissue in our study. Tiniakos *et al*[30] reported that adrenergic nerves form an intrinsic plexus around the walls of blood vessels but less frequently in relation to BD radicles in PTs in their rodent study. They also showed in their human study a rich neural supply of PTs, mainly around HA and PV branches with less in relation to intralobular BDs[14]. Our study also indicated that NF-HA contacts occur more frequently than NF-BD contacts in the early period of PT development. In adult peripheral tissues, nerves often run along larger blood vessels, reflecting their need for oxygen and nutrients, as well as their physiological control of vasoconstriction and dilation[35]. Similarly, NFs in PTs also regulate hemodynamics in the liver[26]. Arterial vessels are reported to secrete neurotrophic factors such as NGF, neurotrophin 3 (NTF3), and brain-derived neurotrophic factor in some organs[5,36]. A study by Mukouyama *et al*[37] suggested that VEGF secretion from peripheral nerves provides a template that determines the organotypic pattern of blood vessel branching and arterial differentiation in the skin. Brunet *et al*[38] indicated that the sympathetic innervation of arteries was facilitated by secretion of the axon guidance molecule netrin-1 by arterial vascular smooth muscle cells in the mesentery. As already mentioned, several key molecules which guide NFs and HA development have already been determined in the BECs[15,17]. On the other hand, the molecules that co-regulate NF and HA development still have not been found. However, the present study showed NF-HA contacts found in an earlier phase than NF-BD contacts and our previous microarray data indicated elevation of *Ntf3* and *Netrin1* RNA level around birth (Supplementary Figure 1). These results suggest that not only BECs but also HAs guide the NF development and the NF-HA interaction strongly influences PT morphogenesis and liver development. Small blood vessels such as capillaries are thought to be stabilized by covering endothelial cells with pericytes[4]. Considering that the BD formation was followed by the development of a neural network, NF may also play a role in stabilizing PT structure by contacting intralobular vessels and BDs within the PT.

Finally, hepatic autonomic nerves are thought to play a role in the liver regeneration. Denervation of the liver NF by vagotomy, operations, or transplantation causes no significant problems in liver functions. This indicates that the autonomous nervous system is not very important regarding lifestyle and life expectancy. However, Hamada *et al*[39] reported that total hepatic denervation enhanced liver regeneration after a partial hepatectomy. On the contrary, Ikeda *et al*[40] reported that the parasympathetic system (vagus nerve) contributed to liver regeneration after hepatectomy by stimulating

interleukin-6 release from Kupffer cells followed by signal transducer and activator of transcription 3 activation in hepatocytes using mouse experiments. Izumi *et al*[41] also showed that a vagus-macrophage-hepatocyte link regulates acute liver regeneration after liver injury in mice. Histologically, the DP is directory innervated in a report of Terada. The neural cell adhesion molecule, chromogranin, and synaptophysin are among the markers of hepatic stem/precursor cells (HSPCs). In that study, DP cells were labeled by these molecules, indicating that the DPs contained many HSPCs; therefore, the development of HSPCs might be controlled by NF in the fetus life[22]. Zanchi *et al*[42] reported the possibility of a widespread interface between nerves and the smallest branches of the proximal biliary tree in human. HSPCs are also thought to be located in the canal of Hering, which is included in the proximal biliary tree in human adult liver. These results suggest that hepatic autonomic nerves impact the development of the liver.

CONCLUSION

In conclusion, hepatic NFs first emerge at the center just before birth and extend toward the periphery with the IHBDs mainly after birth in mice. Our work and previous reports provide the possibility that the nerve network in the liver plays an important role not only in liver function but also in liver morphogenesis and stabilization of PT structures by interaction between NFs, BDs, and HAs. Furthermore, NFs in the liver may regulate liver progenitor cells. Therefore, nerve progenitor cells may be an additional cell source, along with hepatic, endothelial, and mesenchymal progenitor cells, when liver organoids are constructed.

ARTICLE HIGHLIGHTS

Research background

The hepatic nervous system plays important roles in organisms. However, the role of the hepatic nervous system in liver development remains unclear.

Research motivation

We previously created functional human micro-hepatic tissue in mice by co-culturing human hepatic endodermal cells with endothelial and mesenchymal cells. However, they lacked Glisson's sheath [the portal tract (PT)]. The PT consists of branches of the hepatic artery (HA), portal vein, and intrahepatic bile duct (IHBD), collectively called the portal triad, together with autonomic nerves. In-depth studies of both the differentiation and morphogenesis of the liver are prerequisites for *in vitro* and *in vivo* reconstitution of hepatic tissue for regenerative medicine.

Research objectives

This study describes morphological and immunohistochemical analyses, mainly focusing on the development of mouse intrahepatic nerve networks.

Research methods

Liver samples from C57BL/6J mice were harvested at different developmental time periods, from embryonic day (E) 10.5 to postnatal day (P) 56. Thin sections of the surface cut through the hepatic hilus were examined using protein gene product 9.5 (PGP9.5) and cytokeratin 19 (CK19) antibodies, markers of nerve fibers (NFs), and biliary epithelial cells. The numbers of NFs and IHBDs were separately counted in a PT around the hepatic hilus (center) and the peripheral area (periphery) of the liver, comparing the average values between the center and the periphery at each developmental stage. NF-IHBD and NF-HA contacts in a PT were also counted, and their relationship was quantified.

Research results

Primitive IHBDs at the center partly acquired CK19 positivity at E16.5. PGP9.5-positive bodies were first observed at this time point, and HAs were first detected at P0 in the periportal tissue of the center. Therefore, primitive PT structures were first constructed at P0 in the center. Along with remodeling of the periportal tissue, the number of CK19-positive IHBDs and PGP9.5-positive NFs gradually increased, and PTs also formed in the periphery until P5. The numbers of NFs and IHBDs were significantly higher in the center than in the periphery from E16.5 to P5. The numbers of NFs and IHBDs reached the adult level at P28, with fewer differences between the center and periphery. NFs were more frequently associated with HAs than IHBDs in PTs at the early phase after birth, after which the number of NF-IHBD contacts gradually increased.

Research conclusions

Mouse hepatic NFs first emerge at the center just before birth and extend toward the periphery. The interaction between NFs and IHBDs or HAs plays important roles in the morphogenesis and stabilization of the PT structure by interaction between NFs, BDs, and HAs.

Research perspectives

Nerve progenitor cells may be an additional cell source, along with hepatic, endothelial, and mesenchymal progenitor cells, when liver organoids are constructed.

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FOOTNOTES

Author contributions: Koike N and Taniguchi H designed and coordinated the study; Koike N, Kobayashi T and Okamoto S performed immunohistochemical study and analyzed data; Tadokoro T and Murata S performed animal experiments; Ueno Y performed genetic analysis; Koike N wrote the manuscript; all authors approved the final version of the article.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Takotsubo cardiomyopathy in orthotopic liver transplant recipients: A cohort study using multi-center pooled electronic health record data

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Abstract

BACKGROUND

Takotsubo cardiomyopathy (TCM), or stress-induced cardiomyopathy, is associated with adverse prognosis. Limited data suggest that TCM occurring in orthotopic liver transplant (OLT) recipients is associated with elevated peri-operative risk.

AIM

To characterize the predictors of TCM in OLT recipients, using a large, multi-center pooled electronic health database.

METHODS

A multi-institutional database (Explorys Inc, Cleveland, OH, USA), an aggregate of de-identified electronic health record data from 26 United States healthcare systems was surveyed. A cohort of patients with a Systematized Nomenclature of Medicine-Clinical Terms of "liver transplant" between 09/2015 and 09/2020 was identified. Subsequently, individuals who developed a new diagnosis of TCM following OLT were identified. Furthermore, the risk associations with TCM among this patient population were characterized using linear regression.

RESULTS

Between 09/2015 and 09/2020, of 37718540 patients in the database, 38740 (0.10%) had a history of OLT (60.6% had an age between 18-65 years, 58.1% female). A new diagnosis of TCM was identified in 0.3% of OLT recipients (45.5% had an age between 18-65 years, 72.7% female), compared to 0.04% in non-OLT patients [odds ratio (OR): 7.98, 95% confidence intervals: 6.62-9.63, (P < 0.0001)]. OLT recipients who developed TCM, compared to those who did not, were more likely to be greater than 65 years of age, Caucasian, and female (P < 0.05). There was also a significant association with cardiac arrhythmias, especially ventricular arrhythmias (P < 0.0001).

CONCLUSION

TCM was significantly more likely to occur in LT recipients *vs* non-recipients. Older age, Caucasian ethnicity, female gender, and presence of arrhythmias were significantly associated with TCM in LT recipients.

Key Words: Takotsubo cardiomyopathy; Orthotopic liver transplant; Stress-induced cardiomyopathy; Clinical outcomes

Core Tip: In a large cohort study using de-identified pooled electronic health record data, liver transplant recipients were more likely to develop Takotsubo cardiomyopathy compared to non-recipients. Older age, Caucasian ethnicity, female gender, and presence of arrhythmias were significantly associated with Takotsubo cardiomyopathy in liver transplant recipients.

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INTRODUCTION

Takotsubo cardiomyopathy (TCM) is a stress-induced, reversible cardiomyopathy that occurs in the absence of significant coronary artery disease[1]. The awareness of TCM, which was first reported in Japan in 1990, has increased rapidly and several nomenclatures for this condition have been proposed including stress-induced cardiomyopathy, apical ballooning syndrome, left ventricular ballooning, and broken heart syndrome[2,3]. TCM is believed to be related to the presence of an underlying pathological stress, whether physical or emotional[1]. Despite the enormous attention that this condition has gained in recent years, TCM is still considered to be underdiagnosed, with an underestimated risk and incompletely understood pathogenesis[4].

It is hypothesized that emotional or physical stress may trigger a surge in catecholamine secretion, which in turn can lead to microvascular spasms and myocardial stunning *via* interaction with beta-adrenergic receptors, resulting in left ventricular systolic dysfunction[5]. Patients undergoing major surgery often have increased concentrations of catecholamines, caused by the physical and emotional stress of the perioperative period, which can contribute to the development of TCM[6]. Although TCM is self-limited and resolves completely without an adverse event in the majority of affected patients, it may result in significant morbidity and mortality in critically ill patients, such as liver transplant recipients, with estimated mortality rate of 10%-27%[7].

Small observational studies and case series have reported the occurrence of TCM in liver transplant recipients, but large cohort studies evaluating this association are lacking in the literature[6]. In addition to the stress imposed by the perioperative period, liver transplant candidates are particularly vulnerable to developing TCM due to the impaired stress response in the inflammatory milieu of hepatic cirrhosis

[8,9]. Therefore, we aimed to study TCM in liver transplant recipients, providing information about the demographic characteristics of these patients, and delineating this unique patient population's risk factors for TCM.

MATERIALS AND METHODS

Database

We conducted a retrospective, cohort study using a multicenter analytics and research platform developed by IBM Watson Health (Explorys Inc, Cleveland, OH, USA)[10]. At present, Explorys captures more than 70 million unique patients across all 50 states, and thus provides a broad regional and climatic distribution of source population. Diagnoses, findings, and procedures are arranged into the Systematized Nomenclature of Medicine - Clinical Terms (SNOMED-CT) hierarchy while prescription drug orders are mapped into SNOMED and RxNorm[11,12]. Patients with all types of insurance as well as those who self-pay are represented. Ethical review and informed consent were waived, since there are no identifiers associated with any of the patient data. The Explorys rounds cell counts to the nearest 10 and treats all cell counts between zero and 10 as equivalent in order to protect the identities of patients. The Explorys database has been used in multiple publications in gastroenterology, cardiology, oncology, neurology, and surgery[13,14].

Patient selection

Using the Explorys search tool, we identified all active patients in Explorys between 09/2015 and 09/2020 and selected those who underwent liver transplantation. Subsequently, a cohort of patients with a SNOMET-CT diagnosis of "takotsubo cardiomyopathy" was identified. Cases were compared to those who underwent liver transplantation without a history of TCM. Using SNOMED-CT codes, we identified possible associated medical conditions as well as disease outcomes.

Statistical analysis

Demographics and associated diseases were characterized by descriptive statistics. The overall period prevalence was calculated by dividing the total number of individuals with TCM by the total number of individuals in Explorys who underwent liver transplantation (2015-2020). The odds ratio (OR) for univariate analysis, its standard error and 95% confidence intervals (CI) were calculated according to Altman, 1991, using the MedCalc Statistical Software for Windows, version 19.4 (MedCalc Software, Ostend, Belgium) with a case-control design[15]. For all analyses, a 2-sided P value of <0.05 was considered statistically significant.

RESULTS

There were a total of 37718540 individuals in the database (2015-2020) with 38,740 (0.1%) who underwent liver transplantation. Baseline characteristics of patients with liver transplant and control groups are demonstrated in Table 1. The majority of patient who underwent OLT were adults (18-65 years old), female (58.1%), and Caucasian (77.8%). Among those who underwent liver transplantation, there were 110 patients with a diagnosis of TCM with a period prevalence rate of 0.3%. Rates of TCM among OLT patients and timing of diagnosis are shown in Figures 1 and 2, respectively.

Interval epidemiology and underlying associations of Takotsubo cardiomyopathy in OLT.

Of the 110 patients with the diagnosis of TCM, the majority were elderly (> 65 years old) (54.5%), female (72.7%), and Caucasian (90.9%) (Table 2). Patients with a diagnosis of TCM were more likely to have other medical diseases including hypertension (OR: 1.82, 95%CIs: 1.23-2.68, $P = 0.0027$), hyperlipidemia (OR: 1.68, 95%CIs: 1.14-2.48, $P = 0.009$), atherosclerosis (OR: 2.35, 95%CIs: 1.54-3.58, $P = 0.0001$), coronary artery disease (OR: 2.15, 95%CIs: 1.45-3.18, $P = 0.0001$), chronic kidney disease (OR: 2.27, 95%CIs: 1.56-3.31, $P < 0.0001$), sepsis (OR: 5.90, 95%CIs: 4.05-8.60, $P < 0.0001$), anxiety (OR: 2.76, 95%CIs: 1.90-4.02, $P < 0.0001$), and mood disorders (OR: 2.00, 95%CIs: 1.38-2.92, $P = 0.0003$) (Figure 3).

Outcomes of Takotsubo cardiomyopathy among patients with OLT.

Among patients with a history of OLT, patients who were diagnosed with TCM were more likely to have cardiogenic shock (OR: 12.61, 95%CIs: 6.52-24.4, $P < 0.0001$), and to require mechanical circulatory support with an intra-aortic balloon bump (OR: 19.22, 95%CIs: 7.66-48.21, $P < 0.0001$). These patients were also at a higher risk of developing cardiac arrest (OR: 9.52, 95%CIs: 5.84-15.52, $P < 0.0001$). Other complications include cerebrovascular accidents, liver failure, gastrointestinal bleeding, and an increased requirement of invasive mechanical ventilation and renal replacement therapy (Figure 4).

Table 1 Baseline characteristics of patients with liver transplant versus those without liver transplant (control group)

	Liver transplant		No liver transplant	
	n = 38740	%	*n* = 37679800	%
Takotsubo cardiomyopathy	110	0.3	13430	0.0
Age > 65	14780	38.2	8349380	22.2
Age 18-65	23470	60.6	22449470	59.6
Age < 18	500	1.3	6659540	17.7
Male	16230	41.9	16676780	44.3
Female	22510	58.1	20665100	54.8
Caucasian	30150	77.8	22446830	59.6
African American	6060	15.6	4315410	11.5
Obesity	840	2.2	5018440	13.3
Cardiomyopathy	1770	4.6	762420	2.0
Hypertension	390	1.0	3465120	9.2
Diabetes Mellitus	710	1.8	5484800	14.6
Hyperlipidemia	1220	3.1	10758820	28.6
Atherosclerosis	770	2.0	3848890	10.2
Coronary artery disease	730	1.9	3395870	9.0
Myocardial infarction	430	1.1	1528310	4.1
Ischemic heart disease	620	1.6	2285770	6.1
Chronic kidney disease	530	1.4	2052700	5.4
Alcohol abuse	80	0.2	1013590	2.7
Smoking	290	0.7	6369120	16.9
Sepsis	690	1.8	2101780	5.6
Atrial fibrillation	1030	2.7	2659760	7.1
Supraventricular arrhythmia	200	0.5	75580	0.2
Ventricular arrhythmia	590	1.5	415060	1.1

DISCUSSION

The diagnosis of end-stage liver disease (ESLD) carries a poor prognosis and is associated with increased cardiovascular risk[16]. It is well-known that orthotopic liver transplantation (OLT) is the treatment of choice for patients with irreversible ESLD due to the improved survival after transplantation[17]. Nowadays, with the high prevalence of ESLD, increasing numbers of patients are being referred for OLT[18,19]. Although OLT improves the survival of ESLD patients, post-operative complications that affect the outcomes and survival of this patient group may occur, including cardiac complications, such as TCM[6,20]. As such, it is imperative to perform careful preoperative cardiac risk evaluation prior to the transplantation[21].

The current study is the first national database study to assess the association between liver transplantation and the development of TCM. There are several important findings in this retrospective observational study. We found that liver transplant recipients were more likely to develop TCM compared to non-recipients. It is conceivable that OLT predisposes to TCM from a pathophysiologic standpoint, given the increased levels of stress, and thus, a higher catecholamine surge around the time of liver transplant surgery.

On further analysis of OLT subgroup based on occurrence of TCM, we found that the group of patients who developed TCM were more likely to be female, Caucasian, and elderly. This is consistent with prior epidemiological and clinical studies[22-24]. In a retrospective observational study that looked at various co-morbidities, it was found that patients with certain co-morbid conditions were more likely to have concurrent TCM, compared with age-matched control groups[25]. In that study, it was reported that sepsis, cerebrovascular accident, malignancy, and anxiety disorder increased the risk of TCM with an OR 13.94 (95%CI: 11.69-16.62), 10.81 (95%CI: 8.70-13.43), 1.73 (95%CI: 1.63-1.83), and 2.54 (95%CI:

Table 2 Baseline characteristics of patients with Takotsubo cardiomyopathy following liver transplant versus those without a history of Takotsubo cardiomyopathy following liver transplant (control group)

	Takotsubo cardiomyopathy		No takotsubo cardiomyopathy		*P* value
	n = 110	%	*n* = 38640	%	
Age > 65	60	54.5	14720	38.1	0.0005
Age 18-65	50	45.5	23400	60.6	0.0014
Age < 18	0	0.0	500	1.3	
Male	30	27.3	16200	41.9	0.0022
Female	80	72.7	22440	58.1	0.0022
Caucasian	100	90.9	30060	77.8	0.0016
African American	5	4.5	6060	15.7	0.0042
Obesity	30	27.3	10500	27.2	0.9814
Hypertension	40	36.4	9250	23.9	0.0027
Diabetes mellitus	60	54.5	17910	46.4	0.0867
Hyperlipidemia	70	63.6	19720	51.0	0.009
Atherosclerosis	30	27.3	5320	13.8	0.0001
Coronary artery disease	50	45.5	12280	31.8	0.0001
Chronic kidney disease	60	54.5	13350	34.5	< 0.0001
Alcohol abuse	10	9.1	3060	7.9	0.6499
Smoking	40	36.4	10930	28.3	0.0621
Sepsis	60	54.5	6530	16.9	< 0.0001
Atrial fibrillation	50	45.5	5470	14.2	< 0.0001
Supraventricular arrhythmia	60	54.5	6740	17.4	< 0.0001
Ventricular arrhythmia	5	4.5	210	0.5	< 0.0001
Anxiety disorder	60	54.5	11700	30.3	< 0.0001
Mood disorder	60	54.5	14470	37.4	0.0003
Seizure disorder	10	9.1	1750	4.5	0.025

2.34-2.75), respectively. Patients who developed TCM in our study were also more likely to have chronic medical conditions, which may have possibly predisposed them to this condition *via* coronary microcirculatory dysfunction, one of the mechanisms that was proposed as a contributor in the pathogenesis of TCM[26]. Importantly, patients who developed TCM were found to have higher rates of arrhythmias, including those of atrial and ventricular origin, which is an important finding, and may contribute to higher morbidity and mortality in this group of patients[27,28]. It is well-known that infection and critical illness are associated with development of TCM, which may explain the finding of a higher association with sepsis in our TCM cohort[29-31]. Nonetheless, it is unclear whether sepsis preceded the occurrence of TCM, given the limitations of the database utilized in this study.

Furthermore, we found an increasing prevalence of TCM in our studied population of OLT recipients between 2015 and 2020. This higher event rate may be attributed to the increasing numbers of patients undergoing OLT, and perhaps more importantly, better recognition and improved diagnosis of the syndrome. Additionally, data from observational studies reported that coronavirus disease 2019 (COVID-19) pandemic, which started in early 2020, may have contributed to the higher incidence of TCM[32-34]. The increased incidence was seen in both the general population and COVID-19 patients, which was linked to increased psychological distress as well as heightened sympathetic responses, cytokine storm, and microvascular dysfunction seen in COVID-19 patients[32]. The adverse effects on mental health may be consequences of social distancing, economic worry, and fear of contracting the virus, among other concerns. The association between COVID-19 and TCM may be explained by potential pathophysiological links between the two conditions. Though these direct connections are not fully understood, different mechanisms were proposed for this association. First, the heightened release of pro-inflammatory cytokines and chemokines seen in some COVID-19 patients can trigger myocardial injury that may lead to TCM[35]. Second, the increased sympathetic nervous system activity, noted in

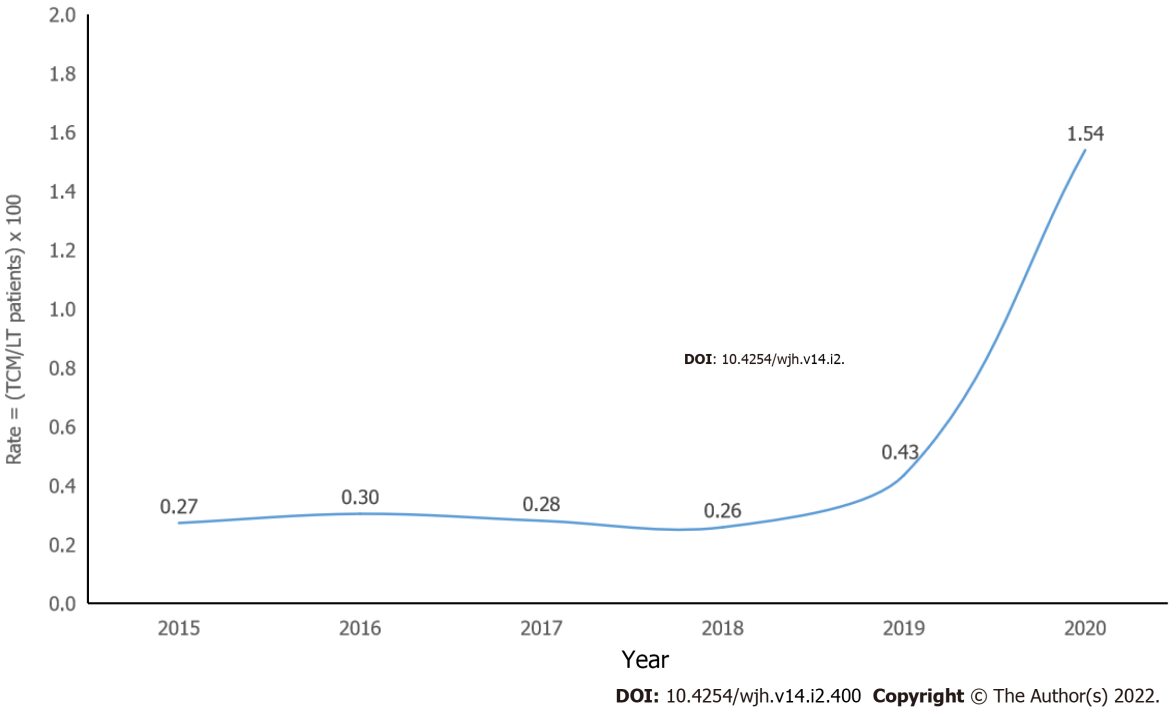

Figure 1 Rates of Takotsubo cardiomyopathy among liver transplant patients between 2015 and 2020. LT: Liver transplant; TCM: Takotsubo cardiomyopathy.

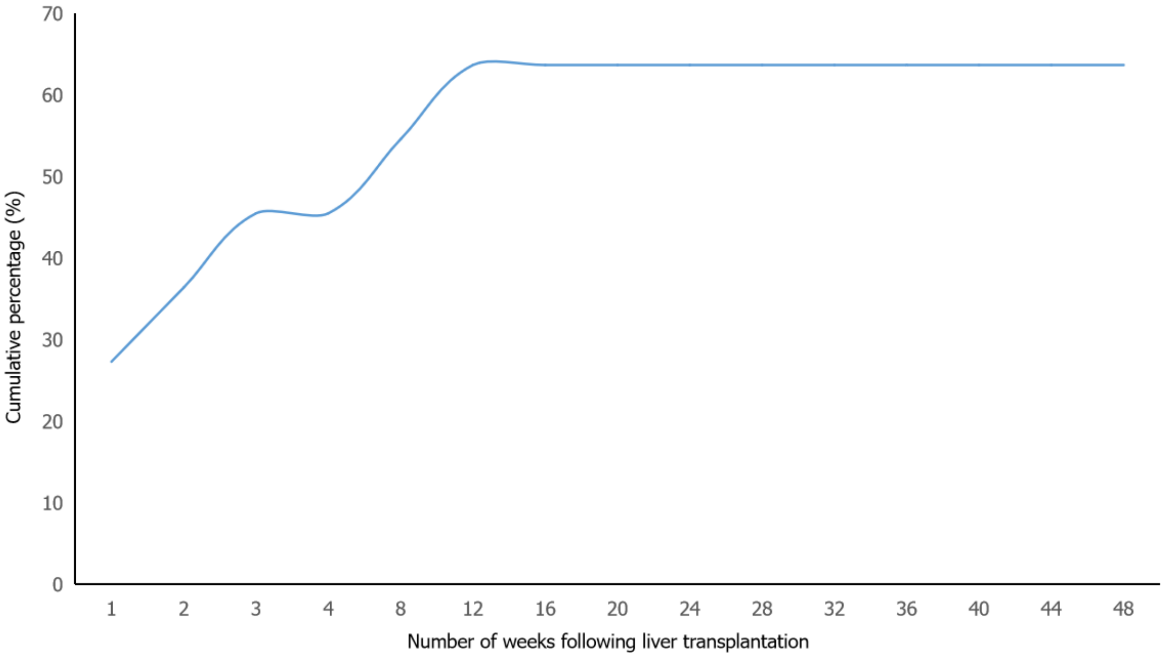

Figure 2 Timing of Takotsubo cardiomyopathy diagnosis after liver transplantation shown as the cumulative percentage of the total number of cases diagnosed in the study period (*n* = 110).

some COVID-19 patients, may result in a catecholamine-induced myocardial stunning, and subsequently stress-induced cardiomyopathy[36]. Last, microvascular dysfunction has been reported in some cases of COVID-19 infection and was attributed to virus-induced systemic inflammatory response and coagulopathy. This microvascular dysfunction has been proposed as a potential mechanism for COVID-associated TCM[37]. Previous reports have shown that patients with COVID-19 infection may demonstrate various histopathological findings on postmortem examinations, including but not limited to, myocyte necrosis, inflammatory cell infiltration, lymphocytic or eosinophilic myocarditis, among

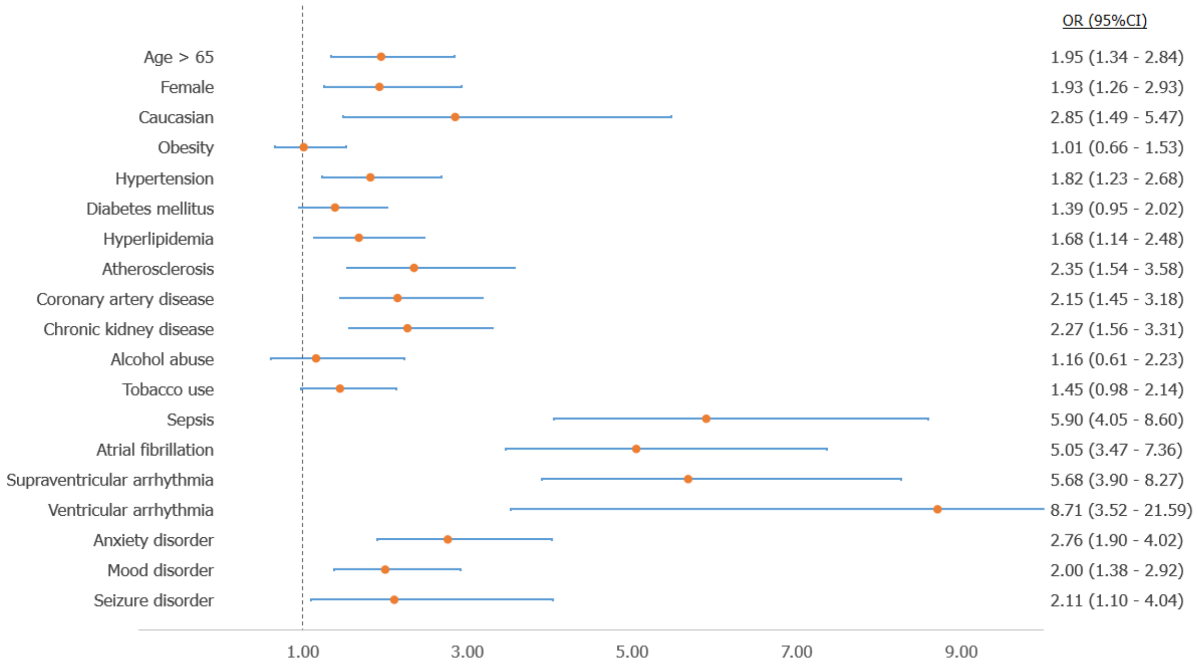

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Figure 3 Predictors of Takotsubo cardiomyopathy for liver transplant patients.

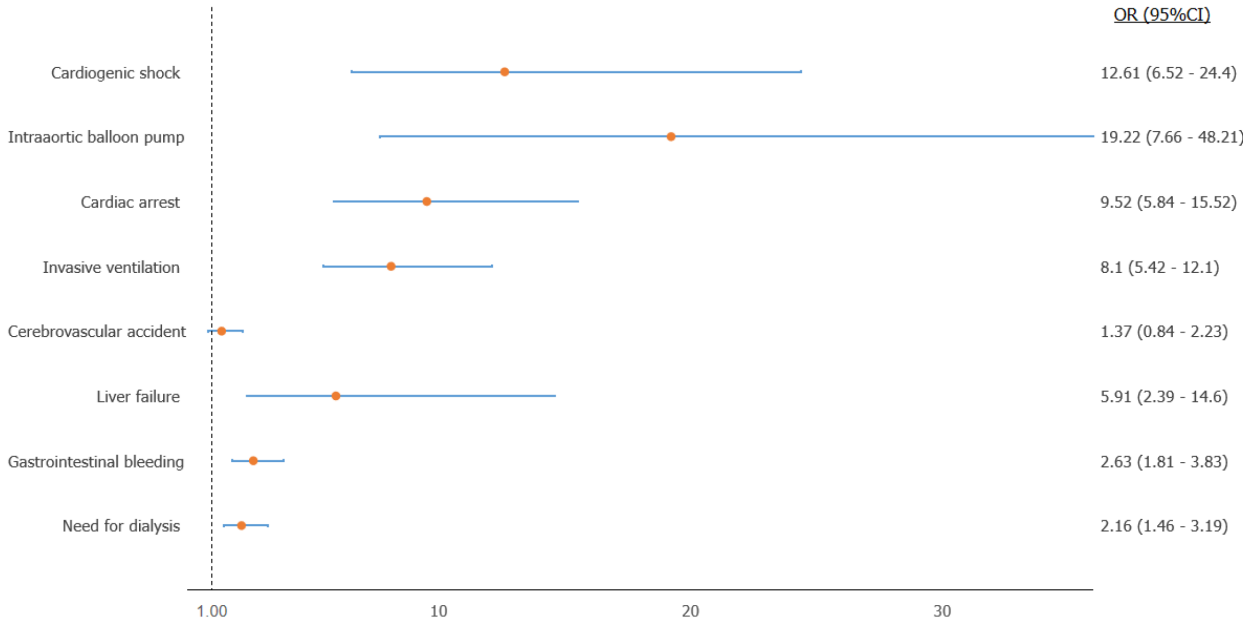

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Figure 4 Clinical outcomes for patients developing Takotsubo cardiomyopathy following liver transplant.

others[38]. Whether these findings are associated with, or increase the risk of, developing TCM remains largely unknown.

Another key finding in our study was that patients who developed TCM had significantly higher rates of life-threatening complications and adverse events, including cardiogenic shock, ventricular arrhythmias, cardiac arrest, respiratory failure requiring invasive mechanical ventilation, acute kidney injury requiring renal replacement therapy, as well as ischemic cerebrovascular accidents. These findings are consistent with prior reports of increased in-hospital complications among patients who developed TCM[1,39-41]. Nonetheless, the observed differences in baseline comorbidities between the two study groups of OLT recipients could also represent a driving factor for the higher in-hospital morbidity and adverse events presenting in patients who developed TCM. Regardless of the exact etiology and pathogenesis, recognition of TCM as a potential postoperative complication in OLT

recipients is pivotal, given its implications on patient outcomes with higher rates of in-hospital complications. Prior studies showed that despite a better recognition of the syndrome, short-term mortality rates remained relatively high[1].

There are several limitations to the current study due to the nature and well□described shortcomings of database studies. First, there is an inability to verify the accuracy of diagnoses or outcomes with potential errors in coding of diseases or procedures. Second, the database does not capture variables related to the severity of TCM (*e.g.*, left ventricular ejection fraction), cardiac-imaging data, use of antithrombotic agents or inotropes, and long-term outcomes. As the database also does not provide information regarding the temporal relationship between diagnoses, it is not possible to reliably distinguish in-hospital complications from comorbidities using this database. Third, owing to the observational nature, our study is subject to traditional biases, such as selection bias. Moreover, the differences reported during comparison of outcomes are subject to residual confounding. Fourth, the lack of follow-up data of patients limited our ability to report outcomes after hospital discharge for patients who developed TCM following OLT.

CONCLUSION

In conclusion, in this large, multicenter retrospective analysis of OLT recipients, transplant recipients had a higher rate of TCM occurrence compared to the general population. The majority of patients who developed TCM following OLT had higher rates of in-hospital complications, including cardiogenic shock, respiratory failure, ventricular arrhythmias, and the need for renal replacement therapy. Hence, TCM development among OLT patients contributes to significant patient morbidity and resource utilization. Multicenter, prospective studies focusing on risk factors and predictors of TCM in OLT recipients are required, in order to fully explore the factors responsible for this disease association and confirm the various outcomes observed in this patient population.

ARTICLE HIGHLIGHTS

Research background

Orthotopic liver transplant recipients are a particularly vulnerable patient population with an elevated risk of developing various complications. Takotsubo cardiomyopathy (TCM) is one of the complications that is thought to have an association with liver transplantation, and can impact the overall prognosis.

Research motivation

Limited data is available regarding the association between orthotopic liver transplantation and TCM. The current research study evaluated this proposed association, and investigated the predictors and outcomes in this specific patient population.

Research objectives

To study the association between orthotopic liver transplantation and TCM, provide details about the demographic characteristics of the patient cohort, and examine the factors that affect the development of TCM in liver transplant patients, with a focus on identifying predictive variables and associated outcomes.

Research methods

Using a multi-center database of de-identified electronic health record data, a cohort of patients who underwent orthotopic liver transplant during the study period was identified. The sample was investigated to reveal the subset of patients who developed TCM. The data was analyzed to evaluate the association of TCM and liver transplantation, and descriptive statistical methods were utilized to demonstrate the specific features pertaining to the cohort of interest.

Research results

The study revealed that TCM is more likely to develop in liver transplant recipients compared to non-recipients. Predictors for the development of this association are described, with older age, female gender, and Caucasian ethnicity being a few notable risk factors. The research study also showed a higher incidence of poor outcomes in liver transplant patients who develop TCM, including but not limited to, cardiogenic shock, cardiac arrest, and multi-organ failure.

Research conclusions

Liver transplant recipients are a vulnerable patient population who have a higher risk of developing TCM. The development of this cardiac complication is associated with a heightened rate of in-hospital

complications. Knowledge of preexisting risk factors may help identify high-risk patients, and can impact management decisions.

Research perspectives

Future multicenter, prospective research studies focusing on risk factors and predictors of TCM in orthotopic liver transplant recipients are required, in order to fully explore this disease association and confirm the various outcomes observed in this patient population.

FOOTNOTES

Author contributions: Zmaili M designed the research study and formulated the idea of the research topic, collected the data, wrote the manuscript, contributed to the statistical analysis and figure formatting; Alzubi J contributed to manuscript writing; Alkhayyat M contributed to manuscript writing, data collection, data analysis, and figure formatting; Cohen J contributed to manuscript writing and revision; Alkharabsheh S contributed to manuscript writing and revision, and data analysis; Rana M contributed to manuscript writing; Alvarez PA contributed to manuscript writing and revision; Mansoor E contributed to manuscript writing and revision; Xu B contributed to manuscript writing and revision, finalizing the research work.

Institutional review board statement: This study is based on a multicenter analytics and research platform developed by IBM Watson Health (Explorys Inc, Cleveland, OH, USA). At present, Explorys database is rich, integrated, and growing living clinical data set that is HIPAA-enabled, including more than 70 million unique patients across all 50 states in the United States, and thus provides a broad regional and climatic distribution of source population. Ethical review and informed consent were waived, since there are no identifiers associated with any of the patient data. The Explorys rounds cell counts to the nearest 10 and treats all cell counts between zero and 10 as equivalent in order to further protect the identities of patients. In other words, the identities of subjects is completely anonymous and there is no risk involved in the study. Additionally, the research presents no risk of harm to subjects and involves no procedures for which written consent is normally required outside the research context.

Conflict-of-interest statement: This statement is to certify that all Authors have seen and approved the manuscript being submitted. We warrant that the article is the Authors' original work. We certify that there is no actual or potential conflict of interest in relation to this article to declare.

Data sharing statement: This study is based on a multicenter analytics and research platform developed by IBM Watson Health (Explorys Inc, Cleveland, OH, USA). At present, Explorys database is rich, integrated, and growing living clinical data set that is HIPAA-enabled, including more than 70 million unique patients across all 50 states in the United States, and thus provides a broad regional and climatic distribution of source population. Ethical review and informed consent were waived, since there are no identifiers associated with any of the patient data. The Explorys rounds cell counts to the nearest 10 and treats all cell counts between zero and 10 as equivalent in order to further protect the identities of patients. In other words, the identities of subjects is completely anonymous and there is no risk involved in the study. Additionally, the research presents no risk of harm to subjects and involves no procedures for which written consent is normally required outside the research context.

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ORIGINAL ARTICLE

Retrospective Study

Cystic fibrosis patients on cystic fibrosis transmembrane conductance regulator modulators have a reduced incidence of cirrhosis

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Abstract

BACKGROUND

Cystic fibrosis transmembrane conductance regulator (CFTR) modulators significantly improve pulmonary function in patients with cystic fibrosis (CF) but the effect on hepatobiliary outcomes remains unknown. We hypothesized that CF patients on CFTR modulators would have a decreased incidence of cirrhosis compared to patients not on CFTR modulators or on ursodiol.

AIM

To investigate the effect of CFTR modulators on the development of cirrhosis in patients with CF.

METHODS

A retrospective analysis was performed using Truven MarketScan from January 2012 through December 2017 including all patients with a diagnosis of CF. Patients were excluded if they underwent a liver transplantation or if they had other etiologies of liver disease including viral hepatitis or alcohol use. Subjects were grouped by use of CFTR modulators, ursodiol, dual therapy, or no therapy. The primary outcome was development of cirrhosis. Kaplan-Meier curves estimated the incidence of cirrhosis and log-rank tests compared incidence curves between treatment groups.

RESULTS

A total of 7201 patients were included, of which 955 (12.6%) used a CFTR modulator, 529 (7.0%) used ursodiol, 105 (1.4%) used combination therapy, and 5612 (74.3%) used neither therapy. The incidence of cirrhosis was 0.1% at 1 year and 0.7% at 4 years in untreated patients, 5.9% and 10.1% in the Ursodiol group, and 1.0% and 1.0% in patients who received both therapies. No patient treated with CFTR modulators alone developed cirrhosis. Patients on CFTR modulators alone had lower cirrhosis incidence than untreated patients ($P = 0.05$), patients on Ursodiol ($P < 0.001$), and patients on dual therapy ($P = 0.003$). The highest incidence of cirrhosis was found among patients treated with Ursodiol alone, compared to untreated patients ($P < 0.001$) or patients on Ursodiol and CFTR modulators ($P = 0.01$).

CONCLUSION

CFTR modulators are associated with a reduction in the incidence of cirrhosis compared to other therapies in patients with CF.

Key Words: Cirrhosis; Ursodiol; Transmembrane; Cystic fibrosis; Market scan; Cystic fibrosis related liver disease

Core Tip: The effect of cystic fibrosis transmembrane conductance regulator (CFTR) modulators on hepatobiliary outcomes in cystic fibrosis (CF) patients remains unknown. Utilizing a nationwide database, the incidence of cirrhosis in CF patients utilizing CFTR modulators, ursodiol, combination therapy or neither therapy was compared. A total of 7201 patients were studied including 12.6% on a CFTR modulator, 7.0% on ursodiol, 6.1% on combination therapy and 74.3% on neither therapy. Patients taking CFTR modulators had a lower incidence of cirrhosis than untreated patients ($P = 0.05$), or patients treated with Ursodiol ($P < 0.001$) or Ursodiol and CFTR modulators ($P = 0.003$). CFTR modulators may reduce the incidence of cirrhosis in patients with CF.

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INTRODUCTION

Dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR) leads to abnormal bicarbonate and chloride transport in the lungs, pancreas, liver, bile ducts and other organs and results in cystic fibrosis (CF). Cystic fibrosis affects approximately 1:2000 people of European descent[1-3]. Historically, disease complications and subsequent early mortality was linked to pulmonary complications including reduced pulmonary function, multidrug resistant infections, and pneumothorax[4]. Since the advent of targeted therapy with CFTR modulators, CF patients have significant improvement in lung function, decreased rates of pulmonary infection, improved patient reported quality of life, and life expectancy[5-8]. This will transition the focus of care to other leading causes of morbidity and mortality including hepatobiliary complications.

CF-related liver disease (CFRLD) that progresses to cirrhosis with concomitant portal hypertension is the third leading cause of death in patients with CF[7,8]. CFRLD ranges from abnormal liver function tests, hepatic steatosis, focal biliary cirrhosis, portal hypertension, and cirrhosis[7-11]. CFRLD affects between 2 to 37% of patients, and clinically significant liver disease is generally diagnosed in childhood

[7,8]. While the pathogenesis of cirrhosis in these patients is poorly understood, it is likely related to alkalization and dehydration of bile given ineffective CFTR channels on the apical surface of bile duct epithelium. This leads to plugging and inflammation of the bile ducts and development of hepatic fibrosis over time[12,13].

Given the proposed mechanism of CFRLD, ursodeoxycholic acid (ursodiol) was thought to be a promising intervention to prevent or slow progression of liver disease. However, there has been mixed results regarding its impact on CFRLD[14,15]. Otherwise, no medical intervention has been effective at reducing the incidence or progression of CFRLD. Given the ability for CFTR modulators to act directly upon the dysfunctional channels, these medications may be effective at improving hepatobiliary outcomes despite the risk of causing abnormal liver function tests in a recent systematic review[16].

This study aims to compare the incidence of cirrhosis during follow up among patients with CF who are treated with CFTR modulators and/or Ursodiol.

MATERIALS AND METHODS

Database

A retrospective analysis of the Truven Health MarketScan database was performed between the years 2012 and 2017. MarketScan is one of the largest, comprehensive, publicly available databases including information from over 100 private insurers representing over 150 million individual patients. Utilizing this database allows the researcher to track a patient through multiple years of inpatient and outpatient care[17]. Funding to utilize this database was obtained through The Ohio State University Center for Clinical and Translational Science (CCTS). The Ohio State University Institution Review Board deemed this study exempt from review.

Study sample

All patients with a diagnosis of CF identified *via* International Classification of Diseases (ICD) codes (ICD-9: 571.2, 571.5, 571.6, ICD-10: K70.3*, K71.7, K74.6*) codes were eligible to be included in this study. Patients were required to have either 2 outpatient appointments or 1 inpatient admission related to CF in order to increase the accuracy of the diagnosis. Patients were excluded if they had cirrhosis at the start of the analysis or within 12 months prior, hepatitis C virus, hepatitis B virus or alcohol use contributing to their liver disease. Patients were also excluded if they did not have at least 12 months of follow up after initiation of a CFTR modulator and/or ursodiol, periods of non-continuous drug claims enrollment, and no prescription coverage for any of the enrollment period. Diagnostic codes for each of these inclusion and exclusion criteria have been widely used in previous publications[18,19].

Outcome of interest

The primary outcome of interest was development of cirrhosis in CF patients taking a CFTR modulator, ursodiol, dual therapy or neither medication. Cirrhosis was defined as the presence of an ICD-9 or ICD-10 code for cirrhosis.

Definition of variables

The index date of evaluation was the medication start date or the start of the second medication if both a CFTR modulator and ursodiol were utilized. If patients were not taking either medication, their start date was considered to be the date they were enrolled in the database with a diagnosis of CF. The study inclusion date also corresponded with continuous Marketscan enrollment without any gaps over 90 days. Other variables evaluated included age, gender, insurance plan type, geographic region and the presence of comorbidities defined by the Carlson Comorbidity Index[20]. CFTR modulators included ivacaftor (Kalydeco), ivacaftor with lumacaftor (Orkambi), ivacaftor with tezacaftor (Symdeko), and ivacaftor, tezacaftor, and elexacaftor (Trikafta).

Statistical analysis

Kaplan-Meier curves were used to display and estimate cirrhosis incidence at select time points and log-rank tests were used to compare incidence curves between treatment groups. Analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC).

RESULTS

Demographics

A total of 7201 patients met inclusion criteria of which 105 patients were taking both a CFTR modulator and ursodiol, 955 patients taking only a CFTR modulator, 529 patients taking only ursodiol and 5612 patients on neither medication (Table 1).

Table 1 Cystic fibrosis patient characteristics, time of enrollment and medication

Variable	CFTR modulator + ursodiol (*n* = 105)	CFTR modulator (*n* = 955)	Ursodiol (*n* = 529)	Neither therapy (*n* = 5612)
Age, mean (SD)	20.1 (12.0)	21.0 (13.0)	20.9 (13.2)	23.8 (17.3)
Age group				
0-5	7	97	63	952
44723	16	142	69	671
44912	30	206	97	709
18-25	27	219	160	1079
26-34	12	148	58	709
35+	13	143	82	1492
Sex				
Male	51	507	290	2613
Female	54	448	239	2999
Region				
Northeast	18	190	85	1138
North central	19	260	115	1341
South	42	374	204	1997
West	24	125	109	1039
Unknown	2	6	16	97
Charlson comorbidity index, mean (SD)	0 (0.0)	0.0 (0.1)	0.0 (0.2)	0.0 (0.3)
Chronic respiratory failure	0	1	0	53
Follow up available (years), median (IQR)	2.0 (1.6-3.1)	2.1 (1.5-3.3)	2.9 (2.0-4.6)	3.0 (2.0-5.0)
Percentage of time on CFTR, median (IQR)	95% (70-100%)	100% (75-100%)	0 (0-0)	0 (0-0)
Percentage of time on ursodiol, median (IQR)	70% (23-92%)	0 (0-0)	51% (19-81%)	0 (0-0)
Percentage of time on ursodiol and CFTR Modulator concurrently, median (IQR)	56% (13-85%)	0 (0-0)	0 (0-0)	0 (0-0)

CFTR modulators: Cystic fibrosis transmembrane regulator modulators; IQR: Interquartile range; SD: Standard deviation.

Follow up and length of time on therapy

Patients without therapy were followed up for a median of 3.0 years [Interquartile range (IQR): 2.0-5.0] compared to 2.9 years (IQR: 2.0-4.6) for patients on ursodiol only, 2.1 years (IQR: 1.5-3.3) for patients on CFTR modulators only and 2.0 years (IQR: 1.6-3.1) for patients on both a CFTR modulator and ursodiol (Table 1). Patients taking ursodiol only were on therapy a median percentage of 51% (IQR: 19-81%) of the studied time compared to a median percentage of 100% (IQR: 75-100%) in patients taking CFTR modulators only. Patients taking both a CFTR modulator and ursodiol were taking both medications concurrently a median percentage of 56% (IQR: 13-85%) of the studied time, though were taking the CFTR modulator a median of 95% (IQR: 70-100%) of the time and ursodiol a median of 70% (IQR: 23-92%) of the studied time (Table 1).

Incidence of cirrhosis

Of the 955 patients on CFTR modulators only, 954 patients remained eligible to be evaluated 1 year after initiating therapy. This decreased to 513 patients at 2 years, 288 patients at 3 years and 74 patients at 4 years after initiating therapy. The incidence of cirrhosis at four years was 0% (Figure 1, Table 2). By log rank testing, patients on CFTR modulators had a lower incidence of cirrhosis than patients on no treatment ($P = 0.05$), Ursodiol alone ($P < 0.001$), or Ursodiol and CFTR modulators ($P = 0.003$).

Of the 529 patients taking only ursodiol, 498 patients remained eligible to be evaluated at 1 year. This decreased to 320 patients at 2 years, 195 at 3 years and 139 patients at 4 years. The incidence of cirrhosis increased yearly and was 5.9% at 1 year, 7.5% at 2 years, 9.1% at 3 years and 10.1% at 4 years (Table 2, Figure 1).

Table 2 Kaplan-Meier estimates for incidence of cirrhosis at select timepoints

Time post-index	CFTR modulator + ursodiol	CFTR Modulator only	Ursodiol only	Neither therapy
3 mo	0.01	0	0.019	0.0002
6 mo	0.01	0	0.032	0.001
1 yr	0.01	0	0.059	0.001
2 yr	0.01	0	0.075	0.004
3 yr	0.01	0	0.091	0.005
4 yr	0.01	0	0.101	0.007

CFTR modulators: Cystic fibrosis transmembrane regulator modulators.

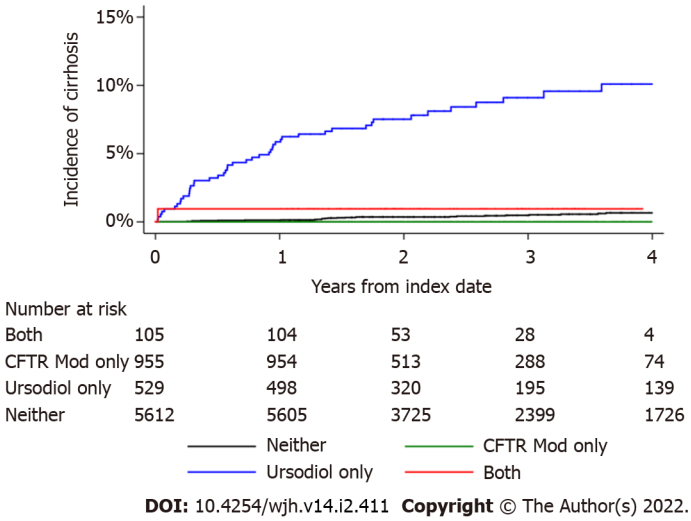

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Figure 1 Kaplan-Meier curves for development of cirrhosis based on medication use.

Of the 105 patients taking both a CFTR modulator and ursodiol, 104 patients remained eligible to be evaluated at 1 year. This decreased to 53 patients at 2 years, 28 patients at 3 years and 4 patients at 4 years. The incidence of cirrhosis increased to 1.0% by 3 mo after initiation of therapy and remained at 1.0% for 4 years (Table 2, Figure 1).

Of the 5612 patients taking neither therapy, 5605 patients remained eligible to be evaluated at 1 year. This decreased to 3725 patients at 2 years, 2399 patients at 3 years and 1726 patients at 4 years. The incidence of cirrhosis increased to 0.1% at 1 year, 0.4% at 2 years, 0.5% at 3 years and 0.7% at 4 years (Table 2, Figure 1).

DISCUSSION

Given the success of CFTR modulators in improving pulmonary function and life expectancy in patients with CF, the focus of care will shift to other leading causes of mortality in these patients, such as CFRLD. The mechanism of cirrhosis and portal hypertension in patients with CF is incompletely understood; therefore, interventions have been ineffective in improving hepatobiliary outcomes. In this retrospective study, we determined that patients with CF using CFTR modulators have a significantly decreased yearly incidence of cirrhosis compared to patients on just ursodiol, dual therapy with a CFTR modulator and ursodiol, or neither therapy.

CFTR modulators are likely effective at reducing the incidence of cirrhosis or delaying the progression of CFRLD due to the ability to directly act upon CFTR channels in the bile ducts[12,21]. While the mechanism of CFRLD is not fully understood, the pathogenesis is currently attributed to lack of chloride secretions from the cholangiocytes leading to intrahepatic cholestasis, biliary cirrhosis, and portal hypertension[9,13]. The CFTR channel is considered to act as a master regulator of the cholangiocyte and plays a crucial role in in the regulation of ductular secretions contributing to the dilution and subsequent flow of bile from the liver into the intestines[12,13,22]. Unlike ursodiol, CFTR

modulators act directly upon these dysfunctional channels to improve flow of bile. Ursodiol is postulated to improve flow of bile in patients with CF by improving the bicarbonate content and by decreasing the resultant inflammation from biliary stasis[23]. Few trials have been completed evaluating the ability of this intervention to reduce the risk of cirrhosis[15]. One study cited improved bile secretion when measured by isotopes[24] and delayed development of portal hypertension when monitored for 6 mo[15,25]. However, ursodiol has not proven to be effective in long term reduction in CFRLD mortality, thus new agents are needed to improve outcomes among patients with CFRLD[7,8].

Our study suggests that CFTR modulators may be effective at reducing the incidence and delaying progression of cirrhosis in patients with CF when compared to ursodiol or dual therapy. Reduction in hepatobiliary complications with the use of CFTR modulators is supported by other smaller studies. Steatosis is considered an early marker of CFRLD though its clinical implications in patients with CF remains unknown[26]. CFTR modulators have been shown to reduce the hepatic fat fraction by half[26]. An observational study of patient registries in the United States and United Kingdom, demonstrated that patients on ivacaftor have significantly less hepatobiliary complications including abnormal liver function tests, cirrhosis, and cirrhosis-related complications[27]. Our study adds to this growing body of evidence that CFTR modulators may improve outcomes among patients with CFRLD.

It should be noted that CFTR modulators carry a risk of causing abnormal liver function tests in all CF patients regardless of underlying CFRLD[16,28]. Elevation in serum aminotransferases were noted in up to 25% of patients while on therapy, although only 5% of patients develop clinically significant elevation greater than 5 times the upper limit of normal. Elevations to this extent typically resulted in a temporary cessation of therapy; however, there is no clear guidance regarding the safety of restarting therapy[28]. Furthermore, there is mixed data in predicting patients that are at highest risk of developing liver function tests abnormalities with on CFTR modulators[16]. Further studies will be necessary to determine whether these transient elevations in transaminases at initiation of therapy predict the development of CFRLD, and whether CFTR modulators have a net benefit in this population.

This study does have limitations. Data was collected from a nationwide database and therefore the information could not be verified in each patient's medical chart. Laboratory testing is not available in this dataset, which limits our ability to assess specific CFTR mutations and serial assessment of liver enzymes. While the ICD-9 and ICD-10 based diagnostic algorithm used here has been used in previous studies on patients with cirrhosis related to alcohol or viral hepatitis, the accuracy has not been determined in patients with CF. However, the prevalence of cirrhosis in our study was similar to the prevalence of cirrhosis in patients with CF, suggesting that the diagnostic coding strategy is valid[10]. In addition, cirrhosis may take many years to develop, and we were only able to include 4 years of follow up due to limited follow up time available in the MarketScan database. Some patients may have developed cirrhosis after the study period. Furthermore, a significant number of patients were lost to follow up during this analysis and were unable to be evaluated for total studied time. Therefore, we are only able to determine correlation not causation given the retrospective utilization of a database[29].

Lastly, since ursodiol is only used in patients with CF who have liver disease, this group is likely enriched for baseline abnormal liver function. Thus, there is a selection bias for this group which likely influenced the increased risk of developing cirrhosis observed in our study. In addition, patients with pre-existing liver disease may have not been started on a CFTR modulators due to the risk of abnormal liver function tests and hepatic decompensation which may have further contributed to this selection bias. However, it is important to note that when CFTR modulators were added to ursodiol, the incidence of cirrhosis was lower. Despite this selection bias, the study still has merit by measuring the open label use of CFTR modulators among patients with CF who may be at risk for hepatic complications.

Despite these limitations, this study has significant strength. This is one of the first longitudinal analyses evaluating a nationwide population of pediatric and adult patients to determine the incidence of cirrhosis among patients with CF. We also provide a direct comparison to ursodiol which is commonly used in patients with CFRLD. The MarketScan dataset is also used to assess compliance based on refill rate, so we are able to include only patients who were consistently taking these medications throughout the study period. We also excluded other causes of liver disease, such as viral hepatitis or alcohol use, which allowed us to include subjects with CF as the main driver of hepatobiliary outcomes.

CONCLUSION

In this large database analysis, we demonstrate that CFTR modulator use is associated with a decreased incidence of cirrhosis compared to no therapy and compared to ursodiol. While concerns exist regarding hepatic side effects of CFTR modulators, we observed improved long term hepatic outcomes compared to other therapies. This study supports the utilization of CFTR modulators in patients with CF to not only improve pulmonary outcomes but also hepatobiliary outcomes.

ARTICLE HIGHLIGHTS

Research background

Due to improvements in pulmonary care in cystic fibrosis (CF), CF-related liver disease (CFRLD) is emerging as a leading cause of morbidity and mortality. Cystic fibrosis transmembrane conductance regulator (CFTR) modulators correct the CFTR dysfunction and dramatically improve pulmonary outcomes, but the effects of CFTR modulators on CFRLD have not been evaluated.

Research motivation

Currently, there is insufficient data examining the impact of CFTR modulators on the incidence of cirrhosis among patients with CF.

Research objectives

To investigate the effect of CFTR modulators on the development of cirrhosis in patients with CF.

Research methods

A retrospective analysis was performed using Truven MarketScan from January 2012 through December 2017 including all patients with a diagnosis of CF. Subjects were grouped by use of CFTR modulators, ursodiol, dual therapy, or no therapy. The primary outcome was development of cirrhosis.

Research results

A total of 7201 patients were included, of which 955 (12.6%) used a CFTR modulator, 529 (7.0%) used ursodeoxycholic acid, 105 (1.4%) used combination therapy, and 5612 (74.3%) used neither therapy. The incidence of cirrhosis was 0.1% at 1 year and 0.7% at 4 years in untreated patients, 5.9% and 10.1% in the Ursodiol group, and 1.0% and 1.0% in patients who received both therapies. No patient treated with CFTR modulators alone developed cirrhosis. Patients on CFTR modulators alone had lower cirrhosis incidence than untreated patients ($P = 0.05$), patients on Ursodiol ($P < 0.001$), and patients on dual therapy ($P = 0.003$). The highest incidence of cirrhosis was found among patients treated with Ursodiol alone, compared to untreated patients ($P < 0.001$) or patients on Ursodiol and CFTR modulators ($P = 0.01$).

Research conclusions

Patients treated with CFTR modulators have a lower incidence of cirrhosis compared to no treatment, ursodiol, or combination therapy.

Research perspectives

The risk of developing cirrhosis is lower among patients treated with CFTR modulators than those not treated with CFTR modulators. Whether this represents a selection bias or represents a treatment effect of CFTR modulators should be studied in a prospective, randomized study.

FOOTNOTES

Author contributions: Ramsey ML and Sobotka LA designed the research project, drafted the manuscript, and provided final approval of the manuscript; Porter K performed the statistical analysis and provided final approval of the manuscript; Wellner MR, Kirkby S, Li SS, Lara LF, Kelly SG, and Hanje AJ made critical revisions related to important intellectual content of the manuscript and provided final approval of the manuscript.

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ORIGINAL ARTICLE

Observational Study

Modified EASL-CLIF criteria that is easier to use and perform better to prognosticate acute-on-chronic liver failure

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Abstract

BACKGROUND

We have recently shown that the European Association for the Study of the Liver-Chronic Liver Failure Consortium (EASL-CLIF) criteria showed a better sensitivity to detect acute-on-chronic liver failure (ACLF) with a better prognostic capability than the North American Consortium for the Study of End-Stage Liver Disease criteria.

AIM

To simplify EASL-CLIF criteria for ease of use without sacrificing its sensitivity and prognostic capability.

METHODS

Using the United Network for Organ Sharing data (January 11, 2016, to August 31, 2020), we modified EASL-CLIF (mEACLF) criteria; the modified mEACLF criteria included six organ failures (OF) as in the original EASL-CLIF, but renal failure was defined as creatinine ≥ 2.35 mg/dL and coagulation failure was defined as international normalized ratio (INR) ≥ 2.0. The mEACLF grades (0, 1, 2, and ≥ 3) directly reflected the number of OF.

RESULTS

Of the 40357 patients, 14044 had one or more OF, and 9644 had ACLF grades 1-3 by EASL-CLIF criteria. By the mEACLF criteria, 15574 patients had one or more OF. The area under the receiver operating characteristic (AUROC) for 30-d all-cause mortality by OF was 0.842 (95%CI: 0.831-0.853) for mEACLF and 0.835 (95%CI: 0.824-0.846) for EASL-CLIF (P = 0.006), and AUROC for 30-d transplant-free mortality by OF was 0.859 (95%CI: 0.849-0.869) for mEACLF and 0.851 (95%CI: 0.840-0.861) for EASL-CLIF (P = 0.001). The AUROC of 30-d all-cause

mortality by ACLF grades was 0.842 (95%CI: 0.831-0.853) for mEACLF and 0.793 (95%CI: 0.781-0.806) for EASL-CLIF ($P < 0.0001$). The AUROC of 30-d transplant-free mortality by ACLF was 0.859 (95%CI: 0.848-0.869) for mEACLF and 0.805 (95%CI: 0.793-0.817) for EASL-CLIF ($P < 0.0001$).

CONCLUSION

Our study showed that EASL-CLIF criteria for ACLF grades could be simplified for ease of use without losing its prognostication capability and sensitivity.

Key Words: Acute on chronic liver failure; Organ failure; 30-d transplant-free mortality; Liver transplantation; EASL-CLIF criteria

Core Tip: There is no consensus on the best definition for acute-on-chronic liver failure (ACLF). The most common definition used in the literature is the one proposed European Association for the Study of the Liver-Chronic Liver Failure Consortium (EASL-CLIF) consortium. One problem with those criteria is that it is not very user-friendly. We have shown that EASL-CLIF criteria for ACLF could be simplified without losing its sensitivity and ability to prognosticate 30-d all-cause and transplant-free mortality. We believe that modified EASL-CLIF criteria; the modified criteria that we propose are easier to use than the EASL-CLIF criteria and also have a better prognostic capability.

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INTRODUCTION

Acute on chronic liver failure (ACLF) is associated with one or more organ failures (OF) and a very high short-term mortality[1-4]. Although more than 13 different definitions of acute-on-chronic liver failure (ACLF) have been proposed, the three commonly used criteria were those proposed by the Asian Pacific Association for Study of Liver Diseases (APASL), the European Association for the Study of the Liver-Chronic Liver Failure Consortium (EASL-CLIF) and the North American Consortium for the Study of End-Stage Liver Disease (NACSELD)[5-9]. In a recent study, we have clearly shown that EASL-CLIF criteria have a better sensitivity and a better ability to predict short-term, all-cause, and transplant-free mortality when compared to the NACSELD criteria[10]. In that study, only 15.3% of those with EASL-CLIF ACLF grade 1-3 met ACLF by NACSELD criteria. Moreover, only less than half of those with EASL-CLIF ACLF grade 3 had ACLF by NACSELD criteria. In addition, the 30-d transplant-free mortality in those with no organ failure by NACSELD was 2.7%, but when the same group was stratified by EASL-CLIF grades 0-3, the mortality rates were 1.5%, 10.5%, 43.5%, and 86%, respectively. There has been a comparative study of EASL-CLIF and APASL criteria using a large Veteran Affairs administrative dataset. In that study, 76.1% of patients with EASL-CLIF ACLF did not fulfill APASL criteria for ACLF[11]. In the same study, the 30-d mortality was 37.6% in those who met the EASL-CLIF criteria suggesting that the APASL criteria missed 75% of patients with ACLF with a very high short-term mortality. Based on the above observations, we believe that EASL-CLIF criteria are superior to NACSELD or APASL criteria to identify ACLF with a very high 30-d mortality.

One of the major criticisms of the EASL-CLIF criteria is that it is not very user-friendly. The EASL-CLIF stratifies patients into four grades (0-3) based on the number of OF, including liver, kidney, brain, coagulation, respiration, or circulation. The differences between EASL-CLIF ACLF grades and EASL-CLIF OF scoring can also result in some confusion, especially with the interpretation of no ACLF and ACLF-grade 1. The objective of our study was to determine whether EASL-CLIF grades of ACLF could be simplified without sacrificing its sensitivity and prognostic capabilities.

MATERIALS AND METHODS

We utilized the national data from the United Network for Organ Sharing (UNOS) for all adults (≥ 18 years) who were listed ($n = 53765$) for liver transplantation (LT) in the United States between January 11, 2016, to August 31, 2020. During this study period, MELD-Na (model for end-stage liver disease-

Table 1 The definitions of modified the European Association for the Study of the Liver-Chronic Liver Failure Consortium organ failures and grades

Definition of mEACLF organ failures	
Liver	Bilirubin ≥ 12 mg/dL
Coagulation	INR ≥ 2.0
Brain	Hepatic encephalopathy grade 3-4
Kidney	Serum creatinine ≥ 2.35 mg/dL or renal replacement
Heart	On vasopressors
Respiration	On mechanical ventilation
Definition of mEACLF grades	
Grade 1	Any one organ failure
Grade 2	Any 2 organ failures
Grade 3	Any 3 or more organ failures

mEACLF: Modified the European Association for the Study of the Liver-Chronic Liver Failure Consortium; INR: International normalized ratio.

sodium) was utilized for organ allocation on January 11, 2016, and there were no major policy changes. Patients were excluded if they were listed as status 1, 1A, or 1B (n = 1264), for multi-organ transplantation (n = 3179), re-transplantation (n = 607) or for live donor LT (n = 1421). We also excluded those who received MELD- exception points (n = 8886) and those with missing information on MELD-Na or serum sodium (n = 14) or hepatic encephalopathy stage (n = 53) (Supplementary Figure 1).

We collected clinical characteristics, biochemical parameters such as albumin, bilirubin, international normalized ratio, creatinine, MELD-Na, and the prevalence of OF as defined by EASL-CLIF criteria. We graded ACLF as described by EASL-CLIF into Grade 0, or those patients without any OF or single non-kidney OF, Grade 1a (renal failure), Grade 1b (single non-kidney OF, creatinine 1.5-1.9 mg/dL, and/or mild hepatic encephalopathy), Grade 2 (two OFs), and Grade 3 (three or more OFs)[3]. For this study, we combined 1a and 1b grades as 1. The only deviation of our definition from EASL-CLIF criteria was on respiratory failure as we did not have access to PaO2 or FIO2 and hence used mechanical ventilation as evidence of respiratory failure. We initially assessed the prevalence of type and frequency of OF using EASL-CLIF. Using the same dataset, we developed modified criteria as described later under 'model development'. Patients were followed until the event date or were censored at the end of 30-ds after listing. Our primary objective was to develop an 'easy to use' model, by modifying the EASL-CLIF criteria, with a better short-term (30-d mortality) prognostic capability.

Outcomes of interest

The primary outcomes of interest were the differences in the prevalence of ACLF grades by EASL-CLIF and mEACLF criteria and the observed 30-d all-cause and transplant-free mortality rates using EASL-CLIF criteria and newly developed modified EASL-CLIF (mEACLF) criteria.

Model development

To improve the EASL-CLIF criteria, we determined the best cutoff values for serum creatinine and international normalized ratio (INR) associated with higher mortality. We used a subset of patients (n = 1445) with information on glomerular filtrations rate (GFR) to determine the best cutoff values for serum creatinine levels. The inclusion of GFR data in the UNOS registry was proposed for those with GFR less than 20 mL/min by the Simultaneous Liver Kidney working group in 2015[12]. We used those GFR values to identify the best cutoff values of serum creatinine by smooth regression analysis. The smooth regression analysis showed that serum creatinine ≥ 2.35 mg/dL is the optimal cutoff value (Supplementary Figure 2).

After identifying the best serum creatinine value, we identified the optimal INR cutoff; INR 2.0 had an area under the receiver operating characteristic (AUROC) of 0.842 (95% confidence interval 0.831-0.853) to prognosticate 30-d all-cause mortality for coagulation failure by logistic regression after fixing serum creatinine values at ≥ 2.35. We further confirmed the best INR value for the coagulation failure by fixing other organ failures as follows (bilirubin ≥ 12 mg/dL, creatinine ≥ 2.35 mg/dL, HE = 3-4, circulation support = yes, respiration support = yes) by logistic regression. Based on these results, INR ≥ 2.0 was chosen to diagnose coagulation failure.

Using the above values, we then developed a modified 6-organ failure criteria mEACLF (Table 1). In the mEACLF, renal failure was defined as serum creatinine ≥ 2.35 mg/dL (instead of ≥ 2.0 mg/dL of EASL-CLIF criteria) or on renal dialysis. Coagulation failure was defined as INR ≥ 2.0 (instead of 2.5 as

Table 2 Characteristics of patients with the European Association for the Study of the Liver-Chronic Liver Failure Consortium and modified the European Association for the Study of the Liver-Chronic Liver Failure Consortium organ failures

Variable	Response	All (*n* = 29618)	EASL-CLIF (*n* = 14044)	mEACLF (*n* = 15574)	*P* value
Age	mean ± SD	52.9 ± 11.6	52.9 ± 11.7	52.9 ± 11.8	0.46
Gender, *n* (%)	Female	11803 (40)	5601 (40)	6202 (40)	0.92
Race, *n* (%)	White	20059 (68)	9484 (68)	10575 (68)	0.95
	Black	2530 (9)	1215 (9)	1315 (8)	
	Hispanic	5242 (18)	2496 (18)	2746 (18)	
	Asian	1129 (4)	540 (4)	589 (4)	
	Others	658 (2)	309 (2)	349 (2)	
Etiology, *n* (%)	HCV	3661 (12)	1730 (12)	1931 (12)	0.87
	Alcohol	12537 (42)	5909 (42)	6628 (43)	
	HCV + Alcohol	353 (1)	165 (1)	188 (1)	
	Cryptogenic	1507 (5)	712 (5)	795 (5)	
	Others	11560 (39)	5528 (39)	6032 (39)	
Bilirubin	mean ± SD	13.8 ± 12.5	14.1 ± 12.7	13.4 ± 12.3	0.001
Creatinine	mean ± SD	2.11 ± 1.79	2.19 ± 1.81	2.03 ± 1.77	< 0.001
INR	mean ± SD	2.30 ± 1.12	2.29 ± 1.15	2.30 ± 1.08	< 0.001
Encephalopathy, *n* (%)	Grade 3-4	7088 (24)	3544 (25)	3544 (23)	< 0.001
Respiration, *n* (%)	Yes	1598 (5)	799 (6)	799 (5)	0.03
Circulatory, *n* (%)	Yes	2684 (9)	1342 (10)	1342 (9)	0.005
MELD-Na	mean ± SD	29.9 ± 8.1	30.2 ± 8.2	29.7 ± 8.0	< 0.001
Albumin	mean ± SD	3.10 ± 0.74	3.12 ± 0.74	3.08 ± 0.74	< 0.001
Ascites, *n* (%)	Moderate	13357 (45)	6456 (46)	6901 (44)	0.02

INR: International normalized ratio; EASL-CLIF: European Association for the Study of the Liver-Chronic Liver Failure Consortium; mEACLF: Modified EASL-CLIF; HCV: Hepatitis C virus; MELD: Model for End-stage Liver Disease.

Table 3 The distribution of patients by the number of organ failure by the European Association for the Study of the Liver-Chronic Liver Failure Consortium and modified the European Association for the Study of the Liver-Chronic Liver Failure Consortium criteria

Number of OF by EASL-CLIF	Number of OF by mEACLF criteria							
	0	1	2	3	4	5	6	Total
0	24227	2086	0	0	0	0	0	26313
1	556	5748	1395	0	0	0	0	7699
2	0	229	2875	653	0	0	0	3757
3	0	0	152	1193	215	0	0	1560
4	0	0	0	52	458	96	0	606
5	0	0	0	0	10	238	63	311
6	0	0	0	0	0	8	103	111
Total	24783	8063	4422	1898	683	342	166	40357

EASL-CLIF: European Association for the Study of the Liver-Chronic Liver Failure Consortium; mEACLF: Modified European Association for the Study of the Liver; OF: Organ failures.

Table 4 The distribution of patients by the European Association for the Study of the Liver-Chronic Liver Failure Consortium and modified European Association for the Study of the Liver-Chronic Liver Failure Consortium grades of the acute-on-chronic liver failure and their 30-d all-cause mortality

EASL-CLIF grade	Number of patients mEACLF Grade (all-cause mortality%)				
	0	1	2	3	Total
0	24227 (1.2)	5114 (3.3)	1372 (10.2)	0	30713 (2.0)
1	556 (4.1)	2720 (6.5)	23 (10.2)	0	3299 (6.1)
2	0	229 (13.1)	2875 (11.8)	653 (15.0)	3757 (12.5)
3	0	0	152 (21.7)	2436 (24.5)	2588 (24.4)
Total	24783 (1.3)	8063 (4.7)	4422 (11.7)	3089 (22.5)	40357 (4.7)

EASL-CLIF: European Association for the Study of the Liver-Chronic Liver Failure Consortium; ACLF: Acute-on-chronic liver failure.

per EASL-CLIF criteria). We further simplified the mEACLF grades to directly reflect the number of OF without over-emphasizing serum creatinine levels and without sub-classifying grade 1 into 1a and 1b. The proposed mEACLF grade are as follows: Grade 1 = 1 OF, Grade 2 = 2 OF and Grade 3 = 3 or more OF (Table 1).

We compared our new mEACLF criteria with the original EASL-CLIF criteria by looking at the distribution of OF, ACLF grades, and 30-d all-cause and transplant-free mortality rates.

Statistical analysis

The demographic characteristics are summarized as mean and standard deviation for continuous variables or frequency for categorical variables. Logistic regression was performed to compare AUROC for 30-d all-cause and transplant-free mortality based on the EASL-CLIF and mEACLF criteria.

Being a de-identified national dataset, institutional review board (IRB) approval was waived.

RESULTS

Of the 40357 patients who were eligible for the study, 14044 had one or more OF and 9644 ACLF grades 1-3 by EASL-CLIF criteria. Patients' characteristics stratified by ACLF and no ACLF are shown in Supplementary Table 1.

Modified ACLF criteria for OF and ACLF grades

Using the mEACLF criteria, 15574 patients had one or more OF. The comparative clinical characteristics of patients with and without ACLF stratified by mEACLF criteria are shown in Table 2. The direct comparison of patients with one or more OF identified by mEACLF (n = 15574) and EASL-CLIF (n = 14044) is shown in Table 2.

Comparison of EASL-CLIF and mEACLF organ failures

The comparative prevalence of OF by EASL-CLIF and mEACLF criteria are shown in Table 3. There were some differences in the number of patients with OF between EASL-CLIF and mEACLF; 2086 patients with no OF by EASL-CLIF criteria were identified with one OF by mEACLF, and this resulted from a lower threshold for INR with the revised criteria. The 30-d mortality in these 2086 (one OF by mEACLF) patients was 3.4% compared to 1.4% in the EASL-CLIF no OF (n = 26313) group. Similarly, 556 patients with one OF by EASL-CLIF were identified with no OF by the revised criteria, and this resulted from a higher threshold for serum creatinine with the new criteria. The 30-d mortality in these 556 patients was 4.1% compared to 5.8% in the EASL-CLIF one OF (n = 7699) group.

The 30-d mortality rates by OF by both criteria are shown in Figure 1A. The 30-d transplant free mortality rates are shown in Figure 1B. The AUROC for 30-d all-cause mortality by OF was 0.842 (95%CI: 0.831-0.853) for mEACLF and 0.835 (95%CI: 0.824-0.846) for EASL-CLIF (AUROC contrast estimation 0.0072, 95%CI: 0.00208 - 0.0123, P = 0.006) (Figure 1C). AUROC for 30-d transplant-free mortality by OF was 0.859 (95%CI: 0.849-0.869) for mEACLF and 0.851 (95%CI: 0.840-0.861) for EASL-CLIF (AUROC contrast estimation 0.0085, 95%CI: 0.00329 - 0.0136, P = 0.001) (Figure 1D).

Comparison of EASL-CLIF and mEACLF grades

There were some discrepancies between the EASL-CLIF grades and mEACLF grades and their corresponding 30-d all-cause mortality rates. 1372 patients who were classified as grade 0 by the EASL-CLIF ACLF were grades mEACLF grade 2, and 30-d all-cause mortality of these 1372 patients was 10.2% as

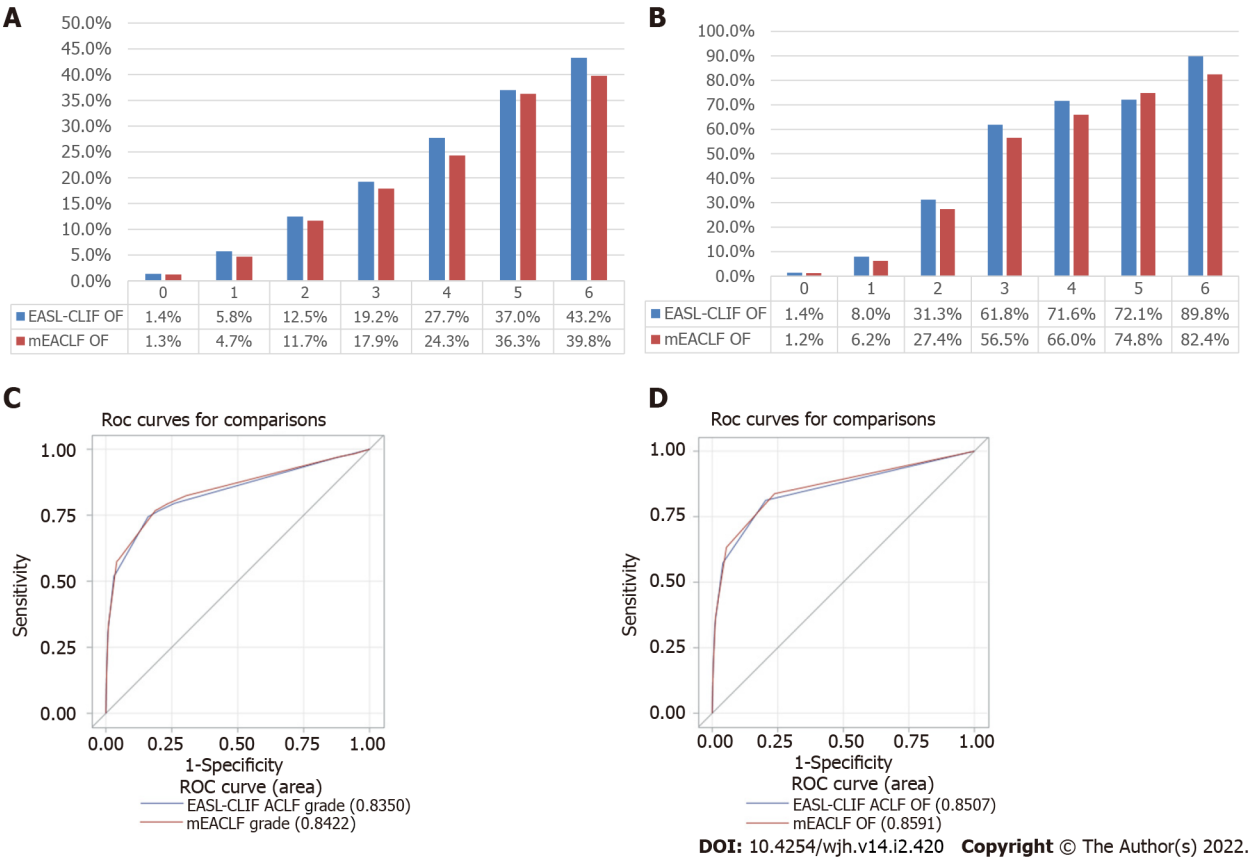

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Figure 1 All-cause and transplant free mortality rates, and the area under the receiver operator characteristics, stratified by the number of organ failures by European Association for the Study of the Liver-Chronic Liver Failure Consortium acute-on-chronic liver failure and modified European Association for the Study of the Liver-Chronic Liver Failure Consortium criteria. A: All-cause mortality stratified by the number of organ failures by European Association for the Study of the Liver-Chronic Liver Failure Consortium (EASL-CLIF) acute-on-chronic liver failure (ACLF) and modified EASL-CLIF (mEACLF) criteria; B: Transplant-Free Mortality stratified by the number of organ failures by EASL-CLIF ACLF and mEACLF criteria; C: Area under the receiver operating characteristic (AUROC) for all-cause mortality by the number of organ failures by EASL-CLIF ACLF and Meaclf; D: AUROC for transplant free mortality by the number of organ failures by EASL-CLIF ACLF and mEACLF. EASL-CLIF: European Association for the Study of the Liver-Chronic Liver Failure Consortium; mEACLF: modified EASL-CLIF; ROC: Receiver operating characteristic.

compared to 2.0% in those with EASL-CLIF grade 0 group (n = 30,713) (Table 4). There were outliers in the mEACLF group in terms of 30-d mortality, including 229 patients with grade 1 (EASL-CLIF grade 2) with a mortality of 13.1%, which was higher than grade 1 mEACLF mortality of 4.7% and 152 patients with mEACLF grade 2 (grade 3 by EASL-CLIF) with a mortality of 21.7% which was higher than mEACLF grade 2 mortality of 11.7%.

The 30-d mortality rates by grades by both criteria are shown in Figure 2A. The 30-d transplant-free mortality rates are shown in 2B. The AUROC of 30-d all-cause mortality by grades was 0.842 (95%CI: 0.831-0.853) for mEACLF and 0.793 (95%CI: 0.781-0.806) for EASL-CLIF. These differences were highly significant (P < 0.0001, Figure 2C). The AUROC of 30-d transplant-free mortality was 0.859 (95%CI: 0.848-0.869) for mEACLF and 0.805 (95%CI: 0.793-0.817) for EASL-CLIF (P < 0.0001, Figure 2D).

DISCUSSION

Our study showed that EASL-CLIF criteria for ACLF grades could be simplified for ease of use without losing its sensitivity. The mEACLF criteria that we propose are also better than the EASL-CLIF grades to prognosticate 30-d all-cause and transplant-free mortality. Both criteria showed low 30-d mortality in those with 0-1 OF, and the mortality increased progressively with an increase in the number of OF. Similar observations were also made for ACLF grades with low mortality with grade 1 and a two-fold difference in mortality between grades 2 and 3.

Few patients in our study will be graded zero by EASL-CLIF but grade 1-2 by mEACLF. This discrepancy is mainly because the EASL-CLIF will grade a single non-kidney organ failure patient as EASL-CLIF grade zero if the serum creatinine < 1.5 mg/dL. The differences in the threshold for INR to classify as coagulation failure also may have contributed to some of the discrepancies. Interestingly, the 30-d all-cause mortality in the group (n = 1372) with grade 2 mEACLF and grade 0 by EASL-CLIF was

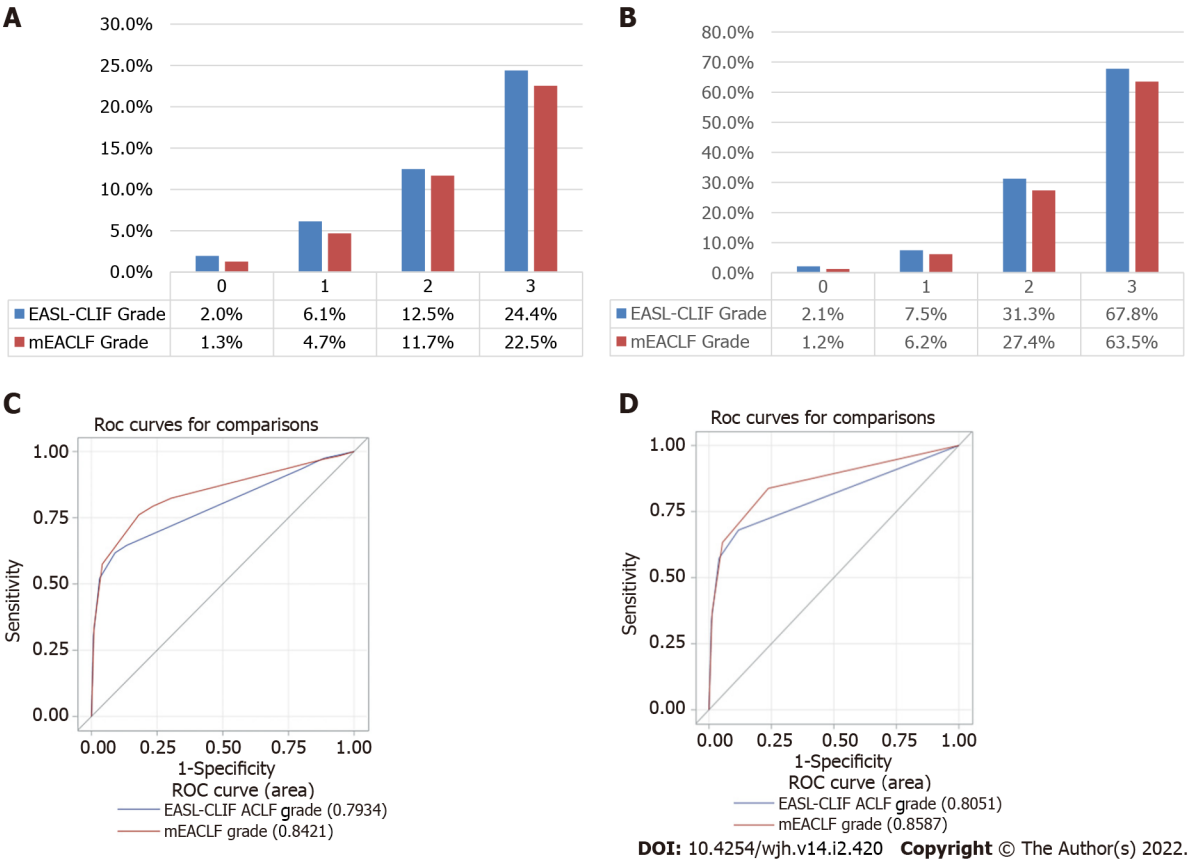

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Figure 2 Figure title All-cause and transplant free mortality rates, and the area under the receiver operator characteristics, stratified by acute-on-chronic liver failure grades by European Association for the Study of the Liver-Chronic Liver Failure Consortium acute-on-chronic liver failure and modified European Association for the Study of the Liver-Chronic Liver Failure Consortium criteria. A: All-cause mortality stratified by European Association for the Study of the Liver-Chronic Liver Failure Consortium (EASL-CLIF) acute-on-chronic liver failure (ACLF) grades and modified EASL-CLIF (mEACLF) grades; B: Transplant-free mortality stratified by EASL-CLIF ACLF grades and mEACLF grades; C: AUROC for all-cause mortality by the EASL-CLIF ACLF grades and mEACLF grades; D: AUROC for transplant free mortality by the EASL-CLIF ACLF grades and mEACLF grades. EASL-CLIF: European Association for the Study of the Liver-Chronic Liver Failure Consortium; mEACLF: Modified EASL-CLIF; ROC: Receiver operating characteristic.

10.2%, 5-fold higher than the mortality rates of 2.0% for the cohort with EASL-CLIF grade 0. The number of outliers with higher mortality than their group mortality was fewer in the mEACLF cohorts when stratified by EASL-CLIF grades. These observations may suggest that mEACLF is perhaps as accurate or perhaps better in terms of mortality risk stratification. The AUROC showed consistently better prognostic ability with mEACLF than EASL-CLIF by organ failures or grades for both 30-d all-cause mortality and transplant-free mortality.

There are a few limitations to our study. Our observations are based on a retrospective analysis of an administrative dataset. Therefore, our observations need to be corroborated in a large and independent dataset. Nevertheless, we had an opportunity to develop the model based on approximately 15000 patients with organ failures from a prospectively maintained administrative dataset that included approximately 40000 patients with end-stage liver disease awaiting a liver transplant. It could be argued that these patients were selected after extensive workup for liver transplantation and may not be a true reflection of ACLF patients seen in the community. Moreover, liver transplantation is a confounder in this study. We believe that these are legitimate limitations of our study and it is also true for most studies of ACLF as they are done in mostly academic centers. It is also challenging to do a study of this nature in patients who are not listed for liver transplantation. Our study population came from the entire country and therefore truly reflects the transplant population with ACLF. The UNOS dataset did not have information about PaO2, FIO2, or mean arterial pressure (MAP), and we had to use the predefined variables in the UNOS dataset for the respiratory and circulatory system failure. We do not believe that the availability of those data would have made any meaningful differences in our observations[10].

CONCLUSION

In summary, we have shown that EASL-CLIF criteria for ACLF could be simplified without losing its

sensitivity and its ability to prognosticate 30-d all-cause and transplant-free mortality. We and others have recently shown that EASL-CLIF criteria are far more sensitive to detect ACLF than both APASL and NACSELD criteria. We believe that the mEACLF criteria that we propose are easier to use than the EASL-CLIF criteria and also have a better prognostic capability. We hope our mEACLF criteria could be adopted by the hepatology community to advance this field.

ARTICLE HIGHLIGHTS

Research background

There is no consensus on the definition of acute on chronic liver failure. We had recently shown that the definition proposed by the European Association for the Study of the Liver-Chronic Liver Failure Consortium (EASL-CLIF) is more sensitive to identify acute on chronic liver failure and has a better ability to predict all-cause and short-term mortality than that were proposed by the North American Consortium for the Study of End-Stage Liver Disease.

Research motivation

One of the major criticisms of EASL-CLIF criteria is that it is more complicated to use in clinical practice.

Research objectives

In this study, using a large dataset, our objective was to develop an easier to use model that will be easier to use in clinical practice.

Research methods

We initially assessed the prevalence of type and frequency of organ failures (OF) using EASL-CLIF. Using the same dataset, we developed modified criteria as described later under 'model development'. Patients were followed until the event date or were censored at the end of 30-ds after listing. To improve the EASL-CLIF criteria, we determined the best cutoff values for serum creatinine and international normalized ratio (INR) that were associated with higher mortality. We used a subset of patients (n = 1445) with information on glomerular filtrations rate to determine the best cutoff values for serum creatinine levels. After identifying the best serum creatinine value, we identified the optimal INR cutoff. Using the above values, we then developed a modified 6-organ failure criteria modified EASL-CLIF (mEACLF). We compared our new mEACLF criteria with the original EASL-CLIF criteria by looking at the distribution of OF, acute-on-chronic liver failure (ACLF) grades, and 30-d all-cause and transplant-free mortality rates.

Research results

The area under the receiver operating characteristic (AUROC) of 30-d all-cause mortality by ACLF grades was 0.842 (95%CI: 0.831-0.853) for mEACLF and 0.793 (95%CI 0.781-0.806) for EASL-CLIF (P < 0.0001). The AUROC of 30-d transplant-free mortality by ACLF was 0.859 (95%CI: 0.848-0.869) for mEACLF and 0.805 (95%CI: 0.793-0.817) for EASL-CLIF (P < 0.0001).

Research conclusions

Our study showed that EASL-CLIF criteria for ACLF grades could be simplified for ease of use without losing its prognostication capability and sensitivity.

Research perspectives

To advance ACLF research in a meaningful manner, it is essential to have easy-to-use criteria. We believe that the modified EASL-CLIF criteria are an important step in that direction.

FOOTNOTES

Author contributions: Thuluvath PJ contributed to the study concept, design, analysis, interpretation of data, and drafting of the manuscript; Li F did the statistical analysis, contributed to the interpretation of data, and drafted the statistical part of the manuscript.

Institutional review board statement: The study was done using a national database (UNOS) that is publicly available. The datasets are de-identified and therefore exempt from IRB approval.

Informed consent statement: This study is based on a de-identified national database (UNOS) and informed consent is not applicable.

Conflict-of-interest statement: There are no conflicts of interest to report.

Data sharing statement: Available to public from the UNOS.

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.

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ORIGINAL ARTICLE

Prospective Study

β-arrestin-2 predicts the clinical response to β-blockers in cirrhotic portal hypertension patients: A prospective study

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Abstract

BACKGROUND

Portal hypertension, a common complication associated with liver cirrhosis, can result in variceal bleeding, which greatly impacts patient survival. Recently, β-arrestin-2 has been shown to predict the acute hemodynamic response to nonselective β-blocker therapy for cirrhotic portal hypertension. However, more data is needed on the long-term effects of and changes in β-arrestin-2 following nonselective β-blocker therapy.

AIM

To investigate the expression and role of β-Arrestin-2 in predicting the long-term response to nonselective β-blockers in cirrhotic portal hypertensive patients.

METHODS

We prospectively enrolled 91 treatment-naïve patients with cirrhotic portal hypertension. Baseline clinical and laboratory data were obtained. Gastroscopy was performed for grading and treating varices and obtaining gastric antral biopsies. We measured the serum and antral expression of β-arrestin-2 and obtained Doppler measurement of the portal vein congestion index. Treatment with nonselective β-blockers was then started. The patients were followed up for 18 mo, after which they have undergone a repeat antral biopsy and re-evaluation of the portal vein congestion index.

RESULTS

A higher serum level and antral expression of β-arrestin-2 was associated with longer bleeding-free intervals, greater reduction in the portal vein congestion index, and improved grade of varices. Among patients with a low β-arrestin-2 expression, 17.6% were nonselective β-blocker responders, whereas, among those with high expression, 95.1% were responders ($P < 0.001$). A serum β-arrestin-2 value ≥ 2.23 ng/mL was associated with a lower likelihood of variceal bleeding (90% sensitivity and 71% specificity). β-arrestin-2 expression significantly decreased after nonselective β-blocker therapy.

CONCLUSION

β-arrestin-2 expression in cirrhotic portal hypertension predicts the clinical response to long-term nonselective β-blocker treatment. Serum β-arrestin-2 is a potential noninvasive biomarker for selecting the candidate patients for nonselective β-blockers.

Key Words: β-arrestin-2; Portal hypertension; Variceal bleeding; Nonselective beta-blockers; Portal congestion index; Variceal ligation

Core Tip: Gastric antral β-Arrestin-2 (β-Arr-2) expression correlates to portal hypertension in terms of esophageal varices and portal gastropathy. A stronger β-Arr-2 expression is associated with a sustained clinical response to nonselective β-blockers (NSBB) with a longer variceal bleeding-free interval. Patients who experienced variceal bleeding while on NSBB had lower baseline serum and tissue expression of β-Arr-2. In patients with responded to NSBB, the expression of β-Arr-2 was reduced after long-term treatment. The serum level of β-Arr-2 correlates to its antral expression and showed high sensitivity and specificity for defining the subgroup of patients who will respond to NSBB.

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INTRODUCTION

Portal hypertension (PHT) is a risk factor for esophageal varices (EV). Variceal bleeding can significantly affect patient survival. The main pathophysiology underlying PHT is the increased resistance and/or blood flow in the portal circulation[1,2]. Once the hepatic venous pressure gradient (HVPG) exceeds 12 mmHg, variceal bleeding occurs[3], with a 30%-50% mortality risk after the first episode and a 70% rate of early rebleeding. Therefore, the prevention of formation, growth, and rupture of varices is important in PHT management[4].

Nonselective β-blockers (NSBB) are the standard of care for primary or secondary prophylaxis against variceal bleeding[5,6]. The risk of bleeding or rebleeding is greatly diminished when HVPG is reduced by ≥ 20% or to < 12 mmHg[7]. However, there are special concerns regarding NSBB use in patients with refractory ascites or spontaneous bacterial peritonitis (SBP), in terms of patient survival and quality of life[8].

Only approximately 40% of patients with PHT show a clinical response to NSBB[9]. Therefore, several patients are exposed to unfavorable side effects without clinical benefit. Identifying who will respond to NSBB is an important unresolved question having a clinical impact. Currently, the only way to identify responders is by HVPG measurement, which is an invasive technique with limited access[10]. Therefore, the search for noninvasive predictors for NSBB response is clinically desirable.

Recently, β-arrestins-2 (β-Arr-2) has been studied as a predictor using the acute propranolol challenge in a few patients[11]. However, the long-term impact of β-Arr-2 expression on portal hemodynamics is not yet clear. Moreover, the changes in β-Arr-2 expression after long-term NSBB treatment have not been studied. We designed our study to investigate the long-term changes in β-Arr-2 and its predictive accuracy for identifying potential responders to NSBB treatment.

MATERIALS AND METHODS

Patient selection

We prospectively enrolled 120 patients with cirrhotic PHT having no previous history of endoscopic or NSBB treatment for varices. The enrollment was done between December 2017 and November 2019, with the last follow-up being performed on April 2021. We also included 40 healthy volunteers to assess normal serum β-Arr-2 levels. The study was conducted at the Internal Medicine Department, Main University Hospital, Faculty of Medicine, as well as the Endoscopy Unit at the Medical Research Institute, Alexandria University. The study was approved by the local ethics committee [Institutional Review Board: 00007555; Review Number: 0303608]. The study was conducted following the 1975 Helsinki Declaration (as revised in 2008), and the Good Clinical Practice guidelines. Informed consent was obtained from all study participants.

Exclusion criteria

We excluded patients with non-cirrhotic PHT, portal vein thrombosis, endoscopic stigmata of active or recent bleeding, previous endoscopic or NSBB variceal treatment, contraindications for NSBB treatment, bradycardia with < 50 beats/minute, SBP of < 90 mmHg, coagulopathy, malignancy, or cardiorenal disease.

Patient assessment

A history of melena or hematemesis was recorded. We performed a complete clinical examination and laboratory investigations, including complete blood count and assessment of aminotransferases, serum albumin and bilirubin levels, and international normalization ratio. The severity of liver disease was assessed using Child-Pugh classification. The Aspartate aminotransferase (AST)/platelet ratio index (APRI) was calculated. Serum β-Arr-2 levels were measured using an ELISA kit (Human ARRB2, Catalog #MBS765831, My BioSource, Inc., CA, United States) following the manufacturer's instructions [12].

Doppler ultrasound of the portal circulation was obtained using the Acuson X-300™ color Doppler machine (Siemens, CA, United States) to measure the portal vein congestion index (PVCI) at both baseline and last follow-up visits[13].

Esophagogastroduodenoscopy (EGD) was performed to evaluate the presence and grade of EV, the risk signs for variceal bleeding, presence of gastric varices (GV), and presence and grade of portal hypertensive gastropathy (PHG)[5,14]. Mucosal biopsies from the gastric antrum, body (corpus), and duodenum were taken during baseline endoscopic evaluation.

Patients with nonbleeding small varices with red wale marks, cherry-red spots, or decompensated cirrhosis and those with medium or large EV were started on propranolol primary prophylaxis (40 mg/d over 2 doses)[5,15]. The dose was then increased on alternate days to reach a target pulse of 55 beats/minutes or a maximally tolerated dose (but not exceeding 360 mg/d). The dose was maintained until the study ended if it was tolerated and an SBP > 90 mmHg was sustained.

The follow-up duration of the study was 18 mo (540 d), calculated from the first dose of NSBB. During this period, EGD was performed every 12 wk or whenever variceal bleeding occurred.

Primary endpoint

The primary endpoint was the occurrence of variceal bleeding necessitating intervention, such as endoscopic variceal ligation or sclerotherapy (EVL/EST). Patients who bled were designated as "NSBB non-responders". Conversely, patients who did not experience variceal bleeding in the 540 days of follow-up were designated as "NSBB responders".

At the end of the study (either when variceal bleeding occurred or the end of follow-up), EGD was performed for EV grading/treatment and obtaining a second antral mucosal biopsy to re-assess β-Arr-2 expression. Variceal bleeding was defined as hematemesis, melena, or hematochezia with endoscopic evidence of the variceal source after excluding nonvariceal sources, including the biopsy site.

The histopathological expression of β-Arr-2 was evaluated by immunohistochemical staining using human ARRB2 antibody (B-arrestin-2, Cat. #PA002135LA01HU, CUSABIO, United States) following the manufacturer's instructions. The degree of β-Arr-2 expression was semiquantitatively expressed as (+) low, (++) moderate, and (+++) strong staining[16]. The endoscopic and pathologic evaluations were blinded.

The response to NSBB was evaluated clinically (signs of bleeding during the follow-up), endoscopically (changes in variceal grade), and by Doppler ultrasound assessment of changes in the PVCI *i.e.* ΔPVCI.

Statistical analysis

The sample size was calculated using Stata/MP v.15.1 software (StataCorp LLC, United States) with a statistical power of 90%, a two-tailed α level of 0.05, and assuming a value of 0.5 as a hazard ratio. Normality of distribution was assessed, and data were expressed as a mean ± SD or proportions. The student's *t*-test or the ANOVA test was used as appropriate. The Chi-square (χ^2), Fisher's Exact (*FET*), or

McNemar (Bowker's) test was used to compare proportions. The sensitivity and specificity of serum β-Arr-2 were assessed by receiver-operating characteristic (ROC) curve. Correlations between variables were analyzed by Pearson's or Spearman's test as appropriate. Multivariate regression, Cox regression, Kaplan-Meier analysis were done.

RESULTS

During the study, 29 patients were lost to follow-up (during the COVID-19 pandemic). The analysis was done for 91 patients (per-protocol analysis). In total, 31 (34.1%) patients experienced variceal bleeding (NSBB non-responders), and 60 (65.9%) patients did not bleed (NSBB responders). At baseline, small, medium, and large EVs were present in 17 (18.7%), 48 (52.7%), and 26 (28.6%) patients, respectively. Mild and severe PHG was detected in 32 (35.2%) and 33 (36.2%) patients, respectively. GV was seen in 14 (15.4%) patients. Table 1 shows the baseline clinical and laboratory data.

Baseline serum and tissue expression of β-Arr-2

The serum β-Arr-2 levels in patients were higher than those in healthy controls (mean ± SD), 2.57 ± 0.48 *vs* 1.59 ± 1.29 ng/mL, respectively, $P < 0.001$). At baseline, serum β-Arr-2 levels in the responders were higher than those in the non-responders (mean ± SD, 2.79 ± 0.40 *vs* 2.13 ± 0.28 ng/mL, respectively, $P < 0.001$, 95%CI: -0.80 to -0.51) (Supplementary Figure 1). The tissue expression of β-Arr-2 in the gastric antrum was significantly different between subgroups [(5%, 30% and 65% among responders *vs* 45.2%, 48.4% and 6.4% among non-responders) for low (+), moderate (++), and strong (+++) expressions, respectively, $\chi^2 = 30.1$, $P < 0.001$]. No significant difference was found in terms of β-Arr-2 expression in the gastric body ($P = 0.23$) or duodenum ($P = 0.40$). Therefore, the statistical analysis subsequently focused on β-Arr-2 expression in the gastric antrum (Figure 1, Figure 2A).

β-Arr-2 vs esophageal and GV at baseline

Patients were stratified according to the baseline grade of antral β-Arr-2 expression ($n = 91$). The comparison between these strata showed that stronger antral β-Arr-2 expression was associated with a higher EV grade at baseline (43.9% of patients with strong β-Arr-2 expression ($n = 41$) had large EV *vs* 29.4% and 9.1% for low ($n = 17$) and moderate ($n = 33$) β-Arr-2 expression, respectively, $\chi^2 = 14.2$, $P = 0.007$) (Figure 2B). However, there was no significant difference between these strata as regards the presence of GV ($P = 0.11$).

The serum β-Arr-2 levels were higher in patients with large EVs than in those with medium and small EVs and patients with medium EVs than in those with small EVs (mean ± SD, 2.90 ± 0.42 *vs* 2.50 ± 0.44 and 2.24 ± 0.42 ng/mL respectively, $P < 0.001$) (Supplementary Figure 2). Patients with GV showed a higher mean serum β-Arr-2 levels than patients without GV (2.85 ± 0.37 *vs* 2.51 ± 0.48 ng/mL, $P = 0.007$). However, the number of patients with GV in the current study was too small ($n = 14$) for detailed analysis.

β-Arr-2 vs PVCI at baseline

Patients with strong antral β-Arr-2 expression showed higher mean values of PVCI than those with moderate and low expression (0.566 ± 0.09 *vs* 0.517 ± 0.11 and *vs* 0.483 ± 0.08 cm^2/s, $P = 0.04$ and $P < 0.005$ respectively). There was no difference between patients with low and moderate expression of β-Arr-2 in terms of mean values of PVCI ($P = 0.24$).

PVCI and EV before and after NSBB therapy

The mean value of PVCI after NSBB treatment in NSBB-responders significantly decreased compared with the baseline (0.492 ± 0.11 *vs* 0.545 ± 0.10 cm^2/s, $P < 0.001$, 95%CI: 0.04 - 0.06), whereas in non-responders, there was no difference (0.511 ± 0.09 *vs* 0.509 ± 0.08 cm^2/s, $P = 0.76$). Also, the mean value of ΔPVCI among responders was higher than that among non-responders (0.0538 ± 0.06 *vs* 0.002 ± 0.04 cm^2/s, $P < 0.001$).

At baseline, the frequency of small, medium, and large EV between patients ($n = 91$) was 18.7%, 52.7%, and 28.6% of cases, respectively. At the end of the study, the frequency of small, medium, and large EV was changed to 31.9%, 45.1%, and 23.1% of cases, respectively ($P = 0.049$, $\chi^2 = 7.6$, Supplementary Figure 3).

Before NSBB treatment, the EV grades were not significantly different between responders and non-responders (Table 1). However, after treatment, a significant difference in favor of the responders appeared (the frequency decreased from 36.7% to 21.7% and from 46.7% to 36.7% for large and medium varices, respectively, $P = 0.003$). Among non-responders, there was a progression in the EV grades compared with baseline ($P = 0.03$) (Figure 2C).

Antral β-Arr-2 expression after NSBB treatment

There was a significant change in the antral expression of β-Arr-2 after treatment with NSBB compared

Table 1 Baseline clinical and laboratory data of the study population

	PPA (*n* = 91)	NSBB responders (*n* = 60)	NSBB non-responders (*n* = 31)	*P* value
Male sex *n* (%)	65 (71.4)	44 (73.3)	21 (67.7)	0.37[1]
Age (years)	55.16 ± 4.0	55.43 ± 3.74	54.64 ± 4.57	0.38[2]
Ascites *n* (%)	29 (31.9)	19 (31.7)	10 (32.3)	0.57[1]
HB (g/dL)	11.07 ± 1.44	11.10 ± 1.66	11.02 ± 0.91	0.81[2]
Platelets (× 10^3/mm^3)	83.13 ± 12.33	85.32 ± 15.46	78.90 ± 12.19	0.02[2]
WBCs (× 10^3/mm^3)	4.85 ± 1.05	4.88 ± 1.11	4.78 ± 0.96	0.66[2]
ALT (IU/L)	40.01 ± 9.74	40.75 ± 9.98	38.58 ± 9.23	0.32[2]
AST (IU/L)	54.40 ± 14.00	55.48 ± 14.24	52.29 ± 13.47	0.31[2]
APRI-score	1.94 ± 0.46	1.92 ± 0.30	1.97 ± 0.56	0.63[2]
Albumin (g/dL)	2.94 ± 0.18	2.93 ± 0.17	2.97 ± 0.18	0.25[2]
Bilirubin (mg/dL)	1.73 ± 0.62	1.65 ± 0.55	1.89 ± 0.72	0.11[2]
INR	1.35 ± 0.18	1.34 ± 0.17	1.39 ± 0.16	0.19[2]
CTP A/B/C	48/36/7	34/22/4	14/14/3	0.56[3]
Baseline PVCI	0.533 ± 0.10	0.545 ± 0.11	0.509 ± 0.09	0.11[2]
Baseline esophageal varices grade: *n* (%)				
Small	17(18.7)	10 (16.7)	7 (22.6)	0.06[1]
Medium	48 (52.7)	28 (46.6)	20 (64.5)	
large	26 (28.6)	22 (36.7)	4 (12.9)	
Baseline portal hypertensive gastropathy grade: *n* (%)				
No	26 (28.6)	13 (21.7)	13 (41.9)	0.012[1]
Mild	32 (35.2)	19(31.7)	13 (41.9)	
Severe	33 (36.2)	28 (46.6)	5 (16.2)	
GV presence *n* (%)	14 (15.4)	12 (20.0)	2 (6.5)	0.08[3]
NSBB dose (min-max): (mg/d)	64.84 ± 14.63 (30-100)	65.33 ± 15.45 (30-100)	63.87 ± 13.08 (40-80)	0.65[2]

[1]Chi-square test.
[2]Independent sample *t*-test.
[3]Fisher's exact test.
ALT: Alanine aminotransferase; APRI: Aspartate aminotransferase/platelet ratio index; AST: Aspartate aminotransferase; β-Arr-2: β-Arrestin-2; CI: Congestion index; CTP: Child-Turcotte-Pugh class; GV: Gastric varices; HB: Hemoglobin; INR: International normalization ratio; ITA: Intention to treat analysis; Max: Maximum; Min: Minimum; NSBB: Nonselective beta-blocker; PPA: Per-protocol analysis; WBCs: White blood cells.

with baseline. At baseline, 18.6% of patients showed low expression, 36.3% showed moderate expression, and 45.1% showed strong expression. After NSBB treatment, 42.9% of patients showed low expression, 37.4% showed moderate expression, and 19.7% showed strong expression (McNemar Bowker's $\chi^2 = 16.18$, $P = 0.001$). Among the NSBB-responders, the frequency of strong β-Arr-2 expression significantly decreased (20% strong expression post-treatment *vs* 65% at baseline, $\chi^2 = 26.6$, $P < 0.001$); whereas in the non-responders, there was no significant difference between baseline and post-treatment expression ($P = 0.54$) (Figure 2D). Multivariate regression showed that NSBB dose ($P = 0.02$, OR = 1.01, 95%CI: 0.91-1.05) and the ΔPVCI ($P = 0.005$, OR = 1.58, 95%CI: 0.001- 0.002) were the only independent predictors of reduced β-Arr-2 expression (Table 2).

Correlations between β-Arr-2 and study parameters

Serum and antral expression of β-Arr-2 were directly correlated to each other (rs = 0.72, $P < 0.001$). Both serum and antral expression of β-Arr-2 showed a direct correlation with baseline EV grade, baseline PHG, PVCI, and APRI score. They also showed a negative correlation with platelet count and serum AST ($P < 0.05$) (Table 3). In addition, β-Arr-2 expression intensity after NSBB therapy was directly correlated with the severity of PHG (rs = 0.35, $P < 0.001$).

Table 2 Univariate and multivariate analysis for predictors of diminished β-Arrestin-2 expression after nonselective β-blockers

Parameters	Univariate analysis		Multivariate analysis		
	P value	OR	*P* value	OR	95%CI
ALT	0.51	-	-	-	-
AST	0.20	-	-	-	-
Platelets	0.26	-	-	-	-
APRI	0.06	-	-	-	-
Albumen	0.25	-	-	-	-
Bilirubin	0.69	-	-	-	-
Child-Turcotte-Pugh class	0.73	-	-	-	-
Baseline EV grade	0.003	1.35	-	-	-
Baseline β-Arr-2 expression	< 0.001	2.41	-	-	-
NSBB dose	0.049	6.5	0.02	1.01	0.91-1.05
ΔPVCI	< 0.001	10.34	0.005	1.58	0.001- 0.002

ALT: Alanine aminotransferase; APRI: Aspartate aminotransferase/platelet ratio index; AST: Aspartate aminotransferase; β-Arr-2: β-Arrestin-2; ΔPVCI: Delta (change in) portal vein congestion index; EV: Esophageal varices; NSBB: Nonselective beta-blockers.

Table 3 Correlations between serum and antral expression of β-arrestin-2 and different study parameters

	Serum β-Arr-2 level		β-Arr-2 expression in the gastric antrum	
	rs	*P* value	rs	*P* value
Age	0.10[1]	0.43	0.16	0.14
Child-Pugh class	0.11	0.34	0.08	0.45
INR	0.12[1]	0.19	0.11	0.34
Serum albumin	-0.19[1]	0.07	-0.12	0.26
Serum bilirubin	0.12[1]	0.19	0.03	0.77
Presence of ascites	0.11	0.30	0.14	0.18
Platelets count	-0.38[1]	< 0.001	-0.28	0.008
Baseline EV grade	0.48	< 0.001	0.30	0.004
Baseline PHG grade	0.33	0.002	0.38	< 0.001
Baseline congestion index	0.36[1]	< 0.001	0.32	0.002
Antral β-Arr-2expression	0.72	< 0.001	-	-
Corpus β-Arr-2 expression	0.17	0.10	0.11	0.28
Duodenal β-Arr-2 expression	0.19	0.07	0.13	0.23
AST	-0.23[1]	0.03	-0.25	0.019
APRI score	0.34[1]	< 0.001	0.25	0.039
Non-selective B-blocker dose	0.15[1]	0.15	0.03	0.73

[1]Pearson's correlation. APRI: Aspartate aminotransferase/platelet ratio index; AST: Aspartate aminotransferase; β-Arr-2: β-Arrestin-2; EV: Esophageal varices; INR: International normalization ratio; PHG: Portal hypertensive gastropathy.

Variceal bleeding and baseline expression of β-Arr-2

At baseline, 17 patients had low β-Arr-2 expression; among them, 14 patients (82.4%) experienced variceal bleeding. Further, 33 patients had moderate β-Arr-2 expression; among them, 15 (45.5%) patients experienced variceal bleeding. Similarly, 41 patients had strong β-Arr-2 expression, and among them, 2 (4.9%) patients experienced variceal bleeding ($\chi^2 = 35.10$, $P < 0.001$).

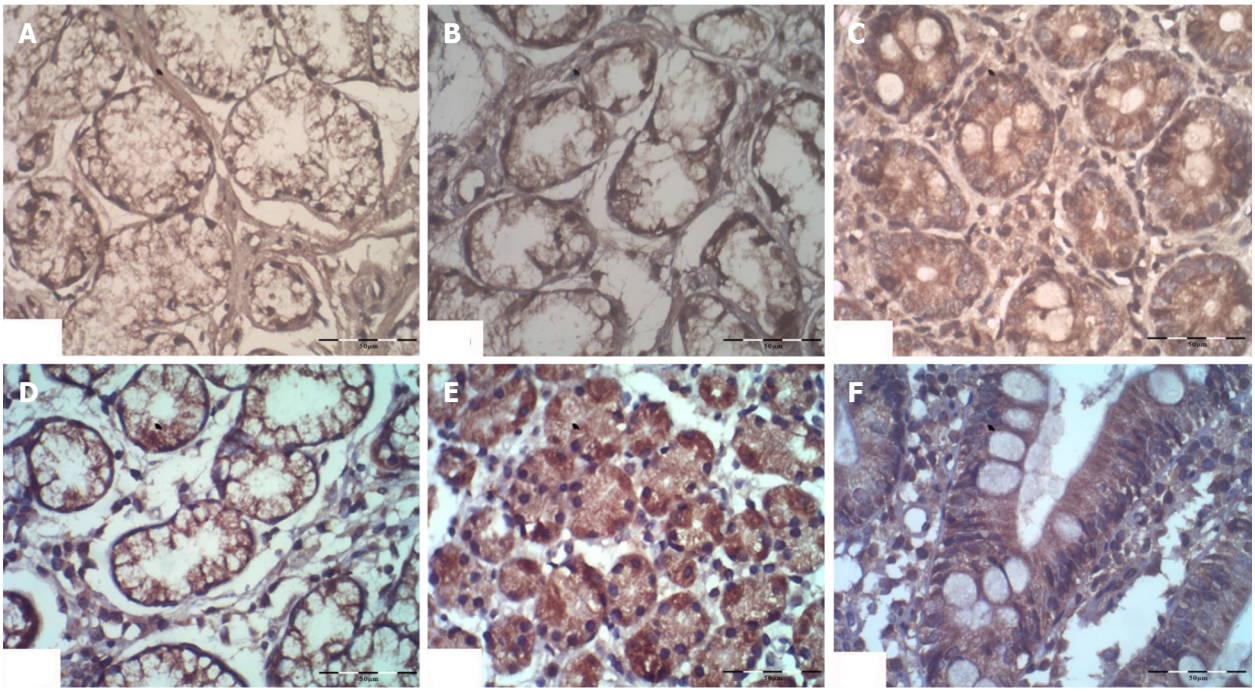

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Figure 1 The statistical analysis subsequently focused on β-Arr-2 expression in the gastric antrum. Cytoplasmic staining of β-Arrestin-2 (× 40) showing (A, B, C) low, moderate, and strong expression in the gastric antrum; (D, E) moderate and strong expression in the gastric body; and (F) strong expression in the duodenum respectively.

At a cut-off value of ≥ 2.23 ng/mL, serum β-Arr-2 could be used to identify patients at low risk of variceal bleeding with a sensitivity and specificity of 90% and 71%, respectively. The positive predictive value was 79%, and the negative predictive value was 85.7% (AUC = 89.2%, $P < 0.001$, 95%CI: 0.83-0.96) (Figure 3).

Kaplan-Meier and regression analysis

Kaplan-Meier analysis with log-rank test was performed ($n = 91$). Patients with strong baseline antral β-Arr-2 expression and receiving NSBB treatment had a longer variceal bleeding-free interval. The mean (median) time interval before variceal bleeding for low, moderate, and strong antral β-Arr-2 expression was 351.7 (290), 481.6 (540), and 538.5 (540) days, respectively ($\chi^2 = 62.02$, $P < 0.001$) (Figure 4A).

In addition, patients with a serum β-Arr-2 level of ≥ 2.23 ng/mL (as obtained from ROC analysis, $n = 63$) had a longer bleeding-free interval compared with patients who had a serum β-Arr-2 level of < 2.23 ng/mL ($n = 28$). The mean (median) variceal bleeding-free interval was 527.5 (540) and 382.8 (360) days respectively, ($\chi^2 = 57.6$, $P < 0.001$) (Figure 4B).

Via Cox-regression analysis, serum β-Arr-2 level ($P < 0.001$, OR = 0.13, 95%CI: 0.09-0.13), the intensity of β-Arr-2 expression in the gastric antrum ($P < 0.001$, OR = 0.15, 95%CI: 0.1-0.3), and platelet count ($P = 0.006$, OR = 0.93, 95%CI: 0.85-0.99), were the only independent predictors for variceal bleeding (Table 4).

Adverse events during the follow up (SBP and refractory ascites)

Among patients who completed the follow-up ($n = 91$), we had 7 (7.7%) patients who developed SBP. This subgroup of patients had Child-Pugh class B (2 patients) and C (5 patients). One patient (14.3%) of them had a strong β-Arr-2 expression, four (57.1%) patients had a moderate expression, and two (28.6%) patients had low expression. They also have a mean serum β-Arr-2 of 2.15 ± 0.3 ng/mL (*vs* 2.60 ± 0.47 ng/mL for patients without SBP, $P = 0.006$). This subgroup of patients received a mean dose of NSBB = 60 ± 19.1 mg (*vs* 65.23 ± 14.26 mg for patients without SBP, $P = 0.36$).

In addition, we have 5 (5.5%) patients who developed refractory ascites. This subgroup of patients had Child-Pugh class B (1 patient) and C (4 patients). Two (40%) patients had low β-Arr-2 expression and 3 (60%) patients had moderate expression (no patients showed strong expression). They had a mean serum β-Arr-2 of 2.10±0.19 ng/mL (*vs* 2.59 ± 0.48 ng/mL for patients without SBP, $P = 0.002$). This subgroup of patients received a mean dose of NSBB = 70 ± 10 mg (*vs* 64.5 ± 14.85 mg for patients without refractory ascites, $P = 0.42$).

Table 4 Cox-regression analysis for predictors of variceal bleeding among patients

Baseline parameters	*P* value	OR	95%CI
Alanine aminotransferase	0.32	-	-
Aspartate aminotransferase	0.19	-	-
Platelets count	0.006	0.93	0.85- 0.99
APRI score	0.11	-	-
Child-Turcotte-Pugh class	0.54	-	-
Baseline esophageal varices grade	0.29	-	-
Baseline portal vein congestion index	0.49	-	-
B-arrestin-2 antral expression	< 0.001	0.15	0.1- 0.3
Serum β-arrestin-2	< 0.001	0.13	0.09-0.13

APRI: AST/platelet ratio score; CI: Confidence interval; OR: Odd's ratio.

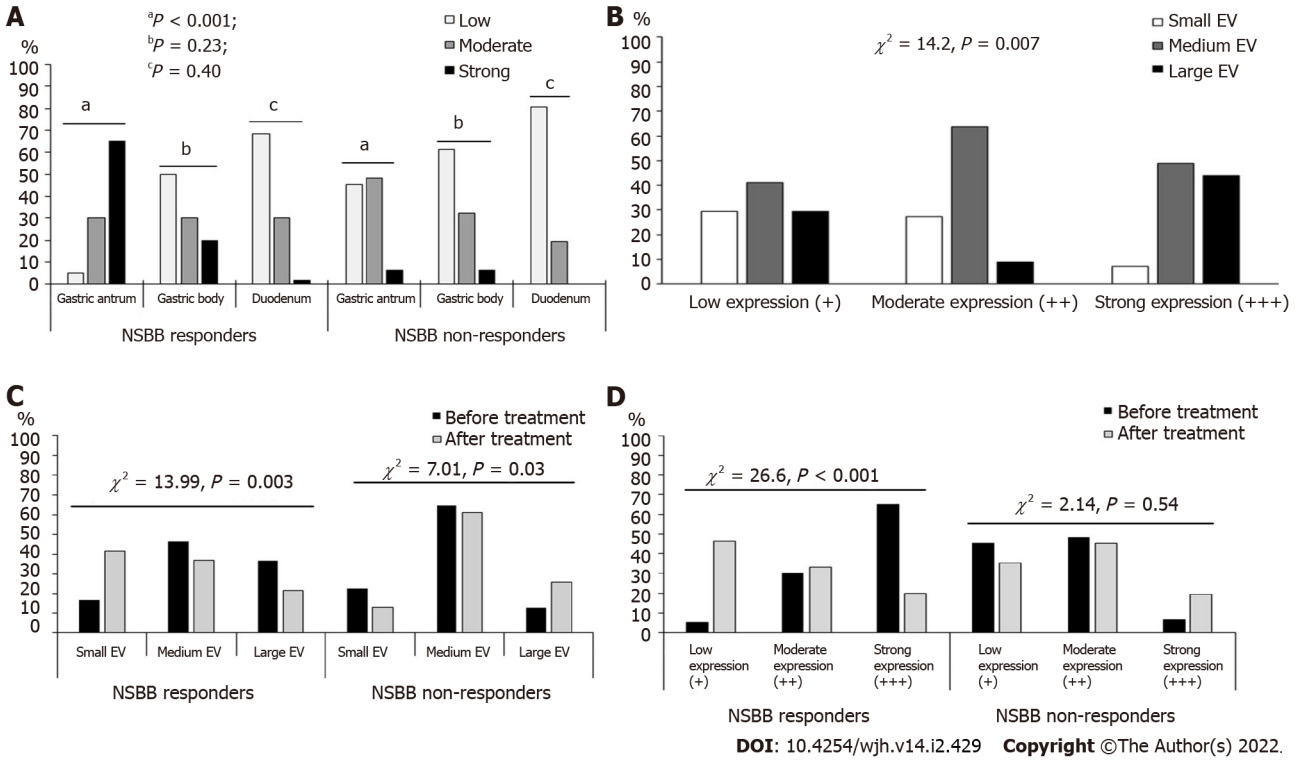

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Figure 2 The statistical analysis subsequently focused on β-Arr-2 expression in the gastric antrum. A: Comparison between nonselective β-blockers (NSBB) responders and non-responders as regards β-Arrestin-2 expression, [a]*P* < 0.001; [b]*P* = 0.23; [c]*P* = 0.40; B: The frequency of small, medium, and large esophageal varices according to different intensities of β-Arrestin-2 expression at baseline; C: Comparison between NSBB responders and Non-responders as regards the changes in the frequency of low, medium, and large esophageal varices before and after treatment; D: Comparison between NSBB responders and Non-responders as regards the changes in the frequency of low, moderate, and strong antral β-Arrestin-2 expression before and after treatment. EV: Esophageal varices; NSBB: Nonselective beta-blockers.

DISCUSSION

Nonselective β-blockers reduce portal pressure by minimizing the cardiac output *via* the blockade of β1 cardiac receptors and enhancing splanchnic vasoconstriction *via* the blockade of β2 receptors, leaving an unopposed α-adrenergic activity[17].

The core findings in our study were as follows: (1) Gastric antral β-Arr-2 expression is more related to the portal hemodynamics than corpus or duodenal expression; (2) β-Arr-2 expression correlates with the degree of PHT in terms of EV and PHG grades; (3) Stronger β-Arr-2 expression is associated with sustained clinical response to NSBB, decrease in the PVCI, better EV control, and longer variceal bleeding-free interval; (4) Patients who experienced variceal bleeding while on NSBB therapy had a

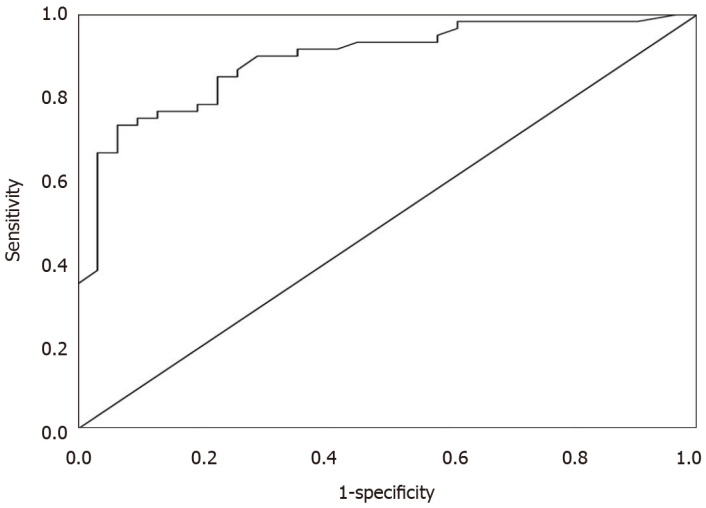

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Figure 3 A receiver operating characteristic curve analysis of serum β-Arrestin-2 Levels to identify patients with a low likelihood of variceal bleeding.

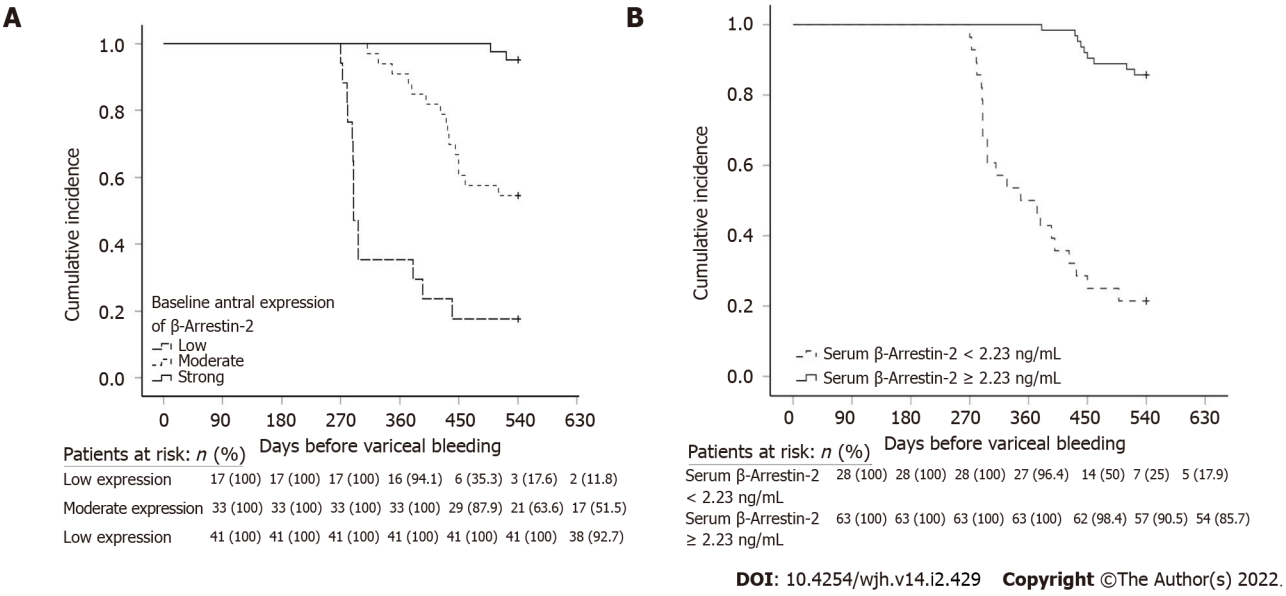

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Figure 4 The cumulative incidence rates of variceal bleeding among NSBB non-responders group concerning. A: Baseline β-Arrestin-2 (β-Arr-2) antral expression; B: serum β-Arr-2 levels.

lower baseline serum and tissue expression of β-Arr-2; (5) Patients who did not bleed during NSBB therapy (NSBB responders) showed a reduction in the expression of β-Arr-2 after long-term treatment, highlighting the link between PHT dynamics and β-Arr-2 expression; and (6) the serum level of β-Arr-2 directly correlates with the antral expression of β-Arr-2 and show high sensitivity and specificity for defining the subgroup of patients who will respond to NSBB with a low likelihood for variceal bleeding. These results suggest that serum and gastric antral β-Arr-2 are potentially simple and minimally invasive markers for PHT patients who may show a favorable response to NSBB.

Although NSBB minimizes the risk of variceal bleeding, this is observed only in approximately 40% of cases, leaving 60% of patients vulnerable to the drug's adverse effects without any benefits. Therefore, identifying such patients is essential, especially among patients with refractory ascites and spontaneous bacterial peritonitis[8,18].

The pathophysiology of PHT involves the down-regulation of vasoactive proteins [RhoA/Rho kinase (ROCK)] and up-regulation of vasodilators [*e.g.* Nitric oxide (NO)]. This contributes to splanchnic vasodilation and induction of the renin-angiotensin-aldosterone signaling (RAAS) pathway[19]. However, the vasoconstrictor angiotensin II (AT-2) and other vasoactive substances [*e.g.*, endothelin 1 (ET-1)] fail to induce splanchnic vasoconstriction due to the down-regulation of downstream pathways involving RhoA and ROCK. This probably extends to the mucosal vasculature and is not limited to the

large splanchnic vessels[19].

β-Arr-2 expression is increased in splanchnic vessels of animals and humans with cirrhosis and could suppress the vasoactive signaling *via* the desensitization of the AT-2 and ET-1 receptors[19,20]. This explains the direct correlation between the serum levels and tissue expression of β-Arr-2 on one side and the grade of varices and PHG on the other side, which was demonstrated in our study, corroborating previous research[11,21,22].

As it determines the probability of achieving more benefit than damage, the timing of initiation of NSBB therapy is clinically important. In the early stages of PHT, RAAS system activation is minimal. This results in a milder form splanchnic and systemic hyperdynamic state; therefore, the splanchnic circulation sensitivity to NSBB remains turned off[23,24]. This clinically important aspect can be identified indirectly *via* a minimally invasive technique through the correlation between β-Arr-2 expression and PHT severity. Also, serum and tissue expression of β-Arr-2 can aid in selecting patients who will benefit from NSBB in cases of advanced cirrhosis, in which life-threatening complications of NSBB can occur[8,24].

The mechanisms by which NSBB lowers the portal pressure[17] are driven by their increased affinity to β adrenergic receptors-1 and 2 (β1 and 2-AR)[25]. β-Arr-2 signaling has been linked to β1-AR up-regulation. In mice, β-Arr-2 overexpression has been found to restore the inotropic properties of β1-AR. In patients with heart failure, β-Arr-2 could upregulate β1-ARs (so that they are more ready for β-blocker binding). Also, β-Arr-2 can bind and inhibit β1-AR through the kinase pathway (synergistic effect with β-blockers)[26-28].

With regards to β2-AR, the overexpression of β-arr-1 or βarr-2 in human airway smooth muscle (ASM) cultures causes β2-AR desensitization and β agonist-stimulated signaling attenuation[29]. In *in vivo* and *ex vivo* murine models of ASM contractile regulation, β-Arr-2 appeared to antagonize β agonist-mediated ASM relaxation[30]. β-Arr-2 can preferentially bind to PIP5K-Iα and μ2-adaptin proteins, which regulate G protein-coupled receptor trafficking and enhance β2-AR endocytosis. These effects synergize in the attenuation of the physiological functions of β1 and β2-AR[31].

These interactions might also apply for splanchnic β2-AR and may explain the enhanced response of patients with PHT, who have a stronger expression of β-Arr-2, to NSBB treatment. With the lack of a correlation between NSBB dose and β-Arr-2 expression in our study, we suggest that β-Arr-2 might manipulate portal hemodynamics through a direct synergistic effect and by enhancing the affinity of β2-AR for binding NSBB rather than a dose-related effect. This is supported by the absence of a significant difference in the mean dose of NSBB between responders and non-responders in our results.

In the current study, few patients developed SBP and refractory ascites. The lack of significant difference in NSBB dose in these events among subgroups may support the possible hypothesis of β2-AR receptors readiness for NSBB therapy rather than a dose-dependent effect. This again, emphasizes the role of β-Arr-2 as a marker to select patients with PHT who will tolerate NSBB therapy without complications. However, the number of cases with SBP and refractory ascites in our cohort is too low to provide a conclusion as regards this point and more validation on a wide scale is reco-mmended.

In the current study, the antral expression of β-Arr-2 decreased significantly in the NSBB responders. Similarly, Trebicka *et al*[11,21] found in their study that β-Arr-2 expression in the gastric antrum decreased after performing a trans-jugular intrahepatic portosystemic shunt. Following a decrease in the portal pressure, they found a reversal of the vasoactive protein expression toward normal. In another study, however, β-Arr-2 expression remained unchanged despite HVPG reduction. Further investigation of the changes in β-Arr-2 expression is recommended to resolve these inconsistencies.

This study has some limitations. We did not perform HVPG measurement due to its invasiveness and unavailability in our institute. Further, we did not assess other vasoactive substances, such as NO and RhoA, due to financial constraints. Nevertheless, the current study still has notable strengths. In addition to the prospective design, we studied a relatively large number of patients and had a relatively long follow-up period. This is also the first study to provide the measurement of β-Arr-2 in the serum of patients with PHT with good sensitivity and specificity.

CONCLUSION

Antral β-Arr-2 expression in PHT patients correlates to the severity of PHT. Stronger expression is associated with a better response to NSBB and longer variceal bleeding-free interval. We suggest assessing serum β-Arr-2 level as a potential, noninvasive biomarker for identifying PHT patients who are good candidates for NSBB therapy. In addition, we recommend future studies to validation of the current results on a larger scale of patients.

ARTICLE HIGHLIGHTS

Research background

Variceal bleeding is a life-threatening complication of portal hypertension (PHT). Nonselective β-blockers (NSBB) are used as primary or secondary prophylaxis in patients with PHT. The use of NSBB has been associated with the development of refractory ascites and spontaneous bacterial peritonitis in a subgroup of patients. β-arrestin-2 (β-Arr-2) has been shown to predict the short-term response to NSBB in a few studies.

Research motivation

There is a gap of knowledge still present. The previous research about β-Arr-2 was about the acute hemodynamic response to NSBB infusion, but no data about the long-term effects. About two-thirds of patients with PHT fail to respond to NSBB, with the exposure to undesirable side effects. Identifying this subset of patients noninvasively is of clinical importance. Again, the long-term changes in β-Arr-2 expression after NSBB therapy have not yet been investigated.

Research objectives

We aimed to investigate the role of both serum and tissue expression of β-Arr-2 as a minimally invasive to predict the long-term clinical response of PHT to NSBB therapy, as well as to investigate the long-term changes in β-Arr-2 expression after NSBB therapy.

Research methods

We prospectively enrolled 120 patients with cirrhotic PHT. Full history and clinical evaluation were done. Laboratory investigations including serum β-Arr-2 were done. Doppler ultrasound of the portal circulation to measure the portal vein congestion index (PVCI) was obtained. Esophagogastroduodenoscopy (EGD) was performed to evaluate the presence and grade of varices and to obtain mucosal biopsies to define the expression of β-Arr-2. NSBB therapy was initiated. A follow-up for 18 mo (540 d) was done. Another endoscopic biopsy was obtained at the end of the study to re-assess the tissue expression of β-Arr-2. Patients were designated as "NSBB responders" if they didn't experience variceal bleeding until the end of follow-up; or "NSBB non-responders" if they had bled. PVCI was re-evaluated at the end of the study.

Research results

A higher serum level and antral expression of β-Arr-2 were associated with better clinical response to NSBB (longer bleeding-free intervals, and improved grade of varices). Only 17.6% of patients with low baseline β-arr-2 expression responded to NSBB, whereas, 95.1% of patients with strong β-arr-2 expression were responders ($P < 0.001$). A serum β-Arr-2 value ≥ 2.23 ng/mL was associated with a lower likelihood of variceal bleeding with 90% sensitivity and 71% specificity. β-arrestin-2 expression significantly decreased after nonselective β-blocker therapy. Serum β-Arr-2 level ($P < 0.001$), the intensity of β-Arr-2 expression in the gastric antrum ($P < 0.001$), and platelet count ($P = 0.006$), were the only independent predictors for variceal bleeding

Research conclusions

The serum level and tissue expression of β-Arr-2 in the gastric antrum are correlated to the severity of PHT. The lower β-Arr-2 expression can predict non-response to NSBB therapy. Stronger expression is linked to a better long-term clinical response to NSBB in terms of variceal bleeding-free interval. We introduce serum β-Arr-2 level as a potential, noninvasive biomarker for selecting patients with PHT who are potentially good candidates for NSBB therapy.

Research perspectives

Future studies are needed to validate the results of our study on a wider scale of patients. Prospective research is needed to explore the relation between the expression of β-Arr-2 and the development of spontaneous bacterial peritonitis and hepatorenal syndrome in cirrhotic PHT.

FOOTNOTES

Author contributions: Lashen SA drafted the manuscript and performed data analysis, participated in study design, was involved with data collection, and performed the endoscopic assessment; Shamseya MM drafted the manuscript, participated in study design, was involved with data collection, and performed the endoscopic assessment; Madkour MA was involved with data collection, drafted the manuscript, performed the Doppler evaluation, and assisted in the data analysis; Abdel Salam RM and Mostafa SS equally drafted the manuscript, were involved with data collection, and performed the pathological analysis; all authors read and approved the final manuscript.

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SYSTEMATIC REVIEWS

Timing of surgical repair of bile duct injuries after laparoscopic cholecystectomy: A systematic review

Patryk Kambakamba, Sinead Cremen, Beat Möckli, Michael Linecker

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Abstract

BACKGROUND

The surgical management of bile duct injuries (BDIs) after laparoscopic cholecystectomy (LC) is challenging and the optimal timing of surgery remains unclear. The primary aim of this study was to systematically evaluate the evidence behind the timing of BDI repair after LC in the literature.

AIM

To assess timing of surgical repair of BDI and postoperative complications.

METHODS

The MEDLINE, EMBASE, and The Cochrane Library databases were systematically screened up to August 2021. Risk of bias was assessed *via* the Newcastle Ottawa scale. The primary outcomes of this review included the timing of BDI repair and postoperative complications.

RESULTS

A total of 439 abstracts were screened, and 24 studies were included with 15609 patients included in this review. Of the 5229 BDIs reported, 4934 (94%) were classified as major injury. Timing of bile duct repair was immediate (14%, n = 705), early (28%, n = 1367), delayed (28%, n = 1367), or late (26%, n = 1286). Standardization of definition for timing of repair was remarkably poor among studies. Definitions for immediate repair ranged from < 24 h to 6 wk after LC

while early repair ranged from < 24 h to 12 wk. Likewise, delayed (> 24 h to > 12 wk after LC) and late repair (> 6 wk after LC) showed a broad overlap.

CONCLUSION
The lack of standardization among studies precludes any conclusive recommendation on optimal timing of BDI repair after LC. This finding indicates an urgent need for a standardized reporting system of BDI repair.

Key Words: Bile duct injury; Major bile duct injury; Laparoscopic cholecystectomy; Surgical repair; Immediate repair; Early repair; Delayed repair; Late repair; Biliary reconstruction; Standardization of bile duct injury repair reporting

Core Tip: Bile duct injury after laparoscopic cholecystectomy is a relevant iatrogenic complication, which urgently needs attention. In this systematic review, we would like to focus on surgical repair and particularly on the timing of repair. This literature search reveals that the ideal timing of repair is reported remarkable poorly, indicating an urgent need for standardization to better direct treatment of this condition.

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INTRODUCTION

Bile duct injury (BDI) remains the most serious and challenging adverse event after laparoscopic cholecystectomy (LC)[1-5]. If not recognized and treated properly, BDI may lead to severe morbidity and even death of the patient due to biliary peritonitis and sepsis[6-8].

The management of BDI requires multidisciplinary input, demanding close collaboration of surgeons, gastroenterologists, and interventional radiologists[9-13]. Endoscopic or interventional strategies may suffice in the treatment of minor BDI such as cystic stump leakage or partial laceration[14,15]. However, major BDI often requires surgical repair[10,16]. Due to the anatomical complexity of the biliary tree, surgical BDI repair requires a certain expertise in biliary reconstruction and therefore referral to a tertiary center with a division specialized in hepatobiliary (HPB) surgery is strongly recommended[17-21].

Alongside the extent of injury and surgical experience of those managing BDI, it has been suggested that timing of BDI repair may be a significant prognostic factor for clinical outcomes[10,20-24]. To date, the timing of BDI repair is controversial, with discussions in the literature failing to reach clear recommendations. Whereas several groups claim superiority of early BDI repair[25,26], other publications report beneficial outcome measures if BDI repair was delayed[27-29]. Inconsistent methods of reporting and a plethora of distinct definitions for time intervals create difficulties in comparing study outcomes and draw conclusions on the best timing of BDI repair[29,30].

Therefore, the aim of this systematic review was first to investigate the existing literature on outcome after BDI repair according to timing of repair and second to analyze the standardization concerning definitions of timing of BDI repair among studies.

MATERIALS AND METHODS

Search strategy

A systemic electronic search for studies published until August 2021 was preformed, which screened different databases such as Medline, EMBASE, and Cochrane. The search strategy was designed to screen for publications reporting timing of BDI repair and outcome according to timing. Related key phrases and MESH subject headings were combined. The initial search was completed by an objective librarian (Supplementary Table 1).

Inclusion and exclusion criteria

All studies reporting BDI repair after LC, including information on timing and postoperative outcome, were included. Abstracts, reviews, case reports, letters to the editor, and articles only available in non-English language were excluded from analysis. Additionally, studies not reporting postoperative outcome according to timing of BDI repair were excluded.

Data extraction and risk of bias assessment

The following data was extracted: Study period, number of patients, age, number of BDI, classification of BDI, presence of concomitant vascular injury, timing and type of BDI repair, and postoperative outcome after BDI repair. The primary outcome of this study was the definition of timing of BDI repair. Postoperative complications were considered as the secondary outcome.

Two independent reviewers (Kambakamba P and Linecker M) screened all articles and checked the extracted data for accuracy. The Newcastle-Ottawa scale was used to assess included papers for risk of bias[31,32].

Statistical analysis

Variables are described as the median and interquartile range (IQR), unless specified differently. The Mann-Whitney U Test or the one-way ANOVA tests was used.

Due to the fact that point estimates from most of the studies (*e.g.*, odds ratios or risk ratios for binary outcomes, or mean difference for quantitative outcomes including 95% confidence interval) were missed, a statistical analysis by pooling the data according to the meta-analysis methods could not be performed. Significance was set at $P = 0.05$ and statistical trend was defined as $P \leq 0.1$. Statistical analyses were performed with the software package SPSS 22 (SPSS Inc., Chicago, Ill) and Graph Pad Prism Software Version 6.0.

RESULTS

Search results

From all the databases searched, 539 studies were identified through screening of Medline ($n = 296$), EMBASE ($n = 200$), and Cochrane ($n = 43$, Figure 1). After excluding duplicates, a total of 539 studies remained for abstract reviewing. Of these, 275 studies were excluded because of reporting of interventional management of BDI only (*i.e.*, endoscopy), or representing review articles or case series < 10 patients. Finally, after critical reading of 127 articles, 24 studies were considered for the final analysis (Figure 1)[18,20,25-28,33-51].

All 24 studies were assessed for the criteria selection (case definition, representativeness of cases, selection of controls, and definition of controls), comparability (age and sex, and other factors), and exposure (ascertainment of exposure, follow-up, and adequacy of follow-up; Supplementary Figure 1).

Descriptive cohort

A total of 24 studies met the inclusion criteria and reported sufficient information on timing of surgical BDI repair and postoperative outcome after BDI repair. Overall, 15609 patients undergoing cholecystectomy were enrolled. Out of 5229 described injuries of the bile duct, 94% ($n = 4934$) were classified as major BDI with the need for surgical repair (Table 1).

Three different classifications were used to characterize the type of major BDI: The Strasberg classification, the Bismuth classification, and the Stewart Way System. Fifteen studies, accounting for 49% ($n = 2440$)[26,33,34,36-43,45-47,49,50] of BDIs, used the Strasberg classification system, three studies, including 8% ($n = 395$)[43,47,51] of BDIs, used the Bismuth classification, and one study, reporting 6% ($n = 307$)[18] of BDIs, used the Stewart Way System (Table 2). Five studies, including 36% ($n = 17924$)[25,27,28,35,49] of patients, did not identify which classification system was used. Of note, one study including 12 patients used both the Bismuth and the Strasberg classification[39]. Concomitant vascular injury was reported in 4% ($n = 222$)[28,33,34,36,37,43-45,47] of included patients.

Timing of BDI repair

Details on timing of surgical BDI repair were available in 98% ($n = 4879$) of analyzed major BDIs. Among all studies, the timing of repair was categorized as "*immediate*", "*early*", "*delayed*", or "*late*". In the literature, all four strategies were used in comparable frequencies: 14% ($n = 705$) of BDI repairs were classified as immediate and 28% ($n = 1367$) as early, whereas delayed and late repair represented 28% ($n = 1364$) and 26 % ($n = 1286$) of BDI repairs, respectively (Table 3).

The most common type of surgery was biliodigestive reconstruction with a hepaticojejunostomy (median, 95%, IQR: 88%-100%; Table 4). Additionally, the late BDI repair group included nine (0.2%) cases of hepatic resections and 32 (0.6%) patients who were treated by liver transplantation.

Table 1 Descriptive cohort, *n* (%)

Ref.	Study period	Study population (*n*)	Major BDI (*n*)
de Reuver *et al*[20], 2007	1991- 2005	500	151 (30)
Mushtaq *et al*[35], 2020	1974- 2004	5000	11 (100)
Goykhman *et al*[28], 2008	2002- 2007	29	29 (100)
Silva *et al*[34], 2008	-	22	22 (100)
Stewart *et al*[18], 2009	-	307	307(100)
Sahajpal *et al*[42], 2010	1992-2007	69	69 (100)
Huang *et al*[25], 2011	1984- 2009	282	41 (15)
Perera *et al*[26], 2011	1991- 2007	200	157 (79)
Barauskas *et al*[41], 2012	2000- 2007	4438	23 (53)
Iannelli *et al*[27], 2013	-	640	543 (85)
Lubikowski *et al*[38], 2012	2002- 2011	300 (TPL)	5 (100, TPL)
Parrilla *et al*[37], 2014	1987- 2010	27	27 (100, TPL)
Stilling *et al*[44], 2015	1995-2010	139	139 (100)
Arora *et al*[46], 2015	2000- 2010	10	10 (100)
Perini *et al*[36], 2015	2000 – 2011	148	9 (16, LR)
Sulpice *et al*[33], 2014	1992- 2010	60	38 (63)
Felekouras *et al*[40], 2015	1991- 2011	67	67 (100)
Kirks *et al*[45], 2016	2008- 2005	61	61 (100)
Dominguez-Rosado *et al*[43], 2016	1989- 2014	699	614 (88)
E-AHPBA[47], 2019	2000-2016	913	913 (100)
Battal *et al*[48], 2019	2012-2017	13	13 (100)
Sweigert *et al*[49], 2021	2006-2015	1168	1168 (100)
El Nakeeb *et al*[50], 2021	2015-2020	412	412 (100)
Anand *et al*[51], 2021	2013- 2020	105	105 (100)
Total		15.609	4934

BDI: Bile duct injury; LR: Liver resection; TPL: Liver transplantation.

Postoperative outcome after BDI repair

Thirteen studies, including 94% (n = 4643) of BDI repairs, defined postoperative outcome according to various timing groups of BDI repair, which included immediate *vs* early *vs* delayed in four studies (n = 745); immediate *vs* delayed *vs* late in four (n = 661); immediate *vs* early *vs* delayed *vs* late in three (n = 335); early *vs* delayed in three (n = 335); early *vs* delay *vs* late in three (n = 2695); and early *vs* late in one (n = 105) (Table 3). Overall, 11 studies (n = 4006) proposed a recommendation for timing of BDI repair. Two manuscripts were in favor of delayed (n = 572, 12%)[27,28], while two other groups (n = 153, 3%)[25,26] recommended early repair of BDI (Figure 2). The other eight studies (n = 3281, 66%) postulating a recommendation for timing found equal results for early or delayed BDI repair[42,43,45].

Median overall morbidity after bile duct repair was 28% (IQR: 19-38) and did not vary significantly between the different timings of BDI repair (P = 0.789; Table 4). Further, mortality was low and was not different among groups (P = 0.832). A detailed list of reported complications can be found in Table 5.

Standardization of reporting of timing of repair

Among 14 studies, we found 14 different definitions of immediate repair (n = 705; Figure 3), ranging from a surgical repair during initial LC (n = 435/705, 62%) to BDI repair within 2 d (n = 27/705, 4%), 3 d (n = 179/705, 7%), 2 wk (n = 34/705, 5%), or within 6 wk (n = 15/705, 2%) after cholecystectomy (Figure 3). Six various definitions for early BDI repair (n = 1367) were provided. Early repair was described as surgery within 1 wk (n = 1053/1367, 67%), 2 wk (n = 80/1367, 5%), 3 wk (n = 43/1367, 3%), 4 wk (n = 12/1367, 1%), 6 wk (n = 223/1367, 16%), or 12 wk (n = 32/1367, 2%). Similar, definitions of

Table 2 Classification systems of bile duct injury

Ref.	BDI (*n*)	Classification	Bismuth classification					Strasberg classification									Vascular injury
			I	II	III	IV	V	A	B	C	D	E1	E2	E3	E4	E5	
de Reuver *et al*[20]	151	Bismuth	37	37	37	-	-	-	-	-	-	-	-	-	-	-	-
Mushtaq *et al*[35]	11	None	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Goykhman *et al*[28]	29	None	-	-	-	-	-	-	-	-	-	-	-	-	-		5
Silva *et al*[34]	22	Strasberg	-	-	-	-	-	-	1	-	1	1	7	7	3	2	2
Stewart *et al*[18]	307	Stewart	16	72	187	32	-	-									-
Sahajpal *et al*[42]	69	Strasberg						1	-	-	2	22	16	22	4	2	-
Huang *et al*[25]	41	None	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Perera *et al*[26]	157	Strasberg	-	-	-	-	-	19	2	9	26	20	65	37	19	3	-
Barauskas *et al*[41]	23	Strasberg	-	-	-	-	-	(4)	-	-	(17)	1	21	-	1	-	-
Iannelli *et al*[27]	543	None	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lubikowski *et al*[38]	5	Strasberg	-	-	-	-	-	1	2	-	-	-	1	1	-	-	-
Parrilla *et al*[37]	27	Strasberg	-	-	-	-	-	-	-	-	-	-	4	11	12	-	7
Stilling *et al*[44]	139	Bismuth	49	49		35											26
Arora *et al*[46]	10	Strasberg	-									1	3	5		1	0
Perini *et al*[36]	9	Strasberg	-	-	-	-	-	-	-	-	-	-	-	2	7	-	9
Sulpice *et al*[33]	38	Strasberg						-	-	-	-	6	14	6	9	3	10
Felekouras *et al*[40]	67	Strasberg						7	-	4	18	10	26	22	4	1	-
Kirks *et al*[45]	61	Strasberg						2	1	7	4	10	16	11	6	1	12
Dominguez-Rosado *et al*[43]	614	Strasberg											448		166		22
E-AHPBA[47]	913	Strasberg											757				126
Battal *et al*[48]	13	Strasberg									3	4	1		1		
Sweigert *et al*[49]	1168	None															
El Nakeeb *et al*[50]	412	Strasberg										59	234	80	33	6	
Anand *et al*[51]	105	Bismuth	10	37	43												3
TOTAL	4934		112	195	267	67	0	34	6	20	71	134	1613	204	265	19	222

Various classification systems were used among studies and a relevant portion of studies did not declare any classification system of BDI.

delayed (n = 1364) and late repair (n = 1286) suffered from inconsistent reporting and were described in six and three distinct ways, respectively. The term "delayed" ranged from after 2 d (n = 34/1364, 3%) to within 3 d (n = 5/1364, 0.5%) to within 6 wk (n = 994/1364, 73%), to a minimum delay after cholecystectomy of 2 wk (n = 22/1364, 5%), 6 wk (n = 308/1364, 22%), or 12 wk (n = 22/1364, 3%). Late BDI repairs (n = 1286) were defined as BDI repair 6 wk (n = 1142/1286, 88%), 8 wk (n = 10/1286, 1%), 12 wk (n = 84/1286, 7%), or 2 years (n = 9/1286, 1%) after LC. In 3% (n = 41/1286) of patients undergoing late repair, the time interval was not further specified at all.

As described above, the standardization of timing of repair was remarkably poor among[8,29] studies. Based on the included literature, most commonly used definitions for immediate and early BDI repair were < 24 h and < 1 wk after (Figure 3). Both delayed and late repairs were equally described as BDI repair 6 wk after index surgery in the majority of reported cases (Figure 3). Overall, the lack of standardized reporting leads to a broad overlap of time intervals (Figure 4A), which precludes any conclusive comparison of different studies.

Nonetheless, the provided data allowed the formation of two groups without being confronted by an overlap. In an attempt to standardize the population according to timing of BDI repair, a cut off of 14 d was proposed (Figure 4B). This subgroup analysis revealed increased complications for a BDI repair within 14 d (n = 1757)[2,11,15,16,20,27,29,30,37,38,42,43,45-49] when compared to surgical repair after this interval (n = 2031)[18,20,25-28,33,40,42,43,47,49,51]. Nevertheless, this difference did not reach statistical significance, implicating that outcome is not dependent on timing of repair only. Therefore, based on the present literature, no recommendation can be given on whether early or delayed BDI repair should be preferred. Moreover, there are many inconsistencies in the reporting of timing intervals for BDI repair following LC in the identified literature.

DISCUSSION

The analysis of this systematical review revealed that standardization of definitions for timing of repair is remarkably poor among studies. This lack of standardized reporting precludes any conclusive recommendation on optimal timing of BDI repair after LC and claims for a uniform reporting system.

Despite single reports postulating reduced occurrence of BDI, it remains a major concern after LC[8,29]. The repair of major BDI requires exact preoperative characterization of lesions and sufficient expertise in HPB surgery[18,26]. As a result, there are numerous studies that investigate factors influencing outcome following biliary reconstruction for BDI[11,18,29]. Both patient-associated factors, such as septic complications and complexity of BDI, and surgical technique are known prognostic factors for outcome of BDI repair[10,11,18]. Additionally, several authors attach great importance to the optimal timing of surgical BDI repair[23-25,27,29,40]. Whereas immediate repair requires early identification of the injury and potentially shortens patient's cumulative hospital stay, delayed reconstruction may provide optimal planning and enable the eradication of intra-abdominal infection prior to surgery. Both strategies are equally supported and opposed by various groups and therefore a conclusive

Table 3 Timing of bile duct injury repair, *n* (%)

Ref.	Surgical repair (*n*)	Definition IR	IR (*n*)	Definition ER	ER (*n*)	Defintion DR	DR (*n*)	Definition LR	LR (*n*)	In favor of
de Reuver *et al*[20]	151	< 6 w	15 (10)	-	-	> 6 w	96 (64)	> 6 w (R)	40 (27)	-
Mushtaq *et al*[35]	11	Initial surgery	11 (100)	-	-	-	-	-	-	-
Goykhman *et al*[28]	29	Initial surgery	14 (48)	-	-	24-72 h	5 (17)	> 8 w	10 (35)	Delay
Silva *et al*[34]	22	Initial surgery	22 (100)	-	-	-	-			
Stewart *et al*[18]	300	Initial surgery	163 (53)	1-2 w	61 (20)	3-6 w	33 (11)	> 6 w	43 (14)	
Sahajpal *et al*[42]	69	≤ 3 d	13 (19)			3d- 6w	34 (49)	> 6 w	22 (32)	Immidiate or delay
Huang *et al*[25]	41	-	-	< 14 d	19 (46)	14-24 w	22 (54)	-	-	Early
Perera *et al*[26]	112	Initial surgery	28 (18)	< 21 d	43 (27)	> 21 d	41 (26)	-	-	Early
Barauskas *et al*[41]	23	Initial surgery	3 (13)	-	7 (30)	-	13 (57)	-	-	-
Iannelli *et al*[27]	543	Initial surgery	194 (35)	< 45 d	216 (39)	> 45 d	133 (24)	-	-	Delay
Lubikowski *et al*[38]	5 (TPL)	-	-	-	-	-	-	-	5 (100)	-
Parrilla *et al*[37]	27 (TPL)	-	-	-	-	-	-	-	27 (100)	-
Stilling *et al*[44]	139	-								
Arora *et al*[46]	10	≤ 3 d	10 (100)							
Perini *et al*[36]	9 (LR)	-	-	-	-	-	-	-	9 (100)	-
Sulpice *et al*[33]	35	< 3 d	15 (43)	3 d–6 w	7 (20)	> 6 w	4 (11)	> 24 mo	9 (26)	-
Felekouras *et al*[40]	67	< 14 d	34 (51)	2-12 w	11 (16)	> 12 w	22 (33)	-	-	-
Kirks *et al*[45]	61	≤ 2 d	27 (44)			> 2 d	34 (56)			Equal
Dominguez-Rosado *et al*[43]	614			< 7 d	61 (10)	1-6 w	152 (26)	> 6 w	374 (63)	Early or late
E-AHPBA[47]	913			< 7 d	339 (37)	1-6 w	261 (28.6)	6 w-6 mo	313 (34)	Equal
Battal *et al*[48]	13			< 3 d	13 (100)					
Sweigert *et al*[49]	1168			< 3 d	569 (48)	4 d-6 w	439 (38)	> 6 w	169 (15)	Early or late
El Nakeeb *et al*[50]	412	< 3 d	156 (38)			4-6 w	75 (18)	> 6 w	181 (44)	Early or delayed
Anand *et al*[51]	105	-	-	< 12 w	21 (20)	-	-	> 12 w	84 (80)	NS
TOTAL	4879		705 (14)		1367 (28)		1364 (28)		1286 (26)	

IR: Immediate repair; ER: Early repair; DR: Delayed repair; LR: Late repair; TPL Transplantation; LR: Liver resection.

recommendation on timing of BDI repair remains unclear[25-28,42,43,45].

Inconsistent methods of reporting the timing of BDI is a major reason for these continued inconsistencies in recommendations[30]. Substantial variability in presentation of data makes comparison of results difficult and precludes a synoptic statement. In line with our findings, a recent study by the group of Strasberg highlighted the weaknesses of irregular formats of observational studies in the field of BDI repair[30]. Likewise, our systematic review found a multitude of definitions for timing of BDI repair in the literature, resulting in a broad overlap of time intervals among studies. As a result, BDI

Table 4 Outcome according to timing of bile duct injury repair

Timing of repair	Immediate *n* = 705	Early *n* = 1378	Delayed *n* = 1364	Late *n* = 1286	*P*
Type of surgery					
HJS	89 (72-100)	77 (75-91)	100 (96-100)	95 (91-100)	0.132
End-to-end	32 (19-57)	21 (13-47)	3 (2-10)	-	0.265
Complications	34 (21-41)	21 (20-29)	25 (16-36)	28 (19-38)	0.789
Bile leak	21 (12-36)	7 (5-12)	12 (0-24)	5 (5-14)	0.653
Wound infection	23 (12-35)	-	10 (7- 28)	9 (7-12)	0.456
Liver dysfunction	8 (5-11)	-	-	-	-
Cholangitis	11 (10-31)	11 (7-25)	13 (8- 59)	10 (4-18)	0.684
Jaundice	9 (4-14)	-	-	-	-
Redo HJS	33 (3-43)	8 (3-31)	20 (10-31)	3 (1-8)	0.642
Intervention	16 (14- 17)	5 (2-12)	24 (23- 25)	-	0.035
Stricture	17 (13-41)	29 (14-39)	25 (15- 62)	13 (11- 19)	0.821
Time to stricture	11 (4-29)	50 (12-89)	14 (14-30)	-	0.642
Mortality	1 (0-5)	2 (0-1)	3 (1-5)	0	0.832

HJS: Hepaticojejunostomy.

Table 5 Outcome after bile duct injury within 14 d or later

Timing of repair	≤ 14 d (*n* = 1757)	> 14 d (*n* = 2031)	*P*
Type of surgery			
HJS	100 (73-100)	100 (68-100)	0.842
End-to-end	0 (0-27)	0 (0-5)	0.352
Complications	33 (21-41)	22 (18-25)	0.085
Bile leak	11 (5-21)	6 (0-12)	0.453
Wound infection	5 (0-14)	9 (6-18)	0.593
Abnormal liver function	11 (0-21)	10	-
Cholangitis	11 (10-35)	9 (9-10)	0.348
Jaundice	13 (5-21)	-	-
Redo HJS	23 (4-42)	0	-
Intervention	16 (14-18)	17	-
Stricture	18 (12-43)	13 (5-23)	0.352
Time to stricture (mo)	17 (10-62)	3	-
Mortality	2 (0-3)	4 (3-5)	0.203

After exclusion of overlapping definitions of timing, two groups at a cut off of 14 d were formed. Again outcome was comparable between early (≤ 14 d) and delayed (> 14 d) BDI repair. HJS: Hepaticojejunostomy.

repair may be considered as "early" in one study, whereas the same time interval may be classified as "delayed" or even "late" in another paper. This lack of standardized definition for BDI timing repair means that a conclusion on superiority of either one of the strategies cannot be reached. Hence, two studies included in this review proposed the early[25,26], while another two recommended the delayed [27,28] approach as treatment of choice. This goes in line with the findings of two recent meta-analyses that BDI repair should be undertaken either early or in a delayed fashion after 6 wk, whereas the time frame between 2-6 wk seems to be associated with increased morbidity[23,24].

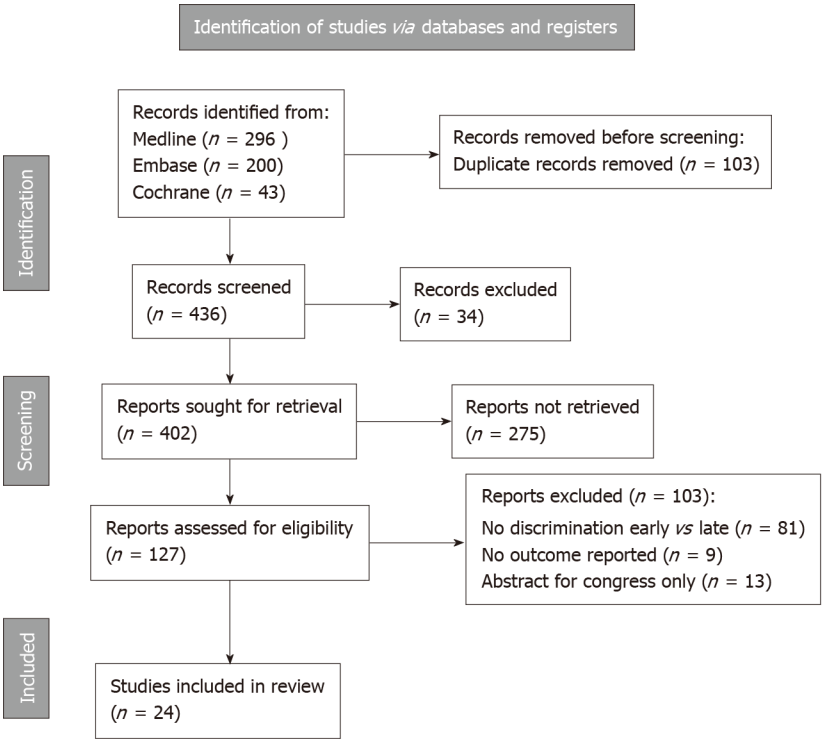

Figure 1 Flowchart of literature research.

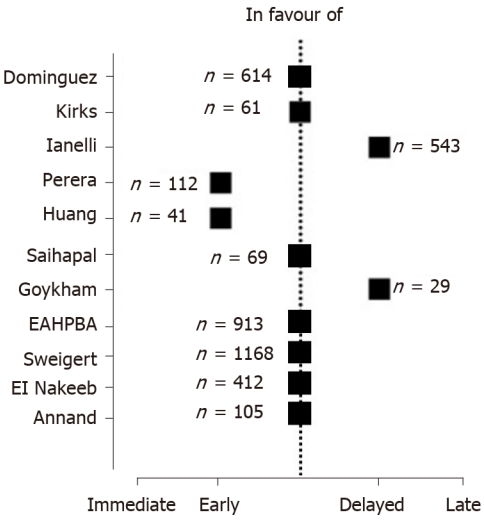

Figure 2 Recommendation of bile duct injury repair. Eleven studies postulated recommendations for optimal timing of bile duct injury repair.

In order to overcome this inconsistency in reporting of timing, the population was divided into two subgroups based on BDI repair within 14 d and after 14 d. Admittedly, the subgroup analysis failed to reveal a significant difference for outcome. This result emphasizes that outcomes after BDI repair are influenced by multiple variables and not just by timing of repair. Likewise, a multivariate analysis including 307 major BDIs concluded that timing of BDI repair plays a subordinate prognostic role for outcome[18]. In contrast, sepsis control, accurate characterization of the BDI, and surgical experience seem to be the major factors influencing the postoperative course.

Many of the studies included in this review were retrospective, which accounts for a major limitation of this systematic review. The retrospective study design does not allow conclusions on patients' condition prior to surgery and the reason for surgeon's choice for one strategy or the other. Surgeons' decision was likely driven by extent of BDI, concomitant vascular injury, and inflammatory status than by standardized protocols. Subsequently, a retrospective comparison of early and delayed BDI repair group leads to clustering of two fundamentally heterogeneous populations. Nevertheless, the low incidence and the unpredictable course of BDI complicate the design of a prospective randomized

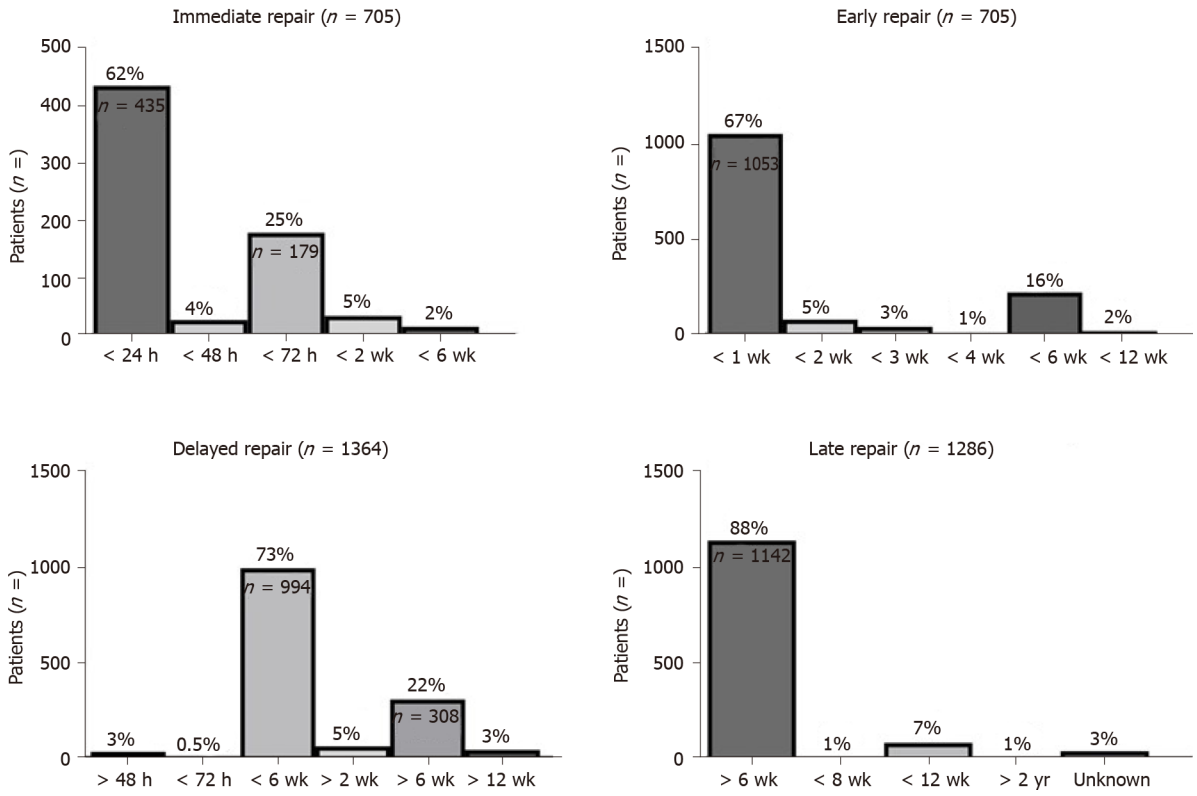

Figure 3 Distribution of definitions of bile duct injury repair timing. Definitions of timing were heterogeneous among publications. Immediate, early, delayed, and late repairs were defined in four, five, six, and five different manners.

control trial.

Likewise, the value of the attempt to standardize the groups according to a BDI repair within 14 d or more than 14 d is diminished by the above-mentioned limitations in data reporting. Still, this allowed a more precise pooling of patients undergoing BDI. In line with other publications, timing alone did not predict outcome in this subgroup analysis. Nevertheless, caution should be taken in interpreting these results based on the quality of provided data and heterogeneity of populations.

In this context, original raw data of the included studies was not available and all analyses were based on provided medians. Therefore, the analysis was limited by data quality, which precluded pooling the data according to the methods of a meta-analysis. However, this study has certain strengths, including the systematical character with providing a comprehensive review of studies declaring outcome according to timing of bile duct repair.

CONCLUSION

In conclusion, based on clinical practice, it is assumable that immediate BDI repair is reasonable if detected intraoperatively and sepsis control should be guaranteed before delayed BDI repair. Nevertheless, only standardized reporting can help to answer the ongoing debate of influence of timing on outcome and provide solid fundament for a recommendation. Therefore, based on the findings of this review, a consensus in the field of timing of BDI repair is urgently needed.

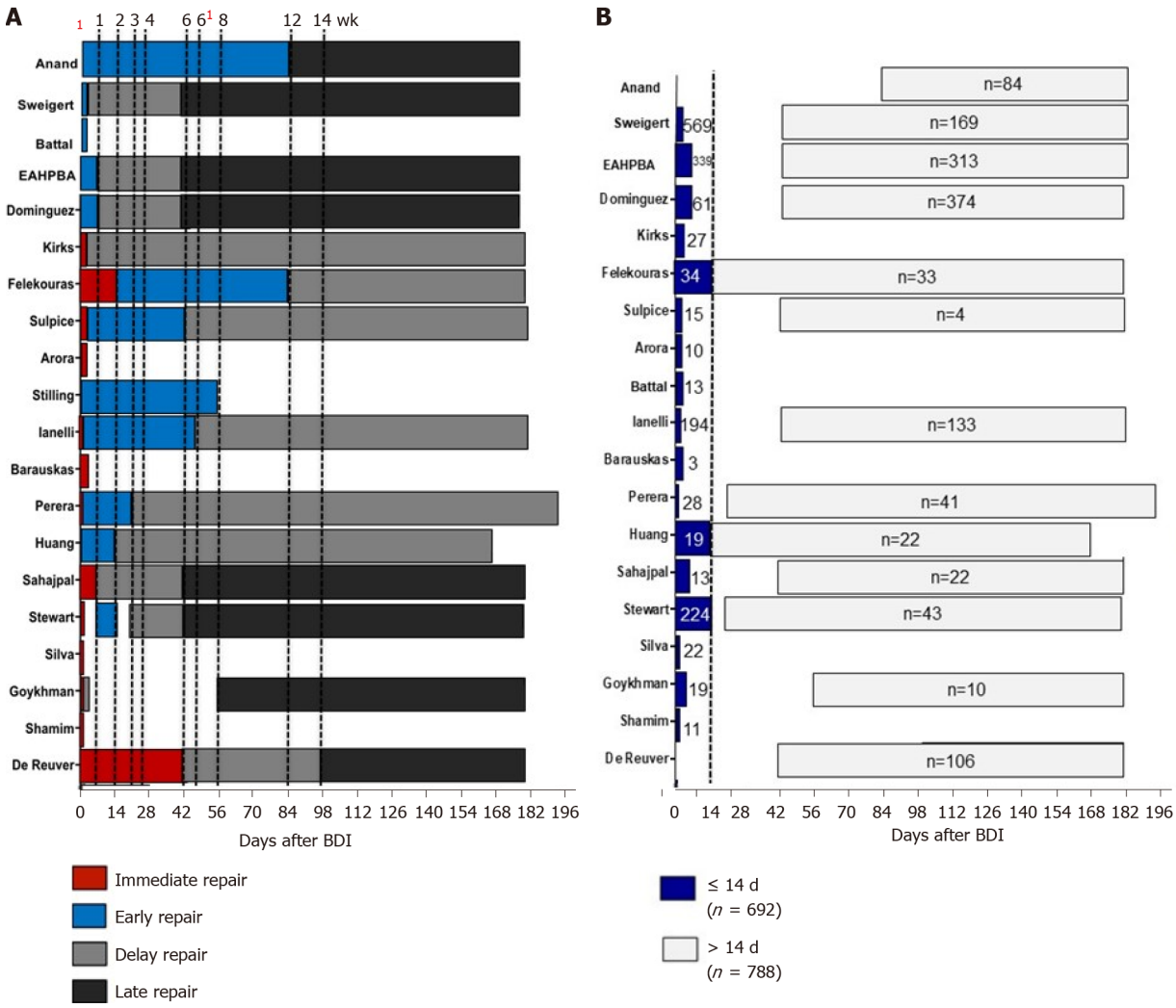

Figure 4 Overlapping definitions of timing of bile duct injury repair. A: Dotted lines indicate different cut offs according to heterogeneous definitions of timing; B: Subgroup analysis after exclusion of overlapping definitions. Dotted lines indicates cut off for BDI repair at 14 d after LC. [1]: 24 h and 72 h. 6[1]: 45 d.

ARTICLE HIGHLIGHTS

Research background

Bile duct injuries (BDIs) are an important topic for the practicing hepatobiliary (HPB) surgeon. While it is widely agreed that most major BDIs after laparoscopic cholecystectomy (LC) should undergo surgical repair, the timing of repair is still controversially discussed in the literature.

Research motivation

Our research motivation was: (1) To bring clarity into the terms "immediate", "early", "delayed", and "late" repair; and (2) to assess postoperative complications.

Research objectives

The objective of this study was to assess timing of bile duct repair after BDI and postoperative complications.

Research methods

A systematic review of the literature was performed using the databases MEDLINE, EMBASE, and The Cochrane Library. These databases were systematically screened up to August 2021. Bias assessment was performed using the Newcastle Ottawa scale.

Research results

A total of 439 abstracts were screened, and 24 studies were included with 15609 patients included in this review. Of the 5229 BDIs reported, 4934 (94%) were classified as major injury. Timing of bile duct repair was immediate (14%, *n* = 705), early (28%, *n* = 1367), delayed (28%, *n* = 1367), or late (26%, *n* = 1286).

Standardization of definition for timing of repair was remarkably poor among studies.

Research conclusions

The lack of standardization among studies precludes any conclusive recommendation on optimal timing of BDI repair after LC. This finding indicates an urgent need for a standardized reporting system of BDI repair.

Research perspectives

Future perspectives include the establishment of a clear definition for the terms "immediate", "early", "delayed", and "late" repair. Only such a definition can make comparisons of study outcomes possible.

FOOTNOTES

Author contributions: Kambakamba P, Cremen S, Möckli B, and Linecker M all contributed in creating this manuscript.

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CASE REPORT

Learning from a rare phenomenon — spontaneous clearance of chronic hepatitis C virus post-liver transplant: A case report

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Abstract

BACKGROUND

Hepatitis C virus (HCV) can lead to chronic liver damage resulting in cirrhosis and hepatocellular carcinoma. Spontaneous clearance of HCV has been documented after an acute infection in 20%-45% of individuals. However, spontaneously resolved chronic hepatitis C following liver transplant (LT) is rare and has been documented only in a few case reports. The phenomenon of spontaneous clearance of chronic hepatitis C occurs together with other meaningful events, which are typically associated with significant changes in the host immunity.

CASE SUMMARY

We report three cases of spontaneous resolution of chronic hepatitis C following liver transplantation. These patients either failed or had no HCV treatment prior to transplant, but had spontaneous resolution of HCV post-LT as documented by undetectable polymerase chain reaction (PCR). Diagnosis of HCV was based on viremia through PCR or liver biopsy. All three patients currently undergo surveillance and have no recurrence of HCV.

CONCLUSION

Examining each patient's clinical course, we learned about many viral, host and cellular-factors that may have enhanced the host's immunity leading to spontaneous clearance of HCV. Though HCV treatment has excellent cure rates, understanding this mechanism may provide clinicians with insights regarding timing and duration of treatment.

Key Words: Spontaneous resolution of hepatitis C; Liver transplantation; Hepatitis C; Immunosuppression; Viral load; Case report

Core Tip: Spontaneous resolution of chronic hepatitis C virus (HCV) following liver transplant is a rare phenomenon. In this case report, we examined three cases and completed a literature review thereby examining thirty cases. Spontaneous resolution may be related to host, viral and other factors resulting in enhancement of the host's immunity. Host factors include younger age, female sex, HLA, DQBI, IL28 gene and pregnancy. Viral factors include a low viral load. Lastly, other factors include infections, rejection episodes, medications, and surgery. Even though HCV treatment is excellent, understanding this phenomenon will be beneficial to determine timing and duration of treatment.

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INTRODUCTION

Chronic infection with hepatitis C virus (HCV) leads to progression of liver disease, cirrhosis, hepatocellular carcinoma, and is a common indication for liver transplant (LT). Whereas spontaneous clearance of acute HCV occurs in 20%-45% of individuals[1], spontaneous resolution of chronic HCV has been documented only in case reports. The latter is rare as HCV has already successfully managed to evade immune control for a prolonged period. Spontaneous clearance of HCV following LT is unusual due to ongoing immunosuppression use. Indeed, studies have shown HCV recurrence following LT to be universal and associated with poor graft and patient survival[2]. Immunosuppression use is associated with accelerated disease with up to a third of patients developing cirrhosis at 5 years[2].

It has been hypothesized that viral, host and cellular-factors change the host's immunity by enhancing it whereby leading to spontaneous clearance of HCV. These protective factors include HLA alleles[3], co-infection with hepatitis B[4], infection with other viruses[4], immunosuppressive therapy withdrawal[5], immune reconstitution after highly active antiretroviral therapy (HAART)[6,7], pregnancy[8] and surgery including LT and gastrectomy[9]. The mechanism by which all these factors lead to spontaneous resolution of HCV is not completely understood but likely involves alteration of the host immune response. We report three cases of patients on immunosuppression who have had spontaneous HCV clearance post-LT.

CASE PRESENTATION

Chief complaints

The chief complaints of ALL the presented case reports are HCV viremia following LT.

History of present illness

Case 1: A 57-year old Caucasian male who had been HCV positive (genotype 1a) for 9 years. Clinic notes showed that HCV viremia was diagnosed based on polymerase chain reaction (PCR). He was treated with ribavirin and pegylated interferon but did not achieve sustained virologic response (SVR). This treatment led to significant side effects including decompensation. He required an urgent LT in 2006. Unfortunately, pre-transplant HCV viral load was not available.

Case 2: Another case was a 63-year-old Caucasian male diagnosed with HCV positive (genotype 1) in 2004. He was treated for HCV but did not achieve SVR (HCV viral load 311 IU/mL in 2014).

Case 3: Our third case was a 57-year-old male with HCV (genotype 1a) as a result of a blood transfusion in 1994.

History of past illness

Case 1: He had a past medical history of schizophrenia, dyslipidemia, and diabetes.

Case 2: His past medical history included osteoarthritis and hepatocellular carcinoma diagnosed in December 2012. He had transarterial chemoembolization as well as selective internal radioembolization (SIRT) in April 2013. Unfortunately, he developed decompensated liver cirrhosis and required LT in July 2015.

Case 3: His past medical history included a kidney transplant in 2001 for IgA nephropathy that failed in 2007. He also had HCV liver cirrhosis, requiring LT in 2012. Due to his kidney transplant, his

medications included mycophenolate mofetil 750 mg twice daily, tacrolimus 0.5 mg twice daily, pantoprazole 40 mg daily and amlodipine 5 mg daily.

Personal and family history

They have no special personal and family history.

Further diagnostic work-up (including relevant labs)

Case 1: Post-LT immunosuppression included prednisone, sirolimus, and mycophenolate mofetil. Immediately after LT, he had a mild episode of cellular rejection that was treated with oral prednisone. One-year post-LT, he had a second episode of mild cellular rejection with liver biopsy showing a superimposed recurrent HCV (Metavir A1, F2). HCV viral load was positive in 2007 (unknown viral load). A liver biopsy was done in February 2007 showing mild acute cellular rejection with superimposed recurrent Hepatitis C (Metavir Grade 3, Fibrotic Stage 2).

Case 2: He had HCV viral load of less than 12 IU/mL following LT, consistent with untreated HCV. Post-LT immunosuppression included tacrolimus and mycophenolate mofetil. The donor's liver was hepatitis B core antibody-positive and the patient was started on Entecavir. His post-transplant course was remarkable for cytomegalovirus (CMV) viremia in 2016 with a peak of 1376 IU/mL, which cleared without antiviral therapy; subsequent CMV viral load testing was negative.

Case 3: He was never treated for HCV; liver biopsy done in 2007 showing stage 4 fibrosis and he had a positive HCV viral load in 2008 (viral load unknown). Unfortunately, HCV viral load was not available pre-transplant. His post-transplant course was complicated by biliary anastomotic strictures requiring ERCP stent placement.

FINAL DIAGNOSIS

Cases 1-3

Recurrent HCV following liver transplant.

TREATMENT

Case 1

Unfortunately, at the time, he was not considered for re-treatment due to the potential for adverse psychiatric side-effects of using Interferon-regimens especially in the setting of paranoid schizophrenia. In June 2007, he developed CMV viremia from which he recovered. A repeat liver biopsy was done in January 2009 showing chronic hepatitis, consistent with recurrent Hepatitis C (Metavir Grade A1, Stage F1).

Case 2

No treatment for HCV was provided.

Case 3

No HCV treatment was provided following LT.

OUTCOME AND FOLLOW-UP

Case 1

Despite no additional treatment for his recurrent hepatitis C, repeat HCV PCR in 2013, 2015, 2016 and 2017 all showed undetectable viral load consistent with spontaneous clearance of HCV. Presently, he undergoes surveillance for cirrhosis and has normal serum liver tests.

Case 2

Subsequent HCV viral load testing in October 2015 and January 2016 were negative thereby suggesting spontaneous resolution of HCV following liver transplant. Presently, he has normal serum liver test and is on tacrolimus for immunosuppression.

Case 3

Repeat testing for HCV viral load in 2013, 2014, and 2015 were negative, consistent with spontaneous

clearance following LT. Presently, he has normal serum liver tests while being on tacrolimus and mycophenolate mofetil.

DISCUSSION

Spontaneous clearance of chronic HCV following LT is a rare phenomenon that is poorly understood. Only a small number of cases exist, which makes it difficult to understand host and viral factors influencing chronicity or to identify predictors of spontaneous clearance. Nevertheless, certain viral and host factors seem to be associated with clearance. Scott *et al*[10] completed a prospective study in Alaskan natives and found the rate of spontaneous HCV clearance among patients with chronic disease to be 1.15 cases per 100 persons per year. A low viral load and young age at onset of disease were associated with spontaneous clearance.

We performed a retrospective review of patients who underwent a liver transplant at the University of Alberta Hospital, Edmonton, Canada from 2000 to 2015 to identify cases of spontaneous HCV clearance. Among the 191 patients transplanted for HCV, we only found the three cases described above (1.5%). We also performed a literature review to identify additional cases of spontaneous HCV resolution post-LT to better understand factors associated with this phenomenon. We used a similar strategy as Tamaki *et al*[11], but did not exclude patients on HAART, interferon or ribavirin. We completed a systematic review using PubMed from August 2015 to January 2020 by including keywords of LT and spontaneous clearance of HCV. No additional case reports were found. We have presented all the case reports since 2000 (Table 1) and reviewed the literature to consolidate the protective factors that may be associated with spontaneous clearance of HCV.

Host factors

Female sex and younger age have been associated with spontaneous clearance[10] (Table 2). Younger age may be protective due to lower likelihood of advanced fibrosis. It may also mean a more robust immune system. Though it is unclear what benefit these factors have in the post-LT setting. The mean age of cases included was 49 years (SD 9.95 years) and the majority were men (75%). The beneficial effect of female sex may be related to gender-based differences in immunity. For instance, polymorphisms of interleukin-28B gene (IL28B), specifically IL28B-CC genotype associated with spontaneous clearance of chronic HCV have a much greater effect in females. These polymorphisms are also associated with response to treatment with pegylated interferon (PEG-IFN), simeprevir, sofosbuvir, and ribavirin[12]. Interestingly, two of the patients with spontaneous resolution of HCV had donors with IL28B-CC genotype; it was felt this altered host immune response to HCV and led to spontaneous clearance[12]. Host HLA class II genotype plays an important role in host susceptibility. In a recent meta-analysis by Gauthiez *et al*[3], HLA alleles DQB1*03, DQB1*03:01, DQB1*11 and DRB1*11:01 were thought to be protective due to effective presentation of HCV epitopes to CD4+ T lymphocytes. On the other hand, HLA allele DQB1*02 was associated with failure to spontaneously clear HCV[3].

Host immune response: HCV infection causes an immediate induction of interferons and cytokins[13]. The outcome of HCV infection is determined by the quality of the adaptive and humoral immune response[14]. Firstly, innate immunity consists of activation of T-cells by natural killer (NK) cells leading to interferon-gamma production and cytotoxic killing of hepatocytes that are infected[13]. Chronic HCV leads to a decline in NK cells thereby promoting persistent infection of hepatocytes[13]. Secondly, the humoral immunity consists of a T-cell response that develops between 5 wk to 12 wk after infection[13]. Studies in humans and chimpanzees suggest that control of HCV viremia is observed after emergence of a robust $CD4^+$ T-cell proliferation[13-15]. Indeed, in cases where anti-$CD4^+$ antibody treatment was used HCV immune evasion was seen with persistent infection[14]. Additionally, $CD8^+$ T-cells are thought to be important in controlling viremia but require simultaneous $CD4^+$ T-cells to maintain response. Therefore, HCV persistence is hypothesized to be caused by $CD4^+$ exhaustion followed by $CD8^+$ phenotypic exhaustion. A study by Smyk-Pearson *et al*[14] found that there is a quantitative T-cell threshold that exists by which spontaneous HCV occurs. Hence, a robust T-cell activation is needed for a spontaneous HCV clearance.

Other factors

The spontaneous clearance of HCV post-LT is unique as patients are on immunosuppression. Segev *et al*[16] performed a meta-analysis and meta-regression comparing steroid-free and steroid-based immunosuppression and found corticosteroids increased the ability of HCV to enter cells and led to a dramatic increase in spread of infection. Lower rates of HCV recurrence were seen when using steroid-free regimes, which was also corroborated by Fafi-Kremer *et al*[17]. Of note, half (15/30) of the cases with spontaneous HCV clearance had experienced rejection following transplant. This observation is in contrast to the findings of Segev *et al*[16] as corticosteroids are used for the management of rejection. One theory may be that rejection leads to stimulation of the immune system, which alters the host's immune response to HCV eventually leading to spontaneous clearance.

Table 1 Summary of cases of spontaneous hepatitis C clearance post-liver transplant

ID	Ref.	Age (yr)	Sex	Preoperative HCV RNA (IU/mL)	HCV genotype	Rejection episode	Concomitant Issues	Immunosuppression	HCV clearance time
1	Neumann and Neuhaus [20], 2004	54	M	+	1b	1	HAT, retransplant	TAC, MMF, CS	3 mo
2	Samonakis *et al*[21], 2005	48	M	250000	1a	3	Renal failure	TAC, AZA, CS	75 mo
3	Samonakis *et al*[21], 2005	55	M	121000	4	3	Renal failure	TAC, AZA, CS	15 mo
4	Bhagat *et al* [7], 2008	43	M	564000	NA	3	HIV/HAART	MP, TAC, MMF	1 mo
5	Bhagat *et al* [7], 2008	44	M	450000	NA	3	HIV/HAART	MP, TAC	1 mo
6	Suneetha *et al* [22], 2008	69	F	+	3a	3	Renal failure/dialysis	MP, Il2a, CSA, CS	12 yr
7	Weber and Trotter[23], 2009	53	M	2.5 million	1a	3	-	TAC, CSA, MMF to CSA	28 mo
8	Dale *et al*[24], 2009	32	F	3.2 million	NA	1	Dialysis/renal tx	Basiliximuab, TAC, MMF, CS	5 mo
9	Haque *et al* [19], 2010	66	F	+	2a/2c	3	IVC thrombosis	TAC, MMF, CS	11 mo
10	Seetharam *et al*[18], 2011	48	M	675000	1	0	-	MP, MMF, CS	2.25 mo
11	Gutiérrez-Moreno *et al* [26], 2012	38	M	2564	1	0	HIV	CSA, MMF, CS	5 mo
12	Chin *et al*[12], 2012	40	M	24	1a	1	Alcohol	Daclizumab, TAC, CS, MMF	34 mo
13	Chin *et al*[12], 2012	41	M	+	1	0	Alcohol	TAC, CS, AZA	9 years
14	Elsiesy *et al* [25], 2015	32	F	65553	4	0	AIH, DM	FK, CS, CSA, CS	1 mo
15	Urzúa *et al* [27], 2015	51	M	+	NA	1	Colon Cancer	CSA, MMF, TAC	18 mo
16	Urzúa *et al* [27], 2015	48	M	280998	3a	0	D2M, alcohol	CSA, IL2a	56 mo
17	Kogiso *et al* [28], 2015	50	F	19952	1	NA	-	TAC, MMF, MP, CS	Approximately 3 mo
18	Tamaki *et al* [11], 2015	66	M	199526	1b	0	Sepsis	Rituximab, TAC, MMF, MP, CS	5 mo
19	Tamaki *et al* [11], 2015	61	M	199	2	Yes	Sepsis	TAC, MMF, MP, CS	3.6 mo
20	Tamaki *et al* [11], 2015	55	M	125	1b	0	-	TAC, MMF, MP, CS	5.8 mo
21	Tamaki *et al* [11], 2015	55	M	316227	1b	Yes	-	TAC, MMF, MP, CS	0.5 mo
22	Our Case 1	57	M	+	1a	2	CMV infection	Sirolimus, MMF, CS	15 yr
23	Our Case 2	64	M	+	1	0	Donor HBV core +	TAC, MMF	2 mo
24	Our Case 3	57	M	+	1a	0	Renal tx	TAC, MMF	1 yr

F: Female; M: Male; LT: Liver transplant; NA: Not available; HBV: Hepatitis B virus; HCV: Hepatitis C virus; CMV: Cytomegalovirus; HAT: Hepatic artery thrombosis; HAART: High activity anti-retroviral therapy; IVC: Inferior vena cava; HIV: Human immunodeficiency virus; AIH: Autoimmune hepatitis;

DM: Diabetes melilites; MP: Methyl prednisone; AZA: Azathioprine; CSA: Cyclosporine; CS: Corticosteroid; ATG: Anti thymocyte globulin; MMF: Mycophenolate mofetil; Tac: Tacrolimus; IL2a: Interleukin 2 receptor antibody.

Table 2 Factors associated with spontaneous hepatitis C virus clearance

Host factors	Viral factors	Other factors
Younger age	Low viral load (< 1 million IU/mL)	Infection (CMV, HBV, HIV, sepsis)
Female sex		Rejection episode
HLA: DQB1*03, DQB1*03:01, DQB1*11 and DRB1*11:01		Medication related HAART; Withdrawal of immunosuppression
IL28 gene polymorphism		Surgery (transplant, gastrectomy)
Pregnancy		

HBV: Hepatitis B virus; CMV: Cytomegalovirus; HAART: High activity anti-retroviral therapy; HIV: Human immunodeficiency virus.

It has been postulated that activation of Th2 cytokines may predominate in high stress situations including pregnancy, and post-gastrectomy[8,9]. Undergoing a liver transplant, also a high stress situation may lead to spontaneous clearance by restoration of the HCV-specific T-cell response. Similarly, infection of the allograft might engage the host's immune system and lead to activation of Th-17 cells that contribute to clearance[18]. The patient in Case 1 likely had resolution as he had both episodes of rejection and CMV reactivation, which may have led to a boost in the immune system. A third of the patients (9/30) who had spontaneous resolution experienced concomitant infections (co-infection with hepatitis B or human immunodeficiency virus; CMV or sepsis) following LT. Interestingly, almost half of the patients (13/28) who had spontaneous HCV clearance had a negative HCV PCR documented within 6-months of LT.

Viral factors

A low viral load has been shown to be associated with spontaneous clearance of HCV[10]. In the cases presented, half of the patients (14/30) had low viral load defined as < 1 million IU/mL (mean ± SD, 451088 ± 224854 IU/mL).

CONCLUSION

In conclusion, spontaneous resolution of chronic HCV following LT is a rare phenomenon and seems to be related to immunomodulatory effects. Though the small number of cases prevents identification of predictors of clearance some factors have emerged. Some may argue the impact of these findings is low as patients can be treated with direct-acting antivirals (DAAs). Nevertheless, these findings are beneficial in settings where there is no access to DAAs due to cost.

These findings may also help clinicians with management. Determining the presence of IL28B polymorphisms may help determine response to treatment (or presence of resistance). The viral load could be used to determine the duration of treatment with a shorter duration in those with low viral load. The median time to spontaneous HCV clearance was 11 mo (IQR 3.6, 66 mo) with almost half of the patients achieving spontaneous clearance within 6 mo (13/30). Treatment could therefore be started after 6 mo. This would provide an additional advantage of limiting drug-drug interactions early in the post-transplant setting. In patients without evidence of fibrosing cholestatic hepatitis, episodes of rejection or concomitant infections may warrant further delay in treatment; these episodes may lead to immune modulation facilitating spontaneous clearance. The number of cases of spontaneous resolution may be underestimated as we do not always get repeat HCV PCR prior to treatment. Learning from this rare event may be the first step to individualized medicine. Further studies to elucidate the mechanisms of spontaneous HCV clearance are warranted to explore new potential therapeutic strategies in this special population.

FOOTNOTES

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CASE REPORT

Step-up approach in emphysematous hepatitis: A case report

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Abstract

BACKGROUND

Emphysematous hepatitis (EH) is a rare, rapidly progressive fulminant gas-forming infection of the liver parenchyma. It is often fatal and mostly affects diabetes patients.

CASE SUMMARY

We report a case of EH successfully managed by a step-up approach consisting of aggressive hemodynamic support, intravenous antibiotics, and percutaneous drainage, ultimately followed by laparoscopic deroofing. Of 11 documented cases worldwide, only 1 of the patients survived, treated by urgent laparotomy and surgical debridement.

CONCLUSION

EH is a life-threatening infection. Its high mortality rate makes timely diagnosis essential, in order to navigate treatment accordingly.

Key Words: Emphysematous hepatitis; Septic shock; Step-up approach; Percutaneous drainage; Laparoscopic deroofing; Case report

Core Tip: Emphysematous hepatitis (EH) is a very rare, rapidly progressive fulminant gas-forming infection of the liver parenchyma. There is a paucity of literature with regard to pathogenesis, involved organisms, imaging appearance, and management of this condition. We report the successful treatment of a patient diagnosed with EH by adopting a multimodal step-up approach including rigorous fluid resuscitation, early hemodynamic support, broad-spectrum antimicrobial therapy, and percutaneous radiologically guided drainage followed by minimal invasive surgical treatment.

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INTRODUCTION

Emphysematous hepatitis (EH) is a rare life-threatening condition that results from a necrotizing gas-forming infection of the liver parenchyma. The pathogenesis is poorly understood, although rare published case series have shown that diabetes mellitus was present in most patients[1-6]. Diagnosis of EH is based on radiological findings on computed tomography demonstrating hepatic intraparenchymal gas without the typical fluid-air level seen in pyogenic abscesses. Early recognition is crucial in attempts to decrease mortality, although there is still discussion regarding the appropriate management, as almost all documented cases evolved unfavorably[1-4,6-11]. This report presents the successful management of a critically ill patient with EH using a step-up approach.

CASE PRESENTATION

Chief complaints

A 70-year-old woman presented to the emergency department with acute epigastric pain of 1 h duration.

History of past illness

The patient had a history of well-controlled diabetes mellitus, cholecystectomy, and heterozygote alpha-1 antitrypsin deficiency.

Physical examination

Clinical examination revealed the patient to be in no distress, fully alert and oriented, and presenting with epigastric tenderness without signs of peritonitis. She had no fever, a pulse rate of 82 beats/min, blood pressure of 128/68 mmHg, and normal respiratory rate and oxygen saturation.

Laboratory examinations

Laboratory investigation performed within a few hours after onset of pain showed a total bilirubin of 0.28 mg/dL, glutamic oxaloacetic transaminase of 45 U/L, glutamic pyruvic transaminase of 30 U/L, γ-glutamyltransferase of 91 U/L, alkaline phosphatase of 99 U/L and lipase of 3518 U/L. Complete blood count, C-reactive protein, coagulation, and renal function were within normal limits. Cardiac evaluation by electrocardiogram and cardiac enzymes confirmed normal findings. As acute pancreatitis was suspected, the patient was initially managed conservatively, *via* intravenous fluid resuscitation and pain relief. However, within hours after admission, the patient deteriorated rapidly, developing signs of severe septic shock. She was transferred to the intensive care unit, requiring mechanical ventilation and aggressive hemodynamic support.

Imaging examinations

After initial resuscitation, a computed tomography (CT) scan was performed, showing a large (9 cm) air-filled cavity in the right liver lobe (Figure 1A and B). The bile duct was only mildly dilated in this cholecystectomized patient but nonradiopaque choledocholithiasis could not be ruled out. No apparent inflammation surrounding the pancreas was visible on scans.

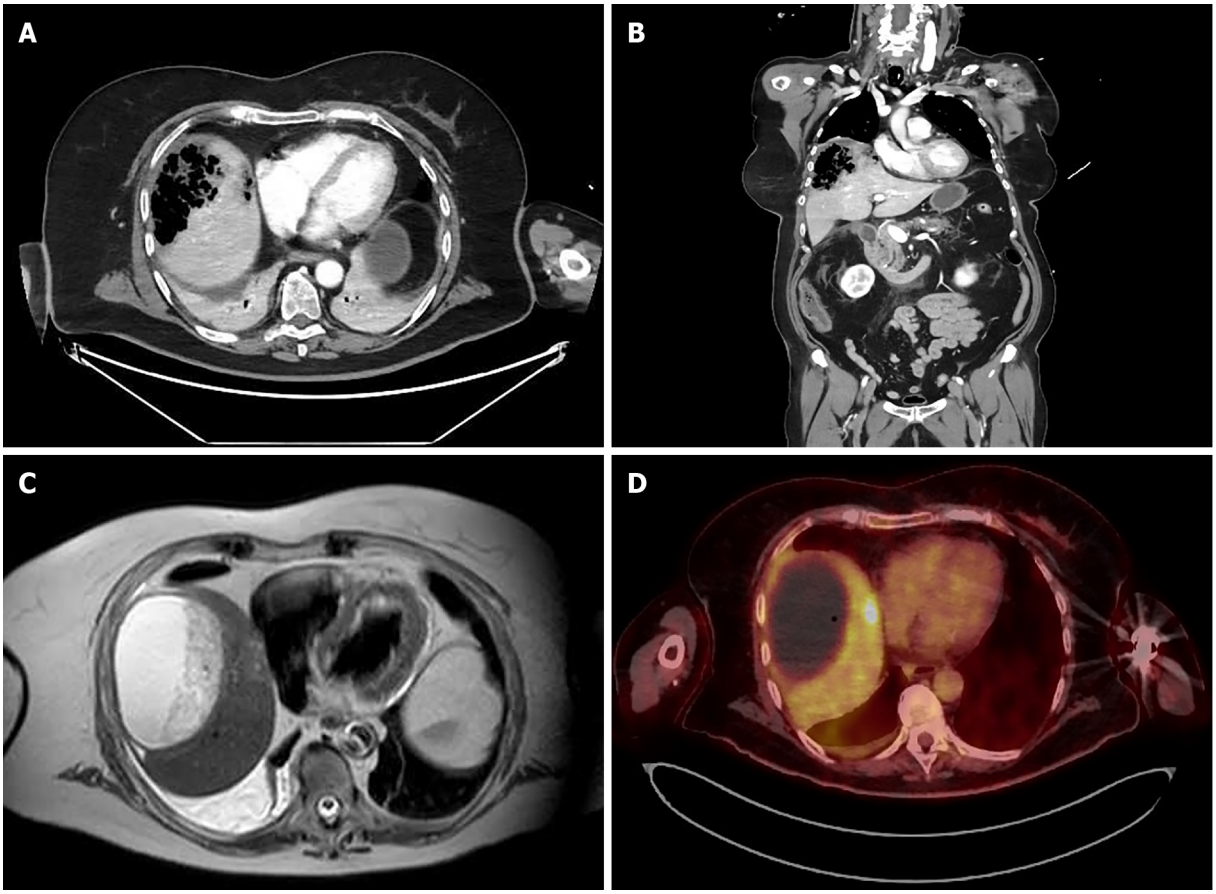

Figure 1 Imaging of emphysematous hepatitis. A and B: Axial (A) and coronal (B) computed tomography scans on admission, showing a 9 cm air-filled cavity in the right liver lobe; C: Magnetic resonance imaging, showing a 10 cm fluid-filled collection in the right liver lobe with heterogeneous content at 6 wk after initial presentation; D: Positron emission tomography performed at 9 wk after initial presentation, showing no metabolic activity in the large collection in the right liver lobe and a 2-cm nodule with positive metabolic activity in segment VIII.

MULTIDISCIPLINARY EXPERT CONSULTATION

As soon as the patient clinically deteriorated, multidisciplinary consultations between gastroenterology, hepatobiliary surgery, intensive care, interventional radiology and microbiology were performed repeatedly.

FINAL DIAGNOSIS

Based on the CT-graphic findings, a diagnosis of EH was made.

TREATMENT

The patient was treated with broad-spectrum intravenous antibiotics (meropenem, vancomycin, and amikacin). Subsequent CT-guided percutaneous pigtail catheter drainage yielded no significant amount of fluid or pus. The pigtail drain was then flushed by continuous irrigation of 1 L saline solution per 24 h. Because of elevated serum lipase suggesting pancreatitis and a mildly dilated bile duct, albeit without biochemical cholestasis, an endoscopic retrograde cholangiopancreatography was performed in this rapidly deteriorating patient. A cholangiogram showed normal biliary anatomy, and clear bile was visible after endoscopic sphincterotomy of the papilla Vateri.

Antibiotics were rationalized to ceftriaxone and metronidazole after blood and fluid cultures revealed *Escherichia coli*, *Streptococcus anginosus*, and *Klebsiella oxytoca* as microbial pathogens. Continuous pigtail irrigation was stopped after 3 d, and the drain was removed after 5 d because of no output. The patient was weaned from the ventilator after a week and transferred to the ward after 2 wk in intensive care.

Table 1 Emphysematous hepatitis case reports in the literature

Ref.	Year	Age/sex	History	Imaging	Treatment	Pathogen(s)	Outcome
Blachar *et al* [1]	2002	43/F	Diabetes mellitus, hyperlipidemia, short-gut duet of multiple ischemic episodes, peripheral vascular disease	CT: Extensive hepatic gas right lobe without fluid collection	IV antibiotics; Radiological drainage	Blood and liver aspirate: *Klebsiella pneumoniae*	Died 3 d after admission
Lopez Zarraga *et al* [2]	2006	72/F	Diabetes mellitus	CT: Total gas content in multiple abscesses	NA	Culture of liver lesion post mortem: *Klebsiella oxytoca*	Died 24 h after admission
Létourneau-Guillon *et al* [7]	2010	53/M	Three mo before admission: Left hepatectomy with hepaticojejunostomy for hilar cholangiocarcinoma; No adjuvant chemotherapy1 wk before admission: Cellulitis at surgical incision treated with oral cephalexin	CT: 8 cm air-filled cavity in the right lobe, no fluid collection	IV antibiotics	Blood culture: *Enterobacter cloacae, Clostridium perfringens*	Died 36 h after admission
Chauhan *et al* [3]	2012	77/F	Diabetes mellitus	CT: Air collection in segment VI and VII without fluid collection	IV antibiotics; Radiological drainage	NA	Died 3 d after admission
Jung Ho *et al* [8]	2012	80/F	Hilar cholangiocarcinoma; ERCP + stenting was performed 3 mo before admission followed by radiotherapy for 17 d after admission	CT: Hepatic parenchymal gas 6.3 cm × 4.4 cm in the right liver (sVII/sVIII)	IV antibiotics; Radiological drainage	Blood culture: *Clostridium perfringens, Escherichia coli*	Died 3 d after admission
Dimitriou *et al*[4]	2014	72/M	Diabetes mellitus	CT: Replacement of liver parenchyma by gas without fluid collection	IV antibiotics	NA	Died within hours after admission
Nada *et al*[9]	2017	73/F	Pancreatic adenocarcinoma; Whipple performed 8 mo before admission. Lung- and liver metastasis diagnosed 6 wk prior to admission. COPD, hypertension, chronic hepatitis C, pulmonary embolism	CT: Hepatic gas in the right liver lobe, sparing the hepatic metastasis	IV antibiotics	Blood culture: *Streptococcus mutans, Enterococcus faecalis*	Died within 24 h after admission
Ghosn *et al*[5]	2019	38/F	Diabetes mellitus, cholecystectomy	CT: Mixed collection 8 cm × 7 cm × 5.5 cm, containing necrotic debris and air	IV antibiotics; Laparotomy urgent	Perioperative fluid: *Escherichia coli, Enterococcus faecium*	Survived. Discharged 13 d after admission
Calderon *et al* [6]	2020	80/F	Hypertension, diabetes mellitus, chronic kidney disease	CT at presentation: Indeterminate, scattered, hypo-enhancing lesions in the liver. CT 5 h after admission (clinical deterioration): Gas in the right liver lobe	IV antibiotics	Blood culture: *Clostridium perfringens*	Died within 16 h after admission
Azri *et al*[7]	2020	75/F	Hilar cholangiocarcinoma; ERCP + stenting 14 mo prior to admission. Followed by stereotactic radiotherapy until 4 mo prior to admission	CT: Left hepatic parenchymal emphysema and pneumoperitoneum	NA	Blood culture: *Klebsiella pneumoniae, Escherichia coli, Enterococcus faecalis, Clostridium perfringens, Aeromonas ichtiosmia*	Died
Gonçalos *et al* [11]	2020	74/M	Hypertension, gastroesophageal reflux	CT: Two areas of gas within the right lobe of the liver	IV antibiotics	Blood culture: *Escherichia coli*	Died 3 d after admission

COPD: Congestive obstructive pulmonary disease; CT: Computed tomography; ERCP: Endoscopic retrograde cholangiopancreatography; F: Female; IV: Intravenous; M: Male; NA: Not available.

OUTCOME AND FOLLOW-UP

The patient continued to recover favorably and was discharged 1 mo after admission, with intravenous antibiotics at home. During her stay, a colonoscopy and transthoracic echocardiogram were performed to rule out other potential etiologies. At the time of discharge, a CT scan showed a 90 mm × 47 mm liquefied collection in segment VII. Follow-up included magnetic resonance imaging (MRI) and positron emission tomography (PET). MRI, performed 6 wk after initial admission, showed a 10-cm cystic formation in liver segments VII and VIII that contained both fluid and necrotic debris (Figure 1C) that were not metabolically active on the PET scan. However, in segment VIII, a 2-cm PET-positive nodule was detected (Figure 1D). Surgical drainage was performed because of the heterogenous content of the large intrahepatic collection in segments VII-VIII and the undetermined nature of the 2-cm lesion in segment VIII. Three mo after onset, the patient underwent laparoscopic deroofing and debridement of the hepatic collection in segment VII and partial hepatectomy of the 2-cm lesion in segment VIII (Figure 2). No malignancy was found in the resected specimens, and microbiological cultures were sterile. Hence, antibiotics were discontinued after a total treatment of 14 wk. The patient had an uneventful recovery but was hospitalized again after a few weeks for coronavirus disease 2019 infection. To date, the patient is asymptomatic and without recurrence on follow-up imaging.

DISCUSSION

EH is a severe, life-threatening infection of the liver parenchyma by gas-forming bacteria. To the best of our knowledge, 11 cases of EH have been previously reported in the literature (Table 1)[1-11]. Remarkably, only 1 of those patients, treated by urgent laparotomy and surgical debridement, was reported to have survived this dismal clinical entity[5]. However, a mixed collection of necrotic debris and air was diagnosed by CT and intraoperatively, making the probability of a pyogenic liver abscess or at least coexistence of both entities more likely. The other patients all died within 3 d of severe multiple organ failure in the setting of fulminant septic shock. This case report describes the favorable outcome of a patient diagnosed with EH and managed by a step-up approach consisting of initial aggressive resuscitation, systemic antimicrobial therapy, and percutaneous radiologically guided drainage followed by laparoscopic surgical debridement.

EH occurs predominantly in women, and diabetes mellitus seems to be a predisposing condition. Abdominal pain and fever are the most common clinical manifestations of the disease. Diagnosis of EH is confirmed by the presence of parenchymal gas in the liver on CT in the absence of intrahepatic fluid collection. CT is the imaging modality of choice for diagnosing EH, as it permits early detection, evaluation of the extent and location of liver involvement, and excludes other etiologies of acute abdominal pain causing septic shock. Importantly, parenchymal gas in the liver has to be differentiated from air in other liver structures. Air can be observed within bile ducts (*e.g.*, following endoscopic sphincterotomy), portal veins (*e.g.*, as a result of bowel infarction), in infarcted liver (*e.g.*, after liver transplantation), and in pyogenic liver abscesses. In contrast to EH, characteristics of pyogenic liver abscesses on CT scans include peripheral enhancing and centrally hypoattenuating (dense or) liquefied collections containing gas bubbles or air-fluid boundaries[12].

Emphysematous infections in the abdomen are known to occur in the gallbladder, stomach, pancreas, and urinary tract[13]. Clinically, pathologically, and radiologically, EH shares features with emphysematous pyelonephritis. The latter is defined as an acute necrotizing, gas-forming infection in the kidney associated with a poor prognosis. Bacterial pathogens cultured in emphysematous pyelonephritis include *Escherichia coli* and members of the genera *Klebsiella*, *Enterobacter*, *Pseudomonas*, *Proteus* and *Streptococcus*[1,14]. As shown in Table 1, the same causative pathogens can be found in EH.

The pathophysiology of emphysematous infections is believed to be caused by mixed acid bacterial fermentation from tissue necrosis resulting in the production of hydrogen (15%), nitrogen (60%), oxygen (5%), and carbon dioxide (5%). Diabetes is known to predispose to emphysematous infections by providing high levels of glucose used as a substrate by the microorganisms[3,15]. Last, diabetes as well as other risk factors for microangiopathy, may contribute to slow transport of catabolic products, leading to accumulation of gas[3,15]. Similar to most previously published cases of EH, our patient was diabetic. Noteworthy was the well-controlled disease state, with glycated hemoglobin of 5.8%, suggesting that factors other than circulating glucose levels may have been of importance.

With advances in cross-sectional imaging and localization, percutaneous drainage has now become the treatment of choice of pyogenic abscesses of the liver. By analogy, less invasive means seem to be a first-line approach in EH as well. Although often ineffective in previously reported cases, our patient responded to early aggressive medical management and radiologically guided drainage. Surgical intervention in this case was not intended as a salvage therapy but rather as a step-up to initial conservative management. Laparoscopic deroofing and debridement of necrosis was undertaken 3 mo after the initial presentation. Given the dorsal localization of the hepatic area involved in our patient, a semi-prone position was chosen to allow laparoscopic visualization of posterior segments and partial hepatectomy of segment VII and VIII, avoiding laparotomy *via* a right subcostal incision.

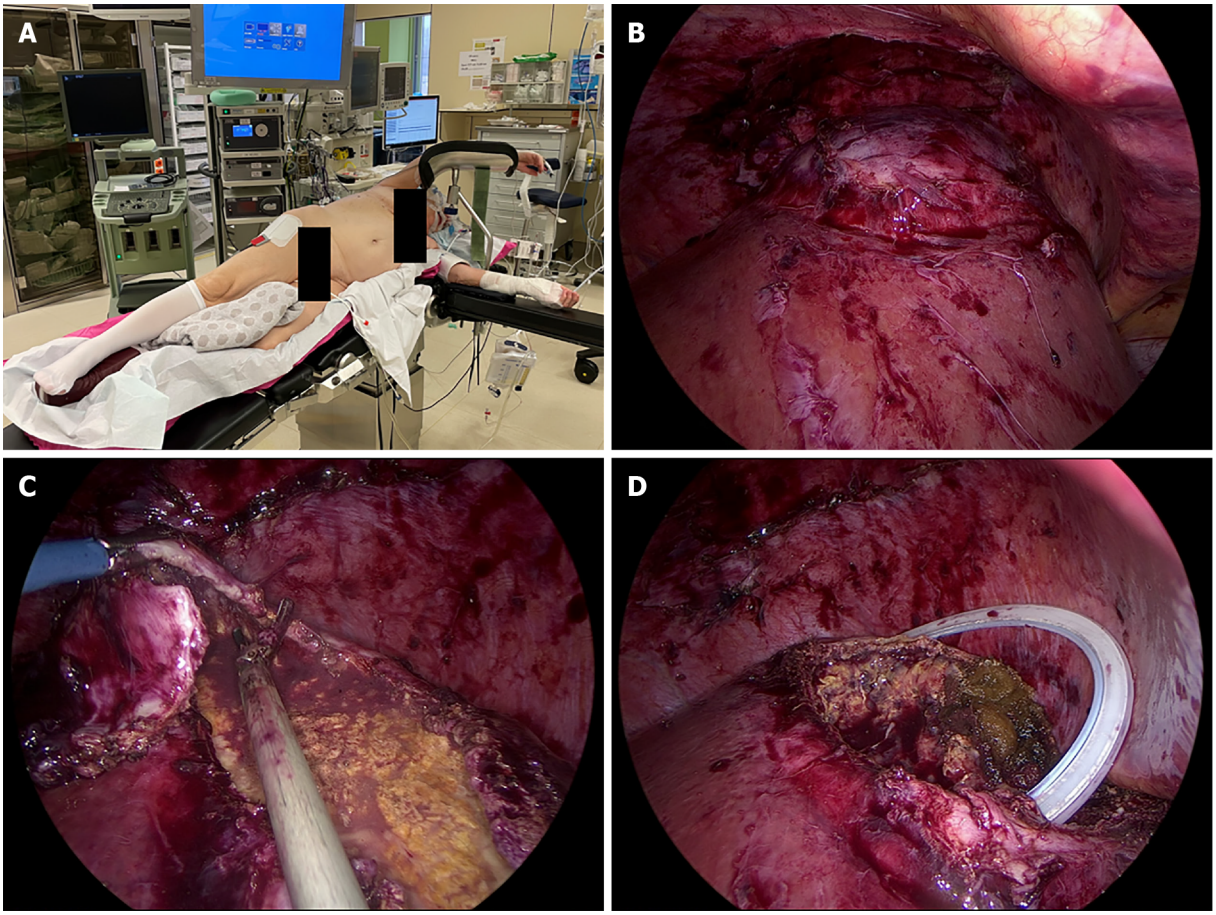

Figure 2 Laparoscopic treatment of emphysematous hepatitis. A: Semi-pone positioning of the patient; B: Laparoscopic view of liver segments VII and VIII after mobilization of the right liver lobe; C: Laparoscopic deroofing of the liver capsule of segments VII and VIII; D: Partial hepatectomy of segment VIII and placement of a surgical drain in the remaining cavity.

CONCLUSION

In conclusion, EH is a serious potentially life-threatening infection of the liver. We report the successful treatment of a patient diagnosed with EH by adopting a multimodal step-up approach including rigorous fluid resuscitation, early hemodynamic support, broad-spectrum antimicrobial therapy and percutaneous radiologically guided drainage followed by minimally invasive surgical treatment.

FOOTNOTES

Author contributions: Francois S designed the report and collected the patient's clinical data; Francois S and Messaoudi N wrote the manuscript; Aerts M, Reynaert H, Van Lancker R, Van Laethem J and Kunda R read and approved the final manuscript.

Informed consent statement: Consent was obtained from the patient (on 09/06/2021) for publication of this anonymized case details and accompanying images.

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CASE REPORT

Glycogen hepatopathy in type-1 diabetes mellitus: A case report

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Abstract

BACKGROUND

It has been studied that fluctuating glucose levels may superimpose glycated hemoglobin in determining the risk for diabetes mellitus (DM) complications. While non-alcoholic steatohepatitis (NASH) remains a predominant cause of elevated transaminases in Type 2 DM due to a strong underplay of metabolic syndrome, Type 1 DM can contrastingly affect the liver in a direct, benign, and reversible manner, causing Glycogen hepatopathy (GH) - with a good prognosis.

CASE SUMMARY

A 50-year-old female with history of poorly controlled type 1 DM, status post cholecystectomy several years ago, and obesity presented with nausea, vomiting, and abdominal pain. Her vitals at the time of admission were stable. On physical examination, she had diffuse abdominal tenderness. Her finger-stick glucose was 612 mg/dL with elevated ketones and low bicarbonate. Her labs revealed abnormal liver studies: AST 1460 U/L, ALP: 682 U/L, ALP: 569 U/L, total bilirubin: 0.3mg/dL, normal total protein, albumin, and prothrombin time/international normalized ratio (PT/INR). A magnetic resonance cholangiopancreatography (MRCP) demonstrated mild intra and extra-hepatic biliary ductal dilation without evidence of choledocholithiasis. She subsequently underwent a diagnostic ERCP which showed a moderately dilated CBD, for which a stent was placed. Studies for viral hepatitis, Wilson's Disease, alpha-1-antitrypsin, and iron panel came back normal. Due to waxing and waning transaminases during the hospital course, a liver biopsy was eventually done, revealing slightly enlarged hepatocytes that were PAS-positive, suggestive of glycogenic hepatopathy. With treatment of hyperglycemia and ensuing strict glycemic control, her transaminases improved, and she was discharged.

CONCLUSION

With a negative hepatocellular and cholestatic work-up, our patient likely had GH, a close differential for NASH but a poorly recognized entity. GH, first described in 1930 as a component of Mauriac syndrome, is believed to be due to glucose and insulin levels fluctuation. Dual echo magnetic resonance imaging

sequencing and computed tomography scans of the liver are helpful to differentiate GH from NASH. Still, liver biopsy remains the gold standard for diagnosis. Biopsy predominantly shows intra-cellular glycogen deposition, with minimal or no steatosis or inflammation. As GH is reversible with good glycemic control, it should be one of the differentials in patients with brittle diabetes and elevated transaminases. GH, however, can cause a dramatic elevation in transaminases (50-1600 IU/L) alongside hepatomegaly and abdominal pain that would raise concern for acute liver injury leading to exhaustive work-up, as was in our patient above. Fluctuation in transaminases is predominantly seen during hyperglycemic episodes, and proper glycemic control is the mainstay of the treatment.

Key Words: Glycogen; Mauriac; Hepatic; Steatosis; Diabetes; Type 1; Case report

Core Tip: Glycogen hepatopathy is a poorly understood complication of type 1 diabetes mellitus patients who have poor glycemic control. Its presentation can closely mimic non-alcoholic fatty liver disease creating a diagnostic enigma in patients with diabetes. After excluding other common causes of hepatitis, one must keep this elusive diagnosis in mind. Hepatic biopsy has been the mainstay for diagnosis, however, with recent advancements sequential magnetic resonance imaging and computed tomography scans are also sensitive but limited by availability.

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INTRODUCTION

Ensemble of poorly controlled type 1 diabetics presenting with marked elevation in serum aminotransferases that corresponds with serum glucose fluctuation, and the defining histological changes on liver biopsy help clinch the diagnosis of glycogenic hepatopathy (GH)[1]. GH was first defined by Mauriac in a child with brittle diabetes. It was considered as a component of Mauriac syndrome, accompanied by delayed development, hepatomegaly, cushingoid appearance, and delayed puberty[2]. Additionally, GH can also be observed in adult type 1 diabetic individuals without other components of Mauriac syndrome[3,4]. Hyperglycemia and corresponding spike in insulin levels are believed to be the main culprits in GH. The treatment of GH is *via* establishing glycemic control. Often these findings in patients with diabetes may be dismissed as NAFLD; however, rigorous glycemic control *via* intensive insulin therapy provides complete remission of clinical, laboratory, and histological abnormalities[5]. Here, a 50-year-old female with poorly controlled type 1 diabetes mellitus is presented with a discussion referenced to the medical literature.

CASE PRESENTATION

Chief complaints

Abdominal pain, diarrhea and fatigue

History of present illness

A 50-year-old female with a long-standing history of uncontrolled type 1 DM, presented with fatigue and nausea for a day to a local hospital, where a basic workup was unrevealing, and she was discharged on symptomatic management. Her symptoms progressed and she began experiencing concomitant abdominal pain and diarrhea. Following this, she presented to our emergency room, with the above symptoms. Her abdominal pain was 6/10 in severity, cramping in nature that was relieved on lying down and predominantly on the left half. She did not have pruritus, jaundice, night sweats, fever, easy bruising, or bleeding. A review of systems was negative for headaches, chest discomfort, chest pain, shortness of breath, orthopnea, or paroxysmal nocturnal dyspnea. In an initial interview, she mentioned that she was admitted 6 mo ago with similar symptoms and at that time her appendix was removed. The prior hospital course was complicated by elevated liver function tests according to the patient. A list

of medications at the time of admission included amlodipine, aspirin (low dose-81mg)), gabapentin, insulin glargine, and lispro (was not taking it for 2 days prior to presentation), metoprolol succinate, oxycodone, and pravastatin (taking a statin for several years, low dose).

History of past illness

Type 1 DM complicated by peripheral neuropathy, diabetic retinopathy, diabetic gastroparesis, coronary artery disease, deep vein thrombosis, gastroesophageal reflux disease.

Personal and family history

Personal history: Currently not working, Nonsmoker, never smoked, no vaping used, denied alcohol intake, denied recreational drug use.

Family history: Type 1 diabetes in aunt; Type 2 diabetes in both parents; Fatty liver disease in both parents and aunt.

Physical examination

The patient was obese, anicteric not in acute distress. She was afebrile; her pulse was 80 beats *per* minute, blood pressure 147/94 mmHg, BMI 31.39 kg/m^2, oxygen saturation 97% on room air. There was no elevation of jugular venous pressure. Examination of the heart and lungs was unremarkable. The abdomen was non-distended and soft, with normal bowel sounds. She had mild tenderness on palpation in the left lower quadrant with no rebound or guarding. Murphy's sign was absent. The liver was palpable 4 cm below the costal margin, with a measured span of 13 cm at the midclavicular line. The edge of the liver was smooth. The spleen was not palpable, and there was no evidence of ascites. There were no stigmata of chronic liver disease either including vesicular lesions, palmar erythema, pedal edema, or spider angiomata. The results of the neurologic examination, including mental status, were normal.

Laboratory examinations

A workup for acute abdomen was initiated. Initial labs showed blood glucose: 610 mg/dL, AG: 13 U/L, CO2:23 ppm, pH: 7.35, and 1+ ketones in the urine. Diabetic ketoacidosis protocol ensued with insulin drip and IV fluids and frequent fingerstick glucose checks.

Hospital laboratory workup showed elevated liver enzymes viz. aspartate aminotransferase:1460 U/L, alanine aminotransferase: 682 U/L, alkaline phosphatase: 569 U/L, elevated LDH: 823 U/L, GGT:436 U/L total bilirubin 0.2 mg *per* deciliter, total protein 6.4 mg *per* deciliter, and albumin 3.7 g *per* deciliter. Levels of sodium were 135 mmol *per* liter, potassium 4.7 mmol *per* liter, chloride 103 mmol *per* liter, bicarbonate 20.9 mmol *per* liter, blood urea nitrogen 25 mg *per* deciliter, creatinine 0.85 mg *per* deciliter, and glucose 225 mg *per* deciliter. Amylase and lipase levels were normal. The white-cell count was 3900 *per* cubic millimeter, with an unremarkable differential count; hematocrit: 38.5%, hemoglobin: 10.9; and platelet count 221,000 *per* cubic millimeter. Levels of vitamin B12 and folic acid were normal. The international normalized ratio was 1.

Serum iron level was 76 µg *per* deciliter, total iron-binding capacity 384 µg *per* deciliter, ferritin 165 µg *per* liter, and thyroid stimulation hormone 0.71 µIU *per* liter. Her total cholesterol level was 122 mg *per* deciliter, triglycerides 104 mg *per* deciliter, high-density lipoprotein cholesterol 49 mg *per* deciliter, and low-density lipoprotein cholesterol 52 mg *per* deciliter. The glycated hemoglobin level was 11.4%. The erythrocyte sedimentation rate was 34 mm *per* hour. Serologic tests for viral hepatitis were negative for hepatitis B surface antibody, negative for hepatitis B surface antigen and core antibody, negative for hepatitis C antibody, negative for cytomegalovirus IgM+IgG antibody, negative for herpes simplex virus PCR. Tests for antimitochondrial antibody, anti-smooth-muscle antibody, and antinuclear antibody screen were negative. The serum ceruloplasmin and urinary copper levels were normal, as were the results of an ophthalmologic examination. The level of alpha1-antitrypsin was also normal. Salicylates, Tylenol levels, and a urine toxicology screen were all negative.

Imaging examinations

CT abdomen and pelvis with IV contrast showed diffuse colonic wall thickening from previous involving the hepatic flexure, transverse colon, splenic flexure, and descending colon, suggestive of colitis. There was no pneumatosis or bowel obstruction or free air under the diaphragm. No focal hepatic lesions were seen either. There were post-cholecystectomy changes causing mild intrahepatic and extrahepatic biliary ductal dilation without significant interval change. No obstructing calculus or lesions were visualized in the hepatobiliary system. The size, shape, and morphology of the liver, spleen, and pancreas were normal.

Doppler ultrasonography (USG) of the abdomen showed a surgically absent gallbladder. The common bile duct measured 10 mm. There was mild central intrahepatic biliary ductal dilatation, likely cholecystectomy related with nil fatty infiltration or vascular abnormalities, and normal echogenicity. An echocardiogram of the heart showed regular biventricular size with an ejection fraction of 65-70%.

Further, an MRCP showed mild intrahepatic and extrahepatic biliary ductal dilation without evidence of choledocholithiasis. There were no focal hepatic lesions. No diffuse parenchymal signal abnormality.

Hospital course

During the course of the hospital, it was noticed that initial rise in transaminases on admission, there was a sharp drop without any specific intervention. Strict glucose control ensued and hepatotoxic medications were held.

By the third day, transaminases dropped to the mid 300-400 range (Figures 1 and 2).

Due to the above negative results, a liver biopsy was pursued to clinch the diagnosis.

Liver biopsy findings

Liver biopsy showed a subset of hepatocytes, slightly enlarged with PAS-positive glycogen, suggestive of mild glycogenic hepatopathy. No significant glycogenated nuclei were seen - portal tracts with minimal inflammation composed of mononuclear cells, neutrophils, eosinophils with occasional ceroid-laden macrophages. A minority of bile ducts show mild epithelial injury. Bile ducts were present with focal mild bile ductular reaction (CK7 immunostain examined). No steatosis, cholestasis, hepatocyte ballooning degeneration, acidophil bodies, congestion, or confluent necrosis were identified. Trichrome stain showed no increased fibrosis. PAS/D stain is negative for intracytoplasmic globules. Iron stain is negative for iron deposition. A Gomori methenamine silver stain was negative for fungal organisms. Immunohistochemistry for CMV, HSV-1, HSV2, and adenovirus was negative. EBV-encoded RNA in situ hybridization was negative. The copper stain was negative. Reticulin stain showed an intact reticulin framework.

FINAL DIAGNOSIS

Glycogen hepatopathy secondary to poorly controlled type 1 DM.

TREATMENT

With treatment targeting aggressive glycemic control with insulin and a strict carbohydrate-restricted diet, her transaminases improved. Her colitis resolved with conservative management following which, she was discharged.

OUTCOME AND FOLLOW-UP

The patient was advised to monitor blood sugars at home and was advised about the importance of a diabetic diet with the help of a diabetes educator. She was taught how to use an insulin pen, and was discharged with an insulin kit containing Insulin glargine (long-acting) along with Insulin lispro (short-acting to be taken with meals).

When she was seen a month later at the primary care physician's office, she was asymptomatic. Her laboratory tests revealed a normal biochemical profile, with transaminases well under the normal range.

DISCUSSION

The findings of increased liver enzymes have increased amongst patients with diabetes. The observation of increased alanine aminotransferase levels is 9.5% among type 1 as compared to 12.1% among type 2 diabetics. These percentages are higher than those expected in our general population (2.7%)[6,7]

A disease like GH develops due to hepatic glycogen accumulation. It is characterized by hepatomegaly and elevated liver function tests including AST and ALT[8,9]. GH was first defined as glycogen accumulation in 1930, as a component of Mauriac syndrome (type 1 diabetes, delayed development, hepatomegaly, cushingoid appearance, and delayed puberty)[2]. Interestingly, type 1 diabetic individuals without other components of Mauriac syndrome can have isolated manifestation of GH[10, 3]. Type 1 diabetes patients formulate a major chunk of the case reports of this rare condition.

Elevated glucose levels with corresponding insulin spikes are believed to be metabolic preconditions for hepatic glycogen accumulation in GH. Hyperglycemia signals glycogen synthase by inhibiting glycogenesis by glycogen phosphorylation inactivation. Insulin also activates glycogen synthase which further increases glycogen accumulation[11]. A study conducted in rats with insulin deficiency has shown that glycogenesis continues for a significant amount of time after blood glucose levels return to

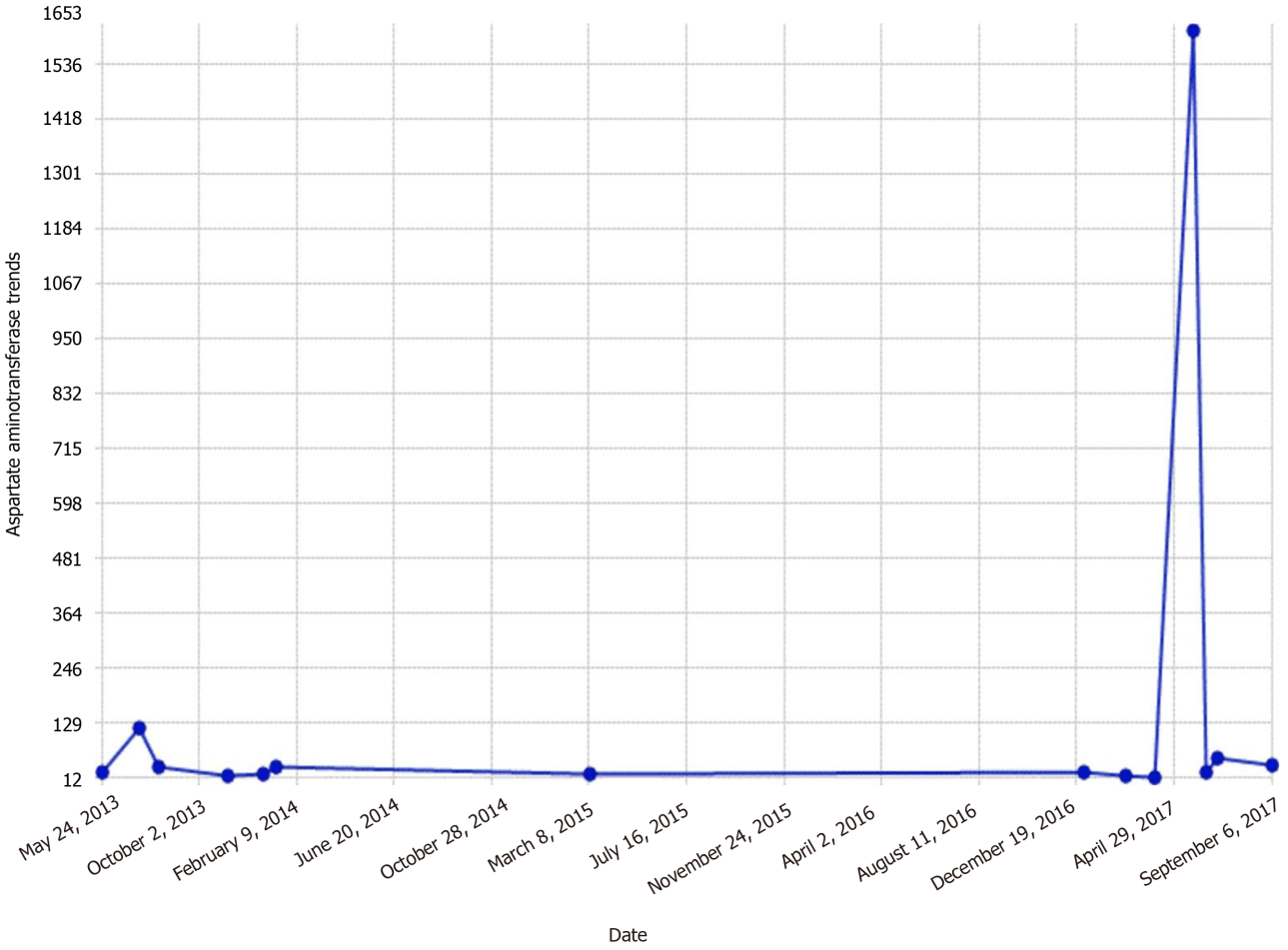

Figure 1 Aspartate aminotransferase trends.

the preinjection levels after a single dose of insulin injection[12]. In essence, accumulation of glycogen continues to occur in the liver, despite the high cytoplasmic glucose concentration in the presence of insulin. Hence, oscillating hyperglycemic episodes and the following insulin therapies to chase the elevated glucose levels are believed to be the primary pathogenetic mechanisms of hepatomegaly and abnormal liver chemistries that develop in T1DM patients due to glycogenic deposition. However, it is not clear why this pathogenetic mechanism develops specifically in a small patient group. Several theories have been proposed on the matter, one of which was the defect in genes coding the proteins that regulate the glycogen synthase and/or glucose 6-phosphatase activity[13]. Mainstay of managing GH is by establishing strict glycemic control *via* intensive insulin therapy. This modality of approach results in full remission of clinical, laboratory, and histologic abnormalities[5]. Several medical case reports exhibit remission in cases of GH by a continuous insulin infusion pump implanted under the skin[3]. Similarly, in our presented case, we attained blood glucose regulation, that was followed by a reduction in the liver size and significant decreases in ALT and AST levels using intensive insulin therapy.

Furthermore, after GH diagnosis, the treatment should aim for intense glycemic control as it is considered the backbone of its management[14-16]. Anomalously, the resolution of GH has also been described after minimal glucose control[15-17]. The disease has a benign course with an excellent prognosis and symptoms abate using above therapeutics within a few weeks, as also observed in our patient (Figure 3).

CONCLUSION

GH is a rare complication of diabetes mellitus, particularly in type 1 diabetes mellitus (T1DM) patients with poor glycemic control and the presentation can closely mimic non-alcoholic fatty liver disease creating a diagnostic enigma in patients with diabetes.

Clinicians should be aware of this rare complication of diabetes mellitus in T1DM patients with poor glycemic control.

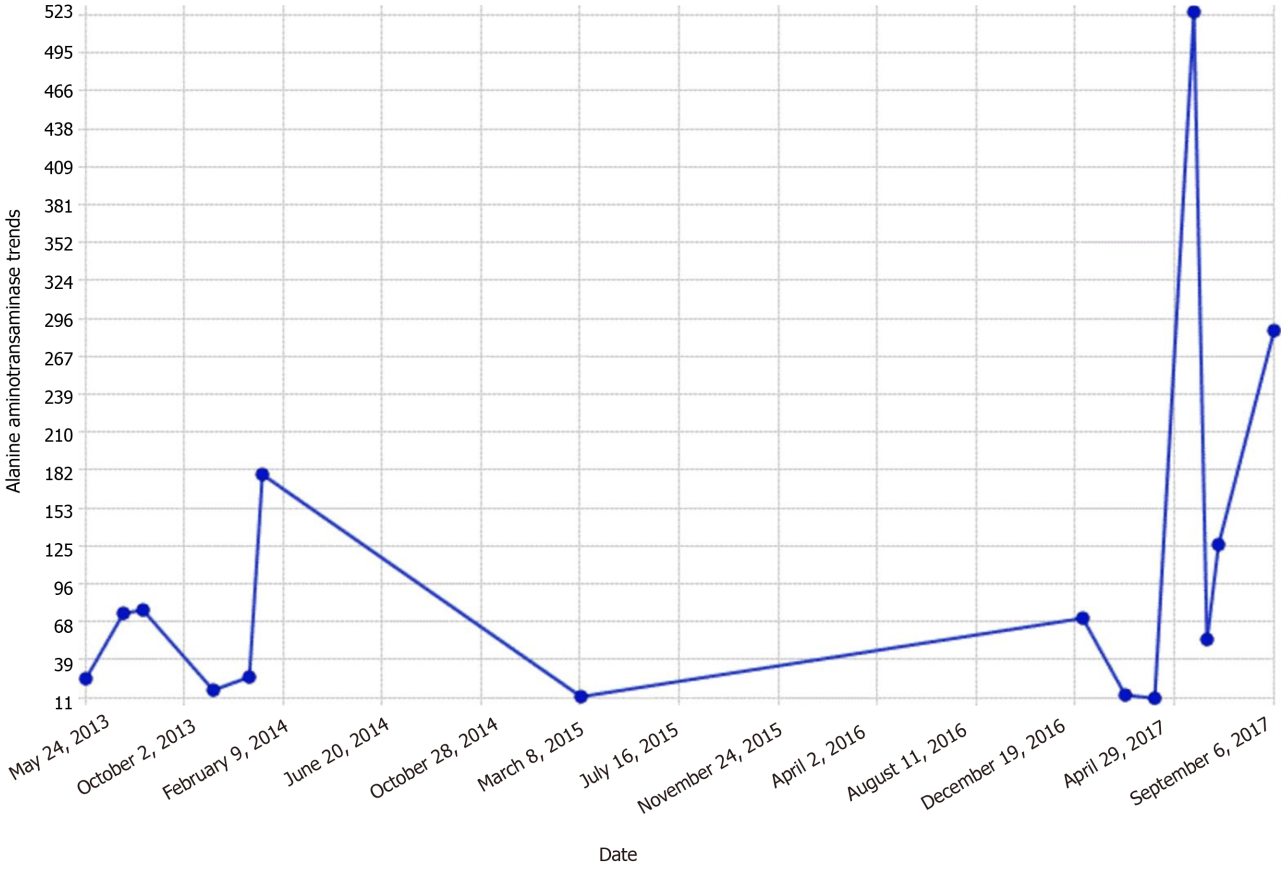

Figure 2 Alanine aminotransaminase trends.

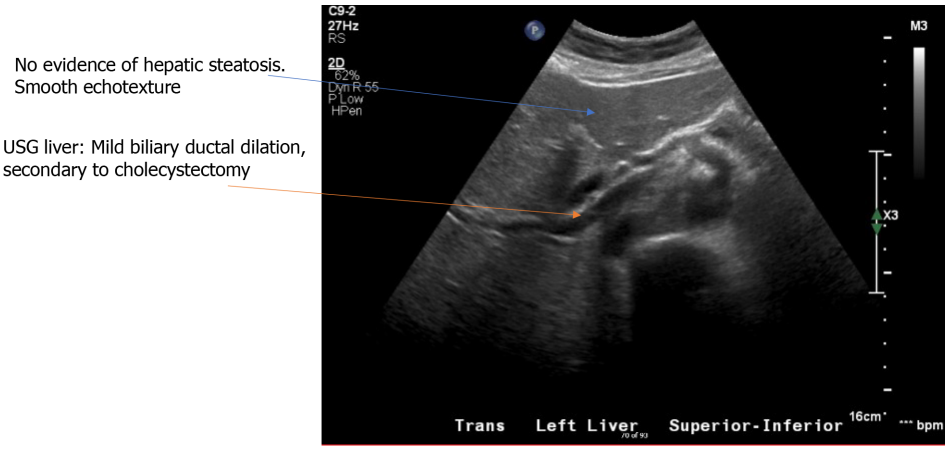

Figure 3 Ultrasonography liver.

After excluding other common causes of hepatitis, including viral and autoimmune hepatitis or celiac disease, an in-depth investigation for GH should be performed.

Liver biopsy has been the mainstay for diagnosis, however, with recent advancements, sequential magnetic resonance imaging and computed tomography (Figure 4) scans are also sensitive but limited by the availability.

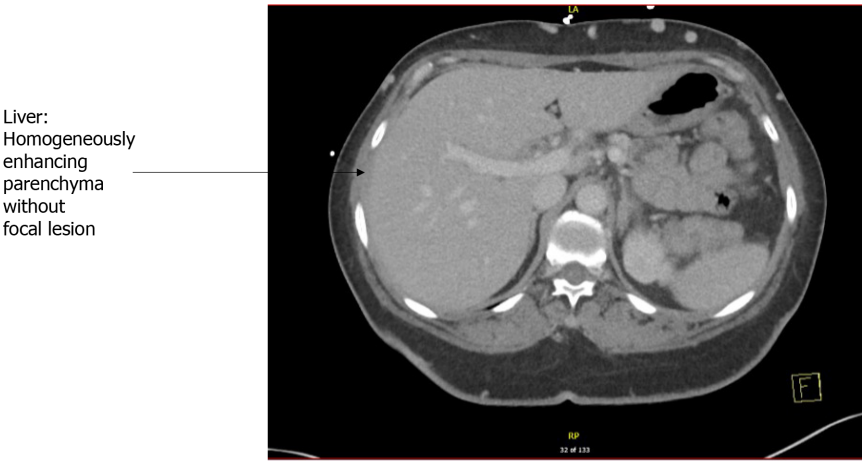

Figure 4 Computed tomography scan of abdomen.

FOOTNOTES

Author contributions: Singh Y wrote down the manuscript, collected data, directly involved in patient care; Gogtay M collected imaging of the patient, assisted in procuring biopsy specimen of the patient; Gurung S contributed to collecting patients past medical history and literature review on the diagnosis; all authors have read and approved the final manuscript.

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LETTER TO THE EDITOR

COVID-19 and liver disease: Are we missing something?

Tarana Gupta

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Abstract

Since the coronavirus disease 2019 (COVID-19) has hit the world as a pandemic, researchers all over the world have worked on its diagnostics, prognosticating factors, *etc.* The present study showed liver enzymes, especially aspartate aminotransferase (AST) levels, to be high in non-survivors with raised AST/alanine aminotransferase ratio. Considering the non-specific nature of AST with its presence in organs other than liver such as muscle, heart, kidney and brain makes it difficult to interpret. Even pre-existing metabolic syndrome and non-alcoholic fatty liver disease are confounding factors for deranged liver functions detected during COVID-19 disease. Therefore, the results of the study should be taken with caution.

Key Words: COVID-19; Liver disease; Transaminases; Non-alcoholic fatty liver disease; Hepatocytes; Cholangiocytes

Core Tip: The presence of angiotensin converting enzyme 2 receptors in liver endothelial cells makes it susceptible to severe acute respiratory syndrome coronavirus 2 injury. The authors have suggested raised aspartate aminotransferase (AST) levels in almost a third of non-survivors along with high AST/alanine aminotransferase ratio. Considering the presence of AST in organs other than liver such as muscle, red blood cells, heart and kidney, makes the interpretation difficult. Additionally, pre-existing non-alcoholic fatty liver disease has also been documented as a risk factor for severe coronavirus disease 2019 (COVID-19) disease. Therefore, more studies are needed for evaluation of AST as a predictive factor for severe COVID-19 disease.

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TO THE EDITOR

We read with great interest the article by "Madian *et al*[1]". Limited data is available for hepatic injury in coronavirus disease 2019 (COVID-19) disease. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus causes direct cytopathic effect on hepatocytes. It enters the cell through angiotensin converting enzyme 2 (ACE2) receptors which are ubiquitously present on alveolar epithelium, liver, kidney and blood vessels *etc.* ACE2 receptors are present on endothelium of smaller blood vessels in liver, however, sparse on sinusoidal endothelium. Chai *et al*[2] reported higher ACE2 expression on cholangiocytes (59%) than hepatocytes (2.6%). They also suggested that liver dysfunction in COVID-19 is predominantly due to cholangiocyte dysfunction. The profound cytokine storm generated by lung injury also results in liver dysfunction. The drug-related hepatotoxicity related to the use of acetaminophen, remdesivir, lopinavir/ritonavir, azithromycin *etc.* during treatment of COVID-19 disease plays an additive role in causing deranged liver functions[3]. In addition, use of steroids especially in moderate to severe cases can also cause hepatitis B flare in occult hepatitis B patients.

Only a few studies could highlight liver function tests in patients with COVID-19 in non-cirrhotic patients. Limited studies have shown acute liver injury in 14%-53% of COVID-19 cases. In the present study, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were elevated in 31% and 3% among non-survivors respectively with 48% having AST/ALT ratio > 1.2. Zhang *et al*[4] showed raised AST levels more frequently in intensive care unit (ICU) patients (62%) than in non-ICU (25%) settings. Interestingly, the present study revealed an increased in-hospital mortality up to 11-and 13-fold with AST levels > 1 ULN and > 2 ULN respectively. Chen *et al*[5] analyzed biochemical profile of 113 deceased and 161 recovered patients in Wuhan, and found abnormal AST levels in 59 (52%) *vs* 25 (16%) and lower serum albumin in 74 (65%) *vs* 22 (14%) patients respectively. In the present study, authors also mentioned the possibility of raised AST levels due to muscle injury resulting from profound cytokine storm in COVID-19 illness, though they have documented normal creatine kinase (CK) values indicating liver involvement. AST is an enzyme which is found in liver, muscle, heart, kidney and brain. Therefore, raised AST levels should be taken with caution. The pattern of liver injury in COVID-19 disease is elevated AST levels more than ALT levels with higher gamma-glutamyl-transferase (transpeptidase) (GGTP) values which is similar to alcoholic hepatitis.

We should not forget that non-alcoholic fatty liver disease (NAFLD) and obesity are associated with symptomatic, severe and complicated COVID-19 disease and is a potential confounder[6]. The studies assessing the role of liver injury on the course of COVID-19 illness have not screened patients for NAFLD, and we are not aware about their basic liver function tests before COVID-19. A pooled analysis of 8 studies in NAFLD and COVID-19 by Sachdeva *et al*[7] revealed NAFLD being a predictor of severe COVID-19 disease after adjustment of presence of obesity (OR: 2.3; 95%CI: 1.9-2.9, $P < 0.001$). Therefore, it may be too early to depend alone on AST levels for the severity and outcome of COVID-19 illness.

FOOTNOTES

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