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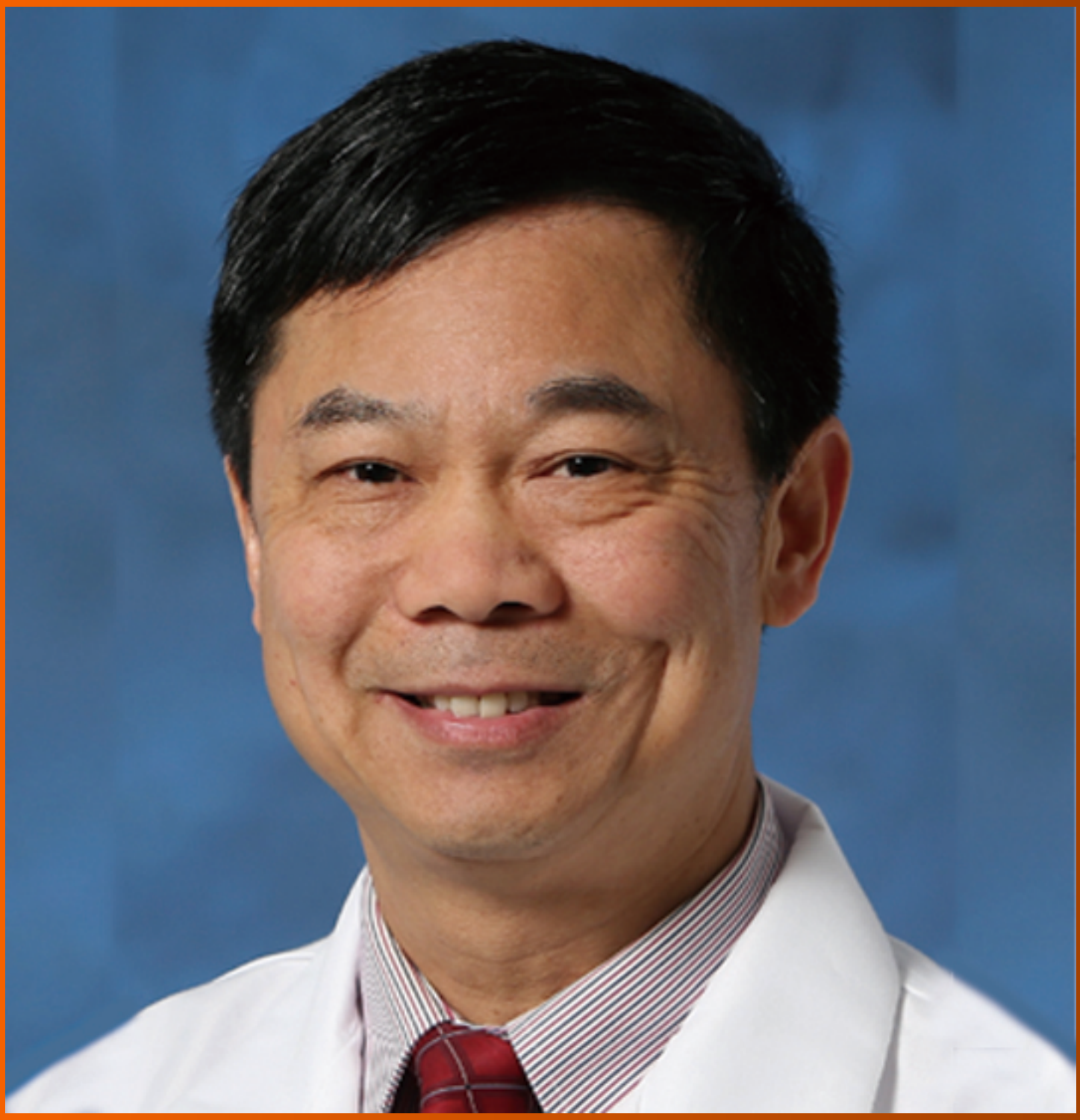

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World Journal of Hepatology

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ABOUT COVER

Editor-in-Chief of *World Journal of Hepatology*, Dr. Ke-Qin Hu is Director of Hepatology Services and Professor of Medicine in the Division of Gastroenterology and Hepatology, University of California, Irvine School of Medicine (United States). Dr. Hu's career efforts emphasize bridging research advances to bedside patient care. His clinical research has focused on the natural history and outcomes of various liver diseases and healthcare disparity. His basic science research has focused on molecular virology and diagnosis of hepatitis B and C virus infection, and chemoprevention of liver cancer. Dr. Hu has coauthored more than 150 research papers, book chapters, and review articles. He is Deputy Editor-in-Chief for Frontiers of Medicine. He is dedicated to community outreach, public health education, and reduction of healthcare disparity. (L-Editor: Filipodia)

AIMS AND SCOPE

The primary aim of *World Journal of Hepatology* (*WJH*, *World J Hepatol*) is to provide scholars and readers from various fields of hepatology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

WJH mainly publishes articles reporting research results and findings obtained in the field of hepatology and covering a wide range of topics including chronic cholestatic liver diseases, cirrhosis and its complications, clinical alcoholic liver disease, drug induced liver disease autoimmune, fatty liver disease, genetic and pediatric liver diseases, hepatocellular carcinoma, hepatic stellate cells and fibrosis, liver immunology, liver regeneration, hepatic surgery, liver transplantation, biliary tract pathophysiology, non-invasive markers of liver fibrosis, viral hepatitis.

INDEXING/ABSTRACTING

The *WJH* is now abstracted and indexed in PubMed, PubMed Central, Emerging Sources Citation Index (Web of Science), Scopus, China National Knowledge Infrastructure (CNKI), China Science and Technology Journal Database (CSTJ), and Superstar Journals Database. The *WJH*'s CiteScore for 2019 is 5.8 and Scopus CiteScore rank 2019: Hepatology is 22/61.

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EDITORIAL

New Year's greeting and overview of *World Journal of Hepatology* in 2021

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Abstract

The *World Journal of Hepatology* (*WJH*) was launched in October 2009. It mainly publishes articles reporting research findings in the field of hepatology, covering a wide range of topics, including viral hepatitis B and C, non-alcoholic fatty liver disease, alcoholic liver disease, autoimmune and chronic cholestatic liver disease, drug-induced liver injury, cirrhosis, liver failure, hepatocellular carcinoma, coronavirus disease 2019-related liver conditions, *etc*. As of December 31, 2020, the *WJH* has published 1349 articles, among which, the total cites is 18995 and the average cites per article is 14. In celebrating the New Year, we are pleased to share with you special a New Year's greeting from the *WJH* Editors-in-Chief, along with a detailed overview of the journal's submission, peer review and publishing metrics from 2020. In all, we are appreciative for the substantive support and submissions from authors worldwide, and the dedicated efforts and expertise provided by our invited reviewers and editorial board members.

Key Words: *World Journal of Hepatology*; New Year's greeting message; Editors-in-Chief; Editorial Board; Highly influential scientists; Baishideng Publishing Group Inc

States

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Core Tip: The *World Journal of Hepatology* (*WJH*) mainly publishes articles reporting research results obtained in the field of hepatology and covering a wide range of topics, including a variety of different liver diseases, cirrhosis, hepatocellular carcinoma, and more recently coronavirus disease 2019-related liver conditions and management, and so on. Since its launch in October 2009, the *WJH* has published 1349 articles. As of December 31, 2020, the total cites among these articles is 18995 and the average cites per article is 14. The enthusiastic and excellent support and submissions from authors worldwide, complemented by the dedicated efforts and expertise of our invited reviewers, Editorial Board members, and Editorial Office staff, have been invaluable.

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INTRODUCTION

The *World Journal of Hepatology* (*WJH*, ISSN 1948-5182, https://www.wjgnet.com/1948-5182/index.htm) is a high-quality, monthly, online, open-access, single-blind peer-reviewed journal published by the Baishideng Publishing Group Inc (BPG). The primary aim of *WJH* is to provide scholars and readers from various fields of hepatology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online. The *WJH* is abstracted and indexed in PubMed, PubMed Central, Emerging Sources Citation Index (Web of Science), and Scopus.

Since its launch in October 2009, the *WJH* has published 1349 articles[1]. As of December 31, 2020, the total cites among these articles is 18995 and the average cites per article is 14.

A NEW YEAR'S GREETING FROM THE *WJH* EDITORS-IN-CHIEF

For all of us, 2020 was a very tough year due to coronavirus disease 2019 (COVID-19). As Editors-in-Chief of *WJH*, it is now our great pleasure to take this opportunity to wish all our authors, readers, Editorial Board members, independent expert referees, and staff of the Editorial Office a very Happy New Year. On behalf of the Editorial team, we would like to express our gratitude to all authors who contributed their valuable manuscripts, as well as all independent referees and readers for their continuous support, dedication, and encouragement. Together with an excellent team effort by our Editorial Board members and staff of the Editorial Office and BPG, *WJH* was able to advance in 2020 despite the ongoing COVID-19 pandemic.

As the chief editors, we strive to work with the journal's Editorial Office and BPG staff to make the manuscript submission process as simple as possible and ensure an efficient communication with the authors to provide our support and answer their questions. We are also open to any suggestions that could improve *WJH*'s operation and publication. Please feel free to contact us at (editorialoffice@wjgnet.com) with any question on your submission or suggestions for the journal in general.

OVERVIEW OF THE *WJH* IN 2020

In celebrating *WJH*'s 12-year anniversary and the 2021 New Year, we are very proud to share with you that we completed the following endeavors in submission, peer review and publishing in 2020.

Submission and acceptance

From 2013 to 2020, the *WJH* has received 2302 manuscripts, including invited manuscripts and unsolicited manuscripts, and the average submissions per year is 288. The submissions of unsolicited manuscripts are stable in recent years (Figure 1).

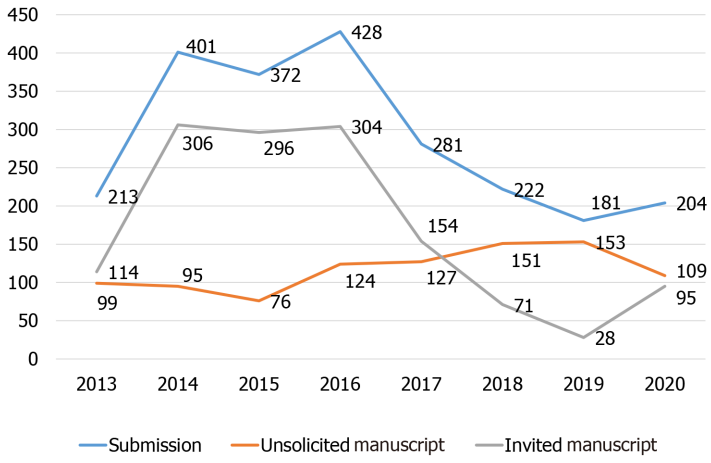

Figure 1 Annual submissions of *World Journal of Hepatology* from 2013 to 2020.

In 2020, we received 204 submissions from authors around the world and published 112 articles in 12 issues. Among those 112 articles, 57 (50.9%) were original articles, 31 (27.7%) were review articles, 1 was an editorial (0.9%), 15 (13.4%) were case reports and 8 (7.1%) were articles of 'other' types (Figure 2). The authors hailed from 32 countries, including 32 (28.6%) from the United States, 10 (8.9%) from Brazil, 6 (5.4%) each from Italy, Japan and Spain, and 5 (4.5%) each from the United Kingdom and France; the remaining 26 (23.2%) were from various individual countries (Figure 3).

Invitation for 2021

In November and December, invitations to contribute high-quality articles to *WJH* were sent out to distinguished scientists in the field of hepatology. As of December 31, 2020, *WJH* has accepted a total of 327 proposed titles for those invited manuscripts; these articles, to be submitted for publication in 2021, include 85 (26.0%) original articles, 215 (65.7%) review articles, 15 (4.6%) editorials, and 12 (3.7%) 'other' types (Figure 4). We are currently inviting highly influential scientists to submit Topic Highlight articles, commenting on and discussing hot topics in the field of hepatology. As of December 31, 2020, we have already received 14 submissions online.

Conducting peer review statistics

As of December 31, 2020, *WJH* had sent out 6120 invitations to peer reviewers and Editorial Board members to conduct peer review of manuscripts. Among the peer reviewers and Editorial Board members who accepted the invitation, 428 (35.0%) submitted the peer review report on time, 425 (34.7%) failed to submit the peer review report on time, and 370 (30.3%) have not submitted the peer review report yet.

Editorial Board members of WJH

The 2020 Editorial Board of *WJH* was composed of 195 members[2]. Among them, 3 were Editors-in-Chief (Professor Ke-Qin Hu, Professor Koo Jeong Kang, and Professor Nikolaos Pyrsopoulos), 5 were Associate Editors, and 187 were Editorial Board Members. The members were based in 45 countries and areas, including 23 (11.8%) in China, 22 (11.3%) in Italy, 19 (9.7%) in the United States, 18 (9.2%) in Turkey, 11 (5.6%) in Egypt, and 102 (52.3%) in various other countries (Figure 5). A total of 86 (44.1%) of the Editorial Board Members served as peer reviewers in 2020.

We are pleased to have received 71 applications for Editorial Board membership (up to December 2020), which are currently under evaluation.

Journal metrics

According to data from the Web of Science (up to January 4, 2021), *WJH* published 258 articles between 2017 and 2018. These articles were cited 830 times in 2019, with a mean citation of 3.217 for each. On behalf of *WJH*, BPG will submit an application to Clarivate Analytics for abstracting and indexing in Science Citation Index Expanded (SCIE), in the near future. *WJH*'s Scopus CiteScore for 2019 is 5.8, ranking 22/61 in the category of Hepatology.

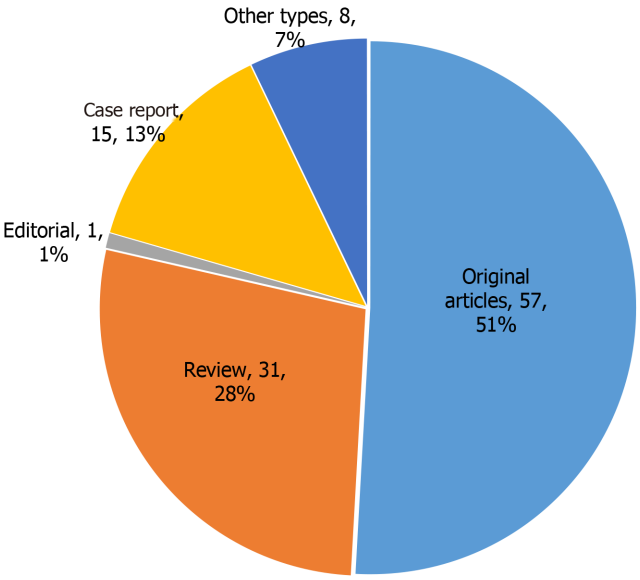

Figure 2 Article types among the 112 manuscripts published by *World Journal of Hepatology* in 2020.

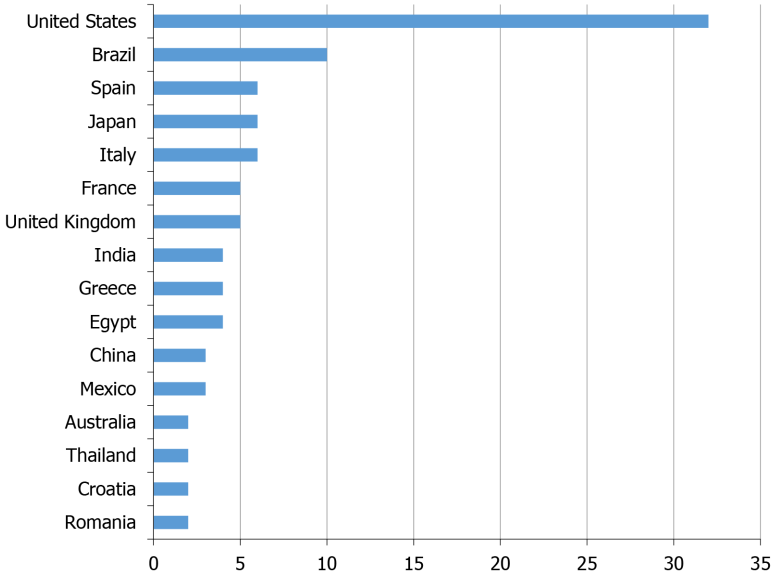

Figure 3 Top 16 countries by number of *World Journal of Hepatology* published manuscripts in 2020.

Accurately pushing WJH articles and authors ahead

To enable more peers to read, share, and cite *WJH* authors' published research results and to help enhance their global academic influence and reputations, thereby also promoting the overall development of the field of hepatology, BPG sends *WJH*'s published articles to 1000-10000 highly influential experts in a topically-accurate manner. After completing this outreach activity, BPG formally notifies the paper's authors of the number of experts to whom their manuscript was sent *via* email. As of December 31, 2020, *WJH* articles included in the push email campaign were sent to 19905 in October, 5308 in November, and 11023 in December.

Challenges facing WJH in 2021

The development and growth of *WJH* rely on a large amount of high-quality manuscripts. We appreciate and encourage all authors to submit their topically-relevant manuscripts to *WJH*, to enjoy the benefits of this great platform and sharing resource in disseminating their medical research results. Our Editorial Board members are encouraged to continue their support by actively serving as peer reviewers, authors contributing articles, and journal representatives inviting high-quality articles from others. *WJH* Editorial Board members are also encouraged to communicate with

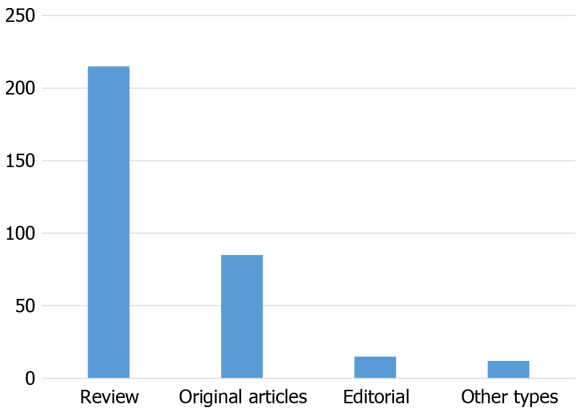

Figure 4 Article types of *World Journal of Hepatology* invited manuscripts for 2021.

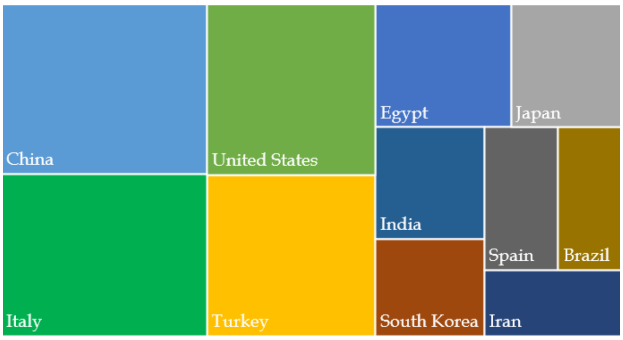

Figure 5 Countries of *World Journal of Hepatology* Editorial Board Members in 2020. Top 11 countries by the number of editorial members, where no less than five members are located in each country.

the Editors-in-Chief actively, provide suggestions and analyze discipline hotspots to promote their academic influence through the *WJH*.

CONCLUSION

In 2021, *WJH* will publish more high-quality original and review articles, consistently improving its academic influence and moving closer towards its next goal of inclusion in the SCIE as soon as possible, which will ultimately promote the overall development of the field of hepatology. *WJH*'s Editors-in-Chief and Editorial Office staff expect to be more productive and have committed to working diligently with all of you to raise the academic rank of *WJH* in 2021. In order to achieve these goals, we recognize the importance of substantive support and submissions from authors like you in tandem with the dedicated efforts and expertise of our invited reviewers, many of whom also serve on our Editorial Board. Please feel free to contact our Editorial Office (editorialoffice@wjgnet.com) if you have further questions, need support, or wish to share your suggestions.

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1 ***World Journal of Hepatology.*** All published articles of *World Journal of Hepatology* from its launch in October 2009 to present. Available from: https://www.wjgnet.com/1948-5182/archive.htm
2 **The 2020 Editorial Board of *World Journal of Hepatology***. Available from: www.wjgnet.com/1948-5182/editorialboard.htm

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REVIEW

Autophagy in liver diseases

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Abstract

Autophagy is the liver cell energy recycling system regulating a variety of homeostatic mechanisms. Damaged organelles, lipids and proteins are degraded in the lysosomes and their elements are re-used by the cell. Investigations on autophagy have led to the award of two Nobel Prizes and a health of important reports. In this review we describe the fundamental functions of autophagy in the liver including new data on the regulation of autophagy. Moreover we emphasize the fact that autophagy acts like a two edge sword in many occasions with the most prominent paradigm being its involvement in the initiation and progress of hepatocellular carcinoma. We also focused to the implication of autophagy and its specialized forms of lipophagy and mitophagy in the pathogenesis of various liver diseases. We analyzed autophagy not only in well studied diseases, like alcoholic and nonalcoholic fatty liver and liver fibrosis but also in viral hepatitis, biliary diseases, autoimmune hepatitis and rare diseases including inherited metabolic diseases and also acetaminophene hepatotoxicity. We also stressed the different consequences that activation or impairment of autophagy may have in hepatocytes as opposed to Kupffer cells, sinusoidal endothelial cells or hepatic stellate cells. Finally, we analyzed the limited clinical data compared to the extensive experimental evidence and the possible future therapeutic interventions based on autophagy manipulation.

Key Words: Autophagy; Lipophagy; Mitophagy; Fatty liver disease; Fibrosis; Liver sinusoidal cells

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S-Editor: Gao CC
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Core Tip: Extensive investigation of autophagy is mostly based on experimental data. However there is now enough evidence to support the notion that autophagy is not only the waste recycling mechanism of the hepatocyte, but is strongly involved in the pathogenesis of almost all liver diseases. It can be either a defensive mechanism against various insults or a detrimental machinery aggravating the underlying disease. Modulation of autophagy has different consequences in the hepatocyte than in the liver macrophages, the sinusoidal endothelium or the hepatic stellate cells. There is also an opportunity for future treatment applications of autophagy manipulation.

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INTRODUCTION

Autophagy in the liver

Autophagy (from the Greek self-eating) is a process crucial for cell survival[1,2]. Autophagy is a lysosomal degradation pathway that controls the disposition of intracellular waste including damaged organelles or invading pathogens. It can be characterized as the recycling energy system of the cell.

Under basal conditions autophagy degrades 1.5% of total hepatic protein per hour but in starvation, protein degradation increases to 4.5% of liver protein per hour[3]. When rodents are starved for 48 h, autophagy degrades up to 40% of liver protein[4].

Although It is accepted that the term "autophagy" was introduced in 1963 by the Belgian researcher Christian René de Duve, in fact the term autophagy was used almost a century earlier by Anselmier in a French journal[5].

However the modern era of autophagy started with the pioneer work of de Duve and Novicoff in the 1950s when acid phosphatase positive lysosomes were described in the rat liver[6-9] and the term lysosome was used for the first time[10].Later de Duve introduced the term autophagosome and Arstila and Trump proved that the autophagosomes originate from the endoplasmic reticulum (ER)[11]. The next important progress came when Takeshige *et al*[12] identified approximately fifteen Autophagy related genes (Atgs) involved in Saccharomyces cerevisiae autophagy[12-14]. Today, more than 40 Atgs in various animal and human cells have been identified and unified[15-17]. The importance of autophagy was recognized by the award of two Nobel Prizes for Physiology or Medicine, the first to Cristian De Duve in 1974 and the second to Yoshinori Ohsumi in 2016[18,19]. Landmarks of autophagy were recently described[20]. During the period 2008-2018 more than 33000 papers related to autophagy were published[21,22].

Autophagy has certain discrete stages including induction, phagophore formation, autophagosome formation, autolysosome formation and degradation[23-25]. Atg molecules are involved in various complexes essential for autophagy induction and autophagosome formation[26]. Initiation starts with activation of the unc-51-like kinase 1 complex (ULK1, Atg1 in yeast) followed by beclin 1 (Atg6 in yeast) and a subsequent cascade of Atg proteins leading to autophagosome formation where LC3 (Atg8 in yeast) is implicated[27]. LC3 is further processed to form initially LC3-I and then LC3-II[28]. Once the autophagosome is formed, a blockage of autophagic flux at late steps will downregulate the clearance of autophagosomes. A blockage of autophagic flux finally results in autophagy dependent cell death[29]. Detailed descriptions of the complex molecular steps of each stage of autophagy were recently published[20,28,30].

A commonly used marker for estimating autophagosome formation is the fusion protein green fluorescent protein-LC3 (GFP-LC3)[31]. Of the three members LC3A, LC3B, and LC3C of the human LC3 gene family, LC3B and LC3-II are mostly used for autophagy assays[32-34]. Autophagic flux into the lysosomes is estimated by measuring p62/SQSTM1 degradation. p62/SQSTM1 is a protein complex that binds to LC3 and is efficiently degraded by autophagy[35]. The total cellular level of p62/SQSTM1 inversely correlates with autophagic activity. Thus in autophagy-deficient cells, p62/SQSTM1 levels are increased after starvation in contrast to cells with normal autophagy[36].

It should be stressed that he level of LC3 is related to the induction of autophagy

but might not reflect the final stages of autophagy and should not be used as a general marker of autophagy[34-36]. Further progress of autophagy is detected by a low level of p62 since p62 degradation depends on the function of the autophagosome-lysosome fusion[37]. Therefore an increase of both LC3 and p62 indicates formation of autophagosomes without lysosomal degradation[38].

As mentioned before, a major breakthrough in autophagy was the identification of Atgs. Evidence for the importance of autophagy in liver homeostasis was provided by the generation of of Atgs-knockout mice models[39]. Livers of mice with deletion of the autophagy gene Atg7 were markedly enlarged, up to 30% of the body weight of the animal and hepatocytes were characterized by structural alterations of mitochondria and peroxisomes and aggregation of ubiquitinated proteins. These aggregates disappeared when the ATg7- knockout mouse was bred to a mouse null for SQSTM1/p62indicating that SQSTM1 is important to direct damaged cytosolic proteins into the autophagic pathway[40,41].

To date, three major types of autophagy, namely, macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA), have been described[22,42,43].

Macroautophagy is the classical pathway that engulfs the cytosolic components targeted for lysosomal degradation. Initiation of autophagy is controlled by two metabolic sensors the mammalian target of rapamycin complex 1 (mTORC1) and the AMP-activated protein kinase (AMPK). mTORC1 negatively regulates autophagy by direct phosphorylation of ULK1 thus inhibiting ULK1. AMPK suppresses mTORC1 activity by phosphorylation of tuberous sclerosis 2 and raptor, two essential regulators of mTORC1[44,45]. Recently it was reported that the final step in this activation process of mTOR is dependent on Rheb, a small GTPase that binds to mTOR and allosterically activates its kinase activity[46]. The long-term regulation of autophagy is carried out by transcription factor EB (TFEB)[47], the main regulator of lysosomal biogenesis and autophagy. Under nutrient-rich conditions, mTORC1 phosphorylates TFEB and retains TFEB in the cytosol[48-50]. Nutrient deprivation on the other hand leads to mTORC1 inhibition, dephosphorylation of TFEB and its translocation to the nucleus to initiate the rapid transcription of autophagy genes[51,52]. All subsequent series of complex events leading to the final degradation in lysosomes have elegantly been described[2,24,53].

A simplified scheme of macroautophagy is presented in Figure 1.

Microautophagy is the least studied type of autophagy where compounds or membranous vesicles are directly taken up by lysosomes[54]. Microautophagy is important during amino acid starvation[55,56] and possibly three different types can be recognized[57].

Chaperone Mediated Autophagy (CMA) is a selective engulfment process of substrates containing the pentapeptide "Lys-Phe-Glu-Arg-Gln" (KFERQ) motifs. They are recognized by, the cytosolic chaperone heat-shock cognate protein of 70 kDa (HSC70), and transported into the lysosomes through the lysosomal membrane protein 2A (LAMP2A)[58,59]. CMA is induced by DNA damage, hypoxia and oxidative stress, among others[60-65].

Today macroautophagy is also divided into non selective autophagy and selective macroautophagy targeting special organelles or specific compounds for degradation[43,66,67]. Thus new names have appeared according to the coumpounds involved: Ribophagy (ribosomes)[68], pexophagy (peroxisomes)[69], ferritinophagy (iron-based compounds)[70] and most importantly reticulophagy (ER)[71] lipophagy (lipids)[72] and mitophagy (mitochondria)[73]. The last two are practically involved in every form of fatty liver.

Reticulophagy: Multiple receptors directly interact with LC3 and form autophagosomesduring reticulophagy, a very important form of macroautophagy thatpreserves the size and function of the ER in different conditions like starvation, non-alcoholic fatty liver disease (NAFLD), viral infections and fibrosis[74-79].

Lipophagy: Lipophagy is implicated in lipid homeostasis and metabolism in liver diseases. It is usually down-regulated in steatosis of either alcoholic or non-alcoholic liver disease[80-84], but it is up-regulated when fibrosis, cirrhosis or hepatocellular carcinoma are evolving[85-87]. Comprehensive reviews of lipophagy in liver disease were recently presented[88-91].

Mitophagy: The first step of mitophagy in mammals requires the induction of canonic Atg-dependentautophagy with either mTOR suppression induced by mitochondrial generated reactive oxygen species (ROS), or AMPK activation induced by adenosine triphosphate (ATP) depletion. The second step is the priming of the mitochondria involving molecular modifications leading to their recognition by the autopha-

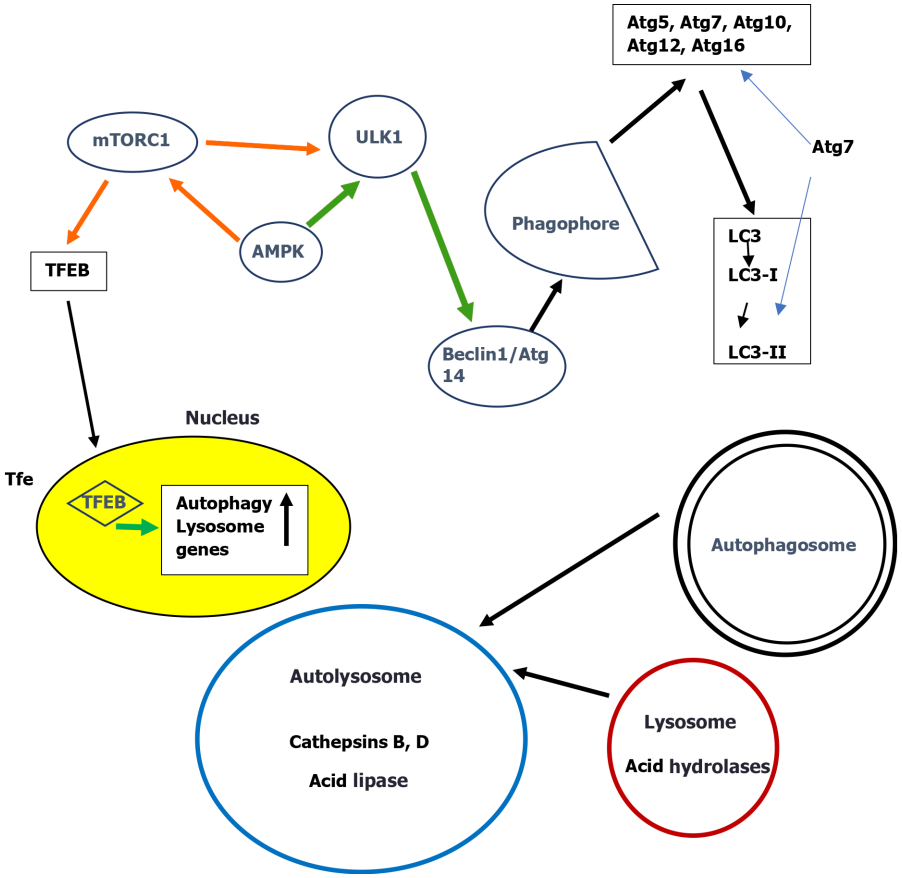

Figure 1 A simplified scheme of the macroautophagy pathways in the liver. Initiation starts with activation of the unc-51-like kinase 1 complex (ULK1, Atg1 in yeast) followed by beclin 1(Atg6 in yeast) and a subsequent cascade of Atg proteins leading to autophagosome formation where LC3 (Atg8 in yeast) is implicated. LC3 is further processed to form initially LC3-I and then LC3-II. Fusion of the autophagosomes with lysosomes form the autolysosome where acid proteases (among which cathepsins are important) and lipases degrade proteins and lipids. Initiation of autophagy is controlled by two metabolic sensors the mammalian target of rapamycin complex 1 (mTORC1) and the AMP-activated protein kinase (AMPK). mTORC1 negatively regulates autophagy inhibiting ULK1. AMPK suppresses mTORC1 activity. The long-term regulation of autophagy is carried out by transcription factor EB (TFEB), the main regulator of lysosomal biogenesis and autophagy. Under nutrient-rich conditions, mTORC1 phosphorylates TFEB and retains TFEB in the cytosol. Orange arrows: Inhibition. Green arrows: Positive regulation. For details see Ref.[21,29,31]. mTORC1: Mammalian target of rapamycin complex 1; TFEB: Transcription factor EB; ULK1: Unc-51-like kinase 1 complex.

gosomes[92,93]. Even in the healthy liver, worn out mitochondria with a half-life of 10 to 25 d are removed by mitophagy[94,95]. Elimination of aged or damaged mitochondria protect cells from release of pro-apoptotic proteins, generation of toxic ROS and non proper hydrolysis of ATP[96-99]. When oxidative stress appears, autophagy rapidly acts to remove oxidized proteins or damaged mitochondria that generate more ROS. Recent data show that in autophagy deficiency there is acummulation of ROS and p62 probably mediated by the loss ofFOXO1/3. It has been reported that the p62-FOXO1/3 axis is the molecular basis for the reduction of antioxidant defense in autophagy deficiency[100]. Three different types of mitophagy have been described based in the different molecular pathways involved[101,102]. An extensive review of molecular mechanisms of mitophagy in liver diseases has been recently published[103].

New players in liver autophagy: It is clear today that apart from the known pathways regulating liver autophagy, there are additional mechanisms involved. The most important are the long non-coding RNAs (lncRNAs), microRNAs (miRNAs) and exosomes. Many recent studies have presented strong evidence that ncRNAs influence autophagy by regulating various autophagy pathways[104-110]. Equally, miRNAs regulate autophagy influencing the core autophagy pathways[111].

Evidence from experimental animals with liver specific deletions of Atgs has demonstrated the role of High mobility group box 1 (HMGB1)[112] and Yes-associated protein (YAP)[113] in the pathological changes induced by autophagy. Nuclear receptors were also reported to control autophagy. Activation of the farnesoid X receptor (FXR), occurs during feeding and suppresses Atgs expression. On the other hand during starvation, fasting-activated nuclear receptors, the peroxisome proliferator-activated

receptor alpha (PPAR), and the cAMP response element-binding protein (CREB), induce expression of Atgs and therefore increase autophagy[114-116].

An association of autophagy with the formation and function of exosomes has also been described. Exosomes are extracellular vesicles originating from late endosomes, which do not fuse with lysosomes but are released extracellularly by exocytosis. Exosomes can either activate autophagy pathways or transfer extracellular vesicles to the lysosomes[117].The interplay between autophagy and exosome biogenesis has been recently described[118].

Most researchers have studied either the early or the late stages of autophagy. However equally important is the final stage, namely the lysosome reformation (ALR), leading to regeneration of functional lysosomes from autolysosomes. A series of proteins including clathrin, the motor protein KIF5B, and dynamin 2 are sequentially involved up to the maturation of functional lysosomes. Early lysosomes are pH-neutral but eventually they gain acidity and luminal proteins[119-122]. Accumulating evidence suggests that most, if not all, components of the molecular machinery for autophagy also mediate autophagy-independent functions. Autophagy is involved in various cell functions like endocytosis, phagocytosis, DNA repair, centrosome function, cell proliferation, cell death and immunological response including memory. Details were recently reported[123].

Autophagy and immunity: The implication of autophagy with the immune system has been investigated in the last few years[124-131]. Non-canonical forms of macroautophagy were described, resulting in the formation of autophagosomes that fuse with the lysosomes[132]. Only a subset of the Atgs machinery is used. Among these, LC3-associated phagocytosis (LAP) has been extensively studied because of its implication in immune regulation. LAP recruits LC3-II to the phagosomal membrane[133-135] and is taken up by macrophages through innate immune receptors such as Toll-like receptors. In contrast to autophagy the LAPasome is a single membrane vacuole. In contrast to autophagy, ULK1 is not required for LAP[133]. Chaperone-mediated autophagy has also attracted attention because of its central role in antigen presentation and aging[136,137]. Autophagy is also implicated in the function of innate immunity interfering with macrophage autophagy. There is interplay between autophagy and innate immunity as interferon (IFN)-γpromotes autophagy in macrophages[138]. Mice fed with high fat diet had impaired autophagy in bone marrow-derived macrophages and peritoneal macrophages[139]. Mice with Atg5 deficient macrophages, developed hepatic inflammation when stimulated with lipopolysaccharide (LPS) after a high fat diet feeding. Acquired immunity is primarily a defense function against specific pathogens and is brought about by the different subsets of T cells and B cells. Interestingly there is evidence that high autophagic activity maintains the differentiation and function of important T-cell subsets such as regulatory T (Treg)-cells[140] and γ δ T-cells[141].

Autophagy and cell death: It has been proven that autophagy can be either a protective mechanism or a contributor to cellular death in certain instances[142-144]. Autophagy is involved in cellular death mostly by its effects on apoptosis. Autophagy is connected to apoptosis and these two cellular destructive phenomena are affecting each other[145-148]. This is particularly important in hepatic cell death[149].

Generally autophagy blocks the induction of caspase-dependent apoptosis, and apoptosis-associated caspase activation stops the autophagic process. Yet, in special cases, autophagy may induce apoptosis or necrosis, and autophagy has been shown to degrade the cytoplasm, leading to 'autophagic cell death'[150-152].

Autophagy is also implicated in caspase-independent cell death, leading to necrosis and necroptosis[153]. Induction of apoptosis eliminates cells damaged through the action of the tumor suppressor gene p53[154]. Apoptosis is counteracted, among others, by the mTOR/AKT pathway also involved in autophagy. The balance between p53 and AKT/mTOR is crucial for the fate of injured cells[155,156]. In addition, autophagy induces a particular mechanism of cell death named ferroptosis. It was initially reported as a specific iron-dependent form of malignant cell death. It soon became clear that ferroptosis is a more general form of cell death[157,158]. Many proteins implicated in autophagy (like Atgs and BECN1) were also involved in ferroptosis. Moreover activators of ferroptosis, like erastin, induced autophagosome accumulation and activation of autophagy led to ferroptotic cell death possibly by the turnover of ferritin through ferritinophagy[159-161].

A recent study has shown that ferroptosis is also interconnected with lipophagy. Lipids released during lipophagy and subsequent peroxidized increase ferroptosis. Therefore it might be that ferroptosis is a mechanism of cellular death in NAFLD[162].

Autophagy and inflammation: Autophagy is also closely associated with the inflammatory response in the liver. Inflammasome and autophagy regulate each other by the same inhibitory mechanisms which however are controlled by different input pathways. The NLRP3 inflammasome activation, usually through the stimulation by pathogen- and/or danger-associated molecular patterns[163,164], induces procaspase-1 activation which promotes interleukin interleukin (IL)-1β and IL-18 production leading to pyroptotic cell death. These events are counteracted by caspase-1-mediated activation of autophagy. In addition autophagy reduces inflammasome activation degrading the inflammasomes in the autophagosomes but also eliminating damaged cytoplasmic organelles that otherwise would produce DAMPS increasing activation of inflammasomes[165,166].

On the other hand, the negative correlation between inflammasomes and autophagy[167-169] leads to an increased production of the pro-inflammatory IL-1β[170] when autophagy is decreased[128].However, the relationship between NLRP3 and autophagy has not been fully clarified, and recent studies have reported that nuclear factor-κappa beta (NF-κB) activation can modulate the NLRP3 and autophagy towards the same direction[171].

In view of the above is not surprising that many reviews on autophagy use the term "double-edged sword" stressing the fact that autophagy may have opposite effects on the same biological phenomenon[172]. Prominent general paradigms are cancer[173,174] and viral infections[175].

Another characterization pertinent to the liver is that autophagy behaves like Jekyll and Hyde depending on the cells involved. In hepatocytes, macroautophagy [in NAFLD and alcoholic liver disease (ALD)] and CMA (in NAFLD) is protective. It reduces fat accumulation and oxidative stress, it removes damaged mitochondria and favors regeneration. In macrophages, macroautophagy inhibits liver inflammation and fibrosis but it enhances fibrosis activated stellate cells. It is protective in early phases of hepatocellular carcinoma, but may be detrimental in late phases[176,177].

Autophagy in hepatocytes but also in the non-parenchymal sinusoidal cells of the liver is a key for liver physiology[178,179] and defects of autophagy are implicated in the pathophysiology of most liver diseases[180]. Both common diseases like alcoholic and non-alcoholic fatty liver or viral hepatitis and rare entities like Wilson's disease and a1 antitrypsin deficiency are related to autophagy defects[30,41,57,181-184]. Defective autophagy also leads to accumulation of detrimental hepatocyte byproducts due to the fact that hepatocytes have a long half life of 6-12 mo [143]. Moreover, the liver is responsible for handling of a large number of xenobiotics and autophagy is a cytoprotective mechanism[99,185] (Figure 2).

OBESITY, STEATOSIS AND NAFLD

NAFLD is the commonest liver disease worldwide. Recently it was suggested that it should be renamed as metabolic dysfunction-associated fatty liver disease (MAFLD)[186,187]. Pathological lesions in the liver vary from simple steatosis to non-alcoholic steatohepatitis (NASH) and cirrhosis. Current pathogenesis of NASH is mainly focused on the effects of insulin resistance and lipotoxicity in hepatocytes[188]. The abnormalities reported in Kupffer cells, stellate cells and endothelial cells are regarded as secondary events[189,190].

Obesity and insulin resistance are well documented risk factors for NAFLD development. Defects in liver autophagy have been established as fundamental abnormalities in both conditions.

Hepatic autophagy in obesity and insulin resistance

In the hepatocyte, lipids are catabolized by two major pathways. The first involves cytoplasmic neutral lipases and the second is lipophagy and acid lipases and hydrolases of the lysosomes. The end result is the production of free fatty acids that are further broken down by βI-oxidase in the mitochondria[191].

Lipid droplets have a core of lipids enwrapped in a phospholipid layer characterized by proteins called perilipins directing them to the autophagosome[72]. A crucial protein mediating lipolysis and autophagy is the adipose triglyceride lipase (ATGL). Cytoplasmic lipolysis and lipophagy are interconnected. The degradation of perilipins by autophagy facilitates actions of ATGL which in turn induces autophagy *via* sirtuin1 deacetylation of certain Atgs and activation of the transcription factors FoxO1 and FoxO3 thus promoting autophagy[192-194].

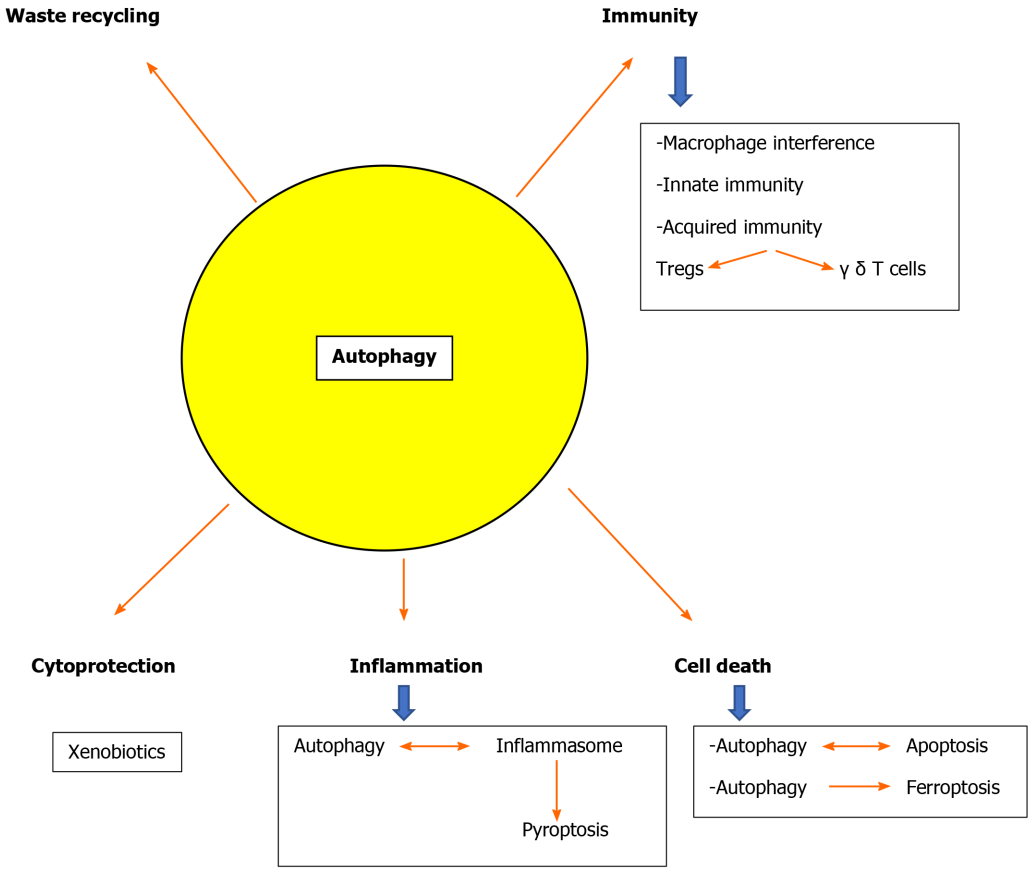

Figure 2 Implications of autophagy in critical cellular functions in the liver. For details see text.

Lipophagy can prevent lipid accumulation in hepatocytes, while the inhibition of lipophagy promotes lipid droplets (LDs) accumulation, resulting in hepatocellular steatosis[195].

Characteristic changes of the metabolic syndrome like obesity, hyperglycemia, and dyslipidemia have been shown to exert a negative effect on autophagy because the regulatory control of forkhead box O1 (FoxO1) on the expression of *Atg* genes is lost leading to autophagy malfunction[196]. Macroautophagy and CMA are also down-regulated by increased intracellulal lipids due to either interference with the lysosomal stability of the CMA receptor or to the reduction of the ability of autophagosomes to fuse with lysosomes leading to the reduction of macroautophagic flux[196-198].

The severity of steatosis is related to the expression of three proteins, the damage regulated autophagy modulator (DRAM), BAX and p53. In mice livers, p53 expression increased in mild and severe steatosis. A DRAM expression increase was observed in mild hepatosteatosis, whereas high BAX expression was identified in severe hepatosteatosis[199].

A clinical study has confirmed the link between induction of autophagy and liver steatosis[200]. Autophagy-related genes (Atg5, LC3A, and LC3B) were overexpressed in obese patients compared with non obese patients.

Experimental evidence also suggests that defective autophagy is crucial in the development of obesity, oxidative stress, and the metabolic syndrome[201-203].

Insulin is intimately involved in autophagy regulation as the mTOR inhibitor of the FoxO and TFEB controllers of the transcription of autophagic genes is insulin-inducible[204]. Overactivation of mTOR in turn leads to insulin resistance[205,206]. Several mechanisms might explain this defect in obesity. Obesity increases calpain-2 by a still unknown signal pathway. Calpain is a protease that degrades Atg7 and modulates autophagy[201]. Autophagosome-lysosome fusion is also defective in livers of obese mice due to alterations of the lipids in cellular membranes induced by the high-fat diet[198]. A defective liver autophagy and the associated decrease of lysosomal degradation contribute to an additional increase in the ER stress which leads to insulin resistance and a vicious circle is completed[201,207,208]. Hyper-insulinemia decreases liver autophagy and reduced hepatic autophagy aggravates ER stress and insulin resistance.

An additional mechanism is a defect in acidification of lysosomes. Impaired substrate degradation in autolysosomes has also been reported for obese ob/ob mice. Activities of lysomal cathepsins were implicated in obesity. Cathepsin L was decreased in obese adipose tissue, while Cathepsin B was significantly elevated. Interestingly in obese adipose tissue inflammasomes were activated and further upregulation of cathepsin B resulted in additional activation of inflammasomes[209-212].

A study of the expression of 322 lysosomal/autophagic genes was recently reported in adipose tissue of lean and obese patients. Among 35 significantly expressed genes, 34 were upregulated. In isolated murine cells, tumor necrosis factor alpha (TNFα) stimulation resulted in upregulation of lysosomal/autophagic genes accompanied by upregulation of the autophagy associated SQSTM1/p62 receptor leading to increased degradation of perilipin 1. It seems that local inflammatory cytokines may impair lipid storage *via* autophagy induction[213].

An extensive review of lysosomal enzyme abnormalities in both adipose and liver tissue was recently published[214]. A recent report suggests an additional mechanism contributing to obesity-associated abnormalities. Obesity increases lysosomal iNOS and NO production leading to exacerbation of lysosomal nitrosative stress, impairment of lysosomal function, defective autophagy and insulin resistance[215].

There is also evidence that mitophagy is negatively regulated by liver insulin resistance. Mitophagy can promote mitochondrial fatty acid oxidation to inhibit hepatic fatty acid accumulation and improve hepatic insulin resistance. Fundc1 is a recently characterized mitophagy receptor and mice lacking this receptor develop severe obesity and insulin resistance when maintained in a high-fat diet[216,217].

However, when autophagy is defective an alternative mechanism protects the liver from steatosis. An induction of fibroblast growth factor 21 (FGF21) was reported in mice with subsequent amelioration of insulin resistance and decreased diet-induced obesity[218,219]. This has been corroborated in a clinical study of overweight NAFLD patients, where increased FGF21 levels were correlated with steatosis grade, fibrosis and lobular inflammation. NASH patients had the highest levels[220]. An analogue of FGF21 has been tested in experimental animals and obese diabetic patients with promising results[221-223]. Nevertheless, the control of adipose tissue biology is very complex and is elegantly described in a recent publication[224].

NAFLD-NASH

Not surprisingly autophagy is strongly associated with NAFLD pathogenesis[179]. Diet-induced NAFLD in mice blocks hepatic autophagy and leads to oxidative stress and mitochondrial dysfunction[225], also reducing thyroid hormone-induced mitophagy[226]. The potential molecular pathways and possible therapeutic implications of thyroid hormones in NAFLD have been recently reviewed[227].

Mitophagy abnormalities are strongly implicated in NAFLD[228-230]. In particular an impairment of mitophagy seems to activate the NLRP3 inflammasome favoring the progression of NAFLD to NASH[38]. Accordingly, recent evidence indicates that restoration of mitophagy may improve NAFLD[231-234].

In addition to mitophagy, reticulophagy is also implicated in NAFLD. An extensive reticulophagic response is evident in hepatocytes after induction of NAFLD by oleic acid[228,235]. It is suggested that reticulophagy and mitophagy are independent, events involved in NAFLD progression[228].

Impaired lipophagy and lipotoxicity are also strongly involved in NAFLD[72,192,236,237]. Lipid accumulation in hepatocytes blocks autophagic flux and impaired autophagic flux favors the progress of NAFLD[30].

This impaired flux and the subsequent ER stress can be improved by inhibition of the sterol regulatory element-binding protein 2 (SREBP-2) whose activation promotes accumulation of cholesterol in NAFLD. This improvement is associated with upregulation of autophagy genes[238].

Intracellular lipid trafficking is also regulated by store operated calcium entry and enhanced lipophagy is observed in cells defective in this system[239]. Moreover, the detrimental effects of diets rich in saturated FFA were increased bysirtuin-3, which enhanced lipotoxicity, reducing the autophagic flux[240]. The effect of lipophagy in liver steatosis is further supported by experimental evidence that various chemicals are involved in steatosis by interfering with autophagy. Caffeine reduces lipid content and stimulates beta-oxidation in hepatocytes through autophagy in mammalian liver cells in NAFLD[17]. In essence caffeine protects against fatty liver through the co-ordination of the induced lipophagy and mitochondrial β-oxidation[241,242]. Epidemiologic studies

demonstrated that coffee consumption reduced the development of fatty liver, fibrosis, and hepatocellular carcinoma in NAFLD patients[243,244] supporting thus the experimental evidence.

Methionine is a well known inactivator of autophagy and lipophagy. The correlation between lipophagy and methionine in the liver from patients with liver steatosis has been studied. Increased levels of methionine inhibit autophagic catabolism of lipids and contribute to liver steatosis in NAFLD[83]. Mice fed with a methionine/choline deficient diet developed steatosis, inflammation, fibrosis and ER stress associated with mitochondrial dysfunction. The administration of the autophagy enhancer rapamycin ameliorated these lesions while chloroquine, a well established autophagy inhibitor, aggravated the liver injury[245]. Resveratrol, another autophagy activator, also attenuated liver lesions induced by a similar diet[246,247]. Consistent with these findings is a recent report that a traditional Chinese herb increased autophagy and considerably improved steatohepatitis induced by methionine/choline deficient diet in rats[248].

Other diet-supplied molecules affect autophagy and are possibly beneficial in NAFLD including the purple sweet potato color[249]. Likewise, the caffeic acid of vegetables has been reported to ameliorate hepatic steatosis[250] while curcumin, an antioxidant polyphenol of Curcuma longa, has been shown to inhibit apoptosis and induce autophagy with a potential protective effect on hepatocellular carcinoma[251].

A finding that might be useful in future treatment of NAFLD was recently reported. Celecoxib, a COX-2 inhibitor, attenuated steatosis and restored autophagic flux in cells treated with palmitate and rats fed a high fat diet[252].

Other lipids like the sphingolipid ceramide may be implicated in NAFLD as it is increased in Atg7 knockout mouse liver in parallel with the impaired autophagy[253]. Autophagy increased when sphingolipid de novo synthesis was upregulated, indicating that lipid degradation was activated to prevent excessive sphingolipid accumulation.

Interestingly, autophagic activity seems to be upregulated when the renin angiotensin system is overexpressed. The underlying mechanisms and its role in NAFLD have yet to be clarified as there are many controversial issues to be solved[254]. Overall there is extensive evidence that inhibition of lipophagy is detrimental for the liver in NAFLD[198,222,238,255].

Summarizing the above studies, a therapeutic approach against NAFLD would be the activation of lipophagy[90]. However, it is noteworthy that there is one study indicating the opposite, as suppression of autophagy through inhibition of c-Jun N-terminal Kinase (JNK) ameliorates insulin resistance in a rat NAFLD model[256].

Extensive reviews on the mechanisms of autophagy deregulation in NAFLD were recently published[183,257,258]. Not only impaired macroautophagy but also reduced liver chaperon mediated autophagy (CMA) favors steatosis due to failure in the timely removal of perilipins[259,260] and therefore an increase in lipogenic enzymes. When oxidative stress is increased in the liver, an upregulation of CMA occurs to selectively remove damaged proteins[62]. Loss of CMA leads to impairment of proteostasis and accumulation of oxidized protein aggregates perpetuating thus chronic oxidative stress[261].

Autophagy and NASH

Involvement of autophagy in the progression of NAFLD to NASH has not yet been clarified and molecular mechanisms are not fully understood.

One of the histological characteristics of NASH used in diagnosis and scoring systems is the formation of Mallory-Denk bodies (MDB)[262-264]. There is experimental evidence that inhibition of autophagy and accumulation of p62 is related to their formation while autophagy activation with rapamycin leads to their resolution[265]. Further support of the involvement of autophagy in NAFLD evolution to NASH was reported in a clinical and experimental study where a decrease of autophagic flux in parallel with an increase in ER stress was demonstrated both in the livers from NAFLD patients and mice models of NAFLD, and in lipid-overloaded human hepatocytes[266]. However tests for measurements of autophagic flux used in this paper are not full-proof as they can be influenced by autophagy independent factors. Therefore these findings should be corroborated in a different set up.

Patients with NASH and murine models of steatotic inflammation had reduced expression of Atg7 and TFEB while the autophagy inhibitor rubicon was increased[139,177,255].

In contrast, steatosis and liver injury were improved in parallel with restoration of autophagy and reduction of ER stress in mice with a deletion of the Rubicon or adenoviral delivery of Atg7[202,251]. Recent evidence also indicates that impaired

mitophagy may contribute to liver injury during progression of NAFLD and formation of megamitochondria[229].

Transition of NAFLD to NASH also implicates Kupffer cells. These cells, constitute 80%-90% of tissue macrophages in the body and are critical cells in liver inflammation[20]. They are the main site of NLRP3 inflammasome activation and production of the pro-inflammatory cytokines compared to hepatocytes and stellate cells[267,268]. Activation of the NLRP3 inflammasome plays an important role in the transition from NAFLD to NASH[269].

An earlier report demonstrated that cathepsin B, a lysosomalcysteine protease, is released in the cytosol in response to FFAs and that this redistribution of cathepsin B is present in the liver of patients with NAFLD related to disease severity. Importantly in a dietary mouse model of NAFLD, inhibition of Cath B significantly decreased steatosis, liver inflammation and insulin resistance[270].

These findings were recently elaborated in more detail as it was reported that cathepsin B and activation of the NLRP3 inflammasome are interconnected in a murine model of NASH but also in isolated Kupffer cells stimulated with palmitate. Expression of cathepsin B and activation of NLRP3 inflammasome were increase in NASH animals. Moreover, an inhibition of Cathepsin B decreased liver inflammation, ballooning, and the pro-inflammatory cytokines IL-1β and IL-18. *In vitro* stimulation of Kupffer cells showed identical results in inflammasome activation, expression of Cath.B and cytokine production before and after Cath.B inhibition. These results indicate that NASH pathogenesis probably depends in part to inflammasome activation which in turn is regulated by the activity of aprotease tightly connected to autophagy[271].

Additional supporting evidence for the role of autophagy in NASH pathogenesis is the fact that impaired autophagy in obese mice is critical for macrophage polarization. M2 macrophage polarization relies on energy provided by FFA oxidation, suggesting a potential implication of autophagy in this process. Macrophages change to a pro-inflammatory phenotype due to both increased M1 and decreased M2 polarization[132] with a resultant upregulation of liver inflammation, a prominent feature of NASH.

The situation is controversial when adipose tissue macrophages from obese mice are concerned. Increased rather than decreased autophagy of macrophages has been demonstrated in adipose tissue[272,273]. Another cathepsin mostly found in Kupffer cells seems to be implicated in NASH. Lysosomal cholesterol accumulation inside murine Kupffer cells leads to increased liver Cathepsin D activity which is related to liver inflammation[274]. Kupffer cell cathepsin D may therefore be an additional key player in hepatic inflammation of NASH[275]. The impairment of macrophage autophagy with aging may explain in part the increased prevalence of the metabolic syndrome and steatohepatitis of older age in humans[276,277].

The oxidative stress is also involved in the progression to NASH. Hepatocytes exposed to palmitate concentrations similar to those found in patients with the metabolic syndrome and NAFLD showed mitochondrial membrane permeabilization and production of ROS. Similarly, an inhibition of Cathepsin B ameliorated mitochondrial dysfunction and oxidative stress, indicating an additional mechanism of NASH progression[229,278].

Under normal conditions, damaged mitochondria are removed through mitophagy. In certain cases of NAFLD however mitophagy is defective and the oxidation of biomolecules by mitochondrial ROS starts a vicious cycle of increasing mitochondrial dysfunction and aggravation of hepatocellular oxidative damage. This ultimately leads to hepatic inflammation and liver failure[279,280], since impaired mitophagy triggers liver NLRP3 inflammasome activation *in vivo* and *in vitro* in isolated murine hepatocytes[38].

Impairment of autophagy in other liver sinusoidal cells may also participate in the progression of NAFLD to NASH. Decreased autophagy has been observed in the liver endothelial cells of patients with NASH or in mice with endothelial deletion of Atg5 and features of inflammation[180,190,281]. A very recent study has convincingly shown that impaired autophagy of liver endothelial cells (LSECs) occurs in NASH patients but not in simple steatosis. Deficiency in autophagy in LSECs induces endothelial inflammation ultimately leading to liver inflammation and fibrosis. This defective autophagy, in part due to inflammatory mediators of the portal blood, might well be one of the missing links of the progression of simple steatosis to NASH and cirrhosis[282].

A further mechanisms leading to NASH involves multivesicular bodies (MVBs), a form of endosomes, whose contents are transported into lysosomes[283]. The MVB-lysosomal pathway was shown to participate in the development of steatohepatitis through lysosomal degradation of Toll-like receptor 4 reported to be critical for the progression of NASH[284].

Finally a role of the chemokine CXCL10 in the development of steatohepatitis has been proposed. Upregulation of CXCL10 impairs autophagic flux decreasing thus autolysosome formation. Autophagic protein degradation is inhibited followed by the accumulation of ubiquitinated proteins with ultimate development of steatohepatitis[285].

ALD

The liver is the organ mostly responsible for ethanol metabolism. Oxidation of ethanol happens through three pathways namely alcohol dehydrogenase in the cytosol, cytochrome P450 (CYP2E1) in the ER and microsomes and the enzyme catalase in peroxisomes[286]. Ethanol oxidation also produces ROS, including superoxide anion, and hydroxyl radicals that may damage hepatocytes[287].

Ethanol induces autophagosome formation in the liver. Reduction of autophagy results in the accumulation of lipid droplets and apoptosis of hepatocytes[288]. On the other hand activation of autophagy by rapamycin attenuates steatosis and injury induced by a combination of ethanol and lipopolysaccharide[289].

Induction of autophagy by acute ethanol exposure is mediated through many mechanisms Ethanol-induced autophagy requires ethanol oxidation to acetaldehyde and ROS generation[290,291]. ROS activates autophagy by suppressing mTOR and proteasome activity[292,293] and inactivation of Atg4[294].

Oxidants differentially influence the activities of the proteasome (the other major pathway of protein degradation.) Proteasomes are reduced when autophagosomes are increased[295]. Proteasome inhibition further triggers ER stress activates autophagy through JNK activation. Ethanol may also suppress Akt and mTOR through the upregulation of PTEN[296,297]. Metals, like zinc, are also implicated in autophagy alterations after ethanol treatment[298].

A caution should be exercised on CYP2E1 ethanol oxidation as oxidative products resulting from the expression of CYP2E1 may in fact impair autophagy leading to lipid accumulation in the liver. In cells expressing CYP2E1, hepatocyte lipids and generation of ROS were increased by an inhibitor of autophagy and decreased when a stimulator of autophagy was used[299]. Similar results were found after acute alcohol in CYP2E1 knockout mice[291]. These findings also support the idea that autophagy protects against ethanol/CYP2E1-dependent hepatic injury.

It has also been shown that hepatic autophagy depends on the level of acetaldehyde produced during ethanol metabolism. Mice expressing the ALDH2 isoenzyme, clear acetaldehyde more rapidly and have increased autophagy and lower levels of hepatic triglycerides[300]. Cannabinoid receptor 2 can also induce macrophage autophagy to protect from alcoholic liver damage[301].

It should be stresses however that acute and chronic ethanol exposure may have different effects in liver autophagy[302]. Increased autophagosome formation and autophagy flux were shown in cultured hepatocytes after short term incubation with ethanol or in livers of mice after acute alcohol administration[288,302]. Enhanced autophagy parallel a higher hepatocyte nuclear content of TFEB, the main transcriptional regulator of genes involved in lysosome biogenesis[49,50].

Alcohol also has an effect on the transcription factor forkhead box O3a (FoxO3a) that modulates liver autophagy[303]. The activity of FoxO3a is largely controlled by multiple post-transcriptional modifications, including phosphorylation and acetylation[304]. Acute ethanol exposure increases nuclear translocation of FoxO3a inducing its dephosphorylation and acetylation.

However, results are not uniform for the chronic ethanol effect. Chronic ethanol administration (Lieber-DeCarli model) for 4 wk or 10 wk increased autophagosome numbers in murine livers, suggesting the induction of autophagy[305]. In another similar murine model, mice were given gradually increasing ethanol ethanol concentrations for 10 d and autophagic flux was reduced[302].

The discrepancy seems to be solved by the report that autophagy response was dependent on the alcohol concentration used. In a murine model on Lieber-DeCarli diet with different levels of alcohol for 4 wk, autophagy is increased by a lower dose of alcohol (29% of the caloric need), but decreased by a higher dose (36% of the caloric need). Liver injury was aggravated by further reduction of autophagy and attenuated by autophagy activation[306].

Earlier studies have also demonstrated that chronic alcohol exposure disrupts lysosome function[307]. Overall results have demonstrated that autophagy is suppressed in chronic alcohol consumption due to either the defect of lysosomal function and

biogenesis from TFEB suppression[302,308] or to a reduction in AMPK activity and inhibition of autophagosome formation[309,310].

After ethanol-induced reduction of autophagy, there is accumulation of aggregated proteins and SQSTM1/p62, leading to activation of nuclear factor erythroid 2-related factor 2 (Nrf2) and damage to the mitochondria and cell death[309,311].

How the other autophagy-related transcriptional factors, such as TFEB and farnesoid X receptor (FXR) are interconnected with FoxO3a in the expression of autophagy genes is unknown. Moreover, how ROS generation in acute or chronic alcoholic condition systematically affects the mTORC1 activation or TFEB translocation is unclear.

Autophagy is also protective against CYP2E1-dependent liver lesions in a chronically ethanol-fed murine model[312]. Autophagy in ALD can be further affected by additional factors identified in various experimental models. Augmenter of liver regeneration (ALR) is a factor that can promote liver growth. It was reported to protect mice from ethanol-induced liver injury through inhibition of mTOR and therefore activation of autophagy[313]. Moreover an interesting recent study used many genetic models of autophagy impairment, with different functional levels and different alcohol regimens. Deficiencies of either Atg7 or Atg5 demonstrated variable responses to ethanol feeding according to the timing of autophagy dysfunction, the gene being affected, and the alcohol scheme used[314].

It should be stressed that in acute alcohol administration, ethanol-induced autophagy may protect the liver by three basic mechanisms namely mitophagy[80,102,315,316], lipophagy[72,293,317] and clearance of Mallory-Denk bodies by proteophagy[265,318,319].

However, chronic alcohol exposure impairs autophagy and lipophagy[308,320] most likely due to the activation of mTOR signaling and a decrease in lysosomal biogenesis. Administration of the mTOR inhibitor Torin- 1 restores lysosomal biogenesis and attenuates liver lesions[308]. An additional pathway through which chronic alcohol exposure could reduce liver autophagy is the inactivation of the guanosine triphosphateRab7 and reduction of dynamin 2 activity leading to depletion of lysosomes and inhibition of hepatocyte lipophagy[320,321].

Ethanol Induced steatosis activates mitophagy by elevating PINK1 expression on mitochondria[305]. PINK1-dependent mitophagy was correlated with the mitochondrial expression of Parkin and the level of an indicator of oxidative mtDNA damage[322-325]. Mitophagy has a dominant role in protection of the hepatocyte from alcohol-induced hepatic injury as evidenced by a report that enhancement of mitophagy by quercetin, a natural flavonoid, attenuated ethanol-induced mitochondrial damage[326].

Regulation of mitophagy is related to three receptors namelyFUN14 domain containing 1 (FUNDC1), BCL2 interacting protein 3 (Bnip3), and Parkin[327].

DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is a newly described housekeeper of liver mitochondrial fission. DNA-PKcs is overexpressed in murine livers after exposure to ethanol and was positively correlated with steatosis, mitochondrial damage andfibrosis. On the other hand this over expression repressed FUNDC1-required mitophagy[328].

An additional significant point is the effect that ethanol might have on the different sinusoidal cell subpopulations. There is strong evidence that autophagy in macrophages is crucial to protect the liver from ethanol-induced damage. Investigations were mostly performed in macrophage specific deletions of either Atg7 or Atg5. The cannabinoid CB2 receptors of macrophages were found to have a protective rolein ALD, which was abrogated by Atg5-deletion in macrophages[301]. Increased mortality in Atg5 deleted mice was also demonstrated after chronic ethanol feeding plus LPS challenge[329]. Similar findings were reported after Atg7 deletion[330]. Both studies demonstrated an activation of the inflammasome and an augmented IL-1 production.

In contrast to hepatocytes and macrophages the effect of autophagy in hepatic stellate cells after ethanol exposure has not been clarified. A recent study in immortalized rat stellate cells demonstrated that autophagy could contribute to ethanol-induced stellate cell activation[331]. Induction of fibrosis by alcohol in current murine models is not feasible unless accompanied by steatosis induced by a high-fat diet[332].

Most autophagy studies in ALD are focused on the involvement of macroautophagy. Recent evidence however indicates that CMA is also important in alcoholic liver disease through the CMA negative regulator sorting nexin 10 (snx10). Snx10 knockout mice fed with Lieber-DeCarli diet were resistant to alcohol-induced liver injury associated with an increase of lysosome-associated membrane protein 2A (LAMP2A) and CMA activation through inhibition of the enzyme Cathepsin A which

is responsible for LAMP2A degradation[333]. Deficiency therefore of a CMA negative regulator, protects animals from ALD. Deficiency of another CMA negative regulator, Lipocaline-2 (LCN2), also maintains hepatic CMA activity in murine livers after chronic alcohol administration[334] verifying the idea that impaired CMA may be responsible at least in part in alcohol-induced liver injury.

Involvement of miRNAs is an additional factor in the regulation of autophagy in ALD that has emerged from recent evidence. Several miRNAs were reported to alter autophagy and alcoholic steatosis[335]. miR-26a ameliorates alcohol-induced acute liver injury by two MAPKs inhibitors thus inducing Beclin-1 expression and autophagy[335]. Another report provided evidence that miR-155 is a mediator of alcohol-related exosome production and autophagy impairment in both hepatocytes and macrophages[336]. Deletion of miR-155 protected mice from alcoholic steatosis and inflammation. Interestingly in this study serum levels of exosomes were increased in ALD patients and alcohol exposed mice, whereas miR-155 deficient mice had significantly reduced exosome release from both hepatocytes and Kupffer cells. It was suggested therefore that autophagy is an atypical promoter of exosome release in ALD.

Clinically important observations indicate that withdrawal of ethanol from ethanol-fed rats resolves steatosis[337] suggesting that removal of ethanol oxidation and restoration of lipophagy may be the mechanism of steatosis resolution observed in humans after ethanol abstinence[338,339]. Informative reviews of autophagy in ALD were recently published[90,181,182,340-342].

In view of the fundamental role of lipophagy in the pathogenesis of ALD, it is not surprising that pharmacological inducers of lipophagy like carvamazepine, rapamycin, resveratrol and simvastatin were tested in alcohol-fed animals with a resultant attenuation of liver lesions. By contrast chloroquine exacerbated hepatic steatosis[312,343,344]. Recently plant-derived agents were also used to activate lipophagy. Thus, corosolic acid[345], quercetin[346] and Salvianolic acid A[347] all had a favorable result on alcohol-induced liver lesions activating lipophagy through different pathways.

Summarizing, it is evident that whether ethanol causes an increase or decrease of autophagy depends on the duration of ethanol consumption/exposure, the amount of alcohol given, and the manner in which it is administered[290,302]. Moreover, lipophagy and mitophagy cannot act as defensive mechanisms in the long term as they do in acute ethanol consumption as they are inhibited by chronic alcohol exposure[102,348].

VIRAL HEPATITIS

In the past decade, hepatic autophagy has been implicated in viral infection with either hepatitis B (HBV) or hepatitis C (HCV).

HBV

Recent studies have shown that autophagy is involved in the life cycle of Hepatitis B. Inhibition of autophagosome formation could reduce HBV production, while stimulation of autophagy could significantly contribute to HBV production[349,350].

However, the mechanism by which HBV activates autophagy is not clear. Previous reports have implicated either the HBx[351,352] the large HBsAg protein[353] or a mutant with a deletion in the preS2 region[354,355] as inducers of ER stress which in turn increases autophagy.

In contrast it was shown that HBx does not play a significant role in the induction of autophagy compared to the small HBsAg protein also increasing autophagy *via* the induction of ER stress. An HBV genome unable to express small HBsAg does not activate autophagy[356]. To reconcile the discrepancy, it has been suggested that autophagy can be stimulated both by HBx and the small surface HBsAg protein through upregulation of beclin-1 expression[357,358]. In addition HBx induces autophagy through its effect on the cytoplasmic high-mobility group box 1 (HMGB1), identified as a a positive regulator of autophagy. HBx binds to HMGB1 and triggers autophagy in hepatocytes[359]. This observation may be clinically relevant. Spontaneous and induced autophagy of peripheral Treg cells from 98 patients with chronic hepatitis B were assessed[360]. No difference of spontaneous autophagy was found between patients and normal controls but induced autophagy was significantly higher in patients. It was also related to HMGB1 as it was significantly decreased when HMGB1 was blocked with a neutralizing antibody.

HBx further impairs lysosomal acidification with a final result the accumulation of immature lysosomes. Moreover immature lysosomal hydrolase cathepsin D was

shown in human liver tissues with chronic HBV infection suggesting that a repressive effect of HBx on lysosomes may be responsible for the inhibition of autophagic degradation[350]. Interestingly, although HBV could impair lysosomal acidification it was unable to induce autophagic protein degradation, due to the inability of HBV to increase the sequestration of proteins destined for degradation by autophagy[350]. Therefore, it is usually stated that HBV induces incomplete autophagy. In addition, it was clearly shown that HBV specifically targets damaged mitochondria and mitophagy. Either the whole HBV genome or HBx alone were able to induce Parkin-mediated mitophagy[361,362]. In addition, HBx-induced autophagy inhibited mitochondrial apoptosis increasing the survival of HBV DNA-transfected cells[349]. Another clinically important observation is that different HBV genotypes have a variant effect on autophagy. HBV genotype C was a more potent inducer of autophagy than HBV genotype B. HBV-C is associated with more severe disease than HBV-B but however attractive such an association between autophagy and severity of liver disease may be, it has to be verified[363,364].

It is important to realize that many viruses, including HBV, have developed strategies to hijack autophagy to benefit their replication and dissemination[356,365,366]. So far, HBV is the only DNA virus known to exploit autophagy for its own replication as it is RNA, but not DNA viruses, that commonly use autophagic function to promote replication[367].

HBV infection induced the early-stage formation of autophagic vacuoles increasing the PI(3)K enzyme activity to promote HBV DNA replication. HBx can directly bind and activate the PI3KC3 complex[368,369]. Ablation of Atg5 has been shown to inhibit autophagy and impair nuclear localization of the HBV core protein. HBV DNA level in sera was decreased by more than 90% accompanied by practically undetectable levels of the HBV DNA replicative intermediate in the liver[370].

Autophagy was responsible for the degradation of an oncogenic microRNA-224 in the liver of HBV patients with hepatocellular carcinoma (HCC) and HBx-transgenic mice. In HCC patients, the combination of low-Atg5 expression and high miR-224, was significantly correlated with a poor overall survival rate[371]. The list of the mechanisms used by HBV to subvert autophagy and the detrimental consequences in the liver is by no means complete as new factors are constantly reported including release of pro-inflammatory cytokines and chemokines and inhibition of neutrophil extracellular trap[372-375].

Further evidence of autophagy subversion by HBV was recently reported. In HBV-replicating hepatocyte cultures, the silencing of Atg5, Atg12, and Atg16L1, interfered with viral core/nucleocapsid (NC) formation/stability and significantly reduced virus yields. It was further demonstrated that a covalent conjugation of Atg12 to Atg5 was essential for HBV replication. In addition the virus required Atg10 and Atg3 which are necessary for Atg5-12 conjugation. Deletion of Atg10 and Atg3 decreased HBV yields, while Atg3 overexpression increased virus production. HBV was associated with the Atg5-12/16L1 *via* interaction of HBV core protein with the Atg12 unit of the complex. Subsequent autophagosome maturation events were not necessary for HBV replication. These data indicate that HBV subverts early, non degradative autophagy components avoiding thus autophagosomal destruction[178,376,377].

Death receptors of TNFSF10 (tumor necrosis factor superfamily member 10) participate in the immune defense against several viruses by promoting apoptosis. HBx impairs TNFSF10 receptor signaling through autophagy mediated lysosomal and not proteasomal degradation. Importantly a significant reduction of the protein TNFRSF10B was demonstrated not only in cell lines but also in the liver of chronic HBV patients[378].

It was very recently reported that the hepatitis D virus also utilizes autophagy to assist its life cycle as it increases autophagosome accumulation and impairs autophagic flux. Both the small HDAg and large HDAg proteins are capable to disturb the autophagy machinery, in particular the proteins Atg7, Atg5, and LC3 involved in the early elongation stage of autophagy. Unexpectedly, deletion of Atg5 and Atg7 reduced the intracellular HDV RNA level in hepatocyte cell lines without an effect on HDV secretion[379]. Reviews of autophagy in HBV have recently been published[366,380].

HCV

Reported data have shown that HCV could induce autophagy to support its own replication[381,382]. Several mechanisms for HCV induction of autophagy have been investigated using hepatocyte cell lines[383,384]. HCV infection initiates the formation of phagophores after induction of the localization of Atg5 to the ER. Phagophores fuse to form autophagosomes. HCV-induced autophagosomes were further reported to be required for viral RNA replication as the autophagosomal membrane provided a

platform containing HCV NS5A, NS5B, and viral RNA for replication[385-387] but subsequently HCV blocks the fusion of autophagosomes and lysosomes through Rubicon overexpression. As a result autophagosomes accumulate and HCV RNA replication and assembly of infectious virions[385,388,389,390,391] are supported.

However, several studies have contradicted the need for co-localization of viral proteins in the autophagosomal membrane suggesting that this is not a necessity for viral replication[392-395].

Autophagy favors HCV replication with an additional mechanism. The entire autophagic process may be manipulated leading to the suppression of the HCV associated innate antiviral response[393,396]. After silencing different Atgs, HCV viral infectivity was suppressed in parallel with an upregulation of interferon-stimulated gene expression[390]. Moreover, HCV seems to activate autophagy to degrade the tumor necrosis factor receptor -associated factor 6 (TRAF6), thus subverting innate host immunity[389,397-399]. HCV induced unfolded protein response strongly activates autophagy to sustain viral replication through inhibition of cellular apoptosis[396]. Different HCV genotypes may have variable influence on autophagy[391,400].

HCV was also found to selectively activate lipophagy to counteract the HCV induced lipid abnormalities. This may be clinically important as the levels of autophagy in the liver of chronic HCV patients were inversely correlated to steatosis[401]. Inhibition of autophagic degradation of lipophagy may account for the characteristic occurrence of hepatic steatosis in chronic HCV infection. Mitophagy is also selectively activated *via* the PINK1–Parkin axis in infected cells, thereby promoting HCV viral RNA replication[361,402]. Virus-activated mitophagy further attenuates apoptosis and favors persistent viral infection[403]. In agreement with this finding, the viral non-structural protein 5A (NS5A) was shown to disrupt mitochondrial dynamics, thus increasing ROS production and mitophagy[404].

On the other hand, the viral core protein interacts with Parkin inhibiting its translocation to mitochondria. Mitophagy is suppressed and mitochondrial injury of infected hepatocytes is sustained and viral persistence is maintained[405].

Syntaxin 17 is an autophagosomal protein required for the fusion of autophagosomes with lysosomes and also the release of HCV. The amount of syntaxin 17 was reduced in HCV-replicating cells indicating that HCV impairs the late stages of autophagy affecting the equilibrium between the release and the lysososomal degradation of viral particles[406].

Recently CMA was also demonstrated to be activated by HCV leading to degradation of IFN-alpha receptor-1[407]. Moreover the HCV NS5A was found to interact with Hsc70, recruiting Hsc70 to hepatocyte nuclear factor 1 alpha thus targeting HNF-1α for CMA degradation[408]. Taken together these studies indicate that HCV induced CMA also facilitate HCV replication.

However, an opposite less permissive effect of the manipulation of autophagy by HCV has been suggested as a result of recent studies. Atg10 is critical for autophagy as it promotes the Atg5-Atg12 complex formation. Two isoforms of the Atg10 protein were described, namely Atg10 (a longer one) and Atg10S. They have a similar amino acid sequence except for an absence of a 36-amino acid fragment in Atg10S. Yet they differ in their effects on HCV genome replication. Atg10 with deleted or mutated two cysteins, (Cys^{44} and Cys^{135}) could trigger the expression of anti-HCV immunological genes combating the HCV replication[409,410].

Taken together these results indicate that autophagy is required for initiation of the HCV replicative phase but not for further replication[393]. However this might not be entirely true, as chloroquine an inhibitor of lysosomal acidification inhibits HCV replication offering an additional evidence for the permissive role of autophagy in HCV infectivity in the late phase[411].

Autophagy may additionaly be involved in HCV replication through the regulation of the exosomal pathway[390] and apolipoprotein transport[412], both critical steps in the egress of the HCV virion. The virion is associated to apolipoprotein E (ApoE) and its infectivity is enhanced. Autophagy has a central role in the trafficking of ApoE in HCV-infected cells leading to partial autophagic degradation of ApoE, but also to the interaction between ApoE and the viral protein E2 to increase the production of infectious viral particles[412].Molecular details of how HCV is using autophagy to its own advantage were recently published[380,413].

In summary, the life cycles of HBV and HCV in liver cells can be subdivided into 7 steps: Endocytosis, uncoating, genome replication, translation, envelopment, assembly and release. Both HBV and HCV drive autophagy largely by the ER stress response resulting from uncontrolled translation of viral proteins[414-416]. In addition HBx modulates autophagy for the benefit of HBV replication[357], while multiple HCV proteins including p7, NS3/4A and NS4B, modulate autophagy by direct or indirect

association with moieties of the early autophagy machinery in favor of its replication[417-419]. Pharmacological or genetic manipulation of autophagy may limit the viral yield[183,369,420], making autophagy a feasible target for HBV and HCV treatment.

FIBROSIS-CIRRHOSIS

The liver responds to practically any insult with only a limited number of pathological lesions: Hepatitis (hepatocyte death), cholestasis, fibrosis-cirrhosis or a combination of the three. Autophagy participates in all liver pathological responses.

Liver fibrosis is a complex and dynamic cellular process implicated in the evolution of the majority of chronic liver disease towards cirrhosis. Most review articles have broadly concentrated on the role of autophagy in liver diseases, with restricted information on cell types implicated in liver fibrosis. Not unexpectedly, most research has focused on hepatic stellate cells (HSCs) and myofibroblasts, because they are the central elements in extracellular matrix production[421]. However, other liver cells, including hepatocytes, macrophages, sinusoidal endothelial cells (LSECs), infiltrating immune cells and the so-called ductular reaction (DR) are also important[422,423]. DR significantly correlates with the degree of fibrosis and involves cholangiocyte-like cells that dominate an interplay of extracellular matrix and inflammatory infiltrate[424-427].

HSC and autophagy

The fundamental event in fibrosis is the transformation of hepatic stellate cells into myofibroblasts and this is closely related to autophagy. Typical autophagosomes that contained LDs were found in cultured HSCs indicating a connection of liver fibrosis and lipid autophagy[428]. Increasing evidence supports the notion that inhibition of lipophagy in hepatocytes reduces HSC activation and fibrosis progression[429,430]. Inhibition of the activation of HSCs and the formation of autophagosomes have been reported and these seem to be connected with the downregulation of transforming growth factor beta 1/Smads pathway as an increase in TGFb/Smad3 Leads the transcription of Beclin-1, which is a critical player in the autophagy process[431-433].

In rat-derived HSCs, cytoplasmic LDs are degraded followed by fibrogenic genes expression. Moreover induced lipid accumulation by an alkaloid, was associated with quiescent HSCs due to autophagy blockade[434]. Inhibition of autophagy by chloroquine improved CCl4-induced liver fibrosis affecting the activation of hepatic stellate cells as expected[435]. On the other hand, dihydroceramide an inhibitor of autophagy promoted the progression of liver steatosis to fibrosis[436]. Similarly, inhibition of YAP degradation also led to liver fibrosis[113].

In addition, it has been suggested that the IL-17A/STAT3 signaling pathway is important in the evolution of liver fibrosis through suppression of hepatocellular autophagy since neutralization of IL-17A promotes the resolution of experimental fibrosis[437].

Based therefore on current evidence, it has been stated that autophagy at least in murine hepatocytes is a selective survival mechanism through clearance of excessive fat leading to attenuation of lipotoxicity[438]. This is certainly not the case for HSCs autophagy where lipid droplets are digested to supply energy for the activation of HSCs, promoting thus liver fibrosis. Non specific inhibition of stellate cell autophagy or specific inhibition of Atg5 or Atg7, blocked HSCs activation[439-441]. Lipophagy in HSCs is induced by ER stress[442] and is mediated through Rab25 in a ROS dependent manner as antioxidants were effective in stopping autophagy[87]. In agreement with experimental data, clinical research found that cirrhotic patients had significantly increased levels of several autophagy- related genes compared with non cirrhotics accompanied by increased maturation of lysosomal cathepsin D[85]. Furthermore, serum lipids were evaluated in patients with cirrhosis of viral etiology and compared to non cirrhotics. Low serum lipids were found in HCV and HBV cirrhosis which were negatively correlated with lipophagy[443].

Micro-RNAs interfere with the activation of stellate cells. miR-16 inhibits the expression of guanine nucleotide-binding -subunit 12 (G12) which is overexpressed during fibrogenesis and facilitates Atg12-5 formation, thus activating stellate cells[444]. Also miR-181-5p transferred to mouse HSCs *via* exosomes from engineered adipose derived stem cells led to inhibition of fibrosis[445].

Several signals can induce autophagy in HSCs[180], including hypoxia-inducible factor-1alpha[446], transforming growth factor 1[447], as well as the danger-associated pattern molecule high-mobility group box-1 (HMGB-1)[448]. Additional signals like ROS-JNK1/2 and the XBP1 arm of the Unfolded Protein Response have also been

identified as necessary requirements of HSCs activation through autophagy[449,450]. TGF-β1 has also been reported to mediate autophagy[440]. Similarly, HSCs in cell culture with depleted Atg2A fail to spontaneously trans-differentiate[451]. Quercetin attenuated hepatic fibrosis in mice through inhibition of hepatic HSC activation and autophagy[452].

Selective activation of mitophagy in HSCs also favors fibrosis. PM2.5 is an air pollutant that activates HSCs and initiates liver fibrosis. This is due to increased ROS production and induction of mitophagy through activation of the Pink1/Parkin pathway[453]. In contrast, inhibition of mitophagy was shown to promote inflammation[454] due to dissemination of inflammatory signals from HSCs production of inflammatory cytokines[455]. However very recently it was reported that selective inhibition of mitophagy in macrophages attenuates fibrosis. Mice Kupffer cells from CCL4-induced acute injury showed increased ROS production, activated mitophagy and increased TGF-β1 secretion. T-cell immunoglobulin domain and mucin domain-4 (TIM-4) interference in Kupffer cells inhibited Akt1-mediated ROS production and decreased mitophagy and TGF-β1 secretion through suppression of PINK1/Parkin, to ameliorate CCl4-induced hepatic fibrosis[456]. Seemingly in disagreement with this notion, is the finding that the autophagic proteinp62/SQSTM1, a negative controller of HSC activation is downregulated in trans-differentiating HSCs associated with hepatocellular carcinoma. P62 ablation increases fibrogenesis but this is not related to autophagy but rather to the reduction of p62-dependent activation of the vitamin D receptor (VDR) and the resultant loss of repression of HSC by VDR agonists[457,458].

Even in HSCs the characterization of autophagy as a double-edged sword has been justified. A novel molecular mechanism of selective autophagy in HSCs indicates that autophagy may also protect from liver fibrosis. The RNA-binding protein ELAVL1/HuR plays a crucial role in regulating ferroptosis in liver fibrosis. ELAV1 enhances ferritinophagy leading to ferroptosis of HSCs and attenuation of liver fibrosis[459]. Despite this report, most existing evidence indicate that activation of HSCs autophagy is pro-fibrogenic, therefore a selective block of autophagy in fibrogenic cells might be an attractive future anti-fibrotic therapy[90].

The opposite seems to happen in hepatic macrophages[55] where activation of autophagy is anti-fibrogenic[460]. Mice macrophages with specific deletion of atg5, secreted increased levels of ROS-induced IL-1A and IL-1B. In addition, liver myofibroblasts incubated with the conditioned medium of Atg5(-/-) macrophages expressed increased pro-fibrogenic genes. Attenuation of fibrosis was achieved after IL-1 neutralization indicating that IL1A/B are critical mediators of the profibrotic effects of autophagy inhibition in macrophages[461-463]. Autophagy in Kupffer cells is counteracted by the enzyme monoacylglycerol lipase catalyzing the production of arachidonic acid leading to inflammatory macrophage activation and fibrosis[464].

On the other hand deletion of Atg7 in sinusoidal endothelial cells (LSECs) demonstrated that the selective loss of their autophagy led to cellular dysfunction and decreased intrahepatic nitric oxide. Impairment of autophagy after CCL4-induced acute liver injury in rats, also impaired handling of oxidative stress by LSECs and amplified liver fibrosis[465].

Similarly, autophagy defective sinusoidal endothelial cells (LSECs) as demonstrated in patients with NASH favor advancement of fibrosis[282]. At the same time, even excessive autophagy activation may lead to caveolin-1 degradation, thus worsening the LSECs defenestration and ultimately promoting fibrosis[466]. Therefore, any dysregulation of autophagy in LSECs may aggravate liver fibrosis[467].

An elegant immunofluorescence study of cirrhotic livers linked autophagy with an additional population of fibrogenic cells other than HSCs, the reactive ductular cells (RDC) which were characterized as cholangiocyte-like epithelial cells positive for cytokeratin 19[85]. They are responsible for ductular reaction (DR), a common response to various insults of the liver implicated in the pathogenesis of cirrhosis[432]. Administration of chloroquine, reduced the expression of CK19 positive RDC and blunted liver fibrosis[86]. DR parallels HSC activation in many liver diseases[430]. Reactive ductular cells secrete soluble pro-fibrogenic factors targeting HSCs and myofibroblasts[468]. Recently it was demonstrated that in cirrhotic human livers, RDCs with activated autophagy also had upregulated expression of TGF and fibroblast specific protein-1[469] making autophagy a necessary requirement during the DR process. The role of autophagy in liver fibrosis is therefore complex and the end result depends on the cell population involved. In general, HSCs and RDCs have a pro-fibrogenic effect. On the contrary, autophagy counteracts fibrogenesis acting in hepatocytes, macrophages and LSECs[470].

HCC

The role of autophagy in tumor cell biology has not been fully elucidated. Autophagy has both pro-and anti-tumorigenic roles. For example, it can either inhibit inflammation acting as an anti-oncogen or protect tumor cells from ROS damage acting as a pro-oncogen[471,472].

Opposing effects have been reported. Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis[473]. On the other hand, Ras-induced expression of two proteins Noxa and Beclin-1 promotes autophagic cell death, limiting thus the oncogenic potential of deregulated Ras signals[474]. Drugs like ursodexocycholic acid can efficiently eliminate resistant to other drugs cancer cells through induction of autophagic death[475].

HCC is one of the most common types of liver cancer[476]. Most of the HCC cases are accompanied by cirrhosis that results from long-standing chronic inflammation due to viral hepatitis or non-viral etiologies including heavy alcohol intake, NAFLD, autoimmune hepatitis, primary biliary cholangitis, and hemochromatosis[477].

Mice with impaired autophagy are unable to develop HCC even after of strong challenge. This was related to the induction of tumor suppressors like p53[478]. However, after initiation of HCC, the presence of autophagy is required to degrade tumor suppressors promoting thus the development of HCC[86]. Both macroautophagy and CMA are implicated as a double edge sword in liver tumorigenesis[479].

Autophagy has a dual role in hepatocellular carcinoma (HCC). It is an anti-cancer mechanism in the dysplastic stage of HCC initiation, while it favors HCC development and confers resistance to treatment[480,481]. This is possibly due to the maintenance of mitochondrial integrity and protection of cells against oxidative stress during HCC initiation, followed by the downregulation of tumor suppressors to promote the development of HCC[86,482].

In a study of 156 HCC patients increased levels of the autophagy marker LC3B are associated with a dismal prognosis[483]. Higher levels of LC3-II were associated with lymph nodes metastasis, higher vascular invasion and reduced 5-year survival[484].

Macroautophagy may also have an anti-oncogenic function, as reduction of either Atg5 or Atg7 Levels lead to appearance of multiple liver tumors[485]. Similarly, low levels of autophagic proteins and activity are associated with bad prognosis of human HCC[486,487]. Beclin-1 Levels are lower in HCC tissue samples compared to normal tissue from the same patient. Beclin-1 expression was studied in 300 HCC patients. A correlation with disease-free survival and overall survival was found only in the Bcl-xL+ve patients. It was suggested therefore that a synergy of defective autophagy and altered apoptotic activity lead to tumor progression and reduced survival[488].

Inhibition of autophagy leads to the accumulation of SQTSM1/p62. Accumulation of p62 on the one hand may protect from HCC initiation as it blocks the antioxidant functions of nuclear factor erythroid-2- related factor 2 (Nrf2)[489-492]. On the other hand, accumulation of p62 also contributes to carcinogenesis through persistent activation of Nrf2[493]. Nrf2 expression promotes the development of HCC[493]. Deletion of p62 in autophagy defective livers counteracts tumorigenesis. Therefore an accumulation of p62 is partly responsible for the increase in hepatic tumors, *via* the activation of Nrf2[492-494]. The activation of Nrf2 turns glucose and glutamine into anabolic pathways supporting tumor cell proliferation[176,495]. In addition, autophagy inhibits malignant transformation in the liver through Yes associated protein 1 (YAP1) degradation, a protein with a crucial role in hepatic oncogenesis[113,496].

Aberrant activation of the Wnt/β-catenin signaling is another critical pathway in the onset and development of HCC. A recent study reported that the Wnt/β-catenin inhibitors exert anti-tumor effects on HCC cells by regulating autophagy[497]. However this is in disagreement with a previous report where interfering with Wnt secretion in HCC cell lines does not affect autophagy or the level of β-catenin signaling despite cell growth suppression indicating that other mechanisms might underlie the growth-suppressive effect[498].

Furthermore, the activation of autophagy was shown to mediate inhibition of proliferation and induction of apoptosis of hepatoma cell through several mechanisms[499-506]. The induction of autophagy by concanavalin A or different chemotherapeutic drugs in murine livers inhibit hepatoma cell growth and prolongs survival[507-519]. On the other hand suppression of autophagy was reported to enhance the susceptibility of hepatocellular cancer cells towards a variety of chemotherapeutic agents[108,520-529].

Several microRNAs (mirRNAs) have been implicated in HCC tumorigenesis. miR-204 reduces tumor autophagy in HCC[530]. Moreover autophagy degradation of miRNA-224 suppressed the growth of HBV-related HCC[371], while miR-375 which is

downregulated in HCC was reported to inhibit autophagy by decreasing the expression of Atg7 and autophagic flux. Up-regulation of miR-375 inhibits mitophagy of HCC cells, reduces the elimination of damaged mitochondria, and decreases cell viability[99]. miR-26 could inhibit autophagy and enhance chemosensitivity of HCC cells[531].

LncRNAs are another set of ncRNAs with a length exceeding 200 nucleotides without translation into proteins[109]. Several lncRNAs, like Hnf1a-as1, Hotair and Hulc promote autophagy and function as oncogenes in HCC[106-109].

The role of mitophagy and lipophagy is also important in HCC growth acting as a double edge sword. Increased mitophagy by concanavalin A, adriamycin or curcumin was shown to suppress hepatoma cell growth[507,510,517,530] while melatonin increased the sensitivity of human hepatoma cells to sorafenib by triggering mitophagy[532]. A recent study also demonstrated that inhibition of inflammasome activation and induction of mitophagy suppressed HCC growth[533]. On the other hand it has been demonstrated that increased mitophagy may facilitate HCC cell survival either through ROS production or attenuation of p53 activity[534,535].

Lipophagy can also act both ways. On the one hand, it can allow tumor cells to have access to a supply of energy critical to their growth[536] and on the other hand, lysosomal acid lipase, the lipase that facilitates lipophagy, exhibits tumor suppressor activity[537]. Lipophagy was also reported to induce apoptosis *in vitro*, *via* induction of ER and mitochondrial stress[538]. CCAAT enhancer binding protein a, a protein that is upregulated in HCC patients, increases resistance to energy starvation and favors carcinogenesis through lipophagy[539].

In addition to the general characteristics of autophagy implication in HCC, there are certain points to be mentioned in specific liver disease associated HCC. As mentioned before, autophagy is activated by the HBx protein[357,369] and this may be related to HBV carcinogenesis. Increased autophagosome formation by HBx was accompanied by decreased degradation of LC3 and SQSTM1/p62 and greatly impaired lysosomal acidification and accumulation of immature Cathepsin D. These data may indicate that repression of lysosomal function by HBx could be important for the initiation and progress of HBV-associated HCC[350].

CMA and cancer metabolism are also interconnected. Once malignant transformation occurs, CMA activity is significantly increased in cancer cells so that the new metabolic requirements are maintained[64]. Blockade of CMA which is upregulated in several cancers reduces progression and metastatic potential of solid tumors because the characteristic increased rates of aerobic glycolysis are reduced in a p53-dependent manner[540]. Macroautophagy and CMA seems to be interconnected and often substitute for one another as in the case of HCC. Under physiological conditions there is no expression of p62 in normal livers pointing to macroautophagy as the main mechanism facilitating cell survival. However in a recent study of 46 cirrhotic livers it was shown that p62 was increased indicating an impairment of macroautophagy, but LAMP-2A and heat shock protein 70 were uniformly increased indicating that an upregulated CMA was trying to compensate for the reduced macroautophagy and therefore promote HCC survival. Moreover, hydroxychloroquine inhibition of lysosomal degradation led to induction of the tumor suppressor p53 and promotion of apoptosis[541]. HCV is also an inducer of HCC. During HCV infection, increased cellular stress has been reported. Severe stress promotes Nrf2 transcription which in turn is responsible for CMA activation resulting in the suppression of hepatic innate immunity and possible degradation of tumor suppressors. The subsequent oncogenic cell programming initiated by a cytoplasmic virus like HCV, has been recently described in detail[542].

Defective autophagy is linked to MAFLD-related HCC, because the accumulated p62/SQSTM1, induces the oncogenic NF-κB activity while retained damaged mitochondria and produced ROS to damage cellular DNA[543]. A novel mechanism was recently reported in ethanol induced liver disease and HCC. Tumor necrosis factor-α-induced protein 8 (TNFAIP8) has been associated with tumor progression in several cancer types including the initiation of HCC. TNFAIP8 induced autophagy in liver cancer cells through blocking of AKT/mTOR signaling and direct interaction with ATtg3-Atg7 proteins. This mechanism is operative in alcohol related liver disease in mice and humans but not in high-fat-fed obese mice or patients with MAFLD[544]. Details of the molecular mechanisms of autophagy in both protection and promotion of HCC were recently published[545-547].

An additional aspect of HCC biology where autophagy plays an important role is the involvement of tumor-associated macrophages and tumor microenvironment. They are polarized after implication of sensing factors from tumor environment and autophagy[130,548]. Deficiency of TLR2 decreased the liver production of TNFα, IFN

gamma and IL1a/b accompanied by reduction of autophagy flux and increase in oxidative stress and p62 aggregates in liver tissue. These changes were associated with increased carcinogenesis and progression of HCC[549]. Enhancement of autophagy in tumor-associated macrophages leads to M1 polarization which reduces tumor progression while M2 polarization is permissive for tumorigenesis[550]. The mTOR-TSC2 pathway, a key negative regulator of autophagy, is crucial for macrophage polarization since its activation leads to M2 phenotype. It was recently shown that the coagulants tissue factor (TF) and factor VII (FVII) produced in tumor microenvironment, are implicated in HCC growth promotion by suppression of autophagy mediated through mTOR activation and Atg7[551].

In view of the variable functions of autophagy, there should be an individualized approach of autophagy manipulations for HCC treatment. Thus, various lysosomal inhibitors including chloroquine and hydroxychloroquine have been used as treatment either as sole agents or in combinations with other treatment modalities in a variety of murine HCC models[523,552,553]. Interference with autophagy may be a sound therapeutic option for the treatment of HCC[554,555]. Based on the fact that autophagy is upregulated in metastatic HCC[556] use of autophagy inhibitors like chloroquine and hydrochloroquine in combination with other drugs may be a better option for treating metastatic HCC in humans. A combination of a number of drugs with autophagy inducers have been used to target cancer cells. A combination of percutaneous transarterial chemoembolization with chloroquine, was associated with increased tumor cell necrosis and apoptosis[557] and might counteract the presence of residual hepatocellular carcinoma cells[558,559]. Sorafenib, a multikinase inhibitor approved for HCC treatment, induces autophagy[560] and data show that a combination with autophagy inhibitors increase tumor response[537,561].

Cholangiocarcinoma

Xenografts in nude mice are widely used models of cholangiocarcinoma (CCA). Activated autophagy has been reported in tumor cells from such a model and in specimens from CCA patients[562]. LC3B, Beclin 1, and p62/SQSTM1 expressions were additionally found to be increased at the initial stage of the multistep cholangiocarcinogenesis[563]. However, a lower Beclin 1 expression was associated with metastatic lymph node disease and poor survival of patients with intrahepatic CCA[564,565]. Apoptosis was induced in cholangiocellular cell lines and tumor development was suppressed in a mice xenograft model after interference with autophagy[562]. Similarly, suppression of autophagy by chloroquine increased the chemosensitivity of cisplatin-treated CCA cells[566] and increased apoptosis of CCA cells through ER stress[567]. Chloroquine blockade of autophagy inhibited the tumor growth in Kras/p53 intrahepatic CCA[568,569]. CCA is extremely resistant to chemotherapy. 5-fluorouracil (5-FU) induced autophagy in CCA cells[570] while autophagy inhibition by capsaicin was followed by repression of malignant cell growth[570], indicating that autophagy may be implicated in the multidrug resistance of this tumor. Autophagy was also induced after incubation of CCA cells with the sphingosine kinase 2 inhibitor, ABC294640. Inhibition of autophagy by chloroquine potentiated ABC294640-induced apoptosis[571]. Modulation of autophagy therefore may be helpful in CCA treatment.

INHERITED METABOLIC DISEASES

A1 antitrypsin deficiency and fibrinogen storage disease

Autophagy is also implicated in other types of liver injury like the inherited metabolic diseases. Alpha 1-antitrypsin deficiency is the most extensively studied. Alpha 1-AT is a glycoprotein inhibitor of destructive neutrophil proteases[572,573]. Several naturally occurring mutants of alpha1-AT, have been shown to participate in the pathogenesis of human diseases, such as chronic liver-associated diseases[574-576]. The Z mutation resulting from a single G->A transition in codon 342, generates a mutant protein that forms aggregates in the hepatocytes[577]. Liver injury is caused by the retention of this polymerized mutant alpha1-ATZ molecule in the ER of hepatocytes followed by an induction of autophagic response. Removal of the insoluble alpha-1 anti-trypsin by the autophagosome is the mechanism by which the activation of autophagy protects the liver in alpha1-antitrypsin deficiency[578-581]. In earlier studies, liver injury was associated with mitophagy indicating that the ER retention of alpha(1)-ATZ led to involvement of the mitochondria, with specific patterns of mitochondrial dysfunction and mitochondrial injury[582,583].

Genetic studies in mice have shown that deletion of Atg5 led to an increased retention of alpha 1-ATZ[584] and that deficiency of Atg6 and Atg14 in yeasts inhibited alpha1-ATZ degradation[585]. Similarly, the induction of autophagy in mice by rapamycin reduced liver alpha1-ATZ aggregation and liver injury[582,586-589]. These findings have been repeated and verified when enhancement of autophagy[590] with either carbamazepine[591], gene transfer of the autophagy regulator TFEB[592] or an analog of glibenclamide[593] reduced the toxic protein. Recent preclinical studies have also demonstrated that an exogenous bile acid like norursodeoxycholicacid may be clinically useful in this condition[594,595].

Fibrinogen storage disease is a very rare autosomal-dominant ER storage disease presented with hypofibrinogenemia, elevated transaminases, accumulation of fibrinogen aggregates in the ER of hepatocytes and several fold increase of autophagocytic vacuoles. Some patients progress to cirrhosis similar to alpha-1-AT deficiency. A clinical study of eight patients has showed that administration of carbamazepine at low anticonvulsive dosage led to rapid normalization of alanine-aminotransferase indicating a critical role of autophagy in this disease[596].

Wilson's disease

Wilson disease is an inherited disease of copper metabolism linked to hundreds of mutations in the ATP7B gene[597]. Recent evidence based on studies from hepatocytes of patients and ATP7b deleted mice has shown the presence of an increased number of autophagosomes, indicating the activation of an autophagic response to prevent copper associated cell death[598]. Moreover, inhibition of autophagy accelerated hepatocyte death whereas increased autophagy by either starvation or TFEB overexpression had a cytoprotective effects[598]. Autophagy therefore seems to be a major protective mechanism for hepatocytes in copper accumulation. These findings may lead to the use of autophagy inducers like carbamazepine as a future potential treatment of Wilson's disease.

Glycogen storage disease

Glycogen storage disease type 1a (GSD1a) is an inherited hepatic disease associated with decreased autophagic flux as a consequence of defects in the glucose-6-phosphatase a, that converts glucose-6-phosphate into glucose. These abnormalities lead to glycogen and lipid accumulation in hepatocytes[599]. GSD1a is associated with the down regulation of several components of the autophagy machinery[600].

GSD1a has also been associated with defective sirtuin 1 (SIRT1) signaling leading to impairment of TFEB activity. As in other storage diseases, pharmacological or genetic activation of autophagy reduces the accumulation of glycogen and lipids in cellular and animal models[601].

VARIOUS DISEASES

Autophagy in the liver is implicated in other diseases as well. An important point that should always be remembered is that the liver is the site of almost 80% of body macrophages and therefore innate immunity can be deeply involved in liver and other organ abnormalities through impaired autophagy of Kupffer cells. Sepsis is the main paradigm of this notion.

Sepsis and liver autophagy

Infection can lead to a systemic multi-organ inflammatory response. Macrophages, play a critical role as they are the most important cells of the innate immunity. Autophagy induction is protective in sepsis through regulation of macrophage polarization. Negative regulation of macrophage activation inhibits inflammasome activation[602]. Autophagy also interferes with macrophage apoptosis. Uncontrolled autophagy however may lead to autophagy death of macrophages with additional aggravation of inflammation and the so called cytokine storm[603].A current example is possibly the SARS-Cov-2 pandemic[604]. Interestingly, autophagy-deficient macrophages after LPS stimulation over-secrete macrophage migration inhibitory factor and aggravate inflammation[605]. Other mechanisms are also involved including signaling pathways such as NF-κB, mTOR, and PI3K/AKT[603].

Mitophagy and mitochondrial dysfunction seem to be also a fundamental factor in multiple organ failure caused by sepsis[606]. It has been shown that mtDNA liberated from damaged mitochondria, induces a cascade of inflammatory responses[607-609]. Mitophagy therefore is of great importance for the protection against oxidative stress

during sepsis. It should be noted however that mitophagy defects in the liver, are not the only cause of organ or cell damage during sepsis[610]. Nonetheless, the liver is the main organ responsible for sepsis-induced damage[611]. Autophagy is an important protective mechanism in septic liver injury. Increased autophagy can play a protective role in liver function in septic conditions where the activation of autophagy is mediated through activating transcription factor 4 (ATF4). ATF4 is inhibited 48 h after LPS-induced acute liver injury and reversed after obeticholic acid treatment[612]. Autophagy inhibitors or AMPK inhibitors administration reduced the protective mitochondrial function in LPS-induced human hepatocyte injury[613,614]. Mitophagy is also involved in apoptosis of CD4+ve T cells which is the main mechanism of immune inhibition during sepsis. Mitofusin 2 (Mfn2) is a mitochondrial outer membrane protein and a negative regulator of autophagy which is increased in sepsis leading to inhibition of autophagy and increase in apoptosis of CD4ve+ T cells[615]. Autophagy defects can affect antigen presentation by T cells leading to immunosuppression as in the case of Atg5 deficiency[616]. The role of autophagy in sepsis has been recently reviewed[617].

Acetaminophen liver damage

Autophagy is also implicated in acetaminophen induced liver disease. There is evidence that increased autophagy is protective against acetaminophen (APAP)-induced liver damage[618,619]. Pathogenetically, APAP was reported to form APAP-protein adducts in hepatocytes of mice and humans[620]. Adducts localized in mitochondria contribute to APAP-induced mitochondrial dysfunction and subsequent oxidant stress[621,622]. Therefore, it is plausible that removal of APAP-adducts will help to ameliorate APAP-induced mitochondrial damage and maintain hepatocyte integrity[41,623,624]. Experimental evidence indicates that autophagy is mostly responsible for the removal of APAP-adducts[625]. Moreover administration of adiponectin was found to attenuate APAP-induced injury activating AMPK mediated autophagy[626]. Activation of autophagy by rapamycin also attenuates APAP-induced liver injury, whereas inhibition of autophagy by chloroquine or deletion of Atg7 in hepatocytes deteriorates liver damage[153,627]. There is also evidence that autophagy is activated after APAP overdose in specific liver zones[53].

Somewhat different results were recently presented. Unc-51-like autophagy activating kinase 1 and 2 (Ulk1/2) are important autophagy initiation regulators. Unexpectedly, Ulk1/2 double knockout mice have normal autophagic activity after fasting, but are exceptionally resistant to APAP-induced liver injury possibly indicating that autophagy-dependent and independent ULK1/2 pathways have opposing effects in APAP-induced liver injury[628].

Reduction of ROS and repression of apoptosis by autophagy is also essential for hepatic regeneration after APAP-induced acute liver failure[520,627]. A very recent report confirmed that increased autophagy by rapamycin protects mice against APAP hepatotoxicity while chloroquine enhanced liver injury. Importantly it was demonstrated that APAP overdose activated PINK1/Parkin-mediated mitophagy and increased the expression of NF-kB and NLRP3 inflammasome signaling. These findings were reversed by rapamycin and augmented by chloroquine indicating the critical role of mitophagy in APAP hepatotoxicity[629].

Interestingly it was reported that infusion of human amniotic mesenchymal stromal cells ameliorated the APAP liver injury through promotion of Kupffer cell M2 polarization and reduction of Kupffer cell autophagy. These results suggest that Kupffer cell autophagy has an opposite effect on APAP hepatotoxicity compared to hepatocytes. This last observation may be useful for future therapeutic exploitation[630].

Acute liver failure

Acute liver failure (ALF) is a serious syndrome of different etiologies with high mortality[631]. HSCs implication is significant in ALF. Temporarily increased fibrosis in ALF is probably beneficial serving as scaffolding that maintains regenerating hepatocytes and hepatic integrity[437,632,633]. Data from a murine APAP induced ALF model have demonstrated that mortality was significantly increased in HSCs depleted animals[633]. HSCs cannot usually regenerate during ALF due to the submassive necrosis. Autophagy seems to be implicated[634]. The significance of HSCs survival has been verified in a study of patients with HBV induced acute liver failure. ALF was accompanied by fibrosis and HSCs activation and autophagy induction. It was shown for the first time that the High Mobility Group Box 1 (HMGB1) protein is a powerful inducer of autophagy responsible for HSCs survival[635].

As mentioned before, autophagy is crucial for HSCs activation which in turn maintains the liver architecture thus preventing the liver scaffold collapse during ALF.

Nitric oxide induces HSCs apoptosis through generation of ROS[636]. There is evidence however that nitric oxide is also involved in the regulation of autophagy in ALF. Observations in human liver tissue showed an inhibition of autophagy in HSCs while further *in vitro* experiments demonstrated that nitric oxide inhibited autophagy and increased apoptosis of HSCs. These findings were reproduced by chloroquine and reversed by the autophagy inducer rapamycin. Therefore, nitric oxide impairment of HSCs survival may be a decisive factor for the devastating effects of ALF[637].

An additional clinical and experimental study verified the significance of intact mitophagy in ALF. One of the measurements of oxidative stress is the level of superoxide dismutase (SOD). The serum superoxide dismutase was significantly increased in ALF patients, correlating with the MELD-Na score. SOD levels returned to normal in the remission stage of ALF. In liver tissue from ALF patients and mice models, manganese-dependent SOD was overexpressed and mitophagy in HSCs was inhibited by ROS. Inhibition of mitophagy promoted inflammation in HSCs which was reversed by a mitophagy inducer[454].

Acute liver damage

Autophagy also protects hepatocytes from acute liver injury, a characteristic of viral hepatitis and acute alcoholic and non-alcoholic steatohepatitis. Mechanisms and cells involved are different as both direct and indirect effects on hepatocytes and macrophages are implicated. Direct effects include autophagy dependent inhibition of caspase 8 in hepatocytes[638], while indirect effects on macrophages involve limitation of NF-kB-mediated inflammation and inflammasome-dependent IL-1b production through p62-dependent mitophagy[462,639]. Reduced macrophage autophagy can induce pro-inflammatory macrophage polarization and increase the immune mediated acute damage in obese mice[131]. The TAM family of RTKs (receptor tyrosine kinases), which is expressed in macrophages, has been reported to alleviate inflammation. AXL is the only member of the TAM family that induces autophagy in macrophages and ameliorates hepatic inflammatory responses inhibiting the NLRP3 inflammasome activation in murine macrophages[640].

The role of Kupffer cells (Kcs) is significant in the pathogenesis of acute liver injury. In a murine model of thioacetamide induced acute liver injury it was shown that hyperglycemia aggravated the liver lesions activating the NLRP3 inflammasome of Kupffer cells *via* inhibition of AMPK/mTOR-mediated autophagy. Interestingly, AMPK activation or mTOR signaling deletion restored autophagy and subsequently inhibited inflammasome activation in Kupffer cells[641]. Spermine is an anti-oxidative polyamine with autophagy induction properties. In a model of acute liver injury, spermine pre-treatment ameliorated liver injury and intrahepatic inflammation by promoting M2 polarization of Kupffer cells.

Furthermore, spermine increased autophagy in KCs. Deletion of Atg5 in spermine treated KCs greatly increased pro-inflammatory cytokines and reduced the anti-inflammatory cytokine IL-10[642].

LSECs are also involved in acute liver injury. Selective impairment of autophagy in liver endothelial cells increases oxidative stress, thus leading to fibrosis in acute injury[465].

Ischemia/reperfusion injury

The central role of autophagy in ischemia/reperfusion injury (I/R) injury has been verified by the fact that pharmacological or genetic stimulation of autophagy ameliorate the liver reperfusion injury[643-645].

I/R impairs hepatocellular autophagy[646] through I/R-induced ATP depletion leading to energy shortage and malfunction of the autophagic machinery. Moreover Ca^{2+} overloading during I/R results in calpain overproduction and ultimate loss of key autophagy proteins like Atg7. Interestingly the autophagy suppressor chloroquine attenuated liver injury when administered in early phases of I/R but aggravated the lesions, as expected, when given in late phases[647].

Hepatic encephalopathy

Ammonia is an important mediator of hepatic encephalopathy. Increased ammonia levels rapidly induce an autophagic response that preferentially targets mitophagy[648-650]. Ammonia induced autophagy may in fact be a protective mechanism against encephalopathy as suggested by a recent report. Deletion of Atg7 or loss of functional TFEB deteriorated ammonia detoxification in mice. By contrast activation of liver autophagy either by rapamycin administration or genetic TFEB expression reduced ammonia levels in acquired hyperammonaemia[651].

Autoimmune hepatitis

The role of autophagy in autoimmune hepatitis (AIH) has not been adequately studied. It is suggested that autophagy is implicated in AIH through its involvement in antigen processing and presentation to T cells[652] and its well proven role in liver fibrosis[653], but the exact pathways have not been delineated.

Concanavalin A-induced hepatitis is an extensively used model for immune-mediated liver injury. Comparative proteomic results in this model have shown that the activation of immune system resulted in hepatitis with deregulation of autophagy as indicated by an increase in p62 and LC3B. Arctigenin is a biologically active lignan with antioxidant and anti-inflammatory properties. Pretreatment with arctigenin alleviated autophagy as well as apoptosis verifying that immunity and autophagy are interconnected in AIH pathogenesis[654].

A group of researchers recently used the same model of concanavalin (conA) induced experimental hepatitis to clarify the role of autophagy in AIH. Methyl prednisolone (MP) treatment significantly decreased inflammation in the liver and activated the Akt/mTOR pathway to inhibit hepatocyte apoptosis and autophagy. Reduced numbers of autophagosomes were present in the MP treated group compared to the conA group. It was further shown that MP attenuated the mitochondria-mediated autophagy and apoptosis[655]. In a second report on the same experimental model, accumulation of mature conventional dendritic cells (cDCs) was observed in the liver. *In vitro*, ConA treatment induced the expression of autophagy proteins and the formation of autophagosomes in dendritic cells. A further blockade of autophagy flux inhibited the maturation of DCs and the proliferation and differentiation of CD4+ T cells when ConA-induced DCs were co-cultured with CD4+ T cells. Taken together these studies elegantly showed that autophagy is critically implicated in AIH and aberrant autophagy and defective maturation of cDCs are involved in AIH immunopathogenesis[656].

A recent clinical study using immunohistochemistry in liver biopsy samples from chronic HCV and AIH patients confirmed the central role of autophagy in AIH. Activated but impaired autophagy and less efficient elimination of damaged mitochondria were demonstrated in AIH as compared with HCV. Increased p62 levels significantly correlated with necroinflammation in AIH[657].

Biliary disease

The mechanisms of liver damage in cholestasis are incompletely understood. Autophagy and protein degradation were shown to be impaired in cholestasis induced in bile duct ligated mice[658-660]. Moreover, defective autophagy after chloroquine inhibition or deletion of Atg7 and Atg5 led to increased cholestatic liver injury[661,662].

Accumulated toxic bile acids lead to ER stress, mitochondrial dysfunction with increased oxidative stress, inflammasome activation and apoptosis leading to liver fibrosis[663]. These events should in fact activate autophagy in cholestasis but instead, at least in mice, it appears that autophagy is inhibited in cholestasis[664,665]. Bile acids can inhibit autophagy in mice either *via* the farnesoid X receptor (FXR) during the feeding-fasting cycle[114,115] or independently of FXR[666]. How autophagy is affected in human cholestasis is under investigation.

In human disease autophagy was initially associated with the pathogenesis of primary biliary cholangitis (PBC)[667-669]. As mentioned before autophagy is also involved in the processing and presentation of various antigens. It is only logical therefore that an interesting hypothesis implicating deregulated cholangiocyte autophagy connected to cholangiocyte senescence has been proposed to explain not only the pathogenesis of PBC but of the other fibrosing cholangiopathies including primary sclerosing cholangitis (PSC) and biliary atresia as well[670].

An upregulation of autophagy was reported along with senescence in PBC[668,671]. LC3B and p62 proteins were accumulated in damaged bile ductular cells in association with senescence markers[68,125] suggesting that autophagy could induce and facilitate cholangiocyte senescence[664,665,671-674]. Mitophagy may be specifically involved in PBC as granular expression of the mitochondrial protein PDC-E2 was co-localized with LC3[667].

Autophagy has also been implicated in the treatment of PBC. Ursodeoxycholic acid (UDCA) is still the first line treatment of PBC while obeticholic acid (OCA) is a second-line treatment[675-677]. Hydrophobic bile acids, such as glycochenodeoxycholic acid impair autophagy *in vitro* and induce abnormal expression of mitochondrial antigens and cellular senescence in cholangiocytes, possibly through induction of ER stress. Pretreatment with UDCA reduced ER stress and partially restored deregulated autophagy and cellular senescence[678]. It is not clear how UDCA stimulates autophagy.

UDCA has been reported to be an FXR antagonist[679] but this may not be the explanation[680]. On the contrary, OCA is a semi-synthetic FXR agonist with anti-cholestatic functions including the suppression of endogenous bile acid synthesis and interference with hepatocellular bile acid transporter systems[681]. OCA impairs autophagic flux *in vitro* and also *in vivo*. A favorable effect of treatment with OCA in a cholestatic disease like PBC would be incompatible with data, indicating that cholestasis progresses when autophagy is blocked[661,662]. However, the other potent, anti-cholestatic properties of OCA can overcome the negative effects of reduced autophagy.

A recent paper offers an interesting explanation. Autophagy seems to be also impaired in human cholestatic conditions where accumulated bile acids induce Rubicon in an FXR-dependent fashion. Rubicon induction suppresses autophagosome-lysosome fusion and inhibits proper autophagolytic breakdown. Rubicon was also induced after treatment with the FXR agonist OCA. Genetic inhibition of Rubicon reversed the impairment of autophagic flux. In contrast, UDCA reduced Rubicon levels, enhanced autophagic flux and autophagolysosome formation independently of FXR[680].

An overview of autophagy abnormalities is presented in Table 1.

CONCLUSION

Autophagy is an important process through which intracellular parts are degraded in the lysosomes. It is a fine example of effective cellular recycling mechanism, connecting cellular quality control with energy saves. There are three types of autophagy with various pathways of delivery to the lysosomes: Macroautophagy (which is further divided into non selective autophagy and selective macroautophagy targeting special organelles or specific compounds for degradation), microautophagy and chaperon-mediated autophagy. Autophagy is related to major physiologic processes as cell death, inflammation and immunity. It is increasingly recognized that it is implicated in almost every aspect of liver diseases, and this can be the basis for future pathophysiologically based and targeted management.

Table 1 Overview of autophagy abnormalities in liver disease

Disease	Abnormalities of autophagy	Results	Ref.
Obesity	↓Autophagy; Hepatocytes: ↓Mitophagy, ↓Lipophagy; HSCs: ↓Autophagy	↑ER stress, →↑Lipids, ↑Insuline resistance, → Anti-fibrotic	Liu *et al*[203], Lavallard *et al*[204], Gual *et al*[205], Tremblay *et al*[206]
NAFLD	↓Lipophagy; ↓CMA	Lipotoxicity, ↑Lipogenic enzymes	Madrigal-Matute *et al*[30], Zhou *et al*[234], Niso-Santano *et al*[235], Singh *et al*[236]
NASH	Hepatocytes: ↓Autophagy, ↓Mitophagy; Kupffer cells: ↓Autophagy; LSECs: ↓Autophagy	↑Mallory-Denk bodies, ↑Inflammasome activation; ↑Cathepsins B,D, ↑M1 polarization, ↓M2 polarization; ↑Inflammation, fibrosis	Xu *et al*[272], Noureddin *et al*[277], Zhang *et al*[285], Dey *et al*[287]
Alcoholic liver disease	Acute ETOH administration: ↑Autophagy, ↑Mitophagy, ↑Lipophagy, ↑Proteophagy; Chronic ETOH administration: ↑ Autophagy (low dose), ↓Autophagy (high dose); Kupffer cells: ↓Autophagy, ↑Autophagy; HSCs: ↓Autophagy, ↑Autophagy	Protection, protection, protection, →Clearance of Mallory-Denk bodies; →Protection, →Mitochondrial damage, Cell death; Liver damage, protection; Reduced fibrosis, increased fibrosis	Chao *et al*[308], Komatsu *et al*[311], Yan *et al*[314], Harada *et al*[318]
HBV	↑Autophagy, ↓Lysosomal acidification, ↑Mitophagy	↑Virus replication, ↓HBV degradation	Li *et al*[356], Tang *et al*[357], Luo *et al*[372], Wang *et al*[383]
HCV	↑Autophagy, ↓Lipophagy, ↑Mitophagy; ↑CMA	↑Virus replication, steatosis, ↑Virus replication, ↓Apoptosis, persitent infection, ↑Virus replication	Ferraris *et al*[387], Paul *et al*[395], Jassey *et al*[404], Ren *et al*[406]
Fibrosis-Cirrhosis	Hepatocytes: ↓Autophagy, ↓Lipophagy; Kupffer cells: ↓Mitophagy, or, ↑↑Mitophagy; HSCs: ↓Mitophagy, ↓Lipophagy, or, ↑Lipophagy, ↑Mitophagy; LSECs: ↑↓Autophagy; Ductular reaction: ↑Autophagy	↑Fibrosis, ↑Lipotoxicity, ↓TGFb, ↓Fibrosis; ↑TGFb, ↑Fibrosis; Pro-inflammatory anti-fibrotic: →Pro-fibrotic, →Pro-fibrotic, ↑Fibrosis, ↑Fibrosis	Zhang *et al*[437], Singh *et al*[438], Li *et al*[448], Sun *et al*[463]
HCC, "Double edge sword"	Induction stage: ↑CMA, ↑Autophagy; Late stages: ↑Autophagy, or, ↓Autophagy, ↑Mitophagy, ↑Lipophagy	Anti-oncogenic: ↓YAP1, ↓proliferation, ↑Apoptosis→Anti-oncogenic, ↓Tumor suppressors; ↑Tumor progression, ↓↑Progression↑↓Progression	Wang *et al*[558], Zhao *et al*[559], Prieto-Domínguez *et al*[560]; Niture *et al*[544], Yang *et al*[547]; Lin *et al*[549], Chen *et al*[550], Chen *et al*[551]
Cholangiocarcinoma	↑Autophagy	↑Tumor progression	Marciniak *et al*[580], Teckman *et al*[581]
A1 antitrypsin deficiency	↓Autophagy		Yamamura *et al*[590], Pastore *et al*[592]
Fibrinogen storage disease	↓Autophagy		Hu *et al*[609]
Wilson'S disease	↓Autophagy		Oami *et al*[611]
Glycogen storage disease	↓Autophagy		Xing *et al*[613]
Sepsis	Kupffer cells: ↑Autophagy, ↑↑Autophagy, ↓Mitophagy	M2 polarization, ↓Inflammasome activation; Kupffer cell apoptosis→Cytokine storm, ↓Apoptosis of CD4+ve T cells	Ying *et al*[615], Neumann *et al*[616], Sun *et al*[628], Shan *et al*[629]
Acetaminophene liver damage	↓Autophagy, ↓Mitophagy, ↑Kupffer cell autophagy	↑APAP-Protein adducts	Sydor *et al*[618], Kim *et al*[643], Biel *et al*[644]
Acute liver failure	↑Autophagy, ↓Autophagy, ↓HSCs Mitophagy	HMGB1→HSCs activation (protective); ↑NO,ROS→↓HSCs→Devastation	Cheong *et al*[649], Sridhar *et al*[652]
Ischemia/reperfusion injury	↓Autophagy		Kwak *et al*[658], Huang *et al*[659]

Hepatic encephalopathy	↑Autophagy (NH4)	Protection	Woolbright *et al*[663], Manley *et al*[666]
Autoimmune hepatitis	↑Autophagy, ↓ Mitophagy	Defective maturation of dendritic cells	Sasaki *et al*[671], Sasaki *et al*[672], Young *et al*[673]
Biliary disease (experimental)	↓Autophagy	Possibly through increased bile acids	Sasaki *et al*[665], European Association for the Study of the Liver[675], Lindor *et al*[676], Panzitt *et al*[680]
Primary biliay cholangitis	Deregulated autophagy	Cholangiocyte senescence	Van de Graaf *et al*[669], Sasaki *et al*[665], Sasaki *et al*[674]

Note the double edge sword behaviour of autophagy, particularly evident in hepatocellular carcinoma. Autophagy refers to macroautophagy. HSCs: Hepatic stellate cells; LSECs: Liver sinusoidal endothelial cells; CMA: Chaperone mediated autophagy; ER: Enoplamic reticulum; ASH: Acute alcoholic hepatitis.

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MINIREVIEWS

Post-liver transplant biliary complications: Current knowledge and therapeutic advances

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Abstract

Liver transplantation is the current standard of care for end-stage liver disease and an accepted therapeutic option for acute liver failure and primary liver tumors. Despite the remarkable advances in the surgical techniques and immunosuppressive therapy, the postoperative morbidity and mortality still remain high and the leading causes are biliary complications, which affect up to one quarter of recipients. The most common biliary complications are anastomotic and non-anastomotic biliary strictures, leaks, bile duct stones, sludge and casts. Despite the absence of a recommended treatment algorithm many options are available, such as surgery, percutaneous techniques and interventional endoscopy. In the last few years, endoscopic techniques have widely replaced the more aggressive percutaneous and surgical approaches. Endoscopic retrograde cholangiography is the preferred technique when duct-to-duct anastomosis has been performed. Recently, new devices and techniques have been developed and this has led to a remarkable increase in the success rate of minimally invasive procedures. Understanding the mechanisms of biliary complications helps in their early recognition which is the prerequisite for successful treatment. Aggressive endoscopic therapy is essential for the reduction of morbidity and mortality in these cases. This article focuses on the common post-transplant biliary complications and the available interventional treatment modalities.

Key Words: Post-transplant biliary complications; Endoscopic retrograde cholangiopancreatography; Cholangioscopy; Percutaneous biliary interventions; Liver transplantation; Living-donor liver transplantation

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Core Tip: Liver transplantation is the current standard of care for end-stage liver disease. Biliary complications are the leading cause of morbidity and mortality among recipients and despite the advances in surgical techniques they are seen in up to 25% of cases. Surgery, interventional endoscopy and percutaneous approaches are the available therapeutic options. Endoscopic retrograde cholangiography when possible is the most recommended therapeutic modality, replacing more aggressive surgical interventions. New techniques such as cholangioscopy overcome many of the limitations of conventional endoscopy. This article discusses the most common post-transplant biliary complications and the advances in treatment modalities.

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INTRODUCTION

Liver transplantation (LT) is the widely endorsed method for treatment of end- stage liver disease, acute liver failure and primary liver cancer. The advances in surgical techniques, postoperative care, immunosuppression, and antiviral therapy have led to remarkable progress in survival of these patients. The currently reported 5-year survival rate is 70%-75%[1,2].

Biliary complications are a significant source of morbidity in the early and long-term period after LT (Table 1). Their overall incidence ranges between 15% and 25%. With associated mortality of 10%, they remain a major problem in post-transplant patients. Timely identification and treatment play a significant role in preserving the graft and improving the overall survival rate of patients[3,4].

The most common current treatment is focused on interventional endoscopic (ERC) and percutaneous (PTC) procedures[4-6].

ERC provides minimal invasion with great long-term results and is a preferred method when surgical reconstruction allows this. ERC has been proven to be safe and highly effective in dealing with most of the early as well as late post-LT biliary complications. Procedural-related adverse events in post-LT cases are comparable with those among the general population[6].

The complication rate in patients after living donor liver transplantation (LDLT) in particular is about 10%, which is 2-fold higher than the standard[7,8].

PTC is an effective alternative in patients with altered anatomy which impedes endoscopy access. There is growing evidence that cholangioscopy could be a beneficial tool in the diagnostics and therapy of selected cases[9].

Surgery is available for cases when endoscopic and PCT methods have failed.

Biliary reconstruction techniques

The two major options for biliary reconstruction are bilio-enteric (hepatico-jejunostomy or choledocho-jejunostomy) and duct-to-duct anastomosis. Duct-to-duct anastomosis is the method of choice for biliary reconstruction in any type of transplantation: Cadaveric liver transplantation (DDLT), split transplantations, LDLT (left lobe or right lobe) transplantations[10,11].

Hepatico-jejunostomy is currently used only for selected cases such as those with primary sclerosing cholangitis, prior bilio-digestive surgery, significant ductal size mismatch, and insufficient length of recipient bile ducts[12].

Many benefits motivate the preference for direct duct-to-duct suturing: Preserved sphincter-of-Oddi function, lower risk of cholangitis, and reduction in the number of anastomoses. Besides, the preserved intestinal continuity ensures an endoscopic access to the biliary tree in case of potential complications[13,14].

T-tube placement has been widely abandoned over the last decades[15]. It has been proven that its usage increases the rate of biliary complications. A single-center retrospective review of 1041 transplantations reported that cholestatic liver disease, Roux-en-Y anastomosis, donor risk index > 2, and T-tubes were independent predictors of post-LT complications[16].

Table 1 Risk factors for the most common biliary complications

	Anastomotic	Non-anastomotic
Strictures	Advanced recipient age; Female donor; Failure to flush the donor duct; Preceding bile leakage; Acute rejection; Chronic rejection; Hepaticojejunostomy reconstruction	HAT; Chronic ductopenic rejection; Blood type ABO incompatibility; PSC, autoimmune hepatitis prolonged warm and cold ischemia times prolonged donor exposure to vasopressors
Leaks	Active bleeding at the bile duct end excessive dissection of periductal tissue tension on ductal anastomosis	T-tube tract, excessive use of electrocautery incorrect suture of the cystic duct stump
Stones and clots	Ischemia, stricture, infection	
Biliary cast syndrome	Acute cellular rejection, bile stasis, ischemia, infection, sepsis, HAT	
Haemobilia	Alcoholic liver disease, high body mass index of recipient; Iatrogenic: PTC, liver biopsy	

PSC: Primary sclerosing cholangitis; PTC: Percutaneous; HAT: Hepatic artery thrombosis.

LDLT and DDLT

The rising number of LTs augments the need for liver grafts. This has led to the widespread tendency of LDLT. Multiple factors related to LDLT techniques contribute to the increased incidence of biliary complications[11].

Hepatic resection of the donor liver in LDLT requires dissection of the hilum, which could cause bile duct devascularization or subsequent bile leak from the cut surface of the liver. Excessive use of coagulation diathermy is another risk factor for the occurrence of bile leak. On the other hand, the need for dissection of the recipient's left or right hepatic duct could prolong the ischemic time. Bringing the recipient's hepatic duct to the graft's hilum to ensure tension-free anastomosis could cause additional disturbance of the blood supply. In general, the reported biliary complication rate is 2-3-fold higher in LTLD than in DDLT. Furthermore, the treatment is usually more complicated due to the smaller size of the ducts or the presence of multiple anastomoses. Therefore, the success rate of treatment for complications is lower in LDLT[14,17-19].

Classification

The most common complications are strictures, leaks, and biliary stones. According to the timeframe of their occurrence, post-LT complications can be divided into early (occurring within the first 4 wk after transplantation) and late. Biliary leaks are the most common complication in the early postoperative period, while biliary strictures are the predominant complication as a whole. According to the lesion location, strictures and leaks are divided into anastomotic and non-anastomotic[20-22].

It is appropriate to make a distinction between biliary stricture and biliary obstruction. While the obstruction can be caused by external compression (biloma, haematoma), luminal cast, stones or tube remnants, the stricture is narrowing of the duct lumen, causing bile outflow disturbance.

Multiple factors can play a role in the occurrence of biliary complications. Anastomotic lesions are mostly due to technical issues, while non-anastomotic lesions are the result of ischemia or immune reactions[23].

With respect to the etiology, some authors divide the complications into five groups[21]: (1) Hepatic artery thrombosis-related; (2) Technical biliary complications; (3) Ischemic-type biliary lesions; (4) Infectious biliary complications; and (5) Uncommon: Sphincter of Oddi dysfunction (SOD), bile cast syndrome, haemobilia, lymphoproliferative disease, and other neoplasms.

Biliary strictures

Up to 50% of post-LT biliary complications consist of biliary strictures[24]. They are divided into two major morphological types: Anastomotic (AS) and non-anastomotic (NAS).

Most frequently, the strictures are anastomotic. AS appear more often in LDLT than in DDLT. They are short, single narrowings, located at the anastomotic site. The incidence ranges between 5%-15% in DDLT and 13%-36% in LDLT[21-26]. They occur mostly during the first year after transplantation within a mean time of 5-8 wk[23,27].

The most common factors associated with AS are surgical issues over the first months and ischemia leading to fibrous healing at the later stages. Additionally, ABO

incompatibility, advanced recipient age, small bile duct caliber, prolonged warm and cold ischemia time, and cytomegalovirus infection are reported to be significant risk factors[25,28-30].

Endoscopic retrograde cholangiography (ERCP) is the standard of care for AS treatment, whenever anatomy allows it. The overall reported success rate ranges between 70%-100%[31-33].

For patients with hepatico-jejunostomy, different scopes such as single or double balloon enteroscope, spiral enteroscope or pediatric colonoscope are used. These techniques are time-consuming and complex; they require additional expertise and are related to higher risk and higher cost[32,34-37].

For all these reasons, PTC is a widely accepted approach in cases of altered anatomy[38]. Surgical therapy is now used as salvage therapy and is required in about 1% of cases[39].

AS treatment aims to normalize bile outflow through the anastomosis. The endpoint of ERC is lack of narrowing during occlusive cholangiography or free contrast outflow during fluoroscopy (Figure 1). Clinical and laboratory resolution of cholestasis are the most reliable measures of successful treatment.

The standard treatment includes guidewire insertion across the stricture, followed by balloon dilation and stent insertion. Most commonly 10Fr or 7Fr plastic stents are used. These stents can be easily removed or replaced. Balloon dilation in itself is effective as a non-invasive technique, which has shown less than promising long-term results with a 30%-40% success rate[34,40] (Figure 2).

Numerous large studies have proven that the combination of balloon dilation plus stent placement is more effective than dilation or stenting alone[41].

Several endoscopic strategies are applied in the management of anastomotic strictures. The most frequently used technique is balloon dilation with placement of a maximum number of 10Fr plastic stents with subsequent stent exchange until full resolution of the stricture on fluoroscopy (Table 2).

The initial dilation requires 4-10 mm balloons. In rare cases of tight strictures a Soehendra catheter can be used to overcome the stricture. The progressive increase in the number of stents with every subsequent procedure has ensured more sustained resolution of the stricture[42,43].

Different time intervals between stent exchanges were investigated. In a study from 2008, a short-term stent exchange of every 2 wk was investigated. The reported resolution rate was 87%, achieved for a mean period of 107 d and a mean number of stents inserted of 2.5. More often stents are replaced every three months to prevent occlusion and cholangitis. The reported success rate in many large studies is 80%-95%[39,41-43]. In a review of 440 post-LT patients with AS, the success rate of stent therapy was 84%. The resolution rate was established to be dependent on therapy duration and was highest (94%-100%) when therapy lasted 12 mo or more[44].

The time it takes for the structure to evolve has also been proved to be a predictive factor for healing. Strictures manifested within the first 6 mo after LT have better prognosis for sustained resolution[25,31,45].

Due to elevated rupture risk, it is preferable for ERC to be postponed at least one month after the transplantation. When necessary, a 7-8.5 Fr stent is applied without balloon dilation. In tight strictures, a 4 mm angioplasty balloon may be considered[46].

Some new dilation balloons have been tested in order to improve bile stricture resolution. There are few published data on the usage of peripheral cutting balloons[47].

Paclitaxel-eluting balloons have been investigated, due to the fact that paclitaxel can suppress fibrotic proliferation[48]. The latter two are not in common use.

An available alternative to the standard multiple-plastic-stent therapy is the placement of fully covered self-expanding metal stents (fSEMS). Their major benefits are a reduction in the number of procedures and cost-effectiveness[49-52].

In a substantive study with 200 cases, the reported success was 80%-95%[51]. Eight and 10 mm SEMS are available according to the stricture size. FSEMS are not considered suitable for AS smaller than 5 mm[24].

Stent migration is the major limitation of this technique. The main strategies to prevent migration include skipping dilation of the stricture, using stents with flaps, and leaving the stent in the duodenum for a long period[49-51].

A large systematic review, published in 2013, reported a migration rate of fSEMS of 16%; the authors also mentioned a low risk of stent ingrowth and stent impaction. The comparison analysis in that study showed that neither technique was superior[49,53].

Management in LDLT is more challenging due to the frequent presence of multiple anastomoses with a smaller size (Figure 3). According to Coté *et al*[24], significant risk factors for treatment failure in LDLT are higher LT recipient age, longer operation duration, and a pouched morphology of the AS.

Table 2 Studies on the effectiveness of maximal stent therapy in post-operative biliary strictures

Ref.	Patients	Treatment duration	Mean number of stents	Number of ERCPs	Success rate
Costamagna *et al*[41], 2001	45	12.1 mo (range 2-24 mo)	3.2 (range 1-6)	4.1 (range 2-8)	89% (40/45)
Hsieh *et al*[23], 2012	41	5.3 (range 3.8-8.9)	7.0 (range 4-10)	4.0 (range 3.0-5.3)	100% (41/41)
Morelli *et al*[43], 2008	38	107 d (range 20-198 d)	2.5 (range 1-6)	3.4 (range 2-6)	87% (33/38)
Pasha *et al*[90], 2004	25	3.3 mo (range, 2.2-7 mo)	2.0 (range 1-4)	3.5 (range 1-9)	88% (22 of 25)
Tabibian *et al*[42], 2010	69	15 mo (range 12-60 mo)	3.0 (range 2-7)	2.5 (range 2-5)	94% (65/69)

ERCP: Endoscopic retrograde cholangiography.

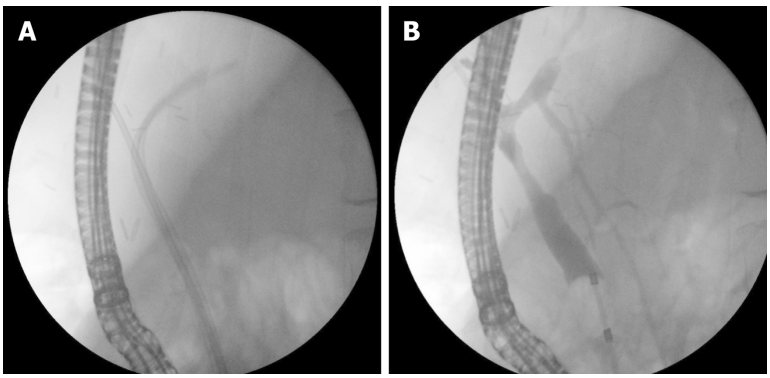

Figure 1 Endoscopic treatment of anastomotic stricture after living donor liver transplantation. A: Two plastic stents; and B: Occlusive cholangiogram after treatment.

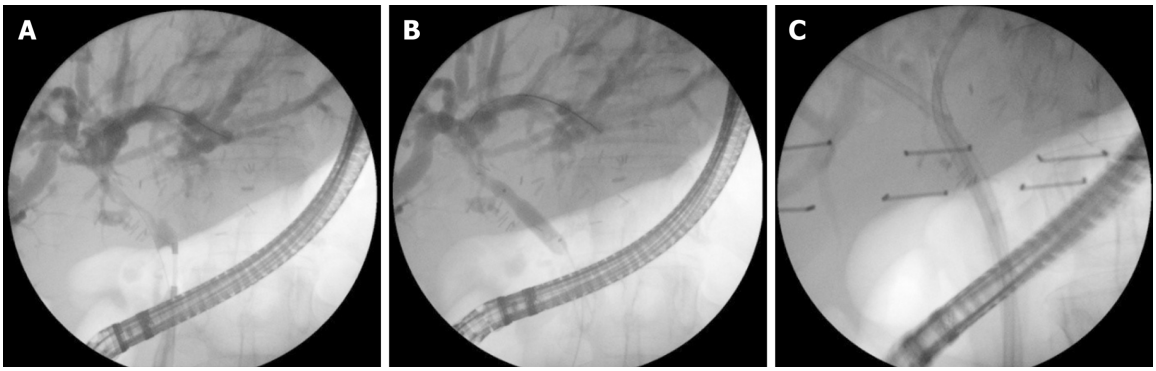

Figure 2 Anastomotic stricture. A: Cholangiogram; B: Balloon dilation; and C: Multiple stent treatment.

Non-anastomotic strictures consist of one or more duct narrowings proximal to the anastomosis. They are longer, complex, and usually multiple, and can affect intra- and extrahepatic ducts. NAS are more rarely observed: 5%-10% of biliary complications[54]. Ischemia and immunological reactions are the main aetiological mechanisms. The most common risk factors reported in the literature are hepatic artery thrombosis, prolonged cold and warm ischemia, prolonged exposure to vasopressors of the donor, ABO incompatibility, chronic ductopenic rejection, PSC or autoimmune hepatitis in the recipient[55,56]. In the case of acute hepatic artery thrombosis, early revascularization therapy is required to prevent multiple stricture formation.

Cases with NAS could benefit from mini-invasive (endoscopic and percutaneous) treatment, but the estimated results are significantly worse than in cases with AS. In cases with dominant strictures and extrahepatic localization ERC is the first treatment option. Endoscopic access to NAS is much more challenging due to the small caliber and relatively proximal location[53]. Cases with angulated, complex strictures, not suitable for ERC passage benefit from percutaneous approaches, followed by hybrid procedures such as the rendezvous technique. When intrahepatic strictures are

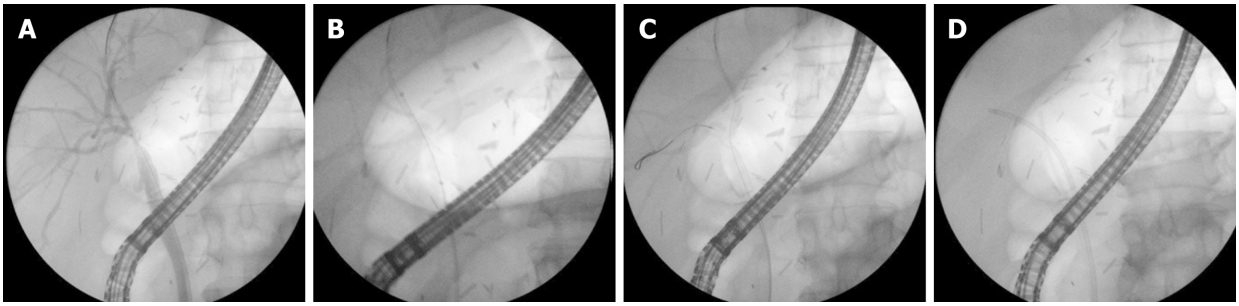

Figure 3 Anastomotic stricture after living donor liver transplantation (right lobe). A: Guidewire insertion; B: Balloon dilation; C: Second guidewire insertion; and D: Stent placement (7Fr + 5Fr).

present, PTC with direct radiology-guided percutaneous stent insertion could be in order[57,58].

Stricture recurrence and continued stricture formation are possible even after successful endoscopic therapy. Long-term observation (MRCP and laboratory) of these patients is required to evaluate the disease course and the response to treatment. Cases resistant to stent treatment, or those with diffuse bile duct injury, must be listed for re-transplantation. Percutaneous drainage could be a bridging therapy to the operation[54,58].

The reported success rate of stenting therapy in the literature is 50% to 75% for DDLT and 33%-50% for LDLT[26,50]. In most NAS cases, the treatment process also takes longer than with AS[11,59]. Passing a guidewire through the stricture is considered the most critical moment. Occlusion balloons and swing-tip catheters for selective cannulation are used for this purpose[54]. The rendezvous technique could also be used to deal with this issue[59,60].

After successful cannulation, the standard technique of balloon dilation followed by plastic stent insertion is performed. For this type of stricture, 4-6 mm balloons with a subsequent increase in caliber are used. Even when cannulated, the width, angulation and proximal location of the strictures often limit the stent insertion. The stents used are usually 7 or 8.5Fr and carry a high migration risk due to rigidity of the plastic[58,59].

A working group from Minnesota reported their treatment for NAS with long (12-20 cm), 10Fr flexible stents with side fenestration. They provide better bile drainage through the stent and through the side holes and could be inserted higher due to their flexibility[61].

Cholangioscopy provides direct visualisation of the biliary tree. This allows visual assessment of the biliary epithelium at the stricture and tissue sampling if needed. In cases of strictures, not suitable for standard cannulation, cholangioscopy enables guidewire insertion under visual control (Figure 4). This facilitates guidewire placement in tight, angulated strictures. Cholangioscopy has been proven to increase the stricture cannulation rate and the success rate of endoscopy treatment as a whole (Figure 5). The implementation of cholangioscopy in stricture therapy could spare the need for percutaneous drainage and surgical interventions[62,63].

Bile leaks

Bile leaks are the second most frequent biliary complication after LT. Bile leaks are also divided into anastomotic and non-anastomotic. Most of them are anastomotic and occur early - within the first 4 wk after LT[8,10,64].

The reported incidence in the literature ranges from 2% to 25%[13,22].

Their occurrence is slightly higher in patients with bilio-enteric reconstruction than with cases of duct-to-duct anastomosis. A systematic review, including data from 61 studies, reported the incidence of bile leaks to be 9.5% in LDLT and 7.8% in DDLT[64]. The presence of a bile leak is an independent risk factor for further development of a stricture[65].

Early bile leaks are usually caused by technical issues related to surgery, such as tension of the anastomosis, incomplete cystic stump suture, excessive use of diathermy, bleeding from the cut ends of ducts, premature T-tube extraction, and the cut surface of the graft. Ischemic injury is the other major cause of bile leaks[8,18,19,52]. Large studies have shown double and triple hepatico-jejunostomy and warm ischemia time as independent risk factors for the occurrence of bile leaks[17,18].

Bile diversion is the key to bile leak healing. Therapeutic options include ERC followed by stenting or nasobiliary drainage, percutaneous drainage, and surgical

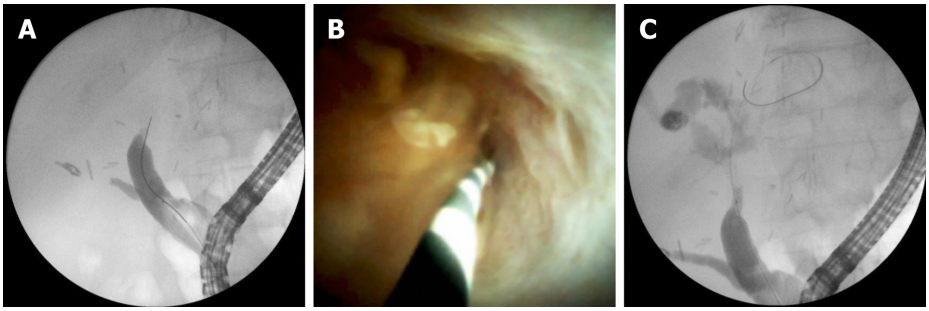

Figure 4 Complex anastomotic stricture. A: Impossible insertion of guidewire through a stricture; B: Guidewire insertion under direct visual control; and C: Guidewire inserted above anastomosis.

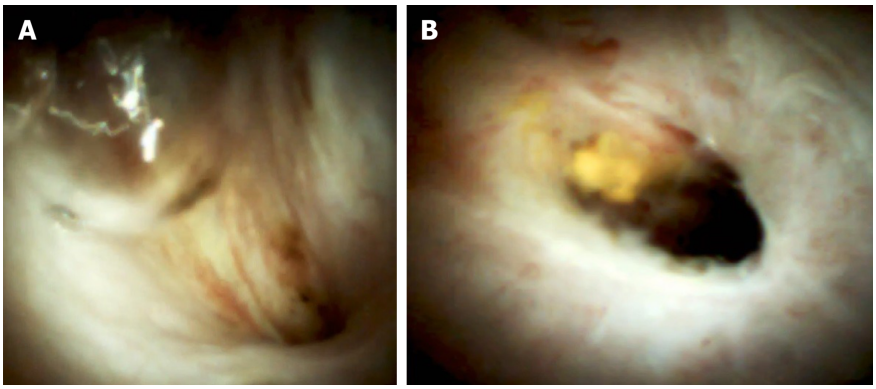

Figure 5 Digital cholangioscopy image of an anastomotic stricture.

revision. Sphincterotomy with endoscopic stenting leads to reduction in the transpapillary pressure, usually followed by fast lesion closure. Stent placement leads to successful treatment in over 90% of cases with early leaks (Figure 6)[13,66]. Simple defects like T-tube exit, cystic duct remnant or small anastomotic leaks usually close in 2-5 wk. The biliary stent is usually extracted after no less than 3 mo due to potentially delayed tissue healing on account of immunosuppression. Some centers prefer the placement of nasobiliary drainage for early small defects. This allows close fluoroscopic follow-up of the defect closure and avoids the need for a second stent extraction procedure. Given the low patient tolerance, displacement risk, and prolonged hospital stay, this practice is currently of limited use[19,66].

In cases with defects, refractory to plastic stent treatment, fcSEMS usage could be considered. Small studies have reported good closure success rate[67-69].

According to a study including 35 cases treated with 8 mm and 10 mm fcSEMS, the achieved leak resolution was 94%[68].

In some studies, a high incidence of stricture was observed after stent removal[70].

In cases with bilio-enteric anastomosis, percutaneous access to the biliary tree is used for bile diversion. An internal-external drainage placement for 3-6 mo is an effective alternative to the endoscopic approach. A technique with EUS-guided gastrostomy, used for ERCP access, is also reported in a small study from 2011[33].

In cases with a T-tube, drainage unclamping is sufficient. When bile juice is diverted outside the body (nasobiliary, percutaneous, T-tube drainage), the level of immunosuppression medication, in particular cyclosporine, should be closely monitored. If a significant collection is formed, the latter must be drained to prevent infection, sepsis, and late adhesion. Large or complex leaks often require surgical revision due to a high probability of intra-abdominal abscess formation[54,55].

Bile stones and sludge

Formation of sludge, clots, casts and stones can cause bile obstruction. The reported incidence after LT ranges widely between 4%-10%[71,72].

Cyclosporine therapy, mucosal damage due to ischemia or infection and cholesterol supersaturation (often seen post-LT) could predispose to lithogenesis. In many cases, there is an underlying stricture. Usually, an ERC and sludge/stone extraction procedure is sufficient for definitive treatment with a success rate over 90%[72-76].

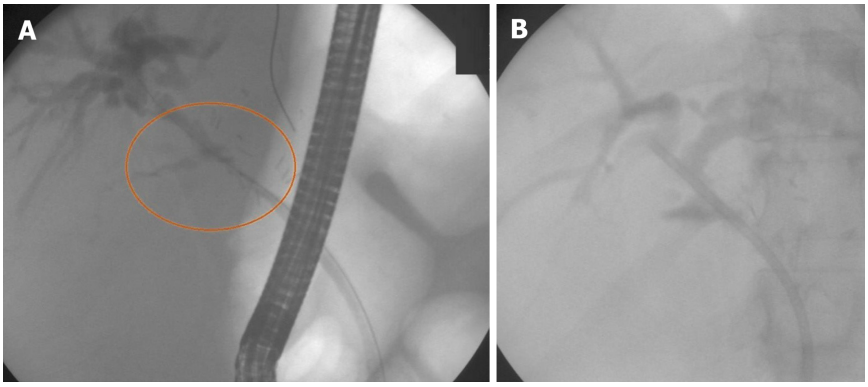

Figure 6 Anastomotic leak. A: Guidewire insertion; and B: Stent placement (10Fr).

According to Alazmi *et al*[45], there is a 17% incidence rate of recurrence within the first 6 mo after the procedure.

Well-known techniques, such as large balloon dilation and mechanical lithotripsy, are used in cases of large stones. In cases of difficult lithiasis such as multiple, large or intrahepatic stones, as well as stones over the stricture, extracorporeal lithotripsy could be applied. A study in 2015 reported six cases of difficult lithiasis that could not be treated with standard ERCP. Five of the six cases were managed with ECSL with no reported adverse events[77].

The limitations of endoscopic therapy can be overcome by means of digital cholangioscopy. Cholangioscopy provides an opportunity for visually controlled fragmentation of large biliary stones with little risk of biliary injury. Advanced intraductal techniques such as Holmium laser or Electrohydraulic lithotripsy achieve outstanding results in difficult cases, not suitable for ERC treatment (Figure 7)[62,78,79].

A research team from South Korea (Nam *et al*[79]) reported a case series of 15 patients (intrahepatic lithiasis n = 10, biliary cast syndrome n = 3, stones over the stricture n = 2) treated with percutaneous intrahepatic cholangioscopy. Eleven patients were successfully managed and no procedure-related adverse events were observed[79].

Biliary cast syndrome

This disorder represents multiple filling defects in intra- and extrahepatic bile ducts, caused by casts adherent to the biliary epithelium. The reported incidence varies between 2.5% and 18%[80,81].

The pathogenetic mechanism is considered to be cell injury as a result of ischemia, acute cellular rejection, chronic rejection, infection, or bile stasis. The desquamated epithelial cells combined with bile components may form hard casts[82].

ERC with bile tree flushing and cast extraction will suffice in many cases. Balloon extractors and Dormia baskets are used for this purpose[81]. In cases of extended intrahepatic involvement or altered anatomy, a percutaneous procedure could be needed. In a study of 10 patients with biliary cast syndrome, mini-invasive (endoscopic/percutaneous) treatment was successful in 60% of cases[83].

Several studies noted good outcomes following cholangioscopy-guided therapy of bile cast syndrome (Figure 8). Nam *et al*[79] reported three cases, treated by percutaneous cholangioscopy.

Ursodeoxycholic acid is considered to have a role in the prophylaxis of bile cast syndrome. In cases refractory to mini-invasive therapy, surgery is required.

Sphincter of Oddi dysfunction

Chronic injury, denervation of the recipient's common bile duct (CBD), or fibrotic tissue formation could cause impaired ampullary relaxation and hypertension of the papilla of Vater. The role of biliary manometry in the diagnosis of SOD after transplantation is uncertain. Sphincterotomy is usually sufficient to resolve the obstruction[27,29]. In cases of firm fibrosis, stent placement could be in order[8,21].

Mucocele

In rare cases, the donor's cystic duct could be incorporated in the suture line of the anastomosis. As a consequence, a blind mucosa-lined sac is formed. Due to accumulation of mucin, this sac can increase in size and cause bile obstruction due to external compression. Endoscopy could not provide sustainable resolution in such

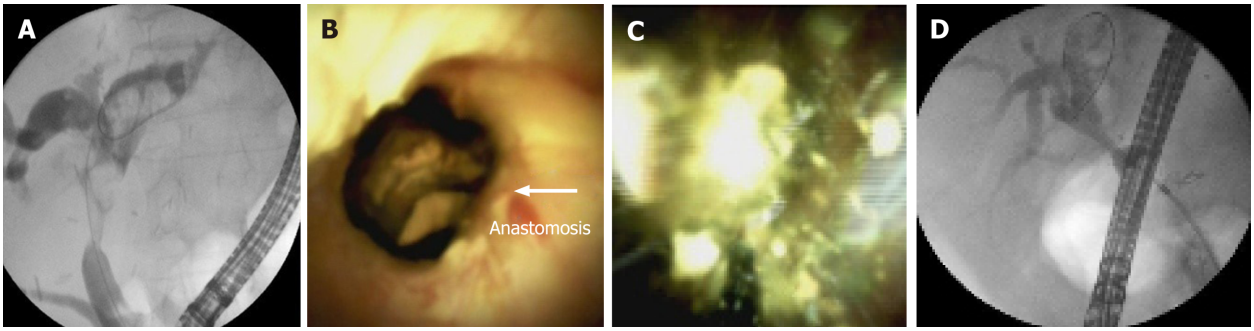

Figure 7 Multiple intrahepatic stones above anastomotic stricture. A: Fluoroscopic image; B: Digital cholangioscopic image; C: Electrohydraulic lithotripsy performance; and D: Fluoroscopic image after treatment.

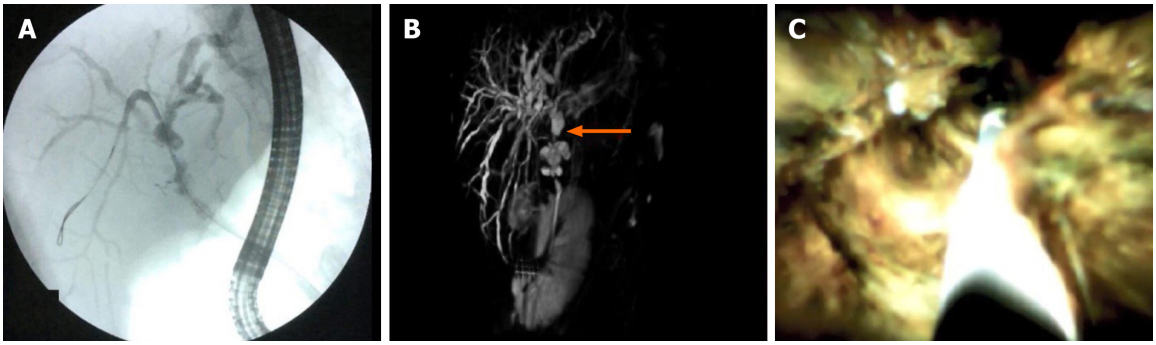

Figure 8 Biliary cast syndrome. A: Fluoroscopic image; B: Magnetic resonance cholangiopancreatography; and C: Digital cholangioscopic image.

cases. Percutaneous drainage or surgical resection are effective treatment options. The differential diagnosis of mucocele is made with any type of fluid collection such as biloma, abscess, hemorrhage, and aneurysm[8,84,85].

Redundant CBD

The excessive length of the donor's common hepatic duct could lead to a sigmoid-shaped deflection of the CBD. This could entail bile outflow deterioration. The reported incidence is 1.6% in all LT. ERC with long plastic stent placement usually resolves cholestasis. In very rare cases, surgery with a new biliodigestive anastomosis is needed[86].

Haemobilia

Spontaneous haemorrhage in the biliary tree after LT occurs rarely with a reported frequency of 1.2%. There are reported cases of haemobilia associated with large biliary stones over the stricture. More often, haemobilia is iatrogenic, *i.e.* subsequent to percutaneous biliary drainage or liver biopsy. Rupture of a hepatic artery pseudoaneurysm can cause severe biliary haemorrhage. Recipient high BMI and alcoholic liver disease were significant risk factors for spontaneous haemobilia reported in a study including 2701 post-LT patients[87].

ERC with clot extraction and nasobiliary drainage placement is the first-choice therapy. Nasobiliary drainage ensures an opportunity for biliary lavage, which prevents the development of cholangitis and indicates the presence of recurrent bleeding. In most cases, the combination of endoscopic desobstruction therapy, coagulation correction, and supportive medication yields good results. In cases of severe haemorrhage, selective embolization techniques are reported to be successful. Plastic biliary stents or fSEMS were reported to be effective haemostatic tools in studies of non-transplant patients with significant haemobilia[88-90].

Due to low incidence, there are not enough data regarding post-transplant patients with severe haemobilia.

Foreign bodies

Suture materials or T-tube remnants could form a nidus for bile sediment and stones. ERC and PTC are effective methods for detection and clearance of bile duct

remnants[59].

CONCLUSION

Known as the Achilles' heel of liver transplantation, biliary complications are observed in one quarter of all patients. Their prevalence has increased due to the worldwide increase in liver transplantation. Living donor liver transplantations have a higher complication rate and presuppose more complicated treatment scenarios with lower success rates. Endoscopic stent insertion is the key treatment for most biliary complications. Percutaneous or EUS-guided puncture and cholangioscopy are feasible options for biliary access when standard fluoroscopic cannulation fails. A wide variety of accessories have been developed to overcome the complexity of living donor liver transplantation complications, but the treatment success rate remains unsatisfactory. Early recognition and aggressive management are essential for the reduction of morbidity and mortality in patients with biliary complications.

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MINIREVIEWS

Shifting perspectives – interplay between non-alcoholic fatty liver disease and insulin resistance in lean individuals

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Abstract

Non-alcoholic fatty liver disease (NAFLD) has become a significant public health burden affecting not only obese individuals but also people with normal weight. As opposed to previous beliefs, this particular subset of patients has an increased risk of all-cause mortality and worse outcomes than their obese counterparts. The development of NAFLD in lean subjects seems to be interconnected with metabolic phenotype, precisely visceral fat tissue, sarcopenia, and insulin resistance. Here, we summarize available data focusing on the co-dependent relationship between metabolic phenotype, insulin resistance, and development of NAFLD in lean individuals, suggesting more appropriate tools for measuring body fat distribution for the screening of patients at risk.

Key Words: Non-alcoholic fatty liver disease; Metabolic phenotype; Lean individuals; Insulin resistance; Visceral fat tissue; Sarcopenia

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Core Tip: The prevalence of non-alcoholic fatty liver disease among non-obese (overweight or lean) individuals seems to be much higher than previously reported, affecting almost 20% of the non-obese population. Non-alcoholic fatty liver disease is no longer considered solely an obesity-related disorder since non-obese individuals participate significantly in this entity. The metabolic phenotype is the key role-player in the development of non-alcoholic fatty liver disease in lean individuals. The detection of lean patients with non-alcoholic fatty liver disease is particularly challenging since the body-mass index is not a good indicator of metabolic health.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), recently known as metabolic-associated fatty liver disease[1], is one of the most common causes of chronic liver disease. NAFLD was traditionally associated with metabolic syndrome encompassing obesity, insulin resistance, hypertension, and atherogenic dyslipidemia[2]. Recently, a new clinical entity, including NAFLD in non-obese/lean individuals has emerged. It soon became apparent that the existence of NAFLD in non-obese subjects should not be neglected since its prevalence has significantly increased. According to a recently published meta-analysis, up to 40% of NAFLD patients are non-obese, with the highest prevalence in western countries as opposed to previous findings dominantly allocating this entity in Asian regions[3]. The clinical consequences of NAFLD can be detrimental; for instance, progression to significant fibrosis remains uncertain as well as long-term cardiometabolic complications and mortality[4-7]. However, prevalence data and terminology are quite variable since definitions used to determine lean and obese patients differ among various studies, depending on Asian or Caucasian cutoff values. In addition, a body mass index (BMI) cutoff value of 25 kg/m^2 is frequently used to differ between lean and obese individuals, thus excluding the overweight population (Table 1). Here, we decided to use terms "non-obese" or "lean NAFLD" depending on the study in question and definitions used.

The recognition of NAFLD in lean individuals is associated with a concept known as the metabolic phenotype. There are separate subgroups of individuals divided according to their phenotype and metabolic profile to metabolically unhealthy normal weight (MUHNW) and metabolically healthy obese (MHO), the latter being disputable due to higher incidence of cardiovascular disease (CVD) in long-term studies[8]. Distinguishing between those phenotypes is based on BMI, an inadequate surrogate marker for determining the quantity of skeletal muscle mass and adipose tissue, especially in the visceral area[9]. As a consequence, a MUHNW individual could be a person with sarcopenia and a high proportion of fat tissue, with a high probability of developing insulin resistance and/or metabolic syndrome (MetS), subsequently leading to the development of NAFLD[10]. In addition, other factors could be involved in the pathogenesis of NAFLD in lean subjects such as genetics [*e.g.*, patatin-like phospholipase domain-containing 3 (*PNPLA3*) variant (rs738409 C/G)][11], environmental factors including dietary habits[12,13] and physical activity[14], changes in gut microbiota[15], and secondary causes such as hypothyroidism or polycystic ovary syndrome.

Lean NAFLD patients were traditionally considered to have milder metabolic disturbances, thus carrying a lower risk for the development of CVD and progression to non-alcoholic steatohepatitis (NASH) and fibrosis[6,16,17]. However, recent data suggest that progression to diabetes as well as NASH and fibrosis is higher in lean NAFLD individuals, undoubtedly linking visceral fat tissue with undesirable consequences of MUHNW phenotype[5,10,18,19]. Still, a contribution of specific components of MetS to fibrosis remains unclear, although insulin resistance seems the most probable culprit[20-22], Table 1.

In this critical review, we summarized available data and addressed practical issues

Table 1 Prevalence, characteristics, and outcomes in lean/non-obese individuals with non-alcoholic fatty liver disease

Author, year	Population, study design, sample size	Prevalence of NAFLD in lean subjects	Main findings
Zou *et al*[4], 2020	Mixed population, 1999-2016 NHANES databases	32.3% overall NAFLD prevalence; 22.7% obese and 9.6% non-obese; Amongst NAFLD patients, 29.7% were non-obese (Caucasian BMI 25-30 kg/m^2, Asian BMI 23-27 kg/m^2), of which 13.6% had lean NAFLD (Caucasian BMI < 25 kg/m^2, Asian BMI < 23 kg/m^2)	Non-obese NAFLD individuals had higher 15-year cumulative all-cause mortality (51.7%) than obese NAFLD (27.2%) and non-NAFLD (20.7%)
Huang *et al*[20], 2020	2483 Asian participants, community based study	44.5% NAFLD and 15.8%, MetS prevalence; Among NAFLD subjects, 48.8%were obese (BMI ≥ 24 kg/m^2)	IR is predictive of NAFLD irrespective of BMI; CV risk calculated by Framingham Risk Score may exist in lean NAFLD subjects
Tobari *et al*[18], 2020	Asian, biopsy-proven 762 NAFLD patients, cross sectional study	Over 25% men and almost 40% women were non-obese, but most of them had visceral fat obesity and/or IR; BMI cutoff 25 kg/m^2	NAFLD was not milder in non-obese patients; Histological steatosis was associated with BMI; Advanced fibrosis was not associated with BMI and showed a significant sex difference
Kim *et al*[10], 2020	664 Asian subjects with biopsy-proven NAFLD and controls, cross sectional study	542 subjects with biopsy-proven NAFLD132 non-obese NAFLD (BMI < 25 kg/m^2) ; 410 obese NAFLD (BMI > 25 kg/m^2) ; 122 controls	Non-obese subjects with NAFLD displayed a similar severity of histological liver damage; Sagittal abdominal diameter was independently associated with significant fibrosis among subjects with non-obese NAFLD
Alferink *et al*[71], 2019	4609 elderly European, population based study	1623 had NAFLD (n = 161 normal-weight and n = 1462 overweight, BMI cutoff 25 kg/m^2)	Both high fat mass and low SMI were associated with normal-weight NAFLD; Fat distribution (assessed by AGR) could best predict NAFLD prevalence
Denkmayr *et al*[19], 2018	European, 466 patients diagnosed with NAFLD, cross sectional study	Lean (BMI ≤ 25.0 kg/m², n = 74) ; Overweight (BMI > 25.0 ≤ 30.0 kg/m², n = 242) ; Obese (BMI > 30.0 kg/m², n = 150)	Lean NAFLD patients had a histological picture similar to obese patients but more severe compared to overweight patients.
Gonzalez-Cantero *et al*[21], 2018	European, cross-sectional study 113 non-obese, non-diabetic individuals	55 patients diagnosed with NAFLD; NAFLD defined as hepatic triglyceride content > 5.56% (quantified by 3T H1-MRS) ; BMI cutoff 25 kg/m^2	Lean-with-NAFLD group had significantly higher HOMA-IR and lower serum adiponectin than the overweight-without-NAFLD group; IR was independently associated with NAFLD but not with waist circumference or BMI
Hagström *et al*[5], 2017	European, prospective cohort study of 646 patients with biopsy-proven NAFLD	19% lean NAFLD; 52% overweight NAFLD; 29% obese NAFLD; BMI cutoff 25 and 30 kg/m^2	Lean NAFLD had lower stages of fibrosis and higher risk for severe liver disease development compared to patients with NAFLD and a higher BMI, independent of available confounders (follow-up 19.9 years)
Leung *et al*[6], 2017	Asian, prospective, 307 NAFLD patients	23.5% were non-obese; BMI cutoff 25 kg/m^2	Non-obese NAFLD patients have less-severe disease and may have a better prognosis than obese patients; Hypertriglyceridemia and higher creatinine are the key factors associated with advanced liver disease in non-obese patients
Fracanzani *et al*[11], 2017	European, retrospective cohort study of 669 patients with biopsy-proven NAFLD	143 patients had BMI < 25 kg/m^2 and NAFLD	20% of patients with lean NAFLD have NASH, fibrosis scores of 2 or higher, and carotid atherosclerosis
Feldman *et al*[22], 2017	Caucasian, cross sectional, 187 subjects with hepatic steatosis on ultrasound	Lean healthy (BMI ≤ 25 kg/m^2, no steatosis, n = 71) ; Lean NAFLD (BMI ≤ 25 kg/m^2, steatosis, n = 55) ; obese NAFLD (BMI ≥ 30 kg/m^2, steatosis; n = 61)	Lean NAFLD have impaired glucose tolerance, low adiponectin concentrations and an increased rate of PNPLA3 risk allele carriage
Feng *et al*[7], 2014	Asian, population based, 1779 participants	The prevalence of NAFLD was 18.33% in the lean group and 72.90% in the overweight-obese groupBMI cutoff 24 kg/m^2	Lean-NAFLD was more strongly associated with diabetes, hypertension, and MetS than overweight-obese-NAFLD; NAFLD patients were more likely to have central obesity especially in lean groups
Younossi *et al*[17], 2012	Mixed population, 1988-1994 NHANES databases	2185 (18.77% ± 0.76%) of subjects had NAFLD; 7.39% ± 0.65% had lean NAFLD; 27.75% ± 1.00% had overweight/obese NAFLDBMI cutoff 25 kg/m^2	Lean NAFLD was independently associated with younger age, female sex, and a decreased likelihood of having IR and hypercholesterolemia
Margariti *et al*[16], 2012	European, cross sectional, 162 NAFLD patients	Normal BMI was present in 12% of patients; BMI cutoff 25 kg/m^2	Lean NAFLD patients do not have IR-associated metabolic disorders, but they have higher levels of ALT/AST than the overweight or obese NAFLD patients

3T H1-MRS: 3Tesla H1-magnetic resonance spectroscopy; ALT: Alanine aminotransferase; AGR: Android gynoid ratio; AST: Aspartate aminotransferase; BMI: Body mass index; CV: Cardiovascular; IR: Insulin resistance; MetS: Metabolic syndrome; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-

alcoholic steatohepatitis; SMI: Skeletal muscle index.

of whether it is time to shift perspectives away from the scale and how to screen for non-obese patients with a metabolically unhealthy profile.

METABOLIC PHENOTYPE - THE KEY ROLE PLAYER IN THE DEVELOPMENT OF NAFLD IN LEAN INDIVIDUALS

Obesity is generally associated with severe health consequences, mainly related to increased cardiovascular risk[8]. However, a subset of obese patients will never develop cardiovascular disease and is therefore considered an MHO. Conversely, metabolically unhealthy patients exist even in the group of normal-weight people, the category known as the MUHNW. People with this phenotype seem to have 1.5 to 3-times higher risk for cardiometabolic complications than metabolically healthy normal-weight people and even higher risk than MHO[23,24], but unfortunately often go under the radar for cardiovascular screening and primary outcome prevention.

Generally, the assessment of cardiovascular risk, regardless of the patient's BMI, was historically mainly based on the presence of the MetS. However, according to data from prospective studies, only a smaller proportion of individuals in the normal-weight category with cardiovascular events have MetS compared to patients with cardiovascular events who were overweight or obese (20% compared to 52% and 76%, respectively)[25]. Although MetS as such might not be an accurate predictor of CV risk in normal-weight individuals, its components, especially, lipids and glucose level, as well as waist circumference and waist-to-hip ratio might be useful for risk stratification[9,26,27]. On the other hand, up to 30% of normal-weight individuals can be classified as metabolically obese normal weight having an increased cardiometabolic risk.

It seems that the distribution and health of fatty tissue, rather than its amount, is likely the major determinant of disease risk. For example, higher amounts of visceral fat compared to peripheral and subcutaneous fat comprise a higher metabolic risk and are directly linked to both liver inflammation and fibrosis, independently of insulin resistance and hepatic steatosis[24,28-30].

Some previously published studies have failed to show an association of insulin resistance and NAFLD in lean individuals[16,17]. However, more recently published studies have demonstrated the opposite, linking insulin resistance with the development of NAFLD, irrespective of BMI[10,20-22].

In a study published by Kim *et al*[10] comparing non-obese with Mets and obese without MetS, the ratio of visceral adipose tissue area-to-subcutaneous adipose tissue area (VAT/SAT) was independently linked with NASH or fibrosis in a dose-dependent manner, confirming that metabolic phenotype is crucial in the progression of liver disease, irrespective of the presence of obesity. Lean with MetS were non-obese, had insulin resistance, and an increased VAT area[10]. Another community-based study in the Asian population demonstrated that insulin resistance was a significant predictive factor for NAFLD in both obese and lean subjects[20].

Obviously, metabolic disturbances are responsible for disease progression, with insulin resistance being a key role player (Figure 1). The mechanisms involved seem to be similar as in obese individuals[22]. Higher levels of free fatty acids, enhanced adipose tissue lipolysis, and decreased fat storage capacity of subcutaneous fat tissue overcome fatty acid oxidation and triglyceride secretion leading to the accumulation of triglycerides in hepatocytes[23,31]. An increase in lipotoxicity causes pronounced oxidative stress[32], whereas chronic inflammation is continuously being fueled by changed adipokine secretion from visceral adipocytes, primarily decreased adiponectin secretion together with mitochondrial dysfunction leading to further liver injury[23,31].

Some of the major game-changers determining the nature of metabolic profiles are dietary intake and physical activity. To date, published data indicate a correlation between weight gain in non-obese individuals with the development of NAFLD[12,13], suggesting that calorie intake and modest weight gain in non-obese individuals have deleterious effects on metabolic disturbances primarily through an increase in visceral adipose tissue. Conversely, waist circumference and body weight reduction achieved through lifestyle intervention were independent predictors of NAFLD resolution in lean subjects[33]. Furthermore, sarcopenia is positively correlated to insulin resistance in

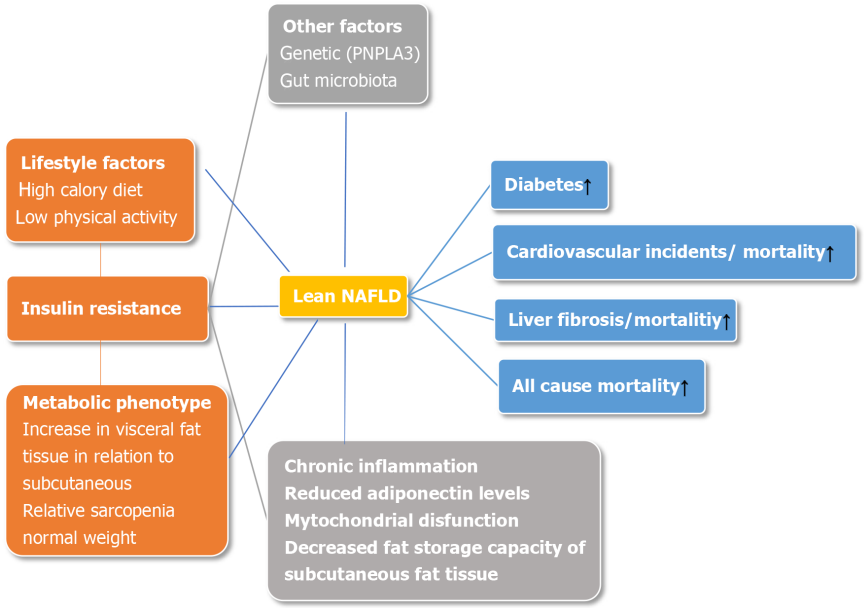

Figure 1 Pathophysiological mechanisms and outcomes of non-alcoholic fatty liver disease in non-obese individuals. NAFLD: Non-alcoholic fatty liver disease.

obese patients and is considered one of the major factors responsible for the obesity paradox[14]. The potential mechanisms involved are the accumulation of intramyocellular lipid and intermuscular adipocytes, chronic inflammation, and loss of insulin sensitivity to protein synthesis preceding insulin resistance to glucose metabolism[34]. Thus, we could hypothesize that the unfavorable ratio of skeletal muscle mass and visceral adipose tissue in non-obese individuals is one of the main determinants of insulin resistance. Indeed, it has been shown that physical activity increases skeletal muscle mass, thus improving sarcopenia and lean/fat tissue mass ratio advancing metabolic health in non-obese individuals through the reduction of insulin resistance[18,35].

OTHER RISK FACTORS INVOLVED IN THE DEVELOPMENT OF NAFLD IN LEAN INDIVIDUALS

Compared to obese and overweight NAFLD patients, some clinical, biochemical, and histological distinctions have been observed in lean NAFLD subjects, going far beyond the simple differences in the BMI. Specifically, low adiponectin levels and high concentrations of proinflammatory cytokines suggest a pronounced degree of adipose tissue dysfunction and distinct metabolic and gut microbiota profiles[11,19,36-38]. Additionally, impaired glucose metabolism and carriage of the *PNPLA3* minor allele was seen in lean Caucasian NAFLD patients[22].

Genetic factors

Several genes and single-nucleotide polymorphisms (SNPs) associated with NAFLD have been identified, of which transmembrane 6 superfamily member 2 (*TM6SF2*)[39-41] and the patatin like *PNPLA3*[42-44] are the most investigated ones.

The rs58542926 genetic variant of *TM6SF2* gene, which encodes the E167K aminoacidic substitution and determines neutral fat accumulation in the liver, has been implicated in NAFLD development. Previous studies suggested a significant association between the *TM6SF2* polymorphism and disease severity and/or progression[39,41].

The rs738409 genetic variant of the *PNPLA3* gene, which takes part in lipid transformation, is now recognized as the major genetic determinant of NAFLD. A meta-analysis based on 23 case-control studies involving 6071 NAFLD patients and 10366 controls showed that *PNPLA3* rs738409 polymorphism is associated with disease severity and progression and that these changes were not influenced by the ethnicities or age of subjects[45]. In addition, Shen and al. demonstrated that the G allele in

PNPLA3 rs738409 increases the risk of NAFLD, especially in subjects without MetS, independent of dietary pattern and metabolic factors[46].

Genetic background for developing NAFLD in the absence of obesity has also been investigated in different populations. Initial reports on NAFLD in lean individuals originated mostly from an Asian background[7,47,48], and implicated Asian ethnic preponderance. However, "non-obese" NAFLD makes just over 40% of the NAFLD population and is common in both eastern and western countries[3].

Earlier studies in Asian populations found that the G allele at the *PNPLA3* rs738409 mutation has been more common in lean than obese NAFLD patients (78.4% *vs* 59.8%; $P = 0.001$)[49]. However, a study investigating the prevalence of metabolic co-morbidities and *PNPLA3* risk alleles (GG) in the Japanese population did not confirm the difference among the non-obese, obese, and severely obese groups of both sexes[18]. Similarly, a recently published study in the Chinese population found no difference in the SNPs of several genes (*SIRT1*, *APOC3*, *PNPLA3*, *AGTR1*, and *PPARGC1A*) between lean subjects with and without NAFLD[50].

In the Caucasian population, Feldman *et al*[11] showed a high rate of *PNPLA3* risk alleles (CG/GG) in the lean NAFLD group compared with lean controls (odds ratio [OR] 2.676, $P = 0.007$), but at a comparable rate to obese NAFLD subjects (OR 0.759, $P = 0.464$)[22]. Another study investigating gene polymorphisms in the Caucasian population demonstrated that in lean NAFLD subjects, the only independent variable associated with NASH and significant fibrosis (≥ 2) was the GG *PNPLA3* polymorphism[11]. In addition, in lean NAFLD patients, a significantly higher prevalence of *TM6SF2* E167K variant carriers was associated with more severe steatosis, inflammation, and NASH.

Gut microbiota

The human gut microbiota (GM) forms a complex ecosystem involving different microorganisms (bacteria; dominated by four bacterial phyla: Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria[51], viruses, uni/pluricellular eukaryotes) that have been implicated in various physiological processes[52]. The impact of diet on GM composition and function is well established, and alterations in the microbiome composition have been associated with the development of obesity, diabetes, MetS and NAFLD[15,53,54]. Previous studies have identified that NAFLD patients have altered microbiome with fewer proportions of Bacteroidetes and higher proportions of Porphyromas and Prevotella than healthy individuals[55,56]. Moreover, an increase in *Lactobacillus, Escherichia, Streptococcus* abundance, decrease in *Ruminococcaceae*, and *Faecalibacterium prausnitzii,* have also been identified in NAFLD patients[57-59].

In addition, substantial differences in fecal and blood microbiota profiles between obese and lean individuals with NAFLD have been identified in the Asian population[18]. Similarly, a Brazilian study confirmed a specific gut microbiota composition in lean NASH patients, showing a lower abundance of *Faecalibacterium* and *Ruminococcus*, and a deficiency in Lactobacillus compared with overweight and obese NASH patients[60]. These differences in microbiota composition between lean and obese NAFLD patients may serve as biomarkers for identifying the specific metabolic NAFLD phenotype.

AVOIDING PITFALLS IN THE DIAGNOSIS OF LEAN NAFLD

After publishing a meta-analysis on metabolic health, which suggested the highest CV risk among individuals of normal weight who are metabolically unhealthy (response rate [RR] 3.14, 95% confidence interval [CI] 2.36-3.93)[61], Kramer *et al*[61] raised the need to phenotype metabolically unhealthy individuals.

Currently, definitions of metabolic health are not unique (Table 2). Sometimes they include either the absence of insulin resistance[62,63], or the absence of insulin resistance and low C-reactive protein (CRP) levels as a surrogate marker for inflammation, in combination with up to any two parameters of MetS[64,65]. In clinical practice, only the latter are used[66,67].

The study by Stefan *et al*[23] (2017) was the first head-to-head comparison of cardiometabolic risk phenotypes suggesting that metabolically unhealthy lean people mainly have insulin secretion failure, insulin resistance, and increased carotid intima-media thickness. Among the aforementioned, insulin resistance is the most widely used cardiovascular risk marker. Metabolically unhealthy normal-weight subjects (defined by a BMI < 25 kg/m^2 and presence of insulin resistance), compared to their

Table 2 Definitions of metabolic health in non-obese

Definitions of metabolic health in non-obese individuals:	
Absence of insulin resistance	Meigs *et al*[62]; Stefan *et al*[63]
Absence of insulin resistance and low CRP levels as a surrogate marker for inflammation, in combination with up to any two parameters of metabolic syndrome	Wildman *et al*[64]; Karelis *et al*[65]
Combination with up to any two parameters of metabolic syndrome	Stefan *et al*[66]; Phillips[67]
Definition of metabolically unhealthy non-obese individuals:	
BMI < 25 kg/m^2 and presence of insulin resistance	Stefan *et al*[23]
Waist circumference adjusted for BMI and/or android gynoid ratio and presence of insulin resistance	Suggested by authors

BMI: Body mass index; CRP: C-reactive protein.

healthy counterparts, in addition to elevated CV risk, have an elevated risk of colorectal cancer (OR = 1.59, 95%CI: 1.10-2.28)[68].

As already mentioned, BMI is an inadequate surrogate marker of metabolic health, especially in determining the ratio of visceral and subcutaneous fat tissue, the most important risk factors of NAFLD's insulin resistance and progression in lean individuals[10]. In addition, data on muscle mass are missing, thus providing no information on sarcopenia[69], which is clinically relevant in the development of NAFLD in lean patients. Thus waist circumference and/or waist-to-hip ratio might be a better tool. However, waist circumference is mostly dependent on BMI, meaning that normal-weight patients could have waist circumference in the normal range, but still have higher visceral fat tissue and increased cardiometabolic risk[9]. This issue could be avoided by using waist circumference adjusted for BMI, which has shown a strong linear increase in risk for cardiovascular mortality[70], but no data are available on the association of adjusted waist circumference and NAFLD in lean individuals.

Additionally, in an elderly population-based study, both high-fat mass and low skeletal muscle index were associated with normal-weight NAFLD, although fat distribution assessed by the android gynoid ratio was the best predictor of NAFLD prevalence[71].

CLINICAL AND THERAPEUTIC IMPLICATIONS OF NAFLD IN LEAN INDIVIDUALS ASSOCIATED WITH INSULIN RESISTANCE

The liver-related and general outcomes of patients with NAFLD depend on a number of factors including the presence of metabolic risk factors, especially type 2 diabetes mellitus and hypertension, severity of fibrosis, genetic predisposition, age, diet and other environmental factors.

Metabolic consequences

Regarding metabolic health and clinical outcomes, cardiometabolic complications take the most prominent place in driving the mortality. It seems that metabolically unhealthy, regardless of BMI, including individuals within the normal range of BMI category, have the highest risk of cardiometabolic consequences[72]. Moreover, in a recently published study, normal-weight patients with central adiposity and coronary artery disease had a worse survival rate than normal, overweight, or obese subjects without central obesity[73]. However, long term studies in lean NAFLD patients and cardiovascular health are lacking. In a retrospective study of lean Caucasian patients with biopsy-proven NAFLD *vs* obese or overweight individuals, 20% of patients who were lean developed NASH, significant fibrosis, and carotid atherosclerosis[11].

A study by Feng and coauthors addressed the question of metabolic consequences and laboratory discrepancies in lean subjects with NAFLD. Compared to obese and overweight NAFLD counterparts, lean Chinese NAFLD individuals had a higher risk of developing diabetes (OR = 2.47, 95%CI: 1.14-5.35), hypertension (OR = 1.72, 95%CI: 1.00-2.96) and MetS (OR = 3.19, 95%CI: 1.17-4.05), making them prone to the development of cardiovascular disease[7].

In terms of mortality, the higher fat mass could be associated with better nutritional

state associated with higher survival rates (also known as obesity paradox); thus, lean individuals with the more severe and advanced liver disease could have a poor prognosis, especially if sarcopenia is present[74]. This was confirmed in a recently published meta-analysis, encompassing 93 studies including lean NAFLD individuals, demonstrating that all-cause mortality, liver-related mortality, and cardiovascular-related mortality in non-obese individuals with NAFLD was higher than that of obese individuals with NAFLD (12.1 *vs* 7.5 per 1000 person-years; 4.1 *vs* 2.4 per 1000 person-years; 4.0 *vs* 2.4 per 1000 person-years respectively)[3].

In addition, NHANES based study demonstrated that non-obese NAFLD individuals had increased 15-year cumulative all-cause mortality (51.7%) compared to obese NAFLD (27.2%) and non-NAFLD (20.7%) patients[4].

Therefore it seems that NAFLD in lean individuals has serious cardiometabolic complications leading to an increase in mortality, even higher than in their obese counterparts.

Liver consequences - fibrosis, cirrhosis and cancer

Non-alcoholic fatty liver disease encompasses a spectrum of histological changes with different evolution and outcomes, ranging from simple steatosis to NASH with varying degree of fibrosis. The later entity is characterized by lobular inflammation and hepatocyte ballooning degeneration accompanied by various stages of fibrosis that more often progresses to cirrhosis. However, fibrosis can be found in liver biopsy specimens in the absence of significant inflammation; in a recent multicenter study from Italy and Finland, 34% of patients with significant fibrosis did not have NASH and 10.0% had no inflammation[75].

Currently there are no published data on the specific inflammatory pathways or hepatic stellate cells activation pathways that would be unique to the development of NASH in lean patients as opposed to obese NASH patients. It is therefore believed that progression of NASH in lean individuals follows pathways similar to those demonstrated in obese patients with NASH, and that rate of progression probably depends on the similar risk factors as in their obese counterparts[76].

In general, NAFLD is a slowly progressive disease, but more rapid progression occurs in 20% of patients[77]. In a meta-analysis of over 400 patients with paired liver biopsy, 34% of NAFLD patients had fibrosis progression, 43% had stable fibrosis, and 22% showed an improvement in the fibrosis stage during follow-up[77]. The rate of progression was doubled in the presence of arterial hypertension[77]. The data on the natural history and prognosis of lean patients with NAFLD remains conflicting. Although better or similar metabolic and histological profiles than in obese NAFLD patients are mainly suggested, long term liver related outcomes remain an open question[5,6,19].

In a retrospective cohort study from Italy, significantly lower proportions of lean NAFLD patients had NASH (17% *vs* 40% of obese or overweight patients), and significant fibrosis of F2 or more (17% *vs* 42% for obese/overweight NAFLD patients)[11]. However, lean patients with high waist circumference had increased risk of significant fibrosis of F2 or more, compared to overweight/obese subjects with the same waist circumference[11]. A study from two university centers from Sweden with a median follow-up of 20 years reported that 50% of lean patients had NASH compared to 65% and 80% of overweight and obese subjects[5]. Yet, lean patients with NAFLD had slightly more events of severe liver disease (defined as decompensated liver disease, liver failure, hepatocellular carcinoma, or cirrhosis) compared to overweight patients (16% *vs* 9%), but similar to obese patients (14%)[5]. The main finding of the study was that although lean patients had a better prognostic profile at baseline with less advanced fibrosis and NASH, an increased risk for the development of severe liver disease was found compared to patients with a higher BMI[5].

In a study from Hong Kong, non-obese patients had lower NAFLD activity score and lower fibrosis stages compared to obese patients[6]. In a recently published meta-analysis, 39% of non-obese or lean NAFLD patients had NASH (compared to 53% of obese individuals), 25% had significant lobular inflammation (compared to 36% of obese), 29% had significant fibrosis of F2 or more (compared to 38% of obese individuals), and 3% had cirrhosis in one study[3]. However liver related mortality was higher in non-obese NAFLD subjects compared to obese equivalents (4·1 per 1000 person-years *vs* 2·4 per 1000 person-years)[3].

Additionally, in a study published by Kim *et al*[10] progression to NASH and fibrosis was equally present in non-obese patients with MetS and obese patients without MetS (55%-60%) linking metabolic phenotype with the liver disease progression.

Cirrhosis of any etiology is a well-known risk factor for the development of hepatocellular carcinoma (HCC); the same is true for NAFLD-induced cirrhosis. The

reported incidence of HCC development in patients with NAFLD varies significantly depending on the study population, ranging from 0.25% to 11% after 5 years[78,79]. Furthermore, in a significant proportion of patients, ranging from 23% to 46%, HCC has been reported to develop in the earlier stages of the disease, before the development of cirrhosis[80,81]. Except for the study of Hagström *et al*[5] where the incidence of hepatocellular carcinoma was collectively reported with other liver-related outcomes, no data on the incidence and risk of HCC development in the subgroup of lean patients with NAFLD has been published. Until new data becomes available, no conclusions can be drawn on the risk for HCC development in lean individuals with NAFLD.

MANAGEMENT

As 3%-25% of lean/non-obese and non-diabetic individuals are diagnosed with NAFLD, with potential for progression to NASH and subsequently liver fibrosis with metabolic dysfunction, it is of interest to find pharmacological modalities and lifestyle interventions to treat this specific phenotype[82-84]. Animal studies on obese rats and mice showed significant reductions in hepatic steatosis and oxidative stress when glucagon-like peptide-1 receptor agonists (GLP-1RAs) were used to treat liver steatosis with no or mild fibrosis[85,86]. Moreover, randomized control trial investigating the role of liraglutide (daily GLP-1RA) reported on histological resolution of NASH after 48 wk of treating obese and overweight NASH patients[87]. Data on lean NAFLD/NASH counterparts are lacking, but recently published animal study gave promising results. Ipsen and colleagues reported on liraglutide effects in reducing both inflammation and hepatocyte ballooning in advanced NAFLD in an animal model. The treatment was more effective than dietary intervention, and when the two were combined, they led to rapid weight loss[88].

Still, available data on the treatment and management of lean subjects with NAFLD are practically non-existent, and further studies are needed to evaluate the effects of lifestyle changes and pharmacotherapy in this vulnerable population.

CONCLUSION

NAFLD in lean individuals presents a severe global burden with detrimental clinical consequences. Determining metabolic phenotype is crucial for detecting normal-weight patients at risk of developing NAFLD and preventing possible long-term complications, such as the cardiometabolic, liver, and all-cause mortality, which may be even more pronounced than in the obese individuals. The main characteristic of MUHNW seems to be insulin resistance associated with visceral adiposity; thus, waist circumference or the android gynoid ratio along with HOMA IR could be better predictors of NAFLD in lean subjects than traditionally used BMI and other components of metabolic syndrome. Insulin resistance is undoubtedly associated with the development of NAFLD in lean individuals irrespective of BMI and the presence of MetS; however, is it causality or correlation remains an open question.

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ORIGINAL ARTICLE

Basic Study

Integrative analysis of layers of data in hepatocellular carcinoma reveals pathway dependencies

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Abstract

BACKGROUND

The broader use of high-throughput technologies has led to improved molecular characterization of hepatocellular carcinoma (HCC).

AIM

To comprehensively analyze and characterize all publicly available genomic, gene expression, methylation, miRNA and proteomic data in HCC, covering 85 studies and 3355 patient sample profiles, to identify the key dysregulated genes and pathways they affect.

METHODS

We collected and curated all well-annotated and publicly available high-throughput datasets from PubMed and Gene Expression Omnibus derived from human HCC tissue. Comprehensive pathway enrichment analysis was performed using pathDIP for each data type (genomic, gene expression, methylation, miRNA and proteomic), and the overlap of pathways was assessed to elucidate pathway dependencies in HCC.

RESULTS

We identified a total of 8733 abstracts retrieved by the search on PubMed on HCC

Manuscript source: Unsolicited manuscript

Specialty type: Gastroenterology and hepatology

Country/Territory of origin: Canada

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

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for the different layers of data on human HCC samples, published until December 2016. The common key dysregulated pathways in HCC tissue across different layers of data included epidermal growth factor (EGFR) and β1-integrin pathways. Genes along these pathways were significantly and consistently dysregulated across the different types of high-throughput data and had prognostic value with respect to overall survival. Using CTD database, estradiol would best modulate and revert these genes appropriately.

CONCLUSION
By analyzing and integrating all available high-throughput genomic, transcriptomic, miRNA, methylation and proteomic data from human HCC tissue, we identified EGFR, β1-integrin and axon guidance as pathway dependencies in HCC. These are master regulators of key pathways in HCC, such as the mTOR, Ras/Raf/MAPK and p53 pathways. The genes implicated in these pathways had prognostic value in HCC, with Netrin and Slit3 being novel proteins of prognostic importance to HCC. Based on this integrative analysis, EGFR, and β1-integrin are master regulators that could serve as potential therapeutic targets in HCC.

Key Words: Hepatocellular carcinoma; Gene expression; miRNA; Methylation; Proteomics; High throughput data

Core Tip: Analyzing all available high-throughput genomic, transcriptomic, miRNA, methylation and proteomic data from human hepatocellular carcinoma tissue, we identified master regulators of key pathways in hepatocellular carcinoma, such as the mTOR, Ras/Raf/MAPK and p53 pathways.

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INTRODUCTION

The molecular basis of hepatocellular carcinoma (HCC) has been elusive, given the significant heterogeneity of this tumor that arises in the context of various chronic liver diseases[1]. HCC remains a high-fatality cancer, despite large-scale efforts to better characterize and therapeutically target this malignancy. Since prevalence of cirrhosis due to hepatitis C and fatty liver disease is increasing in North America, HCC continues to rise[2]. Five-year survival remains poor at 18% due to late diagnosis and inability to tolerate chemotherapy in patients with cirrhosis[2]. Consequently, there is an urgent need to better understand the molecular basis of this highly fatal cancer.

Clinical management of HCC is optimized based on disease stage[3]. Curative treatment with resection, radiofrequency ablation or transplantation is possible in early stage disease[4]. When HCC is diagnosed at a later stage, sorafenib is the first-line chemotherapy, which is directed against the Ras/Raf/MAPK pathway[4]. This is associated with a very modest improvement in overall survival of 3 additional months as compared to placebo (10.7 mo *vs* 7.9 mo)[5].

The cancer genome atlas (TCGA) is a large-scale project that has enabled improved characterization of cancers with several layers of data. The TCGA multi-platform analysis of 196 HCC tumors described this cancer as highly heterogeneous and difficult to characterize, although certain key pathways did emerge including the Ras/Raf/MAPK, mTOR, Wnt/B-catenin, and Sonic Hedgehog pathways[1,6]. Integration of various types of data has previously been performed to map interaction networks. By integrating genomic, transcriptomic and proteomic data, one can understand potential interactions that contribute to a disease condition or process[7,8].

These interactions may otherwise not be uncovered, on the basis of a single type of data. This systems biology approach has been especially important in cancer, given that alterations in one gene can have a ripple effect on proteins in the rest of a protein-protein interaction network. Therefore, elucidating the layers of data in a disease can provide additional insights into the pathways that drive cancer[9].

In the current study, we aim to characterize the landscape of high-throughput data profiling in HCC and determine the patterns in key dysregulated genes and pathways across these different layers of data. The patterns that emerge could help in better understanding the pathways that drive HCC and could be considered as therapeutic targets.

MATERIALS AND METHODS

Data collection, analysis and database compiling

We downloaded all available high-throughput genomic, transcriptomic, microRNA, methylation, and proteomic datasets related to human HCC samples from published datasets (PubMed, http://www.ncbi.nlm.nih.gov/PubMed and Gene Expression Omnibus (GEO), https://www.ncbi.nlm.nih.gov/geo).

Using PubMed, the following search was performed for whole exome sequencing data on HCC: ("carcinoma, hepatocellular" [MeSH Terms] OR ("carcinoma" [All Fields] AND "hepatocellular" [All Fields]) OR "hepatocellular carcinoma" [All Fields] OR ("hepatocellular" [All Fields] AND "carcinoma" [All Fields])) AND (whole [All Fields] AND ("exome" [MeSH Terms] OR "exome" [All Fields]) AND sequencing [All Fields]). The following MeSH terms were used to identify gene expression papers: ("carcinoma, hepatocellular" [MeSH Terms] OR ("carcinoma" [All Fields] AND "hepatocellular" [All Fields]) OR "hepatocellular carcinoma" [All Fields] OR ("hepatocellular" [All Fields] AND "carcinoma" [All Fields])) AND ("gene expression" [MeSH Terms] OR ("gene" [All Fields] AND "expression" [All Fields]) OR "gene expression" [All Fields]) AND ("humans" [MeSH Terms] OR "humans" [All Fields]) AND English [All Fields] NOT ("review" [Publication Type] OR "review literature as topic" [MeSH Terms] OR "reviews" [All Fields]). To identify suitable papers regarding methylation in HCC, we used the following terms: ("methylation" [MeSH Terms] OR "methylation"[All Fields]) AND ("carcinoma, hepatocellular" [MeSH Terms] OR ("carcinoma" [All Fields] AND "hepatocellular" [All Fields]) OR "hepatocellular carcinoma" [All Fields] OR ("hepatocellular" [All Fields] AND "carcinoma" [All Fields]) AND ("humans" [MeSH Terms] AND English [lang]). Proteomics papers were retrieved using the following search: [("proteomics" [MeSH Terms] OR "proteomics" [All Fields]) AND high [All Fields] AND throughput [All Fields]] AND ("carcinoma, hepatocellular" [MeSH Terms]) OR ("carcinoma" [All Fields] AND "hepatocellular" [All Fields]) OR "hepatocellular carcinoma" [All Fields] OR ("hepatocellular"[All Fields] AND "carcinoma"[All Fields]). MicroRNAs reported in HCC were identified using these MeSH terms: ("micrornas" [MeSH Terms] OR "micrornas"[All Fields] OR "mirna" [All Fields]) AND profile [All Fields] AND ("carcinoma, hepatocellular" [MeSH Terms] OR ("carcinoma" [All Fields] AND "hepatocellular" [All Fields]) OR "hepatocellular carcinoma" [All Fields] OR ("hepatocellular" [All Fields] AND "carcinoma" [All Fields]).

We considered for inclusion all datasets available in PubMed.

The datasets publicly available on the GEO, a public functional genomics data repository of high-throughput array data (https://www.ncbi.nlm.nih.gov/geo) were retrieved and analyzed using GEO2R (https://www.ncbi.nlm.nih.gov/geo/info/geo2r.html), a web tool available on the portal, identifying genes differentially expressed between samples of HCC and the non-tumoral liver portion. GEO2R compares original submitter-supplied processed data tables using the GEOquery and limma R packages from the Bioconductor project. Following instructions available online at (https://www.ncbi.nlm.nih.gov/geo/info/geo2r.html), we retrieved all dysregulated genes. Only those with an adjusted P value < 0.05, and expression fold change value below ≤ 0.5 or above ≥ 1.5 were considered for further analysis (Table 1, Supplementary Table 1). The genes included in our list from WES papers were reported as affected by nonsynonymous mutations, and synonymous mutations were not considered. Putative microRNA gene targets were identified using an online database, mirDIP 4.1[10], (http://ophid.utoronto.ca/mirDIP). The most stringent predictive search option (top 1%) was used to obtain the list of putative targets of all differentially expressed miRNAs.

From the selected 11 methylation datasets, raw data from eight studies were

Table 1 List of the final 85 selected publications for each layer of data. For each publication the number of hepatocellular carcinoma samples and controls and the platform used for the analysis are reported

Gene expression					
No.	**year**	**PMID**	**HCC (*n*)**	**Controls (*n*)**	**GEO dataset**
1	2004	17393520	35	13	GSE6764
2	2008	18504433	11	2	GSE6222
3	2008	18923165	80	82	GSE10143
4	2009	19098997	47	58	GSE14323
5	2009	19861515	16	47	GSE17967
6	2011	21320499	34	34	GSE20140 (GSE10141, GSE10140)
7	2011	21712445	40	40	GSE28248
8	2013	23691139	15	15	GSE17548
9	2013	23800896	GSE36376_276; GSE25097_211	GSE36376_247; GSE25097_283	GSE36376, GSE25097
10	2014	24498002	46	46	GSE47595
11	2014	24564407	45	45	GSE45114
12	2014	25093504	39	40	GSE57958
13	2014	25141867	11	11	GSE55092
14	2014	25376302	18	18	GSE60502
15	2014	25536056	72	72	GSE39791
16	2015	25666192	132	132	GSE54236
17	2015	25645722	228	168	GSE63898
18	2016	27499918	60	60	GSE64041
19	2016	25964079	26	20	GSE54238
Proteomics					
No.	year	PMID	HCC (*n*)	Controls (*n*)	
1	2004	14726492	8	8	
2	2008	19003864	12	12	
3	2005	15759316	10	10	
4	2005	16097030	14	14	
5	2007	17627933	12	12	
6	2014	23621634	3	3	
7	2009	19562805	3	3	
8	2016	26709725	24	12	
9	2013	23589362	20	20	
10	2012	22813877	10	10	
11	2012	22082227	11	11	
12	2011	21631109	69	123	
13	2010	20230046	5	5	
14	2010	19956837	20	20	
15	2009	19715608	18	18	
16	2009	19535095	3	3	
17	2009	19161326	80	80	

18	2004	15221772	20	20	
19	2003	14673798	21	21	
20	2003	14654528	21	21	
21	2002	12481271	11	11	
22	2013	23462207	7	7	
23	2005	16335951	8	8	
24	2006	16342242	10	10	
25	2011	22034872	3	3	
26	2005	15852300	7	7	
27	2011	21913717	3	3	
28	2007	17203974	25	28	
29	2007	17586277	10	10	
Whole exome sequencing					
No.	year	PMID	HCC (*n*)	Controls (*n*)	GEO dataset
1	2013	23912677	3	3	N/A
2	2014	24055508	4	7	N/A
3	2017	28323123	5	5	N/A
4	2014	24798001	231	231	GSE54504
5	2012	22561517	24	24	N/A
Epigenetic_miRNAs					
No.	year	PMID	HCC (*n*)	Controls (*n*)	GEO dataset
1	2015	26190160	9	7	N/A
2	2014	24789420	10	9	GSE31383
3	2014	24564407	45	45	GSE10694
4	2011	21298008	73	73	GSE21362
5	2008	18649363	78	10	N/A
6	2012	22135159	20	20	N/A
7	2011	21319996	94	94	N/A
8	2009	19473441	20	20	N/A
9	2009	19173277	35		N/A
10	2007	18171346	10	10	N/A
11	2006	16331254	25	25	N/A
12	2015	26062888	30	30	N/A
13	2015	26046780	327	43	N/A
14	2015	25861255	66	66	GSE54751
15	2015	25500075	6	6	GSE54537
16	2014	24875649	24	24	
17	2013	23812667	166	166	GSE31384
18	2013	23390000	9	17	GSE40744
19	2012	23082062	18	18	N/A
20	2014	24586785	29	29	N/A
21	2013	24417970	78	78	N/A
Epigenetic methylation					

No.	year	PMID	HCC (*n*)	Controls (*n*)	GEO dataset
1	2011	21500188	13	12	N/A
2	2014	24306662	45	45	N/A
3	2014	25376292	22	22	N/A
4	2015	25945129	8	8	GSE59260
5	2011	21747116	12	12	GSE29720
6	2010	20165882	20	20	GSE18081
7	2012	22234943	62	62	GSE37988
8	2013	24012984	20	8	GSE44970
9	2013	23208076	66	66	GSE54503
10	2014	25093504	59	59	GSE57956
11	2014	25294808	27	27	GSE60753

HCC: Hepatocellular carcinoma; GEO: Gene Expression Omnibus; N/A: Not applicable.

available on the GEO website (https://www.ncbi.nlm.nih.gov/geo/). We selected the CpG sites or genes reported to be hyper-or hypo- methylated in these publications. The genomic region was considered differentially methylated between HCC tissue and the adjacent non-tumoral sample, if the FDR corrected *P* value < 0.01. Furthermore, we filtered out everything that did not satisfy the criteria: $\Delta\beta \geq 0.20$ or $\Delta\beta \leq -0.20$, where $\Delta\beta = \beta$HCC - βadjacent was the difference in methylation between above specified groups. When the CpG sites were considered, the Illumina HumanMethylation450K and 27K platforms were used for mapping to the genes. When multiple sites or genes were found to have the same sense of differential methylation, the mean value of $\Delta\beta$ was calculated. Only the CpGs in the 5'UTR, 1st Exon, TSS200, TSS1500 or in CpG islands were considered in our analysis. Proteomic results were retrieved and included only if protein abundance was reported as different in HCC liver samples compared to control samples.

Figure 1 outlines our study workflow. Papers were excluded from each specific search for the following reasons: Data from cell lines, or animal models, studying efficacy or drugs, or the presence of long non-coding RNA, mechanistic studies not performing high-throughput or evaluating the role of one molecule, papers focused on liver diseases but not HCC or liver tissue, not original data such as review articles, or those studies using already selected datasets, not reporting the modulation of the molecules, and papers without data available.

Available patient data, including etiology of liver disease (hepatitis C, hepatitis B, alcohol, fatty liver disease) on the basis of which the HCC tumors developed, presence of cirrhosis, the Model for End-stage Liver Disease score (MELD score, an assessment of the severity of liver dysfunction), tumor histology, stage of cancer, alpha-fetoprotein level, overall and recurrence-free survival following treatment were also documented (Supplementary Table 2).

Pathway enrichment analysis

The key dysregulated genes from each type of data (genomic, miRNA, methylation, transcriptomic, and proteomic) were fed into the Integrated Interactions Database[11] (IID, http://ophid.utoronto.ca/iid), to obtain a list of the protein-protein interactions. For the miRNA dataset, we determined the target genes of the differentially expressed miRNAs in tumors using the miRNA Data Integration Portal mirDIP v4.1[10]. The individual lists derived from each type of data were then fed into the pathway Data Integration Portal, pathDIP v3.0 (http://ophid.utoronto.ca/pathDIP)[12], in order to determine the significantly dysregulated pathways in HCC. pathDIP integrates data from 20 major pathway databases, and computationally predicts gene association to curated pathways using protein-protein interactions from IID significance of their connectivity[12]. We used this comprehensive pathway enrichment analysis portal to obtain a list of significantly enriched pathways using literature curated (core) pathway memberships *P* value (FDR: BH-method) less than 0.05.

The lists of pathways from each type of data were then assessed for overlap using Venny 2.1, an online tool for Venn diagram design (http://bioinfogp.cnb.

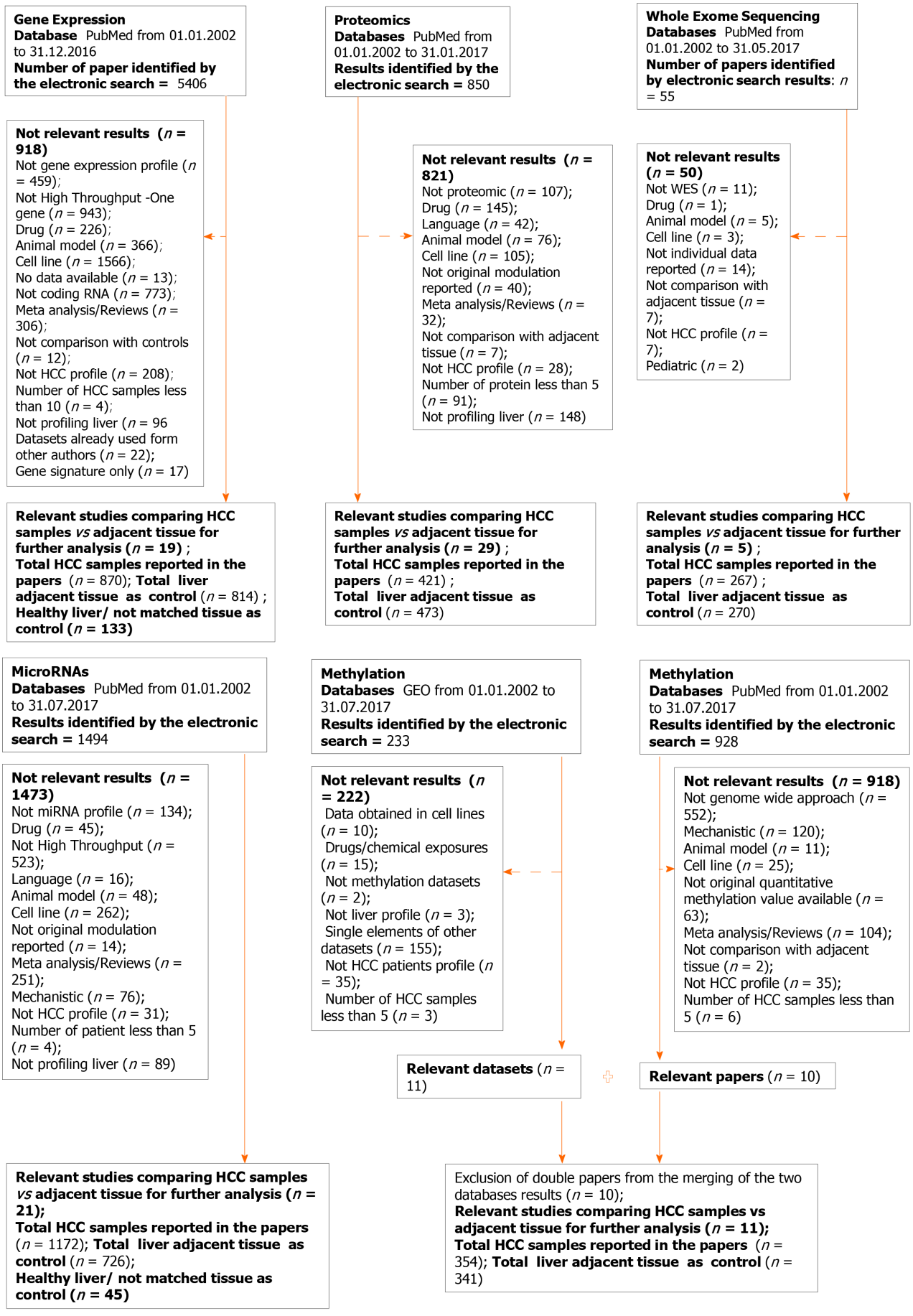

Figure 1 Flow chart showing the paper selection process and exclusion criteria for each data type: Gene expression, proteomics, whole

exome sequencing, microRNAs and methylation.

csic.es/tools/venny/index.html).

Retrospective validation on independent dataset

In order to determine whether key differentially expressed genes along the overlapping pathways had prognostic value, we used KMplotter, a web-based tool that enables survival analysis across multiple cancers and datasets[13]. Patient samples were split into two groups per autoselection of the best cutoff for each gene, in order to assess its prognostic value. We ran multivariate overall survival analysis based on the high *vs* low expression of each gene in HCC tumors. The two groups were compared by a Kaplan-Meier survival plot, and the hazard ratio with 95% confidence intervals and log-rank *P* value were calculated.

Drug identification by CTD

The identification of putative therapeutic agents able to revert the modulation of genes of interest based on their modulation associated with a worse prognosis was obtained using the online Comparative Toxicogenomics Database http://ctdbase.org[14]. This database provides manually curated information about chemical–gene/protein interactions, chemical–disease and gene–disease relationships.

RESULTS

We identified a total of 8733 abstracts retrieved by the search on PubMed on HCC for the different layers of data on human HCC samples, published until December 2016. The flow chart outlining the selection process is detailed in Figure 1.

The number of samples included in our analysis are as follows: (1) Whole exome sequencing: 267 HCC and 270 control samples; (2) Gene expression: 870 HCC and 814 control samples; (3) miRNA: 1172 HCC and 771 control samples; (4) Methylation: 354 HCC and 341 control samples; and (5) Proteomics: 421 HCC and 473 control samples. The methodologies and platforms used to obtain these high-throughput data are reported by type of data (genomic, transcriptomic, miRNA, methylation and proteomic) in Table 1. Clinical data, regarding etiology of liver disease (hepatitis C, hepatitis B, alcohol, fatty liver disease) were frequently reported, on the other side serum levels of liver enzymes, AST and ALT, frequently used to assess liver functions were not available. Pathological details relative to differentiation or stage were frequently absent as well as other crucial variables in the clinic setting, such as Child Pugh/MELD score (Supplementary Table 2).

Integrative analysis reveals most important pathways in HCC

There were 188 overlapping dysregulated genes/proteins across the different types of data. Independently for each type of data, we obtained a list of pathways using pathDIP. We merged the list of dysregulated pathways in miRNA and methylation, given that these epigenetically regulate gene expression, in order to assess for overlapping pathways across the datasets.

This resulted in a list of 3 common, overlapping pathways among the different types of data: EGFR, β1-integrin, and axon guidance pathways, as depicted in Figure 2. From the previous list of 188 common dysregulated elements in all different layers of data (Figure 3), we were able to identify 35/188 genes that were involved in these 3 shared pathways across the layers of data (Supplementary Table 1).

Prognostic value of pathways in HCC

We then examined the prognostic value of the deregulated genes associated to pathways of interest in HCC using TCGA RNA seq dataset, as listed in Table 2. Median survival of 364 patients in the TCGA, which was used for validation purposes regarding the prognostic value is reported. KMplotter HR results from TCGA RNA seq data reflected the altered modulation identified for these 9 genes in the 19 HCC papers relative to the gene expression data (Table 2). Among the five upregulated genes associated with positive HR values, CDK5, was reported with the highest HR value (1.85, $P = 0.0035$) and involved in cell cycle (Table 3). The other 4/9 genes reported as upregulated, COL2A1, LAMC1, RPS6KA3 and ITGB1 were identified with

Table 2 Prognostic value of the 9 dysregulated genes associated with the 3 common dysregulated pathways (EGFR, epidermal growth factor, β1-integrin and axon guidance) among the 4 types of data in obtained with KMplotter

Gene	Modulation in the 19 HCC papers	Probe-ID	HR	CI	Log-Rank *P* value	Median survival low (mo)	Median survival high (mo)	Estradiol gene modulation predicted by CTD
COL2A1	Up	1280	1.49	1.05-2.11	0.0229	61.7	54.1	N/A
FGA	Down	2243	0.52	0.35-0.77	0.0009	49.7	70.5	+
FGG	Down	2266	0.56	0.39-0.79	0.0009	38.3	70.5	+
LAMC1	Up	3915	1.43	0.98-2.09	0.06	56.5	38.3	N/A
CDK5	Up	1020	1.85	1.22-2.81	0.0035	81.9	6.2	N/A
EPHB1	Down	2047	0.72	0.048-1.08	0.1135	54.1	70.5	N/A
RPS6KA3	Up	6197	1.2	0.8-1.78	0.3743	54.1	56.5	-
EGFR	Down	1956	0.61	0.43-0.89	0.0085	31	70.5	+
ITGB1	Up	3688	1.37	0.95-1.97	0.0924	82.9	49.7	N/A

CTD based prediction identified Estradiol to efficiently affect the expression of the 4/9 genes based on their hazard ratios values. HR: Hazard ratios; HCC: Hepatocellular carcinoma; CI: Confidence interval; N/A: Not applicable.

positive HR value by KM plotter analysis and involved in cellular migration (Table 2 and Table 3).

Four out of 9 genes were reported as downmodulated in the 19 HCC gene expression papers. Among these four, two genes, FGA and FGG, were identified as the top statistically significantly (P = 0.0009) associated with a protective role in HCC (HR values 0.52 and 0.59, respectively). FGA and FGG were consistently reported as downmodulated in about 45% of our 19 selected gene expression papers (Table 3). The other two downmodulated genes, EPHB1 and EFGR with negative HR values (Table 2) are reported to be affected by missense mutation leading to a loss of their protective role against cell migration.

Estradiol is a therapeutic agent that appropriately targets HCC genes

Using CTD, we found that estradiol was able to appropriately down- or upmodulate 4 out of 9 cancer-related genes (Table 2). Particularly, CTD reported estradiol capabilities to upregulated FGA, FGG and EGFR reported downmodulated in HCC (Table 2) and counteracting the upregulation of RPS6KA3 in HCC, suggesting a possible role for this hormone in HCC treatment.

DISCUSSION

In this study, we evaluate the molecular pathogenesis of HCC using a unique approach, that of combining all publicly available high-throughput data from patient HCC tumors. This encompasses all miRNA, methylation, genomic, transcriptomic and proteomic profiling data present in the literature, and represents the first effort to derive a consensus molecular model of HCC through analysis of these different types of data. Although these datasets originated from different patient cohorts, presented integrative analysis offers the opportunity to explore common key pathway dependencies of HCC. Starting with the initial generation of genomics and whole exome sequencing data, previous high-throughput studies have brought forth different lists of dysregulated genes, depending on the type of data evaluated. Dysregulated genes may affect different parts of a pathway. Therefore, a pathway-based approach when evaluating different types of high-throughput data offers the ability to assess the pathways most commonly affected in a given cancer. Additionally, the integrative analysis in our study encompasses a large number of patient samples.

Using this integrative approach, we confirm the importance of EGFR, β1-integrin and axon guidance as pathways critical in hepatocarcinogenesis. EGFR activates the signaling cascades of the Ras/Raf/MAPK and mTOR pathways, two pathways that were identified as key to HCC pathogenesis in the TCGA study[6]. The

Table 3 Modulation of the 9 dysregulated genes associated with the 3 common dysregulated pathways (EGFR, epidermal growth factor, β1-integrin and axon guidance) identified in the 19 hepatocellular carcinoma gene expression papers. Their genetic alteration in hepatocellular carcinoma and their mechanism in cancer are reported

Gene	Modulation in the 19 HCC papers	PMID	Mutation in HCC (PMID)	Role in cancer (PMID)
COL2A1	Up (2/19)	23800896/25666192	(rs3917) polymorphism is associated with higher risk of HCC (21665180)	COL2A1 promotes migration in HCC (29858962)
FGA	Down (9/19)	21320499/23800896/25093504/25536056/25141867/25376302/25666192/25645722/25666192	Deleted in HCC patients (27511114)	FGA is a positive predictor of survival in gastric cancer patients (15756001)
FGG	Down 8/19	21320499/23800896/25093504/25536056/25141867/25376302/25645722/24498002	Allelic loss (16980951)	FGG is involved in amino acid and redox metabolism pathway in HCC (28089356)
LAMC1	Up (4/19)	23800896/25536056/25141867/25645722	Not identified	LAMC1 promotes tumor cell invasion and migration in HCC (28928891)
CDK5	Up (2/19)	25141867/25376302	Not identified	CDK5 promotes proliferation in HCC (29312535)
EPHB1	Down (2/19)	23800896/25141867	Missense mutation (19469653)	EPHB1 inhibits cell migration(22242939)
RPS6KA3	Up 1/19	25141867	Somatic mutation and copy number variations (22561517)	RPS6KA3 increases cell proliferation (15833840)
EGFR	Down (2/19)	19098997/25141867	Missense mutation (26436086)	EGFR promotes cell adhesion (31465839)
ITGB1	Up (1/19)	25141867	Somatic number variations (24512821)	ITGB1 promotes migration (30664185)

HCC: Hepatocellular carcinoma.

identification of β1-integrin as being commonly dysregulated in HCC is novel, and its significance is confirmed through its consistent dysregulation across types of data. β1-integrin is a cell surface receptor that senses the extracellular matrix, thereby modulating the hallmarks of cancer such as proliferative signaling with continuous activated cell replication, evasion of growth suppressors, resistance to angiogenesis as well as cancer cell invasion and metastasis[14]. Ras/Raf/MAPK and mTOR are established pathways in hepatocarcinogenesis, and are integrin-dependent signaling pathways[15]. Additionally, β1-integrin is known to crosstalk with EGFR. In fact, the downregulation of β1-integrin was found to decrease phosphorylation of EGFR and c-Met in hepatocytes during liver regeneration[16]. A synergistic relationship between integrins and EGFR has also been demonstrated in tumor progression[17]. The finding of axon guidance pathway-related proteins as being dysregulated across types of data, thereby establishing consistent dysregulation of this pathway in HCC, is also novel. Netrin-1 is the best studied protein in the axon guidance pathway, and is known to be overexpressed in various cancers[13]. It is responsible for regulation of apoptosis, with increased presence of netrin-1 leading to inhibition of apoptosis. The tumor suppressor p53, frequently mutated in the TCGA HCC study, regulates the cell cycle through netrin-1. The axon guidance pathway has previously been identified as a pathway that is significantly mutated in HCC based on integration of all genomic data in HCC[18]. This analysis revealed mutations along the axon guidance pathway as being prognostic of a higher rate of HCC metastasis. We were able to additionally validate the prognostic importance of dysregulated proteins in these pathways proteins using TCGA data.

HCC is a cancer that develops in the context of various chronic liver diseases, which may influence the molecular characteristics of HCC. Additionally, the underlying cirrhosis and liver dysfunction that are often concurrent may influence HCC development and behavior[2]. Patients are often diagnosed at an advanced stage of disease, when it is too late for curative treatment. A unique consideration in HCC is the inability to tolerate hepatotoxic chemotherapy in patients with liver dysfunction,

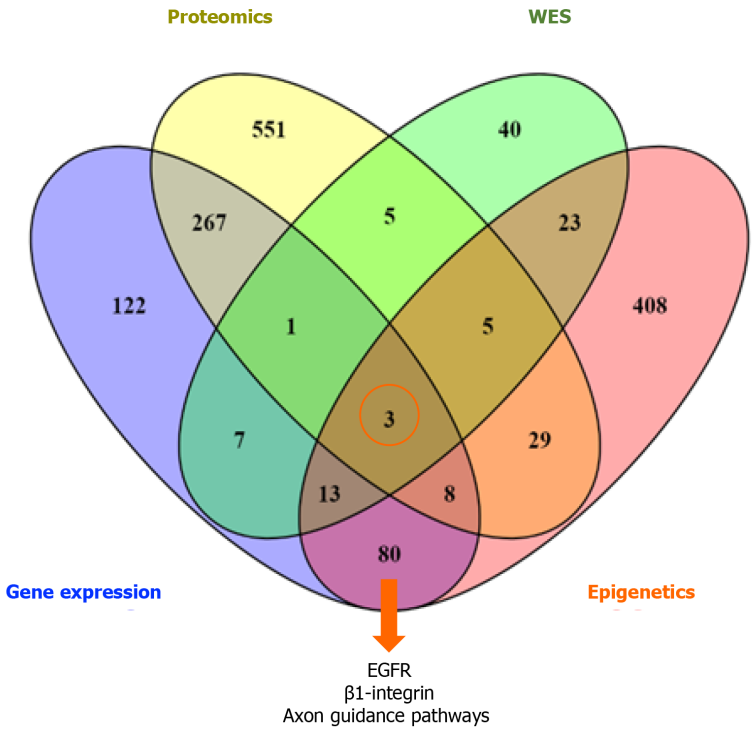

Figure 2 Venn diagram shows the three common pathways (EGFR, epidermal growth factor, β1-integrin, and axon guidance pathways) across the four different types of data.

as it is often patients with cirrhosis who develop HCC[19,20]. Therefore, liver function must be considered prior to, during, and after any form of treatment for HCC.

Thus, especially for HCC, it has been suggested that a multi-pronged approach to HCC therapy jointly targeting different pathways be adopted.

Omics technologies are essential in the progress towards elucidating the molecular basis of HCC. The current study represents the largest integration of all publicly available genomic, gene expression, methylation, miRNA and proteomic data in HCC, covering 85 studies and 3355 patient sample profiles. We identified consistently deregulated pathways associated with hepatocarcinogenesis across different types of data using integrative analysis tools, thereby confirming the importance of these genes in HCC pathogenesis. EGFR (activator of Ras/Raf/MAPK and mTOR) and β1-integrin (also modulator of the aforementioned pathways) were clearly identified as pivotal to HCC[5,21-23]. This is in keeping with the efficacy of the Ras/Raf/MAPK inhibitors sorafenib and regorafenib in HCC[24].

Even beyond this, we found these consistently deregulated genes across pathways to be appropriately modulated by estradiol. HCC is less common in women, and there have been clinical studies demonstrating that hormone therapy and female sex are protective against HCC as described earlier in this thesis.

Other integrative multi-omics studies have been recently performed for other tumors with high mortality such as breast and ovarian cancer[6,25]. Several breast cancer studies emphasizing how data integration of genomic/transcriptomic and proteomic has improved the molecular characterization of subtypes of breast cancer and elucidate its heterogeneity and its interaction with the microenvironment and aggressiveness[26,27]. A single source of data was used in the ovarian cancer multi-omics mathematical integration performed by Bhardwaj *et al*[25]. Copy number variation gene expression and methylation data from TCGA data portal were integrated using mathematical algorithm and identified 32 co-expressed genes and 6 pathways associated with survival.

The main limitation of our study is the different patient samples represented by the various types of data. Nonetheless, there is a large amount of high-throughput data, which allowed us to detect pathway dependency patterns that are compatible with the current HCC literature. Additionally, HCC tumors arise in the setting of various chronic liver diseases. We could not assess for etiology-specific genes and pathways in this study, given that the clinical and genetic data to evaluate these differences were not fully available for all the studies. Therefore, we could only evaluate gene

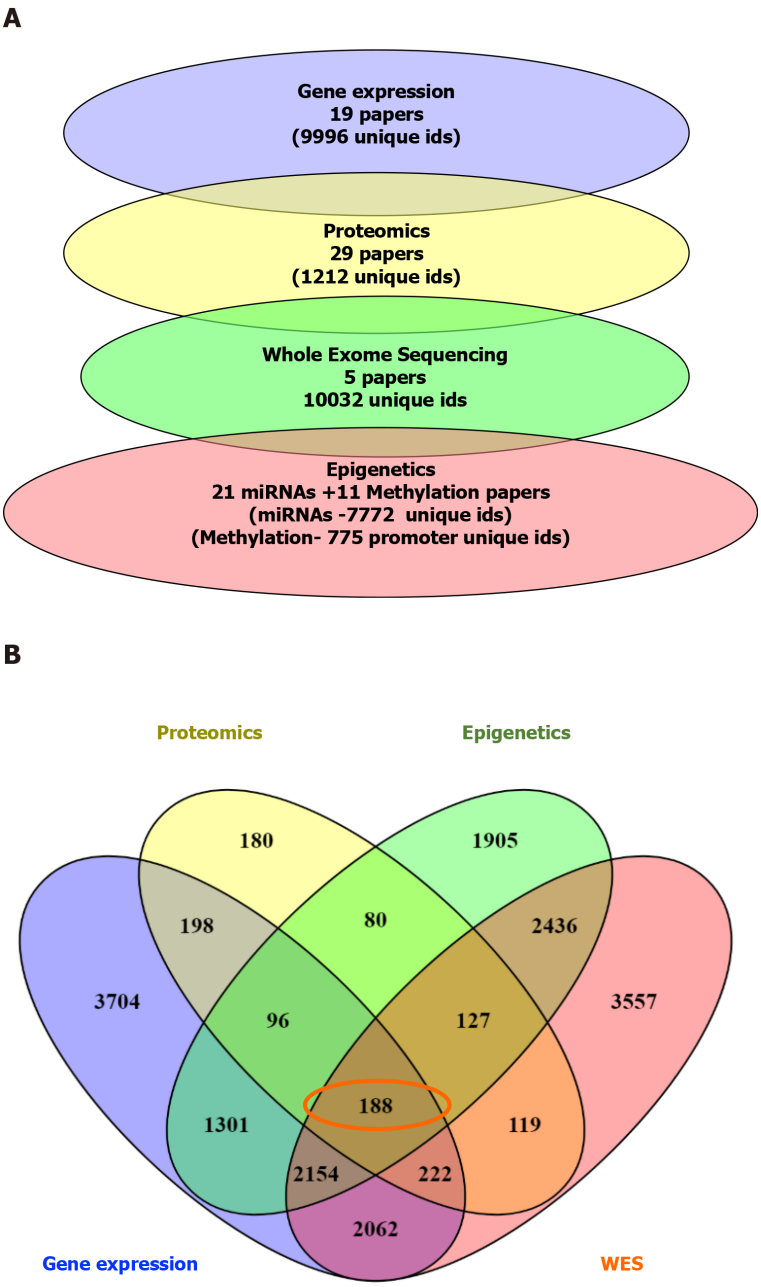

Figure 3 From the previous list of 188 common dysregulated elements in all different layers of data. A: Number of genes/proteins identified in each data type; B: Venn diagram showing the 188 genes identified as commonly deregulated across the 4 different type of data.

differences over whole datasets, rather than individual patients, due not complete individual annotation of the samples available on GEO for each specific dataset. The HCC samples in this integrative analysis all came from patients who had undergone hepatectomy. There were no specimens from patients who were candidates for ablation therapy (early stage), those who were undergoing liver transplantation, or those with advanced HCC. One might anticipate that the molecular features of such tumors differ, given the different stages of HCC captured, but there is unfortunately scarcity of data in this regard.

CONCLUSION

In conclusion, our study represents the largest integrative analysis of all publicly available data in HCC, spanning different types of high-throughput data. Pathway enrichment analysis elucidated EGFR, β1-integrin and axon guidance as pathway dependencies in HCC. These are proteins known to serve as master regulators of key

pathways in HCC such as Ras/Raf/MAPK, Wnt/β-catenin and mTOR[28], and may serve as potential overarching therapeutic targets in HCC. The axon guidance pathway was identified as being of potential importance to HCC for the first time, with prognostic value suggested in patient sample validation with TCGA. Estradiol affects a large number of deregulated genes across data with appropriate modulation and may be a therapeutic agent that helps in HCC. A combined therapeutic approach conjointly targeting different pathways may be more optimal in the treatment of HCC, especially when underlying hepatic dysfunction compromises the ability to tolerate optimal chemotherapeutic doses.

ARTICLE HIGHLIGHTS

Research background

Hepatocellular carcinoma (HCC) is highly heterogeneous, difficult to characterize and the molecular basis of HCC has been elusive.

Research motivation

The Cancer Genome Atlas is a large-scale project that has enabled improved characterization of cancers with several layers of data. Elucidating the layers of data in a disease can provide additional insights into the pathways that drive cancer.

Research objectives

A novel integrative approach of all publicly available high-throughput data from patient HCC tumors was used to delineate critical pathway dependencies in HCC.

Research methods

A comprehensive analysis and characterization of all publicly available genomic, gene expression, methylation, miRNA and proteomic data in HCC covered 85 studies and 3355 patient sample profiles and identified the key overlapping dysregulated genes and pathways affected.

Research results

We identified the prognostic value of these genes in HCC genes, specifically with Netrin and Slit3 being novel proteins of prognostic importance to HCC.

Research conclusions

Our large integrative analysis of all publicly available data in HCC and our pathway enrichment analysis has elucidated epidermal growth factor, β1-integrin, and axon guidance as pathway dependencies in HCC.

Research perspectives

Based on our integrative analysis, epidermal growth factor, and β1-integrin are master regulators that could be considered as potential therapeutic targets in HCC.

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ORIGINAL ARTICLE

Case Control Study

Association of interferon lambda-4 rs12979860 polymorphism with hepatocellular carcinoma in patients with chronic hepatitis C infection

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Abstract

BACKGROUND

Hepatitis C virus (HCV) infection is a public health concern worldwide. Several factors, including genetic polymorphisms, may be evolved in the progression of HCV infection to liver diseases. Interferon lambdas (IFNLs) modulate the immune response during viral infections. IFNLs induce antiviral activity, interfering in the viral replication by promoting the expression of several genes that regulate immunological functions. The interferon lambda-4 (*IFNL4*) rs12979860 polymorphism, which is characterized by a C to T transition in intron 1, is associated with spontaneous and treatment-induced clearance of HCV infection and may play a role in the development of HCV-associated liver diseases, including hepatocellular carcinoma (HCC).

AIM

To investigate the association of *IFNL4* rs12979860 polymorphism with fibrosis, cirrhosis, and HCC in patients with chronic HCV infection.

METHODS

This study was comprised of 305 chronic HCV-infected patients (53 fibrosis, 154 cirrhosis, and 98 HCC cases). The control group was comprised of 260 HCV-negative healthy individuals. The *IFNL4* rs12979860 polymorphism was genotyped using the TaqMan assay. Fibrosis was diagnosed based on liver biopsy findings, while cirrhosis was diagnosed through clinical, laboratory,

Committee of Hospital de Clínicas de Porto Alegre under the protocol 15-0126.

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anatomopathological, and/or imaging data. HCC was diagnosed through imaging tests, tumor, and/or anatomopathological markers.

RESULTS

The T allele was observed in the three groups of patients (fibrosis, cirrhosis, and HCC) at a significantly higher frequency when compared with the control group ($P = 0.047$, $P < 0.001$, and $P = 0.01$, respectively). Also, genotype frequencies presented significant differences between the control group and cirrhosis patients ($P < 0.001$) as well as HCC patients ($P = 0.002$). The risk analysis was performed using the codominant and dominant T allele models. In the codominant model, it was observed that the CT genotype showed an increased risk of developing cirrhosis in comparison with the CC genotype [odds ratio (OR) = 2.53; 95% confidence interval (CI): 1.55-4.15; $P < 0.001$] as well as with HCC (OR = 2.54; 95%CI: 1.44-4.56; $P = 0.001$). A similar result was observed in the comparison of the TT *vs* CC genotype between the control group and cirrhosis group (OR = 2.88; 95%CI: 1.44-5.77; $P = 0.001$) but not for HCC patients. In the dominant T allele model, the CT + TT genotypes were associated with an increased risk for progression to cirrhosis (OR = 2.60; 95%CI: 1.63-4.19; $P < 0.001$) and HCC (OR = 2.45; 95%CI: 1.42-4.31; $P = 0.001$).

CONCLUSION

These findings suggest that the T allele of *IFNL4* rs12979860 polymorphism is associated with the development of cirrhosis and HCC in chronic HCV-infected patients.

Key Words: Hepatitis C; Hepatitis C virus; Cirrhosis; Hepatocellular carcinoma; Genetic polymorphism; Interferon-lambda

Core Tip: Hepatitis C virus (HCV) infection is a major public health problem worldwide as the infection progresses to severe chronic liver diseases in many patients. Interferon lambdas modulate the immune responses against infections, including the antiviral activity by promoting the expression of several genes related to immunological functions. The interferon lambda-4 rs12979860 (C/T) polymorphism, which is associated with spontaneous and treatment-induced clearance of HCV, plays a pivotal role in the host response to HCV-associated liver diseases. In this case-control study, the rs12979860 T allele was found to be associated with the development of cirrhosis and hepatocellular carcinoma in chronic HCV-infected patients.

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INTRODUCTION

Hepatitis C virus (HCV) infection is a public health concern worldwide as it is associated with increased morbidity and mortality[1,2]. HCV, a hepatotrophic virus, is the etiological factor for chronic hepatitis C. Patients with HCV infection can develop cirrhosis and hepatocellular carcinoma (HCC) and may need liver transplantation[2-4]. According to the World Health Organization report on viral hepatitis, 71 million people were infected with hepatitis C in 2015[2].

Generally, acute HCV infections are clinically silent infections. Among the patients with HCV infection, 15%-45% can eliminate the virus spontaneously, with the highest recovery rates observed in children and young women[5]. However, a vast majority of infected patients develop chronic hepatitis C, which is characterized by the persistence of HCV in the serum for more than 6 mo. Chronic HCV infection is associated with

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slow progression, and the patients may remain asymptomatic for several decades. Thus, the persistence of HCV in the organism can cause continuous damage to the liver and can progress to fibrosis, cirrhosis, and HCC[5,6].

HCC, which accounts for 80% of all primary liver cancers, is associated with high mortality rates. Globally, HCC is the third leading cause of cancer-related deaths. HCC is a complex disease with a variety of etiologies and may be associated with different risk factors, such as chronic hepatitis B virus (HBV) and HCV infections, alcoholic liver disease, and nonalcoholic steatohepatitis[7,8]. HCV infection, which is the second most common risk factor for HCC, accounts for 10%-25% of all HCC cases. Additionally, 80%-90% of HCC cases are reported in patients with cirrhosis[9,10].

The pathogenesis of HCV infection and its progression to chronic liver disease vary among individuals. Several factors, including viral, environmental, and host characteristics, such as age, sex, ethnicity, and genetic factors, contribute to the pathogenesis of HCV[11]. The immune system-related genes, such as interferon lambdas (IFN-λs), are directly related to modulate viral infections with the ability to induce antiviral activity in target cells and interfere with HCV replication within the host cells. The binding of IFN-λ to its receptor activates the signal transducer and activator of transcription phosphorylation-dependent signaling cascade, inducing hundreds of IFN-stimulated genes and consequently regulating various immune functions[12-14].

The interferon lambda-3 gene (*IFNL3*), which is located on chromosome 19q13.13, encodes IFN-λ3 protein, a cytokine with antiviral properties. Genome-wide association studies have demonstrated the association of single nucleotide polymorphisms, such as rs12979860 and rs8099917, near the *IFNL3* gene (formerly known as interleukin-28B gene; *IL28B*), both with spontaneous virus elimination after acute infection and with sustained virological response in patients with chronic hepatitis C treated with pegylated interferon plus ribavirin combination therapy[15-18].

Prokunina-Olsson *et al*[19] demonstrated that the rs12979860 polymorphism, commonly referred as an *IL28B* or *IFNL3* variant, is in an independent loci and should be called an interferon lambda-4 (*IFNL4*) variant. The *IFNL4* gene is controlled by rs368234815 ΔG-TT polymorphism, in which the ΔG allele creates an open reading frame for *IFNL4*, while the TT allele does not. Furthermore, the ΔG allele (rs368234815) is reported to be in linkage disequilibrium with the T allele of rs12979860 polymorphism[13,19].

The rs12979860 polymorphism has a relevant and well-known role in the spontaneous and treatment-induced clearance of HCV infection[20]. However, the importance of this polymorphism in the progression of HCV-associated liver diseases is still unclear. Therefore, the objective of our study was to investigate the potential role of the variants from *IFNL4* rs12979860 polymorphism in the progression to hepatic fibrosis, cirrhosis, and HCC in chronic HCV-infected patients.

MATERIALS AND METHODS

Study population

This case-control study was conducted using a convenience sampling strategy. The case group was comprised of 305 patients who visited the outpatient clinic of the Gastroenterology-Hepatology Service of the Hospital de Clínicas de Porto Alegre in Brazil. HCV-positive patients diagnosed with fibrosis, cirrhosis, or HCC were included in the case group. Fibrosis (METAVIR F1-F3) was diagnosed based on liver biopsy findings, while cirrhosis was diagnosed based on liver biopsy or clinical evidence, such as liver imaging (abdominal ultrasonography, computed tomography, and magnetic resonance) abnormalities or endoscopic findings as well as current or past clinical evidence of decompensation, including Child-Pugh B or C classification (score of > 6), ascites on physical examination, hepatic encephalopathy, or variceal bleeding. HCC was diagnosed through liver biopsy (64/98; 65.3%) or in cirrhotic patients through dynamic computed tomography or magnetic resonance by the presence of a nodule of at least 1 cm featuring arterial phase enhancement with decreased enhancement during the portal venous phase as recommended by international guidelines. Patients with HCV/human immunodeficiency virus and/or HCV/HBV coinfection were excluded as well as patients with other causes of liver diseases such as HBV, metabolic associated fatty liver disease, alcohol abuse (more than 20 or 30 g daily consumption of ethanol for females and males, respectively), and/or hemochromatosis. The control group was comprised of 260 samples obtained from the donors at the Hospital de Clínicas de Porto Alegre blood bank. As Brazilian laws for blood donation requires, all have been tested negative for HBV, HCV, human

immunodeficiency virus, syphilis, and Chagas disease. This study was approved by the Research Ethics Committee of the Hospital de Clínicas de Porto Alegre (protocol number: 15-0126). All participants provided their written informed consent to participate in the study.

Molecular analysis

DNA was extracted from the blood samples using the salting-out method as described previously[21]. The polymorphism was genotyped using the validated pre-designed real-time PCR TaqMan® Assays (Applied Biosystems Inc., Foster City, CA, United States; catalog 4351376, assay ID: C___7820464_10) in the StepOnePlusTM Real-Time PCR Systems (Applied Biosystems Inc.). PCR was performed in an 18 μL reaction volume containing 10 mmol/L Tris-HCl (pH 8.5), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.0625 mmol/L dNTPs, 0.25 μM of each primer, 0.045 μM of each probe, 1 U Taq DNA polymerase (Cenbiot Enzimas, Porto Alegre, Brazil), and 1 μL extracted DNA (10-200 ng). The PCR conditions were as follows: 95 °C for 10 min (initial DNA denaturation), followed by 40 cycles of 95 °C for 15 s (denaturation) and 60 °C for 1 min (annealing and extension).

Statistical analyses

All statistical analyses were performed using SPSS® software (Statistical Package for the Social Sciences 17.0 version, Chicago, IL, United States). The normal distribution of the quantitative variables was examined using the Kolmogorov-Smirnov test with Lilliefors correction. The quantitative variables, which were expressed as mean ± SD, were analyzed using analysis of variance, followed by Tukey post-hoc test. For the categorical variables, the frequencies were calculated and expressed as percentages. Gene frequencies were determined by direct allele counting. Hardy-Weinberg equilibrium (HWE) deviation and the gene frequencies between groups were compared using the Chi-square test. Yates' correction for continuity was used to analyze the 2 × 2 contingency tables. Odds ratio (OR) was estimated with 95% confidence interval (CI). The differences were considered significant at $P < 0.05$ (two-tailed). Potential confounding factors were entered in the logistic regression models based on statistical criteria (only if the variable was associated with the study factor and with the outcome at $P < 0.20$). The statistical methods used in this study were reviewed by Dr. D. Simon from the Human Molecular Genetics Laboratory, Universidade Luterana do Brasil (Canoas, Brazil).

RESULTS

The sociodemographic and clinical characteristics of patients are described in Table 1. Patients were stratified into the following three groups: Fibrosis ($n = 53$), cirrhosis ($n = 154$), and HCC ($n = 98$). The mean age of the patients was 59.85 ± 8.83 years, with a statistically significant difference among the groups studied ($P = 0.019$). A significant statistical difference ($P = 0.024$) was also observed in the frequency of males in the HCC group (58.2%) when compared to the fibrosis (37.7%) and cirrhosis groups (43.5%). The mean value of body mass index presented a statistically significant difference between the groups with cirrhosis and HCC (27.80 ± 5.39 and 26.34 ± 4.15 kg/m^2, respectively; $P = 0.038$). Blood transfusion was the most frequent possible infection source among patients (41.0%). The frequencies of HCV 1 and 3 genotypes, which were the most common, were 40.7% and 36.7%, respectively.

Table 2 shows the allele and genotype frequencies of the *IFNL4* rs12979680 polymorphism in the patient and control groups. The success rate for genotyping *IFNL4* rs12979680 polymorphism was 100% in all studied groups. Statistically significant differences were observed regarding the allele frequencies, in which the frequency of the T allele was significantly higher in the three groups of patients analyzed when compared to the controls: [fibrosis group *vs* control group (OR = 1.57; 95%CI: 1.03-1.68; $P = 0.047$), cirrhosis group *vs* control group (OR = 1.75; 95%CI: 1.30-2.36; $P < 0.001$), and HCC group *vs* control group (OR = 1.57, 95%CI: 1.11-2.23; $P = 0.01$)].

Compared with those in the control group, the *IFNL4* genotype frequencies were significantly higher in the cirrhotic and ($P < 0.001$) HCC groups ($P = 0.002$). The genotype distribution in the control and fibrosis groups was in agreement with those expected from HWE ($P = 0.81$ and $P = 0.88$, respectively). In contrast, the genotype frequencies in the cirrhosis and HCC groups deviated from those expected from HWE ($P = 0.02$ and $P = 0.01$, respectively). When the genotype distribution was analyzed in

Table 1 Sociodemographic and clinical features of chronic hepatitis C virus positive patients

Characteristics	Total, *n* = 305	Fibrosis, *n* = 53	Cirrhosis, *n* = 154	HCC, *n* = 98	*P* value
Age in yr	59.85 ± 8.83	57.89 ± 10.43	59.29 ± 8.43	61.78 ± 8.22	0.019
Male	144 (47.2)	20 (37.7)	67 (43.5)	57 (58.2)	0.024
Ethnicity, Caucasian	218 (71.5)	35 (66.1)	110 (71.4)	73 (74.5)	0.547
BMI in kg/m²	27.08 ± 4.85	26.39 ± 4.14	27.80 ± 5.39	26.34 ± 4.15	0.038
Level of education					0.366
Completed primary education or less	196 (62.0)	31 (56.6)	100 (62.3)	65 (64.3)	
Secondary or higher education	102 (24.9)	20 (34.0)	51 (25.3)	31 (19.4)	
Smoker	59 (19.3)	16 (30.2)	31 (20.1)	12 (12.2)	0.001
Alcohol consumption					0.004
No	260 (85.2)	49 (92.5)	137 (89.0)	74 (75.5)	
Former	45 (14.8)	4 (7.5)	17 (11.0)	24 (24.5)	
Illicit drug use					0.164
No	243 (79.7)	43 (81.1)	122 (79.2)	78 (79.6)	
Yes	9 (3.0)	4 (7.5)	4 (2.6)	1 (1.0)	
Former user	53 (17.4)	6 (1.1)	28 (18.2)	19 (19.4)	
Coffee drinker	213 (69.8)	39 (73.6)	112 (72.7)	62 (63.3)	0.226
Age at infection of HCV in yr	27.43 ± 9.75	28.47 ± 9.12	27.48 ± 9.77	26.64 ± 10.26	0.735
Age at diagnosis of HCV in yr	49.11 ± 11.11	46.88 ± 12.99	49.17 ± 10.97	50.24 ± 10.11	0.223
HCV infection *via* blood transfusion	125 (41.0)	24 (45.3)	64 (41.6)	37 (37.8)	0.706
HCV-RNA as log_{10}UI/mL	6.05 ± 0.86	-	6.11 ± 0.87	5.86 ± 0.78	0.141
HCV genotypes					0.060
1	124 (40.7)	-	86 (55.8)	38 (38.8)	
2	7 (2.3)	-	4 (2.6)	3 (3.1)	
3	112 (36.7)	-	61 (39.6)	51 (52.0)	
Antiviral treatment	178 (58.4)	-	115 (74.7)	63 (64.3)	0.077
Diabetes	85 (27.9)	-	50 (32.5)	35 (35.7)	0.595
Steatosis	24 (7.9)	-	13 (8.4)	11 (11.2)	0.431
Ascites	66 (21.6)	-	31 (20.1)	35 (35.7)	0.005
Portal hypertension	146 (47.9)	-	72 (46.8)	74 (75.5)	< 0.001
Esophageal varices	156 (51.1)	-	91 (59.0)	65 (66.3)	0.231
Upper gastrointestinal bleeding	49 (16.0)	-	26 (16.9)	23 (23.5)	0.184
Spontaneous bacterial peritonitis	13 (4.3)	-	7 (4.5)	6 (6.1)	0.568
Hepatic encephalopathy	24 (7.9)	-	13 (8.4)	11 (11.2)	0.431
Child-Pugh					0.083
A	137 (44.9)	-	95 (61.7)	42 (42.9)	
B	43 (14.1)	-	28 (18.2)	15 (15.3)	
C	9 (3.0)	-	3 (1.9)	6 (6.1)	
Number of tumors					
1		-	-	62 (63.37)	
2		-	-	17 (17.35)	
≥ 3		-	-	18 (18.37)	

Tumor size in cm		-	-	2.8 ± 1.81	
Portal vein thrombosis		-	-	10 (10.20)	
Extrahepatic metastases		-	-	7 (7.14)	
Liver transplantation		-	-	47 (47.96)	
Deaths	14 (4.59)	-	8 (5.19)	6 (6.12)	0.754

Characteristics expressed as number and percentage or mean ± SD. BMI: Body mass index; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus.

Table 2 Allele and genotype frequencies of interferon lambda-4 rs12979860 polymorphism in patients with hepatitis C virus-associated liver diseases and healthy control subjects

rs12979860	Control, *n* = 260	Total patients, *n* = 305	Fibrosis, *n* = 53	Cirrhosis, *n* = 154	HCC, *n* = 98	*P* value				
						Fibrosis *vs* Control	Cirrhosis *vs* Control	HCC *vs* Control	Fibrosis *vs* Cirrhosis	Cirrhosis *vs* HCC
Allele						0.047	< 0.001	0.010	0.708	0.618
C	345 (66.3)	331 (54.3)	59 (55.7)	163 (52.9)	109 (55.6)					
T	175 (33.7)	279 (45.7)	47 (44.3)	145 (47.1)	87 (44.4)					
Genotype						0.113	< 0.001	0.002	0.541	0.665
CC	115 (44.2)	76 (24.9)	16 (30.2)	36 (23.4)	24 (24.5)					
CT	115 (44.2)	179 (58.7)	27 (50.9)	91 (59.1)	61 (62.2)					
TT	30 (11.6)	50 (16.4)	10 (18.9)	27 (17.5)	13 (13.3)					

Variables expressed as number (percentage). HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus.

the total sample of patients (n = 305), deviations from HWE were maintained (P = 0.001).

The risk of developing fibrosis, cirrhosis, and HCC was calculated using the following two genetic models: Codominant and dominant T allele models (Table 3). In the codominant model, it was observed that the CT *vs* CC genotype conferred an increased risk of developing cirrhosis in HCV patients when compared with the control group (OR = 2.53; 95%CI: 1.55-4.15; P < 0.001). Additionally, the CT *vs* CC genotype conferred an increased risk for HCC (OR = 2.54; 95%CI: 1.44-4.56; P = 0.001). A similar result was observed in the comparison of the TT *vs* CC genotype between cirrhosis patients and controls (OR = 2.88; 95%CI: 1.44-5.77; P = 0.001) but not for HCC. In the dominant T allele model, the CT + TT genotypes conferred an increased risk of developing cirrhosis (OR = 2.60; 95%CI: 1.63-4.19; P < 0.001) and HCC (OR = 2.45; 95%CI: 1.42-4.31; P = 0.001) when compared with the CC genotype. The observed associations remained significant when logistic regression models were analyzed controlling for potential confounding factors (data not shown).

Table 4 presents the distribution of the *IFNL4* rs12979680 polymorphism genotypes regarding clinical features of HCC patients. A significantly higher frequency of the T allele in the dominant T allele model was observed among patients with HCV genotypes 1 and 3 with a frequency of 92% and 67%, respectively (P = 0.017). In addition, a higher frequency of the TT genotype was observed among patients with hepatic encephalopathy (P = 0.03).

Table 3 Genetic models of association between interferon lambda-4 rs12979860 polymorphism and hepatitis C virus-associated liver diseases

rs12979860	Fibrosis *vs* Control		Cirrhosis *vs* Control		HCC *vs* Control		Fibrosis *vs* Cirrhosis		Cirrhosis *vs* HCC	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Codominant model										
CC	1.00 (Ref.)	-	1.00 (Ref.)	-	1.00 (Ref.)	-	1.00 (Ref.)	-	1.00 (Ref.)	-
CT	1.69 (0.82-3.54)	0.126	2.53 (1.55-4.15)	< 0.001	2.54 (1.44-4.56)	0.001	1.50 (0.67-3.28)	0.277	1.01 (0.52-1.95)	0.986
TT	2.40 (0.87-6.27)	0.053	2.88 (1.44-5.77)	0.001	2.08 (0.86-4.83)	0.068	1.20 (0.43-3.45)	0.702	0.72 (0.28-1.80)	0.447
T allele dominant model										
CC	1.00 (Ref.)	-	1.00 (Ref.)	-	1.00 (Ref.)	-	1.00 (Ref.)	-	1.00 (Ref.)	-
CT + TT	1.83 (0.94-3.71)	0.061	2.60 (1.63-4.19)	< 0.001	2.45 (1.42-4.31)	0.001	1.42 (0.66-2.97)	0.325	0.94 (0.50-1.79)	0.840

CI: Confidence interval; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; OR: Odds ratio; Ref.: Reference.

DISCUSSION

This study investigated the association of the *IFNL4* rs12979860 polymorphism with the development of fibrosis, cirrhosis, and HCC among patients with chronic HCV infection. The frequency of the T allele in the case group was higher than that in the control group. Additionally, the risk analyses indicated that patients with HCV infection harboring the T allele were more susceptible to develop cirrhosis and HCC.

The studies on the role of *IFNL4* rs12979860 polymorphism in HCV-related liver diseases have yielded controversial results. A recent meta-analysis of 18 studies involving different ethnicities attempted to elucidate the global association of this polymorphism with HCV and HBV[22]. The meta-analysis revealed that the *IFNL4* rs12979860 polymorphism is a risk factor for both HCV-and HBV-related HCC. Although the meta-analysis enhanced our understanding of the role of *IFNL4* rs12979860 polymorphism in the outcomes of liver diseases with viral etiologies, the results must be carefully analyzed. Some limiting factors, such as ethnic differences, discrepancies in clinical characteristics among different studies, genotyping methods, HCV genotypes, nonuniform controls in case-control studies, and the influence of confounding factors should be considered.

Various studies have evaluated the role of *IFNL4* rs12979860 polymorphism in the development of HCC. De la Fuente *et al*[23] examined the association of rs12979860 polymorphism with the development of HCC in both chronic HCV infection and nonviral cirrhosis. The authors reported that the TT genotype is highly prevalent in cirrhotic patients infected with HCV genotype 1 who were subjected to liver transplantation. However, there was no significant association between polymorphism variants and hepatocarcinogenesis.

The risk of developing HCC in patients responding to pegylated interferon plus ribavirin treatment is lower than that in nonresponders. Chang *et al*[24] evaluated 800 patients who received pegylated interferon plus ribavirin combination therapy but did not respond to treatment to evaluate the risk factors for HCC. The CT + TT genotypes of rs12979860 polymorphism were an independent risk factor for the development of HCC in these patients, which further indicated the importance of this polymorphism in the progression to HCC. Similarly, a study on 200 patients with advanced fibrosis revealed that the *IFNL4* rs12979860 TT genotype was significantly associated with HCC development after direct-acting antiviral therapy for chronic hepatitis C[25].

A large international study involving 2916 patients, mostly the European Caucasian population, revealed that the increased number of the T allele was significantly associated with the prevalence of cirrhosis/transition to cirrhosis in patients infected with HCV genotype 1. This association was evident in Caucasian European patients but not in Asian, Latin American, or Middle Eastern patients infected with HCV genotype 1[26].

The genetic background of populations can contribute to variable results among

Table 4 Distribution of the interferon lambda-4 rs12979860 genotypes based on the clinical features of patients with hepatocellular carcinoma, *n* = 98

		Genotypes		Codominant model	T allele dominant model
Variable	**CC, *n* = 24**	**CT, *n* = 61**	**TT, *n* = 13**	***P* value**	***P* value**
HCV genotypes				0.052	0.017
1	3 (14.3)	27 (46.6)	8 (61.5)		0.004
2	1(4.8)	2 (3.4)	-		
3	17 (81.0)	29 (50.0)	5 (38.5)		0.007
Diabetes	10 (41.7)	19 (31.1)	6 (46.2)	0.463	0.484
Steatosis	1(4.2)	8 (13.3)	2 (16.7)	0.409	0.195
Ascites	10 (41.7)	20 (32.8)	5 (41.7)	0.679	0.511
Portal hypertension	17 (70.8)	48 (78.7)	9 (75.0)	0.741	0.469
Esophageal varices	17 (70.8)	39 (63.9)	9 (75.0)	0.682	0.646
Upper gastrointestinal bleeding	8 (33.3)	10 (16.4)	5 (41.7)	0.075	0.201
Spontaneous bacterial peritonitis	1 (4.2)	5 (8.2)	-	0.500	0.636
Hepatic encephalopathy	3 (12.5)	2 (3.3)	3 (25.0)	**0.030**	0.383
Child-Pugh				0.209	0.156
A	8 (61.5)	26 (63.4)	8 (88.9)		
B	2 (15.4)	12 (29.3)	1 (11.1)		
C	3 (23,1)	3 (7.3)	-		
Number of tumors				0.325	0.684
1	17 (70.8)	39 (65.0)	6 (46.2)		
2	3 (12.5)	12 (20.0)	2 (15.4)		
≥ 3	4 (16.7)	9 (15.0)	5 (38.5)		
Portal vein thrombosis	4 (16.7)	4 (6.6)	2 (16.7)	0.286	0.238
Extrahepatic metastases	1 (4.2)	5 (8.6)	1 (7.7)	0.780	0.487

Variables expressed as number (percentage). HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus.

different studies as the allele frequencies of *IFNL4* rs12979860 polymorphism vary among populations. In this study, the minor allele frequencies of the *IFNL4* rs12979860 polymorphism, represented by the T allele, in the case and control groups were 0.46 and 0.34, respectively. The minor allele frequencies reported for European, Japanese, and Chinese populations in the 1000 Genomes database were 0.28, 0.10, and 0.06, respectively.

The role of IFN-λ4 in the pathophysiology of chronic HCV infection-mediated liver diseases is still under investigation. IFN-λ4 activates interferon-stimulated genes, induces cell death, and inhibits cell proliferation[27]. In the IFN-λ4-expressing cells, enhanced cell death may cause tissue inflammation, while the antiproliferative effect of IFN-λ4 could decrease the capacity of tissue remodeling[27,28]. In this sense, our study may provide significant information about the association of the genetic variants of the *IFNL4* rs12979860 polymorphism with disease progression and clinical features of hepatitis C, demonstrating that this polymorphism has relevance in the HCV spontaneous and treatment-induced clearance of HCV infection. Also, the present study can stimulate the clarification of this issue by the analyses of large samples as well as the correlation of genetic variants with gene expression and protein interactions.

This study has some limitations. The sample size of this study is relatively small. A more representative sample could enhance the statistical power to detect genetic differences. In this study, the fibrosis group, which had the lowest sample number, exhibited a trend of association with the TT genotype and the T allele when compared with the control group. A larger sample size could clarify the role of this

polymorphism in the development of fibrosis. In addition, some data are missing in the liver fibrosis group (such as HCV RNA, HCV genotype, number of patients on antiviral treatment, diabetes, and steatosis), which precluded a more detailed comparison with the other groups. Besides, the analysis of a nonfibrotic (F0) HCV-infected group would be important because it makes the study more comprehensive. The analysis of a single polymorphism is insufficient to fully explain the genetic basis of HCC. In the cirrhosis and HCC groups, the genotype frequencies of the *IFNL4* rs12979860 polymorphism did not concur with those expected from HWE. The deviations from HWE can be due to the population stratification and selection or may indicate disease association[29,30]. As population stratification may have caused disequilibrium among the cirrhosis and HCC groups, HWE analysis was performed on the case group. However, the genotype frequency in the case group deviated from that expected from HWE. Thus, the observed imbalance could be explained by the effective role of this polymorphism in the sample of patients with HCV-related liver diseases.

CONCLUSION

The findings of this study suggest that the T allele of *IFNL4* rs12979860 polymorphism is a potential genetic factor that determines the susceptibility to cirrhosis and HCC development among patients with chronic HCV.

ARTICLE HIGHLIGHTS

Research background

As a serious public health problem worldwide, hepatitis C virus (HCV) infection has unfavorable trends in morbidity and mortality. Due to high hepatotrophic potential, HCV may cause chronic complications, such as fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). Progression to chronic liver disease usually varies and is influenced by different factors, including genetic factors. The interferon lambda-4 (*IFNL4*) rs12979860 polymorphism, characterized by a C to T transition in the intron 1, has been associated with spontaneous and treatment-induced clearance of HCV infection and may play a role in HCV-associated liver diseases, including HCC.

Research motivation

Although the rs12979860 polymorphism has a relevant and well-known role in the spontaneous and treatment-induced clearance of HCV infection, the importance of genetic variants of this polymorphism in the progression of HCV-associated liver diseases is still unclear.

Research objectives

We aimed to investigate the potential role of the variants in the progression to hepatic fibrosis, cirrhosis, and HCC in chronic HCV-infected patients. In addition, the distribution of the rs12979860 *IFNL4* genetic variants was analyzed in accordance with clinical features of patients.

Research methods

This case-control study included 305 patients with chronic HCV infection patients (53 with fibrosis, 154 with cirrhosis, and 98 with HCC), and 260 HCV-negative healthy individuals as controls. Diagnosis of fibrosis (METAVIR F1-F3) was performed by liver biopsy findings, while the diagnosis of cirrhosis was performed through clinical, laboratorial, anatomopathological, and/or imaging data. Lastly, diagnosis of HCC was performed through dynamic imaging tests, and/or anatomopathological markers. Patients with HCV/human immunodeficiency virus and/or HCV/ hepatitis B virus coinfection were excluded. Molecular analysis was performed using validated pre-designed real-time PCR TaqMan® Assays.

Research results

A higher frequency of the T allele was observed among the groups of patients (fibrosis, cirrhosis, and HCC) as compared to the controls: (P = 0.047; P < 0.001; and P = 0.01, respectively). Also, significant differences were observed concerning genotype

frequencies between HCC (P = 0.002) and cirrhosis patients (P < 0.001) in comparison with controls. Two genetic models were tested in the risk analysis: Codominant model and dominant T allele model. In the codominant model, it was observed that the CT genotype was related to an increased risk of cirrhosis [odds ratio (OR) = 2.53; 95% confidence interval (CI): 1.55-4.15; P < 0.001] and HCC (OR = 2.54; 95%CI: 1.44-4.56; P = 0.001) as compared to CC genotype. In the comparison of the TT *vs* CC genotype, a significant difference was observed between the control group and cirrhosis group (OR = 2.88; 95%CI: 1.44-5.77; P = 0.001) but not the HCC group. In the dominant T allele model, the CT + TT genotypes confer an increased risk for the progression to cirrhosis (OR = 2.60; 95%CI: 1.63-4.19; P < 0.001) and HCC (OR = 2.45; 95%CI: 1.42-4.31; P = 0.001). Finally, a significant higher frequency of the T allele among patients with HCV genotypes 1 and 3 (92% and 67%, respectively; P = 0.017) and a higher frequency of TT genotype among patients with hepatic encephalopathy (P = 0.03) was observed.

Research conclusions

This study suggests that the T allele from *IFNL4* rs12979860 polymorphism is associated with the development of cirrhosis and HCC in chronic HCV-infected patients.

Research perspectives

As an important factor related to spontaneous and treatment-induced clearance of HCV infection, the analysis of *IFNL4* rs12979860 polymorphism in the present study may provide a better understanding of the genetic variants with disease progression and clinical features. In order to clarify this issue, large samples are needed to verify the association of genetic polymorphisms with hepatitis C as well as the correlation of genetic variants with gene expression and protein interactions.

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ORIGINAL ARTICLE

Retrospective Study

Immunization status and hospitalization for vaccine-preventable and non-vaccine-preventable infections in liver-transplanted children

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Author contributions: Sintusek P designed and oversaw the study, drafted the manuscript, collected, interpreted, and analyzed the data, and made critical revisions related to important intellectual content; Poovorawan Y suggested critical intellectual content and approved the final manuscript.

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Informed consent statement: Patients were not required to give

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Abstract

BACKGROUND

Infections and associated morbidity and mortality may be more frequent in children who have undergone liver transplant than in healthy children. Immunization strategies to prevent vaccine-preventable infections (VPIs) can effectively minimize this infection burden. However, data on age-appropriate immunization and VPIs in children after liver transplant in Asia are limited.

AIM

To evaluate the immunization status, VPIs and non-VPIs requiring hospitalization in children who have undergone a liver transplant.

METHODS

The medical records of children who had a liver transplant between 2004 and 2018 at King Chulalongkorn Memorial Hospital (Bangkok, Thailand) were retrospectively reviewed. Immunization status was evaluated *via* their vaccination books. Hospitalization for infections that occurred up to 5 years after liver transplantation were evaluated, and divided into VPIs and non-VPIs. Hospitalizations for cytomegalovirus and Epstein-Barr virus were excluded. Severity of infection, length of hospital stay, ventilator support, intensive care unit requirement, and mortality were assessed.

RESULTS

informed consent to the study because the analysis used anonymous clinical data that were obtained after each the patient agreed to treatment by written consent.

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

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Seventy-seven children with a mean age of 3.29 ± 4.17 years were included in the study, of whom 41 (53.2%) were female. The mean follow-up duration was 3.68 ± 1.45 years. Forty-eight children (62.3%) had vaccination records. There was a significant difference in the proportion of children with incomplete vaccination according to Thailand's Expanded Program on Immunization (52.0%) and accelerated vaccine from Infectious Diseases Society of America (89.5%) (P < 0.001). Post-liver transplant, 47.9% of the children did not catch up with age-appropriate immunizations. There were 237 infections requiring hospitalization during the 5 years of follow-up. There were no significant differences in hospitalization for VPIs or non-VPIs in children with complete and incomplete immunizations. The risk of serious infection was high in the first year after receiving a liver transplant, and two children died. Respiratory and gastrointestinal systems were common sites of infection. The most common pathogens that caused VPIs were rotavirus, influenza virus, and varicella-zoster virus.

CONCLUSION

Incomplete immunization was common pre- and post-transplant, and nearly all children required hospitalization for non-VPIs or VPIs within 5 years post-transplant. Infection severity was high in the first year post-transplant.

Key Words: Children; Hospitalization; Immunization; Liver transplant; Thailand; Vaccine-preventable infection

Core Tip: Incomplete age-appropriate immunization in children waiting for a liver transplant was expected, and nearly half of them had not caught up with age-appropriate vaccinations post-transplant. Though there was no significant difference in hospitalization from vaccine-preventable infections (VPIs) and non-VPIs in children with complete and incomplete immunizations. At least 13.1% required hospitalization within 5 years post-transplant, and > 10% were admitted to the intensive care unit and required respiratory support. The severity of infections was high during the first year post-transplant. Complete immunization and robust infection control should be prioritized in children both pre and post-liver transplant.

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INTRODUCTION

Infection after a liver transplant is a serious concern due to potential associated morbidity and mortality[1-4], as well as the standard complications and severe symptoms that can be experienced by immunocompetent patients. Such infections can give rise to graft rejection, thus affecting short- or long-term graft survival[4]. Accordingly, strategies to reduce overall post-transplant infection are warranted. Immunization is considered an effective, relatively noninvasive, and affordable way to reduce vaccine-preventable infections (VPIs)[5] such as measles, varicella, influenza, and viral hepatitis A and B, among others. The Infectious Diseases Society of America (IDSA)[6] and the American Society of Transplantation Infectious Disease Community of Practice[7] encourage accelerated vaccination, particularly with regard to live vaccines in immunocompromised children awaiting for solid organ transplantation.

Children awaiting a liver transplant can be at a disproportionate risk of VPIs because they tend not to have undergone a complete series of age-appropriate immunizations, because their serious illness has taken medical priority over vaccination[8]. Verma and Wade[9] reported that in their experience at King's College

Hospital, only 20%-30% of children had undergone a complete series of age-appropriate immunizations prior to liver transplantation. Diana *et al*[10] reported that less than half of a cohort of children who underwent liver transplant at the Children's Hospital of Geneva in Switzerland had undergone a complete series of age-appropriate vaccinations, with rates of 43% for diphtheria-tetanus-acellular pertussis-polio vaccine, 44% for measles-mumps-rubella (MMR) vaccine, 13% for hepatitis B vaccine, and 5% for hepatitis A vaccine at the time of liver transplantation. Feldman *et al*[4,11] investigated morbidity, mortality, and costs associated with VPIs in children after solid organ transplants, and reported a significantly higher rate of VPIs in these children than in the general pediatric population.

Studies conducted in the United States and other western countries have highlighted the effects of VPIs in children after solid organ transplantation[4,9-11], but published data on VPIs in children after liver transplantation in the East are scarce. To improve the quality of life of liver-transplanted children by minimizing the serious complications associated with post-liver transplant infections, strategies to avoid VPIs based on strong evidence should be initiated worldwide, including in Asia.

The aim of the present study was to evaluate immunization status in Thai children at the time of liver transplantation, and for up to 5 years post-liver transplantation. The prevalence and effects of VPIs and non-VPIs during hospitalization were also assessed.

MATERIALS AND METHODS

The current study was a retrospective review of all children who received a liver transplant at King Chulalongkorn Memorial Hospital in Thailand from January 2004 to August 2018. Demographic data, patient characteristics, and immunization records from vaccination books were collated. Hospitalization records pertaining to the liver transplant operation and admission due to infections for up to 5 years post-transplant were included. Hospitalizations for Epstein-Barr virus (EBV) and cytomegalovirus were excluded from the study. Infection etiology and source were investigated by the doctors in charge. Culture from specimens was available for all bacterial origins, and immunological and molecular techniques were available for the diagnosis of both viral and bacterial infections, including polymerase chain reaction panel analysis for respiratory tract infections and gastrointestinal infections, and antibody titers for hepatitis A/B/E, dengue, and measles.

Infections were divided into VPIs and non-VPIs. Length of hospital stay, severity of infections, and mortality from infections were collated and classified into three groups: Intensive care unit (ICU) requirement, ventilator support, and death. Complete immunization was defined as that conducted in accordance with the Expanded Program on Immunization (EPI) in Thailand (Table 1) and the accelerated vaccination recommendations described in the 2013 IDSA Clinical Practice Guideline for Vaccination of the Immunocompromised Host[6], which notes: "... children aged 6-12 mo can receive MMR and varicella vaccine and the second dose should be administered at 12 mo for MMR and ≥ 3 mo apart for varicella vaccine. However, the last MMR or varicella vaccine injection should not be within 4 wk of a liver transplant schedule."

Statistical analysis

Continuous and categorical data are presented as the mean ± SD, medians and interquartile ranges, proportions, or percentages as appropriate. The Mann Whitney *U* test and unpaired *t*-test were used to compare continuous data, and Fisher's exact test and the χ^2 test were used to compare discrete data. $P < 0.05$ was considered statistically significant. Data analyses were performed using Statistical Package for the Social Sciences version 24.0.0 (SPSS, Inc.; Chicago, IL, United States). A biomedical statistician employed at the Department of Statistics Science, Kasetsart University (Bangkok, Thailand) reviewed the statistical analyses conducted in the study.

RESULTS

Patient characteristics and history of immunization

Seventy-seven children with a mean age of 3.29 ± 4.17 years were included in the study, of whom 41 (53.2%) were female. The indications for liver transplantation were biliary atresia (n = 63), indeterminate acute liver failure (n = 3), progressive familial

Table 1 The immunization schedule in Thailand and accelerated vaccines by the Infectious Disease Society of America

	Vaccine	Birth	1 mo	2 mo	4 mo	6 mo	7 mo	9 mo	12 mo	18 mo	24 mo	4 yr	9 yr	11 yr
Thai's EPI vaccines	BCG	1												
	HBV	1	(For positive maternal HBsAg)	2		3								
	DTP, OPV/IPV			1	2	3				4		5		
	MMR					Acc[1]		1	Acc[1]		2			
	JE							1			2			
	Influenza					1	2							
	Tdap													1
	HPV												Acc	1-2[2]
Optional vaccine in Thailand	Rota			1	2	(3)								
	PCV			1	2	3			4					
	Varicella					Acc[1]		Acc[1]	1	2				
	HAV								1	2				
	Dengue												1-3[3]	

[1]Acc denotes accelerated vaccines from the 2013 Infectious Diseases Society of America Clinical Practice Guideline for Vaccination of the Immunocompromised Host in which measles-mumps-rubella (MMR) at 6 and 12 mo of age and varicella at 6 mo of age and 3 mo apart from the first dose.

[2]Indicates 0 and 6 mo.

[3]Indicates 0, 6, 12 mo.

BCG: Bacillus Calmette-Guerin vaccine; DTP: Diphtheria-tetanus-pertussis; EPI: Expanded Program on Immunization; HAV: Hepatitis A vaccine; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B vaccine; HPV: Human papillomavirus vaccine; JE: Japanese encephalitis; OPV/IPV: Oral polio vaccine/inactivated polio vaccine; PCV: Pneumococcal conjugate vaccine; Tdap: Tetanus-diphtheria-acellular pertussis.

intrahepatic cholestasis (n = 2), Alagille syndrome (n = 2), cryptogenic cirrhosis (n = 1), citrin deficiency (n = 1), Budd-Chiari syndrome (n = 1), hepatoblastoma (n = 1), autoimmune hepatitis (n = 1), glycogen storage disease type IV (n = 1), and bile acid deficiency (n = 1). The mean follow-up time was 3.68 ± 1.45 years, and 32 children were followed up for a full 5 years after liver transplantation. Vaccinations were noted in the vaccination books of 48/77 children (62.3%). Substantial proportions of children did not have complete vaccinations in accordance with Thailand's EPI (n = 25, 52%) (Table 1) or accelerated vaccinations in accordance with the IDSA recommendations (n = 43, 89.5%) (P < 0.001). Post-liver transplant, 23 children (47.9%) could not catch up with the appropriate immunizations for age. All children were revaccinated with hepatitis B vaccine if hepatitis B surface antibody was < 10 mIU/mL. Other vaccines

they received after liver transplantation included those for influenza (n = 12), invasive pneumococcal disease (n = 10), Japanese encephalitis (n = 6), diphtheria/tetanus/pertussis-inactivated polio vaccine (n = 6), and hepatitis A (n = 3). A minority of children were not up-to-date with influenza vaccination (n = 18, 37.5%) and pneumococcal conjugate vaccine (n = 22, 45.8%) post-liver transplant compared with pre-liver transplant (n = 30, 62.5% for influenza and n = 36, 75% for pneumococcal conjugate vaccine) (P < 0.001; Table 2). With regard to live vaccines, three individuals were inadvertently vaccinated with MMR at their local hospitals without any serious side effects.

Infections during and after liver transplant

Infection severity and mortality were highest during the first year post-liver transplant. The respiratory and gastrointestinal systems were the most common sites of infection (Table 3). Two children died within 3 mo after liver transplantation, and both had underlying post-transplant lymphoproliferative disorder. One of these two children had mixed infection with bocavirus, mycoplasma, and parvovirus B19. The other exhibited EBV viremia that progressed to respiratory failure with an unidentified infectious origin. Of the 31 hospitalizations for VPIs recorded during the study period the median length of hospital stay was 6 d (range: 3-8 d), and in three cases ICU admission and ventilator support were required; two with influenza and one with *Streptococcus pneumoniae* infection. When the children were divided into complete and incomplete immunization groups based on Thailand's EPI, there were no significant differences in the numbers of hospitalizations for VPIs or non-VPIs (Table 4).

Pathogens causing hospitalization in children post-liver transplant

A total of 237 infections requiring hospitalization were recorded during the study period. The most commonly identified bacterial pathogens were *Escherichia coli* (13.1%), *Salmonella* sp. (8.1%), and *Klebsiella pneumoniae* (6.8%), and the most commonly identified viral pathogens were parainfluenza (5.9%), rotavirus (3.4%), and respiratory syncytial virus (3.4%). In cases of VPIs, the most common pathogens were rotavirus (3.4%), influenza virus (2.5%), and varicella-zoster virus (2.1%) (Table 5 and 6).

DISCUSSION

In this study, incomplete age-appropriate immunization before liver transplantation in children was common, particularly with regard to live vaccines that can be accelerated before liver transplantation. Post-liver transplant in nearly half of the children in the study did not catch up with all age-appropriate vaccines. At least 13.1% of the children in the study required hospitalization for VPIs during the 5 years post-liver transplant, and in these cases, the lengths of hospital stays were up to 1 wk. More than 10% of the children required admission to the ICU and respiratory support from VPIs, reflecting the burden of VPIs during the post-transplant period. With regard to non-VPIs, both bacterial and viral infections of the respiratory and gastrointestinal systems played major roles in hospitalizations with severe infections and mortality, especially during the first year post-transplant.

To the best of our knowledge, the current study is the first to investigate immunization status and infections requiring hospitalization in Asian children who underwent a liver transplant. Compared to previous studies in Europe[9,10] and the United States[4,11], in the present study, there was a higher rate of incomplete age-appropriate immunization before liver transplantation, particularly with respect to the accelerated MMR and varicella vaccination. However, the number of hospitalizations with VPIs (13.1%) was comparable to that in a study conducted in the United States by Feldman *et al*[4,11] (11.3%). Moreover, the VPIs in that study were more severe and required longer hospital stays than those in the current study. Genetic risk factors may explain this phenomenon, as with the more contagious and severe coronavirus disease 2019 infections in Europe and the United States than in Thailand.

Prior to liver transplantation, physicians frequently do not offer patient immunization, particularly with respect to live vaccines[8,12,13]. There is solid evidence of adequate immune responses to varicella and measles vaccination in children aged < 1 year[14-16]; hence, the policy to promote accelerated vaccination in children before immunosuppressant therapy was initiated[6,7,17,18]. It is probable that this is not standard practice in healthy children. Moreover, children waiting for a liver transplant may have had complex and serious illnesses that needed to be given priority. Some physicians may not be familiar with the accelerated immunization program[8,13], and

Table 2 Vaccination history in children at liver transplant and up to 5 years follow-up (*n* = 48)

Vaccines	Incomplete vaccination for age at transplantation		Incomplete vaccination for age after liver transplant, *n* (%)
	Thai EPI program, *n* (%)	Accelerated vaccine from IDSA, *n* (%)	
DTP-OPV/IPV	12 (25)	N/A	6 (12.5)
HBV	6 (12.5)		0
MMR	12 (25)	30 (62.5)[b]	27 (56.3)[b]
JE	16 (33.3)	N/A	10 (20.8)
Varicella	16 (33.3)	34 (70.8)[b]	34 (70.8)[b]
HAV	26 (54)		23 (47.9)
Influenza	30 (62.5)		18 (37.5)[a]
PCV	36 (75)		22 (45.8)[b]
Rota	37 (77)	N/A	37 (77)
All	25 (52)	43 (89.5)[b]	23 (47.9)
	(not included rota vaccine)		(not included lived vaccine)

[a]$P < 0.05$ *vs* Thai Expanded Program on Immunization (EPI).

[b]$P < 0.001$ *vs* Thai EPI program.

DTP: Diphtheria-tetanus-pertussis; HAV: Hepatitis A vaccine; HBV: Hepatitis B vaccine; IDSA: Infectious Diseases Society of America; JE: Japanese encephalitis; MMR: Measles-mumps-rubella; N/A: Not applicable; OPV/IPV: Oral polio vaccine/inactivated polio vaccine; PCV: Pneumococcal conjugate vaccine.

therefore may decide to postpone vaccination. A specific protocol and concerted focus on educational interventions, or the development of specialized team care that is responsible for these issues is crucial to ensure that all candidates receive appropriate vaccinations to minimize complications associated with VPIs[6]. One great benefit of pre-liver transplant vaccination is higher immunogenicity compared with revaccination post-liver transplant[18]. Moreover, pretransplant vaccination of children will likely lead to herd immunity that will be beneficial for other transplant children in inpatient and outpatient clinics during their visits[13].

In the present study, the rate of incomplete age-appropriate immunization after liver transplantation was high, and there was no significant difference between the pretransplant rate (52.0%) and the post-transplant rate (47.9%). In theory, children's vaccination schedules should be postponed for more than 2 mo after liver transplantation because of the possibility of an inadequate immune responses[6]. The high level of immunosuppressants is another factor to consider. In the present study almost half of the children were not up-to-date with their age-appropriate immunizations during up to 5 years of follow-up. The reasons might be relatively low concern over children in a stable condition post-transplant, and a level of immunosuppression that is not low enough to warrant immunization. Notably, only 62.3% of the children's guardians brought vaccination books to visits to the doctor. As well as unawareness, financial problems would likely be a major concern for the children's guardians, especially with regard to vaccines that are not included in Thailand's EPI such as pneumococcal conjugate vaccine, influenza vaccine, hepatitis A vaccine, and varicella vaccine. Fortunately the infectious diseases unit in our department conducted a campaign to promote the administration of pneumococcal conjugate vaccine and influenza vaccine to all immunocompromised children every year at no charge. This afforded the children in the present study the opportunity to access these vaccines, and there was a significant increase in the proportion of children that received these vaccines post-transplant ($P < 0.001$). Long-term provision of these high-cost vaccines by the authorities would be a worthwhile venture. With respect to live vaccines, there has been controversy about whether they should be administered to children after liver transplantation[17,19,20-23]. Thus, further reports and large cohort studies are required in order to clarify the safety of live vaccines in these vulnerable patients, before they are routinely vaccinated posttransplant.

In this study, the rate of hospitalization for VPIs up to 5 years post-transplant was similar to those reported in previous studies[9-11], but significantly higher than that in

Table 3 Characteristics of hospitalization from vaccine-preventable infections and non-vaccine-preventable infections up to 5 years follow-up

Time	Type of infections				Organ specific infections, *n* (%)						The severity of infections, *n* (%)		
	VPIs		Non-VPIs		RS	GI	Blood	Renal	Skin	Others	ICU	Ventilator dependence	Death
	Times, *n* (%)	LOS (d)[1]	Times, *n* (%)	LOS (d)[1]									
During transplant	4 (5.2)	51 (24,79)	73 (94.8)[b]	35 (27,49)	25 (35.2)	24 (31.2)	20 (26)	6 (7.8)	2 (2.6)	0	All	All	0
< 3 mo	2 (6.9)	3 (3,3)	27 (93.1)[b]	12 (7,28)[a]	13 (44.8)	10 (34.5)	2 (6.9)	2 (6.9)	1 (3.4)	1 (3.4)	6 (20.7)	5 (17.2)	2 (6.9)
3-6 mo	5 (17.9)	8 (5,39)	23 (82.1)[b]	10 (4,15)	11 (39.3)	13 (46.4)	2 (7.1)	1 (3.6)	0	1 (3.6)	8 (28.6)	6 (21.4)	0
> 6-12 mo	3 (8.3)	5 (3,5)	33 (91.7)[b]	7 (6,17)	15 (41.7)	11 (30.6)	6 (16.7)	0	2 (5.6)	2 (5.6)	10 (27.8)	6 (8.3)	0
> 12-24 mo	6 (15)	5 (4,9)	34 (85)[b]	7.5 (5,10)	18 (45)	12 (30)	1 (2.5)	1 (2.5)	4 (10)	4 (10)	11 (27.5)	9 (22.5)	0
> 2-5 yr	11 (40.7)	6 (3,8)	16 (59.3)	5 (4,9)	7 (25.9)	10 (37)	1 (3.7)	0	6 (22.2)	3 (1.9)	5 (18.5)	1 (3.7)	0
Total	31 (13.1)	6 (3,8)	206 (86.9)[b]	8 (5,15)	89 (37.6)	80 (33.8)	32 (13.5)	10 (4.2)	15 (6.3)	11 (4.6)	40 (16.9)	27 (11.4)	2 (0.84)

[a]$P < 0.05$ *vs* vaccine-preventable infection (VPI) group.
[b]$P < 0.001$ *vs* VPI group.
[1]Data are presented as median (interquartile range).
GI: Gastrointestinal; ICU: Intensive care unit; LOS: Length of stay; RS: Respiratory system.

the normal population[9]. There was the mortality report of VPIs in children with immunocompromised hosts[1,2,22,24,25], but in this study, there was no mortality from VPIs. The VPIs requiring hospitalization in the current study were due to rotavirus, influenza, varicella, dengue fever, measles, *Streptococcus pneumoniae*, hepatitis B/E, and *Vibrio cholera*. These data should emphasize the value of complete immunization and robust infection control to physicians.

Viral hepatitis is endemic in Thailand, but interestingly in the present study there were no reports of hospitalization for hepatitis A post-liver transplant, and only one case of hepatitis E infection that required hospitalization. Viral hepatitis can be symptomatic and severe in older children and adults, and older children and adults may ingest more contaminated food and water than young children. Consequently, serology testing and immunization may be valuable in these groups. There is a reported case in which *de novo* hepatitis B infection was diagnosed 3 years after a liver transplant despite the recipient having undergone complete hepatitis B immunization pre-transplant[26]. This demonstrates that complete hepatitis B immunization pre-liver transplant does not guarantee post-transplant protection. That case prompted us to instigate a protocol for reimmunization and hepatitis B surface antibody monitoring every 3-6 mo to maintain a protective level of > 100 mIU/mL. *De novo* hepatitis B in the aforementioned boy who had hepatitis B surface antibody > 1000 mIU/mL pre

Table 4 Children with vaccination records who developed vaccine-preventable or non-vaccine-preventable diseases

Age-appropriate immunization	Thai's Expanded Program on Immunization				2013 Infectious Diseases Society of America			
	Infection and hospitalization, *n*			Total	Infection and hospitalization, *n*			Total
	None	VPIs and non-VPIs	Non-VPIs		None	VPIs and non-VPIs	Non-VPIs	
Complete immunization	5	5	12	22	9	9	25	43
Incomplete immunization	5	6	15	26	1	2	2	5
Total	10	11	27	48	10	11	27	48

VPIs: Vaccine-preventable infections.

transplant[26] may reflect waning immunity post-liver transplant. As well as vaccination, research evaluating the humoral and cellular immunity evoked by each vaccine should be conducted to determine vaccination schedules and the antibody parameters required to prevent VPIs more effectively. In the present study, the overall infection rate was high in the first year post-transplant, hence vaccination should be initiated as soon as possible after liver transplanted children are sufficiently stable. Predictors of high immunogenic responsivity to vaccination are needed to enable physicians to decide on optimal timepoints for reimmunization.

The current study had some limitations. It was a single-center study with a relatively small sample size. The true prevalence of VPIs may be lower than the frequency in the study, because the study only included children with severe enough illness to require hospitalization. Almost all children in the present study were referred from distant and rural areas, and it is possible that some of them subsequently attended more local hospitals due to infections. The main strength of the study was the reliable vaccination records obtained directly from the patients' vaccination books, which facilitated comparisons of vaccination status pre-transplant and post-transplant.

CONCLUSION

Incomplete immunization was common in children pre-liver transplant and post-liver transplant. Almost all of the children in the study required hospitalization due to VPIs or non-VPIs within 5 years post-liver transplant. The severity of infections was highest in the first year post-liver transplant.

Table 5 Pathogen causing hospitalization in children after liver transplantation

Time	The rank of the pathogen, *n* (%)			
	Bacteria	Total	Virus, fungus, and unidentified	Total
During transplant	*E. coli* (*n* = 19, 24.7), *K. pneumoniae* (*n* = 12, 15.6), *A. baumannii* (*n* = 11, 14.3), *Enterococcus*/*Staphylococcus* (*n* = 4, 5.2), *Salmonella* (*n* = 3, 3.9), *P. aeruginosa* (*n* = 2, 2.6), *B. cereus*/*Corynebacterium*/*S. pneumoniae*/*Elizabethkingia meningoseptica*/*Stenotrophomonas*/*Streptococcus mirabilis*/*C. difficile* (*n* = 1, 1.3)	62	Rotavirus/adenovirus/bocavirus (*n* = 2, 2.6), parainfluenza/fungus/varicella-zoster virus (*n* = 1, 1.3)	9[b]
< 3 mo	*E. coli*/*K. pneumoniae*/*Enterococcus*/*Salmonella*/*Aeromonas* (*n* = 2, 6.9), *Corynebacterium*/*C. difficile*/*Plesiomonas* (*n* = 1, 3.4)	13	Parainfluenza (*n* = 3, 10.3), coronavirus (*n* = 2, 6.9), rotavirus/bocavirus/RSV/dengue/fungus/norovirus/rhinovirus/parvovirus B19 (*n* = 1, 3.4), unidentified (*n* = 6, 20.7)	19
3-6 mo	*Salmonella*/*E. coli* (*n* = 2, 7.1), *K. pneumoniae*/*Enterococcus*/*S. pneumoniae*/*Staphylococcus* (*n* = 1, 3.6)	8	RSV (*n* = 4, 14.3), influenza (*n* = 2, 7.1), rotavirus/parainfluenza/rhinovirus/measles/HHV6 (*n* = 1, 3.6), unidentified (*n* = 9, 32.1)	20
> 6-12 mo	*E. coli* (*n* = 4, 11.1), *Salmonella* (*n* = 3, 8.3), *A. baumannii*/*Enterococcus*/mycoplasma/*C. difficile* (*n* = 2, 5.6), *Stenotrophomonas*/*Staphylococcus*/*Aeromonas*/*Pseudomonas*/*Plesiomonas*/*P. jirovecii* (*n* = 1, 2.8)	21	*P*arainfluenza (*n* = 3, 8.3), norovirus/herpes simplex virus (*n* = 2, 5.6), fungus/RSV/rhinovirus/influenza/measles (*n* = 1, 2.8), unidentified (*n* = 3, 8.3)	15
> 12-24 mo	*Salmonella* (*n* = 8, 12.5), *E. coli* (*n* = 3, 7.5), *Aeromonas*/*Pseudomonas*/mycoplasma/*Plesiomonas* (*n* = 1, 2.5)	15	Parainfluenza (*n* = 6, 15), rotavirus (*n* = 2, 5), adenovirus/varicella-zoster virus/dengue/rhinovirus/influenza/measles/metapneumovirus/hepatitis E/coxakie AB (*n* = 1, 2.5) unidentified (*n* = 11, 27.5)	28
> 2-5 yr	*Salmonella*/mycoplasma (*n* = 2, 7.4), *E. coli*/*K. pneumoniae*/*Staphylococcus*/*Vibrio cholera*/*B. cereus* (*n* = 1, 3.7)	9	Varicella-zoster virus (*n* = 3, 11.1), rotavirus/RSV/dengue/influenza (*n* = 2, 7.4), fungus/norovirus/herpes simplex virus/hepatitis B (*n* = 1, 3.7), unidentified (*n* = 3, 11.1)	18
Overall	*E. coli* (*n* = 31, 13.1), *Salmonella* (*n* = 20, 8.1), *K. pneumoniae* (*n* = 16, 6.8), *A. baumannii* (*n* = 13, 5.5), *Enterococcus* (*n* = 9, 3.8), *Staphylococcus* (*n* = 8, 3.3), mycoplasma (*n* = 5, 2.1), *C. difficile* (*n* = 4, 1.7), *Plesiomonas Shigelloides*/*Aeromonas* (*n* = 3, 1.3), *Corynebacterium*/*S. pneumononiae*/*Stenotrophomonas*/*P. aeruginosa*/*Aeromonas* (*n* = 2, 0.8), *Bacillus*/*Elizabethkingia meningoseptica*/*Streptococcus mirabilis*/*P. jirovecii*/*Vibrio cholera*/*B. cereus* (*n* = 1, 0.4)	128	Parainfluenza (*n* = 14, 5.9), rotavirus/RSV (*n* = 8, 3.4), influenza (*n* = 6, 2.5), varicella-zoster virus (*n* = 5, 2.1), dengue/norovirus/fungus/rhinovirus (*n* = 4, 1.7), adenovirus/bocavirus/herpes simplex virus/measles (*n* = 3, 1.3), coronavirus (n=2, 0.8), HHV6/metapneumovirus/hepatitis E/coxakie AB/hepatitis B (*n* = 1, 0.4), unidentified (*n* = 32, 13.5)	109[b]

[b]*P* < 0.001; virus *vs* bacterial causes of infections at each time point. *A. baumannii*: *Acinetobacter baumannii*; *B. cereus*: B*acillus* cereus; *C. difficile*: *Clostridium difficile*; *E. coli*: *Escherichia coli*; HHV6: Human herpes virus 6; *K. pneumoniae*: *Klebsiella pneumoniae*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *P. jirovecii*: *Pneumocystis jirovecii*; RSV: Respiratory syncytial virus; *S. pneumoniae*: *Streptococcus pneumoniae*.

Table 6 Vaccine-preventable infections causing hospitalization in children after liver transplantation

Time	During transplant	< 3 mo	3-6 mo	> 6-12 mo	> 12-24 mo	> 2-5 yr	Overall
Rota	2	1	1	0	2	2	8
Influenza	0	0	2	1	1	2	6
Varicella	1	0	0	0	1	3	5
Dengue	0	1	0	0	1	2	4
Measles	0	0	1	1	1	0	3
Streptococcus pneumoniae	1	0	1	0	0	0	2
Hepatitis B	0	0	0	0	0	1	1
Hepatitis E	0	0	0	0	1	0	1
Vibrio cholera	0	0	0	0	0	1	1

ARTICLE HIGHLIGHTS

Research background

Infection after liver transplantation is a serious concern due to potential morbidity and mortality, thus strategies to reduce overall post-transplant infection are warranted. Immunization is an effective and relatively noninvasive and affordable way to reduce vaccine-preventable infections (VPIs).

Research motivation

There is strong evidence that VPIs and non-VPIs post-transplant cause high fatality and increase graft rejection, but published data on VPIs and their effects in children post-liver transplant in Asia are scarce.

Research objectives

To investigate immunization status in children at the time of liver transplantation and up to 5 years thereafter. The prevalence and impact of VPIs and non-VPIs during hospitalization were also evaluated.

Research methods

The current retrospective study included 77 children who underwent liver transplantation and were followed up for up to 5 years thereafter. Demographic data, patient characteristics, immunization details derived from vaccination records, and hospitalizations for VPIs and non-VPIs were analyzed.

Research results

The mean follow-up duration after liver transplantation was 3.68 ± 1.45 years. Of the 77 children in the study, 48 (62.3%) had vaccination records in their vaccination books. There was a significant difference in the proportion of children with incomplete vaccination according to Thailand's Expanded Program on Immunization (n = 25, 52%) and accelerated vaccine from Infectious Diseases Society of America recommendations (n = 43, 89.5%) (P < 0.001). Post-liver transplant, almost half of the children in the study did not catch up with appropriate immunizations for age. There were 237 infections requiring hospitalization during up to 5 years of follow-up post-liver transplant at our hospital. The risks of VPIs and non-VPIs were highest during the first year after liver transplantation, and 2 children died. Respiratory and gastrointestinal systems were common sites of infection. The most commonly identified pathogens that caused VPIs were rotavirus, influenza virus, and varicella-zoster virus.

Research conclusions

Incomplete age-appropriate immunization in children pre-liver transplant and post-liver transplant were common. At least 13.1% of the children in the study required hospitalization for a VPI during a follow-up period of up to 5 years post-transplantation. There was high morbidity, especially during the first year after transplantation. Hence, complete immunization and robust infection control should be

considered in such children.

Research perspectives

The current study suggests that incomplete age-appropriate immunization is a major concern, because a large number of patients with VPIs requiring hospitalization were recorded. Interestingly, waning immunity post-liver transplant can evidently lead to VPIs, as evidenced by a case in which *de novo* hepatitis B infection developed 3 years postliver transplantation in a child who had a hepatitis B surface antibody titer of > 1000 mIU/mL pre-liver transplantation. As well as policies to increase pre- and post-transplant immunization rates, studies investigating humoral and cellular immunity induced by vaccination after liver transplantation are needed.

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ORIGINAL ARTICLE

Observational Study

Endoscopic retrograde cholangiopancreatography and liver biopsy in the evaluation of elevated liver function tests after liver transplantation

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Abstract

BACKGROUND

Abnormal liver function tests (LFTs) in post-liver transplant (LT) patients pose a challenge in the timing and selection of diagnostic modalities. There are little data regarding the accuracy of endoscopic retrograde cholangiopancreatography (ERCP) and liver biopsy (LB) in diagnosing post-transplant complications.

AIM

To evaluate the diagnostic performance of ERCP and LB in patients with non-vascular post-LT complications.

METHODS

This single-center retrospective study evaluated patients undergoing both ERCP and LB for evaluation of elevated LFTs within 6 mo of LT from 2000 to 2017. Diagnostic operating characteristics including accuracy, sensitivity and specificity for various diagnoses were calculated for ERCP and LB. The R factor (ratio of alkaline phosphatase to alanine aminotransferase) was also calculated for each patient.

RESULTS

Of the 1284 patients who underwent LT, 91 patients (74.7% males, mean age of 51) were analyzed. Anastomotic strictures (AS, 24.2%), acute cellular rejection (ACR, 11%) and concurrent AS/ACR (14.3%) were the most common diagnoses. ERCP

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carried an accuracy of 79.1% (95%CI: 69.3-86.9), LB had an accuracy of 93.4% (95%CI: 86.2-97.5), and the combination of the two had an accuracy of 100% (95%CI: 96-100). There was no difference between patients with AS and ACR in mean R factor (AS: 1.9 *vs* ACR: 1.1, P = 0.24). Adverse events did not differ between the two tests (ERCP: 3.1% *vs* LB: 1.1%, P = 0.31).

CONCLUSION
In patients with abnormal LFTs after LT without vascular complications, the combination of LB and ERCP carries low risk and improves diagnostic accuracy over either test alone.

Key Words: Liver transplantation; Endoscopic retrograde cholangiopancreatography; Liver biopsy; Abnormal liver tests; Acute cellular rejection; Anastomotic biliary stricture

Core Tip: Patients commonly develop unexplained elevations in liver function tests after liver transplantation. After cross sectional imaging and basic lab tests, endoscopic retrograde cholangiopancreatography (ERCP) and liver biopsy (LB) are both performed in arbitrary fashion since the diagnostic capacity of each test remains unclear. In this study we found that ERCP and LB are both effective diagnostic tests in the setting of the 2 most common diagnoses, anastomotic biliary stricture and acute cellular rejection. Combining these tests increases the overall diagnostic accuracy to 100%, and both tests carried adverse event rates of < 5%. This study justifies combining ERCP and LB when the diagnosis remains elusive.

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INTRODUCTION

Since 2012, the number of liver transplants (LTs) performed annually in the United States has increased each year, reaching a record number of 8250 in 2018[1]. Just as the field of transplantation has evolved over the past 5 decades, so too have the nuances of post-transplant clinical care. Clinicians commonly face the conundrum of abnormal liver function tests (LFTs) soon after LT which often indicates a transplant-related complication. Practice guidelines provided by the American Association for the Study of Liver Diseases (AASLD), American Society of Transplantation, and the European Association for the Study of the Liver note that the frequency of monitoring LFTs after LT and the subsequent work-up should be individualized to the patient and time after LT, prior complications, stability of serial testing, and the suspected underlying pathology[2,3].

The underlying cause, however, can be challenging to discern. Depending on the pattern of abnormal LFTs, evaluation of the biliary system with transabdominal ultrasound, MRI, CT, and/or endoscopic retrograde cholangiopancreatography (ERCP) may be most appropriate when the LFT pattern is cholestatic, whereas liver biopsy (LB) should be performed first when parenchymal injury is suspected[2]. To date, there are insufficient data regarding the relative accuracy of ERCP and LB in diagnosing specific post-LT complications. Current societal guidelines strongly support both of these tests (Grade 1A recommendations) but provide little guidance on which should be performed initially[2]. The decision to choose LB, ERCP, or both (and in which order) is therefore left to the discretion of the transplant surgeon, hepatologist, or interventional endoscopist. The primary aim of this study was to evaluate the diagnostic performance of ERCP and LB in patients with non-vascular post-LT complications.

MATERIALS AND METHODS

This was a single-center, retrospective review of all patients who underwent LT followed by both LB and ERCP at the University of Colorado Hospital from January 2000 to June 2017.

Patients

Patients undergoing deceased or living donor LT at our center during the study period were identified using the LT database. Inclusion criteria included adult patients post-LT who underwent both LB and ERCP within 6 mo after LT with a primary indication of elevated LFTs. Patients with a clearly identifiable cause of elevated LFTs–such as drug or medication-related hepatitis, vascular liver disease or infectious hepatitis based on the initial history, labs, or imaging studies-were excluded from the analysis. Patients who did not receive post-LT care at our institution were also excluded. Post-LT biliary anatomy types included duct-to-duct (DD) anastomosis and Roux-en-Y hepaticojejunostomy (RYHJ).

Patients with a mixed pattern of liver injury based on LFTs underwent either LB or ERCP initially at the discretion of the provider. ERCP was the first invasive diagnostic test performed when patients had symptoms suggestive of cholangitis or a predominantly cholestatic pattern of elevated LFTs. LB was performed after labs and cross-sectional imaging when hepatocellular disease was suspected. It is our practice to monitor immunosuppressant levels on all post-LT patients. Approval from the Colorado Multi-Institutional Review Board was obtained prior to beginning the study.

ERCP

ERCP was performed under conscious sedation, monitored anesthesia care, or general anesthesia by one of 7 advanced endoscopists who have performed > 1000 ERCPs each. Endoscopists utilized the standard technique in cannulating the bile duct and performing cholangiography. Occlusion cholangiography was used to visualize the entire native and donor biliary tree with particular attention paid to the anastomosis. Biliary sphincterotomy was performed in select cases at the discretion of the endoscopist. If present, strictures were treated with the placement of plastic or fully covered metal stents were placed across strictures according to the endoscopist's judgment. Dilation of strictures *via* balloon or catheter was performed prior to stenting in select cases.

Conventional techniques such as balloon and basket sweeping were used to remove bile duct stones and/or casts, and single or multiple stents were placed across anastomotic bile duct leaks. For patients with DD biliary anastomosis, a standard duodenoscope was used to reach the ampulla. For patients with RYHJ anatomy either a pediatric colonoscope or small bowel enteroscope (single-balloon, double-balloon, or rotational overtube) was used to reach the biliary anastomosis.

LB

While percutaneous (ultrasound-guided) LB represented the preferred route of biopsy, transjugular LB was generally performed in patients with an International Normalized Ratio > 1.5, when intravascular pressure measurements were needed, or when the abdominal anatomy precluded a safe percutaneous approach. Both percutaneous and transjugular LB were performed under conscious sedation. LB techniques are described in detail in an AASLD position paper[4]. Board certified GI pathologists examined all histology samples.

Outcomes and definitions

The study's primary outcome was the accuracy of ERCP and LB in making the ultimate final diagnosis or diagnoses driving the abnormal LFTs, as determined by the GI and Hepatology services. Secondary outcomes included sensitivity and specificity for ERCP and LB in the final diagnosis. Acute cellular rejection (ACR) was defined and graded using a 1-9 scale based on histopathologic findings using the rejection activity index, which was based on inflammatory changes in the portal triads, bile ducts, and venous endothelium (with scores of 1-3 for each of the 3 categories)[5]. A score of 3 or more was classified as definite ACR (Figure 1)[5]. Recurrent hepatitis C infection (HCV) after LT was defined by detectable serum HCV RNA. Anastomotic stricture (AS) was defined as a benign-appearing narrowing in the region of the biliary anastomosis during ERCP, typically within 5-6 mm from the suture line, usually associated with delayed contrast drainage and/or moderate resistance to passage of an inflated 12 mm balloon (Figure 2).

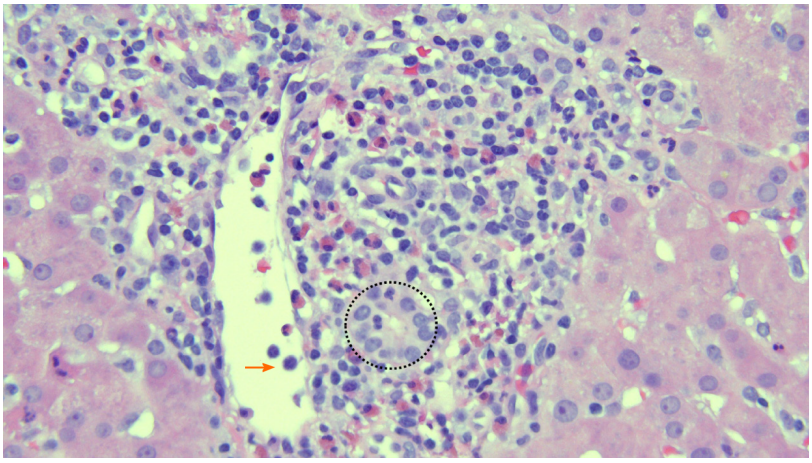

Figure 1 Photomicrograph of representative portal tract in acute cellular rejection. Mixed, lymphocyte predominant portal-based inflammation, bile duct inflammation characterized by lymphocyte infiltration (circle), and a large portal venule with subendothelial lymphocyte infiltration and intraluminal lymphocyte tethering[24] (hematoxylin and eosin stain, 40 ×).

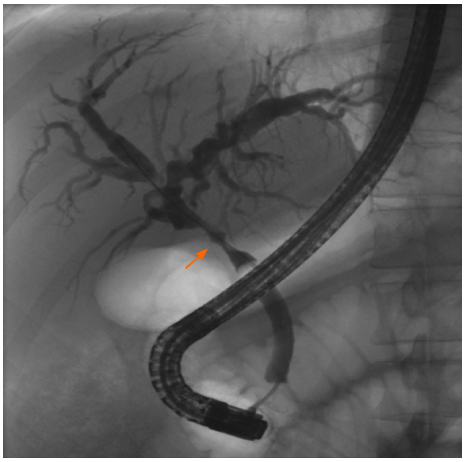

Figure 2 Cholangiogram during endoscopic retrograde cholangiopancreatography demonstrating an anastomotic stricture (arrow).

True positive results for LB or ERCP were defined by findings supportive of at least one of the final diagnosis/es as defined above. True negative results were defined by ERCP or LB results that failed to support the final diagnosis/es with or without supporting an alternative diagnosis. For example, if LB showed signs of a large bile duct obstruction or cholangitis, this was considered a true positive for a final diagnosis of anastomotic stricture or cholangitis, respectively. Conversely, if ERCP did not show biliary pathology, this was considered a false negative when the final diagnosis was a hepatocellular disorder such as ACR or recurrent HCV.

Statistical analysis

Descriptive statistics were used to depict patient demographics, symptoms and laboratory data. An R factor was calculated as the ratio between the degree of elevation of alkaline phosphatase and the degree of elevation of alanine aminotransferase[6]. R factors > 5 were considered to be consistent with hepatocellular damage and R factors < 2 suggested cholestatic patterns of injury, with R factors between 2 and 5 suggesting a mixed pattern of injury. Diagnostic operating characteristics including sensitivity, specificity, and accuracy [(true positive + true negative)/(true positive + false negative + false positive + true negative)] were calculated for both ERCP and LB. Fisher's exact test or the chi square test were used to compare categorical variables between patients with ACR and AS. The student's *t*-test was used to compare continuous variables between patients with ACR and AS. Adverse event rates were compared between ERCP and LB using the Fisher's exact test. All statistical analysis was performed using STATA 15.1 (StataCorp, College Station, TX, United States).

RESULTS

Patients

A total of 1284 patients underwent LT at our center during the study period (Figure 3). Of these, 96 patients (7.5%) received both an ERCP and LB for evaluation of persistently elevated LFTs within the first 6 mo after LT. Ninety-one patients received long-term follow-up at our institution and were included in the final analysis. The mean time interval between the 2 procedures was 9.1 d (SD 6.9).

The mean age of the cohort was 51 (SD 12.1) and 74.7% (n = 68) were male (Table 1). Deceased donor transplants (n = 73, 80.2%) accounted for the majority of transplants, and 73.6% (n = 67) had DD biliary anatomy. Presenting symptoms included jaundice (23.1%, n = 21), abdominal pain (15.4%, n = 14), and fever (12.1%, n = 11), and 21 (25%) patients were asymptomatic. Initial imaging consisted of ultrasound (74.7%), CT (18.7%), and magnetic resonance cholangiopancreatography (MRCP, 6.6%) with a mean donor bile duct diameter of 4.6 (SD 1.9) mm. Imaging revealed a dilated duct in 9 (9.9%, 8 with ultrasound, 1 with MRCP) of patients. LB was performed as the first of the 2 tests in 51 (56%) patients, and 71.4% (n = 65) of LBs were performed *via* the percutaneous route. Nearly 75% of patients were on dual immunosuppression therapy (n = 68) with 22% of patients on monotherapy (n = 20) with the combination of tacrolimus and mycophenolate sodium being the most common combination therapy (n = 21).

Technically, all LB and ERCP procedures were performed successfully. The most common single diagnosis ultimately was AS (34.1%), followed by ACR (11%) with all diagnoses displayed in Table 2. A total of 29 (31.9%) patients had multiple concurrent diagnoses contributing to the elevation in LFTs (and included as final diagnoses), and the most common was a dual diagnosis of AS with ACR (14.3%, n = 13). Four (4.4%) patients had 3 concurrent diagnoses, all of which included ACR and AS (Table 2).

Diagnostic operating characteristics

The diagnostic operating characteristics of LB and ERCP are shown in Table 3. The overall accuracy of ERCP was 79.1% (95%CI: 69.3-86.9). The overall accuracy of LB was 93.4% (95%CI: 86.2-97.5). Combined, the 2 tests had an overall accuracy of 100% (95%CI: 96-100).

For AS, ERCP had an accuracy of 100% (95%CI: 84.6-100) while LB had an accuracy of 72.7% (95%CI: 49.8-89.3). For ACR, LB had an accuracy of 100% (95%CI: 69.2-100) while ERCP had an accuracy of 0% (95%CI: 0-30.9). Sensitivities carried the same values as the accuracy in all cases due to the lack of false positive results. For the same reason, specificity could not be calculated for any of the diagnostic tests.

Liver function tests

The mean R factor (ratio of alkaline phosphatase and alanine aminotransferase) was 2 (SD 2.4), with a mean alkaline phosphatase (AP) level of 392.6 (SD 248.4) IU/L and mean total bilirubin (TB) level of 4.5 (SD 5.4) mg/dL. The mean aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were 200.5 (SD 674.8) and 205.4 (444.2), respectively. Between patients with AS and patients with ACR, there was no significant difference in R factor (AS: 1.9 *vs* ACR: 1.1, P = 0.24), AP (AS: 376.3 *vs* ACR: 452.2, P = 0.48), TB (AS: 4.1 *vs* ACR: 5.5, P = 0.41), AST (AS: 130.9 *vs* ACR: 127.9, P = 0.94), or ALT (AS: 203.1 *vs* ACR: 169.5, P = 0.58). There was also no difference between the 2 diagnoses in terms of bile duct diameter (AS: 4.8 mm *vs* ACR: 3.8 mm, P = 0.36). Patients with concurrent AS and ACR had a mean R factor of 1.06 (0.7).

Adverse events

A total of 3 adverse events occurred after 96 ERCPs (3.1%): 1 case of mild post-ERCP pancreatitis treated conservatively, and 2 cases of post-procedure abdominal pain requiring overnight hospitalization and supportive care. One adverse event occurred after LB, a hepatoportal fistula that required hospitalization and angiography with embolization by Interventional Radiology. There was no significant difference in the adverse event rates due to ERCP or LB (3.1% *vs* 1.1%, P = 0.31).

DISCUSSION

It is common to encounter asymptomatic patients with abnormal LFTs in the post-LT

Table 1 Baseline characteristics reported as *n* (%) or mean (SD)

Variable	Overall cohort (*n* = 91)
Age	51 (12.1)
Sex (male)	68 (74.7)
Presenting symptom	
Jaundice	21 (23.1)
Fever	11 (12.1)
Abdominal pain	14 (15.4)
Asymptomatic	21 (25)
Liver biopsy performed first	51 (56)
Percutaneous liver biopsy	65 (71.4)
Bile duct diameter (mm)	4.6 (1.9)
R factor	2 (2.4), Range: 0.1-6.4
Alkaline phosphatase (international units/liter)	392.6 (248.4)
AST (units/liter)	200.5 (674.8)
ALT (units/liter)	205.4 (444.2)
Total bilirubin (mg/dL)	4.5 (5.4)
Deceased donor	73 (80.2)
Transplant biliary anatomy	
Duct-to-duct	67 (73.6)
Roux-en-Y hepaticojejunostomy	24 (26.4)
Tacrolimus	66 (73.3)
Sirolimus	20 (22.2)
Everolimus	6 (6.6)
Mycophenolate sodium	28 (31.1)
Mycophenolate mofetil	13 (14.4)
Cyclosporine	16 (17.8)
Prednisone	20 (22.2)
Immunosuppression monotherapy	20 (22)
Dual immunosuppression therapy	68 (74.7)
Triple immunosuppression therapy	3 (3.3)

AST: Aspartate aminotransferase; ALT: Alanine transaminase.

setting, as well as symptomatic patients with normal LFTs. It is also common for patients to undergo multiple invasive diagnostic tests as part of the work-up. Abnormal LFTs post-LT are a major cause of unplanned hospital readmissions, and the ensuing work-up may consume significant resources[7]. ERCP is the accepted diagnostic and therapeutic test for suspected biliary pathology and LB is the accepted test for suspected hepatocellular pathology. But in reality, because of the poor specificity of LFT patterns and the limitations of cross-sectional imaging, patients with post-LT LFT elevations will too often undergo both procedures. The timing and order of these procedures is left to the discretion of the transplant surgeon, hepatologist and advanced endoscopist, with little evidence to guide them. Despite the high incidence of immune-mediated and biliary complications following LT, the usual clinical tools (*e.g.*, clinical history, LFT patterns, bile duct diameter on imaging) are poorly specific for any single diagnosis. Besides the main finding of our study, this study demonstrated that patients with AS had no significant difference from patients with ACR in terms of R factor, alkaline phosphatase level, total bilirubin level, AST level,

Table 2 Etiologies of liver function test elevation reported as *n* (%)

Single diagnosis	*n* (%)
Anastomotic stricture	31 (34.1)
Acute cellular rejection	10 (11)
Recurrent primary sclerosing cholangitis	6 (19.4)
Recurrent HCV	5 (5.5)
Biliary cast syndrome	3 (3.3)
Ischemic cholangiopathy	2 (2.2)
Papillary stenosis	1 (1.1)
Posterior reversible encephalopathy syndrome	1 (1.1)
Cholestatic hepatitis	1 (1.1)
Recurrent PBC	1 (1.1)
Venous outflow obstruction	1 (1.1)
Two diagnoses	
Anastomotic stricture and acute cellular rejection	13 (14.3)
Recurrent HCV and anastomotic stricture	6 (19.4)
Bile leak and acute cellular rejection	2 (2.2)
Congestive hepatopathy and anastomotic stricture	1 (1.1)
Anastomotic stricture and suprahepatic cava stenosis	1 (1.1)
Recurrent PBC and anastomotic stricture	1 (1.1)
CMV hepatitis and bile leak	1 (1.1)
Three diagnoses	
Acute cellular rejection, anastomotic stricture, and recurrent HCV	2 (2.2)
Acute cellular rejection, anastomotic stricture, and de novo autoimmune hepatitis	1 (1.1)
Acute cellular rejection, anastomotic stricture, and CMV hepatitis	1 (1.1)

HCV: Hepatitis C virus; PBC: Primary biliary cholangitis; CMV: Cytomegalovirus.

Table 3 Operating characteristics for endoscopic retrograde cholangiopancreatography and liver biopsy in diagnosing post-liver transplant complications

	ERCP	LB	ERCP + LB
Overall accuracy % (95%CI)	79.1 (69.3-86.9)	93.4 (86.2-97.5)	100 (96-100)
Overall sensitivity % (95%CI)	79.1 (69.3-86.9)	93.4 (86.2-97.5)	100 (96-100)
Acute cellular rejection accuracy % (95%CI)	0 (0-30.9)	100 (69.2-100)	100 (91.9-100)
Anastomotic stricture accuracy % (95%CI)	100 (84.6-100)	72.7 (49.8-89.3)	100 (89.4-100)

ERCP: Endoscopic retrograde cholangiopancreatography; LB: Liver biopsy.

ALT level, or bile duct diameter. Hence, additional testing with LB and ERCP was justified.

Ultrasound and MRCP have variable accuracy in diagnosing biliary pathology post-LT, since obstructive ductal dilation in the transplanted liver is variable. Several studies have demonstrated poor sensitivity and specificity of bile duct diameter post-LT[8-11]. While both modalities can detect biliary dilatation, MRCP offers an advantage over ultrasound in being able to detect biliary strictures with a sensitivity ranging from 64%-79%[9,12]. While both of these modalities are first-line options for imaging in the diagnostic work-up of elevated LFTs after LT, we have found that MRCP both

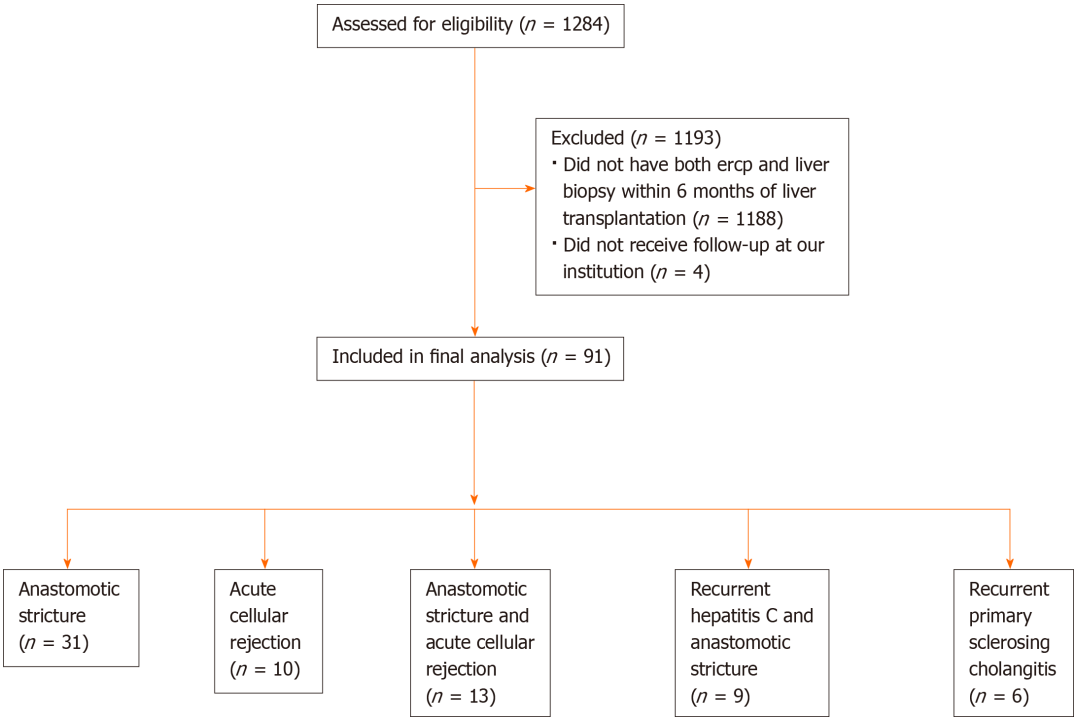

Figure 3 Flow diagram of patients. ERCP: Endoscopic retrograde cholangiopancreatography.

under-estimates and over-estimates stenosis size and severity. Additionally, ERCP permits a real-time accurate assessment of strictures, based on contrast drainage and balloon passage, and the ability to perform stricture therapy. For these reasons, we generally go straight to ERCP and bypass MRCP when there is significant ductal dilation, a cholestatic pattern of LFTs, or a negative LB.

To our knowledge, this is the largest study evaluating the diagnostic performance of combined LB and LT in patients with abnormal LFTs after LT. Our novel finding in this study is the high diagnostic accuracy for ERCP and LB, in contrast to standard laboratory tests or cross-sectional imaging. Diagnostic accuracy was 79.1% overall for ERCP and 93.4% overall for LB. Combined, the 2 tests study had an overall diagnostic accuracy of 100%.

ACR and AS were the most frequent final diagnoses in our patients. These are commonly encountered diseases in the LT population, but the differential diagnosis remains broad (Figure 4) and includes de novo autoimmune hepatitis, recurrent liver disease (HCV, PSC, others), drug toxicity, de novo infection, biliary stones or casts, hepatic artery thrombosis, and more[2]. We recognize that a previously common clinical dilemma–differentiating recurrent HCV from ACR or other etiologies–is less common in the current direct-acting antiviral (DAA) era, and our study included patients in the current and pre-DAA eras.

In the early days of LT, ACR was a near-universal complication resulting in long-term graft failure[13,14]. Advances in immunosuppression have subsequently led to reduced rates of allograft rejection, though the incidence still ranges from 20% to 40% after LT, with most occurring within the first month[15-17]. In addition, ACR remains clinically significant, impacting long-term graft survival and mortality[18]. The incidence of biliary complications after LT is highly variable but still relatively common. The estimated incidence of AS post-LT is up to 20% for patients following deceased donor LT and 19%-40% after living donor liver transplantation. Risk factors include graft ischemia, DD anastomosis, reperfusion injury, deceased donor, and hepatic artery thrombosis. The incidence of non-anastomotic stricture is 0.5% to 10%, while stones/sludge are seen post-LT in approximately 5% of patients. Biliary cast syndrome is less common (2.5%-3%)[19-22].

It is critical to make a prompt and diagnosis when a transplanted patient presents with abnormal LFTs, since graft survival depends on timely and appropriate treatment. While ACR is successfully treated with various combinations of immunosuppressive medication, the management of biliary complications is procedural. AS may be treated successfully with endoscopic placement of multiple plastic stents or a covered metal stent. Recent data suggests that metal stents incur

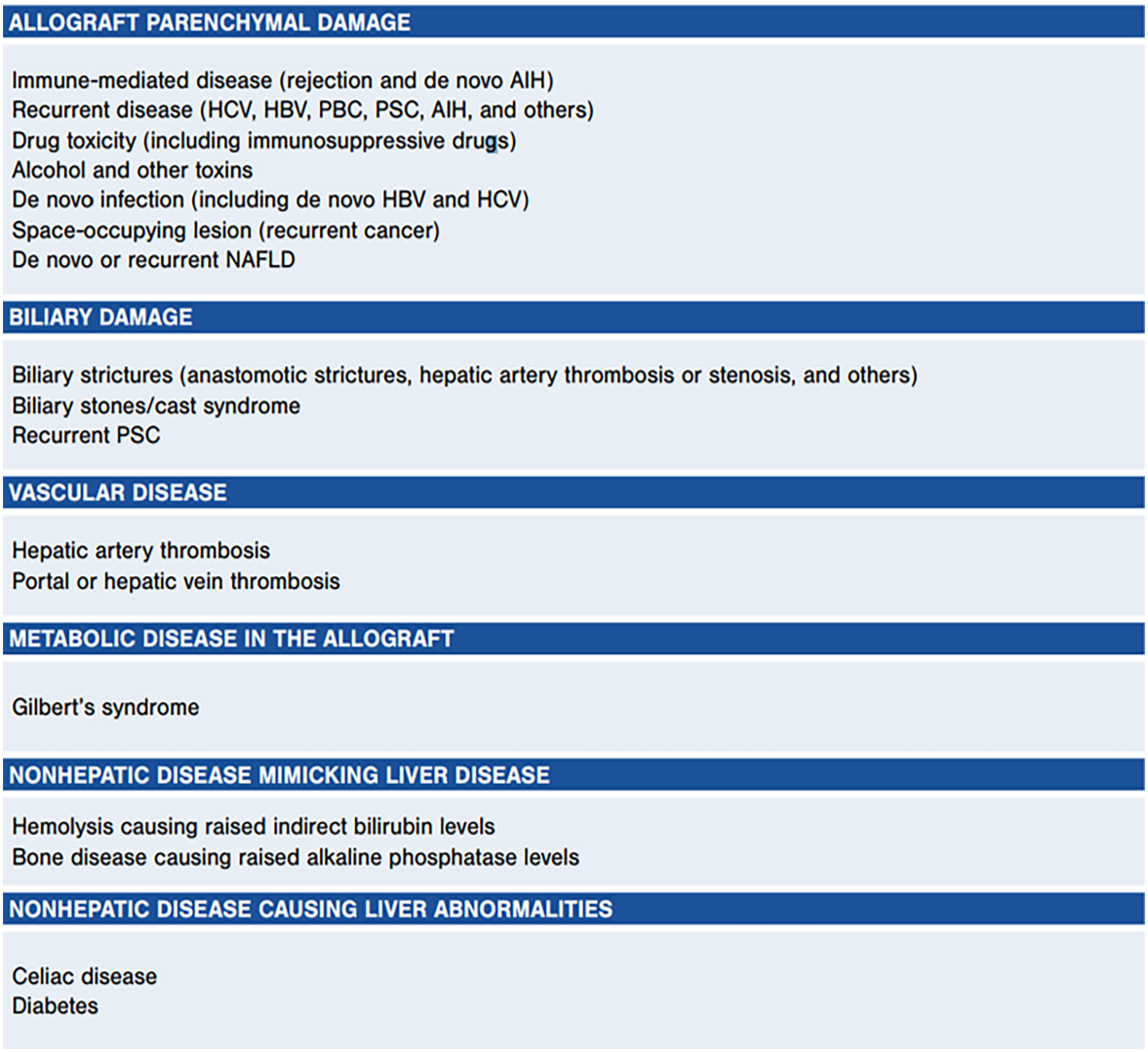

Figure 4 Causes of liver test abnormalities after liver transplantation. Legend: Used with permission from Lucey *et al*[2], 2013. HBV: Hepatitis B virus; HCV: Hepatitis C virus; PBC: Primary biliary cholangitis; PSC: Pulmonary scar cancer.

fewer procedures and costs while leading to stricture resolution similarly to plastic stents[23].

Our study sheds light on the frequency of dual diagnoses in patients with abnormal LFTs post-LT, which is an under-studied phenomenon. In this study, 34 (37.4%) patients had multiple diagnoses, of which the most common combination was AS plus ACR (14.3%). Four patients (4.4%) ultimately received 3 final diagnoses. In practice, patients receive therapy for multiple diseases concurrently (*e.g.* stenting for AS plus corticosteroid bursts for AS), so knowing which diagnosis is dominant can be challenging. Previous studies assessing abnormal LFTs in the post-LT population mostly included patients undergoing LB or ERCP but not both, so our study may represent more complex, sicker patients[7]. Alternatively, some of the various diagnoses in our patients may be clinically silent. AS, for example, is quite subjective and may be diagnosed or treated by endoscopists even though the stricture may not be high-grade or impede bile flow.

Our findings suggest that physicians managing post-LT patients can have a lower threshold to perform both LB and ERCP when evaluating abnormal LFTs within the context of the patient's clinical presentation. While one modality alone has high diagnostic accuracy over lab tests and imaging, LB and ERCP combined have a very high diagnostic accuracy. Ultimately the decision to perform one test over the other depends on clinician experience, but both tests improve the diagnostic accuracy over one test alone. However, despite the high prevalence of multiple final diagnoses (37.4%), only 96 of 1284 transplanted patients at our center underwent both ERCP and LB during the study period, suggesting they are used sparingly overall. Finally, the adverse event rates of ERCP and LB are low, and we demonstrated no significant

difference between the two.

This study was limited by its size and design. It was performed at a single, United States tertiary care hospital with experienced endoscopists and transplant hepatologists, so the results may not be generalizable to other centers. The final diagnosis was determined by review of the medical record and hence may be affected by bias or subjectivity amongst the various treating physicians. Moreover, a reproducible, objective grading score for AS has not been established. The study was also limited by its retrospective nature and by limiting the analysis to patients undergoing ERCP and LB early after LT during the 17-year study period. An additional limitation is the variable time gap between ERCP and LB, although across the entire study population the mean time interval between both procedures was relatively short (9.1 d) suggesting that the diagnostic evaluation typically occurred during a single clinical episode. Despite these limitations, our cohort represents the modern-day practice of ERCP and LB after LT, and the study permits a comparison between the 2 key diagnostic tests in the most common clinical scenarios. Future studies may include a prospective evaluation of abnormal LFTs post-LT or outcomes of post-LT patients who undergo empiric treatment without LB or ERCP.

CONCLUSION

In summary, these results offer insight into the diagnostic and etiology of abnormal LFTs after LT, in which standard lab and imaging studies have poor specificity. Our study shows that LB and ERCP improve diagnostic accuracy over either test alone and carry low risk. Dual diagnoses are relatively common in this population. In the future, prospective and multicenter studies should include patients undergoing LB and ERCP beyond the early post-LT period and establish reproducible, objective criteria for the ultimate diagnosis.

ARTICLE HIGHLIGHTS

Research background

Elevated liver function tests (LFTs) are commonly encountered in the post-liver transplant (LT) setting. When a diagnosis is not made by history, labs, and cross-sectional imaging, endoscopic retrograde cholangiopancreatography (ERCP) and liver biopsy (LB) are commonly performed. However, the diagnostic performance of each of these tests individually and in combination remains unknown.

Research motivation

We first hoped to determine what are the most common diagnoses in the population of patients with elevated LFTs after LT. At the same time, we want to assess the diagnostic performance of both ERCP and LB in these patients so that we can decide which of these tests is safer and more effective at clinching the diagnosis.

Research objectives

We aimed to assess the diagnostic accuracy and safety of ERCP and LB together and in isolation for a final diagnosis in patients with unexplained LFT elevations after LT.

Research methods

In this single-center, retrospective study we evaluated patients undergoing both ERCP and LB for the evaluation of elevated LFTs within 6 mo of LT based on review of existing medical records. Diagnostic accuracy, sensitivity and specificity for the various final diagnoses were calculated for each test.

Research results

Anastomotic strictures (AS), acute cellular rejection (ACR) and concurrent AS and ACR were the most common diagnoses. ERCP carried an accuracy of 79.1%, LB had an accuracy of 93.4%, and the combination of the 2 had an accuracy of 100% (95%CI: 96-100). The pattern of liver chemistries (R Factor) did not diagnostic accuracy of either test. Adverse event rates did not differ between the 2 tests.

Research conclusions

While LB had a higher accuracy than ERCP, the combination of the 2 tests had an accuracy of 100% and a low adverse event rate, suggesting that physicians can have a low threshold in utilizing both modalities for the evaluation of elevated LFTs.

Research perspectives

In patients with elevated LFTs after LT without a diagnosis, neither LB nor ERCP is clearly superior. Both tests can be used and the decision to use one over the other will depend on the clinical context and physician preference. However, when necessary both tests can be used safely together to reach a final diagnosis in nearly all patients.

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META-ANALYSIS

Effectiveness of entecavir in preventing hepatocellular carcinoma development is genotype-dependent in hepatitis B virus-associated liver cirrhosis

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Abstract

BACKGROUND

The oral nucleos(t)ide analogue, entecavir (ETV) was demonstrated to reduce the rate of hepatocellular carcinoma (HCC) in patients with hepatitis B virus (HBV)-associated liver cirrhosis. However, the reduction of HCC differs in various regions of the world.

AIM

To investigate the reduction of HCC development due to ETV therapy by meta-analysis.

METHODS

We surveyed the differences in HCC development following ETV treatment based on published articles using PubMed (2004-2019).

RESULTS

The regions with the most marked reduction in HCC development due to ETV therapy were Spain (1.0%/year) and Canada (Southern part, 1.3%/year), and the most ineffective areas were South Korea (3.6%-3.8%/year), China (3.3%/year), Taiwan (2.4%-3.1%/year), and Hong Kong (2.8%/year). Following ETV administration, the incidence of HCC in genotype D regions (1.89% ± 0.28%/year,

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mean ± SE) was significantly lower than that in genotype C regions (2.91% ± 0.24%/year, $P < 0.01$). With regard to the initial HBV-DNA level, in genotype C patients (average: 5.61 Log_{10}IU/mL) this was almost the same as that in genotype D patients (average: 5.46 Log_{10}IU/mL). Moreover, there was no association between the prevalence ratio of HBV and the incidence of HCC on ETV treatment.

CONCLUSION
The effectiveness of ETV in preventing HCC development in HBV-associated liver cirrhosis is genotype-dependent.

Key Words: Hepatocellular carcinoma; Entecavir; Genotype of hepatitis B virus; Oral nucleos(t)ide analogue

Core Tip: Entecavir was demonstrated to reduce the rate of hepatocellular carcinoma (HCC) in patients with hepatitis B virus (HBV)-associated liver cirrhosis. The reduction of HCC differs in various regions of the world. We surveyed these differences based on published articles using PubMed (2004-2019). Following entecavir administration, the incidence of HCC in genotype D regions (1.89% ± 0.28%/year, mean ± SE) was significantly lower than that in genotype C regions (2.91% ± 0.24%/year, $P < 0.01$). The initial HBV-DNA level in genotype C patients was almost the same as that in genotype D patients. The effectiveness of entecavir in preventing HCC development in patients with HBV-associated liver cirrhosis is genotype-dependent.

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INTRODUCTION

The third-generation nucleos(t)ide analogue, entecavir (ETV) is currently recommended as one of the first-line antiviral therapies for chronic hepatitis B virus (HBV) infection. Moreover, it is generally accepted that long-term ETV treatment may reduce the incidence of hepatocellular carcinoma (HCC) in HBV-infected patients. Wong *et al*[1] demonstrated that the 5-year cumulative incidence of HCC was 13.8% in an ETV cohort *vs* 26.4% in a control cohort.

However, on surveying published reports, the effect of ETV in preventing HCC differed in various regions of the world. In this study, we examined the reduction of HCC development in various regions of the world, and the possible reasons for these differences.

MATERIALS AND METHODS

The PubMed database was searched (2004-2019) for studies published in English regarding the follow-up results of the development of HCC in patients with HBV-associated liver cirrhosis after treatment with ETV for more than 2 years. Studies with follow-up periods shorter than 3 years after ETV treatment were excluded.

In this study, we included only HBV cirrhotic cases. Furthermore, we surveyed the possible reasons for the differences in HCC reduction. We examined the association between the reduction in HCC development and initial HBV-DNA levels, which is a strong accelerating factor for HCC development[2], the prevalence of HBV in these regions, and HBV genotypes.

To compare the incidence of HCC between the main genotypes C and D, we calculated the weighted mean of the HCC incidence rate for each genotype using the

random effect model (ref: Dersimonian R, Laird N. Meta-analysis in clinical trials. Controlled Clinical Trials 1986; 7: 177-188). To assess whether the incidence rate among genotype D patients was lower than that among genotype C patients, we calculated the *P* value using a *Z* test. All reported *P* values correspond to two-sided tests, and those with $P < 0.05$ were considered significant. All analyses were performed with JMP version 12 (SAS Institute, Cary, NC, United States).

RESULTS

The results of HBV-associated cirrhotic patients administered ETV are presented in Table 1.

The regions where HCC development was markedly reduced by ETV therapy were Spain (1.0%/year)[3] and Canada (Southern part) (1.3%/year)[4]. The most ineffective regions were South Korea (3.6%-3.8%/year)[5,6], China (3.3%/year)[7], Taiwan (2.4%-3.1%/year)[8,9], Japan (Ehime, southern part of Japan 2.9%/year)[10], and Hong Kong (2.8%/year)[1]. The regions with a moderate reduction were Turkey (2.2%-2.7%/year)[11,12], the Caucasus (2.2%/year)[13], and Greece (1.8%/year)[14].

With regard to the genotype of HBV, the incidence of HCC in regions where the main prevalent type is D (1.89% ± 0.28%/year, mean ± SE) was significantly lower than that in regions where the main prevalent genotype is C (2.91% ± 0.24%/year, $P < 0.01$) (Table 2).

Moreover, the incidence of HCC in regions where the main prevalent genotype is C was significantly higher than that in regions where the main prevalent genotype was other than C (D + A, 1.61% ± 0.21%/year, $P < 0.0001$).

The initial HBV-DNA levels in genotype C patients (average 5.61 Log_{10}IU/mL) was almost the same as that in genotype D patients (average 5.46 Log_{10}IU/mL) (Table 3).

The association between the prevalence ratio of HBV in various countries and the incidence of HCC with ETV treatment was as follows (Table 1): The incidence of HCC with ETV treatment with a prevalence ratio of HBV of more than 8% was 2.64% ± 0.16%/year (mean ± SE), as compared with 2.39% ± 0.14%/year in regions where the prevalence ratio of HBV was 2%-7% (not significant, $P = 0.576$).

DISCUSSION

We demonstrated that there were marked differences in the impact of ETV treatment on reducing the risk of HCC in patients with HBV-associated cirrhosis in many countries of the world. We must consider why such differences exist.

Firstly, the genotypes of HBV should be considered. Genotype C is seen mostly in Asia, and genotype A in Northwest Europe, North America, India, and Africa. Genotype D is seen in Southern Europe, Middle Eastern Europe, and India. Various cross-sectional studies have found that patients with genotype C have more severe liver disease including cirrhosis or HCC than those with other genotypes[15,16].

In cohort studies of 426 chronic hepatitis B patients from Hong Kong[17] and of 4841 HBsAg-positive men from Taiwan[18], genotype C was associated with a 3-to 5-fold increased risk of HCC, respectively, compared with other HBV genotypes. Moreover, it was reported that the estimated 5-year cumulative incidence of HCC was 17% in East Asia where HBV genotype C is predominant and 10% in Western regions where HBV genotype D or A is predominant[19].

It is considered that the same tendency exists even on long-term treatment with ETV, and the incidence of HCC is higher in genotype C regions than in regions with other genotypes (especially genotype D).

In our studies, we demonstrated that ETV treatment of HBV cirrhotic patients with genotype C was less effective at preventing the occurrence of HCC than in those with other genotypes (chiefly genotype D).

In support of our findings, Kao *et al*[20] demonstrated differences in the response to lamivudine between HBV genotypes. They reported that genotype B showed a better virological response to lamivudine than genotype C in Taiwan.

Another factor that must be taken into account is the association between the prevalence ratio of HBV in various places and the incidence of HCC under ETV treatment. The incidence of HCC under ETV treatment where the prevalence ratio of HBV is more than 8% was 2.64% ± 0.16%/year, as compared with 2.39% ± 0.14%/year in regions where the prevalence ratio of HBV was 2%-7% (not significant, $P = 0.576$).

Another important factor that must be taken into consideration is the initial HBV-

Table 1 Difference in the impact of entecavir treatment on the risk of hepatocellular carcinoma in patients with hepatitis B virus-associated cirrhosis in various regions of the world

Ref.	Region	Main genotype	Prevalence ratio	Entecavir administered to HBV cirrhotics patients	Observation period (yr)	Incidence of HCC (%/yr)
Riveiro-Barciela *et al*[3]	Spain (Caucasian)	D	2%-7%	64	4.6	1.0
Coffin *et al*[4]	Canada (South)	D	< 2%	25	3.2	1.3
Hosaka *et al*[21]	Japan (Tokyo)	C	< 2%	79	5.0	1.4
Papatheodoridis *et al*[14]	Greece	A	2%-7%	69	3.3	1.8
Idilman *et al*[11]	Turkey	D	2%-7%	72	4.0	2.2
Arends *et al*[13]	Caucasus	D	> 8%	155	3.5	2.2
Su *et al*[8]	Taiwan	C	> 8%	1315	4.0	2.4
Köklü *et al*[12]	Turkey	D	2%-7%	73	3.0	2.7
Wong *et al*[1]	Hong Kong	C	> 8%	482	5.0	2.8
Watanabe *et al*[10]	Japan (Ehime)	C	2%-7%	86	5.0	2.9
Chen *et al*[9]	Taiwan	C	> 8%	586	4.9	3.1
Chen *et al*[2]	China (Chinese)	C	> 8%	61	4.0	3.3
Kim *et al*[5]	Korea	C	2%-7%	367	5.0	3.6
Choi *et al*[6]	Korea	C	2%-7%	510	4.0	3.8

HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus.

Table 2 Difference in the incidence of hepatocellular carcinoma under long-term treatment with entecavir between genotype C and genotype D cirrhotic patients

	Incidence of HCC (%/yr)	*P* value
Genotype C group (n = 8)	2.91 ± 0.24 (SE)	$P < 0.01$
Genotype D group (n = 5)	1.89 ± 0.28 (SE)	$P < 0.01$

HCC: Hepatocellular carcinoma.

DNA level. However, we demonstrated that the initial HBV-DNA level in genotype C patients was almost the same as that in genotype D patients.

CONCLUSION

The impact of long-term ETV treatment on reducing the risk of HCC in patients with HBV cirrhosis differs in many countries of the world[1-13,21]. Moreover, it was demonstrated that effectiveness of ETV in preventing HCC development is genotype-dependent in HBV-associated liver cirrhosis.

Table 3 Comparison of initial hepatitis B virus deoxyribonucleic acid levels (log_{10} IU/mL) between genotype C and D cirrhotic patients treated with entecavir

Main genotype	Ref.	Entecavir administered to HBV cirrhotic patientssis	Initial HBV DNA	Average
C	Su *et al*[8]	1315	5.5	5.61
C	Wong *et al*[1]	482	5.0	
C	Watanabe *et al*[10]	86	6.4	
C	Chen *et al*[9]	586	5.9	
C	Chen *et al*[2]	61	5.8	
C	Kim *et al*[5]	367	4.6	
C	Choi *et al*[6]	510	6.7	
D	Riveiro-Barciela *et al*[3]	64	4.9	5.46
D	Coffin *et al*[4]	25	6.5	
D	Idilman *et al*[11]	72	5.5	
D	Arends *et al*[13]	155	5.4	
D	Köklü *et al*[12]	73	5.7	

HBV DNA: Hepatitis B virus deoxyribonucleic acid.

ARTICLE HIGHLIGHTS

Research background

The oral nucleos(t)ide analogue, entecavir (ETV) was demonstrated to reduce the rate of hepatocellular carcinoma (HCC) in patients with hepatitis B virus (HBV)-associated liver cirrhosis. However, the reduction in HCC is different in various countries of the world.

Research motivation

The relationship between the reduction of HCC and HBV genotypes is interesting.

Research objectives

We surveyed the differences in the reduction of HCC development following ETV administration in many countries.

Research methods

We surveyed the differences in the reduction of HCC development following long-term administration of ETV based on already published articles using PubMed (2004-2019).

Research results

The countries which showed the greatest reduction in HCC development following ETV administration were Spain, Canada, and most ineffective countries or regions were South Korea, China, Taiwan, and Hong Kong. With ETV administration, the incidence of HCC in genotype D regions was significantly lower than that in genotype C regions. The initial HBV-DNA levels in genotype C patients was almost the same as that in genotype D patients. No relationship was observed between the prevalence ratio of HBV and the incidence of HCC following ETV treatment.

Research conclusions

The effectiveness of ETV in preventing HCC development in HBV-associated liver cirrhosis is genotype-dependent.

Research perspectives

In countries with low effectiveness of ETV in the prevention of HCC development, frequent surveillance using imaging modalities will be necessary.

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CASE REPORT

Living-donor liver transplantation in Budd-Chiari syndrome with inferior vena cava complete thrombosis: A case report and review of the literature

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Abstract

BACKGROUND

Budd-Chiari syndrome (BCS) is a challenging indication for liver transplantation (LT) due to a combination of massive liver, increased bleeding, retroperitoneal fibrosis and frequently presents with stenosis of the inferior vena cava (IVC). Occasionally, it may be totally thrombosed, increasing the complexity of the procedure, as it should also be resected. The challenge is even greater when performing living-donor LT as the graft does not contain the retrohepatic IVC; thus, it may be necessary to reconstruct it.

CASE SUMMARY

A 35-year-old male patient with liver cirrhosis due to BCS and hepatocellular carcinoma beyond the Milan criteria underwent living-donor LT with IVC reconstruction. It was necessary to remove the IVC as its retrohepatic portion was completely thrombosed, up to almost the right atrium. A right-lobe graft was retrieved from his sister, with outflow reconstruction including the right hepatic vein and the branches of segment V and VIII to the middle hepatic vein. Owing to

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massive subcutaneous collaterals in the abdominal wall, venovenous bypass was implemented before incising the skin. The right atrium was reached *via* a transdiaphragramatic approach. Hepatectomy was performed *en bloc* with the retrohepatic vena cava. It was reconstructed with an infra-hepatic vena cava graft obtained from a deceased donor. The patient remains well on outpatient clinic follow-up 25 mo after the procedure, under an anticoagulation protocol with warfarin.

CONCLUSION
Living-donor LT in BCS with IVC thrombosis is feasible using a meticulous surgical technique and tailored strategies.

Key Words: Liver transplantation; Living donors; Budd-Chiari syndrome; Hepatic veno-occlusive disease; Inferior vena cava; Case report

Core Tip: A right-lobe living-donor liver transplantation (LT) with inferior vena cava (IVC) resection and reconstruction was performed in a patient with liver cirrhosis due to Budd-Chiari syndrome and hepatocellular carcinoma beyond the Milan criteria. It was necessary to remove the IVC because its retrohepatic portion was completely thrombosed, up to almost the right atrium. It was reconstructed with an infra-hepatic vena cava graft obtained from a deceased donor. The patient remains well 25 mo after the procedure. This case highlights the meticulous surgical technique and tailored strategies required for dealing with these challenging procedures in living-donor LT.

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INTRODUCTION

Budd-Chiari Syndrome (BCS) is characterized by the obstruction of hepatic venous drainage that leads to progressive hepatic congestion and, ultimately, portal hypertension and liver cirrhosis[1]. This blockage may be present in the hepatic venules, main hepatic veins, inferior vena cava (IVC) or right atrium[2]. Several nonsurgical therapeutics have been described, such as anticoagulation therapy, percutaneous transluminal angioplasty and interventional radiologic placement of a transjugular intrahepatic portosystemic shunt (TIPS) or direct intrahepatic portocaval shunt[1-3]. Liver transplantation (LT) is indicated in acute cases of fulminant hepatic failure or chronic cases with cirrhosis, which commonly evolve with gastrointestinal bleeding, untreatable ascites, sarcopenia, encephalopathy and hepatocellular carcinoma (HCC)[4]. In such scenarios, TIPS is often unfeasible due to extensive venous thrombosis or advanced liver disease[5].

Venous thrombosis can affect not only the hepatic veins but also a prolonged segment of the retrohepatic IVC, occasionally very close to the right atrium. The association between the severity of the disease, the extension of the venous thrombosis and the massive liver that is frequently present in BCS makes LT a particularly difficult procedure in these cases[1]. The hypercoagulative nature of the syndrome further increases the challenge, owing to vascular complications[6].

The challenge is even greater when considering living donor liver transplantation (LDLT) since the graft does not contain the retrohepatic IVC, as in deceased-donor liver transplantation (DDLT). Therefore, hepatic venous reconstruction is more complex, especially if the IVC is also obliterated[7]. That is the reason why only approximately 70 patients with BCS underwent LDLT worldwide between 1989 and 2015[1,8]. When LDLT is performed and HCC is also present, DDLT may not be possible

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in case of postoperative complications if the patient is beyond the Milan criteria[9], depending on local legislation in some countries, such as Brazil. Thus, performing LDLT for BSC in such a scenario is even more risky.

We report a case of a complex retrohepatic IVC thrombosis due to BCS in a patient with HCC beyond the Milan criteria. As the patient had a good response to transarterial chemoembolization (TACE) and his alfa fetoprotein levels decreased, we decided to perform LDLT.

CASE PRESENTATION

Chief complaints

A 35-year-old cirrhotic male patient was referred for LT evaluation due to BCS and HCC.

History of present illness

The patient had been diagnosed with cirrhosis and BCS four years previously, after presenting with ascites and hematemesis due to esophageal varices. Abdominal computed tomography (CT) scan on this occasion showed hepatic veins thrombosis and signs of chronic hepatopathy with paraumbilical vein recanalization and extensive collateral circulation in the splenic hilum, around the stomach, and in the anterior and lateral abdominal walls. The liver also showed multiple hepatic nodules of up to 1.5 cm in diameter, some them hypervascularized, which in the context of BCS, were compatible with regenerative hepatic nodules. Hepatic biopsy revealed chronic hepatic outflow obstruction. Laboratory testing for autoimmune hepatitis was negative, as were serological markers for hepatitis C and B viruses. The patient also denied previous alcohol abuse. No thrombophilia was diagnosed, despite extensive hematological investigation. The patient was then maintained on oral anticoagulation with warfarin.

History of past illness

The patient had no previous medical history.

Personal and family history

The patient was a smoker (10 cigarettes/day for 20 years). There was no relevant family history concerning this case.

Physical examination

The patient exhibited mild jaundice and extensive subcutaneous collateral veins in the anterior abdominal wall (Figure 1). Further physical examination was unremarkable.

Laboratory examinations

Blood analysis revealed normal hemoglobin, mild leukopenia and mild thrombocytopenia with mildly elevated total bilirubin, direct bilirubin and gamma-glutamyl-transferase (Table 1). Kidney function and electrolytes were normal as well as serum albumin, alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase. The patient's prothrombin time was elevated even without warfarin (Table 1). Considering that the patient did not present encephalopathy or ascites, his Child-Pugh score was A6, and his Model of End-Stage Liver Disease (MELD) score was 15. His alpha-fetoprotein level was 58.7 ng/mL (normal range < 10 ng/mL), although 6 mo earlier, it was 9.4 ng/mL.

Imaging examinations

During outpatient follow-up, an abdominal CT scan showed a heterogeneously vascularized nodule in segment V, which increased from 2 cm to 4 cm in three years (Figure 2A and B). He also showed complete thrombosis of the retrohepatic IVC, up to almost the right atrium, with large subcutaneous veins in his abdominal wall (Figure 2C). Further evaluation with abdominal liver magnetic resonance imaging with hepatobiliary contrast showed two hypervascularized nodules with hypocaptation in the biliary phase in segments V and II, 4 and 2.3 cm in size, respectively (Figure 3). Considering the previous CT scans with multiple regenerative nodules, these 2 specific nodules were classified as indeterminate lesions. Given their growth, the atypical pattern of contrast uptake and the rise in alpha-fetoprotein serum levels, further investigation with biopsy of these nodules was indicated due to the

Table 1 Laboratory tests results and normal range

Laboratory test	Result	Normal range
Hemoglobin	12.6 g/dL	12.5-17.5 g/dL
Leukocytes	3.5 × 10^9/L	4-11 × 10^9/L
Platelets	80 × 10^3/mm^3	150-400 × 10^3/mm^3
Total bilirubin	1.73 mg/dL	0.2-1 mg/dL
Direct bilirubin	0.85 mg/dL	< 0.3 mg/dL
Alanine aminotransferase	20 U/L	< 41 U/L
Aspartate aminotransferase	35 U/L	< 37 U/L
Alkaline phosphatase	78 U/L	40-129 U/L
Gamma-glutamyl-transferase	115 U/L	8-91 U/L
Creatinine	0.79 mg/dL	0.7-1.2 mg/dL
Blood urea nitrogen	31 mg/dL	10-50 mg/dL
Sodium	143 mEq/L	135-145 mEq/L
Potassium	3.9 mEq/L	3.5-4.5 mEq/L
Albumin	4.4 g/dL	3.4-4.8 g/dL
Prothrombin time	21.8 s	9.4-12.5 s
International normalized ratio	1.75	0.95-1.2

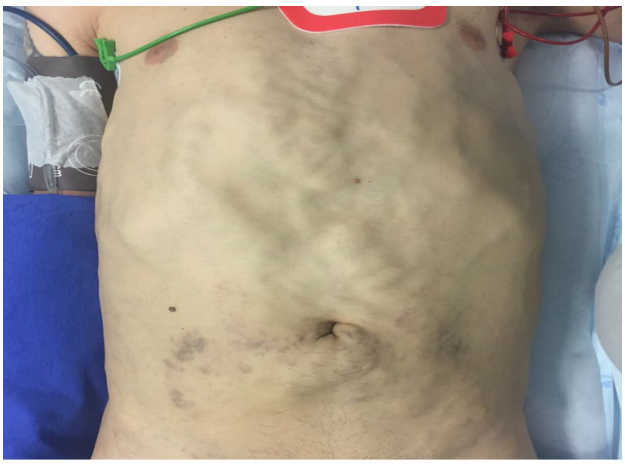

Figure 1 Massive blood return by subcutaneous veins in the anterior abdominal wall, which required the use of venovenous bypass prior to the abdominal incision.

suspicion of HCC.

FINAL DIAGNOSIS

Percutaneous ultrasound-guided biopsy of the largest nodule confirmed a moderately differentiated HCC (grade 3 Edmondson-Steiner grading system). Therefore, the patient presented liver cirrhosis due to BCS with retrohepatic vena cava thrombosis and multicentric HCC beyond the Milan criteria.

TREATMENT

According to Brazilian legislation, the patient could not be listed for DDLT due to

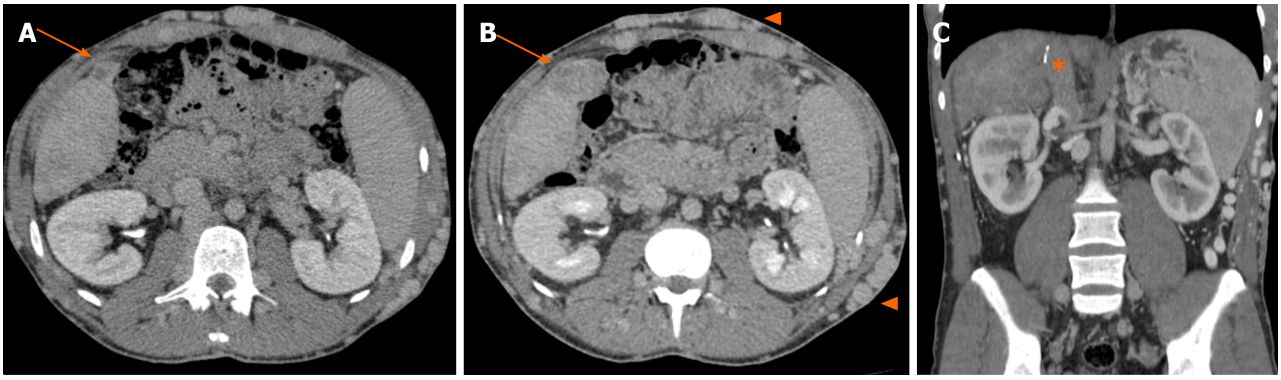

Figure 2 Abdominal computed tomography scans, with a 3-year interval. A: Heterogeneously vascularized nodule in segment V, of 2 cm, more visible in delayed phase due to hypocaptation (arrow); B: Same nodule in segment V in an exam scan performed 3 years later, with 4 cm (arrow). Massive subcutaneous veins in the abdominal wall are noted (arrowhead); C: The retrohepatic vena cava is completely thrombosed, up to almost the right atrium (asterisk).

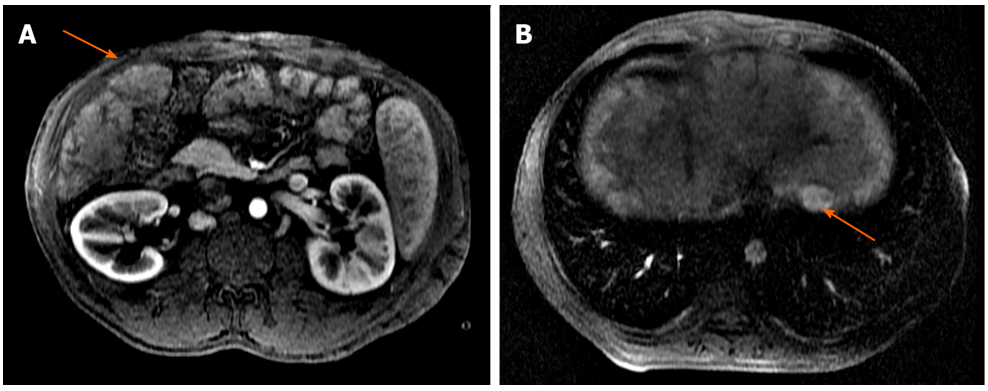

Figure 3 Liver magnetic resonance imaging with hepatobiliary contrast (arterial phase). A: Hypervascularized nodule in segment V of 4 cm (arrow); B: Hypervascularized nodule in segment II of 2.3 cm (arrow).

being beyond the Milan criteria. He underwent 2 TACE procedures in order to downstage the lesions to within the Milan criteria so that he could be listed. Even though the serum alfa-fetoprotein level decreased from 58.7 to 18 ng/mL, the nodules did not decrease in size and the patient remained beyond the Milan criteria. His sister then volunteered for liver donation and the patient was selected for LDLT. She was a healthy 51-year-old female with a body mass index of 22.6 kg/m^2. Liver volumetry revealed a right lobe of 724 cm^3 (66% of the entire organ), and usual biliary tree anatomy was found on magnetic resonance cholangiopancreatography. Liver parenchyma also showed simple cysts.

The patient weighed 71 kg, resulting in a predicted graft-to-recipient weight ratio (GRWR) of 0.81%. Donor operation consisted of a right hepatectomy with middle hepatic vein preservation. The procedure was uneventful, resulting in a 560 g right lobe graft with usual anatomy (GRWR of 0.79%). In the backtable operation, the right hepatic vein and the V5 and V8 branches of the middle hepatic vein were reconstructed to avoid outflow blockage.

For the recipient, the surgical strategy included the use of a venovenous bypass prior to incising the abdomen due to very large subcutaneous collaterals in the abdominal and thoracic walls. The left femoral and left axillary veins were used to implement the venovenous bypass. Hepatectomy was performed with the retrohepatic vena cava, close to the right atrium. The explanted liver weighed 1880 g. The portal vein was then cannulated and added to the venovenous bypass. As the right lobe graft did not include the retrohepatic vena cava, it was reconstructed using an infra-hepatic IVC from a deceased donor (Figure 4A). The graft was then implanted using this newly formed IVC to be anastomosed with the graft venous conduit for the outflow reconstruction. The right portal vein, right hepatic artery and right hepatic duct of the graft were then respectively anastomosed to their counterparts in the recipient (Figure 4B and C). Total and warm ischemia times were 370 and 30 min, respectively.

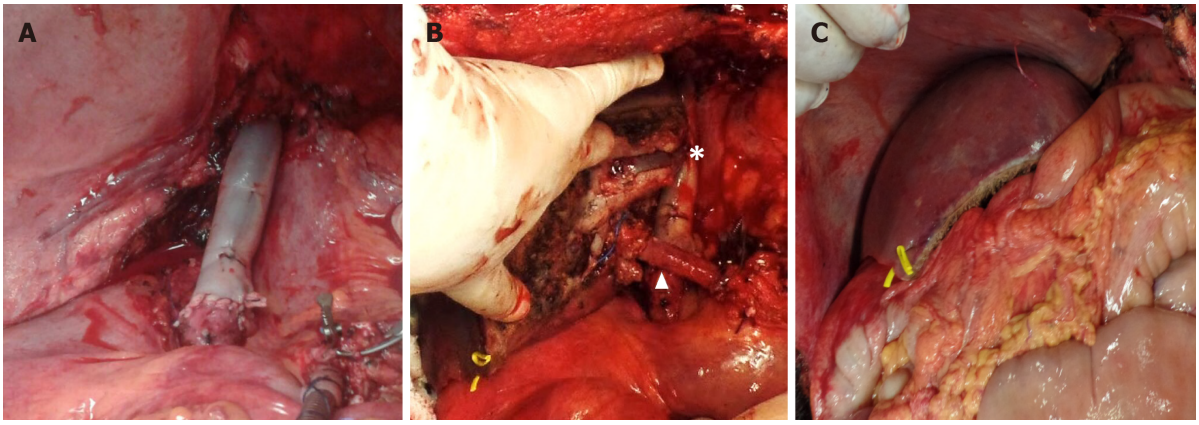

Figure 4 Intraoperative images. A: Reconstructed retrohepatic vena cava using an infrahepatic vena cava graft of a deceased donor; B: Revascularized graft showing the venous conduit anastomosed to the newly formed vena cava (asterisk) and the portal vein anastomosis (arrowhead); C: Graft final aspect after arterialization at the end of transplantation.

OUTCOME AND FOLLOW-UP

The donor's postoperative course was uneventful, and she was discharged home on postoperative day (POD) 5. The recipient was extubated on POD 2, and anticoagulation with enoxaparin was restarted, as well as low-dose aspirin. Liver Doppler ultrasound on POD 1 and 15 showed preserved graft vascularization. Renal function remained preserved, and the patient's condition progressively improved. The patient's immunosuppression regimen included intraoperative corticoid bolus and tapering associated with tacrolimus. The patient was discharged home on POD 19. Everolimus was added to the tacrolimus regimen 3 mo after the transplantation. Low-dose corticoid was maintained for 6 mo.

On histopathological analysis, the explanted liver confirmed hepatic cirrhosis related to chronic BCS and two moderately differentiated HCCs in segment V (4.5 cm) and segment II (2.5 cm).

Routine abdominal CT scan performed 23 mo after transplant showed a patent retrohepatic vena cava and adequate right lobe vascularization (Figure 5). The patient remains well on outpatient clinic follow-up 25 mo after the procedure, under an anticoagulation protocol with warfarin and without signs of HCC recurrence (alpha-fetoprotein 6.5 ng/mL).

DISCUSSION

Despite the numerous treatment modalities available for BCS, LT is performed in 10% to 20% of patients[1,2]. Nevertheless, it is a rare cause for LT, accounting for approximately 1%[10,11]. This a challenging indication for LT due to a combination of massive liver and increased bleeding, caudate lobe enlargement, retroperitoneal diffuse fibrosis, firm retrohepatic IVC adhesions and frequently presents with stenosis and/or thrombosis of the IVC[3]. Especially in LDLT, in which the donor's IVC cannot be used, the retrohepatic IVC dissection performed during the piggyback technique and the venous outflow reconstruction are particularly problematic. Novel alternative techniques, aimed at eliminating stenosis or obstruction in the recipient IVC, are thus needed for LDLT in the context of BCS[6]. Some of them include cross-clamping the supra- and infrahepatic IVC and excising its thickened wall to create a wide orifice for graft implantation[7] or the V-Y plasty technique[12].

Nevertheless, when the IVC is completely occluded, which is known as obliterative hepatocavopathy (OHC), it is advisable to remove the IVC *en bloc* with the native liver[13], as the piggyback dissection becomes technically unfeasible due to dense inflammatory adhesions, enlarged collaterals and hypertrophied caudate lobe. If an LDLT is performed in this situation, it may be necessary to reconstruct the retrohepatic IVC. In 2006, Yan *et al*[14] reported the first LDLT for BCS with IVC reconstruction using an interposed cryopreserved cadaveric IVC graft[14]. Since then, many other studies have addressed IVC reconstruction with interposing autologous veins[15], cadaveric venous allografts[3,7,16-18], cadaveric aortic allografts[7,17-20], synthetic material[12,13,18] or a combination of synthetic material and autologous vein[21,22] or venous allografts[18,23].

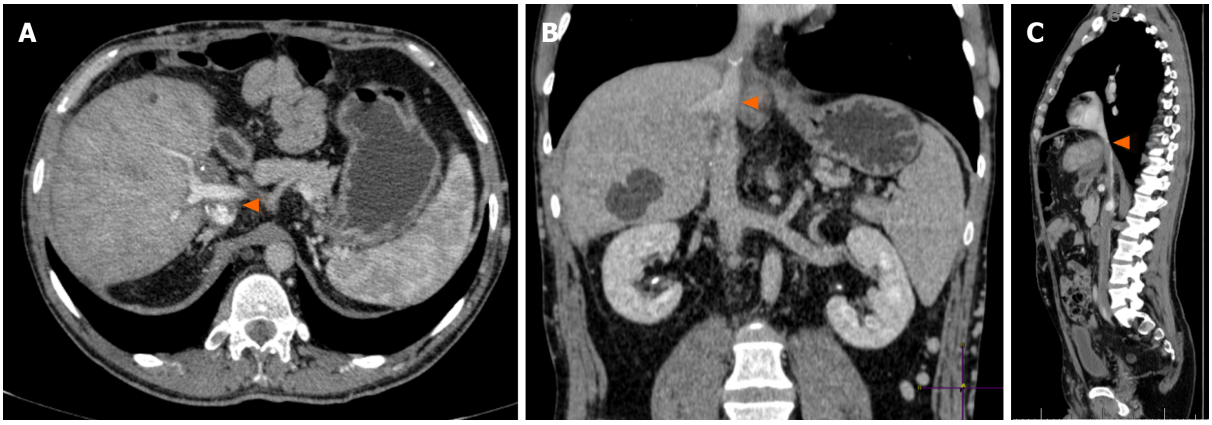

Figure 5 Late postoperative abdominal computed tomography scan, portal phase. A: Graft with adequate aspect and preserved portal inflow (arrowhead); B: Coronal view showing patent retrohepatic vena cava (arrowhead) and preserved graft outflow; C: Sagittal view of patent retrohepatic vena cava (arrowhead).

Table 2 provides a review of all cases found in the literature of LDLT for BCS with IVC resection.

In the present report, we faced three ordeals in the preoperative period. First, the massive liver was associated with extensive IVC thrombosis starting close to the renal veins and progressing up to the transition between the IVC and the right atrium. Second, it was necessary to use a living donor right lobe with the potential risk of postoperative small-for-size syndrome, given the association of extensive thrombosis, portal hypertension and partial graft[12]. Finally, the LDLT was performed in a patient with HCC beyond the Milan criteria, which, according to Brazilian law, prevented the use of a deceased-donor graft in case of postoperative graft dysfunction.

Most authors describe a transdiaphragmatic access to the supradiaphragmatic IVC or even the right atrium, although a rarely performed lower median sternotomy may be helpful in some cases[13,24]. In the present report, through a standard *Makuuchi* incision, the recipient's liver was removed *en bloc* with the retrohepatic vena cava, from just above the renal veins to the beginning of the right atrium. This surgical approach, without thoracic access, was very useful as the patient had no major bleeding or hemodynamic instability. The interposition of a conduit replacing the retrohepatic IVC was necessary because we could observe considerable venous flow from the suprarenal vena cava. There is no consensus in the literature regarding the best material for IVC reconstruction[18]. The use of synthetic material raises concerns regarding the long-term patency of the anastomosis between the hepatic vein from the liver graft and the prosthesis, due to the possibility of thrombosis, deformity of the synthetic orifice and anastomosis kinking consequent to growth of the liver graft[25]. Infection of prosthetic material is also an issue[26]. Many centers, including ours, therefore prefer autologous or allogeneic grafts, which present less thrombosis and infection risk[18,27]. Even cadaveric IVC recovered 25 h after the donor´s circulatory death has been successfully used[28]. As a high-volume center of DDLT, there is great availability of allografts in our institution biobank. Storage of such grafts is feasible and inexpensive, only requiring sterile Ringer Lactate solution and a laboratory freezer[29]. However, in countries with scarce deceased donor organ donation and in centers with a high volume of LDLT, access to these grafts may be difficult[18].

Given the complexity of such procedures, it is paramount to obtain a suitable amount of liver parenchyma[30]. Therefore, we used the right lobe, as in most reported cases; however, some authors have also used the right posterior segment[15], the left lateral segment (pediatric recipients)[7,17,19], the left lobe[2,22,24,25] and dual grafts[13]. Another concern is the possibly elevated portal inflow to the graft[31]. That is the reason why we routinely measure the portal venous pressure by a catheter inserted *via* a jejunal branch. As the portal pressure was below 14 mmHg in this case after graft implantation, we did not implement further strategies to decrease the portal inflow.

In most cases reported, venovenous bypass was not used (Table 2). Due to the chronicity of IVC obstruction, venous return is expected to occur *via* collaterals involving the azygos, hemiazygos, accessory hemiazygos and thoracolumbar veins[24]. In a large series addressing LDLT with IVC resection for various reasons in 29 patients by Gonultas *et al*[18], venovenous bypass was not used in any case, as there was no hemodynamic instability during IVC clamping. In our case, the patient presented a

Table 2 Summary of all reported cases of living-donor liver transplantation for Budd-Chiari syndrome with inferior vena cava resection

Ref.	Number of cases	Technique	Venovenous bypass use	Outcomes
Yan *et al*[14], 2006	n = 1	IVC replacement with cadaveric IVC allograft	Yes	Alive after 3 mo
Yamada *et al*[2], 2006	n = 1	IVC resection without replacement	No	Alive after 10 mo
Shimoda *et al*[15], 2007	n = 1	IVC replacement with autologous internal jugular vein, external iliac vein and suprarenal IVC	No	Alive after 17 mo
Sasaki *et al*[16], 2009	n = 1	IVC replacement with cadaveric IVC allograft	No	N/A
Kazimi *et al*[32], 2009	n = 1	IVC resection without replacement	No	Alive after 3 mo
Choi *et al*[3], 2010	n = 2	IVC replacement with cadaveric IVC allograft (n = 1) and RHV-atrial shunt using preexisting mesoatrial shunt (n = 1)	No	Both alive after a median follow-up of 18 mo
Ogura *et al*[21], 2011	n = 1	IVC replacement with an inverted composite graft (Gore-Tex stretch vascular graft and transposed IVC)	Yes	Alive after 24 mo
Sakçak *et al*[19], 2012	n = 1	IVC replacement with cadaveric aortic allografts	No	Alive after 4 mo
Fukuda *et al*[24], 2013	n = 1	IVC resection without replacement	No	Alive after 60 mo
Yagci *et al*[17], 2015	n = 4	IVC replacement with cadaveric IVC (n = 1), iliac vein (n = 1) and aorta allografts (n = 2)	No	2 patients died due to biliary complications after 5 mo of follow-up
Cetinkunar *et al*[20], 2015	n = 1	IVC replacement by cadaveric aortic allograft	No	Alive after 4 mo
Ara *et al*[7], 2016	n = 7	IVC replacement with cadaveric IVC (n = 4) and cadaveric aorta allografts (n = 2). No replacement in one case	No	2 patients died due to recent HAT after LT, and 2 patients died of sepsis during follow-up
Pahari *et al*[12], 2016	n = 2	IVC replacement with e-PTFE graft	No	Both alive after a median follow-up of 18 mo
Karaca *et al*[6], 2017	n = 3	IVC resection without replacement	No	N/A
Sabra *et al*[25], 2018	n = 1	IVC resection without replacement	No	Alive after 3 mo
Yagi *et al*[22], 2018	n = 1	IVC replacement with an inverted composite graft (e-PTFE graft and transposed IVC)	Yes	Alive after 36 mo
Ionescu *et al*[23], 2018	n = 2	IVC replacement with caval-dacron composite graft	No	Both alive (follow-up not available)
Yoon *et al*[13], 2019	n = 5	IVC replacement with synthetic material (ringed polyester)	Yes (n=3)	All alive after a median follow-up of 10.5 years
Gonultas *et al*[18], 2020	n = 12	IVC replacement with cadaveric IVC allograft (n = 6), cadaveric aorta allograft (n = 1), synthetic material (n = 3, Dacron) and caval-dacron composite graft (n = 2)	No	All alive after median follow-up of 15 mo
Present study	n = 1	IVC replacement with cadaveric IVC allograft	Yes	Alive after 25 mo

N/A: Not available; e-PTFE: Polytetrafluoroethylene; HAT: Hepatic artery thrombosis; IVC: Inferior vena cava; RHV: Right hepatic vein; LT: Liver transplantation.

well-developed collateral circulation; however, we observed that it was mainly composed of a massive subcutaneous plexus in the abdominal and thoracic wall (Figures 1 and 2). Thus, we decided to use the extracorporeal venovenous bypass before the abdominal skin was incised. We feared that an abdominal incision could lead to hemodynamic instability, since it was necessary to ligate the collaterals forming this enormous subcutaneous plexus. Therefore, when we accessed the abdominal cavity and clamped the IVC, the patient was already on venovenous bypass.

Retrohepatic IVC resection without replacement in LDLT for BCS has also been reported[2,6,7,24,25,32], in which the liver graft is anastomosed directly to the right atrium[6,32], to the intrapericardical IVC[24,25] or to the rarely preserved supra-hepatic

IVC[2,6,7]. In one patient, the graft was directly anastomosed to a previous mesoatrial shunt[3]. This raises the question of whether or not it necessary to reconstruct the IVC. As addressed by Gonultas *et al*[18], the venous continuity should be maintained in patients without a venous collateral circulation system or in those with insufficient venous drainage. For patients that have a well-developed venous collateral, on the other hand, the liver graft may be, in theory, anastomosed directly to the suprahepatic IVC without the need for reconstruction. In our case, as the collaterals forming the subcutaneous plexus were ligated during the skin incision, the IVC reconstruction was required. We also observed a significant blood flow in the infra-hepatic IVC after the native liver was removed, suggesting the necessity of venous continuity restoration with an IVC interposition graft.

Despite the complexity of cases, most studies describe successful outcomes after LDLT (Table 2). The literature review identified 2 deaths due to early hepatic arterial thrombosis and another 4 patients died during follow-up due to infectious and biliary complications occurring months after transplant. In the series by Gonultas *et al*[18], 4 patients experienced late thrombosis of the replaced IVC during follow-up that were successfully treated with percutaneous balloon dilatation and/or stenting. The early use of low-dose aspirin and low molecular weight heparin a few days after LDLT is important to prevent the recurrence of thrombosis[12,13,18,32].

CONCLUSION

We describe a novel surgical approach for LDLT in BCS with OHC and HCC beyond the Milan criteria that can be used in highly selected patients. Due to its complexity and rarity, LDLT in such situations is feasible using a meticulous surgical technique and tailored strategies.

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ABOUT COVER

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EDITORIAL

Meeting report of the editorial board meeting for *World Journal of Hepatology* 2021

Li Ma, Xiang Li

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Abstract

The 2021 online editorial board meeting of the *World Journal of Hepatology (WJH)* was held on January 16, 2021. Xiang Li, Director of Production Office on behalf of the Baishideng Publishing Group, organized the meeting. Three Editors-in-Chiefs (EiCs) and 15 Baishideng Publishing Group staff attended the meeting. The meeting goal was to brief EiCs on journal performance and gather ideas for journal development in 2021. In 2020, *WJH* published 204 articles, a 20% increase compared to 2019, authors were from 32 countries and regions, and the average citation per article was three times. However, attracting high quality original article submissions remains a challenge. The EiCs provided feedback and suggestions centered on four topics: (1) Improve journal quality by building editorial; (2) Improve board engagement by establishing a clear policy and consistent internal communications; (3) Improve peer review quality and efficiency; and (4) Refine the current journal marketing strategy to increase visibility and discoverability.

Key Words: *World Journal of Hepatology*; Baishideng; Editorial board meeting; Journal development

Core Tip: The 2021 *World Journal of Hepatology* editorial board meeting was held on January 16, 2021. The meeting goal was to brief board members on journal performance and gather ideas for journal development in 2021. The discussion focused on (1) improving journal quality by building editorial; (2) improving board engagement by establishing a clear policy and consistent communications; (3) improving peer review quality and efficiency; and (4) refining current journal marketing strategy.

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INTRODUCTION

Every year *World Journal of Hepatology* (*WJH*) editorial office organizes the editorial board meeting at the American Association for the Study of Liver Disease annual meeting. Due to the coronavirus disease 2019 pandemic, the 2021 meeting moved to online format. *WJH* editorial office hosted the meeting on January 16, 2021 to review journal performance in 2020 and identify strategies to further *WJH*'s mission, which is to publish high impact research in the field of hepatology. The meeting was moderated by Dr. Li Ma, Company Vice-Editor-in-Chief. The first part of the meeting consisted of presentations on journal status review and plans for 2021, and the second part consisted of open discussions with Editors-in-Chiefs (EiCs) for their feedback and suggestions.

ATTENDEES

This online meeting brought together three EiCs: Namely Dr. Ke-Qin Hu, Dr. Koo Jeong Kang, Dr. Nikolaos Pyrsopoulos, and 15 Editors (Li Ma, Xiang Li, Jin-Lei Wang, Ze-Mao Gong, Ya-Juan Ma, Jia-Ping Yan, Yun-XiaoJian Wu, Dong-Mei Wang, Jia-Ru Fan, Chen-Chen Gao, Le Zhang, Ji-Hong Liu, Yu-Jie Ma, Yan-Liang Zhang, Li-Li Wang) from Baishideng Publishing Group Inc (Figure 1).

REPORTS

WJH year in review 2020

Xiang Li began the meeting by offering an overview of *WJH*'s journal statistics, status quo of editorial board, challenges journal faces, and update from publisher. She showed in 2020 that *WJH* published 204 papers, an 20% increase compared to 2019 (181 papers). The 2020 editorial board consists of 195 members from 45 countries and regions. The top three countries are China, Italy and United States. Forty-four percent of Editorial board members reviewed at least one manuscript in 2020. She highlighted that the main challenge is to attract high quality, high impact original research submissions. Xiang Li finally highlighted new features Baishideng Publishing Group has launched to serve better the authors, including open peer review, shortened peer review time by using artificial intelligence empowered search techniques and post publication promotion by marketing articles to targeted audiences[1].

Dr. Ma presented the journal's 2021 priorities: (1) Commissioning and publishing high impact original articles in the important areas of hepatology; (2) Encouraging editorial board members to recommend and submit to the journal; and (3) Improving the overall quality and relevance of *WJH*.

OPEN DISCUSSION FROM EDITOR-IN-CHIEFS FOCUSED ON FOUR MAIN TOPICS

Peer review

Dr. Hu inquired about the low response rate of peer review invitations; he commented that the current key word matching search using the in-house database is suboptimal. He presented the following possible solutions: (1) Allow authors to suggest reviewers; editor should cross check if suggested reviewers are suitable candidates; and (2) Prioritize editorial board member as peer reviewers. Dr. Ma addressed the above question that the current peer reviewer search strategy offers a fair and unbiased review process, but there is room for improvement. Dr. Pyrsopoulos added that it is also worth improving peer review quality when poor language is used by the peer

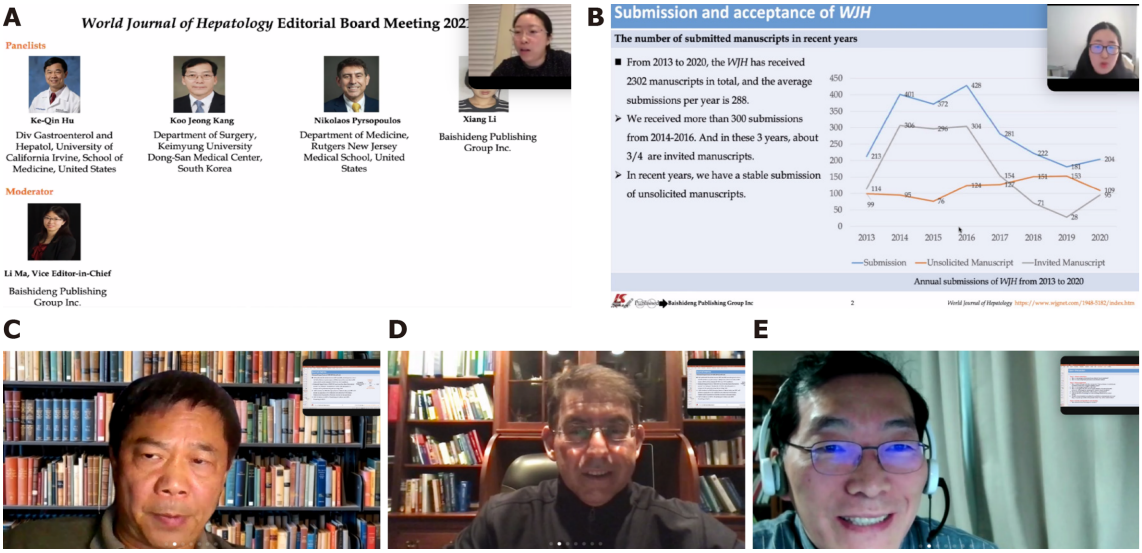

Figure 1 2021 *World Journal of Hepatology* editorial board meeting presenters. A: Dr. Li Ma; B: Xiang Li; C: Dr. Ke-Qin Hu; D: Dr. Nikolaos Pyrsopoulos; E: Dr. Koo Jeong Kang.

reviewer, and the editorial office should contact the reviewer for professional and constructive comment.

Journal quality

Dr. Hu pointed out that *WJH* should aim to build up a strong editorial and suggested that the editorial office should group upcoming manuscripts in topics or inform EiCs in advance so they can help group the topics. EiCs can assist with writing or help finding suitable authors to write an editorial or commentary. Dr. Pyrsopoulos brought up the topic of how to handle better invited manuscript rejection, as authors may feel disappointed when an invited article is rejected. On a different note, he suggested the importance of tracking the evolution of journal citations to monitor journal health.

Maintain an active editorial board

Statistics presented by Xiang Li showed that about 50% of editorial board members are inactive and that they did not review a paper for the journal in 2020. Both Dr. Hu and Dr. Pyrsopoulos suggest that the Editorial Office should send a kind reminder to these editors about the duties and commitments. In addition, internal metrics should be set to monitor editorial board member activities. Along with the above points, a "Dashboard" should be created to show editorial board member their statistics, including the number of invitations sent, response time, time taken to review and review quality grading by the handling editor. Orientation should be prepared for editorial board members to familiarize them with journal history, policy and peer review best practice. In addition, a quarterly editorial board member newsletter can inform everyone about journal news, initiatives and policy updates. Lastly, but most importantly, there should be a recognition mechanism for those editorial board members who contribute to journal growth by submitting their research or performing peer review.

Dr. Kang reminded everyone that a high-quality and active editorial board is more crucial to journal growth. He inquired about the percentage of surgeons serving on the board and surgery related papers published in *WJH* and proposed increasing liver surgeon representation. Dr. Pyrsopoulos emphasized the advantages of having a large editorial board to increase submissions.

Journal marketing

Dr. Pyrsopoulos and Dr. Hu gave a very comprehensive overview on how to increase journal visibility. They commented that the editorial office needs to think about how to market and position the journal. Research online advertising channels, such as social media, increase the discoverability in search engines. Offline channels include society conferences (American Association for the Study of Liver Disease, European Association for the Study of the Liver, Asian Pacific Association for the Study of the Liver), setting up a booth to meet and connect with authors and donating and

supporting the conference.

ACTIONS AND FUTURE PLANS FROM THE EDITORIAL OFFICE

First, the *WJH* editorial office would like to collaborate with EiCs to select two editorial worthy articles in 2021 Q1 and Q2 and to coordinate a suitable candidate to write editorial.

Second, research suitable venues online (website advertising) and offline (leading Hepatology meetings) should be utilized to promote the journal. A volunteer editorial board member will need to be recruited to help manage content of a dedicated *WJH* twitter account.

Third, editorial board member engagement needs to improve and editorial board member review rate should increase from 44% to 70%. An annual editorial board member activity "dashboard" email that contains review number, speed and reviewed article status would be helpful. Thank you letters should be sent to recognize their valuable time. It is important to send bi-annual editorial board newsletters to convey journal news, internal information exchange, publisher update, *etc.*

Fourth, EiCs should collaborate to identify and address author submission pain points in order to improve author publishing experiences.

Fifth, liver surgery editorial board member representation should be expanded and experts from this field should be invited to submit articles.

CONCLUSION

All EiCs expressed commitment and enthusiasm to help the journal grow. Dr. Hu is committed to moving the journal to higher ranking and improving journal quality, and he is interested to provide guidance to make the submission system more streamlined and user friendly. Dr. Pyrsopoulos and Dr. Kang said that they will continue to contribute their research and encourage their peers to contribute; they will work together with the editorial office to have *WJH* recognized by leading databases. They also expressed wishes to continue the in person editorial board meeting when possible at future American Association for the Study of Liver Diseases annual meeting, as it is a great way to catch up with other members and the editorial office. Dr. Li Ma thanked the EiCs for their contributions in the past years[2-6], enlightening ideas and time to attend the meeting. The next online editorial board meeting is tentatively set for June 2021. All editorial members are welcome to attend, stay tuned for more details!

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WJH editorial office thanks all the EiCs for their leadership, guidance and contributions to the journal growth.

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REVIEW

Extrahepatic cholangiocarcinoma: Current status of endoscopic approach and additional therapies

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Abstract

The prognosis of patients with advanced or unresectable extrahepatic cholangiocarcinoma is poor. More than 50% of patients with jaundice are inoperable at the time of first diagnosis. Endoscopic treatment in patients with obstructive jaundice ensures bile duct drainage in preoperative or palliative settings. Relief of symptoms (pain, pruritus, jaundice) and improvement in quality of life are the aims of palliative therapy. Stent implantation by endoscopic

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retrograde cholangiopancreatography is generally preferred for long-term palliation. There is a vast variety of plastic and metal stents, covered or uncovered. The stent choice depends on the expected length of survival, quality of life, costs and physician expertise. This review will provide the framework for the endoscopic minimally invasive therapy in extrahepatic cholangiocarcinoma. Moreover, additional therapies, such as brachytherapy, photodynamic therapy, radiofrequency ablation, chemotherapy, molecular-targeted therapy and/or immunotherapy by the endoscopic approach, are the nonsurgical methods associated with survival improvement rate and/or local symptom palliation.

Key Words: Cholangiocarcinoma; Endoscopic drainage; Endoscopic retrograde cholangiopancreatography; Photodynamic therapy; Radiofrequency ablation; Brachytherapy

Core Tip: Cholangiocarcinoma is an aggressive tumor with a poor prognosis mainly due to its late diagnosis. The development of new minimally invasive techniques provides these patients a chance to relieve symptoms and attain a better quality of life. We herein discuss the palliation of obstructive jaundice by radiofrequency ablation, photodynamic therapy and brachytherapy in advanced extrahepatic cholangiocarcinoma.

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INTRODUCTION

Cholangiocarcinomas (CCAs) have a very high mortality rate worldwide[1,2]. Diagnosis is challenging and delayed in many cases due to the common asymptomatic clinical behavior of early-stage disease, the lack of a standardized screening protocol for early-stage disease and the limitations inherent to using CA19-9 as a cancer marker[3].The ability to achieve a definite cytopathological or histopathological diagnosis in patients with suspected CCA ranges widely in the literature from 26% to 80%[4-8]. Magnetic resonance imaging plus magnetic resonance cholangiopancreatography is the preferred imaging modality as it can assess resectability and tumor extent with a high accuracy[9-15]. Endoscopic ultrasound (EUS) and fine needle aspiration guided by EUS is a useful technique in the diagnosis and staging of CCA (Figure 1 and 2) and should always be taken into consideration for CCA clinical management. For patients with obstructive jaundice, in particular, intraductal ultrasonography has been suggested for the assessment of bile duct strictures and local tumor staging[16] (Figure 3).

CCAs are divided into three types: Intrahepatic CCA, distal CCA (dCCA) and perihilar CCAs (pCCA) or Klatskin tumors. The majority of CCAs are pCCAs (60%-75% of cases). dCCA is present in 15%to 25% of cases, and intrahepatic CCA accounts for 5%to 15% of cases[17-20].

Surgery is the only curative treatment for extrahepatic CCA with the goal of R0 resection. Unfortunately, only a minority of patients (approximately 35%) have early stage disease and are candidates for this curative treatment option[21].Furthermore, only a few patients with pCCA are candidates for liver transplantation following neoadjuvant chemotherapy[22].

More than 50% of patients with jaundice are reportedly inoperable at the time of first diagnosis. Locally advanced, unresectable CCA cases include patients with macroscopic residual disease following resection, locally advanced, categorically unresectable disease at presentation or locally recurrent disease after potentially curative treatment. Prognosis of these patients is poor with a median survival time of < 6 mo[23]. Relief of symptoms (pain, pruritus, jaundice) and improvement in quality of

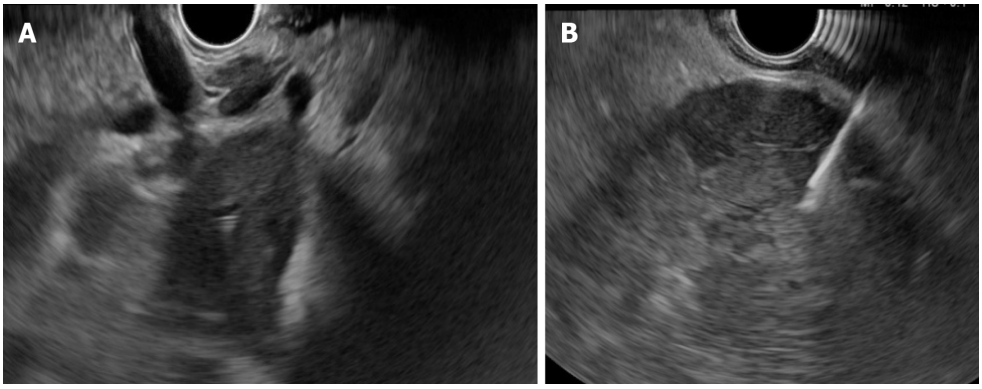

Figure 1 Endoscopic ultrasound for liver evaluation. A: Hilium view of the liver tumoral mass; B: Fine needle aspiration guided endoscopic ultrasound for left lobe intrahepatic cholangiocarcinoma.

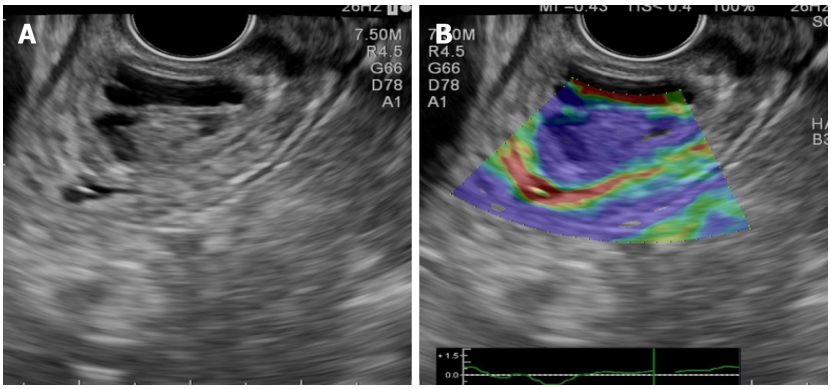

Figure 2 Endoscopic ultrasound of the distal common bile duct. A: Small non-invasive tumoral mass (distal cholangiocarcinoma); B: Elastography: Blue color of the tumor (hard stiffness).

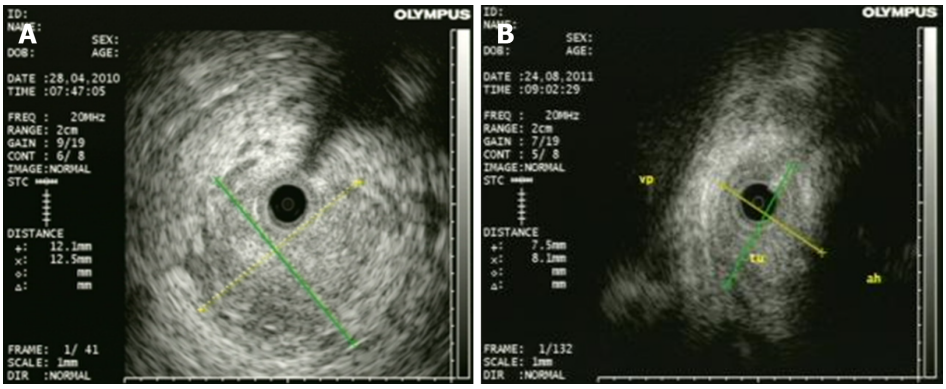

Figure 3 Intraductal ultrasonography for diagnosis of cholangiocarcinoma. A: Large tumoral mass with invasion of surrounding tissue; B: Infiltrative cholangiocarcinoma in proximity of the hepatic artery.

life are the aims of palliative therapy.

Each subtype of CCA has different clinical management[24]. Therefore, an individualized approach is mandatory for pCCA or dCCA. In patients with extrahepatic CCA who are not candidates for surgery or liver transplantation, consideration should be given to enrollment in a clinical trial, particularly those evaluating targeted therapy[25].

Additional treatment measures in locally advanced extrahepatic CCA may include the following: Stenting, radiofrequency ablation (RFA), photodynamic therapy (PDT), radiation therapy, chemotherapy, molecular-targeted therapy and/or immune-therapy[25].

Preoperative or palliative biliary drainage using stents are two main approaches for

extrahepatic CCA[26]. Stents can be placed *via* endoscopic retrograde cholangiopancreatography (ERCP) or percutaneous transhepatic cholangiography to relieve biliary obstruction. Stenting may relieve the jaundice and pruritus and improve the quality of life[26]. In ERCP, a unilateral or bilateral plastic or metallic stent can be used[23,24,26].

RFA and PDT are effective in restoring biliary drainage and improving quality of life in patients with nonresectable disseminated extrahepatic CCA[23,27]. Local radiotherapy combined with metallic stent placement is a new and efficient method in advanced extrahepatic CCA[28]. Several clinical trials are evaluating the effect of specific molecular agents targeting various signaling pathways in advanced extrahepatic CCA[25]. Our proposal is to highlight the utility and the efficiency of different endoscopic techniques and additional measures in extrahepatic CCA.

PALLIATION OF OBSTRUCTIVE JAUNDICE

Endoscopic treatment of CCA with obstructive jaundice ensures bile duct drainage in preoperative or palliative settings[23,26]. Endoscopic procedures are the preferred palliative treatment options for patients with advanced or unresectable CCA. In patients with advanced pCCA, endoscopic biliary drainage *via* ERCP is more difficult than those with dCCA[23,25,26]. If the transpapillary approach failed, then other procedures can be considered: Percutaneous transhepatic biliary drainage (PTBD), endoscopic ultrasound-guided biliary drainage (EUS-BD) or hepatico-gastrostomy or locoregional therapies including transluminal PDT and RFA[27].

Preoperative biliary drainage

There is some controversy in the literature as to how preoperative biliary drainage should be accomplished prior to laparotomy for patients with obstructive jaundice[29-30]. In a European multicenter study, Gouma *et al*[31] showed that the postoperative outcomes in patients with pCCA who underwent surgery and preoperative biliary drainage were not improved. However, the rate of mortality was lower in patients who received en bloc right hepatectomy. In dCCA, preoperative bile duct drainage is not always necessary unless neoadjuvant chemotherapy is planned and might be associated with an increased risk of cholangitis and postoperative infectious complications[32].

Acute cholangitis, sepsis, bilirubin > 10-15 mg/dL, scheduled neoadjuvant therapy and the need for extensive hepatic resection are indications for preoperative biliary drainage. The goal is to reduce peri- and postoperative complications[23,24,26,29]. Cholestasis, liver dysfunction and biliary cirrhosis can develop rapidly with unrelieved obstruction and may influence postoperative morbidity and mortality after surgery[23-26,33]. The definitive operation is deferring until bilirubin levels are less than 2 to 3 mg/dL[33].

Some centers prefer preoperative biliary decompression in order to decrease the total bilirubin level to under 3 mg/dL, whereas others recommend resection in patients without biliary drainage. In our center the decision to perform preoperative biliary drainage is made in the setting of a multidisciplinary team, and it is not generally recommended unless severe liver dysfunction is suspected.

It should be taken into account that the stent may induce different artifacts in subsequent images. Therefore, previous high-quality imaging is required (computed tomography, magnetic resonance imaging, magnetic resonance cholangiopancreatography, endoscopic ultrasound and intraductal ultrasonography) to assess the tumor resectability[23-26,33]. The biliary stent may be a hindrance for the surgeon to find the proximal tumor extent. Resection of pCCA always requires a concomitant major liver resection. Liver segments that will remain after surgery should be drained sufficiently with a plastic stent to improve postoperative liver function and regeneration[29].

There are different data regarding the benefits of preoperative biliary drainage in jaundice patients with pCCA without absolute indications for biliary drainage[29]. The most recent studies concluded that routine biliary drainage does not impart any advantage because it does not improve the morbidity or mortality of patients with resected pCCA[31,34,35]. A recent meta-analysis and a systematic review showed that preoperative biliary drainage have not changed the incidence of postoperative complications, hospitalization time, R0 or survival rate. However, in jaundice patients, preoperative biliary drainage decreased postoperative mortality[36].

In dCCA, a European multicenter study did not find any differences regarding mortality rate in patients with preoperative biliary drainage[37]. Moreover, in a recent

retrospective study, preoperative endoscopic biliary drainage was associated with a decrease in the survival rate[38].

In PTBD, some studies reported that catheter tract recurrence rates were up to 6%[32], and the median time of recurrence was months. Furthermore, the technical success rate regarding the decrease of biliary level is higher with the endoscopic approach than with PTBD[39,40]. In a recent randomized prospective study, the risk of cholangitis in patients who underwent surgery was higher in the PTBD group compared with the endoscopic biliary drainage group (59% *vs* 37%) (P = 0.1)[41].

PTBD is no longer recommended for preoperative biliary drainage in patients with extrahepatic CCA, and an endoscopic approach is currently preferred[24,26]. The risk of endoscopic plastic stent occlusion is 60%. Therefore, there are several groups of experts who recommend preoperative nasobiliary drainage. Kawashima *et al*[42] compared preoperative nasobiliary drainage with endobiliary stenting drainage in 164 patients with pCCA. They found a longer stent patency and a lower risk of cholangitis in the nasobiliary group than the endobiliary stenting group.

Palliative biliary drainage

The relief of symptoms (pain, pruritus, jaundice) and improvement in quality of life are the goals of palliative therapy. Radiotherapy, PDT, RFA, local ablation and embolization are nonsurgical local therapies that can prolong the time to local failure (in patients with macroscopically positive margins) or to palliate local symptoms, pain or jaundice (in patients with unresectable or recurrent disease).

In patients with pCCA and dCCA who are not suitable for surgery or liver transplantation, the guidelines recommend endoscopic bile duct drainage as the first approach[23,24,26,34]. In patients with a good performance status an additional treatment, such as chemotherapy, radiotherapy, molecularly targeted therapy and/or immunotherapy, is recommended[25].

Stenting

Stent implantation by ERCP should be the standard procedure[24-26] (Figure 4 and 5). Placement of a stent is generally preferred for long-term palliation. This approach has similar successful palliation and survival rates and less morbidity compared with the surgical approach[43]. The endoscopic drainage with one or more stents is technically possible in 70% to 100% of cases. The extent of decompression that is necessary to restore sufficient bile flow while avoiding the risk of bacterial cholangitis, the optimal approach to placement of the stents and the use of plastic or metal uncovered/covered stents are the major issues of biliary endoscopic stenting[44].

The goal of palliative drainage is to drain more than one half of the biliary tree, although it has been shown that the jaundice may be clinically improved if only a quarter of the liver is drained[45]. A target stenting using previous superior imaging methods is preferred[44]. In cases of cholangitis, drainage of all suspected infected intrahepatic segmental branches should be performed[24].

In complex and difficult cases a multimodality biliary drainage (transpapillary drainage in combination with PTBD) should be considered[44]. Rendezvous technique, anterograde PTBD and transluminal stenting through the stomach, duodenum or jejunum walls are the procedures using EUS-BD in these cases. This approach can be performed even when a passage of a wire through a biliary stricture is not possible[46]. In a meta-analysis conducted by Leng *et al*[47], the technical success rate of PTBD varied from 60% to 90% and the morbidity rate from 18%to 67%. In some difficult cases, an external drainage has been required. Therefore, the quality of life of these patients is decreased. EUS-BD technical success varied from 70% to 100%, and the rate of complications was up to 77%[48,49]. A few comparative studies are available[50-54] (Table 1). The technical success rates are similar in most studies with a higher incidence of complications for PTBD than EUS-BD[50-54].

Unilateral or bilateral endoscopic stenting

In most cases, unilateral stent placement should be adequate for biliary drainage *via* ERCP because only 25% to 30% of the liver needs to be drained to relieve jaundice[54-56]. However, unilateral drainage alone may not relieve jaundice completely and may increase the risk of cholangitis due to contrast medium injection into undrained bile ducts[45]. Unilateral stenting is technically easier and less expensive than bilateral stenting with reintervention for stent dysfunction also being considerably easier[45]. In our practice, we prefer to place a unilateral self-expandable metallic stent (SEMS) in order to provide good efficacy of biliary drainage with minimum risk of cholangitis. In clinical practice, many endoscopists prefer to place bilateral stents (plastic or metal) in

Table 1 Success rate and complications for percutaneous transhepatic biliary drainage, endoscopic ultrasound-guided biliary drainage, endoscopic retrograde cholangiopancreatography and self-expandable metal stent

Ref.	Procedure	Patients, *n*	Technical success %	Morbidity %
Artifon *et al*[50], 2012	PTBD, EUS-BD	12, 13	100, 100	25.00, 15.30
Bapaye *et al*[51], 2013	PTBD, EUS-BD	26, 25	46.00, 92.00	46.00, 20.00
Khashab *et al*[52], 2015	PTBD, EUS-BD	51, 22	100, 86.40	39.20, 18.20
Dhir *et al*[53], 2015	ERCP SEMS, EUS-BD	104, 104	94.23, 93.26	8.65, 8.65

ERCP: Endoscopic retrograde cholangiopancreatography; EUS-BD: Endoscopic ultrasound-guided biliary drainage; PTBD: Percutaneous transhepatic biliary drainage; SEMS: Self-expandable metal stent.

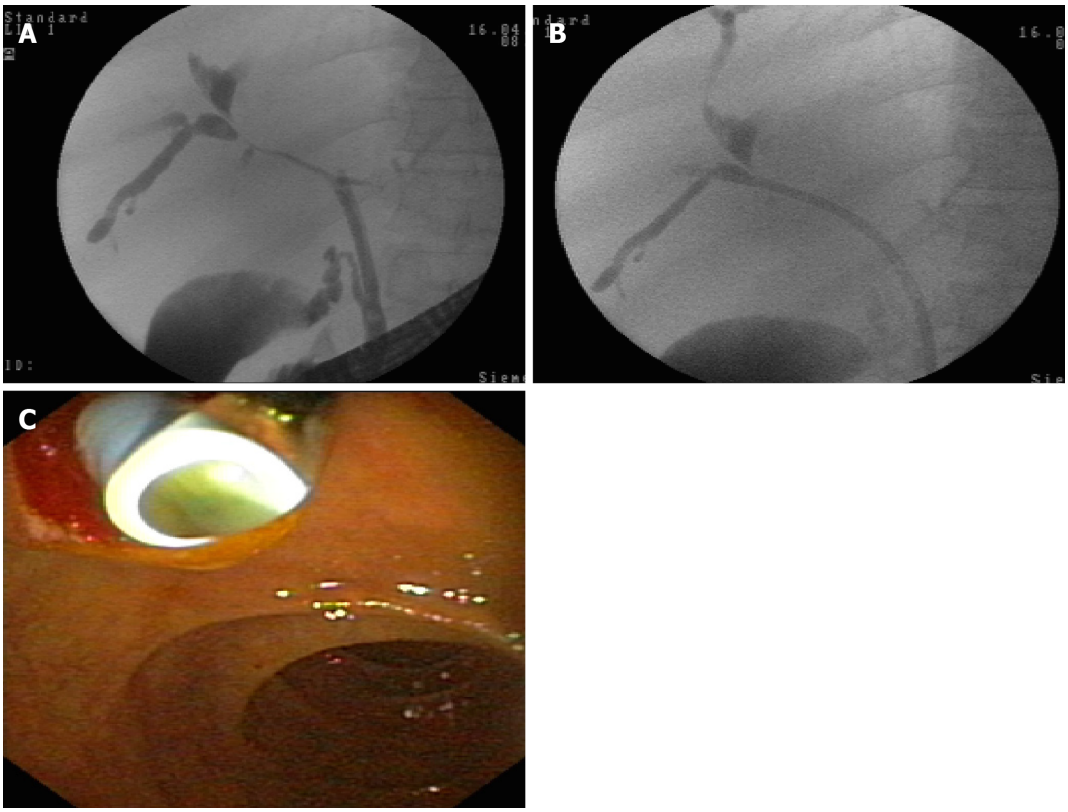

Figure 4 Endoscopic retrograde cholangiopancreatography. Radiologic and endoscopic view. A: Bismuth IV cholangiocarcinoma of the hilum; B: Endoscopic stenting with plastic stent in place; C: Endoscopic view of plastic stent at the level of the papilla.

an attempt to maximize biliary drainage and to prevent cholangitis.

Previous studies have demonstrated that bilateral stenting is associated with longer stent patency compared to unilateral stenting[57,58]. In a recent multicenter prospective randomized study conducted by Lee *et al*[59], the same survival rate in patients with bilateral SEMS biliary drainage but with a longer stent patency *vs* unilateral SEMS biliary stenting were shown. No significant difference between unilateral and bilateral SEMS regarding the technical success or complications was shown[59]. These results highlighted the superiority of bilateral stenting. However, several study results have similarly supported the superiority of unilateral stenting[54-56,60].

In a recent meta-analysis involving 782 patients, bilateral biliary drainage had a lower re-intervention rate compared to unilateral drainage in patients with pCCA with no significant difference in technical success and early or late complication rates[61].

Plastic stents or SEMS

Endoscopic biliary drainage can be performed using plastic or SEMS. There are a variety of plastic and metal stents, covered or uncovered. While some studies showed benefits of metallic stents regarding the successful drainage and early complication

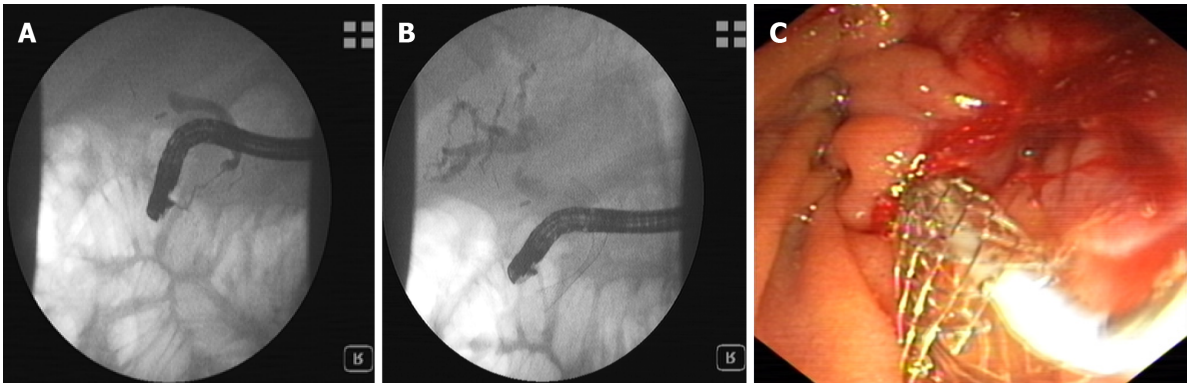

Figure 5 Endoscopic retrograde cholangiopancreatography. Radiologic and endoscopic view. A: Bismuth IV cholangiocarcinoma of the hilum; B: Endoscopic stenting with metallic stent in place; C: Endoscopic view of metallic stent at the level of the papilla.

rate, stent patency and survival rate[55-59,62], a systematic review concluded that neither stent type offered a survival advantage[63]. The decision to use one *vs* another should be guided by the expected length of survival, quality of life, costs and physician expertise. Usually, SEMS should be considered for patients with a life expectancy of longer than 3 mo[44]. The results of different meta-analyses that compared SEMS with plastic stents for endoscopic drainage of distal malignant biliary obstruction are illustrated in Table 2[62-66].

Plastic (polyethylene) stents are inexpensive, effective and easily removable or exchangeable[38-44]. The major disadvantage is a higher rate of occlusion by sludge and/or bacterial biofilm with cholangitis development and necessity of multiple ERCPs[60,62-66]. Instead, metal stents have a longer patency (approximately 8-12 mo *vs* 2-5 mo for plastic stents)[61-66], higher costs and may not be removable. The high occlusion rate of plastic stents (average 42%) can be reduced by changing the stents every 3-6 mo[60,62-66]. Another way is to wait for a complication before changing the stent because many patients will die before the stents will obstruct. The preferred approach for patients who are expected to live beyond a few months is to replace the plastic stent with a metal one as soon as is feasible[44].

In dCCA, uncovered SEMS are used in patients with an intact gallbladder[26]. For patients who have undergone prior cholecystectomy, the choice of a covered *vs* uncovered SEMS is individualized given the location and geometry of the stenosis. Patients with extrinsic compression may be adequately treated with an uncovered SEMS, while those with intrinsic and/or papillary tumors may benefit from a covered SEMS in an attempt to minimize tumor ingrowth[26,67,68]. The patency rates are not higher for covered stents despite showing significantly less tumor ingrowth. Tumor overgrowth and stent obstruction by debris and biliary sludge are associated with a low patency rate for uncovered SEMS[68]. Covered SEMS should be used for pCCA. Deployment may inadvertently result in the occlusion of a major hepatic duct[24,26,44,68].

The stent in stent technique (Y stenting) and the side-by-side technique (Figure 6) are two endoscopic techniques for biliary drainage in CCA. By using the Y stent technique, Hwang *et al*[69] demonstrated an 86.7% technical success rate and a 100% functional success rate regardless of the stent type. For side-by-side stenting technique in pCCA, Lee *et al*[70] reported a 91% technical success rate and a 100% functional success rate with no statistically significant difference between stent patency and median survival of the 8-mm and 10-mm groups.

The reported rate of stent dysfunction following pCCA biliary drainage was 45%-57% due to tumor ingrowth, tumor overgrowth or stent migration[55-58]. Given the fact that SEMS may be successfully revised in the majority of cases and that the second SEMS have a higher patency compared with plastic stents, it seems that SEMS are the best choice in cases of SEMS dysfunction[55-59].

Guidelines recommend prophylactic antibiotics in patients with plastic or metal stents for long-term palliation of obstructive jaundice after the first episode of cholangitis[24,26,44]. In 5%-10% of cases, endoscopic biliary drainage by ERCP will fail or will be incomplete[54-69]. In this case, multimodality drainage should be considered[24,26,44].

Percutaneous vs endoscopic approach

Several studies have shown a higher rate of successful palliation of jaundice and lower

Table 2 Meta-analyses comparing self-expandable metal stents with plastic stents for the endoscopic drainage of distal malignant biliary obstruction

Ref.	Studies included	Patients, *n*	Procedures	Results
Almadi *et al*[62], 2017	20	1713	Endoscopic or percutaneous palliative biliary drainage with plastic stent *vs* SEMS	Stent patency 4.45 mo (95%CI: 0.31-8.59) in favor of SEMS; Overall survival 0.67 (95%CI: 0.66-1.99), no difference
Moole *et al*[33], 2017	11	947	Endoscopic palliative biliary drainage with plastic stent *vs* SEMS	Stent occlusion OR 0.48 (95%CI: 0.34-0.67) in favor of SEMS; Overall survival/time to death: (1) SEMS, 157.3 d (95%CI: 148.9-165.6), (2) Plastic, 120.6 d (95%CI: 114.3-126.9), P = 0.0024
Zorrón Pu *et al*[64], 2015	13	1133	Endoscopic palliative biliary drainage with plastic stent *vs* SEMS	Stent dysfunction, RD -0.26 (95%CI: -0.32 to -0.20) in favor of SEMS; Survival longer in the SEMS group (187 d *vs* 162 d, P < 0.0001)
Sawas *et al*[65], 2015	19	1989	Endoscopic or percutaneous palliative biliary drainage with plastic stent *vs* SEMS	Stent occlusion, HR 0.42 (95%CI: 0.27-0.64) in favor of SEMS; 30-d survival, HR 0.82 (95%CI: 0.45-1.48), no difference
Hong *et al*[66], 2013	10	785	Endoscopic palliative biliary drainage with plastic stent *vs* SEMS	Stent patency, HR 0.37 (95%CI: 0.28-0.48) in favor of SEMS; Survival, HR 0.81 (95%CI: 0.68-0.96) in favor of SEMS

CI: Confidence interval; HR: Hazard ratio; OR: Odds ratio; RCT: Randomized controlled trial; RD: Risk difference; SEMS: Self-expandable metal stent; WMD: Weighted mean difference.

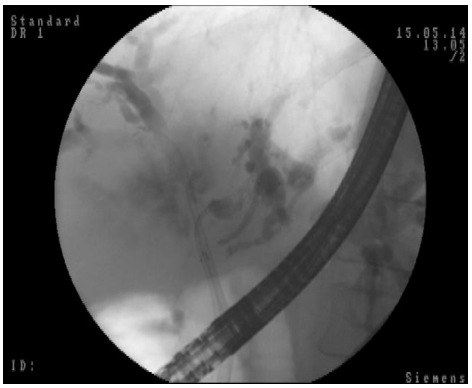

Figure 6 Endoscopic retrograde cholangiopancreatography. Radiologic view. Side-by-side technique (metallic stents in both intrahepatic ducts).

rates of cholangitis in the percutaneous approach rather than the endoscopic approach of biliary drainage in patients with malignant hilar obstruction (pCCA/gallbladder cancer)[71-73]. Bile leaks and bleeding are more frequent and morbidity and mortality are higher than the endoscopic approach[73]. Percutaneous stents are usually left to open drainage externally from the body and are less comfortable for the patient. Another technique is the combination of ERCP with percutaneous drainage.

EUS-BD: EUS-BD has been proposed as an effective alternative for PTBD after failed ERCP[74-80]. The use of EUS-BD is feasible for a left system drainage procedure in patients with advanced CCA who failed transpapillary drainage[74-80]. For extrahepatic CCA, the procedure of choice is EUS-guided hepatico-gastrostomy, which allows left system access only. It is less invasive given that it affords a more accurate control as well as more access sites to the bile duct than the classical alternatives of PTBD or surgery[77]. After the identification of the biliary duct, the technique consists of puncturing and dilatation by EUS with stent placement across the bile duct into the digestive lumen. Literature data showed a 94.0% per-protocol success rate and a 90.2% intention-to-treat basis success rate[75-81].

Peritoneal bile leakage and cholangitis are the most frequent complications[75-81]. Early migration or the clogging of the plastic stents may lead to cholangitis[76]. Bile peritonitis and biloma are more frequent in transmural SEMS placement[77,80]. However, most complications are mild and can be conservatively treated[81]. By combining an uncovered metal stent with a covered metal stent inside, the risk of leakage is minimized. The uncovered stent is initially deployed to provide anchorage and prevent migration. The covered stent is inserted coaxially and dropped in the first

stent. A fully covered SEMS[77] or a double pig-tail stent through the expanded SEMS may be used to prevent stent migration[78].

The advantages of EUS-guided hepatico-gastrostomy over rendezvous or anterograde stent insertion are particularly relevant in patients with prior duodenal or biliary SEMS who experience recurrent biliary obstruction[79,81]. Dhir *et al*[82] compared ERCP-guided biliary drainage with EUS-guided approach in patients with malignant distal obstruction who required SEMS placement. They found that the short-term outcome of EUS-BD is comparable to that of ERCP. Postprocedural pancreatitis rates were higher in the ERCP group[82,83]. Clinical efficacy of a novel technique of EUS-BD for right intrahepatic bile duct obstruction was evaluated[84,85]. Most of the studies have only shown the role of EUS-BD in distal biliary obstruction, and the utility of EUS-BD for pCCA is limited. Recent studies have reported the efficacy of EUS-BD in a setting of failed ERCP for biliary drainage in proximal malignant obstruction[86,87].

Kongkam *et al*[88] proposed a new concept of a combination of ERCP and EUS-BD for biliary drainage in pCCA as a primary biliary drainage method whereby ERCP with a single SEMS is placed into either the right or the left intrahepatic bile duct. In cases of failure of all interventional options, surgical bypass should be considered as the last rescue procedure. It is typically only performed during an unsuccessful attempt at resection, or it may be necessary in jaundice patients in whom stenting is not possible due to tumor location[1,6,7,18].

ROLE OF CHOLANGIOSCOPY

Peroral cholangioscopy (POC) allowing direct visualization of the biliary tract with targeted biopsy of suspicious lesions is a useful diagnostic procedure in the evaluation of biliary strictures (Figure 7). A recent study[89] showed that POC use for the assessment of intraductal spread in potentially resectable pCCA can accurately detect and can change surgical management. In the future, preoperative staging of CCAs should combine radiological with endoscopic (*i.e.* POC evaluation) in order to optimize surgical results.

Another study[90] compared the performance characteristics of single-operator cholangioscopy-guided biopsies and transpapillary biopsies with standard sampling techniques for the detection of CCA. It showed that single-operator cholangioscopy-guided and transpapillary biopsies improved sensitivity for the detection of CCAs in combination with other ERCP-based techniques compared to brush cytology alone. However, it seemed that these modalities did not significantly improve the sensitivity for the detection of malignancy in primary sclerosing cholangitis.

A very recent publication[91] evaluated a newly developed POC classification system by comparing classified lesions with histological and genetic findings. Thirty biopsies were analyzed from 11 patients with biliary tract cancer who underwent POC. An original classification of POC findings was made based on the biliary surface's form (F factor, 4 grades) and vessel structure (V factor, 3 grades). Histological malignancy rate increased with increasing F- and V-factor scores. The system was validated by comparing it to the histological diagnosis and genetic mutation analysis in simultaneously biopsied specimens. F-V classification is the first reported system to quantify and classify biliary tract cancer based on POC findings.

RADIOFREQUENCY ABLATION

Percutaneous image-guided RFA is a potential "new tool" for the endobiliary treatment of pCCA[92]. After selective intrahepatic duct cannulation, the 0.035-inch guidewire is placed across the stricture point. The lesion is identified during cholangiography. After the previous sphincterotomy, the RFA is performed using a specific catheter. It is mandatory for all of tumor area to be caught during the procedure. The coagulated tissue will be removed using a balloon probe, and a stent will be inserted[93]. There are only a few studies regarding the successful therapy with intraductal RFA for pCCA[94,95]. A recent study[95] including 65 patients with unresectable extrahepatic CCA showed that the mean survival time was significantly greater among those who underwent RFA plus stenting compared with stenting alone (13 mo *vs* 8 mo). At 12 mo, the survival rate was 63% in the RFA group compared with 12% in the stenting-only group. Stent patency was also longer in the RFA group (7 mo *vs* 3 mo). The adverse event rate did not differ significantly between groups (6% and 9%).

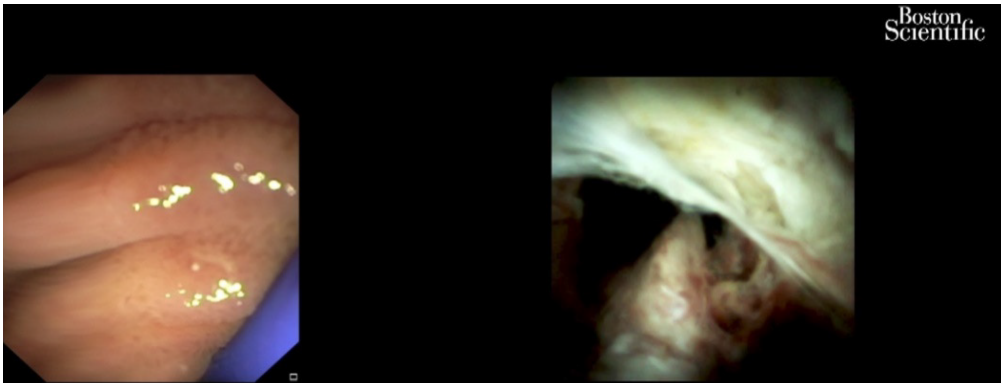

Figure 7 Cholangioscopy: Hilum malignant obstruction.

These results are overlapping with those of a meta-analysis, which was comprised of 505 patients and evaluated the effectiveness of biliary stent placement with RFA on stent patency and patient survival[27]. The pooled weighted mean difference in stent patency was 50.6 d, favoring patients receiving RFA and an improved survival in patients treated with RFA. RFA was associated with a higher risk of postprocedural abdominal pain. There was no significant difference between the RFA and stent placement-only groups with regard to the risk of cholangitis, acute cholecystitis, pancreatitis and hemobilia[27].

A prospective open-label multicenter study included 12 patients with histologically proven endobiliary adenoma remnant (ductal extent < 20 mm) after endoscopic papillectomy for ampullary tumor. RFA was performed during ERCP with biliary ± pancreatic stent placed at the end of the procedure. All underwent one successful intraductal RFA session with biliary stent placement and recovered uneventfully. Five (25%) received a pancreatic stent. The rates of residual neoplasia were 15% and 30% at 6 and 12 mo, respectively. Only two patients (10%) were referred for surgery. Eight patients (40%) experienced at least one adverse event between intraductal RFA and 12 mo of follow-up. No major adverse events occurred. Intraductal RFA of residual endobiliary dysplasia after endoscopic papillectomy can be offered as an alternative to surgery with a 70% chance of dysplasia eradication at 12 mo after a single session and a good safety profile[96].

PHOTODYNAMIC THERAPY

PDT is the use of photosensitizing agents that accumulate into the tumor. The agents are activated by laser light. Free oxygen radicals are released and destroy the neoplastic cells[69]. Apoptotic death of cells is another mechanism produced by PDT with an immunomodulatory effect. Hematoporphyrin derivatives, ∂-aminolevulinic acid and meso-tetra (hydroxyphenyl) chlorin are the photosensitizing agents used for CCA treatment[24,97]. Strong phototoxic skin reactions that can persist for weeks are a disadvantage of the use of photosensitive substances such as photofrin (porfimer sodium). The advantage of the ∂-aminolevulinic acid, which is a second generation photosensitizer, is the lack of prolonged photosensitization and laser light exposure.

The endoscopic PDT technique involves intravenous 48-h administration of the photosensitizing agent prior to the laser light illumination. The specific substance is retained in tumor cells and into the skin longer than 48-72 h like in the normal tissues. With a guidewire and a catheter, the light laser fiber is placed across the tumoral stricture (Figure 8). The power density used is 300-400 mW/cm with a power energy of 180-200 J/cm. The irradiation time is 400-600 s[97]. Due to the fact that light laser fiber is stiff, the breakage may occur in up to one third of the procedures, making the procedure a bit more cumbersome and affecting treatment cost[24,44]. The PDT is only performed in some specialized centers.

In addition to facilitating biliary decompression after stenting in patients with locally advanced disease, survival might be improved in patients who undergo PDT[98-107] (Figure 9). The data showed a survival benefit for this approach with favorable early results including longer survival and quality of life[98,105,106] (Table 3). The survival benefit was related to the prolonged relief of obstruction rather than to a reduction of the tumor. Although the factors that are associated with prolonged

Table 3 Photodynamic therapy in patients with cholangiocarcinoma

Ref.	No. patients	Median survival, d/mo	Adjuvant therapy
Ortner *et al*[106]	PDT 20, Control 19	493 d, 98 d	PDT -, Control -
Zoepf *et al*[103]	PDT 16, Control 16	630 d, 210 d	PDT -, Control -
Dumoulin *et al*[100]	PDT 24, Control 23	9.9 mo, 5.6 mo	PDT -, Control -
Kahaleh *et al*[102]	PDT 19, Control 21	8.0 mo, 5.0 mo	PDT, CTX 11; RTX 9; CTX 11, RTX 10
Witzigman *et al*[99]	PDT 68, Control 56	12.0 mo, 6.4 mo	PDT, CTX 6; RTX 2; CTX 5, RTX 1

CTX: Chemotherapy; PDT: Photodynamic therapy; RTX: Radiation therapy.

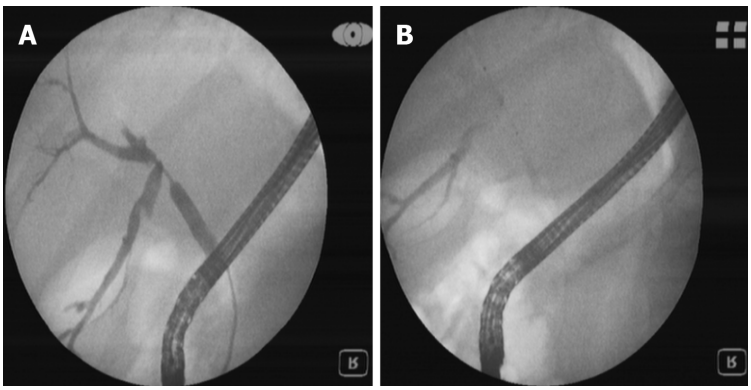

Figure 8 Endoscopic retrograde cholangiopancreatography. Radiologic view. A: Bismuth III cholangiocarcinoma (guidewire is passing through malign stenosis); B: Photodynamic therapy. The laser fiber at the level of stenosis can be seen.

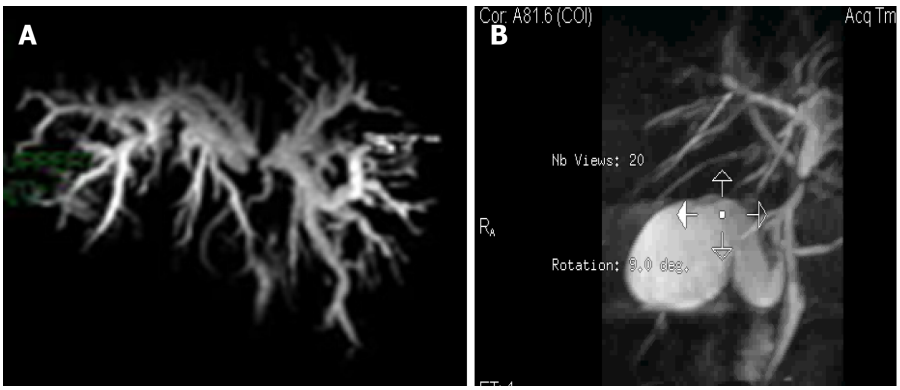

Figure 9 CholangioIRM. A: Before photodynamic therapy: Bismuth III cholangiocarcinoma (large dilatation of intrahepatic ducts can be seen); B: After photodynamic therapy: The stenosis at the level of hilium and intrahepatic dilatation have been reduced.

survival are not completely known, at least some data suggest that the absence of a visible mass on radiographic studies correlates with longer survival after PDT[44,107].

Cholangitis and a liver abscess are the main complications of photodynamic therapy[98-107]. Data suggest that combining photodynamic therapy with systemic combination chemotherapy improved outcomes over PDT alone for patients with nonresectable tumors without increasing toxicity rates, although randomized trials have not been conducted[108-112]. At the moment, PDT is being studied preoperatively as a means of improving the likelihood of achieving a margin-negative resection[113].

In a recent meta-analysis conduct by Lu *et al*[114], overall survival was significantly better in patients who received photodynamic therapy than those who did not. Among the eight trials (642 subjects), five assessed the changes of serum bilirubin levels and/or Karnofsky performance status as other indications for improvement. The incidence of phototoxic reaction was 11.11%. The incidence for other events in photodynamic therapy and the stent-only group was 13.64% and 12.79%, respectively.

A new model of a photosensitizer-embedded self-expanding metal stent (PDT-stent) that provides a photodynamic effect without a systemic injection has been developed. The treatment could be repeated due to the incorporation of the polymeric photosensitizer into the mesh of the stent. The stent maintained its photodynamic power for at least 8 wk. This type of stent after light exposure creates cytotoxic free radical, such as singlet oxygen, in the surrounding tissue and induces destruction of tumoral cells on animal models[115]. Unfortunately, PDT is not widely available and is expensive and uncomfortable for the patient.

BRACHYTHERAPY

The purpose of brachytherapy (BT) is to deliver a high local dose of radiation to the tumoral tissue while sparing healthy tissue around it. It can be adapted for right and left hepatic duct and for common bile duct lesions. It plays a limited but specific role in the curative intent treatment in selected cases of early disease as well as in postoperative small residual tumoral tissue. The indications for BT are as radical or palliative treatment. For radical treatment, it is recommended in small inoperable tumors or in combination with external beam radiation therapy and/or chemotherapy in advanced disease for unresectable tumors. BT may be used as adjuvant treatment after nonradical excision, possibly combined with external beam radiation therapy. The most common indication for BT occurs as palliative in unresectable Klatskin tumors. The purpose is to prevent locoregional disease progression and to facilitate the bile outflow. The major aim is to improve the quality of life and to increase survival. The treatment decision should be personalized[116].

ERCP-directed tumor therapy using iridium-192 ribbons *via* nasobiliary catheters in patients with pCCA as part of a neo-adjuvant treatment protocol that include external beam radiation therapy, radiation-sensitizing chemotherapy and low-dose-rate BT (< 3000 cGy) followed by liver transplant was first described in 2006[117]. High-dose-rate (HDR)-BT using 930-1600 cGy fractionated in 1-4 doses over 1-2 d was introduced in 2009[117]. The benefits of this technique are lack of irradiation of medical staff, lower time span (5-10 min), a better distribution of doses in the tumor and protection of the stomach and duodenum[117]. Using ERCP, an 8.5 Fr or 10 Fr nasobiliary tube is placed into the biliary system with the proximal end of tube at least 2 cm beyond the proximal end of the tumor. In cases of bilateral duct involvement, a second 10 Fr tube is placed. After HDR-BT is completed, the tubes and brachycatheter are removed. Nasobiliary BT catheter displacement, cholangitis, abdominal pain, duodenopathy and gastropathy are possible complications[118,119].

Some studies demonstrated longer survival in patients with CCA due to the BT. Extrahepatic localization of CCA, the absence of metastases, increasing calendar year of treatment and liver transplantation with postoperative radiation therapy were factors significantly associated with improved survival[118,119]. However, another study did not find any benefit regarding the survival in patients treated with PTBD-guided iridium-192, intraluminal BT compared with patients with only PTBD[120]. These results are in accordance with another study that found a correlation only with local tumor control[121].

In a recent study[122], 122 patients with CCA were successfully treated with HDR-BT using the nasobiliary technique. The BT was not completed in three patients because either the catheter migrated between the ERCP and the treatment (two patients) or the HDR after loader was physically unable to extend the source wire into the treatment site (one patient). These three patients benefited from an external beam boost instead of HDR-BT. Intraluminal HDR-BT with a nasobiliary catheter is a minimally invasive method for administering neoadjuvant radiotherapy.

PALLIATIVE AND ADJUVANT CHEMOTHERAPY

The assessment of patients with CCA before starting chemotherapy includes the Eastern Cooperative Oncology Group patient scale used for the evaluation of the patient performance status, disease distribution and accessibility of tumor profiling[123]. The current data support the use of first-line cisplatin and gemcitabine combination regimen chemotherapy. The multicenter phase III ABC-02 study illustrated the superiority of the combination regimen regarding median overall survival (11.7 mo) over the gemcitabine monotherapy (8.1 mo)[124,125].

New combinations and more intensive triple chemotherapy are being explored. The

combinations include: Cisplatin-gemcitabine combined with nab (nanoparticle albumin-bound)-paclitaxel[126]; S1 (tegafur, gimeracil and oteracil)[127]; and FOLFIRI-NOX (5-FU, oxaliplatin and irinotecan; AMEBICA study , NCT02591030). Acelarin is a nucleotide-analogue independent of hENT2 (also known as SLC29A2) cellular transport and is not metabolized by cytidine deaminase, resulting in greater intracellular concentrations. Cisplatin with acelarin was compared with the classic combination regimen of cisplatin and gemcitabine in a phase III study[128].

A recent phase III clinical trial ABC-06[129] randomly assigned 162 patients with advanced biliary cancer (72% with CCA) who obtained symptom control from first-line cisplatin-gemcitabine (81 patients) or second-line chemotherapy with FOLFOX (folinic acid, 5-FU and oxaliplatin) (81 patients). The results showed a benefit from second-line chemotherapy regarding survival at 6 mo (35.5% *vs* 50.6%) and 12 mo (11.4% *vs* 25.9%), but no significant differences regarding overall survival (5.3 mo *vs* 6.2 mo) were observed.

A very difficult to handle and a major issue in the management of patients with CCA is the poor response to pharmacological treatment. A cause could be the poor understanding of the mechanisms of chemoresistance. To identify the so-called "resistome" that includes a set of proteins involved in the lack of response to chemotherapies is required to increase efficacy. Genes involved in mechanisms of chemoresistance are usually expressed by normal cholangiocytes because one of their roles is the protection against potentially harmful compounds present in bile. Their expression during carcinogenesis contributes to intrinsic chemoresistance, and upregulation in response to treatment leads to acquired chemoresistance[130-132].

MOLECULAR TARGETED THERAPY

Recent molecular studies have increased the understanding of the pathogenetic mechanism of CCAs, but to date the clinical data on immune-directed therapies in CCA are limited.

Inhibitors of isocitrate dehydrogenase (IDH) 1, IDH2 and pan-IDH1-IDH2 are currently being tested in patients with intrahepatic CCA. Ivosidenib (IDH1 inhibitor) was tested in 73 patients with IDH1-mutant advanced CCA in a phase I study with no major adverse events reported[133]. A recent preliminary phase III trial showed a benefit for ivosidenib over placebo in terms of progression free-survival. One hundred eighty-five patients with IDH1 mutant CCA were randomly assigned to ivosidenib or placebo. This study highlighted the importance of molecular profiling in CCA[134] .

There are some phase II studies with encouraging preliminary data for fibroblast growth factor receptor inhibitors in patients with CCA. Some fibroblast growth factor receptors inhibitors are currently being evaluated as first-line treatment, for example the FIGHT-302 study (NCT03656536) and the PROOF study (NCT03773302)[135-137].

CONCLUSION

CCAs are heterogeneous and highly aggressive tumors with a poor prognosis despite the progress of the research in this field. Surgical resection is still the only potential curative treatment method. The recent findings on understanding the mechanism of chemoresistance and molecular targeted therapy could bring a new horizon in the approach of these tumors. Currently, endoscopic treatment in patients with CCA and jaundice remain the first choice of biliary duct decompression, either preoperatively or with a palliative purpose. The combination of endoscopic procedures with nonsurgical local methods or additional therapies may increase the quality of life and the rate of survival in patients with locally advanced, unresectable or recurrent disease.

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ORIGINAL ARTICLE

Basic Study

Adult human liver slice cultures: Modelling of liver fibrosis and evaluation of new anti-fibrotic drugs

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Abstract

BACKGROUND

Liver fibrosis can result in end-stage liver failure and death.

AIM

To examine human liver fibrogenesis and anti-fibrotic therapies, we evaluated the three dimensional *ex vivo* liver slice (LS) model.

METHODS

Fibrotic liver samples (F0 to F4 fibrosis stage according to the METAVIR score) were collected from patients after liver resection. Human liver slices (HLS) were cultivated for up to 21 days. Hepatitis C virus (HCV) infection, alcohol (ethanol stimulation) and steatosis (palmitate stimulation) were examined in fibrotic (F2 to

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F4) liver slices infected (or not) with HCV. F0-F1 HLS were used as controls. At day 0, either ursodeoxycholic acid (choleretic and hepatoprotective properties) and/or α-tocopherol (antioxidant properties) were added to standard of care on HLS and fibrotic liver slices, infected (or not) with HCV. Expression of the biomarkers of fibrosis and the triglyceride production were checked by quantitative reverse transcription polymerase chain reaction and/or enzyme-linked immunosorbent assay.

RESULTS

The cultures were viable *in vitro* for 21 days allowing to study fibrosis inducers and to estimate the effect of anti-fibrotic drugs. Expression of the biomarkers of fibrosis and the progression to steatosis (estimated by triglycerides production) was increased with the addition of HCV and /or ethanol or palmitate. From day 15 of the follow-up studies, a significant decrease of both transforming growth factor β-1 and Procol1A1 expression and triglycerides production was observed when a combined anti-fibrotic treatment was applied on HCV infected F2-F4 LS cultures.

CONCLUSION

These results show that the human three dimensional *ex vivo* model effectively reflects the *in vivo* processes in damaged human liver (viral, alcoholic, nonalcoholic steatohepatitis liver diseases) and provides the proof of concept that the LS examined model permits a rapid evaluation of new anti-fibrotic therapies when used alone or in combination.

Key Words: Human liver fibrosis; Hepatitis C virus; Alcoholic liver disease; Nonalcoholic steatohepatitis; *Ex vivo* model; Drugs

Core Tip: In the developed world, about 45% of deaths are due to fibroproliferative diseases. Liver fibrosis is frequently associated with viral infection (Hepatitis C virus and Hepatitis B virus infection), chronic inflammation and excessive alcohol consumption. Despite the availability of effective antiviral drugs, morbidity, and mortality related to viral hepatitis are still increasing. Moreover, the number of non-viral liver diseases such as nonalcoholic steatohepatitis, and alcoholic liver disease is steadily growing. Our studies provide the proof of concept that the three-dimensional *ex vivo* model of human liver slice culture can be used for the molecular investigation of fibrosis as well as to perform follow-up studies of new anti-fibrotic drugs and therapies for a 21-days period.

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INTRODUCTION

Forty five percent of deaths in the developed countries may be attributed to fibroproliferative diseases[1]. Liver fibrosis is frequently associated with viral infection [Hepatitis C virus (HCV) and Hepatitis B virus (HBV)] infection, chronic inflammation, and excessive alcohol consumption. Despite effective antiviral treatment, morbidity and hepatitis-related mortalities are still increasing. Moreover, the number of non-viral liver diseases such as nonalcoholic steatohepatitis (NASH) and alcoholic liver disease (ALD) is steadily growing[2].

Progression to liver fibrosis is a multistep process, whose development time varies. Fibrosis is initiated by the activation of hepatic stellate cells triggered by several signaling pathways[3]. The activation of stellate cells induces cellular matrix production

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and collagen 1 expression This process is stimulated by transforming growth factor-β1 (TGF-β1), which is a crucial element involved in fibrogenesis[4]. The progression of liver fibrosis frequently results in cirrhosis (liver acini are substituted by regeneration nodules surrounded by fibrosis) and, further on, in the development of hepatocellular carcinoma. Liver fibrosis can persist even with effective treatments. In most cases, the necro-inflammation leading to fibrosis can be effectively treated by treatments with antiviral drugs that target HCV, by nucleoside analogs in patients with HBV, by immune suppression in autoimmune hepatitis, by ethanol weaning and other dietary approaches in ALD and NASH, and iron chelation for hemochromatosis. However, if patients are not treated in a timely manner, and fibrosis progresses to decompensated cirrhosis, the only remaining option is liver transplantation The main obstacles (or delays) to liver transplantation are an insufficient number or a shortage of suitable organs, long waiting lists and high cost of this procedure[5]. Thus, mortality remains high in patients on the waiting list and new anti-fibrotic agents and new clinical strategies to manage patients in the different stages of liver fibrosis are needed.

The liver slices (LS) cultures are appropriate models to study liver fibrosis, because they maintain the complex cellular interactions that occur *in vivo*, which cannot be obtained in co-cultures systems[6]. These cultures can be used to study molecular biological events either in the fibrotic liver tissue or in hepatocellular carcinoma tissue. Although the LS cultures from non-fibrotic and fibrotic rat livers have been used to investigate the early and late phases as well as the resolution of liver fibrosis[7,8], the experiments are limited to 3 days in the rat model[7-9], and to 15 days in the human non-fibrotic LS model[10]. In previous studies, we developed a three dimensional (3D) *ex vivo* model of HCV replication using human LS cultures that were followed for 10 days[11] to evaluate a new antiviral drug[12].

Here, for the first time, human fibrotic LS cultures (stages F2-F4) were successfully maintained and evaluated for 21 days. Using the *ex vivo* LS model for a 21-d period makes it possible to explore molecular fibrogenesis in more detail including the role of important factors such as HCV infection, ethanol (EtOH), or steatosis. Thus, this model can improve the understanding of the three of the main causes of liver injury in clinical practice[2]. In addition, it was demonstrated that LS cultures are efficient instruments to study anti-fibrotic drugs and their combination[13,14].

This study provides the proof of concept that the *ex vivo* model of human LS culture can be used for the molecular evaluation of fibrosis and to perform follow-up studies of new anti-fibrotic drugs and therapies for a 21-days period.

MATERIALS AND METHODS

Patients and human liver tissue specimens

Adult human liver tissue samples were obtained from selected patients with different liver pathologies, as previously described[11,12]. Written informed consent was obtained from each patient included in the study and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee. Experimental procedures were carried out in accordance with French laws and Regulations and ethic committees from Pitié-Salpétrière Hospital, Cochin Hospital, and Pasteur Institute (France). The tissue samples from twenty patients were divided into three groups according to their METAVIR score[15]. Liver samples were either non-fibrotic F0-F1, obtained during surgery for colorectal cancer liver metastases or fibrotic ranging from F2 to F4 according to the METAVIR score (Table 1). Significant necrotic inflammation as defined by an activity grade (A) was not always available.

Liver slices preparation, culture and infection

We obtained between 32 to 48 liver slices for each donor sample. On the different days of the kinetic experiments, the results were obtained from the mean of three liver slices from each donor. The liver slices were infected with a same viral stock. The liver slice cultures were inoculated with viral supernatant diluted in fresh medium, at MOI = 0.1 (multiplicity of infection) and incubated overnight at 37 °C. In order to remove free virus, the slices were washed three times with phosphate-buffered saline (PBS) and fresh complete culture medium was added, after which cultures were followed in the absence of additional changes to the media composition or replacement with fresh culture medium. The preparation and culture of the liver slices, HCV RNA transfection, virus production, HCV RNA extraction were performed as previously described[11,12].

Table 1 METAVIR scores and description of clinical liver samples

METAVIR score		Patients (*n*)	Pathology
F0-F1	No fibrosis or mild fibrosis	10	HBV-, HCV-, HIV-seronegative patients who underwent liver resection surgery, mainly for liver metastasis, in the absence of underlying liver disease. A0-F1
F0-F1	No fibrosis or mild fibrosis	1	Prior history: Breast cancer with liver metastases, treated by surgery and radio-chemotherapy. Non-tumoral liver sample: Perisinusoidal and portal fibrosis without septa (F1). No steatosis
		2	Prior history: HCV infection, resected hepato-cholangiocarcinoma. Non tumoral liver samples: A0F0
F2-F3	Moderate to severe fibrosis	2	Cholangiocarcinoma, non-tumor liver samples
		1	Chronic hepatitis B infection, NASH, and two resected hepatocellular carcinoma nodules. Non-tumoral liver sample: Chronic hepatitis with extensive fibrosis A1F3
F4	Cirrhosis	2	HCC, non-tumor liver samples
		1	HCC, treated HCV infection. Non-tumoral liver sample
		1	HCC on untreated HCV infection. Non-tumoral liver sample

A "significant" fibrosis, as defined by a fibrosis grade (F), is greater than 1 by the METAVIR scoring system, with usually a significant necrotic inflammation as defined by an activity grade (A) greater than 1 by the METAVIR scoring system. Fibrosis grade: F0: No fibrosis, F1: Portal fibrosis without septa, F2: Portal fibrosis with few septa, F3: Numerous septa without cirrhosis, F4: Cirrhosis. Activity grade: A0: No activity, A1: Mild activity, A2: Moderate activity, A3: Severe activity. HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; HIV: Human immunodeficiency virus; NASH: Nonalcoholic steatohepatitis.

Liver slices viability

Special attention was paid to the condition of clinical liver samples. It is evident that the condition of the liver sections that we obtained was different. Thus, they were carefully selected for *in vitro* studies. In fact, cell viability was estimated by determining the percentage of viable cells upon microscopic examination 10X, using live/dead fixable dead cell stain kit (Molecular Probes, Invitrogen, ThermoFisher, France) and, as the percentage of ATP production determined by enzyme-linked immunosorbent assay (ELISA) assays, while observing the increasing albumin and urea secretion levels throughout the experiments, which indicates that the physiological and biochemical parameters of the liver slices are normal. On day 15, the immunostaining for Ki67, a cellular marker for proliferation confirmed the cell viability. Only slices with viability greater than 80% were used and allowed to obtain all the presented results. The architecture of human LS cultures was accessed by hematoxylin-eosin (HE) staining performed as following: Cryosections were washed with distilled water for 5-10 min and then stained for 8 min with hematoxylin, followed by a washing step with warm water at 30 °C for 10 min. After a short washing step with distilled water, the slices were counter-stained for 6 min with eosin. Washing was followed by dehydration steps in 2 min intervals in 50%, 60%, 70%, 80% and 90% of ethanol.

Experimental set up

The experimental set up was as follows (Figure 1). Non-infected liver slices obtained either from human non-fibrotic (F0-F1) or fibrotic (F2-F3, F4) liver resection and cut in 350 µm-thick slices (approximately 2.7×10^6 cells per slice), were cultivated for up to 21 days either with or without HCV, ethanol (EtOH) (1 mmol/L, 5 mmol/L, 25 mmol/L) or palmitate (500 µmol/L). Liver slices were infected with hepatitis C virus infection from cell culture (HCVcc) supernatant [Con1/C3 (genotype1b)][16] (MOI = 0.1) (INF LS) in presence or not either of EtOH (1 mmol/L, 5 mmol/L, 25 mmol/L) or palmitate (500 µmol/L). The different concentrations of EtOH were added on days 0, 5, 10, 15 during the kinetic studies. Palmitate (500 µmol/L) was added or not to non-infected and infected liver slices on days 0, 5, 10 and 15 of the kinetic studies. As previously described, infectivity (ffu/mL) was measured on days 1, 5, 10, and 21 post-treatment depending on the experiment[11,12]. All experiments were performed in triplicate. All data were presented in relation to the percentage of viable liver slices in culture. Once the model was validated for the presence of "molecular fibrogenesis" defined as a significant increase in fibrosis biomarkers [TGF-β1, Hsp47, α-SMA, Procol1A1, matrix

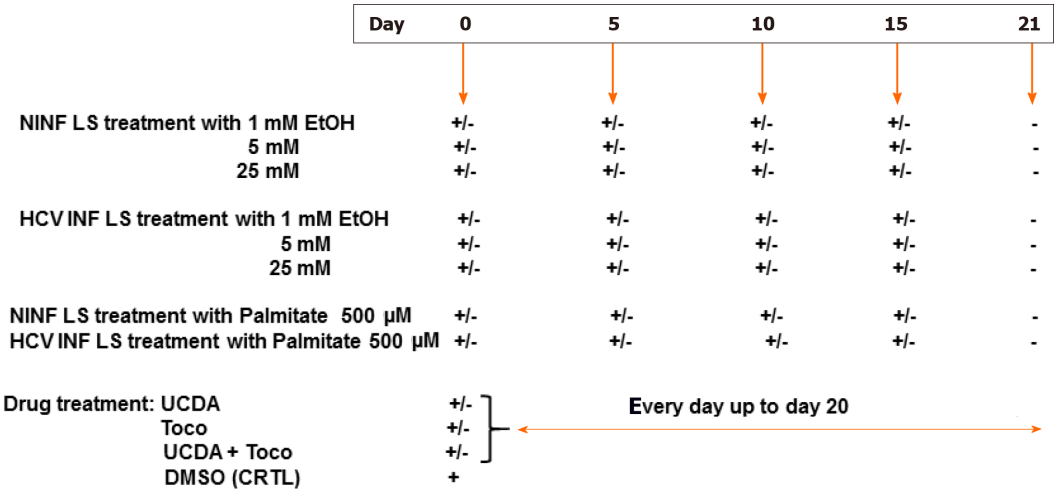


Figure 1 Experimental set up of the different liver slice treatments during the cultures. NINFLS: Non-infected liver slices; EtOH: Ethanol; LS: Liver slices; Toco: Tocopherol; UCDA: Ursodeoxycholic acid; HCV: Hepatitis C virus.

metalloproteinases 2 (MMP-2), MMP-9, and vascular endothelial growth factor (VEGF)], we evaluated the anti-fibrotic properties of two drugs, ursodeoxycholic acid (UCDA) (Sigma-Aldrich, Merck, Germany) and α-Tocopherol (Toco) (Sigma-Aldrich, Merck, Germany). UCDA (240 ng/liver slice) and / or α-Toco (170 ng/liver slice) were added to the culture media from day 0 and every day up to day 20 of the culture. The estimation of the triglyceride content was essential during the different kinetic experiments, since its accumulation in the cytoplasm of hepatocytes indicates cell metabolism disturbances, typical of non-alcoholic fatty liver disease[17].

Quantification of HCV RNA and liver-specific and fibrosis markers genes expression by real-time reverse transcription-quantitative polymerase chain reaction

The liver slices were washed three times in PBS at 4 °C. RNA was extracted from three combined slices using Trizol reagent as described in the protocol (Invitrogen, Cergy Pontoise, France). A strand-specific real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR) technique to quantify the intracellular levels of positive and negative-strand HCV RNA was performed during the experiments with the quantification of 28S rRNA used as an internal standard to quantify HCV in total liver RNA as previously described[11], (detection threshold: 25 copies/reaction). Briefly, reverse transcription was performed using an oligo primer and Moloney murine leukemia virus reverse transcriptase (Promega, Charbonnières, France) according to the manufacturer's instructions. Real-time polymerase chain reactions were performed using Light CylclerR (Roche Applied Science, Grenoble, France) and FastStart DNA Master SYBR Green I kit (Roche Applied Science, Grenoble, France) according to the manufacturer's instructions.

The relative expression of each liver-specific transcript (albumin, HNF-1β, HNF-4α transcription factors, cytochrome P450 enzymes, CYP2E1 and CYP3A4) was quantified by qRT-PCR and normalized to 18S RNA transcripts[11,12]. The relative expression level of the transcripts was then determined in relation to the 18S RNA by the (Ct) method[13]. The PCR conditions were as follows: Denaturation for 10 min at 95 °C, followed by 45 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 20 s, and elongation at 72 °C for 30 s. The specificity of the PCR products was checked by a melting curve analysis after amplification. Primer sequences are listed in Table 2.

The expression of fibrosis markers in either non-infected liver slices (used as controls, CRTL) or in HCV-infected (INF) liver slices with or without the presence of EtOH or palmitate were evaluated by RT-qPCR with the SYBR PrimeScript RT-qPCR Kit (TaKaRa Bio Inc., Japan) and performed with the housekeeping gene, GAPDH as an internal control. Real-Time qPCR reaction for fibrosis markers including TGF-β1, heat shock protein 47 (Hsp47), alpha smooth muscle actin (β-SMA), procollagen1 A1 (Procol1A1), and VEGF was performed as follows: Denaturation for 10 min at 95 °C followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min, and elongation at 72 °C for 30 s. Concerning the MMP-2, MMP-9 gene expression, the Real-Time qPCR reaction was performed as follows: Denaturation for 10 min at 95 °C followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C and 68 °C

Table 2 Primers used for real-time reverse transcription-quantitative polymerase chain reaction analysis

Gene	Forward primer sequence	Reverse primer sequence
CYP2E1	AGCACAAACTCTGAGATATGG	ATAGTCACTGTACTTGAACT
CYP3A4	GCCTGGTGCTCCTCTATCTA	ACAGGCTGTTGACCATCATAAAAG
HNF-1β	ACGTCAGAAAGCAACGAGAGATC	CCCAGGCCCATGGCT
HNF-4α	CCTGGAATTTGAGAATGTGCAG	AGGTTGGTGCCTTCTGATGG
Albumin	ATGAGATGCCTGCTGACTTG	GCACGACAGAGTAATCAGGA
18S RNA	CAGAGCGAAAGCATTTGCCAAG	CGGCATCGTTTATGGTCGGAAC
TGF-β1	CCTGGAAAGGGCTCAACAC	CAGTTCTTCTCTGTGGAGCTGA
HSP 47	GCCACCGTGGTGCCGCA	GCCAGGGCCGCCTCCAGGAG
β-SMA	AGGGGGTGATGGGTGGGAA	ATGATGCCATGTTCTATCGG
Procol1A1	CAATCACCTGCGTACAGAACGCC	CGGCAGGGCTCGGGTTTC
MMP-2	CTT CGCCCC AGG CAC TGG TG	CCTCGCTCCCATGGG GTT CGGT
MMP-9	GGT CCCCCCACT GCT GGC CCTTCTACGGCC	GTCCTCAGG GCACTG GAG GAT GTC ATA GCT
VEGF	TACCTCCACCATGCCAAGTG	ATGATTCTGCCCTCCTCCTTC
GAPDH	ACCAGGGCTGCTTTTAACTCT	GGTGCCATGGAATTTGCC

respectively, for 1 min, and elongation at 72 °C for 30 s. Ct (threshold cycle) values were corrected for the Ct values of the housekeeping gene GAPDH. Primer sequences are listed in Table 2.

Albumin enzyme-linked immunosorbent assay: Human liver albumin concentrations were determined by a competitive ELISA as previously described[18,19]. Purified human albumin and peroxidase-conjugated anti-human albumin were obtained from MP Biomedicals Europe (Illkirch, France). To ensure the specificity of the ELISA, human antibodies were incubated for 2 h at 37 °C with 3% BSA in 0.5% Tween-20 in PBS before the sample addition in order to block any cross reaction.

Urea assays: Urea concentrations were determined by colorimetric assay (640-1, Sigma-Aldrich) according to the manufacturer's recommendations and analyzed with BioPhotometer 6131 (Eppendorf, Hamburg, Germany).

Western blotting and antibodies

Western blotting was performed as previously described[11,12], and the antibodies used are described as following. Mouse monoclonal antibodies (mAbs) to HCV core protein (C7-50; dilution 1/10000, Affinity BioReagents, Golden, CO, United States), HCV nonstructural protein 3 (clone1847, dilution: 1/2000, Viro-Stat, Portland, ME, United States) were used to analyze HCV expression, mouse monoclonal antibodies (mAbs) TGF-β1 (ab 190503, dilution: 1/2000, Abcam, United Kingdom), HSP-47 (M16.10A1, dilution: 1/1000, Enzo life sciences, France), Collagen I alpha 1 (NB600-450, dilution: 1/2000, Novus Biologicals, CO, United States), MMP-9 (ab119906, dilution: 1/2000, Abcam, United Kingdom), VEGF (ab69479, dilution: 1/2000, Abcam, United Kingdom), β-actin (A5316, dilution: 1/5000 Sigma-Aldrich, Merck, Germany), and rabbit polyclonal antibodies (rAbs) to MMP-2 (ab92536, dilution: 1/1000, Abcam, United Kingdom), alpha-smooth muscle actin [alpha-SMA (ab 5694, dilution: 1/2000, Abcam, United Kingdom)] allowing fibrosis analysis, were used as primary antibodies. Horseradish peroxidase-conjugated anti-mouse IgG and horseradish peroxidase-conjugated anti-rabbit IgG (Amersham, GeHealthCare Life Sciences, United Kingdom) secondary antibodies, taken 1:50000, were used as secondary antibodies. The reactions were developed using enhanced chemiluminescence detection reagents (ECL Advance kit, Amersham, GeHealthCare Life Sciences, United Kingdom), followed by exposure to X-OMAT film (Amersham, GeHealthCare Life Sciences, United Kingdom).

Histology and immunohistochemistry

Liver sections (7 μm) were stained with Goldner's trichrome (Electron Microscopy Sciences, United States) or picrosirius red (Abcam, United Kingdom), performed

standard protocols for collagen/connective tissue labelling using two slices per human liver sample and two different human liver samples per group. The images were taken with the EVOS XL Core Imaging System (Invitrogen, Thermo Fisher Scientific, France). The average integrated optical density (OD) of collagen deposition was calculated using the image quantification standard software, ImageJ2[20,21] or inform V2.1 (Perkin Elmer, MA, United States) used routinely in the histology (HISTIM) facilities (Cochin Institute, Paris, France). Immunostaining for TGF-β1 (mAbs, ab92486, Abcam, United Kingdom), MMP-9 (mAbs, ab119906, Abcam, United Kingdom), Ki67 (rAbs, ab15580, Abcam, United Kingdom), and alpha-SMA (rAbs, ab5694, Abcam, United Kingdom) was performed after paraffin removal in xylene, rehydratation in EtOH and then distilled water following the manufacturer's instructions. Unmasking of the antigenic sites was performed at 120 °C in 10 mmol/L citrate buffer, pH 6.0. A solution of 3% H_2O_2 was used to eliminate endogenous peroxidases. The sections were washed 3 times for 5 min. in TBS-Triton 0.1% solution. After incubation in a blocking solution (TBS-Triton 0.1%-3% dry milk) for 1 h at room temperature, they were incubated with the primary antibodies. All primary antibodies were diluted at 1/50 in the blocking solution. After incubation for 2 h at room temperature, the sections were washed 3 times and incubated with secondary antibodies. The nuclei were stained with DAPI. All sections were counterstained with hematoxylin for tissue quality control. Control sections incubated with non-immune serum were used as negative controls.

TGF-β1 and Triglyceride quantification

TGF-β1 and triglyceride quantification were performed according to the manufacturer's instructions (TGF-β1 Quantikine ELISA, RD Systems, United States; Triglyceride assays Kit–Quantification, ab65336, Abcam, United Kingdom). For TGF-β1, cellular lysates and culture supernatants were first treated with acid to lower the pH to 2.0, which denatures the latency-associated peptide and allows the detection of active TGF-β1. The supernatant was then brought back to neutral pH before the ELISA assays.

ATP production quantification and LDH assays

To check viability, the percentages of ATP was assessed at each point of the kinetics studies during the liver slices culture and determined by ELISA assays (CellTiter-Glo® 2.0 Assay, Promega, France)[19]. The viability of liver slices and the potential cytotoxicity[20] (cytoTox 96R Non-Radioactive Cytotoxicity Assay, Promega, France) induced by Ethanol, or Palmitate, or drugs treatments was estimated as described previously[11,12], in accordance with the manufacturers' protocols.

Drugs inhibition of fibrosis markers expression and cytotoxicity assays

Human LS were infected or not with the HCVcc Con1/C3 supernatant as previously described[11,12]. On day 0 of the culture, treatment either with (240 ng/Liver slice) UCDA or (170 ng/Liver slice) Toco or both (the recommended standard of care) or 0.5% of dimethyl sulfoxide (Sigma Aldrich, Merck, Germany) as a control, were added to HCV-infected or non-infected LS culture medium every day to day 20. TGF-β1 and Procol1A1 RNA expression were measured at different time points of the kinetic studies. All experiments were performed in triplicate.

Statistical analysis

Liver specimens from 20 individuals were examined. During the kinetic studies, the quantification of gene expression was determined in relation to the percentage of liver slice viability. The results were obtained from the mean of the three liver slices, on the different days of the kinetic studies. Statistical tests were performed using GraphPad Prism 8.0 software (GraphPad Software, La Jolla, CA, United States). Values are expressed as means ± standard errors of the mean. The data were compared using either the unpaired two-tailed Student's *t*-test or the two-way ANOVA test with multiple comparisons for a given day as compared to the standard LS. A *P* value of 0.05 or less was considered significant.

RESULTS

Maintenance of phenotypic characteristics and viability of three dimensional human non-fibrotic (stages F0-F1) and fibrotic (stages F2-F4) LS cultures for 21 days of cultivation

The viability of human LS cultures during prolonged studies was and is a crucial factor. Liver slices viability (percentage of ATP production) and tissue morphology were assessed daily, until day 21. The architecture of the liver slices was normal (Figure 2A) and human liver slices (HLS) expressed the Ki67 protein, a proliferation marker (Figure 2B). Human LS cultures maintained their differentiation status throughout the entire study period, as previously described (Figure 2A-C)[10,12]. Indeed, LS status was confirmed by analysing various parameters and biomarkers, in particular, albumin content, hepatocyte nuclear factors HNF-1β, HNF-4α, CYP2E1, and CYP3A4 (Figure 2D)[10-12,22-24]. A comparison of the expression of hepatocyte-specific genes in F0-F1 non-infected liver slices and Huh-7.5.1 cells showed increased expression in F0-F1 non-infected liver slices on day 21 compared to that in Huh-7.5.1 cells, either at an exponential growth phase or at the confluence (data not shown). CYP3A4 expression was undetected in Huh-7.5.1 cells whatever the growth stage[25]. Albumin and urea secretion increased throughout, indicating that liver slices had retained normal physiological and biochemical parameters (Figure 2E-F)[11,12]. As previously reported[11], the cell viability and expression of hepatocyte-specific genes were also evaluated post- HCVcc[11]. Results were similar to those in uninfected liver slices, indicating that there was no evident cytopathic effect (Figure 3A-C).

The viability of non-fibrotic (F0-F1) and fibrotic (F2-F4) LS cultures and resistance to EtOH and palmitate treatments were tested during the 21 d follow-up studies by evaluating of the rate of ATP production in the liver slices (Figure 4A, C-E and G)[22], and by quantification of LDH release from liver slices (Figure 4B-F)[23]. On day 21, the F0-F1 and F2-F4 non-infected liver slices had a viability rate of 75% and 50%, respectively (Figure 4A). Following treatment with 25 mmol/L of EtOH, ATP synthesis in F0-F1 infected liver slices was reduced by 55% on day 21 (Figure 4D), and tissue viability decreased by nearly 25% compared to untreated F0-F1 non-infected/infected liver slice cultures (Figure 4C and D). However, the addition of EtOH (25 mmol/L) did not change LDH release in F0-F1 and F4 non-infected LS cultures (Figure 4F). Treatment with palmitate (500 μmol/L) did not reduce significantly the viability rate of F0-F1 non-infected and infected LS cultures, compared to untreated and non-infected LS cultures (55% and 65%, respectively) (Figure 4G). There was no significant difference in LDH release from F0-F1, F2-F3, and F4 non-infected and infected LS cultures after treatment with the combination of UCDA and alfa-Toco (Figure 4H and I). Results of ATP production in F0-F1, F2-F3, and F4 non-infected and infected LS cultures after treatment with the combination of UCDA and alfa-Toco were significantly positive (Figure 4J-K) with increased ATP production in Fibrotic treated liver slices. These results, showing no significant changes in viability (with increasing levels of albumin, urea secretion as well as ATP production throughout the experiments) or morphology (Ki67 marker expression), confirm that the non-fibrotic (F0-F1) and fibrotic (F2-F4) LS cultures can survive for 21 days, and that the 3D LS cultures tolerated the different treatments (Figure 4H-K). Thus, LS cultures from selected donors can be used in extended research.

Evaluation of the expression of fibrogenesis liver biomarkers in 3D LS cultures from non-fibrotic (F0-F1) and fibrotic (F2-F4) livers

Activation or down-regulation of certain biomarkers reflects the process of the transition of the non-fibrotic liver to the fibrotic liver designated as the molecular fibrogenesis. We measured the expression of seven fibrosis biomarkers (TGF-β1, Hsp47, α-SMA, Procol1A1, MMP-2, MMP-9, and VEGF)[1,26] by RT-qPCR to analyse both non-fibrotic (F0-F1) and fibrotic (F2-F3, F4) stages of the liver in human LS cultures.

Induction of fibrogenesis by three exogenous factors: HCV, EtOH, and fatty acids (palmitate) in non-fibrotic (F0-F1) and fibrotic (F2-F4) LS cultures

HCV efficiently replication in LS cultures: A model of the viral liver disease: Robust replication of HCVcc and production of infectious viral particles were detected up to day 21 in human F0-F1 LS (Figure 5). Intracellular replication of the viral genome was assessed by a strand-specific RT-qPCR, as previously described[11]. The HCV RNA negative strand, proof of HCV genome replication, could be detected as early day 1

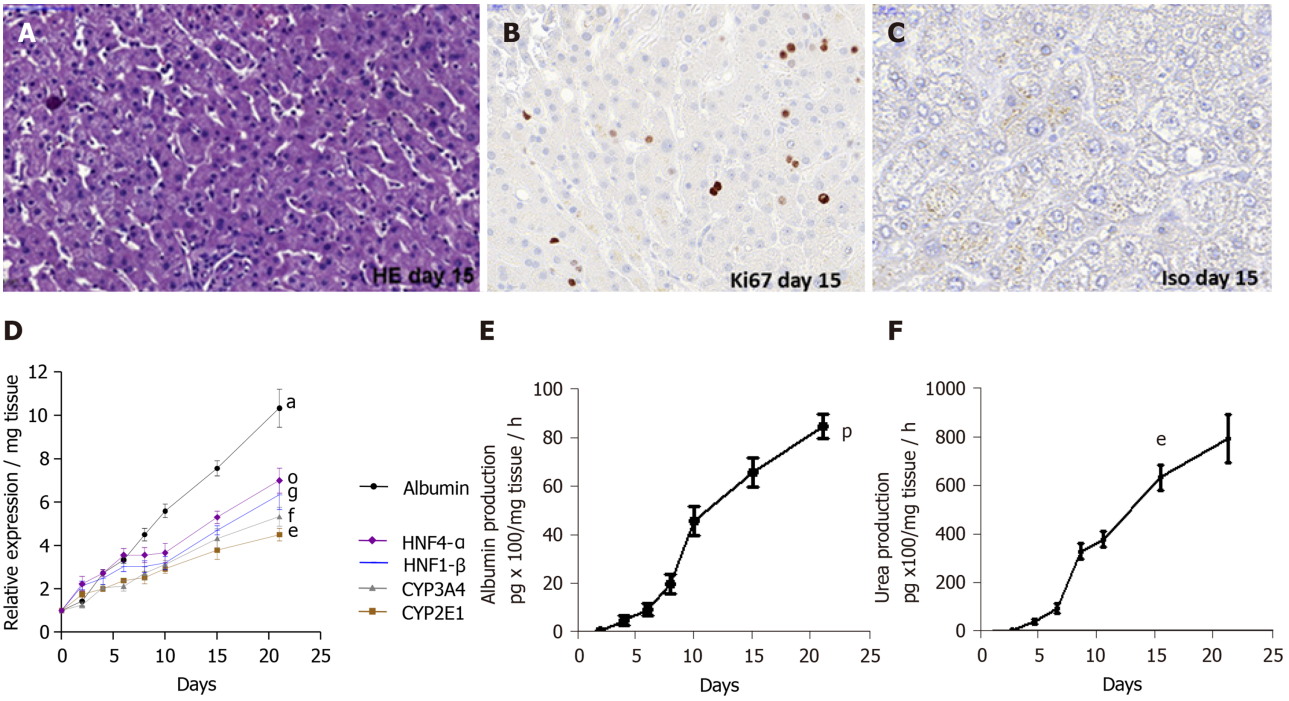


Figure 2 Maintenance of phenotypic characteristics of human non-fibrotic (F0-F1), and fibrotic (F2-F3, F4) liver slices during the culture, demonstrated by histochemistry, real-time reverse transcription-quantitative polymerase chain reaction and biochemical assays. A: Light microscopy of human liver tissue 7 μm-thick section stained with hematoxylin and eosin showing non-fibrotic (F2-F3) liver lobular architecture on day 15, magnification × 20. Scale bars 100 μm; B: Representative human liver tissue 7 μm-thick sections from fibrotic (F2-F3) liver patient showing immunostaining for Ki67, a proliferation marker, on day 15, magnification × 40, Scale bars, 20 μm; C: Representative human liver tissue 7 μm-thick sections from fibrotic (F2-F3) liver patient showing immunostaining with isotype as negative control, on day 15, magnification × 40, Scale bars, 20 μm; D: Hepatocyte-specific gene mRNA expression (relative expression/mg tissue) during the 21 days follow up studies. Maintenance of hepatocyte-specific gene expression patterns in human non-fibrotic (F0-F1) non-infected liver slices during culture. The real-time reverse transcription-quantitative polymerase chain reaction analyses were performed from five independent human non-fibrotic (F0-F1) livers using slices in triplicate from each liver. All liver-specific gene expression values were normalized to 18S RNA as an internal standard and expressed relative to the zero-time point. Values are expressed as mean ± standard errors. The results were compared using the two-paired Student's *t*-test: Albumin: $^{a}P < 0.0001$; CYP2E1: $^{e}P < 0.001$; CYP3A4: $^{f}P < 0.0003$; HNF1-β: $^{g}P < 0.01$; HNF4-α: $^{o}P < 0.008$; E and F: Biochemical functional assays; E: Albumin production (pg x 100/mg tissue/hour) during the 21 days follow up studies; and F: Urea production (pg/mg tissue/hour) during the 21 days follow up studies. Studies were done in triplicate and repeated twice for each liver sample. Values are expressed as means ± standard errors ($n = 5$). The results were compared using the two-paired Student's *t*-test: albumin production ($^{p}P < 0.02$), urea production ($^{e}P < 0.001$).

post-infection, and the intracellular levels of both negative and positive strands increased significantly during LS culture. These results confirmed active viral replication in LS cultures (Figure 5A). The HCV expression level was significantly increased in the LS culture on day 5 post-infection (Figure 5A). HCV protein expression was confirmed by Western blotting. Detection of core and nonstructural protein 3 proteins confirmed effective intracellular processing of the viral protein precursor[11] (Figure 5B and C).

The virus titer was estimated in LS culture supernatants using a classic titration assay on Huh-7.5.1 cells to determine whether progeny virions released from the infected LS could replicate[11]. Infectivity increased during the culture and reached a peak of up to 1.7×10^5 ffu/mL respectively, by day 21 post-infection (Figure 5D). To further confirm that the new progeny virus produced by the human LS called the primary-culture-derived virus was indeed infectious, naive human LS were infected *de novo* with primary-culture-derived virus Con1/C3 at MOI = 0.1. A *de novo* productive infection of LS was obtained with higher infectivity titers on day 21, genotype1b (180000 ffu/mL) (Figure 5E). Thus, HCV RNA replication, the expression of viral proteins, and the production of highly infectious particles were demonstrated.

HCVcc infection of non-fibrotic (F0-F1) LS activated the expression of the main pro-fibrogenic markers. During follow-up studies, in non-fibrotic (F0-F1) LS cultures (Figure 6), RNA, and protein expression of TGF-β1 (Figure 6A-C), α-SMA, Hsp47, Procol1A1, (Figure 6D-F) had increased significantly in non-infected and infected LS on day 21. A marked 2.6 to 3.6 fold increase of α-SMA, Hsp47, Procol1A1 RNA expression was observed in non-fibrotic (F0-F1) HCV infected LS cultures, compared to non-infected LS cultures on day 21. MMP-2 RNA expression was also significantly increased after HCV infection in non-fibrotic F0F1 LS, (Figure 6G). On the contrary,

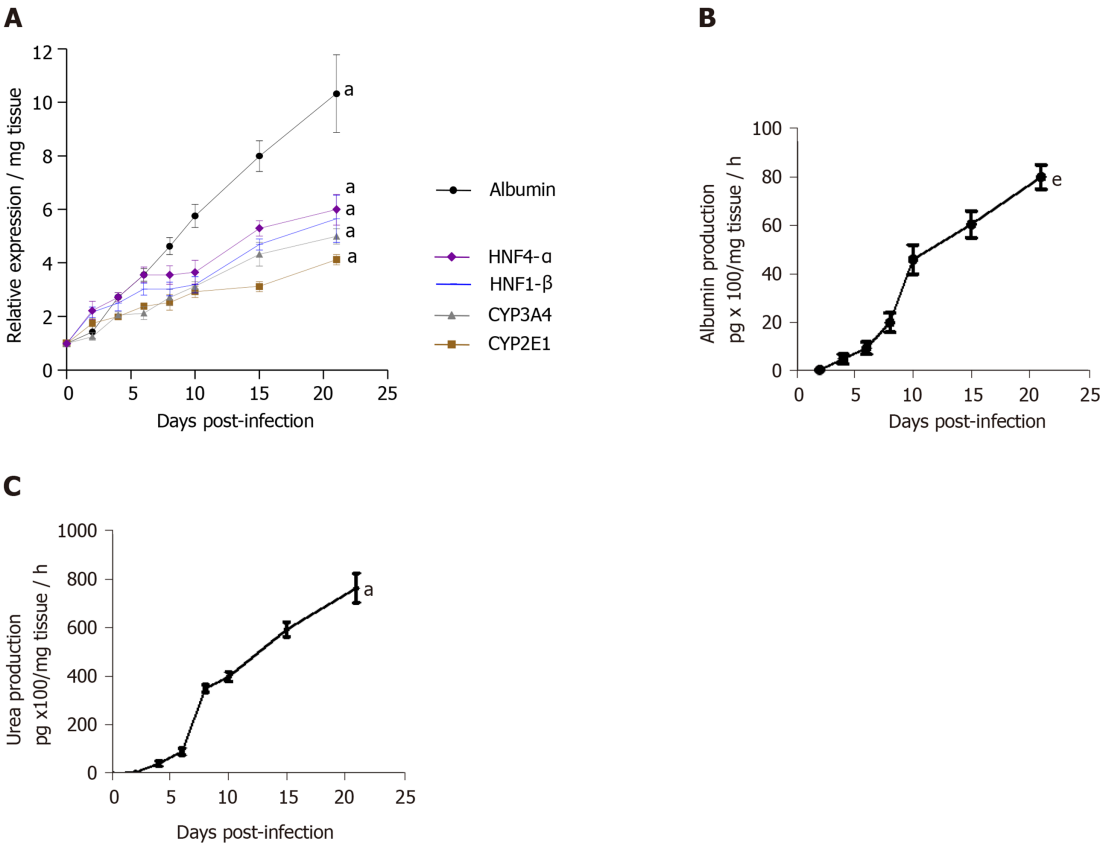
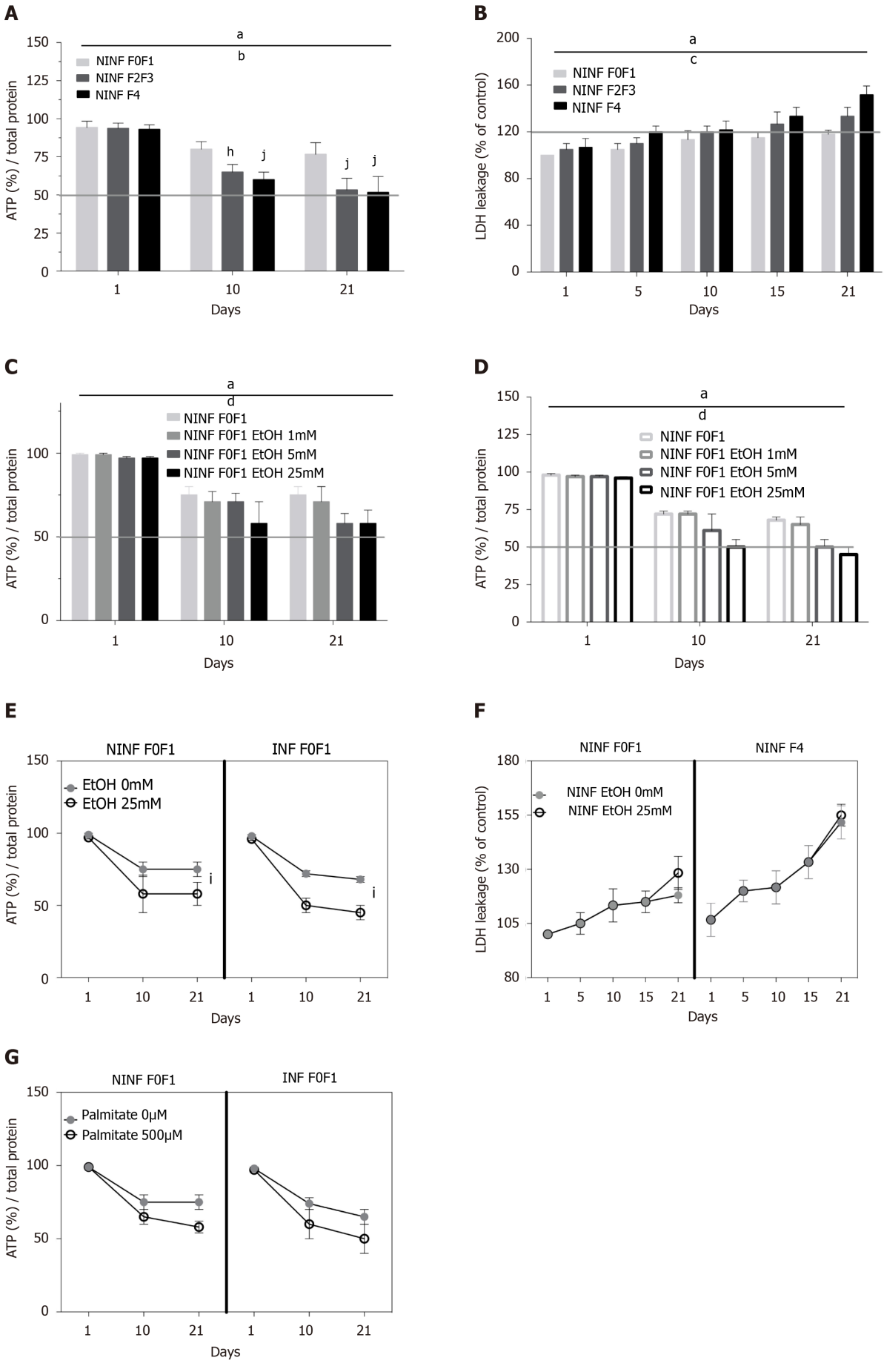


Figure 3 Maintenance of phenotypic characteristics of human non-fibrotic (F0-F1) hepatitis C virus-infected liver slices during the culture, demonstrated by real-time reverse transcription-quantitative polymerase chain reaction and biochemical assays. A: Hepatocyte-specific gene mRNA expression (relative expression/mg tissue) during the 21 days follow up studies. Maintenance of hepatocyte-specific gene expression patterns in human non-fibrotic (F0-F1) hepatitis C virus (HCV) infected liver slices during the culture. The real-time reverse transcription-quantitative polymerase chain reaction analyses were performed from five independent human non-fibrotic (F0-F1) liver samples, using HCV- infected slices in triplicate from each liver. Liver slices were infected with HCVcc, on day 0, at MOI = 0.1. All liver–specific gene expression values were normalized to 18S RNA as an internal standard and expressed in relation to the zero-time point. Values are expressed as mean ± standard errors. The results were compared using the two-paired Student's *t*-test: Albumin, $^{a}P < 0.0001$; CYP2E1: $^{a}P < 0.0001$; CYP3A4: $^{a}P < 0.0001$; HNF1-β: $^{a}P < 0.0001$; HNF4-α: $^{a}P < 0.0001$); B and C: Biochemical functional assays: B: Albumin production (pg x 100/mg tissue/ hour) during the 21d- follow up studies (days). C: Urea production (pg/mg tissue/hour during the 21d- follow up studies (days) by human F0-F1 cultured HCV-infected liver slices ($n = 5$). The assays were performed as previously described[11,12]. Studies were performed in triplicate and repeated twice for each liver sample. Values are expressed as means ± standard errors ($n = 5$). The results were compared using the two-paired Student's *t*-test: Albumin production: $^{e}P < 0.001$; urea production: $^{a}P < 0.0001$.

there was no significant difference in MMP-9 RNA expression between F0-F1 non-infected and infected liver slices (Figure 6H). VEGF RNA expression increased irregularly up to day 21 and seemed to be influenced by HCV infection until day 5 compared to non-infected LS (Figure 6I). The triglyceride production increased in both F0-F1 non-infected and infected LS cultures (Figure 6J) with no significant difference between them.

Expression of fibrosis biomarkers was higher in fibrotic LS culture (stages F2-F3 and F4), than in non-fibrotic LS cultures, with a significant 4 to 8 fold increase compared to controls (day 1) (Figure 7). This mainly concerned TGF-β1 (Figure 7A), Procol1A1 (Figure 7C), α-SMA (Figure 7D), Hsp47 (Figure 7E) as well as an increased triglyceride production in fibrotic LS (approximately 3.2 fold) (Figure 7B). After day 10, RNA expression increased with the progression of fibrosis. MMP-2 RNA expression (Figure 7F), as well as MMP-9 and VEGF expression (Figure 7G and H), did not differ between fibrosis stages F2-F3 and F4. It is interesting to note that HCV infection significantly increased TGF-β1, Hsp47, α-SMA, Procl1A1, MMP-2, MMP-9, VEGF expression as well as triglyceride production in fibrotic (F2-F3, F4) infected LS cultures. A significant 2 to 4 fold increase in fibrosis biomarkers was observed on day 21 in F2-F3 and F4 HCV infected LS compared to F2-F3, F4 non-infected LS. Thus, the TGF-β1 (Figure 6A-C), α-SMA, Hsp47, Procol1A1, MMP-2, MMP-9, VEGF expression increased in non-infected and infected LS cultures with a greater increase in infected LS cultures than in controls. On day 21, a significant 2 to 13 fold increase in fibrosis biomarkers was observed in F2-F3, F4 infected LS cultures compared to F2-F3, F4 non-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ATP (%) / total protein
NINF F0F1
NINF F2F3
NINF F4
Days
B
LDH leakage (% of control)
NINF F0F1
NINF F2F3
NINF F4
Days
C
ATP (%) / total protein
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NINF F0F1 EtOH 1mM
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NINF F0F1 EtOH 25mM
Days
D
ATP (%) / total protein
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NINF F0F1 EtOH 5mM
NINF F0F1 EtOH 25mM
Days
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NINF F0F1
INF F0F1
EtOH 0mM
EtOH 25mM
ATP (%) / total protein
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F
NINF F0F1
NINF F4
NINF EtOH 0mM
NINF EtOH 25mM
LDH leakage (% of control)
Days
G
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INF F0F1
Palmitate 0μM
Palmitate 500μM
ATP (%) / total protein
Days

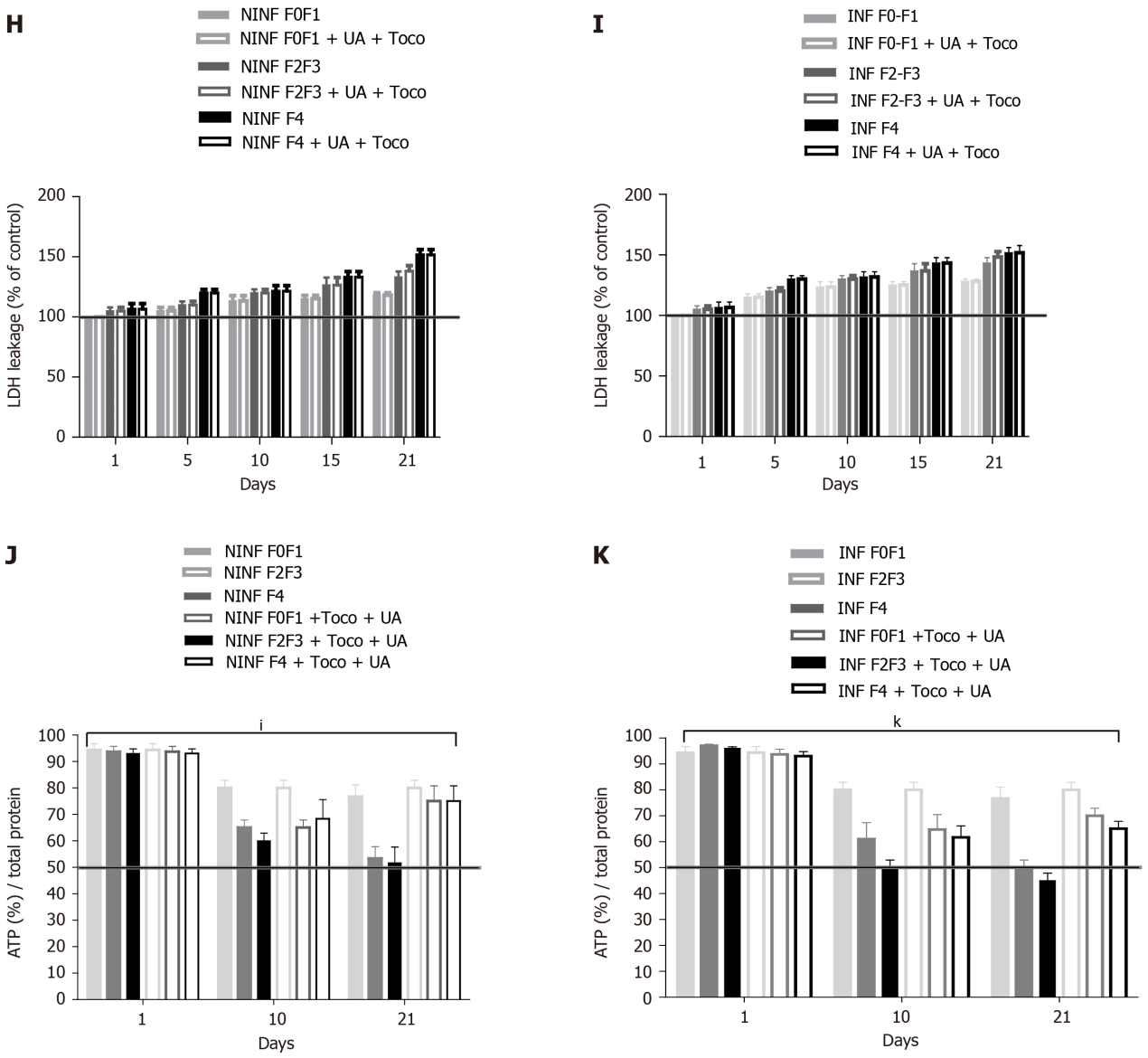


Figure 4 Viability of human non-fibrotic (F0-F1), and fibrotic (F2-F3, F4) non-infected or hepatitis C virus-infected liver slices during the different kinetic studies, with no treatment cytotoxicity as shown by ATP and LDH dosages. A: Percentage of ATP synthesis/total protein in non-infected (NINF) liver slice (LS) with F0-F1 to F4 stage fibrosis during the 21 days-follow up kinetics; B: The percentage of LDH release/control in NINF LS with a F0-F1 to F4 stage fibrosis during the 21d-follow up kinetics (d: days); C and D: The percentage of ATP synthesis /total protein in F0-F1 NINF and hepatitis C virus (HCV)-infected (INF) LS treated with 1 mmol/L, 5 mmol/L and 25 mmol/L of EtOH during the 21d-follow up kinetics; E: The percentage of ATP synthesis / total protein in the presence of 25 mmol/L of EtOH on F0-F1 NINF and INF LS during the 21d-follow up kinetics; F: LDH release (% of control) in F0-F1 non-infected LS cultures treated or non-treated with 25 mmol/L of EtOH compared to F4 non-infected treated or non-treated with 25 mmol/L of EtOH during the 21d-follow up kinetics. Values are expressed as means ± standard errors (SEMs), (n = 5). $^{a}P < 0.0001$ time factor; $^{b}P < 0.01$ fibrosis stage; $^{c}P < 0.05$ fibrosis stage; $^{d}P < 0.05$ alcohol factor; $^{i}P < 0.01$ subject *vs* control (non-treated) (two-way ANOVA test).There is no significant toxic effect of EtOH (25 mmol/L) on F0-F1 NINF and INF LS and F2-F3, F4 NINF LS; G: The percentage of ATP synthesis/total protein during the 21-follow up kinetics showing the viability of F0-F1 NINF or INF LS cultures with or without the presence of palmitate (500 µmol/L); H and I: Absence of drug cytotoxicity (LDH release, (% of control)) on the viability of human F0-F4 LS NINF or infected (INF) by HCVcc Con1/C3 during the treatment with either UCDA (UA) or Toco or both for 21 days. It is important to note that under 150%, there is no cytotoxic effect of the drugs on LS viability. Values are expressed as means ± SEMs, (n = 5); J and K: The percentage of ATP synthesis / total protein during the 21 days follow up kinetics, in F0-F1 to F4 NINF or infected (INF) LS with combined treatment [Toco + UCDA (UA)]. Values are expressed as means ± SEMs, (n = 5); Levels of significance are as follows between: Subject *vs* control, $^{k}P < 0.0001$; $^{i}P < 0.001$; $^{i}P < 0.01$; $^{h}P < 0.05$) (two-way ANOVA test).

infected LS cultures. Triglyceride production increased in both non-infected and infected LS cultures, independent from the stage of fibrosis. After 21 d of the culture, the amount of triglyceride in the supernatant of F2-F3 and F4 LS cultures increased by 1.36 and 2.7 folds, respectively (Figure 7B). Increased expression of the TGF-β1, α-SMA, Procol1A1, MMP-2, MMP-9, and VEGF in F2-F3 LS cultures throughout the 21-d of follow-up was confirmed by Western blotting (Figure 7I and J). On day 10, immunohistochemistry showed that TGF-β1, α-SMA and MMP-9 expression (Figure 7K) was increased by about 20% in F2-F3 LS compared to day 0.

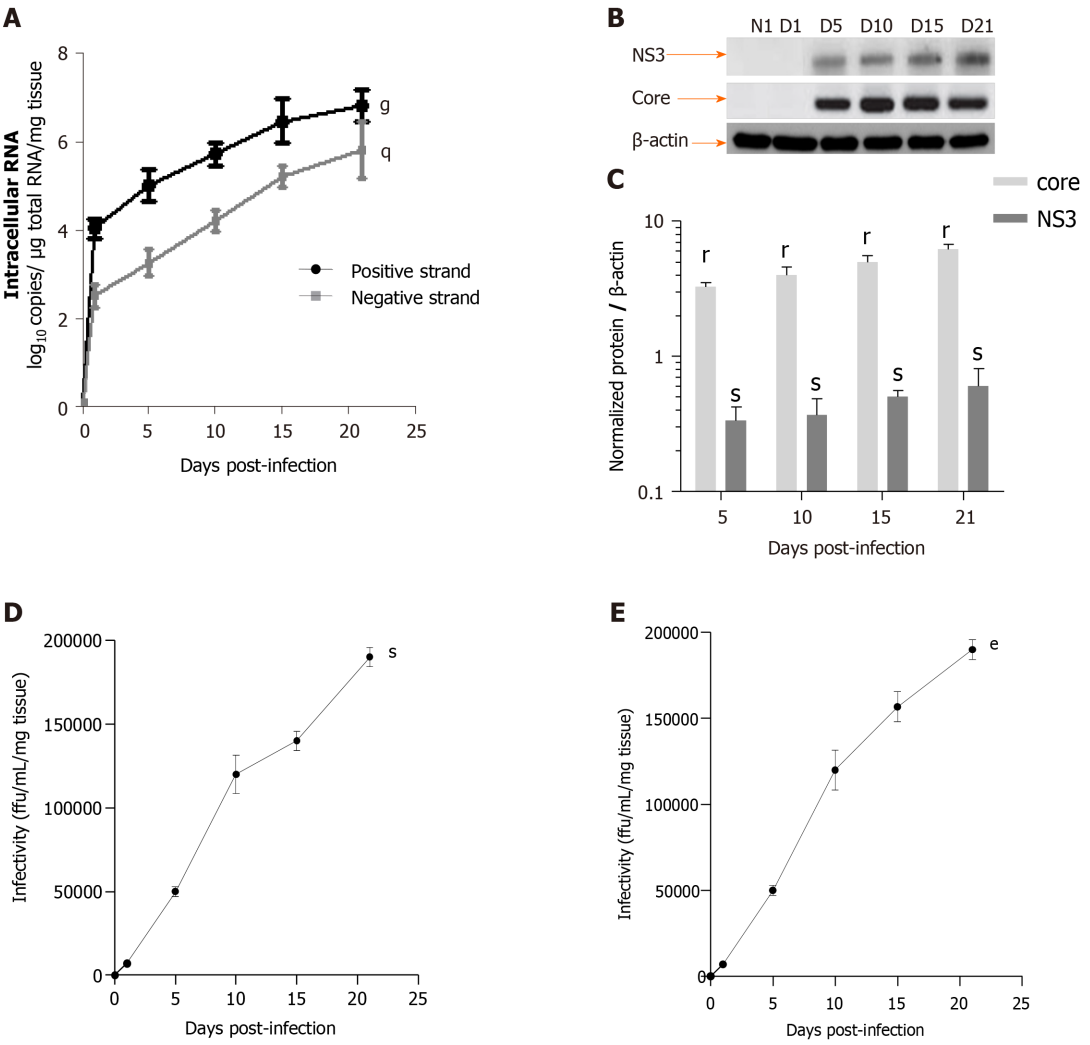
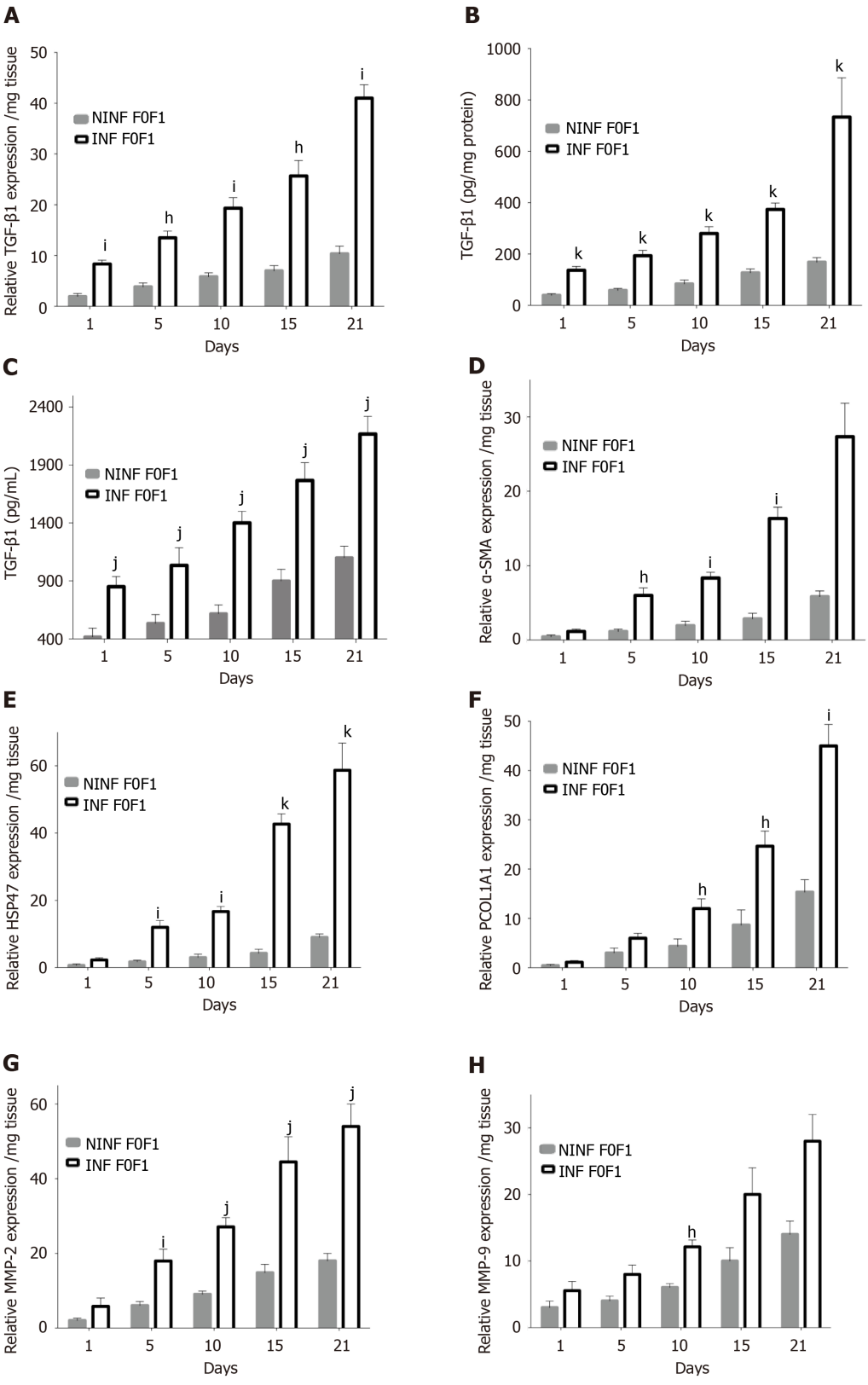


Figure 5 Efficient replication of hepatitis C virus RNA, and hepatitis C virus core and NS3 proteins expression in human F0-F1 liver slice culture as shown by real-time reverse transcription-quantitative polymerase chain reaction and western blotting analysis. A: Quantification of intracellular levels of positive- and negative-strand hepatitis C virus (HCV) RNA (log10 copies/µg total RNA/mg tissue) in primary human F0-F1 HCVcc Con1/C3 -infected liver slice (LS) by specific- strand real-time reverse transcription-quantitative polymerase chain reaction on day 5, day 10, day 15 and day 21 post-infection. Values are expressed as mean ± SEMs. All results were compared using the two-paired Student *t*-test, time factor: Positive strand: $^{g}P < 0.01$; negative strand: $^{q}P < 0.04$, ($n = 3$). Detection of the negative strand of HCV RNA evidences active replication as well as an increase over time of both positive and negative strands of HCV RNA; B: Western blotting analysis of human F0-F1 HCVcc Con-1/C3 -infected LS lysates with mAbs against HCV NS3 or core proteins on day 5, day 10, day 15, and day 21, post-infection (MOI = 0.1) was performed and analyzed ($n = 3$). Lysates of naïve human F0-F1 LS lysates were run in parallel to serve as a negative controls (NI). β-actin was used as a loading control; C: Normalization of Core and NS3 protein expression compared to b-actin expression (Normalized protein / β-actin) during the 21 days follow-up kinetics using the image quantification standard software, ImageJ2[21]. The position of molecular-weight markers is indicated in kDa. Values are expressed as means ± SEMs ($n = 3$): Core $^{r}P < 0.002$; NS3 $^{s}P < 0.02$ (two-paired Student *t*-test); D: Production of HCV infectious particles (genotype 1b) in primary adult human F0-F1 LS: Infectivity titers [*i.e.*, infectivity (ffu/mL/mg tissue)] of culture supernatants from human F0-F1 LS infected by the Con1/C3 virus during the 21 days follow up kinetics. The curve represents the average of three independent infections from 3 different donors. Each kinetic study was performed in triplicate. Values are expressed as means ± SEMs. Results were compared using the two-paired Student *t*-test: $^{s}P < 0.02$; and E: Infectivity titers [*i.e.*, infectivity (ffu/mL/mg tissue)] of culture supernatants of naive F0-F1 LS infected with supernatants from human F0- F1 HCV-infected LS culture (HCVpc) during the 21 days follow up kinetics. The infection of naive F0-F1 LS with supernatants from human F0-F1 HCV-infected LS culture (HCVpc) clearly indicates the infectivity of extracellular viral particles, which are produced by HVCcc Con1/ C3 (genotype 1b) infection. Values are expressed as means ± SEMs ($n = 3$). Levels of significance: $^{e}P < 0.001$ (two-paired Student *t*-test).

Exposition of LS cultures to ethanol: A model of the alcoholic liver disease: The effect of EtOH exposure on LS cultures was estimated using non-fibrotic (F0-F1) HCV infected or non-infected LS cultures (Figure 8A-F and Figure 9) and fibrotic (F2-F4) HCV infected or non-infected LS cultures (Figure 8G-L) and Figure 10A-L). One mmol/L, 5 mmol/L or 25 mmol/L of EtOH was added to F0-F1 HCV infected, or non-infected LS cultures (Figure 8A-F). Only the highest concentration of EtOH was studied in fibrotic (F2-F3 and F4) non-infected or HCV-infected LS cultures, (Figure 8G-L and Figure 10A-L) respectively. During the follow-up studies (Figure 8A-F), EtOH enhanced the RNA expression of fibrosis markers in a dose-dependent manner in F0-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Relative TGF-β1 expression /mg tissue
NINF F0F1
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Days
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TGF-β1 (pg/mg protein)
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TGF-β1 (pg/mL)
D
Relative α-SMA expression /mg tissue
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Relative HSP47 expression /mg tissue
F
Relative PCOL1A1 expression /mg tissue
G
Relative MMP-2 expression /mg tissue
H
Relative MMP-9 expression /mg tissue

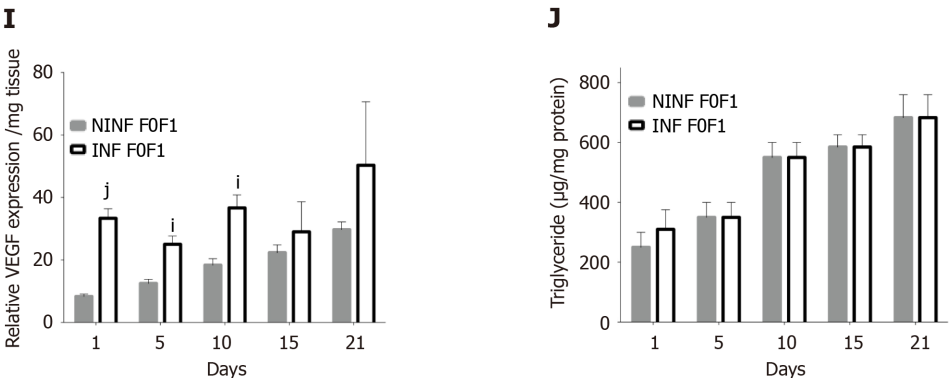
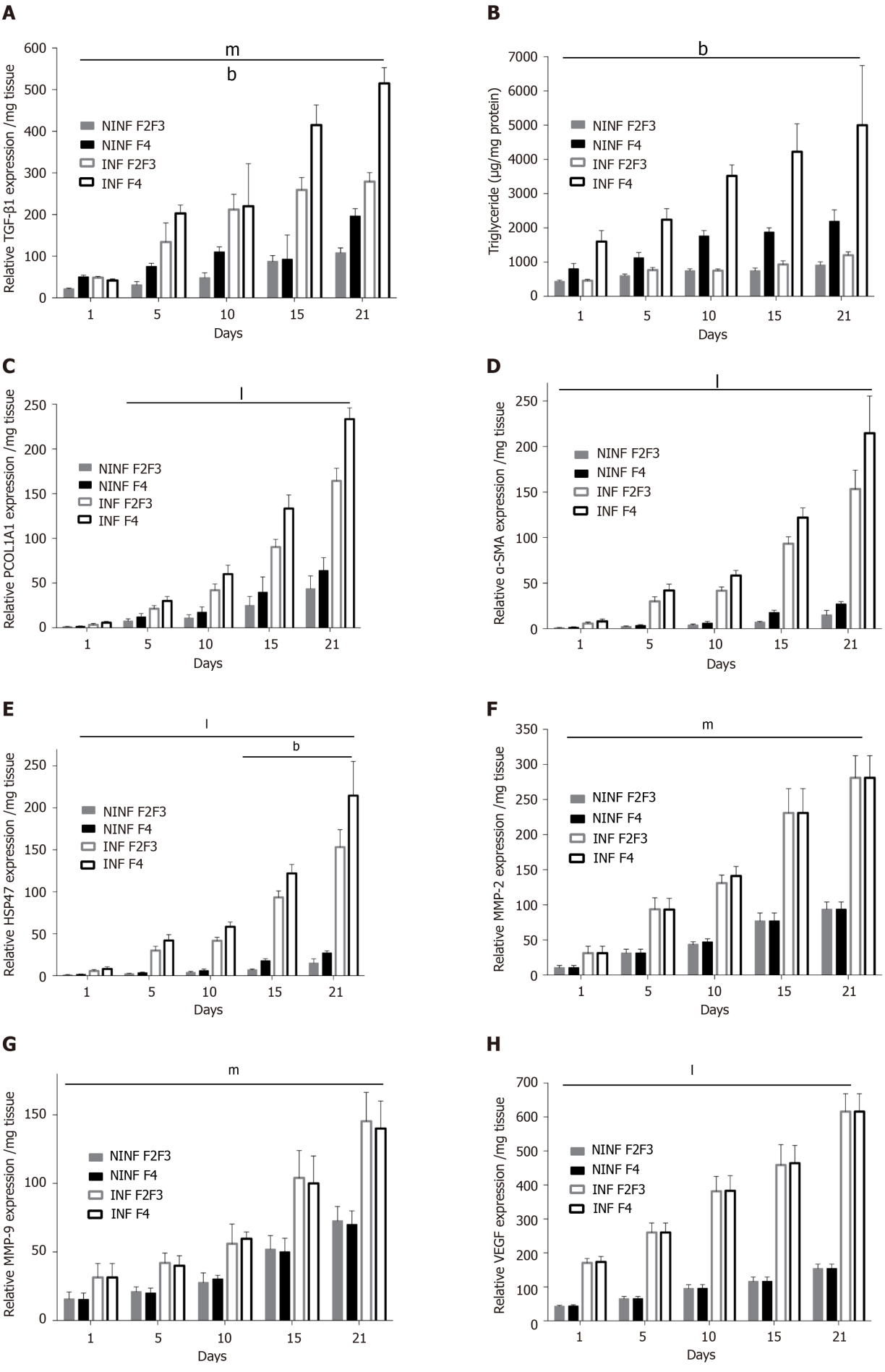


Figure 6 Real-time reverse transcription-quantitative polymerase chain reaction analysis evidencing the significant increase of fibrosis markers expression at the transcriptional level in human F0-F1 non-infected or hepatitis C virus infected liver slice during the kinetics. A: TGF-β1 expression at mRNA level (relative RNA expression / mg tissue) during 21 days follow up kinetics; B: TGF-β1 expression at intracellular protein level (pg/mg protein) during 21 days follow up kinetics; C: TGF-β1 expression at extracellular secretion level (pg/mL) during 21 days follow up kinetics; D-F: mRNA expression (relative RNA expression / mg tissue) of (D) α-SMA, HSP47 (E) and ProCOL1A1 (F) during 21 days follow up kinetics; G and H: MMP-2 and MMP-9 mRNA expression (relative RNA expression / mg tissue) during 21 days follow up kinetics; I: VEGF mRNA expression (relative RNA expression/mg tissue) during 21 days follow up kinetics; J: Triglyceride production (µg/mg protein) raised during the during the 21 days follow up kinetics. All data are presented considering the percentage of viable liver slices in culture. Data are expressed as means ± SD (n = 5), subject vs control, $^{h}P < 0.05$; $^{i}P < 0.01$; $^{j}P < 0.001$; $^{k}P < 0.0001$, (two-way ANOVA test).

F1 LS cultures. Increased expression of TGF-β1, Procol1A1 RNA was further detected in F0-F1 infected LS (Figure 8B, D and F, Figure 9B, Figure 10B), compared to non-infected LS (Figure 8A, C and E, Figure 9A, Figure 10A). Similar results were found in fibrotic F2-F4 LS (Figure 8G-L, Figure 10A-L). Interestingly, there was no significant increase in Procol1A1 or α-SMA RNA expression in F0-F1 non-infected LS except on day 21 when 25 mmol/L of EtOH was added to the culture (Figure 8E and Figure 9C, respectively). However, a significant dose-dependent increase of the Procol1A1 and α-SMA RNA expression occurred whatever the dose of EtOH added to F0-F1 infected LS cultures (Figure 8F, Figure 9D). There was a dose-dependent increase in the RNA expression of the other fibrosis markers such as α-SMA (Figure 9C and D; Figure 10E and F), and HSP47 (Figure 9E and F, Figure 10C and D) with the addition of EtOH in F0-F1 to F4 infected LS which was less marked in F0-F1 to F4 non-infected LS. Analysis of F0-F1 to F4 HCV non-infected or infected LS showed a significant dose-dependent increase in MMP-2, MMP-9, and VEGF expressions in response to EtOH (Figure 10G-L). Masson's trichrome staining showed a significant increase in collagen fibers (%) between day 1 (1.242% of collagen) and day 6 (2.076% of collagen) in F0-F1 HCV infected LS treated with 5 mmol/L of EtOH (Figure 8M) but not in F0-F1 non-infected LS with the same treatment (Figure 8N). Picro Sirius red staining confirmed the significant increase in collagen fibers (%) between day 1 (0.55% of collagen) and day 6 (1.53% of collagen) in F0-F1 HCV infected LS treated with 5 mmol/L of EtOH compared to non-treated LS (data not shown).

Exposition of LS cultures to palmitate: a model of NASH. To imitate NASH, non-fibrotic (F0-F1) LS cultures infected (or not infected) with HCV were exposed to 500 µmol/L of palmitate (Figure 11). More marked triglyceride synthesis was noted in F0-F1 palmitate treated HCV-infected LS cultures, than in F0-F1 untreated non-infected LS cultures (Figure 11A). The F0-F1 infected LS cultures treated with palmitate demonstrated more marked expression of the fibrotic markers such as TGF-β1 (Figure 11B), intracellular expression of TGF-β1 (Figure 11C), and secretion of the extracellular TGF-β1 (Figure 11D). A similar increase was observed with Procol1A1, α-SMA, and HSP47 (Figure 11E-G) on day 21. The expression of markers (RNA) involved in liver fibrolysis, (MMP-2, -9), and VEGF increased significantly in both F0-F1 non-infected LS cultures treated or not with palmitate (Figure 12A). But, the treatment of F0-F1 non-infected LS cultures with palmitate showed a greater significant increase of the expression of MMP-2, -9, and VEGF compared to those of F0-F1 untreated non-infected LS cultures. The treatment of the F0-F1 infected LS cultures with palmitate, increased significantly VEGF, MMP-2, and MMP-9 from day 10, 15, and day 21 respectively (Figure 12B). Fibrotic marker expression increased both in F0-F1 LS cultures HCV infected or non-infected treated with palmitate but with a greater increase in F0-F1 HCV infected LS treated with palmitate.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Days
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Triglyceride (μg/mg protein)
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C
Relative PCOL1A1 expression /mg tissue
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D
Relative α-SMA expression /mg tissue
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Relative HSP47 expression /mg tissue
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Relative MMP-2 expression /mg tissue
G
Relative MMP-9 expression /mg tissue
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Relative VEGF expression /mg tissue

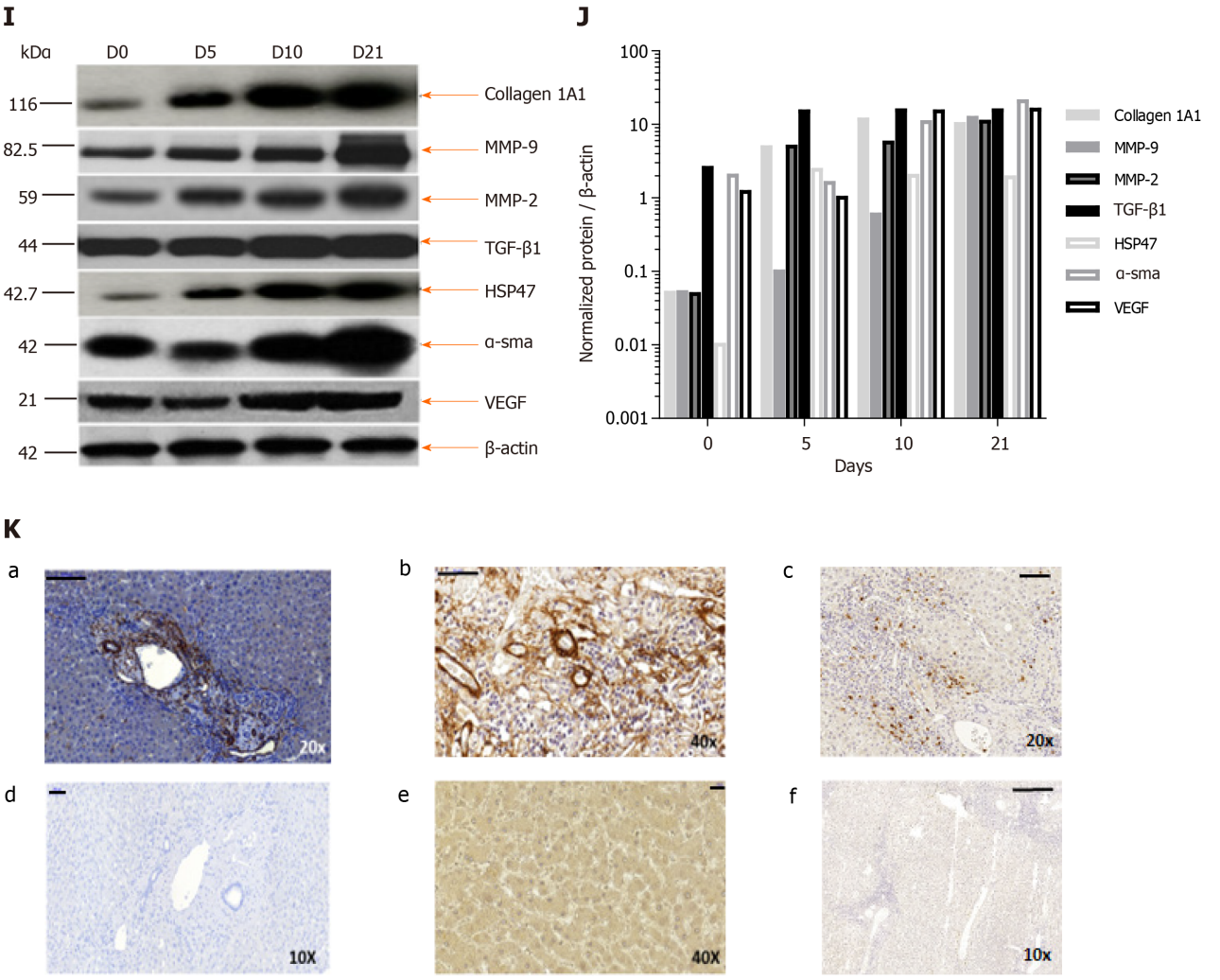
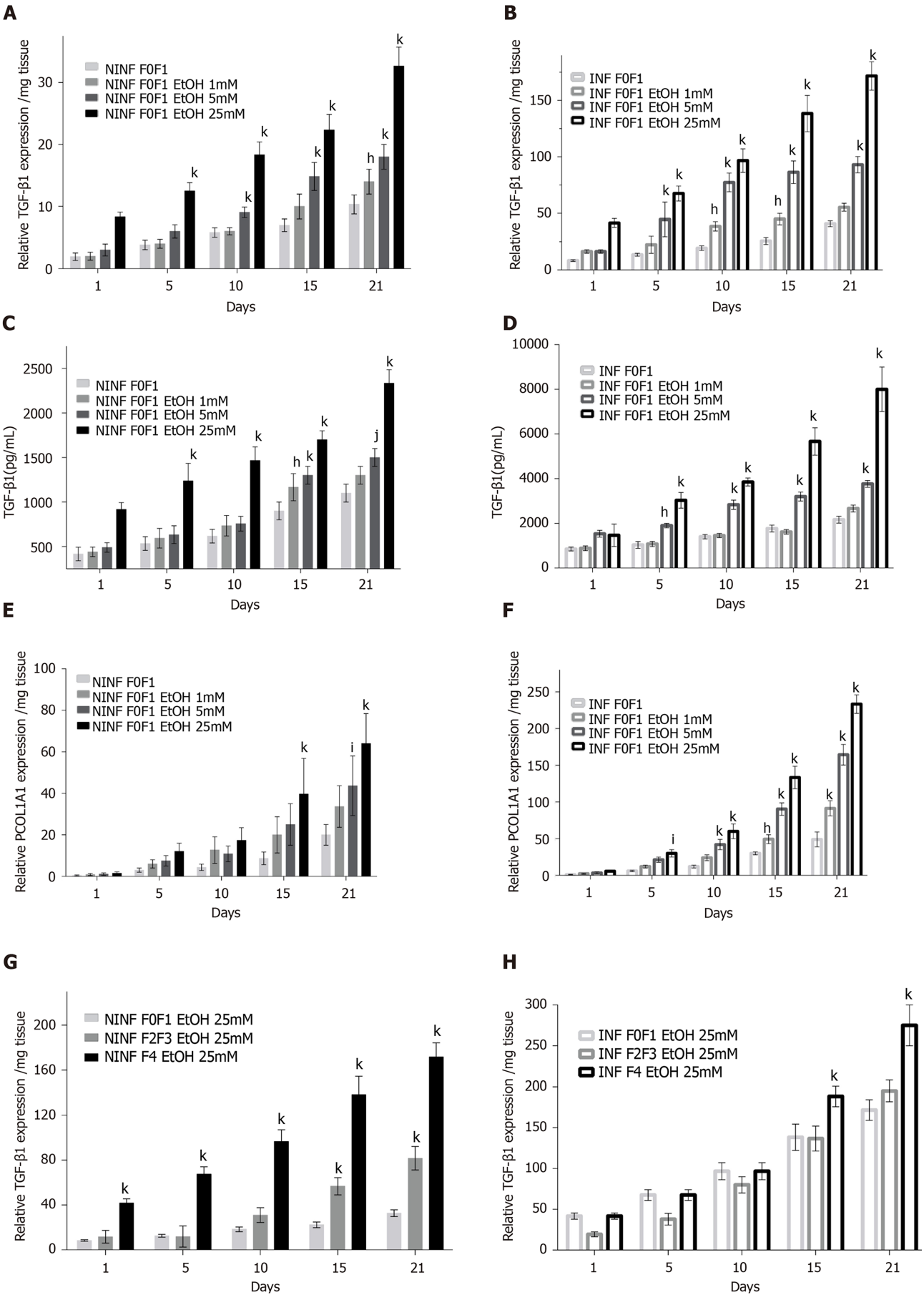


Figure 7 Real-time reverse transcription-quantitative polymerase chain reaction analyses of RNA expression of liver fibrosis markers (TGF-β1, Procol1A1, α-SMA, HSP47, MMP-2, MMP-9, VEGF), and triglyceride production in non-infected or hepatitis C virus infected liver slice cultures from fibrotic liver (F2-F3, F4) showing a significant increase during the kinetics. A: TGF-β1 mRNA Expression (relative RNA expression/mg tissue) during 21 days follow up kinetics; B: Triglyceride production (μg/mg protein); C–E: Procol1A1, α-SMA, HSP47 mRNA expression (relative RNA expression / mg tissue) during 21 days follow up kinetics; F- H: MMP-2, MMP-9 and VEGF mRNA expression (relative RNA expression/mg tissue) during 21 days follow up kinetics. Data are expressed as mean± SD (F2-F3 liver samples, $n = 2$, F4 liver samples, $n = 2$). $^{b}P < 0.01$ fibrosis stage factor; $^{m}P < 0.001$ Infection factor; $^{I}P < 0.0001$ infection factor; (two-way ANOVA test); I: TGF-β1, HSP-47, Collagen I alpha 1, MMP-9, MMP-2, a-SMA, VEGF proteins expression in F2-F3 liver slice performed in western blotting and normalized. Positions of molecular-weight markers are indicated in kDa; J: Normalization of the proteins expression compared to β-actin expression (Normalized protein/b-actin) during the 21 days follow-up kinetics using the image quantification standard software, ImageJ2; and K: Representative human liver tissue 7 μm-thick sections from F2-F3 liver patient showing immunohistochemistry staining for fibrosis markers, TGF-β1 (a), α-SMA (b), MMP-9 (c) on day 10, magnification 20×, Scale bars, 100 μm; 40×, Scale bars 50 μm; 10×, Scale bars 100 μm, respectively. (d-f) isotypes controls staining, magnification 10×, Scale bars, 100 μm; 40×, Scale bars 20 μm; 10×, Scale bars 200 μm, respectively.

LS treatment with a combination of the "hepatoprotective" UCDA and anti-fibrotic α-Toco drugs significantly reducing the expression of the main fibrosis markers TGF-β1, Procollagen1A1, and triglyceride production

To validate the LS culture as a model for drug screening, the "hepatoprotective" (UCDA) and "anti-fibrotic (Toco) drugs were tested on non-fibrotic (F0-F1), or fibrotic (F2-F3, F4) LS cultures, infected or non-infected with HCV. UCDA and Toco were dosed according to the standard of care in humans (Figure 13). On day 0, LS cultures were infected with HCVcc Con1/C3 (MOI = 0.1) and treated either with daily doses of UCDA and /or with Toco for up to day 21.

During the 21-days long follow-up studies of F0-F1, F2-F3, and F4, LS cultures, a significant, 25% to 50%, reduction in TGF-β1 RNA expression was only identified in F4 LS cultures treated with Toco, from day 5 and day 10 of the culture in non-infected and HCV infected LS cultures, respectively (Figure 13A and B). Treatment with UCDA did not induce a significant reduction in TGF-β1 RNA expression in any non-infected F0-F4 LS cultures (Figure 13C). Interestingly, from day 15, at least a two-fold reduction


A
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NINF F0F1 EtOH 5mM
NINF F0F1 EtOH 25mM
Days
B
Relative TGF-β1 expression /mg tissue
INF F0F1
INF F0F1 EtOH 1mM
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Days
C
TGF-β1(pg/mL)
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NINF F0F1 EtOH 5mM
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Relative PCOL1A1 expression /mg tissue
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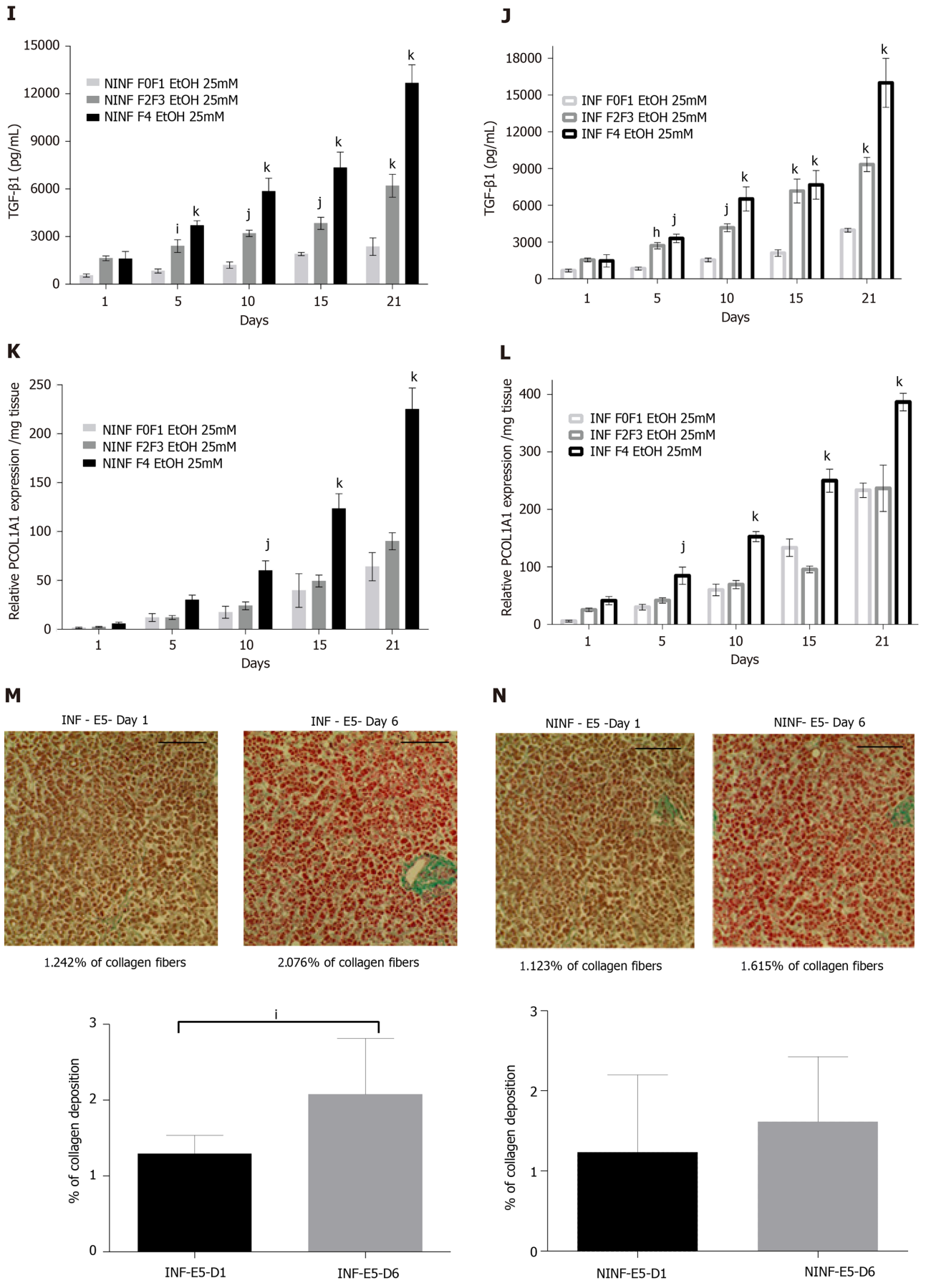


Figure 8 Significant increased expression of TGF-β1 and Procol1A1 mRNA with ethanol (1 mmol/L, 5 mmol/L, 25 mmol/L) treatment of non-infected or hepatitis C virus INF liver slice cultures from non-fibrotic (F0-F1) and ethanol (25 mmol/L) treatment of non-infected or

hepatitis C virus infected liver slice cultures from fibrotic (F2-F3, F4) liver samples as shown by real-time reverse transcription-quantitative polymerase chain reaction and ELISA and histochemistry. A and B: TGF-β1 mRNA expression (relative RNA expression / mg tissue) during 21 days follow up kinetics with ethanol (EtOH) (1 mmol/L, 5 mmol/L, 25 mmol/L) treatment in non-infected (NINF) or hepatitis C virus (HCV) INF liver slice (LS) cultures from non-fibrotic (F0-F1); C and D: Extracellular TGF-β1 protein expression (pg/mL) during 21 days follow up kinetics, with EtOH (1 mmol/L, 5 mmol/L, 25 mmol/L) treatment of NINF or HCV INF LS cultures from non-fibrotic (F0-F1); E and F: Procol1A1 mRNA expression (relative RNA expression/mg tissue) during 21 days follow up kinetic, with EtOH (1 mmol/L, 5 mmol/L, 25 mmol/L) treatment of NINF or HCV INF LS cultures from non-fibrotic (F0-F1); G and H: TGF-β1 mRNA expression (relative RNA expression/mg tissue) during 21 days follow up kinetics, in fibrotic (F2-F3, F4) NINF and HCV INF LS treated with 25 mmol/L of EtOH compared to F0F1 NINF or HCV INF LS cultures in presence of the 25 mmol/L EtOH; I and J: Extracellular TGF-β1 protein expression (pg/mL) during 21 days follow up kinetics, in fibrotic (F2-F3, F4) NINF and HCV INF LS after treatment with 25 mmol/L of EtOH compared to F0F1 NINF or HCV INF LS cultures in presence of 25 mmol/L EtOH; K and L: Relative Procol1A1 mRNA expression (relative RNA expression/mg tissue) during 21 days follow up kinetics, in fibrotic (F2-F3, F4) NINF and HCV INF LS treated with 25 mmol/L of EtOH compared to F0F1 NINF or HCV INF LS cultures in presence of 25 mmol/L EtOH. Data are expressed as means ± SEM (F0-F1, n = 5; F2-F3, n = 2; F4, n = 2). $^{k}P < 0.0001$ subject *vs* control (non-treated); $^{i}P < 0.001$ subject vs control (non-treated); $^{i}P < 0.01$ subject *vs* control (non-treated), $^{h}P < 0.05$ subject vs control (non-treated) (two-way ANOVA test); M: Significant increase of collagen deposition (% of collagen deposition) in F0-F1 HCV INF LS treated with 5 mmol/L of EtOH (E5) on day 6 (D6) compared to day 1 (D1); N: No significant change of collagen deposition (% of collagen deposition) in F0-F1 non-infected (NINF) LS treated with 5 mmol/L of EtOH (E5) on day 6 (D6) compared to day 1 (D1). Data are expressed as means ± SEM (n = 8). $^{i}P < 0.01$ subject *vs* control (non-treated), (unpaired two-tailed Student's *t*-test). Magnification 20X, Scale bars 100 μm.

in TGF-β1 RNA expression, at least, F2-F3, and F4 infected LS cultures was observed (Figure 13D). There was no change in TGF-β1 RNA expression in non-infected LS treated with both UCDA and Toco, whatever the stage of disease (Figure 13E). TGF-β1 RNA expression in F2-F3 and F4 infected LS cultures on days 5 and 15 was reduced by nearly two fold. On day 21, TGF-β1 RNA expression in F4 LS cultures were reduced 2.5 fold (Figure 13F). During the 21-days follow-up studies of infected and non-infected F0-F1, F2-F3, and F4 LS cultures treated with both UCDA and Toco, procollagen1A1 expression was significantly reduced in non-infected and infected F0F1- F4 LS cultures compared to untreated cultures from day 15 (Figure 13G and H). In particular, the significant reduction of procollagen1A1 RNA expression (around two-fold) in treated F2-F3 infected LS cultures was observed from day 10 and from day 15 for treated F4 infected LS cultures. Triglyceride production in HCV non-infected and infected LS from F0-F1, F2-F3 and F4 LS cultures was significantly reduced by the combination treatment from day 10 in F4 HCV non-infected LS cultures (Figure 13I) and from day 1 in F4 HCV infected LS cultures (Figure 13J).

DISCUSSION

For the first time, different stages of human liver fibrogenesis were investigated *ex vivo* and for a relatively long period. Indeed, liver tissue slices remained viable for at least 21 days, as shown by the secretion of albumin and urea, the percentage of ATP production and LDH release observed during the kinetic experiment. However, the secretion of albumin and urea was lower than that in micropatterned hepatocyte co-cultures models[27]. Both fibrotic (stages F2-F4) and non-fibrotic (stages F0-F1) liver samples remained viable *ex vivo* for this period. Twenty one-day follow-up studies of LS cultures significantly improved the investigation of fibrogenesis in general, and fibrotic biomarkers, in particular. We obtained RT-qPCR analyses of the biomarkers (TGF-β1, procol1A1, MMP-2, MMP-9, α-SMA, HSP47, and VEGF) involved in molecular fibrogenesis, and estimation of anti-fibrotic drugs potency, in both non-fibrotic (F0-F1) and fibrotic livers samples (F2-F3, F4). Additional evaluation of fibrotic biomarkers performed by ELISA, histology, and by Western blotting supported RT-qPCR data. With this *ex vivo* model, sustaining hepatocyte-specific gene expression for 21 days, we induced molecular fibrogenesis using HCV, EtOH, or palmitate, thus mimicking human viral, alcoholic, and NASH liver diseases.

The most important property of this LS model is cell viability for a relatively long period of time. The expression of diverse biomarkers of fibrosis was analyzed in the presence of HCV, EtOH or palmitate using non-fibrotic F0-F1 LS cultures. The markers of fibrogenesis and triglyceride production were found to be increased in both non-infected and infected LS cultures. The addition of either EtOH or palmitate significantly increased the expression of fibrotic biomarkers. Moreover, this increase was found to be greater in HCV infected than in non-infected LS with increased triglyceride production higher in infected LS. HCV infection seemed to enhance fibrosis marker expression in the presence of ethanol or palmitate.

It is important to mention, that TGF-β1 expression, the principal marker of fibrogenesis, was higher in non-fibrotic (F0-F1) LS cultures cultivated in the presence

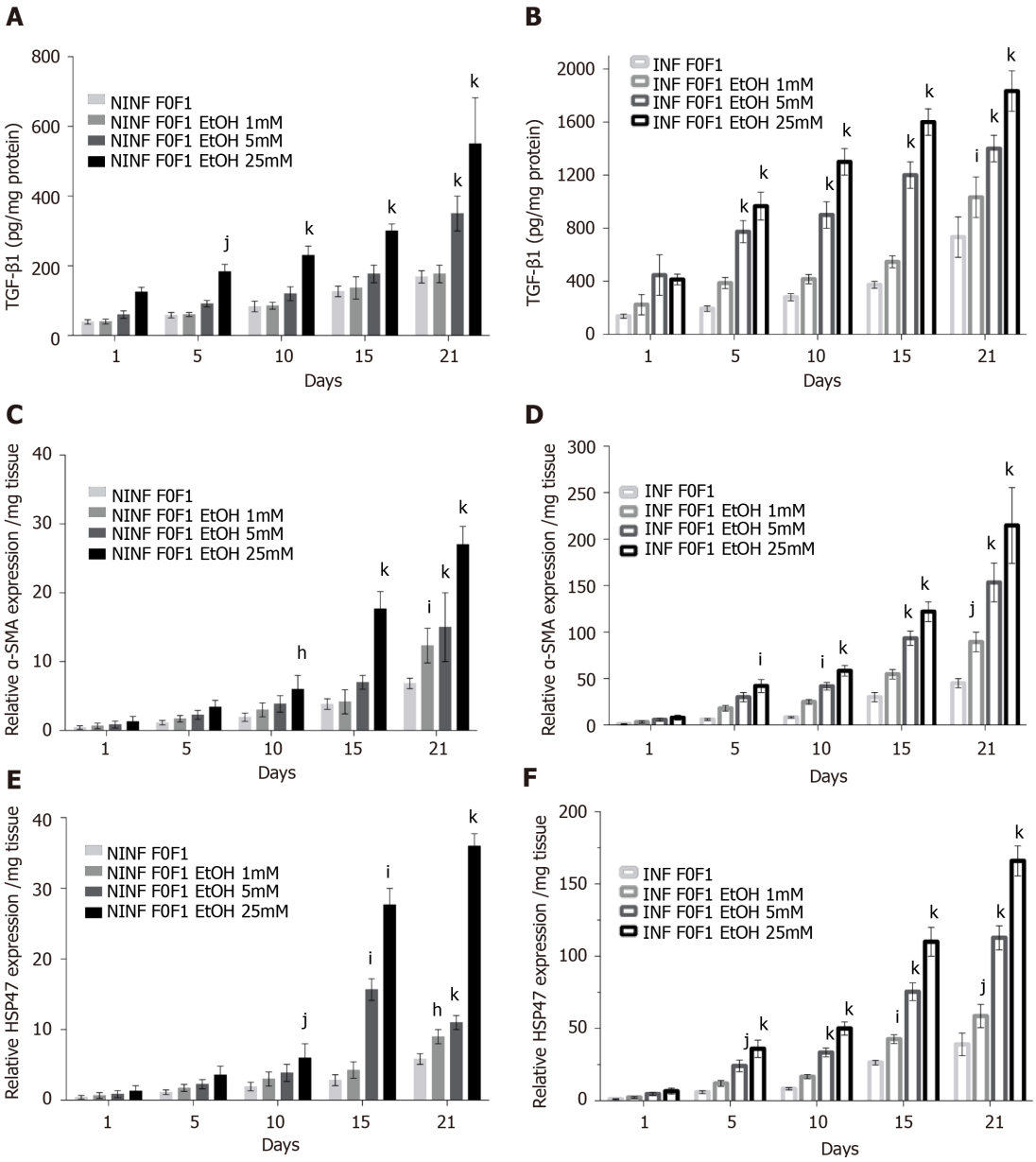
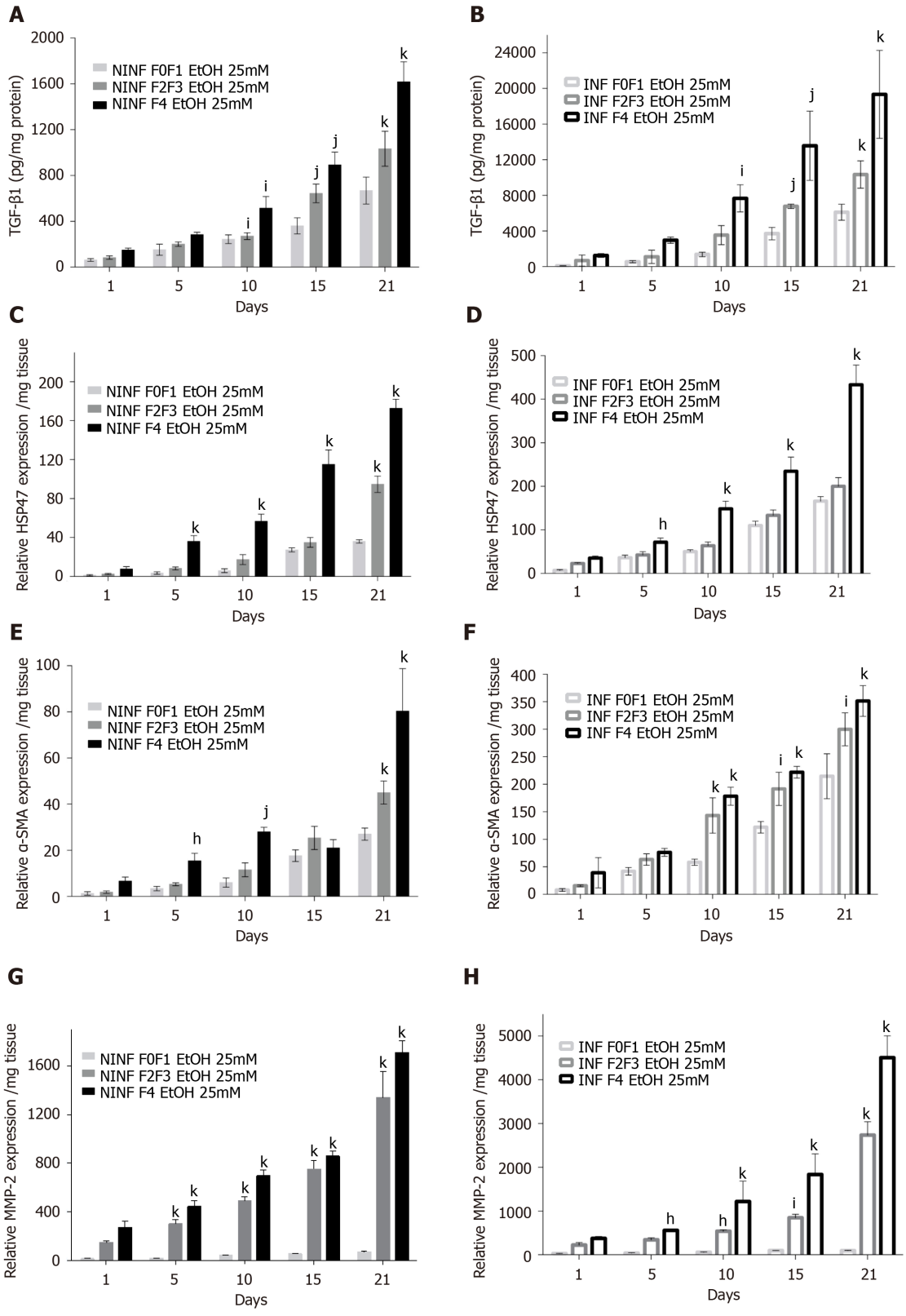


Figure 9 Significantly increase in TGF-β1 protein and RNA expression of α-SMA, and HSP47 in non-infected or hepatitis C virus-infected non-fibrotic (F0-F1) liver slice cultures treated with 1 mmol/L, 5 mmol/L and 25 mmol/L of ethanol was shown by enzyme-linked immunosorbent assay and real-time reverse transcription-quantitative polymerase chain reaction analyses. A and B: TGF-β1 intracellular protein expression (pg/mg protein) during 21 days follow up kinetics, in non-infected (A) and hepatitis C virus (HCV)-infected (B) F0-F1 liver slice (LS) cultures, treated with 1 mmol/L, 5 mmol/L, 25 mmol/L of ethanol (EtOH); C and D: Relative α-SMA RNA expression level (relative RNA expression/mg tissue) during 21 days follow up kinetics, in non-infected (C) and HCV-infected (D) F0-F1 LS cultures treated with 1 mmol/L, 5 mmol/L, 25 mmol/L of EtOH; E and F: Relative HSP47 RNA expression level expression (relative RNA expression / mg tissue) during 21 days follow up kinetics, in non-infected (E) and HCV-infected (F) F0-F1 LS cultures treated with 1 mmol/L, 5 mmol/L, 25 mmol/L of EtOH. All presented data take into account the viability of the liver slice cultures. Values are expressed as means ± SEMs (n = 5); Levels of significance: $^{k}P < 0.0001$ subject *vs* control (non-treated); $^{j}P < 0.001$ subject *vs* control (non-treated); $^{i}P < 0.01$ subject *vs* control (non-treated), $^{h}P < 0.05$ subject *vs* control (non-treated) (two-way ANOVA test).

of HCV and /or EtOH, or palmitate treatment. This effect was greater in fibrotic (F2-F4) LS cultures. Moreover, when fibrotic LS cultures were exposed to EtOH, a significant increase of α-SMA, HSP47, procol1A1 expression as well as the other markers involved in liver fibrolysis such as (MMP-2, -9) and VEGF was identified in both non-infected and infected liver slices.

The increased expression of fibrogenesis biomarkers was throughout the twenty-one days follow-up studies. RT-qPCR showed that the effect was more marked in LS cultures obtained from livers with advanced stages of fibrosis. This was confirmed for the following biomarkers: TGF-β1, α-SMA, HSP47, Procol1A1, MMP-2, -9, and VEGF. These results were further confirmed by Western blot analyses for TGF-β1, α-SMA, Col1A1, HSP47, MMP-2, -9, and VEGF. Thus, analyses of LS cultures revealed, that the


A
TGF-β1 (pg/mg protein)
NINF F0F1 EtOH 25mM
NINF F2F3 EtOH 25mM
NINF F4 EtOH 25mM
Days
B
TGF-β1 (pg/mg protein)
INF F0F1 EtOH 25mM
INF F2F3 EtOH 25mM
INF F4 EtOH 25mM
Days
C
Relative HSP47 expression /mg tissue
NINF F0F1 EtOH 25mM
NINF F2F3 EtOH 25mM
NINF F4 EtOH 25mM
Days
D
Relative HSP47 expression /mg tissue
INF F0F1 EtOH 25mM
INF F2F3 EtOH 25mM
INF F4 EtOH 25mM
Days
E
Relative α-SMA expression /mg tissue
NINF F0F1 EtOH 25mM
NINF F2F3 EtOH 25mM
NINF F4 EtOH 25mM
Days
F
Relative α-SMA expression /mg tissue
INF F0F1 EtOH 25mM
INF F2F3 EtOH 25mM
INF F4 EtOH 25mM
Days
G
Relative MMP-2 expression /mg tissue
NINF F0F1 EtOH 25mM
NINF F2F3 EtOH 25mM
NINF F4 EtOH 25mM
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H
Relative MMP-2 expression /mg tissue
INF F0F1 EtOH 25mM
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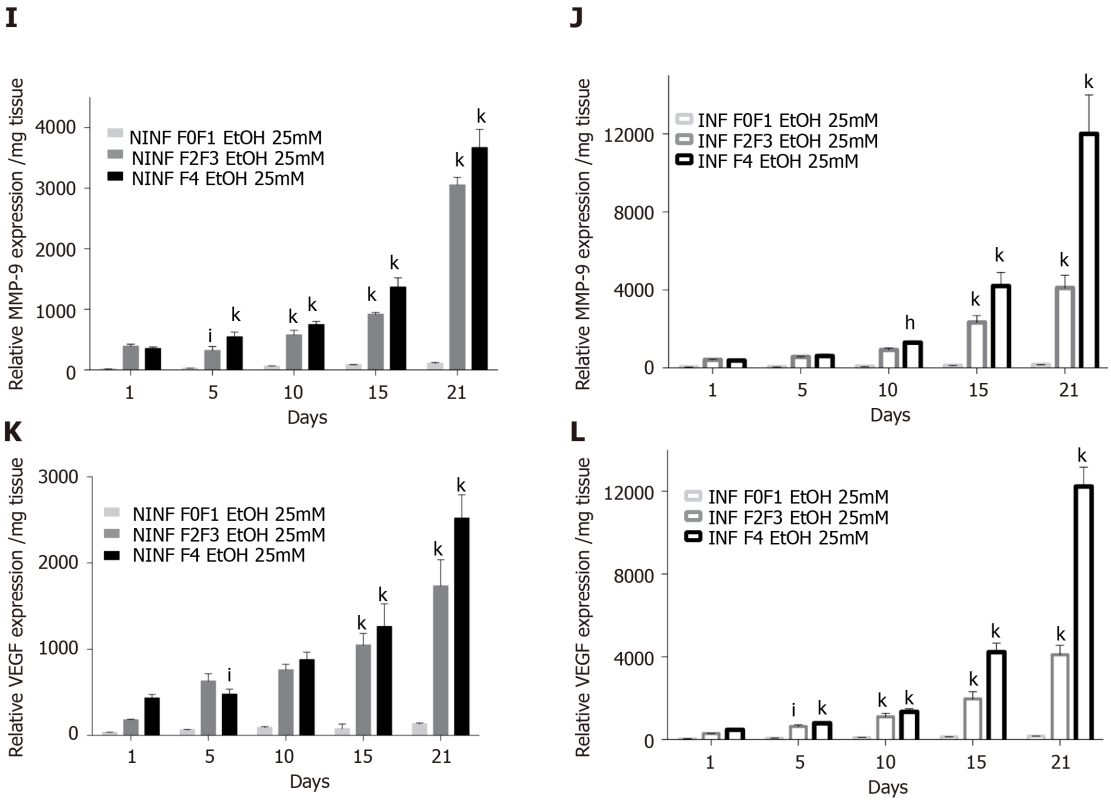

Figure 10 By real-time reverse transcription-quantitative polymerase chain reaction and enzyme-linked immunosorbent assay, significantly increase of TGF-β1 protein and RNA expression of fibrosis biomarkers HSP47, α-SMA, MMP- 2, MMP-9, VEGF increased in non-infected or hepatitis C virus-infected liver slice cultures from stages F0-F1 to stage F4 treated with 25 mmol/L of ethanol. A and B: TGF-β1 intracellular protein expression (pg/mg protein) during the 21 days follow up kinetics, in F0-F1 to F4 non-infected (A) and hepatitis C virus (HCV)-infected (B) liver slice (LS), treated with 25 mmol/L of ethanol (EtOH); C and D: Relative HSP47 RNA expression (relative RNA expression/mg tissue) during the 21 days follow up kinetics, in F0-F1 to F4 non-infected (C) and HCV infected (D) LS treated with 25 mmol/L of EtOH; E and F: Relative α-SMA RNA expression (relative RNA expression / mg tissue) during the 21 days follow up kinetics, in F0-F1 to F4 non-infected (E) and HCV-infected (F) LS treated with 25 mmol/L of EtOH; G and H: Relative MMP-2 RNA expression (relative RNA expression / mg tissue) during the 21 days follow up kinetics, in F0-F1 to F4 non-infected (G) and HCV-infected (H) LS cultures treated with 25 mmol/L of EtOH; I and J: Relative MMP-9 RNA expression (relative RNA expression / mg tissue) during the 21 days follow up kinetics, in F0-F1 to F4 non-infected (I) and HCV-infected (J) LS treated with 25 mmol/L of EtOH; K and L: Relative VEGF RNA expression (relative RNA expression/mg tissue) during the 21 days follow up kinetics, in F0-F1 to F4 non-infected (K) and HCV-infected (L) LS cultures treated with 25 mmol/L of EtOH. Values are expressed as mean ± SEMs (F0-F1: $n = 5$; F2-F3, $n = 2$; F4, $n = 2$). $^{k}P < 0.0001$ subject *vs* control (F0-F1); $^{i}P < 0.001$ subject *vs* control (F0-F1); $^{i}P < 0.01$ subject *vs* control (F0-F1), $^{h}P < 0.05$ subject *vs* control (F0-F1) (two-way ANOVA test).

progression of fibrosis is associated with an increase in the expression of certain biomarkers, in particular, α-SMA expression, and resembles a snowball effect, as shown by histochemistry results with a significant increase of collagen production in F0-F1 EtOH treated HCV infected LS on day 6 compared to day 1. As might be expected, a more marked fibrogenesis reaction was observed in fibrotic (F2-F3, F4) LS cultures, than that in non-fibrotic (F0-F1) LS cultures.

Thus, the LS model well responded to fibrotic inducers and, then, released a set of biomarkers that are usually detected during clinical studies in patients with fibrosis. In particular, this included TGF-β, α-SMA, Procollagen1A1, MMP-2, MMP-9, VEGF, the markers of liver fibrogenesis, whatever the origin of fibrosis. This study also showed the synergistic effect of liver comorbidities (virus, alcohol, and fat) on fibrogenesis and its consequences[28,29]. Finally, the efficacy of hepatoprotective[28] or anti-fibrotic drugs[29] was suggested in the LS cultures model. Recently, Wu *et al*[10] demonstrated that Human liver slices collected from resected livers could be maintained in *ex vivo* culture over a two-week period.

Several anti-fibrotic drugs are now in development[27-29], following validation in animal models[30], in particular, target inhibitors for the treatment of NASH-related fibrosis. This includes NGM282, an FGF19 analog that reduces steatosis, biliary acids injury, and lipotoxicity *via* 2 receptors, the MGL-3196, a THR-β1 agonist that decreases LDL-cholesterol, triglyceride and fatty liver, thus lipotoxicity[30,31]. Randomized controlled trials are known to take time and the results may be disappointing despite the encouraging results of the recent REGENERATE trial, in obeticholic acid[32-36]. For example, Cenicriviroc, a dual CCR2/CCR5 antagonist with positive results in mice,

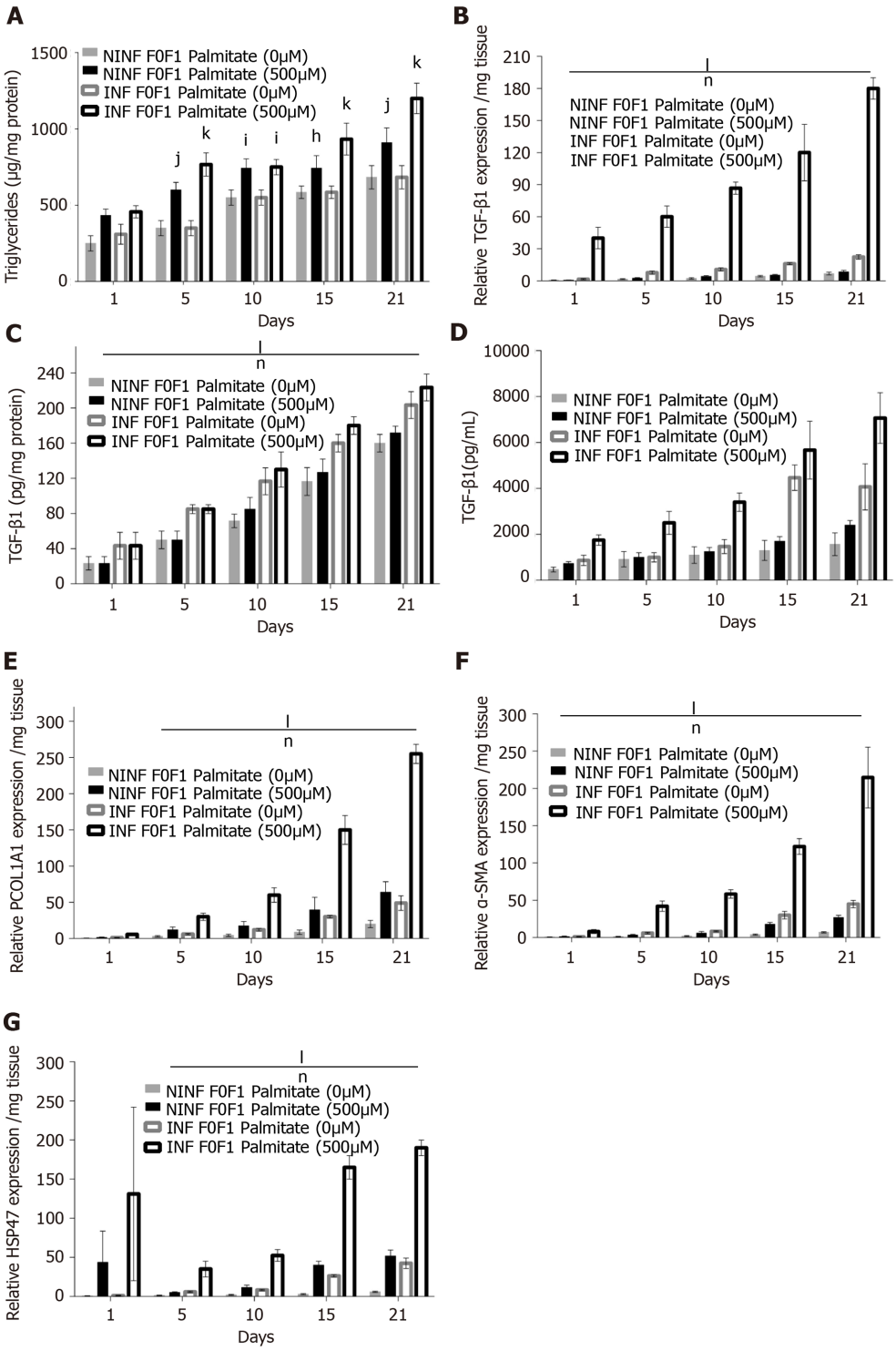

Figure 11 Significant increase of intracellular triglyceride production and RNA expression of fibrosis liver markers in non-fibrotic (F0-F1) hepatitis C virus INF liver slice cultures treated with palmitate (500 µmol/L) compared to non-infected and non-treated liver slice showed by enzyme-linked immunosorbent assay and real-time reverse transcription-quantitative polymerase chain reaction analyses, respectively. A: Triglyceride production (µg/mg protein) during the 21 days follow up kinetics: Non-significant production in hepatitis C virus (HCV) INF liver slice (LS) compared to non-infected (NINF) LS: (ns NINF *vs* INF); significant increase in HCV INF LS treated with palmitate compared to NINF; B: Significant increase of TGF-β1 mRNA expression (Relative RNA expression /mg tissue) during the 21 days follow up kinetics, in HCV INF LS compared to NINF LS and in HCV INF LS treated with palmitate compared to NINF; C and D: (C) Intracellular (pg/mg protein) and (D) extracellular (pg/mL) TGF-β1 protein production during the 21 days follow up kinetics, measured by enzyme-linked immunosorbent assay assays, in F0-F1 NINF and HCV INFLS cultures treated or non-treated with palmitate; Significant increase in HCV INF LS compared to NINF LS; Significant increase in HCV INF LS treated with palmitate compared to NINF; E-G: Intracellular mRNA expression (Relative RNA expression /mg tissue) of the Procol1A1 (E), α-SMA (F), HSP47 (G) during the 21 days follow up kinetics: Significant increase in HCV INF LS compared to NINF LS; Significant increase in HCV INF LS treated with palmitate compared to NINF LS. Data are expressed as mean± SEM (F0-F1, n = 5); ^{l}P < 0.0001 infection factor; ^{m}P < 0.001 infection factor; ^{n}P < 0.0001 palmitate factor (two-way ANOVA test).

did not result in any significant reduction in NASH-related fibrosis after 2 years of studies[37], and Selonsertib, an ASK1 inhibitor with putative anti-fibrotic properties, was recently withdrawn in the Stellar-3 and Stellar-4 Randomized controlled trials (Gilead, Press release, April 2019).

Although randomized clinical trials are the best way to prove the drug efficacy of a drug, there are several important limitations to this approach including the need for serial liver biopsies, suboptimal dosage schedules, or placebo double-blinded controls with a single drug. All of this can require about three years. With existing LS models, anti-fibrotic drug testing can be performed for 2-3 weeks (wk). Testing is possible for single drugs, drug combinations with similar or different agents, dose effects, stability in the liver, *etc*…

In the present study, we used our 3D LS *ex vivo* models to investigate the anti-fibrotic properties of two drugs, being tested in clinical trials. Ursodeoxycholic acid is indicated in the treatment of primary biliary cirrhosis and dissolve radiolucent gallstones in patients with a functioning gallbladder. Alpha-Tocopherol (Toco, vitamin E) is tested currently in patients with high cholesterol and NASH. A meta-analysis in a sub-group analysis of random clinical trials has shown that alpha-tocopherol has an anti-fibrotic effect compared to UCDA[36,38]. These drugs were tested alone and in a combination with the LS model. The combined treatment is not tested during the first phase of the clinical trials. The half-life of UCDA is 3.5 to 5.8 days and that of Toco is 44.5 hours. Patients must be treated daily with UCDA for 2 to 3 months and for 96 wk with Toco to obtain some clinical effects. In the LS model, Toco treatment only reduced the TGF-β1 expression in non-infected and infected LS with stage F4 after day 10. After day 15, UCDA reduced TGF-β1 expression in stage F2 to F4 infected LS. It is interesting to note that with a combination of both drugs, TGF-β1 and Procol1A1 expression was reduced significantly in LS. The level of TGF-β1 decreased nearly 2 fold in F2-F3 infected LS on day 15 and 2.5 fold on day 21, in F4 infected LS cultures. A significant reduction in procol1A1 RNA expression was found with the combination treatment in F2-F3, and in F4 infected and non-infected LS cultures with a two-fold decrease on days 15 and 21. Obviously, to confirm the results, the other dosages and proportions of drugs (in combination) should be tested. In fact, this model showed a clear decrease in the main hepatic fibrogenesis biomarker TGF-β1, in the presence of a combination of anti-fibrotic drugs (UCDA and Toco) in F2-F4 infected LS cultures with a significant decrease in both triglyceride production and Procol1A1 expression. Procol1A1 expression was significantly reduced in F2-F3, and F4 infected or non-infected LS cultures during combined treatment (UCDA and Toco). Thus, these data provide a proof of concept that this proposed 3D *ex vivo* model effectively allows a rapid evaluation of new anti-fibrotic drugs.

CONCLUSION

In summary, the 3D *ex vivo* LS model provides hepatocyte-specific gene expression for 21 days, and effectively reproduces liver fibrogenesis related to HCV infection, EtOH, or lipids exposure, thus, mimicking human viral, alcoholic, and NASH liver diseases. Our study is the proof of concept that this relatively easy model can be used to study human liver fibrogenesis of different origins and evaluate the potency of new anti-fibrotic therapies that are currently under development. In particular, this system might estimate unpredictable side effects when testing certain drug combinations.

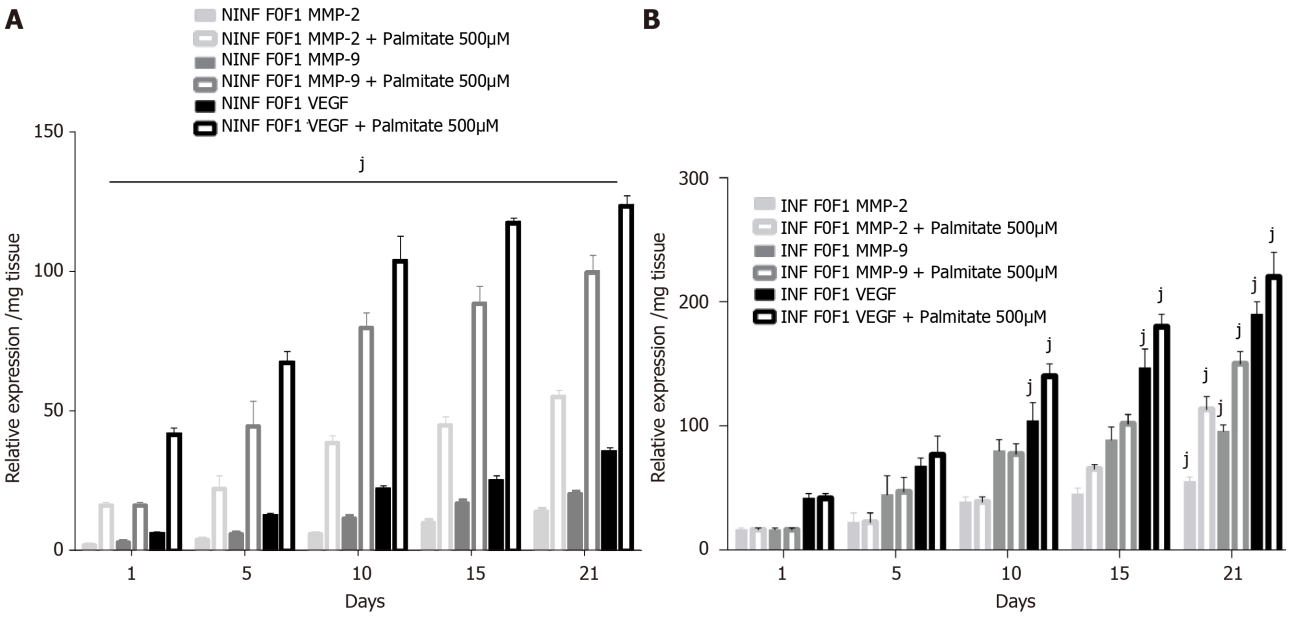

Figure 12 Significant increase of matrix metalloproteinases -2, -9, and vascular endothelial growth factor RNA expression after treatment of F0-F1 non-infected and infected liver slice cultures with palmitate (500 μmol/L). Biomarker expression estimated by real-time reverse transcription-quantitative polymerase chain reaction. A: Matrix metalloproteinases (MMP)- 2, MMP-9 and vascular endothelial growth factor mRNA expression (relative expression /mg tissue) during the 21 days follow up kinetics, in F0-F1 non-infected liver slice (LS) cultures treated without or with palmitate (500 μmol/L); B: MMP- 2, MMP-9 and vascular endothelial growth factor mRNA expression (relative expression /mg tissue) during the 21 days follow up kinetics, in F0-F1 infected LS cultures treated without or with palmitate (500 μmol/L). Real-time reverse transcription-quantitative polymerase chain reaction experiments were performed with five independent human F0-F1 liver samples (n = 5). LS were obtained in triplicate for each liver sample, at each time point in the kinetic studies. Values are expressed as means ± standard errors (n = 5). Levels of significance were as follows: $^{j}P < 0.001$ subject *vs* control (non-treated palmitate), (two-way ANOVA test).

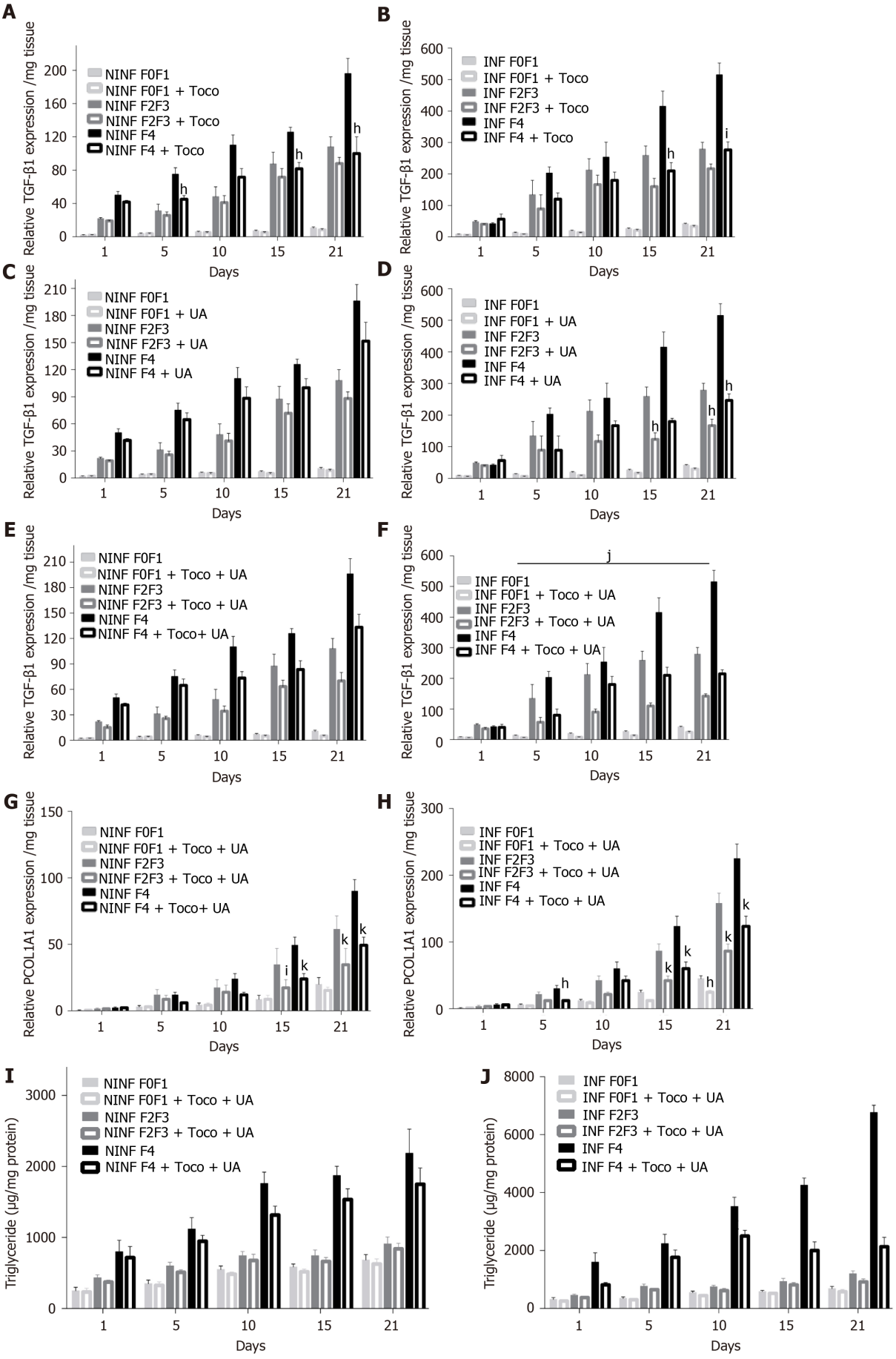

Figure 13 During treatment with alpha-Tocopherol and ursodeoxycholic acid in combination, significant inhibition of the TGF-β1 mRNA

expression of fibrotic (F2-F3, F4) hepatitis C virus INF liver slice cultures from day 5 and significant reduction of Procol1A1 mRNA expression and the triglyceride production in F0 to F4 non-infected and hepatitis C virus INF liver slice cultures during the follow-up kinetics, as evidenced the real-time reverse transcription-quantitative polymerase chain reaction analysis and enzyme-linked immunosorbent assays, respectively. A and B: TGF-β1 mRNA expression (relative TGF-β1 expression /mg tissue) during the 21 days follow up kinetics, in α-Tocopherol (Toco) treated liver slice (LS); C and D: TGF-β1 mRNA expression (relative TGF-β1 expression /mg tissue) during the 21 days follow up kinetics, in ursodeoxycholic acid (UCDA) treated LS; E and F: TGF-β1 mRNA expression (relative TGF-β1 expression /mg tissue) during the 21 days follow up kinetics, in LS during the combined treatment, Toco + UCDA. Data are expressed as means ± SEM (F2-F3 liver samples, n = 2; F4 liver samples, n = 2). $^{k}P < 0.0001$ subject *vs* control (non-treated); $^{j}P < 0.001$ subject *vs* control (non-treated); $^{i}P < 0.01$ subject *vs* control (non-treated), $^{h}P < 0.05$ subject *vs* control (non- treated) (two-way ANOVA test); G and H: Procol1A1 mRNA expression (relative Procol1A1 expression /mg tissue) during the 21 days follow up kinetics, in LS during the combined treatment, Toco + UCDA. Data are expressed as means ± SEM (F0-F1 liver samples, n = 10; F2-F3 liver samples, n = 2; F4 liver samples, n = 2). $^{k}P < 0.0001$ subject *vs* control (non-treated); $^{j}P < 0.001$ subject *vs* control (non-treated); $^{i}P < 0.01$ subject *vs* control (non-treated), $^{h}P < 0.05$ subject *vs* control (non-treated); (two-way ANOVA test); I and J: Triglyceride production (μg/mg protein) during the 21 days follow-up kinetics, in NINF and hepatitis C virus INF LS from F0-F1 to F4 LS cultures significantly reduced by the combined treatment [Toco + UCDA (UA)], more particularly from day 15 in F4 hepatitis C virus infected LS cultures. Data are expressed as means ± SEM (F0-F1, n = 5, F2-F3 liver samples, n = 2; F4 liver samples, n = 2). $^{k}P < 0.0001$ subject *vs* control (non- treated); $^{j}P < 0.001$ subject *vs* control (non-treated); $^{i}P < 0.01$ subject *vs* control (non-treated), $^{h}P < 0.05$ subject *vs* control (non-treated) (two-way ANOVA test).

ARTICLE HIGHLIGHTS

Research background

Liver fibrosis is frequently associated with viral infection [Hepatitis C virus (HCV) and Hepatitis B virus] infection, chronic inflammation, and excessive alcohol consumption. Despite effective antiviral treatment, morbidity and hepatitis-related mortalities are still increasing. Moreover, the number of non-viral liver diseases such as nonalcoholic steatohepatitis and alcoholic liver disease is steadily growing.

Research motivation

In previous studies, we developed a three dimensional (3D) *ex vivo* model of HCV replication using human liver slice cultures that were followed for 10 days to evaluate a new antiviral drug.

Research objectives

We aimed to establish a 3D *ex vivo* liver slice model viable *in vitro* for 21 days allowing us to examine human liver fibrogenesis by fibrosis inducers and anti-fibrotic therapies.

Research methods

The adult human liver tissue samples from twenty patients were collected after liver resection, and divided into three groups according to their METAVIR score (F): Non-fibrotic F0-F1, obtained during surgery for colorectal cancer liver metastases or fibrotic ranging from F2 to F4. HCV infection, alcohol (ethanol stimulation), and steatosis (palmitate stimulation) were examined in non-fibrotic F0-F1 human liver slices (HLS) compared to fibrotic (F2 to F4) liver slices (FLS) infected (or not) with HCV [Con1/C3 (genotype1b)] (INF). HLS of 350 μm (2.7×10^{6} cells per slice) were cultivated for up to 21 days. At day 0, either ursodeoxycholic acid (only choleretic and hepatoprotective properties) and/or α-tocopherol (Toco, anti-oxidant properties which could reduce fibrosis progression) were added to standard of care concentrations on HLS and FLS. The following fibrosis markers expression were assayed in HLS, in FLS and in INF FLS, [tumor growth factor-beta (TGF-β1), Hsp47, Alpha smooth muscle actin, Procol1A1, Matrix metalloproteinases 2, 9 (MMP-2, 9), Vascular endothelial growth factor] and checked by real-time reverse transcription-quantitative polymerase chain reaction and the triglyceride production by enzyme-linked immunosorbent assay assays.

Research results

Here, for the first time, human LS cultures (stages F0-F4) were successfully maintained and evaluated for 21 days allowing to explore molecular fibrogenesis in more detail including the role of important factors such as HCV infection, ethanol (EtOH), or steatosis, three of the main causes of liver injury in clinical practice. In addition, it was demonstrated that LS cultures are efficient instruments to study anti-fibrotic drugs and their combination. We obtained real-time reverse transcription-quantitative polymerase chain reaction analyses of the biomarkers (TGF-β1, procol1A1, MMP-2, MMP-9, Alpha smooth muscle actin, HSP47, and Vascular Endothelial Growth Factor) involved in molecular fibrogenesis, and estimation of anti-fibrotic drugs potency, in

both non-fibrotic (F0-F1) and fibrotic livers samples (F2-F3, F4). Expression of the fibrosis biomarkers and the progression to steatosis (estimated by triglyceride production) increased with the addition of HCV and /or EtOH or palmitate. We observed a significant decrease in both of the expression of TGF-β1, and procollagen1A1 as well as in the production of triglycerides observed in a combined anti-fibrotic treatment applied to the F2-F4 LS cultures infected with HCV.

Research conclusions

The 3D *ex vivo* LS model provides hepatocyte-specific gene expression for 21 days, and effectively reproduces liver fibrogenesis related to HCV infection, EtOH, or lipids exposure, thus, mimicking human viral, alcoholic, and nonalcoholic steatohepatitis liver diseases. Our study is the proof of concept that this relatively easy model can be used to study human liver fibrogenesis of different origins and evaluate the potency of new anti-fibrotic therapies that are currently under development. In particular, this system might estimate unpredictable side effects when testing certain drug combinations.

Research perspectives

Using the *ex vivo* model of human liver slice culture, the perspectives would be to evaluate the potency of new anti-fibrotic therapies alone or in combination and to study the immune components of liver disease.

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ORIGINAL ARTICLE

Clinical and Translational Research

Production and activity of matrix metalloproteinases during liver fibrosis progression of chronic hepatitis C patients

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Abstract

BACKGROUND

Matrix metalloproteinases (MMPs) participate in the degradation of extracellular matrix compounds, maintaining the homeostasis between fibrogenesis and fibrolytic processes in the liver. However, there are few studies on the regulation of liver MMPs in fibrosis progression in humans.

M contributed to the matrix metalloproteinases assessment; Martinez-Castillo M and Gutierrez-Reyes G contributed to the manuscript writing; Gutierrez-Reyes G contributed to the critical revision of the manuscript; All authors read and approved the final manuscript.

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Institutional review board statement: The study was previously approved by the institutional ethics committees of the Hospital General de México (HG/DI/16/107/03/082) and the Universidad Nacional Autónoma de México (FMD/DI/15/2015), guaranteeing its performance in accordance with the ethical principles described in the 1975 Declaration of Helsinki.

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AIM

To assess the production activity and regulation of matrix metalloproteinases in liver fibrosis stages in chronic hepatitis C (CHC).

METHODS

A prospective, cross-sectional, multicenter study was conducted. CHC patients were categorized in fibrosis grades through FibroTest® and/or FibroScan®. Serum MMP-2, -7, and -9 were determined by western blot and multiplex suspension array assays. Differences were validated by the Kruskal-Wallis and Mann-Whitney U tests. The Spearman correlation coefficient and area under the receiver operating characteristic curve were calculated. Collagenolytic and gelatinase activity was determined through the Azocoll substrate and zymogram test, whereas tissue inhibitor of metalloproteinase-1 production was determined by dot blot assays.

RESULTS

Serum concentrations of the MMPs evaluated were higher in CHC patients than in healthy subjects. MMP-7 distinguished early and advanced stages, with a correlation of 0.32 ($P < 0.001$), and the area under the receiver operating characteristic displayed moderate sensitivity and specificity for MMP-7 in F4 (area under the receiver operating characteristic, 0.705; 95% confidence interval: 0.605-0.805; $P < 0.001$). Collagenolytic activity was detected at F0 and F1, whereas gelatinase activity was not detected at any fibrosis stage. Tissue inhibitor of metalloproteinase-1 determination showed upregulation in F0 and F1 but downregulation in F2 ($P < 0.001$).

CONCLUSION

High concentrations of inactive MMPs were present in the serum of CHC patients, reflecting the impossibility to restrain liver fibrosis progression. MMPs could be good diagnostic candidates and therapeutic targets for improving novel strategies to reverse liver fibrosis in CHC.

Key Words: Extracellular matrix; Matrix metalloproteinases; Liver fibrosis; Chronic hepatitis C; Fibrogenesis; Fibrolysis

Core Tip: The relevance of this prospective study was to evaluate the role of matrix metalloproteinases in the pathophysiology of liver fibrosis in chronic hepatitis C patients. Matrix metalloproteinases could be used as possible therapeutic targets and as a monitoring tool in treatment-experienced patients that continue to present with liver fibrosis and develop cirrhosis and/or hepatocellular carcinoma.

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INTRODUCTION

Liver fibrosis is a convergence of repair mechanisms for chronic cellular damage, which can be induced by several etiologies, including the hepatitis B and C viruses, alcoholic liver disease (ALD) and nonalcoholic fatty liver disease, among others[1]. The intricate mechanism of the tissue repair response of extracellular matrix (ECM) compounds has been associated with the balance between fibrogenesis and fibrolysis[2]. In a normal liver, ECM proteins, fibronectin, laminin, proteoglycans and

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collagen types I, III, IV and V comprise approximately 0.5% of the wet weight[2,3]. The unregulated accumulation of ECM can result in chronic liver damage, promoting cirrhosis and hepatocellular carcinoma (HCC), with the subsequent death of patients. The uncontrolled deposition of collagen in the liver parenchyma involves the unceasing activation of hepatic stellate cells (HSCs), which represent approximately 5% to 10% of resident liver cells[4].

Some of the representative features of activated or transdifferentiated HSCs to myofibroblast-like cells are the loss of vitamin A storage, proliferation, inflammation, chemotaxis and ECM production[5,6]. Transforming growth factor beta (TGF-β) is the most potent fibrogenic cytokine and promotes smad-3 protein activation, stimulating the active transcription of collagen type I and III[7]. TGF-β can also activate the MAPK/p38/c-JNK pathway[8], inducing a continuous proinflammatory milieu. Other proliferative HSC inductors have been well described, such as PDGF, CTGF and VEGF[1].

The control of collagen and other ECM elements is known to be regulated by the family of matrix metalloproteinases (MMPs)[9]. The MMPs have been classified into broad groups related to their activity: Collagenases, gelatinases, membrane-type MMPs, stromelysins and matrilysins[10]. MMPs also play important roles in the degradation and activation of immune mediators (*e.g.*, cytokines and antimicrobial peptides)[2]; those biologic regulators are usually released as zymogens that require additional processing in the extracellular space by self-activation, the indirect action of plasminogen and the assistance of transmembrane MMP activity. Thus, some reports state that MMPs may display dual roles in liver fibrosis, depending on the timing of action. Proteolytic activity is mainly controlled by reversible tissue inhibitors of metalloproteinases (TIMPs, 1-4)[11,12]. In fact, activated HSCs have been reported to upregulate TIMP-1, enabling the accumulation of ECM proteins in the extracellular space[13,14].

Approximately 20 years ago, MMP-2 was described to be overexpressed in the liver parenchyma of human fibrotic and cirrhotic patients[15], and a direct association with collagen I expression was also reported in an animal model[14]. In addition, MMP-2 participated in the activation of TGF-β and the modulation of IL-1β, TNF-α and MCP-3 by proteolytic cleavage[16]. In 2000, Lichtinghagen *et al*[17] reported that peripheral blood cells revealed a correlation between the MMP-2/TIMP-1 ratio and the histologic grade of fibrosis in patients with chronic active cirrhosis due to hepatitis C. The authors concluded that said ratio could be used as a progression marker in patients with chronic liver disease[17]. MMP-2 (gelatinases A) and MMP-9 (gelatinases B) were recently suggested as serum biomarkers of ALD severity in a region in Poland[18]. The chronologic expression of MMP-2 and MMP-9 in hepatic fibrosis was proposed using different animal models of fibrosis. Those MMPs were relatively overexpressed and TIMP-1 was downregulated after the fibrosis inductor was eliminated[14].

A recent multi-analysis of serum proteins demonstrated that MMP-7 was directly associated with fibrosis. The authors suggested that MMP-7 could be a valuable indicator of advanced fibrosis and might play a role in liver fibrogenesis, due to its role as a matrix remodeling factor[19]. However, there is little information about MMP-7 and liver fibrosis progression in the liver as well as at the serum level.

The correct determination of liver fibrosis stages is imperative for making the diagnosis and implementing therapeutic decisions. At present, there is no evidence of the production and activity of MMP-2, MMP-7 or MMP-9 or their correlation with fibrosis progression in serum samples from patients. In the present work, we evaluated the serum concentration and proteolytic capacity of MMP-2, -7 and -9 in chronic hepatitis C (CHC) patients according to fibrosis progression.

MATERIALS AND METHODS

Patient selection

A prospective, cross-sectional, observational study was conducted. Patients were carefully selected from the Hospital General de México, "Dr. Eduardo Liceaga," the Universidad Autónoma de Nuevo Leon and the Instituto Nacional de Ciencias Médicas y Nutrición "Salvador Zubirán." The patients included in the study were diagnosed with CHC (n = 119) and were treatment naïve. Fibrosis degrees were classified according to international guidelines by the FibroTest® and/or FibroScan® methods (F0, F1, F2, F3 or F4). The fibrosis stages of patients classified by FibroScan® and FibroTest® were grouped into similar intermediate classifications (F0-F1, F1-F2, F2-F3 and F3-F4). Patients whose tests were concordant or in close stages were included,

whereas patients whose results were discrepant were discarded. Patients with clinical evidence of risk alcohol consumption (AUDIT > 8) and/or systemic infections (*e.g.*, bacteria, flu, autoimmune diseases, *etc.*) and comorbidities (*e.g.*, diabetes and hypertension) were excluded. The control group consisted of blood bank donors from the Hospital General de México with negative serology for HIV and hepatitis A, B and C viruses and classified as non-risk drinkers (AUDIT < 8) (n = 119).

Clinical and biochemical values

The anthropometric variables collected for both sexes were age, height, weight and body mass index (kg/m^2; weight/height^2), and the biochemical parameters were hemoglobin, hematocrit, leukocyte count, platelets, total bilirubin, direct bilirubin, aspartate aminotransferase, alanine aminotransferase and gamma glutamyl transpeptidase.

Sample collection

A total of 30 mL of blood was drawn from all participants for the samples; 20 mL were used for the biochemical tests and 10 mL to obtain serum. The samples were centrifuged at 3500 rpm for 10 min, and the serum was recovered and stored at -80 °C until its use for evaluating MMP concentration and regulation.

Serum MMP-2, -7, and -9 determination by western blot

Serum samples were randomly collected from CHC patients and healthy individuals and incubated at 65 °C for 7 min. Total protein concentration was evaluated by the Bradford method[20]. The sample buffer (Laemmli 2X) and 10% β-mercaptoethanol (Bio-Rad) were then added and the final protein concentration was adjusted to 10 μg/μL for all the samples evaluated.

The proteins were separated using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Electrophoresis was performed at 100 V for 1 h, and the proteins were then transferred to PVDF membranes (Perkin-Elmer) at 400 mA for 60 min. The membranes were blocked with 7% skim milk dissolved in PBS and incubated for 2 h. Solid phase detection was carried out overnight at 4 °C in agitation, utilizing MMP-2, MMP-7, and MMP-9 mouse polyclonal antibodies (1:1000) (Santa Cruz Biotechnology). The membranes were washed three times with 0.05% Tween 20 in PBS and incubated with secondary goat-anti-mouse-IgG peroxidase-conjugated antibodies (1:2500) (Santa Cruz Biotechnology) for 1 h at 37 °C. The membranes were then washed with 0.05% Tween 20 in PBS and exposed to a luminol kit reagent (Santa Cruz Biotechnology) using Kodak film. Densitometry analysis was performed using the ImageJ program (http://rsb.info.nih.gov/nih-image), and the results were expressed as relative optical density.

MMP-2, -7 and -9 serum concentration

MMP concentration was evaluated by multiplex suspension array technology (Millipore®). Nontreated serum samples from patients and controls (25 μL) were evaluated using the HMMP2MAG-55K kit, which allowed the simultaneous determination of MMP-2, -7 and -9 concentrations with no cross-reactivity and minimal intra- and interassay error (% CV < 10) (Merck, Millipore ®, United States). The data were acquired utilizing Luminex200 MAGPIX® Systems equipment, following the supplier's specifications (series number 10294005; Merck, Millipore, United States). The data were validated with internal standards and controls, and minimum and maximum detection values for each protein were obtained using Luminex XPONENT software.

Azocoll quantitative assays

To determinate the collagenolytic activity of MMPs in serum, we used the chromogenic substrate, Azocoll. Two milligrams of Azocoll were incubated with 10 μg/μL of total serum protein and adjusted to 500 μL by the addition of an activation buffer at pH 9.0 (100 mmol/L glycine, 2 mmol/L $CaCl_2$) (J.T. Baker, PA, United States). The serums were incubated overnight with shaking at 37 °C in duplicate. The reaction was stopped with 10% trichloroacetic acid (500 μL) (Sigma-Aldrich, MO, United States), and the samples were centrifuged at 4600 × g for 15 min. The supernatants were collected, and their absorbances were evaluate at 520 nm. A total of 2.5 μg/mL (4.5 U/mL) of collagenase from *Clostridium histolyticum* was used for the positive control. Protease activity was reported as units per milligram; that unit of measure is equivalent to the amount of substrate degraded in 1 min per milliliter[21].

Detection of MMP activity in gelatin-zymogram

MMP activity was analyzed in serum from CHC patients and control subjects by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, copolymerized with 0.1% porcine skin gelatin (type A) (Sigma-Aldrich) or collagen (Collagen Standard from SIRCOL kit assays; Biocolor, United Kingdom) as the substrate. Concentrations of 10 μg of protein were loaded per well. Electrophoresis was performed at 100 V at 4 ^{o}C for 3 h. A total of 5 μg/mL of broad-spectrum collagenase from *Clostridium histolyticum* was used for the positive control (Collagenase P; Roche, United States). After electrophoresis, the gels were washed twice with a 2.5% Triton X-100 (Sigma-Aldrich) solution for 15 min with shaking. For MMP activation, the gels were incubated overnight with buffer solution at pH 9.0 (100 mmol/L glycine and 2 mmol/L $CaCl_2$ with or without 2 mmol/L dithiothreitol). Finally, the gels were stained with 0.5% (w/v) Coomassie brilliant blue R-250 for 30 min. Protease activities were observed after the gels were decolored with methanol-acetic acid-water (%) (50:10:40) until clear bands on a blue background were obtained.

TIMP-1 determination in fibrosis grades by dot blot

To determine TIMP-1 in the different grades of liver fibrosis, we performed dot blot assays. All samples (5 μg/mL) were placed by drops onto PVDF membranes (0.22 μm), and bound protein was determined with Ponceau S solution (Sigma-Aldrich). Dot blots were determined using an anti-TIMP-1 polyclonal antibody (1:500) overnight, followed by incubation with secondary anti-goat antibody (1:2500; Invitrogen, MA, United States). The blots were exposed to a luminol kit reagent (Santa Cruz Biotechnology, TX, United States) using Kodak photographic film. The densitometry analysis was evaluated with the ImageJ program (http://rsb.info.nih.gov/nih-image), and the data were expressed as relative optical density.

Statistical analysis

The continuous variables were described as mean ± standard error of the mean and the qualitative variables as absolute and relative frequencies (%). The qualitative variables were analyzed using the chi-square test, and the continuous parameters were analyzed using the Mann-Whitney U test. Relative optical density data and MMP and TIMP-1 activity from the western blot and Azocoll assays were plotted using GraphPad Prism Software V6 (CA, United States). The *P* values were calculated using two-way ANOVA and Bonferroni's Multiple Comparisons Test. The Pearson correlation was calculated, and the area under the receiver operating characteristic curve for MMP-7 was determined in all the fibrosis stages to determine its relevance as a biomarker. Differences were considered statistically significant when the *P* value was less than 0.05. The statistical analysis was performed using the IBM SPSS Statistics for Windows, Version 22 program (IBM Corp, NY, United States).

RESULTS

Demographic and biometric analyses

A total of 119 patients with CHC were included. Unexpectedly, the CHC group mainly consisted of women. The demographics and biochemical information were contrasted with 119 healthy subjects that were predominantly male (Table 1). Only body mass index did not display differences in the biometric analysis. The data are summarized in Table 1.

Overproduction of MMP-2, -7 and -9 in the serum of CHC patients

To evaluate the presence of MMPs in serum from patients and healthy individuals, we first determined the presence of each of the MMPs by western blot assays. The results showed the presence of bands with molecular weights of 75, 29 and 50 kDa, using specific antibodies against MMP-2, MMP-7, and MMP-9, respectively (Figure 1A). The densitometric analysis of all the MMPs evaluated showed an evident increase in the CHC patients (Figure 1B).

After observing those differences, we evaluated the specific concentration of each MMP by multiplex suspension array technology. Interestingly, the multiplexed determination of MMPs in the CHC patients (n = 119) and controls (n = 119) showed that MMP-2 had higher concentration values compared with MMP-7 and MMP-9 (Figure 2). The results correlated with the western blot assays.

Table 1 Demographic and clinical data of chronic hepatitis C patients compared with healthy individuals

	CHC, *n* = 119	CT, *n* = 119	*P* value
Sex, *n* (%)			< 0.001
Men	53 (45)	75 (63)	
Women	66 (55)	44 (37)	
Age in yr	54 ± 13	37 ± 10	< 0.001
BMI in kg/m^2	27 ± 1	28 ± 1	0.340
Hb in g/dL	14 ± 2	16 ± 1	< 0.001
Leu as 10^3/μL	5.1 ± 1.0	7.4 ± 0.2	< 0.001
Platelets as × 10^3	177 ± 78	272 ± 62	< 0.001
Total bilirubin in mg/dL	2.52 ± 1.70	0.75 ± 0.29	0.001
Direct bilirubin in mg/dL	0.18 ± 0.15	0.07 ± 0.06	0.001
AST in UI/L	60 ± 5	30 ± 11	< 0.001
ALT in UI/L	63 ± 5	26 ± 18	< 0.001
GGT in UI/L	92.50 ± 10.67	30.55 ± 2.43	< 0.001

Data were expressed as the mean ± standard deviation. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; CHC: Chronic hepatitis C; CT: Control; GGT: Gamma glutamyl transpeptidase; Hb: Hemoglobin; Leu: Leukocytes.

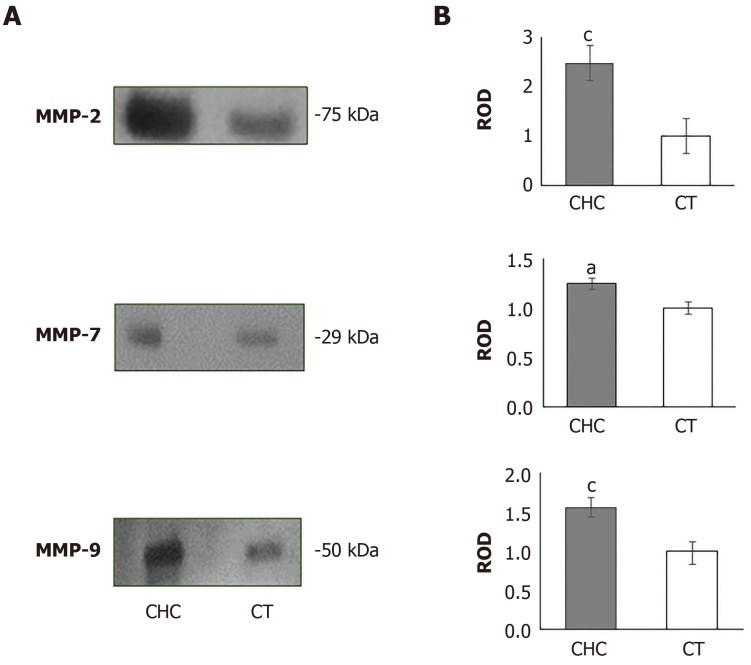


Figure 1 Matrix metalloproteinase-2, -7 and -9 determination in serum. A: Detection of bands at 75, 29 and 50 kDa in chronic hepatitis C (CHC) patients and healthy individuals corresponding to matrix metalloproteinase (MMP)-2, MMP-7 and MMP-9, respectively; B: Densitometric analysis of each of the MMPs in the patients (gray bars) and the control (CT) group (white bars) expressed as relative optical density (ROD). Relative optical density analysis was performed using ImageJ software. Bars display the mean ± standard error of the mean of five independent assays of random samples from CHC samples and CT subjects. $^aP < 0.05$; $^cP < 0.001$.

Differential production of MMPs in fibrosis stages

After determining MMP overproduction, the CHC patients were categorized into fibrosis stages (F0, F1, F2, F3 and F4) (Supplementary Table 1). Mean patient age was between 55 and 60 years, and body mass index was higher in F4 (Table 2). The comparative results of serum MMP-2 concentrations suggested decreases in stages F0, F1, and F2, but no differences were observed in any of the fibrosis grades (Figure 3A).

Table 2 Demographic and biochemical data according to fibrosis stages

	F0 (36)	F1 (11)	F2 (14)	F3 (20)	F4 (38)	Differences
Sex, *n* (%)						
Men	10 (30)	6 (60)	7 (50)	9 (45)	13 (37)	N/A
Women	26 (70)	5 (40)	7 (50)	11 (55)	25 (63)	N/A
Age in yr	50 ± 12	39 ± 7	55 ± 13	59 ± 10	55 ± 13	F0-F1[a], F0-F3[a], F1-F2[b], F1-F3[b], F1-F4[b]
BMI in kg/m^2	25 ± 4	26 ± 4	26 ± 3	27 ± 3	27 ± 5	F0-F4[a]
Hb in g/dL	15.0 ± 1.3	14.0 ± 2.0	14.0 ± 2.0	14.0 ± 2.0	14.0 ± 2.0	N/S
Leu in g/dL	5.6 ± 1.3	6.5 ± 0.8	4.2 ± 1.4	4.7 ± 1.2	4.3 ± 2.2	F0-F4[b]
Platelets as × 10^3	231 ± 56	233 ± 20	193 ± 74	163 ± 54	92 ± 39	F0-F3[b]
						F0-F4[b]
						F1-F4[b]
						F2-F4[b]
						F3-F4[b]
Total bilirubin in mg/dL	0.72 ± 0.31	0.44 ± 0.22	0.73 ± 0.28	1.75 ± 0.85	2.39 ± 2.7	F0-F1[b]
						F1-F4[b]
						F2-F4[a]
						F3-F4[a]
Direct bilirubin in mg/dL	0.18 ± 0.10	0.07 ± 0.05	0.12 ± 0.10	0.23 ± 0.15	0.27 ± 0.3	F0-F1[a]
AST in UI/L	45 ± 7	38 ± 12	49 ± 11	74 ± 10	86 ± 16	F0-F3[b]
						F0-F4[b]
						F1-F3[a]
						F1-F4[a]
ALT in UI/L	56 ± 11	56 ± 8	57 ± 10	69 ± 10	80 ± 13	F0-F4[b]
GGT in UI/L	70.49 ± 15.65	51.30 ± 19.43	52.62 ± 14.10	91.58 ± 18.74	144.00 ± 25.29	F0-F4[a]
						F1-F3[a]
						F1-F4[b]
						F2-F4[a]

[a]$P < 0.05$.

[b]$P < 0.01$. Data are expressed as the mean ± standard deviation. Fibrosis stages: F0, F1, F2, F3 and F4. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index, GGT: Gamma glutamyl transpeptidase; Hb: Hemoglobin; Leu: Leukocytes; N/A: Not applicable; N/S: Not significant.

In contrast, MMP-7 displayed a continuous increase according to fibrosis stage progression. Statistical differences were found in F0 *vs* F1, F0 *vs* F3, F0 *vs* F4, F1 *vs* F3, F1 *vs* F4, F2 *vs* F3 and F2 *vs* F4 (Figure 3B). Finally, the MMP-9 analysis showed no tendency or difference between each fibrosis stage.

MMPs as indicators of fibrosis in CHC patients

After determining that MMP-7 showed differences between fibrosis stages, we performed the Spearman correlation, and MMP-7 displayed moderate correlation (r = 0.32, $P < 0.001$) with CHC. We then determined the area under the receiver operating characteristic curve to evaluate MMP-7 as a candidate marker for fibrosis. Receiver operating characteristic (ROC) values > 0.7 were considered acceptable, whereas values below that point were discarded. The results showed that MMP-7 was not effective for distinguishing F0, F1, F2 or F3 (Figure 4A). However, the ROC values were acceptable in F4, the advanced fibrosis stage (Figure 4A and 4B). The use of MMP-7 as a complementary protein could improve the specificity and sensitivity of

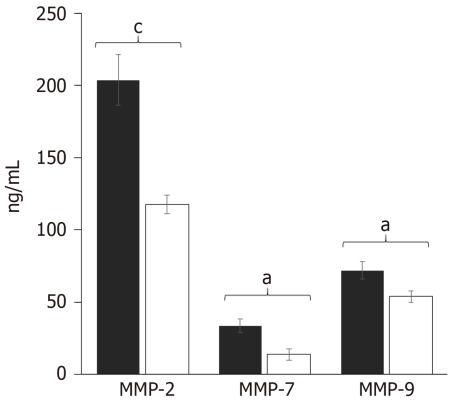


Figure 2 Matrix metalloproteinase concentrations in serum of chronic hepatitis C patients and healthy individuals. The serum concentrations of matrix metalloproteinase (MMP)-2, -7 and-9 were simultaneously determined in chronic hepatitis C patients (black bars) and healthy subjects (white bars) by multiplex suspension array technology, and the values were expressed in ng/mL. Statistical differences were obtained through the Mann-Whitney U test. Data are expressed as mean ± standard error of the mean. $^{a}P < 0.05$; $^{c}P < 0.001$.

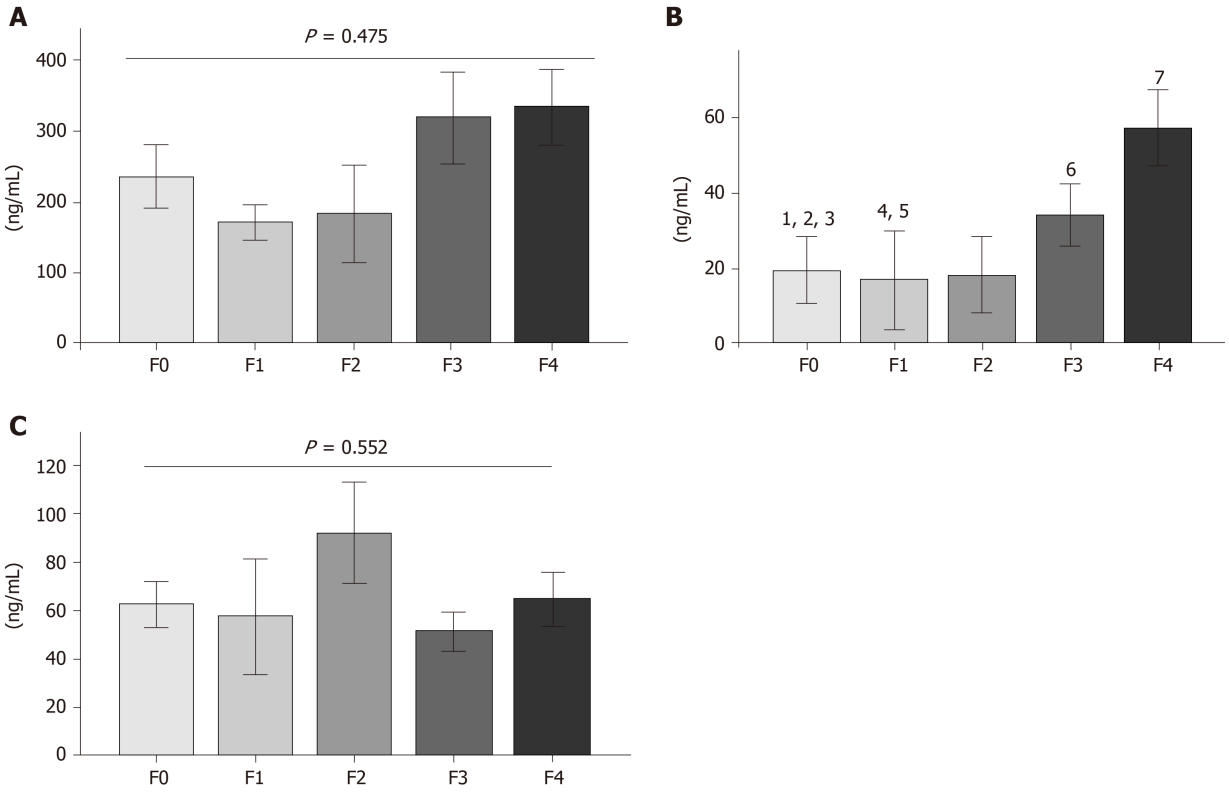


Figure 3 Matrix metalloproteinase productions according to fibrosis stage. Serum concentrations of A: Matrix metalloproteinase (MMP)-2; B: MMP-7 and C: MMP-9 of chronic hepatitis C patients classified according to fibrosis grades F0, F1, F2, F3 and F4. Statistical differences of MMP-7 were observed in 1 = F0 *vs* F1^{a}; 2 = F0 *vs* F3^{b}; 3 = F0 *vs* F4^{b}; 4 = F1 *vs* F3^{b}; 5 = F1 *vs* F4^{b}; 6 = F2 *vs* F3^{a}, 7 = F3 *vs* F4^{b}. Data are expressed as mean ± standard error of the mean. $^{a}P < 0.05$; $^{b}P < 0.01$.

the available methods for determining that fibrosis stage.

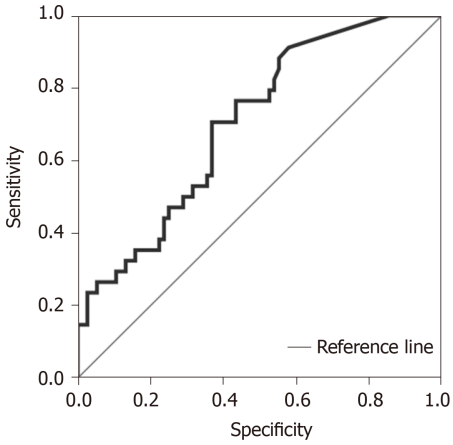
Collagenolytic and gelatinase activity of MMPs in CHC

Zymograms and Azocoll assays were performed to evaluate whether serum MMPs had proteolytic activity. The quantitative analysis of the degradation of Azocoll demonstrated that activity in the CHC patients (n = 119) was slightly higher than in the controls (n = 119) (Figure 5A). The evaluation of activity in relation to fibrosis grades showed that F0 and F1 had collagenolytic activity and that F2, F3, and F4 values were lower than those of F1 (Figure 5B). Activity was adjusted to the positive collagenase activity of *Clostridium histolyticum* reaching absorbance at 4.5 U/mL. The

A

	ROC	*P*	Lower-upper limit 95%CI
F0	0.378	0.044	0.264-0.493
F1	0.296	0.033	0.078-0.504
F2	0.338	0.058	0.190-0.486
F3	0.605	0.144	0.487-0.722

B



F4. ROC: 0.705; *P* = 0.001, 0.605-0.805

Figure 4 Area under the receiver operating characteristic analysis of matrix metalloproteinase-7 in the fibrosis stages. A: Area under the receiver operating characteristic (ROC) values and lower and upper limits of matrix metalloproteinase (MMP)-7 in F0, F1, F2 and F3 were calculated; B: Area under the ROC curve of MMP-7 in F4; area under the ROC value, statistical significance and lower-upper limits are indicated. CI: Confidence interval.

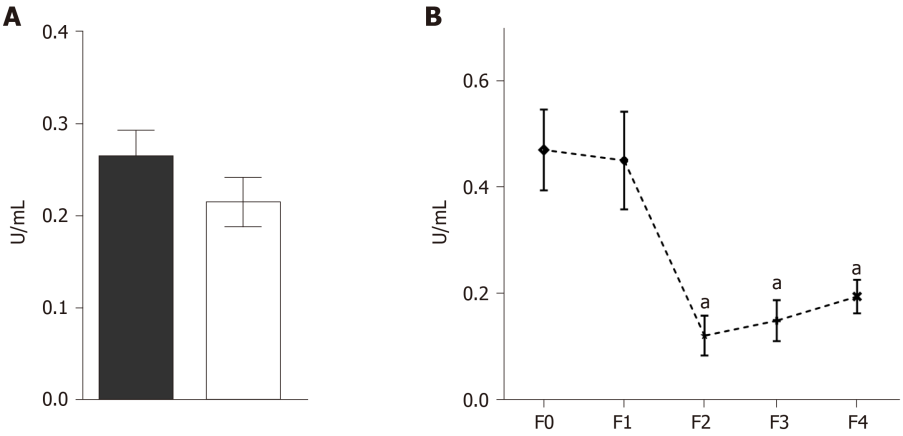


Figure 5 Collagenolytic matrix metalloproteinase activities in serum. A: Representative degradation of the Azocoll substrate reflecting collagenolytic activity in the chronic hepatitis C (CHC) patients and controls (CTs); B: Representative enzymatic activity of the matrix metalloproteinases in F0, F1, F2, F3 and F4. Matrix metalloproteinase activation was carried out with a pH 9.0 buffer at 37 °C for 2 h. Absorbance was obtained through spectrophotometry at 520 nm, and the values were adjusted to the maximal activity reached by *Clostridium histolyticum* collagenase and expressed as U/mL. Bars are expressed as mean ± standard error of the mean. [a]*P* < 0.05.

zymography assays did not detect any apparent activity of gelatinase or collagenase in either the patients or the controls (Figure 6). In contrast, the positive controls showed activity in the range of 150 to 250 kDa and activity close to 62 kDa. Similar results were observed in collagen-zymogram (data not shown). Regarding the zymography assays of fibrosis stages in the CHC patients, no enzymatic activity was shown in the sample evaluated (Figure 6B).

Differential TIMP-1 production in different fibrosis stages

To explore TIMP-1 regulation in the serum from patients with different fibrosis grades, we performed dot blot assays. At the early stages of fibrosis (F0 and F1), the patients had high levels of TIMP-1. However, at F2, TIMP-1 diminished sharply, but the levels were recovered in F3 and F4 (Figure 7B). The data were compared with the control levels. The PVDF membrane was stained with Ponceau S solution as the protein control load (Figure 7A). Statistical differences were determined through the densitometric analysis of TIMP-1 in the different fibrosis grades and the control subjects (Figure 7C).

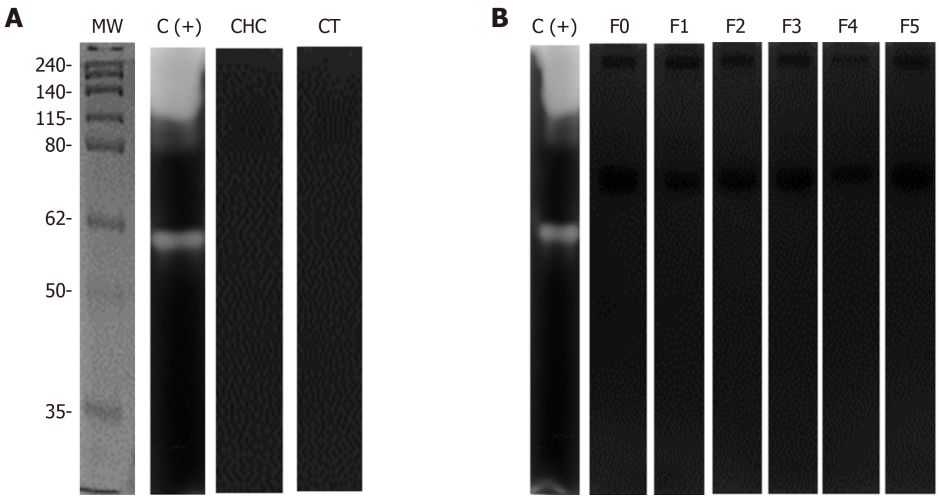


Figure 6 Evaluation of gelatinase activity by zymography. A: Representative zymography assays in 10% polyacrylamide gel electrophoresis copolymerized with 0.1 % (w/v) gelatin (molecular weight pattern). A total of 10 μg/mL of serum from chronic hepatitis C (CHC) patients and healthy individuals (n = 20) was loaded. Zymograms were activated overnight at pH 9.0 at 37 °C; B: Determination of gelatinases in different fibrosis stages (F0, F1, F2, F3 and F4) in the serum of CHC patients. Collagenase from *Clostridium histolyticum* (5 μg/mL) [C (+)] was used as the experimental control (CT). Five samples of each fibrosis stage and CT were evaluated in triplicate. MW: Molecular weight.

DISCUSSION

Up to the year 2010, approximately two million deaths worldwide (an estimated 4% of total deaths) were associated with liver diseases that included acute hepatitis, cirrhosis and liver cancer[22]. Novel antiviral treatments and changes in lifestyle have improved survival and quality of life, albeit treatment is not always successful in cases of chronic hepatitis C virus (HCV) infection. In addition, achieving alcohol abstinence and adherence to diet is no easy task[23-25]. Importantly, some patients that have been treated, and in whom viral infection is clinically eliminated continue to present with liver damage and develop cirrhosis and/or HCC[26]. Fibrosis is a common feature in the wound-healing response for most damage inductors and can be considered the key to adequate or inadequate liver parenchyma function. HSCs activated by TGF-β are thought to be the major source of collagens and TIMPs, but inactivation of that cell line is not enough to restore normal liver function. The degradation of excessive ECM is also necessary[14,27]. Collagens are degraded by MMPs, which are secreted by Kupffer cells and HSCs as proenzymes, and their activation occurs in the extracellular space. In the present work, we evaluated the serum concentration and proteolytic capacity of MMP-2, -7 and -9, in CHC patients according to fibrosis progression.

The demographic data revealed that the CHC population was older than the control group, correlating with progression and evolution time of hepatitis C, which is usually diagnosed in advanced-age patients. In contrast, the control individuals were young, which is the common age range of blood donors (35 to 45 years)[28]. Furthermore, both the CHC and control subjects presented with the obesity criteria with no apparent impact on biochemical values, including platelets and liver enzymes (aspartate aminotransferase, alanine aminotransferase and gamma glutamyl transpeptidase), which showed evident clinical alterations in CHC. Moreover, young patients were mainly identified as F1, which possibly was related to the fact that some of the study subjects then became blood donors (whose common age is from 35 to 45 years). Through the viral panel, these donors were found to be positive for HCV. Advanced stages of fibrosis were found in patients whose age ranged from 55 to 60 years.

Despite the fact that MMP-1, MMP-2, MMP-9 and MMP-13 are the most common MMPs related to liver fibrosis regulation[29], in 2015 MMP-7 was observed to be associated with liver fibrosis in biliary atresia[30]. In our study, the CHC patients had higher concentrations of MMP-7 compared with the healthy individuals, which correlated with the report of upregulation of MMP-7 in cirrhosis[19]. We provided evidence that absolute values of MMP-7 were able to distinguish mild, moderate and advanced fibrosis stages.

After evaluating the regulation of MMP-7 through fibrosis progression and observing the differences according to fibrosis stages, we performed ROC analyses in each of the stages of fibrosis (data not shown). MMP-7 showed acceptable ROC values for distinguishing F4 from the other stages of fibrosis. In previous reports, multiple

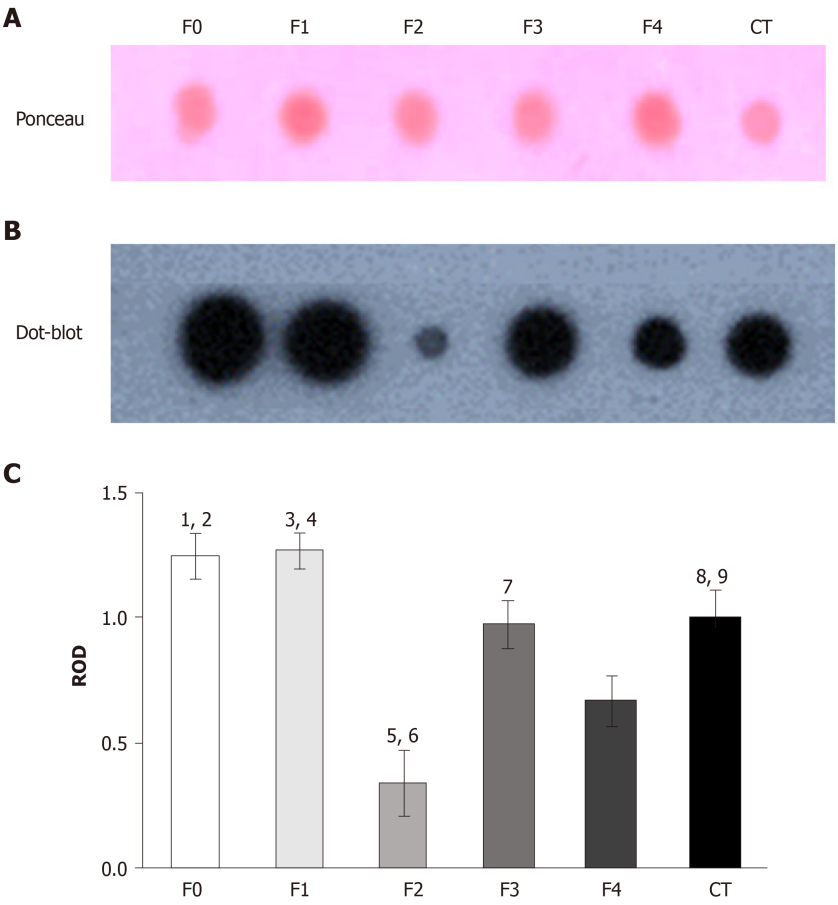


Figure 7 Tissue inhibitor of metalloproteinase -1 evaluations in the fibrosis grades. A: Representative serum protein load (5 μg/μL) onto the PVDF membrane stained with the Ponceau S solution; B: Representative dot blot assay of tissue inhibitor of metalloproteinase-1 in the different fibrosis stages (F0-F4) compared with healthy subjects; C: Densitometric analysis of each fibrosis stage and the controls (CTs; black bars) was expressed as relative optical density (ROD). Random serum samples (n = 5) of each fibrosis stage were evaluated in triplicate. ROD analysis was performed using ImageJ software, and bars display the mean ± standard error of the mean. Group comparisons: 1 = F0 *vs* F2[c]; 2 = F0 *vs* F4[c]; 3 = F1 *vs* F2[c]; 4 = F1 *vs* F4[c]; 5 = F2 *vs* F3[c]; 6 = F2 *vs* F4[c]; 7 = F3 *vs* F4[c]; 8 = CT *vs* F2[c]; 9 = CT *vs* F4[c]; [c]P < 0.001 in all the groups compared.

analyses and multivariate logistic regression modeling of MMP-7 with hyaluronic acid, MMP-1, α-fetoprotein and APRI enhanced diagnostic accuracy to 0.938 in advanced fibrosis[19]. Those types of analyses could possibly improve area under the receiver operating characteristic values in the other fibrosis stages. MMP-7 and other liver proteins in serum (*e.g*, TIMP-1 and IGFBP-7, among others)[19] can potentially improve the available diagnostic methods by enabling the precise discrimination of mild, moderate and advanced fibrosis stages. Those results are promising, and MMP-7 has been considered a predictive biomarker in other fibrogenesis pathologies, such as kidney fibrosis and idiopathic pulmonary fibrosis[31,32]. Thus, the correct clinical evaluation is crucial before using serum markers as diagnostic tools. On the other hand, both MMP-2 and MMP-9 have been proposed as serum biomarkers in ALD, and their concentrations have increased according to Child-Pugh score progression[18]. However, their activity and regulation in fibrosis stages has not been previously evaluated in detail in CHC patients.

MMP-1 is known to degrade collagen I and III, which are typical indicators of liver fibrosis[27,33]. However, in human liver biopsy samples, active MMP-2 expression in the liver parenchyma has been observed[15]. In 2011, MMP-2 was reported to suppress collagen I expression in a murine toxin-induced liver fibrosis model[34]. In the present study, we identified higher concentrations of MMP-2 in the serum of CHC patients compared with healthy subjects. Similarly, HCV-infected patients were reported to have higher circulating levels of MMP-2 than healthy donors[35,36].

Additionally, TIMP-1 levels have been shown to be significantly higher in HCV *vs* healthy donors, suggesting the presence of the inactive form of MMP-2[17]. Interestingly, we found no proteolytic activity of that gelatinase type A in our results. Our findings correlate with the histopathologic events of human liver biopsies of patients with chronic hepatitis and cirrhosis of the liver, in which *in situ* hybridization

showed a strong label of inactive MMP-2 in HSCs located in the lobules and periportal areas and in fibroblasts in the fibrous septa[15]. Our dot blot results showed that TIMP-1 had a pattern like that of MMP-2 production, demonstrating an apparent expression in mild fibrosis (F0-F1) and an important reduction in F2 suggesting that TIMP-1 acts on serum MMP-2 regulation in patients. Perhaps MMP-2 downregulation requires less regulation by its inhibitor. In addition, the analysis of fibrosis grades did not display differences in MMP-2 at any stage evaluated. A recent meta-analysis suggests that lower serum levels of MMP-2 can be found in F2 and F3. The production of MMP-2 has been reported in HSCs, monocytes, lymphocytes, dendritic cells and fibroblasts[37]. However, secretion into serum could be due to alterations in the cellular mechanism caused by HCV (*e.g.*, methylation and acetylation)[38]. Those results support the evidence that inactive MMP-2 is overproduced in CHC, but its role in fibrosis progression is uncertain.

We also found that the behavior of gelatinase B, or MMP-9, was like that of MMP-2. Our results described high levels of MMP-9 in the CHC patients, and the zymography analysis showed no gelatinase activity in the general substrate under physiologic conditions in any of the fibrosis stages evaluated (pH 7.0 and 37 °C). MMP-9 is mainly produced by Kupffer cells, but it can also be produced by lymphocytes and endothelial cells. The inactive presence of MMP-9 in the serum of patients with CHC in our study could be explained by the inactive form of MMP-2, which is its natural activator[39]. Furthermore, denaturalized collagen (Azocoll) was used as the specific substrate for MMP-7[40] showing activity in F0 and F1. However, the collagenolytic activity was drastically reduced after F2, as occurs with TIMP-1 at those stages. TIMP-1 can act as an inhibitor of MMP-7[41], but our results showed strong collagenase activity and higher levels of TIMP-1 at the same stages of fibrosis, suggesting that TIMP-1 could be involved in the partial regulation of MMP-7[38]. Similarly, TIMP-1 has also been reported to directly inhibit MMP-9. In fact, MMP-9 has been suggested as a therapeutic target for fibrosis resolution because it is related to the transdifferentiation process of HSCs and apoptosis of that cell line[29]. A longitudinal study reported an approximate 40% reduction of MMP-9 levels in patients treated with dual and triple antiviral therapies but no changes in MMP-2, TIMP-1, or TIMP-2, which the authors suggested was related to the reduction of liver inflammation[35].

Taken together, our results strongly suggest that MMPs and their activity, when determined in serum, could be complementary indicators in the diagnosis of inflammation and fibrosis, especially MMP-7 in advanced stages. The inactive stage of MMPs could be due to alterations in synthesis and production (acetylation and deacetylation, translation or post-transduction modification) caused by the HCV[38]. The identification of novel strategies or therapeutic targets to induce the fibrolytic function of MMPs could be crucial for improving the recovery from liver damage, preventing patients from progressing to HCC, even after receiving direct-acting antiviral treatment. It is also important to be familiar with the fibrolytic process in other liver diseases (nonalcoholic fatty liver disease and ALD) to understand and distinguish molecular and cellular events so that strategies can be implemented to reduce the exacerbated production of ECM and the consequent development of cirrhosis. In short, our results strongly suggest that serum MMP-7 could be used as a complementary indicator in the diagnosis of advanced fibrosis in CHC. Collagenolytic activity occurred mainly at early fibrosis stages (F0 and F1), but gelatinase activity was not detected at any fibrosis stage. Our study provides novel evidence of the increasing production and downregulation of serum MMP activity in CHC patients during the fibrolytic process and CHC progression.

CONCLUSION

Serum concentrations of MMPs were upregulated in patients with CHC, but their collagenolytic activity was limited to early fibrosis stages, whereas gelatinase functions were inactive during fibrosis progression despite their higher circulating concentrations.

The concentration and activity of MMPs, especially MMP-7, could be complementary indicators in the diagnosis of advanced fibrosis. It is possible that the HCV modulates the cellular and molecular mechanisms of MMP production affecting their correct fibrolytic functions and potentially resulting in progression to HCC. Further studies are needed to determine the exact mechanisms by which the HCV maintains MMPs inactive.

ARTICLE HIGHLIGHTS

Research background

Matrix metalloproteinases (MMPs) maintain the homeostasis between fibrogenesis and fibrolytic processes in the liver. Few studies on the production and activity of liver MMPs and fibrosis progression have been performed in humans.

Research motivation

The correct determination of liver fibrosis stages is imperative for making the diagnosis and implementing therapeutic decisions. At present, there is no evidence of the production and activity of MMP-2, MMP-7 or MMP-9 or their correlation with fibrosis progression in serum samples from patients with liver diseases.

Research objectives

In the present prospective, cross-sectional, multicenter study, we assessed the production, activity and regulation of matrix metalloproteinases in liver fibrosis stages in chronic hepatitis C (CHC).

Research methods

We selected CHC patients from the Hospital General de México, "Dr. Eduardo Liceaga," the Universidad Autónoma de Nuevo Leon and the Instituto Nacional de Ciencias Médicas y Nutrición "Salvador Zubirán." Patients were categorized in fibrosis grades through FibroTest® and/or FibroScan® (F0, F1, F2, F3 or F4). Serum concentrations of MMP-2, -7 and -9 were determined. Differences were validated by the Kruskal-Wallis and Mann-Whitney U tests. Area under the receiver operating characteristic curve was calculated in fibrosis degrees. Proteolytic activity was validated by chromogenic and enzymatic assays and serum concentration, and the regulation of tissue inhibitor of metalloproteinases-1 was tested in fibrosis progression.

Research results

We compared 119 CHC patients with 119 healthy subjects. MMP-2, -7 and -9 concentrations were higher in the patients with CHC than in the control subjects. No differences between the serum concentrations of MMP-2 and MMP-9 were found, but MMP-7 showed differential regulation in accordance with fibrosis stages as well as an acceptable receiver operating characteristic (0.705), in advanced fibrosis (F4). Collagenolytic MMP activity was maintained in F0 and F1 but decreased significantly in F2, F3 and F4. Gelatin activity was not observed in any stage of fibrosis. The concentration of tissue inhibitor of metalloproteinases-1 was lower in F2 and F4 compared with F0, F1 and healthy subjects. Inactive MMPs were found in the serum of the CHC patients.

Research conclusions

Elevated concentrations of inactive MMPs were present in the serum of CHC patients, reflecting the impossibility to restrain liver fibrosis progression. MMPs could be used in the diagnosis of liver fibrosis and the treatment for its reversal in CHC.

Research perspectives

Given that MMP-2, -7 and -9 have not been simultaneously evaluated in the serum from liver fibrosis patients, MMPs could be used to improve the currently available diagnostic methods and as therapeutic targets. They could also be used as a monitoring tool in treatment-experienced patients that continue to present with liver fibrosis and develop cirrhosis and/or hepatocellular carcinoma.

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EVIDENCE-BASED MEDICINE

Awareness of non-alcoholic steatohepatitis and treatment guidelines: What are physicians telling us?

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Abstract

BACKGROUND

There is an acute need to raise awareness of non-alcoholic fatty liver disease/non-alcoholic steatohepatitis (NAFLD/NASH) among primary care physicians, endocrinologists and diabetologists to improve patient identification and address the current difficulties in NASH clinical trial enrollment. We examined the extent of knowledge and practice regarding NASH diagnosis and management guidelines. A randomized online convenience survey of 12869 physicians drawn from a national physician database of primary care physicians (PCPs), and gastroenterology and endocrinology specialists were queried *via* online survey. Our results, based on a cohort of 185 respondents, showed gaps in knowledge and practice between these three groups of practitioners, with primary care providers having the lowest adherence to published guidelines for diagnosis of NASH. Without clear knowledge and patient identification at the point of presentation - which is often in primary care or with specialties other than hepatology–many patients with NAFLD and NASH will remain undiagnosed and untreated, and clinical studies will continue to struggle with patient recruitment, hindering clinical development and optimal patient care.

AIM

To determine knowledge base concerning NASH diagnosis amongst gastroenterologists, endocrinologists and primary care physicians to improve referrals into clinical trials.

METHODS

A randomized online convenience survey of 12869 physicians drawn from a national physician database of PCPs, and gastroenterology and endocrinology specialists was conducted yielding a sample of 185 respondents.

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RESULTS

The survey revealed that many physicians are either unaware of testing options other than biopsy, or do not use them in practice. Only 46% of endocrinologists and 42% of primary care physicians indicated they would refer a patient for specialist workup if they suspected NASH. Risk (25%) and inconvenience to patients (18%) are given as reasons for not referring those with suspected NASH for biopsy. For standard diagnostic algorithms such as Fibrosis-4 score, 18% of PCPs, 30% of endocrinologists and 65% gastroenterologists reported using these tests in clinical practice.

CONCLUSION

Substantial gaps in knowledge of the differences between NAFLD and NASH exist between these physician groups, with knowledge being particularly low among primary care doctors and endocrinologists. The use of a simple non-invasive screening algorithm may help to identify the right patients for clinical trials, which in turn will be vital to the development of effective and well-tolerated treatments for this increasingly ubiquitous condition.

Key Words: Non-alcoholic steatohepatitis; Non-alcoholic fatty liver disease; Enrollment; Screening; Diagnostics; Guidelines

Core Tip: Primary care physician knowledge of non-alcoholic steatohepatitis (NASH) diagnostics guidelines is key for appropriate patient management. We conducted a national online survey of physicians regarding their awareness of NASH guidelines. Endocrinologists and primary care physicians were significantly less likely than gastroenterologists to understand the differences between NASH and non-alcoholic fatty liver disease, as well as undertake diagnostic testing and necessary referrals for NASH. Only 18% of primary care physicians and 30% of endocrinologists were familiar with common indices such as the Fibrosis-4 score. Better education of primary care physicians about NASH could also serve as one way to identify candidates for important NASH clinical trials.

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INTRODUCTION

Prevalence and challenges

Non-alcoholic fatty liver disease (NAFLD), defined as the presence of ≥ 5% steatosis in the absence of secondary causes of fat accumulation in the liver, is the most prevalent chronic liver disease worldwide, and is thought to affect about 25% of the adult population globally[1,2]. There is some variation regionally, from 13% in Africa to more than 30% in South America and the Middle East[3]. An increasing prevalence is being seen in the developed world; NAFLD is closely associated with metabolic syndrome with the conditions being found concurrently in a substantial proportion of patients. Indeed, both the NAFLD phenotype as well as its progression to more serious disease may be viewed as an outgrowth of metabolic alterations in the context of a genetic predisposition associated with higher energy intake[4]. Up to two-thirds of patients with type 2 diabetes, and more than 90% of patients undergoing bariatric (weight loss) surgery to treat obesity present with NAFLD. Similarly, approximately a third of patients with hypertension and half of patients with dyslipidemia show evidence of the condition[5]. In the United States, there also has been an increase in the prevalence of NAFLD in children, with estimated rates up to 17%. The condition is more common in boys and a higher prevalence is seen in Hispanic children compared with white,

Asian or African-American children[6].

The natural history of NAFLD is such that the majority of patients will eventually succumb to closed volume-related mortality. However, it is estimated that up to 20% of patients with NAFLD will, during the clinical course of their disease, progress to non-alcoholic steatohepatitis (NASH), which is associated with liver inflammation and hepatocyte injury[7]. NASH also is associated with significant liver-related outcomes including fibrosis, cirrhosis, hepatocellular carcinoma, liver failure and liver death in 15%-25% of patients[8-11]. The prevalence of NASH is difficult to determine, as an unambiguous diagnosis requires a liver biopsy. In 2016 Younossi *et al*[12] reported rates of NASH among patients with NAFLD ranging from almost 7% for those without an indication for biopsy to 59% in biopsied patients[12]. Similarly, the rates of further progression of NASH are unclear, but it is thought that 10%-20% of patients will develop higher-grade fibrosis and < 5% will progress to cirrhosis[10]. NAFLD is also the most rapidly increasing indication for liver transplant[11]. The substantial prevalence of NAFLD, with an estimated 65 million patients in the United States. And 52 million in Europe (Germany, France, Italy and United Kingdom), is associated with a significant economic burden from direct medical costs estimated at $103 billion and $37 billion, respectively. The burden is significantly higher when indirect and societal costs are included[12].

In spite of its ubiquity, knowledge of NAFLD and NASH is suboptimal in clinical practice. Patients frequently present late in the NAFLD spectrum, as the condition is often silent and asymptomatic. Thus, NASH diagnosis and referral remain low. Although many potential treatment options are in clinical development, it follows that recruitment for clinical trials is extremely challenging. In April of this year, 35 clinical trials of products to treat NASH at Phase II or III were listed as recruiting globally, and requiring at least 13000 patients. However, enrollment rates are typically less than one patient *per* clinical research site *per* month, with less than 25% of recent trials achieving > 0.5 patients *per* site *per* month. Clearly, this dearth of patient enrollment will severely hamper the development and approval of new treatment options.

Knowledge of diagnostics guidelines

Several guidelines for the diagnosis and management of NAFLD and NASH have been published, including by EASL[13] and National Institute for Health and Care Excellence[14]. These guidelines have been reviewed and compared elsewhere[15]. Updated guidelines were published in 2018[1] and a clinical guidelines synopsis followed some months later[2]. However, anecdotal evidence suggests knowledge of the guidelines is poor outside of specialist physicians, and that guidelines are not being followed to the same extent that is seen in other chronic disease settings such as diabetes. The impact of this is far-reaching. Without clear knowledge and patient identification at the point of presentation – which is often in primary care or with specialties other than hepatology – many patients with NAFLD and NASH will remain undiagnosed and untreated, and clinical studies will continue to struggle with patient recruitment, hindering clinical development and optimal patient care.

MATERIALS AND METHODS

To investigate this further, a recent survey carried out by Accelerated Enrollment Solutions (AES) examined the extent of knowledge and practice regarding NASH diagnosis and management guidelines. A randomized online convenience survey of 12869 physicians drawn from a national physician database of primary care physicians (PCPs), and gastroenterology and endocrinology specialists was undertaken, yielding a cohort of 185 (response rate of 1.13%) primary care physicians and medical specialists across a number of disciplines in the United States (Table 1). Respondent physicians in the survey came from 34 states and were generally representative of the population as a whole. When asked how many years the respondents were in practice, 0.5% were in practice 0-5 years, 13.5% for 6-10 years, 38.4% for 11-20 years, 28.1% for 21-30 years and 19.5% for greater than 30 years.

The survey aimed to shed light on medical specialists' and primary care physicians' knowledge and practice regarding NASH diagnosis and management guidelines, and also to identify whether any recommendations could be made to improve adherence to guidelines in clinical practice. To determine "best practice" baseline for comparison purposes, and to identify practices of greatest importance for clinicians, we utilized practice guidelines developed in 2018 by the American Association for the Study of Liver Diseases. Results are presented here for the three largest groups – gastroenter-

Table 1 Number and proportion of participants by specialty

Specialty	*n* (%)
Gastroenterology	64 (35)
Endocrinology	60 (32)
Primary care	
Family practice	39 (21)
Internal medicine	6 (3)
General practice	2 (1)
Other	14 (8)

ologists, endocrinologists and primary care physicians. Statistical work was done in Statistical Analysis Software, with significance determined by chi-squared tests.

RESULTS

Appreciation of disease pathology and progression is important in the identification of at-risk and existing patients. However, the survey revealed substantial gaps in knowledge of the differences between NAFLD and NASH in these physician groups, with knowledge being particularly low with primary care doctors and endocrinologists (Figure 1).

Gastroenterologists were generally well informed, and therefore, it was not surprising that physicians in this group were most likely to undertake diagnostic tests firsthand (blood tests, imaging or liver biopsy) and least likely to refer the patient to another specialist (Figure 2). However, the likelihood of referral was relatively low for other groups, with only 46% of endocrinologists and 42% of primary care physicians indicating they would refer a patient for specialist workup if they suspected NASH. The lack of referral is worrying, considering the low levels of confidence in differentiating NAFLD and NASH, as well as suboptimal disease awareness among these specialties, particularly given the risk that many patients may remain undiagnosed.

Although liver biopsy is the gold standard for diagnosis of NAFLD and NASH, its invasive nature means it is rarely used outside specialist care. Risk (25%) and inconvenience to patients (18%) are given as reasons for not referring those with suspected NASH for biopsy. However, most frequently, physicians in all disciplines fail to recommend biopsy because they believe the outcome will not affect any subsequent treatment plan (34%). With the current lack of treatment availability, this is true, but with many products in the development pipeline, unambiguous identification of NASH patients is fundamental for the clinical trials that ultimately will lead to approval of new, effective and well-tolerated treatments.

Guidelines recommend that patients who are at increased risk of having steatohepatitis and/or advanced fibrosis should routinely be referred for further investigation by biopsy. Many of these patients will be those with concurrent metabolic syndrome–*i.e.*, those presenting at primary care or in endocrinology clinics. The low referral rate for biopsy suggests either a deeper lack of willingness to recommend this procedure on the part of the physician, or a suboptimal knowledge of the guidelines for diagnosis and management of NAFLD and NASH.

The survey also revealed that many physicians are either unaware of testing options other than biopsy, or do not use them in practice. NAFLD is generally recognized through abnormal liver chemistries–most commonly patients have a mildly elevated aspartate transaminase (AST) and/or alanine transaminase (ALT), with an AST:ALT ratio < 1, which in later stages may reverse. Thus, AST:ALT > 1[16,17], although a normal or near normal ALT level, does not preclude NASH.

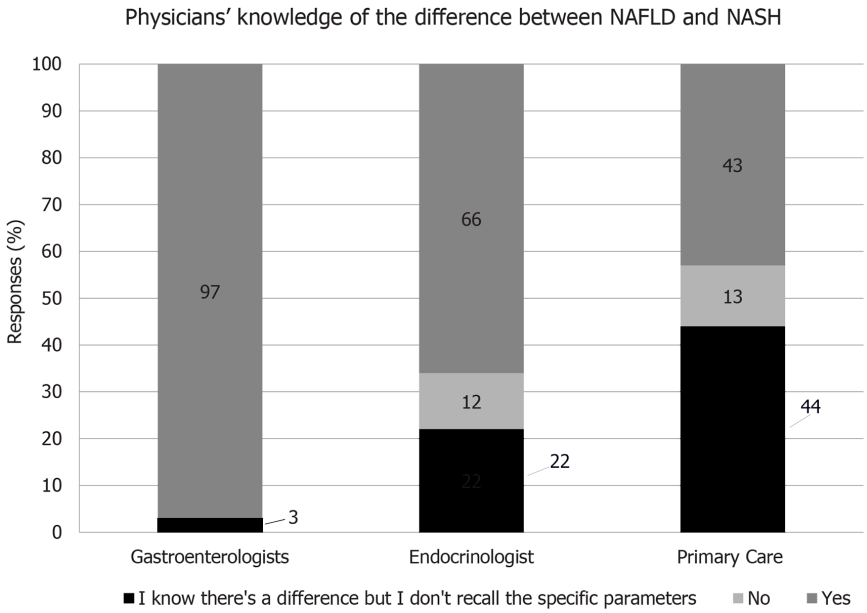

Figure 1 Knowledge of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis among physicians. NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis.

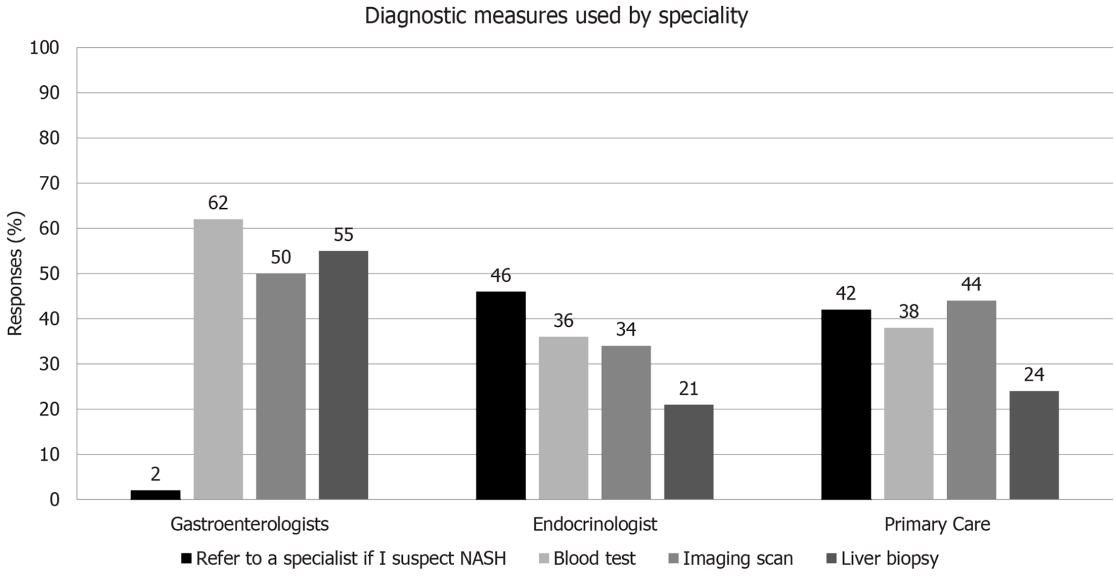

Figure 2 Diagnosis measures for non-alcoholic fatty liver disease/non-alcoholic steatohepatitis by physician specialty. NASH: Non-alcoholic steatohepatitis.

DISCUSSION

How to identify the NASH patient?

The fibrosis-4 (FIB-4) index is a biomarker test that uses outcomes from standard and easily available blood serum tests to generate a score that is correlated with the degree of liver damage in people with a variety of liver diseases. A score can be derived from age, AST and ALT, and platelet counts, and can be used as an indicator of NASH. However, only 36% of PCPs had knowledge of either this or the NAFLD Fibrosis Score (NFS), a non-invasive scoring system that takes into consideration age, hyperglycemia, body mass index, platelet count, albumin and AST/ALT ratio, as diagnostic determinants of NAFLD. In endocrinologists and gastroenterologists, these tests were familiar to 58% and 82%, respectively. However, only 18% of PCPs, 30% of endocrinologists and 65% gastroenterologists reported using these tests in clinical practice. There were significant differences between physician groups ($P < 0.0001$) in both of these cases. Given that many physicians do not opt for liver biopsy, these non-

invasive tests could be crucial to more widespread patient identification. Both of these tests are recommended in the 2018 guidelines[1] as clinically useful tools and decision aids that should be used to differentiate patients at higher risk of advanced fibrosis or cirrhosis. Thus, the lack of awareness in this area is of real concern.

Imaging studies are also an important part of the workup for NAFLD, and while imaging is used by 98% of gastroenterologists, only 83% and 82% of endocrinologists and PCPs, respectively, use the technique. Again, a significant difference ($P < 0.0004$) was seen between physician groups. Outside of liver biopsy in the gastroenterology cohort, vibration-controlled transient elastography [VCTE (FibroScan®)] and computed tomography-guided ultrasound were the techniques most commonly employed (Figure 3).

While the survey sample was small, some clear trends emerged. Although the prevalence of NAFLD and NASH is high in the general population, there are no widely accepted screening processes, even in high-risk patients[7]. As well as exacerbating under-diagnosis and under-treatment, the absence of a standardized screening system contributes to the inadequacy of the numbers of patients available for clinical trials. Currently, trials in NAFLD and NASH tend to recruit and enroll patients who already have, or are very likely to have, a diagnosis. Therefore, to optimize enrollment, an improved process for patient identification would be of great value. This need not involve invasive procedures; rather, the focus should be on identifying those individuals who are most likely to meet clinical trial eligibility criteria. This key subset of patients then can be referred for biopsy to obtain a definitive histological diagnosis. As up to 25% of patients with NAFLD are expected to show evidence of NASH on biopsy, such a screening algorithm is perhaps the most efficient and cost-effective way to identify appropriate patients. It is estimated this process would allow the screening of up to 120000 patients annually, which would greatly aid drug developers and researchers in populating clinical studies in the coming years (Figure 4).

CONCLUSION

Guidelines exist for the diagnosis of NAFLD and NASH. However, disease awareness is low, and therefore, patients are not coming through the referral pathway into the clinical studies required to push forward the development of new treatment options. This will be essential given the rise in the prevalence of NASH and the lack of approved treatment options. The clear association between NAFLD/NASH and metabolic disorders is well known, and reflected in guidance statements. Although routine screening is not recommended, the guidelines indicate physicians should have a high index of suspicion when dealing with patients presenting with these conditions. Furthermore, physicians are advised to use clinical decision aids such as NFS, FIB-4 or VCTE to identify patients who are at risk, and who would benefit from a further referral or more conclusive diagnostic testing[1]. The results from this survey suggest these recommendations are not being implemented in clinical practice, with many physicians having a poor understanding of the stages of disease and the available diagnostic techniques.

The majority of patients with NASH will present at primary care, or specialties other than hepatology. For example, endocrinologists or diabetologists are likely to see a substantial number of high-risk patients. Although it is important to raise awareness across all specialties, there is an acute need to raise awareness and improve the knowledge of NAFLD/NASH among primary care physicians, endocrinologists and diabetologists to improve patient identification and address the current difficulties in NASH clinical trial enrollment. The use of a simple non-invasive screening algorithm may help to identify the right patients for clinical trials, which in turn will be vital to the development of effective and well-tolerated treatments for this increasingly ubiquitous condition.

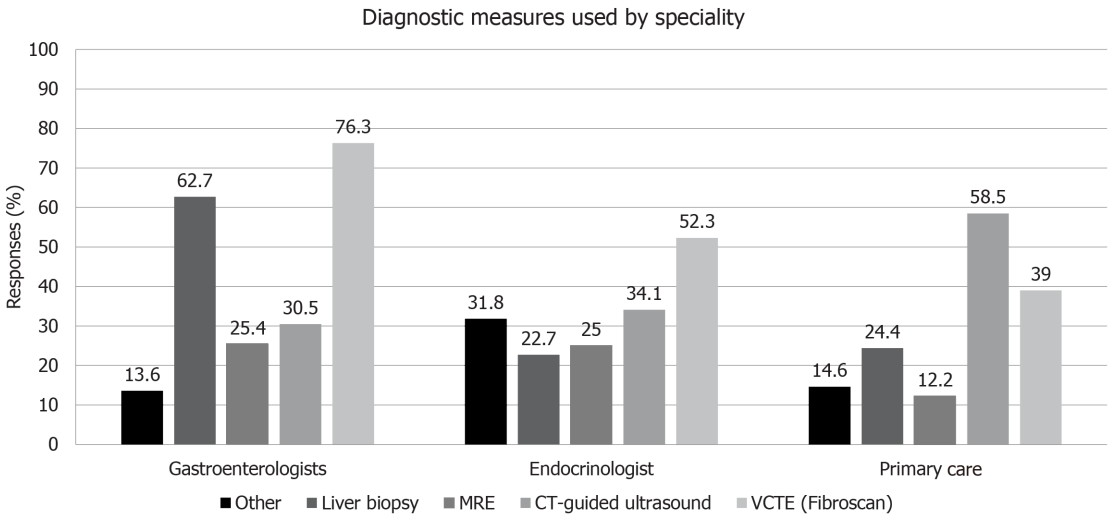

Figure 3 Diagnostic techniques used in non-alcoholic steatohepatitis physicians of different specialties. MRE: Magnetic resonance elastography; VCTE: Vibration-controlled transient elastography; CT: Computed tomography.

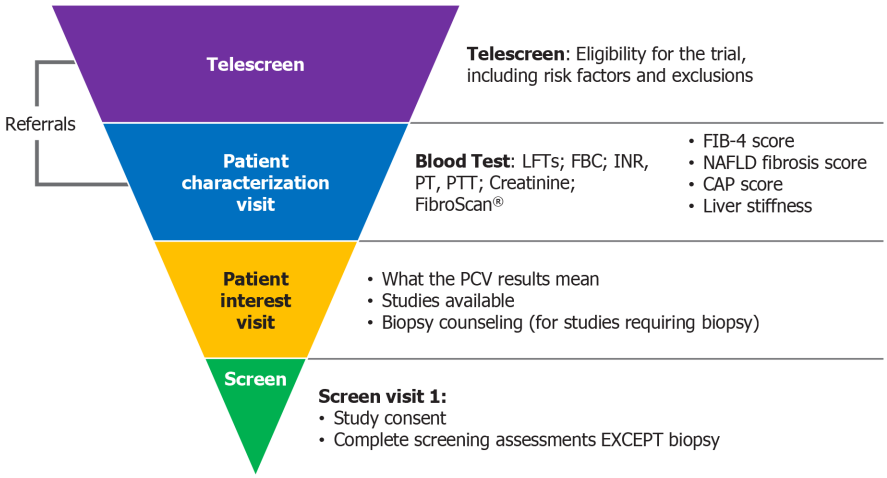

Figure 4 The non-alcoholic steatohepatitis patient recruitment screening pathway. LFT: Liver function test; FBC: Full blood count; INR: International normalized ratio; PT: Prothrombin time; PTT: Partial thromboplastin time; CAP: Controlled attenuation parameter; PCV: Porcine circovirus; NAFLD: Non-alcoholic fatty liver disease; FIB: Fibrosis.

ARTICLE HIGHLIGHTS

Research background

Medical specialist and primary care physician knowledge of non-alcoholic steatohepatitis (NASH) treatments, especially those contained in international guidelines, is important to standardize for the benefit of patient care.

Research motivation

We sought to document to what degree knowledge of NASH diagnostics, as recommended in United States guidelines, varied among United States specialists and primary care providers.

Research objectives

We sought to document to what degree knowledge of NASH diagnostics, as recommended in United States guidelines, varied among United States specialists and primary care providers.

Research methods

We utilized a randomized, online national convenience survey sample of gastroenter-

ologists, endocrinologists, and primary care physicians to inquire about their knowledge and practice regarding NASH.

Research results

While gastroenterologists were relatively well informed, endocrinologists and primary care physicians were less likely to understand the differences between NASH and non-alcoholic fatty liver disease (NAFLD), as well as undertake diagnostic testing and necessary referrals for NASH. Only 18% of primary care physicians and 30% of gastroenterologists were familiar with common indices such as the Fibrosis-4 score by which suspect NASH patients might be identified. Only 46% of endocrinologists and 42% of primary care physicians would refer a patient with a NASH profile for a NASH work-up by a specialist. Risk (25%) and inconvenience to patients (18%) were given as reasons for not referring those with suspected NASH for biopsy.

Research conclusions

Suboptimal knowledge of NASH and NAFLD by primary care physicians and by endocrinologists, both groups to which many NASH patients would be likely to present, may impair the definitive diagnosis of NASH and actions to minimize its effects. Reversing this knowledge gap can help in identification of additional and appropriate patients for enrollment into important NASH clinical trials.

Research perspectives

It is important to raise awareness of NASH among physicians of all kinds. Improved patient identification can not only improve care for the individual patient, but is also necessary to assure sufficient participation of confirmed NASH patients into randomized, placebo-controlled clinical trials for new treatment modalities.

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META-ANALYSIS

Occult hepatitis C virus infection in the Middle East and Eastern Mediterranean countries: A systematic review and meta-analysis

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Abstract

BACKGROUND

The presence of hepatitis C virus (HCV) RNA in liver tissue or peripheral blood mononuclear cells with no identified virus genome in the serum has been reported worldwide among patients with either normal or elevated serum liver enzymes. The characterization of occult HCV infection (OCI) epidemiology in the Middle East and Eastern Mediterranean (M and E) countries, a region with the highest incidence and prevalence rates of HCV infection in the world, would be effective for more appropriate control of the infection.

AIM

To estimate the pooled prevalence of OCI in M and E countries using a systematic review and meta-analysis.

METHODS

A systematic literature search was performed using international, regional and local electronic databases. Some conference proceedings and references from bibliographies were also reviewed manually. The search was carried out during May and June 2020. Original observational surveys were considered if they assessed the prevalence of OCI among the population of M and E countries by examination of HCV nucleic acid in peripheral blood mononuclear cells in at least 30 cases selected by random or non-random sampling methods. The meta-analysis was performed using Comprehensive Meta-analysis software based on heterogeneity assessed by Cochran's Q test and I-square statistics. Data were considered statistically significant at a P value < 0.05.

RESULTS

A total of 116 non-duplicated citations were found in electronic sources and grey literature. A total of 51 non-overlapping original surveys were appraised, of which 37 met the inclusion criteria and were included in the analysis. Data were

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available from 5 of 26 countries including Egypt, Iran, Pakistan, Saudi Arabia, and Turkey. The overall prevalence rate of OCI was estimated at 10.04% (95%CI: 7.66%-13.05%). The lowest OCI rate was observed among healthy subjects (4.79%, 95%CI: 2.86%-7.93%). The higher rates were estimated for patients suffering from chronic liver diseases (12.04%, 95%CI: 5.87%-23.10%), and multi-transfused patients (8.71%, 95%CI: 6.05%-12.39%). Subgroup analysis indicated that the OCI rates were probably not associated with the studied subpopulations, country, year of study, the detection method of HCV RNA, sample size, patients' HCV serostatus, and sex (all $P > 0.05$). Meta-regression analyses showed no significant time trends in OCI rates among different groups.

CONCLUSION

This review estimated high rates of OCI prevalence in M and E countries, especially among multi-transfused patients as well as patients with chronic liver diseases.

Key Words: Occult hepatitis C; Prevalence; Review; Meta-analysis; Middle East; Eastern Mediterranean region

Core Tip: No comprehensive reported data are available in the literature regarding the estimated prevalence rate of occult hepatitis C virus (HCV) infection in the Middle East and Eastern Mediterranean countries. This is the first systematic review and meta-analysis to calculate occult HCV infection rate in this region. We estimated the overall rate as well as the rates among both healthy and high-risk populations such as those infected with human immunodeficiency virus, patients with end-stage renal diseases, cryptogenic liver diseases, cleared or treated HCV infection, lymphoproliferative disorders, and multi-transfused patients.

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INTRODUCTION

The World Health Organization set the global health sector strategy on viral hepatitis in 2015 and established some service coverage targets, including the diagnosis of 90% of persons with chronic hepatitis C and treatment of 80% of the diagnosed cases to eliminate hepatitis C as a public health concern by 2030[1]. Occult hepatitis C virus (HCV) infection (OCI) was introduced as a new and challenging form of this infection in 2004[2]. OCI is characterized by the presence of HCV RNA in the liver samples of patients who were seronegative for the viral RNA[2]. Although liver biopsy is the most accurate way to diagnose OCI cases[3], a reliable and non-invasive alternative method is the examination of the peripheral blood mononuclear cells (PBMCs) for the presence of HCV genome[4,5].

Occult hepatitis C has been proposed to occur in two different clinical conditions. The first category has been described in people reactive to HCV antibodies (anti-HCV) but with normal serum levels of liver enzymes. The majority of these patients are those with HCV infection treated with antiviral drugs or cleared spontaneously. In the second type of OCI, called serologically silent, cryptogenic, or secondary OCI, both anti-HCV and serum HCV-RNA are consistently negative but an increase in liver enzymes is observed[6]. Cryptogenic OCI is found mostly in patients with cryptogenic liver disease; however, the incidence of this type of OCI was also reported among blood donors[7].

Occult hepatitis C might be a long-standing infection[8]. OCI appears to be milder than classic chronic HCV infection; however, it is likely related to the development of

liver cirrhosis or even hepatic cancer[3,9,10]. Additionally, patients with OCI may benefit from antiviral therapies[11]. OCI is a common condition worldwide and all HCV genotypes can be involved in this form of infection[11]. This infection has been described in high-risk populations, such as patients with chronic liver disease, dialysis patients, those infected with HBV or HIV, the family members of patients with HCV infection, and even apparently healthy populations[3].

The Middle East and Eastern Mediterranean (M and E) region has been reported to have the highest rates of HCV infection in the world, with an incidence of 62.5 per 100000 person-years and prevalence of 2.3% among the general population (GP). In 2015, it was estimated that approximately one-fourth of 1.75 million newly HCV-infected persons and one-fifth of 71 million chronically infected individuals in the world resided in M and E countries[12]. The median of the anti-HCV seropositivity rate in the GP of this region ranged broadly from 0.3% in Iran[13] to 13.0% in Egypt[10]. In addition, the rate of HCV viremia among anti-HCV positive individuals in M and E countries varies widely from 9% to 100% with a median of 68.8%; the overall pooled rate was averagely estimated as 67.6% (95%CI: 64.9 ± 70.3%)[14].

The prevalence rate of OCI ranged from zero to 60% among the different studied populations in various M and E countries[15-17]. To our knowledge, no review has yet been performed to provide a pooled estimate for the OCI prevalence rate in this region. In the current systematic review and meta-analysis, we aimed to determine OCI epidemiology among both healthy and risk populations in this region by (1) providing pooled mean estimates for the OCI rate through systematically reviewing and analyzing existing data in various subpopulations; (2) assessing the possible factors contributing to between-study heterogeneity; and (3) evaluating the change in OCI prevalence in different studied populations over time. The results of the present review would help professionals to make appropriate decisions for the detection and management of OCI, particularly in at-risk patients.

MATERIALS AND METHODS

Literature search

We performed this review and meta-analysis following the PRISMA 2009 statement[18]. The main object was the presence of HCV RNA in PBMCs detected by a reverse transcription-PCR (RT-PCR) technique in the blood samples of healthy individuals or different patient categories from M and E countries. The search strategy included "Occult Hepatitis C" or "Occult HCV" along with "Middle East", "Eastern Mediterranean", or the names of M and E countries. In this study, the Middle East and Eastern Mediterranean region consisted of 26 countries: Afghanistan, Algeria, Bahrain, Cyprus, Djibouti, Egypt, Iran, Iraq, Israel, Jordan, Kuwait, Lebanon, Libya, Morocco, Oman, Pakistan, Palestine, Qatar, Saudi Arabia, Somalia, Sudan, Syria, Tunisia, Turkey, United Arab Emirates, and Yemen. The considered terms were searched in the title, abstract, and keywords using Web of Science and SCOPUS and in the text using PubMed, ScienceDirect, and ProQuest databases. Likewise, some regional and local databases were searched as "Occult Hepatitis C" or "Occult HCV" to find the articles published in the English language. These databases included the Index Medicus for the Eastern Mediterranean Region, Scientific Information Database, Iranian Database of publication (Magiran), and Iranian Databank of Medical Literature. In addition, some appropriate available abstract booklets and conference proceedings were manually reviewed. The search was performed from 21 May to 08 June 2020 and then expanded by manual cross-checking all references found from bibliographies of retrieved citations.

Study selection and data extraction

The two authors screened the titles and abstracts of the documents identified in the electronic and grey literature. Duplicate and overlapping surveys (the same studied population, methods, and findings) were excluded. Regarding articles, which reported the OCI prevalence in the region, various methodological aspects of the studies were assessed using a 10-items checklist, specifically developed to evaluate both internal and external validity of the prevalence studies[19]. These aspects encompassed the representativeness of the target population, sampling methods, sample size, data collection methods and instruments, response rate, and statistical analysis. The main inclusion criteria were detection of the HCV genome in PBMCs of at least 30 healthy or high-risk subjects selected by probable or non-probable sampling methods in an original observational survey. Review articles, case reports, editorials, or letters were

removed. Surveys that evaluated OCI in hepatocytes or other samples except PBMCs were not included. In addition, studies were not entered into the meta-analysis if they did not use an acceptable case definition and/or not apply an appropriate numerator and denominator for calculating the event rates.

The full texts, tables, and figures of all relevant articles were reviewed for data extraction by the two authors. The following variables were listed for each study: First author, year of publication, years of data collection, study type, study location, studied population, sampling method, the number of cases with anti-HCV and HCV RNA seropositivity, methods used to assess HCV genome, and the number and demographic features of cases with detectable HCV RNA in their PBMCs samples. Since the surveys in this field were restricted, no specific exclusion criteria were set for the studied population, year of data collection, and patients' age and sex.

Statistical analysis

The meta-analysis was performed using Comprehensive Meta-analysis software 2.2.064 (Biostat, Englewood, NJ, United States). With inverse variance weighting, a random-effect model was applied using the DerSimonian and Laird method if heterogeneity between studies was observed based on Cochran's Q test ($P < 0.05$) and I-square statistics (I^2, values of > 50%). Forest plots were applied to demonstrate the point prevalence rates and the 95% confidence intervals (CIs). Subgroup and meta-regression analyses were implemented to identify the possible factors related to heterogeneity between surveys. HCV serostatus was classified as seronegative (negative results for both anti-HCV and serum HCV RNA) and seropositive (tested positive for anti-HCV but negative for serum HCV genome). All statistical data were considered significant at a P value < 0.05.

RESULTS

Study selection

Among 151 citations retrieved from electronic sources, 107 non-duplicated items were selected to review the titles and abstracts (Figure 1). Fifty-two surveys discussed the prevalence of OCI in M and E countries[15-17,20-68], of which 5 review articles and 2 letters were excluded[62-68]. In addition, 4 pertinent documents were identified following a review of abstracts[69-72], and 5 documents were found by a manual screening of bibliographies[73-77]. After removing 3 overlapping surveys[71,72,75], 51 original articles were chosen for a thorough review of the full-text[15-17,20-61,69,70,73,74,76,77]. Most of the surveys had used a non-probable method to select studied samples and none of them had discussed non-response bias. Fourteen articles were not included owing to methodological difficulties, small sample size, and/or analysis of liver tissue or centrifuged serum[20,24,28,30,32,34,37,40,52,56,61,70,73,77].

Finally, 37 non-duplicate and non-overlapping articles met the inclusion criteria and were included in the analysis[15-17,21-23,25-27,29,31,33,35,36,38,39,41-51,53-55,57,58,59,60,69,74,76]. All studies were cross-sectional investigations and almost all of them were based on consecutive samples selected by a non-random convenience sampling method. The mean sample size was 141 (range: 30-1280); less than 100 cases in 23 studies, 100-200 cases in 11 surveys, and more than 200 cases in three studies.

The overall prevalence of occult hepatitis C in M and E countries

Of the 26 included countries, data were available only from five countries. Egypt (n = 17) and Iran (n = 17) were the countries with the largest number of studies reporting OCI prevalence but Pakistan, Saudi Arabia, and Turkey contributed to only one data point. These five countries had surveyed OCI prevalence among a total of 5200 individuals between 2009 and 2019 (Table 1). The studied population included blood donors, patients for whom HCV infection was resolved following antiviral treatment or spontaneously, patients with cryptogenic chronic liver diseases (LDs), autoimmune hepatitis, thalassemia, hemophilia, lymphoproliferative disorders, or anemia, patients undergoing hemodialysis (HD), HIV positive individuals, and injecting drug users (IDUs). Five surveys had collected data from mixed populations, mainly healthy volunteers as well as those suffering from chronic diseases.

The studied subjects were aged 4 to 89 years and their mean age was between 26 ± 9.31 and 58.9 ± 14.7 years. In 23 studies, 53.2%-98.4% of the participants were males, in 10 surveys, 50.0%-58.1% of them were females, and sex distribution of the samples was not stated in four documents.

As shown in Table 2, the rate of OCI prevalence in this region ranged widely from

Table 1 Selected studies for systematic review and meta-analysis of occult hepatitis C virus infection prevalence in the Middle Eastern countries and Eastern Mediterranean Region

Ref.	Years of data collection	Country	Population	Serostatus	HCV RNA detection method	Sample size	OCI	
							Number	Percent
Makvandi *et al*[21], 2014	2011-2012	Iran	Patients with unexplained abnormal ALT	Seronegative	Is-nested PCR	53	17	32.08
Zaghloul *et al*[22], 2010	2010	Egypt	(1) Patients with unexplained abnormal ALT and AST; (2) Patients with chronic hepatitis C who achieved SVR	Seronegative/seropositive	rRT-PCR	102	11	10.78
El Shazly *et al*[23], 2015	2014	Egypt	Healthy sexual partners of patients with HCV infection	Seronegative	rRT-PCR	50	2	4.00
Bozkurt *et al*[74], 2014	?	Turkey	Hemodialysis patients	Seronegative	rRT-PCR	84	3	3.57
Mohamed *et al*[25], 2017	2017	Egypt	Hemodialysis patients	Seronegative	RT-PCR	60	2	3.33
Donyavi *et al*[26], 2019	2015-2018	Iran	HIV positive injecting drug users	Seronegative/seropositive	RT-nested PCR	77	14	18.18
Ayadi *et al*[27], 2019	2017-2018	Iran	Hemodialysis patients	Seronegative	RT-nested PCR	515	95	18.45
El-Rehewy *et al*[29], 2015	2012-2014	Egypt	Hemodialysis patients	Seronegative	rRT-PCR	75	8	10.67
Sheikh *et al*[31], 2019	2017-2018	Iran	Injecting drug users(negative for HIV)	Seronegative/seropositive	RT-nested PCR	115	11	9.57
Jamshidi *et al*[33], 2020	2015-2019	Iran	HIV positive patients	Seronegative/seropositive	RT-nested PCR	143	14	9.79
Abd Alla *et al*[35], 2017	2015-2017	Egypt	(1) Patients with chronic hepatitis C; (2) Healthy individuals	Seronegative/seropositive	RT-nested PCR	174	41	23.56
Ramezani *et al*[16], 2014	2014	Iran	Hemodialysis patients	Seronegative/seropositive	RT-nested PCR	30	0	0.00
Muazzam *et al*[17], 2011	2007-2009	Pakistan	Patients with chronic hepatitis C who achieved SVR	Seronegative	rRT-PCR	104	0	0.00
Naghdi *et al*[36], 2017	2017	Iran	Hemodialysis patients	Seronegative	RT-nested PCR	198	6	3.03
Abdelrahim *et al*[38], 2016	2013-2014	Egypt	Hemodialysis patients	Seronegative	rRT-PCR	81	3	3.70
Ali *et al*[39], 2018	2014	Egypt	Hemodialysis patients	Seronegative	rRT-PCR	39	9	23.08
Keyvani *et al*[41], 2013	2007-2013	Iran	Patients with cryptogenic cirrhosis	Seronegative	RT-nested PCR	45	4	8.89
Serwah *et al*[42], 2014	2013-2014	Saudi Arabia	Hemodialysis patients	Seronegative	rRT-PCR	84	12	14.29
El-shishtawy *et al*[43], 2015	2015	Egypt	(1) Hemodialysis patients; (2) Healthy volunteers	Seronegative	Strand-specific RT-PCR	63	8	12.70
Nafari *et al*[44], 2020	2017-2018	Iran	Hemophilia patients	Seronegative	RT-nested PCR	450	46	10.22
Eslamifar *et al*[71], 2015	2013	Iran	Hemodialysis patients	Seronegative	RT-nested PCR	70	0	0.00
Bokharaei-Salim *et al*[46], 2011	2007-2010	Iran	Patients with cryptogenic liver disease	Seronegative	RT-nested PCR	69	7	10.14
Rezaee Zavareh *et al*[47], 2014	2012-2013	Iran	Patients with autoimmune hepatitis	Seronegative	RT-nested PCR	35	0	0.00
Ayadi *et al*[48], 2019	2017-2018	Iran	Thalassemia patients	Seronegative	RT-nested PCR	181	6	3.31

Mekky *et al*[50], 2019	2017	Egypt	Patients with chronic hepatitis C who achieved SVR	Seropositive	rRT-PCR	1280	50	3.91
El-Moselhy *et al*[51], 2015	2014-2015	Egypt	Hemodialysis patients	Unknown	RT-PCR/RT-nested PCR	66	18	27.27
Anber *et al*[76], 2016	2015	Egypt	Hemodialysis patients	Seronegative/seropositive	rRT-PCR	63	9	14.29
Abdelmoemen *et al*[53], 2018	2016	Egypt	Hemodialysis patients	Seronegative	rRT-PCR	62	3	4.84
Eldaly *et al*[54], 2016	?	Egypt	Blood donors	Seronegative	RT-nested PCR	138	8	5.80
Youssef *et al*[55], 2012	2010-2011	Egypt	(1) Patients with lymphoproliferative disorders; (2) Healthy volunteers	Seronegative	RT-PCR/RT-nested PCR	87	12	13.79
Bastani *et al*[57], 2016	2015	Iran	Thalassemia patients	Seronegative	RT-nested PCR	106	6	5.66
Farahani *et al*[58], 2013	2010-2011	Iran	Patients with lymphoproliferative disorders	Seronegative	RT-nested PCR	104	2	1.92
Yousif *et al*[59], 2018	2017	Egypt	Patients with chronic hepatitis C who achieved SVR	Seropositive	rRT-PCR	150	17	11.33
Bokharaei-Salim *et al*[60], 2016	2014-2015	Iran	HIV positive patients	Seronegative/seropositive	RT-nested PCR	82	10	12.20
Helaly *et al*[15], 2017	2014-2015	Egypt	(1) Patients with hematologic disorders; (2) Healthy subjects	Seronegative	RT-nested PCR	50	18	36.00
Alavian *et al*[69], 2013	?	Iran	Patients with chronic hepatitis C who achieved SVR	Seropositive	RT-PCR	70	9	12.86
Askar *et al*[49], 2010	?	Egypt	Patients with unexplained persistently abnormal liver function tests	Seronegative	RT-nested PCR	45	20	44.44

ALT: Alanine transaminase; AST: Aspartate aminotransferase; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; Is-PCR: *In situ*-PCR; PCR: Polymerase chain reaction; RT-PCR: Reverse transcription PCR; rRT-PCR: Real time RT-PCR; SVR: Sustained virologic response.

0.0% to 44.44%, with a median of 10.14%. The overall mean prevalence was estimated to be 10.04% (95%CI: 7.66%-13.05%). Across subpopulations, the pooled average OCI rate was highest at 21.70% (95%CI: 11.26%-37.72%) among patients with cryptogenic liver disease, followed closely by 18.18% (95%CI: 11.07%-28.39%) among HIV positive IDUs and 18.15% (95%CI: 10.20-30.20%) in the mixed population. The rate of OCI in Egypt (12.34%; 95%CI: 8.32-17.92%) was higher than in Iran (8.48%; 95%CI: 5.51%-12.84%); however, the difference was not statistically significant (P = 0.157). Subgroup analysis showed that the rate of OCI was probably not related to the disease subpopulations (P = 0.066), year of data collection (P = 0.786), the detection method of HCV RNA (P = 0.507), sample size (P = 0.057), patients' HCV serostatus (P = 0.178) and sex (P = 0.953). Furthermore, meta-regression analysis showed no significant (P = 0.580) time trend in the OCI rate among the total population of this region.

Occult hepatitis C prevalence among healthy populations

Four studies conducted in the M and E area reported OCI prevalence among 300 apparently healthy subjects, such as healthy volunteers, blood donors, and healthy sexual partners of patients with chronic HCV infection. All four studies had been performed among Egyptian HCV-seronegative cases, of which three had studied less than 100 cases, and three had been conducted after 2014. Assessment of HCV RNA in PBMCs had been carried out using RT-nested PCR in three surveys and real-time RT-PCR in another research. Based on the fixed-effect model (Q = 0.77, P = 0.857, I^2 = Zero), the pooled estimation of OCI prevalence among healthy populations was 4.79% (95%CI: 2.86%-7.93%, Figure 2). No evidence was found for a significant trend in the OCI rate in this population over time (P = 0.802).

Occult hepatitis C prevalence among multi-transfused patients

Seventeen studies reported the OCI rate among 2217 multi-transfused patients (MTPs), including 1480 HD patients, 450 hemophilia patients, and 287 thalassemia patients in the region. Fifteen surveys evaluated the presence of viral genome among HCV

Table 2 Subgroup-specific pooled estimates of occult hepatitis C virus infection prevalence across the Middle Eastern and Eastern Mediterranean countries

Prevalence by	Number of studies	Sample sizes	OCI prevalence across studies		Pooled OCI prevalence		Heterogeneity	
			Range (%)	Median	Mean (%)	95%CI	Cochran's Q	I-squared (%)
Studied population								
Blood donors	1	138	-	-	5.80	2.93-11.16	-	-
Hemodialysis patients	13	1427	0-27.27	4.84	9.06	5.83-13.80	64.0[a]	81.2
Healthy sexual partners of patients with chronic hepatitis C	1	50	-	-	4.00	1.00-14.63	-	-
Patients with chronic hepatitis C who achieved SVR	4	1604	0-12.86	7.62	6.70	3.08-13.99	25.4[a]	88.2
Patients with cryptogenic liver disease	4	212	8.89-44.44	21.11	21.70	11.26-37.72	22.6[a]	86.7
Patients with autoimmune hepatitis	1	35	-	-	1.39	0.09-18.67	-	-
Patients with lymphoproliferative disorders	1	104	-	-	1.92	0.48-7.36	-	-
Hemophilia patients	1	450	-	-	10.22	7.74-13.38	-	-
Thalassemia patients	2	287	3.31-5.66	4.49	4.32	2.47-7.46	0.9	0.00
HIV positive individuals	2	225	9.79-12.20	10.99	10.72	7.29-15.50	0.3	0.00
HIV positive injecting drug users	1	77	-	-	18.18	11.07-28.39	-	-
Injecting drug users	1	115	-	-	9.57	5.38-16.45	-	-
Mixed population[1]	5	476	10.78-36	13.79	18.15	10.20-30.20	18.2[b]	78.1
Countries								
Egypt	17	2585	3.33-44.44	11.33	12.34	8.32-17.92	186.2[a]	91.4
Iran	17	2343	0-32.08	9.57	8.48	5.51-12.84	86.5[a]	81.5
Pakistan	1	104	-	-	0.48	0.03-7.15	-	-
Saudi Arabia	1	84	-	-	14.29	8.30-23.49	-	-
Turkey	1	84	-	-	3.57	1.16-10.49	-	-
Temporal duration[2]								
Before 2015	18	1227	0-44.44	9.52	9.58	6.26-14.40	88.5[a]	80.8
2015 and thereafter	19	3973	3.03-36	10.22	10.33	7.21-14.61	192.07[a]	90.6
Method of HCV RNA detection								
RT-nested PCR	22	2833	0-44.44	9.97	11.75	8.52-15.99	159.3[a]	86.8
Real time RT-PCR	12	2174	0-23.08	7.75	7.88	4.96-12.30	58.4[a]	81.2
RT-PCR	2	130	145.71-173.33	159.52	7.75	2.34-22.75	3.3	69.5
Strand-specific PCR	1	63	-	-	12.70	6.48-23.39	-	-
Patients' HCV serostatus								
Seronegative	25	2774	0-44.44	5.80	9.32	6.76-12.73	164.0[a]	85.4
Seropositive	4	1604	6.20-66.50	37.62	6.64	2.94-14.30	25.4[a]	88.2
Seronegative/ Seropositive	7	756	27.27-109.09	73.53	13.58	8.01-22.11	17.8[b]	66.3
Undetermined	1	66	-	-	27.27	17.91-39.19	-	-
Sample size								

Less than 100	23	1440	0-44.44	12.20	12.43	8.87-17.15	102.4[a]	78.5
100 and above	14	3760	0-23.56	7.69	7.43	4.86-11.19	153.0[a]	91.5
Patients' sex								
Female	11	602	0-35.29	8.62	9.92	5.39-17.55	32.4[a]	69.2
Male	11	1785	0-30.56	9.09	10.17	5.85-17.10	70.8[a]	85.9
All studies	37	5200	0-44.44	10.14	10.04	7.66-13.05	284.1[a]	87.3

[a]$P < 0.001$.
[b]$P < 0.01$.
[1]Four studies investigated occult hepatitis C virus infection among healthy volunteers along with hemodialysis patients, patients with chronic hepatitis C, or patients with hematologic and lymphoproliferative disorders. Also one study investigated both the patients with chronic hepatitis C and patients with cryptogenic liver disease.
[2]Based on the last year reported for the data collection. If the collection date was not clear, publication year was considered instead. CI: Confidence interval; Min: Minimum; Max: Maximum; PCR: Polymerase chain reaction; RT-PCR: Reverse transcription polymerase chain reaction; OCI: Occult hepatitis C virus infection.

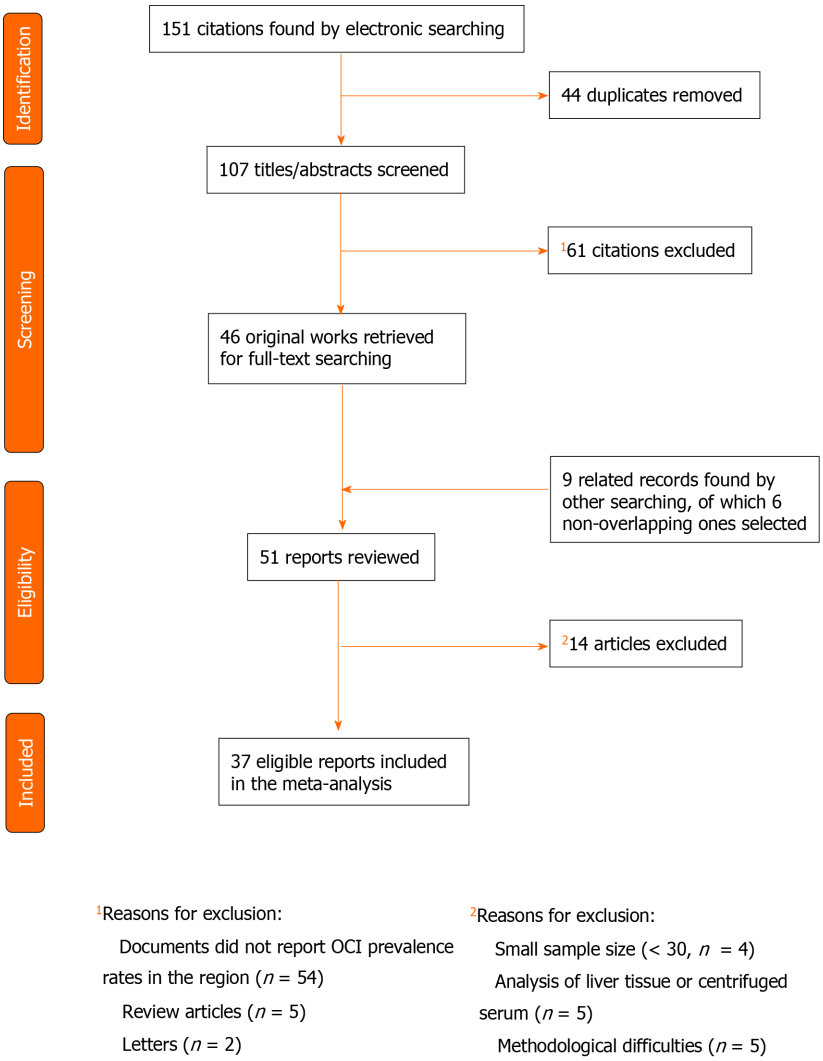

Figure 1 Study selection for the systematic review and meta-analysis of occult hepatitis C prevalence across the Middle East and Eastern Mediterranean countries. OCI: Occult hepatitis C virus infection.

seronegative samples. As shown in Table 3, the prevalence rate was estimated to be 8.71% (95%CI: 6.05%-12.39%) among this population (Figure 3). Based on 14 surveys, the estimated OCI rate among HD patients was 9.52% (95%CI: 6.30%-14.12%).

Subgroup analysis revealed that the rate of OCI in Egypt (11.43%; 95%CI: 6.55%-19.17%) was higher than in Iran (5.93%; 95%CI: 3.09%-11.09%), but the difference was

Table 3 Subgroup-specific pooled estimates of occult hepatitis C virus infection prevalence among multi-transfused patients across the Middle Eastern and Eastern Mediterranean countries

Prevalence by	Number of studies	Sample sizes	OCI prevalenceacross studies		Pooled OCI prevalence (%)		Heterogeneity	
			Range (%)	Median	Mean (%)	95%CI	Cochran's Q	I-squared (%)
Studied population								
Hemodialysis patients	14	1480	0-27.27	7.75	9.52	6.30-14.12	64.0[a]	79.7
Thalassemia patients	2	287	3.31-5.66	4.49	4.32	2.47-7.46	0.9	0.0
Hemophilia patients	1	450	-	-	10.22	7.74-13.38	-	-
Countries								
Egypt	8	499	3.33-27.27	12.48	11.43	6.55-19.17	26.8[a]	73.8
Iran	7	1550	0-18.45	3.31	5.93	3.09-11.09	54.8[a]	89.0
Saudi Arabia	1	84	-	-	14.29	8.30-23.49	-	-
Turkey	1	84	-	-	3.57	1.16-10.49	-	-
Temporal duration[1]								
Before 2015	7	463	0-23.08	3.70	7.89	4.10-14.65	20.6[b]	70.8
2015 and thereafter	10	1754	3.03-27.27	7.94	9.04	5.69-14.09	66.5[a]	86.5
Method of HCV RNA detection								
RT-nested PCR	8	1616	0-27.27	4.49	7.79	4.38-13.47	65.6[a]	89.3
Real time RT-PCR	7	488	3.57-23.08	10.67	9.52	5.26-16.61	17.4[b]	65.4
RT-PCR	1	60	-	-	3.33	0.84-12.37	-	-
Strand-specific PCR	1	53	-	-	15.09	7.73-27.38	-	-
Sample size								
Less than 100	12	767	0-27.27	7.75	9.49	5.85-15.04	40.6[a]	72.9
100 and above	5	1450	3.03-18.45	5.66	7.05	3.61-13.33	47.9[a]	91.6
Participants' sex								
Female	6	322	3.7-33.33	8.86	11.56	6.47-19.81	12.7[c]	60.8
Male	6	506	0-16	6.45	10.74	5.91-18.75	13.5[c]	63.1
All studies	17	2217	0-27.27	5.66	8.71	6.05-12.39	88.56[a]	81.93

[a]$P < 0.001$.
[b]$P < 0.01$.
[c]$P < 0.05$.
[1]Based on the last year reported for the data collection. If the collection date was not clear, publication year was considered instead. CI: Confidence interval; Min: Minimum; Max: Maximum; PCR: Polymerase chain reaction; RT-PCR: Reverse transcription polymerase chain reaction; OCI: Occult hepatitis C virus infection.

not statistically significant ($P = 0.125$). The rate of OCI frequency was not associated with year of study ($P = 0.732$), the detection technique of HCV RNA ($P = 0.618$), sample size ($P = 0.470$), and patients' sex ($P = 0.859$). Moreover, meta-regression analysis showed no significant ($P = 0.520$) changes in the OCI rate among MTPs patients over years.

Occult hepatitis C prevalence among patients with chronic liver diseases

In total, 11 surveys reported the OCI prevalence among 2065 patients with chronic LDs in M and E countries. These patients included 1778 with chronic hepatitis C, including those who achieved sustained virologic response after treatment with antiviral drugs, 252 patients with cryptogenic LDs (persistently abnormal liver tests and/or liver cirrhosis with unknown etiology), as well as 35 patients with autoimmune hepatitis. The rate of OCI prevalence among these patients was estimated to be 12.04% (95%CI:

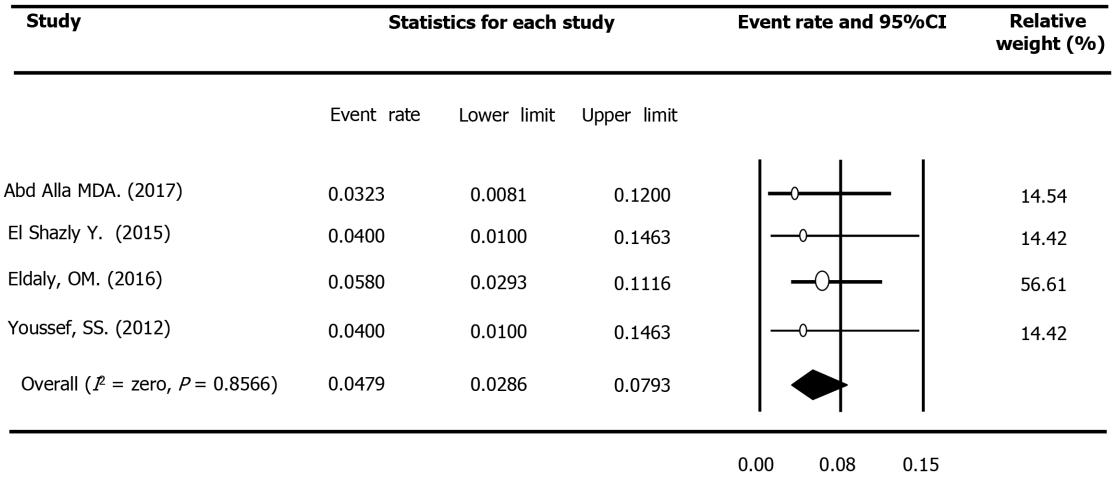

Figure 2 Meta-analysis forest plot of occult hepatitis C among healthy populations across the Middle East and Eastern Mediterranean countries.

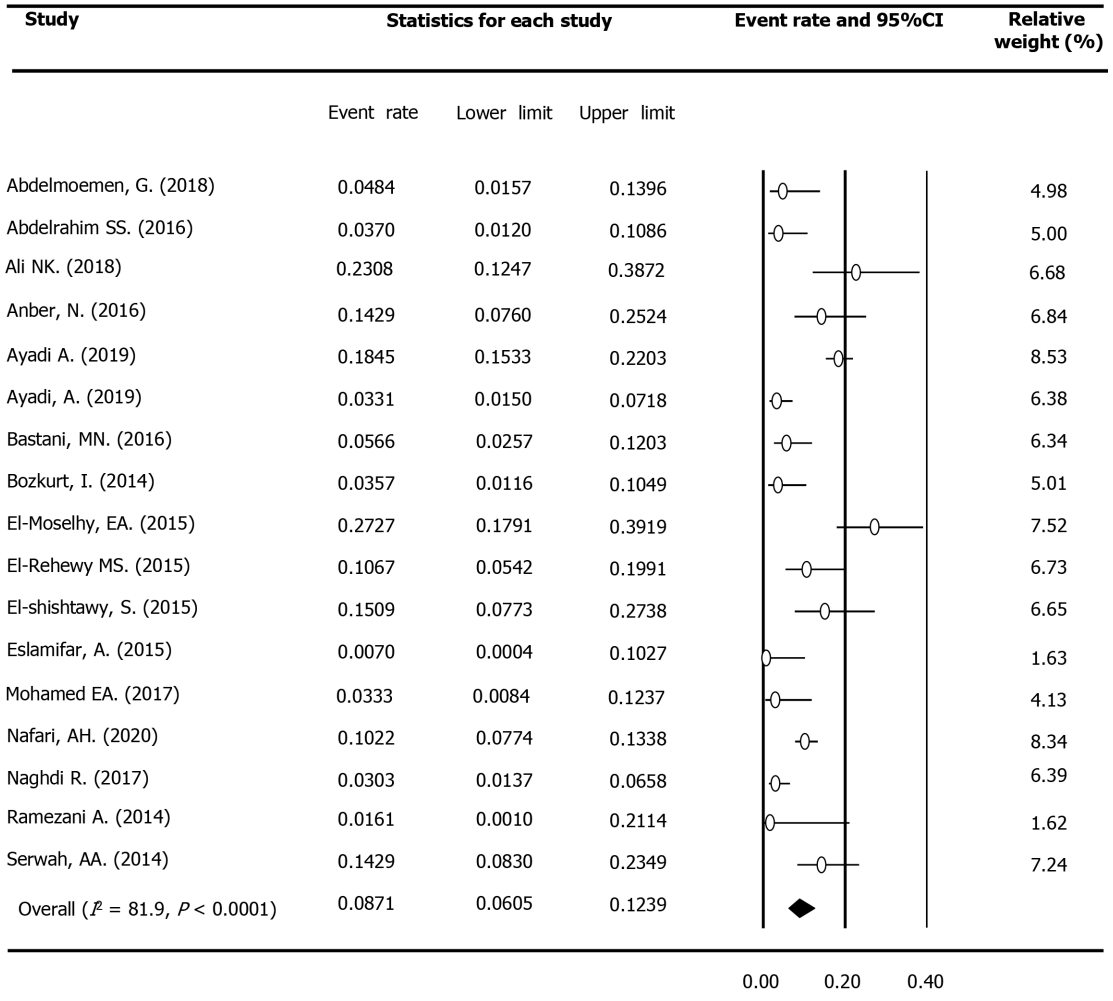

Figure 3 Meta-analysis forest plot of occult hepatitis C among multi-transfused patients across the Middle East and Eastern Mediterranean countries.

5.87%-23.10%, Table 4). The rate in the subgroup of cryptogenic patients (20.81%; 95%CI: 6.87%-48.35%) was double the value calculated for post-HCV non-viremic cases (9.14%; 95%CI: 3.02%-24.53%, P = 0.276). Figure 4 displays the forest plot of OCI among patients with chronic LDs based on the type of the disease. In addition, the rate of OCI among cases detected by RT-nested PCR (21.38%; 95%CI: 11.73%-35.75%) was

Table 4 Subgroup-specific pooled estimates of occult hepatitis C virus infection prevalence among patients with chronic liver diseases across the Middle Eastern and Eastern Mediterranean countries

Variables	Number of studies	Sample sizes	OCI prevalence across studies		Pooled OCI prevalence (%)		Heterogeneity	
			Range (%)	Median	Mean (%)	95%CI	Cochran's Q	I-squared (%)
Countries								
Egypt	5	1689	3.91-44.44	11.33	16.08	5.81-37.35	152.4[a]	97.4
Iran	5	272	0-32.08	10.14	11.46	3.64-30.73	16.6[b]	76.0
Pakistan	1	104	-	-	0.48	00.03-7.15	-	-
Temporal duration[1]								
Before 2015	8	523	0-44.44	10.46	11.83	4.84-26.14	46.0[a]	84.8
2015 and thereafter	3	1542	3.91-34.82	11.33	12.28	3.23-36.99	111.1[a]	98.2
Method of HCV RNA detection								
RT-nested PCR	6	359	0-44.44	21.11	21.38	11.73-35.75	30.5[a]	83.6
Real time RT-PCR	4	1636	0-11.33	7.35	6.29	2.73-13.84	23.9[a]	87.4
RT-PCR	1	70	-	-	12.86	6.83-22.90	-	-
Patients' subpopulation								
Post-HCV non-viremic cases[2]	5	1716	0-34.82	11.33	9.14	3.02-24.53	116.8[a]	96.6
Cryptogenic liver diseases[3]	4	212	8.89-44.44	21.11	20.81	6.87-48.35	22.6[a]	86.7
Chronic HCV infection and Cryptogenic liver diseases[2,3]	1	102	-	-	10.78	6.07-18.43	-	-
Autoimmune hepatitis	1	35	-	-	1.39	0.09-18.67	-	-
Patients' HCV serostatus								
Seronegative	5	247	0-44.44	10.14	16.13	5.41-39.28	27.7[a]	85.6
Seropositive	5	1716	0-34.82	11.33	9.11	2.97-24.69	116.8[a]	96.6
Seronegative/seropositive	1	102	-	-	10.78	6.07-18.43	-	-
Sample size								
Less than 100	6	317	0-44.44	11.50	15.63	6.08-34.67	32.2[a]	84.5
100 and above	5	1748	0-34.82	10.78	8.88	3.04-23.26	116.0[a]	96.5
All studies	11	2065	0-44.44	10.78	12.04	5.87-23.10	179.3[a]	94.4

[a]$P < 0.001$.
[b]$P < 0.01$.
[1]Based on the last year reported for the data collection. If the collection date was not clear, publication year was considered instead.
[2]Including those patients who achieved sustained virologic response after treatment with anti-virals.
[3]Including unexplained persistently abnormal liver enzymes and cryptogenic cirrhosis. CI: Confidence interval; Min: Minimum, Max: Maximum; PCR: Polymerase chain reaction; RT-PCR: Reverse transcription polymerase chain reaction; OCI: Occult hepatitis C virus infection.

considerably higher than cases identified using real-time RT-PCR (6.29%; 95%CI: 2.73%-13.84%, $P = 0.052$). Furthermore, there was no difference in the OCI frequency based on the year of data collection ($P = 0.962$), study location ($P = 0.178$), sample size ($P = 0.416$), patients' HCV serostatus ($P = 0.750$) and sex ($P = 0.749$). According to meta-regression analysis, no significant ($P = 0.943$) link was detected between the OCI rate among this population and data collection time. Figure 4 displays the forest plot of OCI among patients with chronic LDs based on type of the disease.

Occult hepatitis C prevalence among other high-risk categories

Regarding OCI prevalence among HIV-positive subjects in the M and E region, three surveys reported the rate among 417 HCV-seronegative and seropositive samples. All studies had been conducted in Iran after 2014 and evaluated HCV RNA in PBMCs

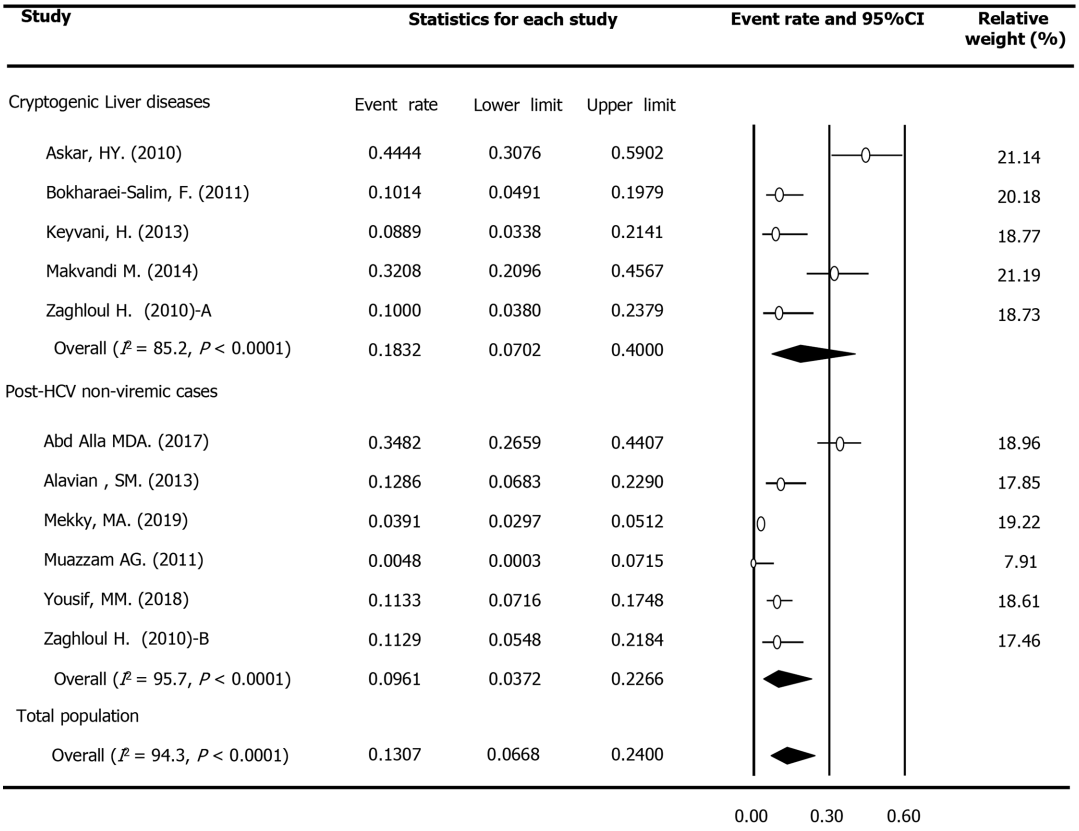

Figure 4 Meta-analysis forest plot of occult hepatitis C among patients with chronic liver diseases across the Middle East and Eastern Mediterranean countries based on type of disease. (Note: Zaghloul's study was considered two surveys, one among patients with cryptogenic liver diseases and one among hepatitis C virus-seropositive patients. Rezaee Zavareh's study among patients with autoimmune hepatitis was not included in this figure).

using the RT-nested PCR method. Using the fixed-effect model (Q = 3.1, P = 0.208, I^2 = 36.3%), the pooled mean prevalence of OCI was estimated at 12.95% (95%CI: 9.56%-17.32%) among this population.

Concerning occult hepatitis C among IDUs, one study recently identified HCV RNA in PBMCs in 18.18% of 77 Iranian HIV-positive IDUs. Moreover, another study from Iran reported an OCI rate of 9.57% among 115 HBV- and HIV-negative IDUs. Both surveys detected HCV genome among both HCV seronegative and seropositive samples by the RT-nested PCR method.

A total of three surveys, including two studies from Egypt and one survey from Iran, focused on OCI among patients with hematologic disorders, such as lymphoma, leukemia, and anemia. All OCI cases were detected by the RT-nested PCR technique among 171 HCV seronegative samples. Using the random-effect model (Q = 29.9, P < 0.001, I^2 = 93.3%), the pooled estimate of OCI among this population was estimated at 19.57% (95%CI: 3.22%-63.99%).

DISCUSSION

Through a comprehensive description and detailed analysis of occult hepatitis C epidemiology among the various populations in M and E countries, we found a considerably high rate of overall OCI prevalence across the region (10.04%; 95%CI: 7.66%-13.05%). The lowest rate (4.79%) was estimated among apparently healthy volunteers and blood donors. On the other hand, the higher rates were estimated for MTPs (8.71%), patients with chronic LDs (12.04%), HIV-positive subjects (12.95%), and those with lymphoproliferative and hematologic disorders (19.57%). Although the rate varied significantly across the studies, the pooled mean rate of OCI was not dissimilar regardless of subpopulation, location and year of study, the detection method of HCV RNA, patients' HCV serostatus or sex. Lastly, meta-regression analysis could not ascertain a declining or rising trend for OCI prevalence as a whole or among the different subpopulations of the region.

The incidence of OCI in each area is affected by various factors, mainly the

prevalence and risk factors of HCV infection in the community as well as in the studied population. Some investigators believed that there is a geographical pattern for OCI that is probably related to HCV endemicity distribution[3]. The majority of all chronically HCV infected people in the M and E region reside in the two countries most affected by the infection, *i.e.* Egypt and Pakistan[78]. In the current review, we noted that the Egyptian population had the highest rates (12.34%; 95%CI: 8.32%-17.92%) of OCI in this region. Likewise, based on four studies from Egypt, we calculated the pooled OCI rate among healthy populations to be 4.79 (95%CI: 2.86%-7.93%). Egypt is one of the countries highly affected by HCV and with high anti-HCV prevalence in almost all population groups[10]. Based on the Egypt Demographic and Health Surveys, anti-HCV prevalence among the adult Egyptian population was 10.0% in 2015[10]. Similarly, a recent systematic review estimated a pooled mean rate of 11.9% (95%CI: 11.1%-12.6%) for anti-HCV prevalence among the general Egyptian population[10]. Another systematic review estimated an average pooled HCV viremic rate of 67.0% (95%CI: 63.1%-70.8%) among anti-HCV positive individuals in this country[14]. In other words, the prevalence of chronic hepatitis C in Egypt is around 8%, which is close to the rate (6.3%) reported previously in 2015[79]. Moreover, four-fifths of hepatocellular carcinoma (HCC) patients in this country are infected with HCV—which ranks first in the world[80]. On the other hand, Iran has one of the lowest rates of HCV infection worldwide, particularly in the M and E region[81]. In this country, where HCV spread is dominated by transmission through injecting drug use[81], the pooled rates of anti-HCV positivity and viremic HCV among the GP have been estimated as low as 0.2%-0.3% and 0.4%-0.6%, respectively[13,79,81,82]. Correspondingly, we identified a lower overall rate of OCI among the Iranian population (8.48%) in comparison with the Egyptian population (12.34%).

Occult hepatitis C is primarily identified among populations at higher risk of health-care-related exposure, such as people who received repetitive transfusions particularly HD patients[66]. Our analysis estimated an average pooled OCI rate among MTPs of 8.7% (95%CI: 6.0%-12.4%); a higher level was calculated for HD patients (9.5%, 95%CI: 6.3%-14.1%) than for thalassemia patients (4.3%, 95%CI: 2.5%-7.5%). The rates of OCI prevalence among HD patients ranged from zero to 45% in different studies across the world[66]. In a survey by Barril *et al*[83], 45% of 109 Spanish HD patients with abnormal serum levels of liver enzymes had detectable HCV-RNA in their PBMCs. The patients with OCI had significantly higher mean levels of serum alanine aminotransferase. In addition, a significantly higher percentage of OCI patients died during the follow-up period compared with patients without OCI (39% *vs* 20%; $P = 0.031$). It is expected that HD patients are at higher risk of HCV infection owing to shared dialysis machines[84]. Some researchers suggested that the duration of dialysis is associated with the increased probability of HCV infection among HD patients[83,85]. In the M and E region, Harfouche *et al*[86] showed that about one-fifth of HD patients are chronic HCV carriers and can potentially spread the infection through the dialysis machine. They suggested that their findings may reflect the higher HCV incidence in the communities along with poor standards of dialysis in this area. Despite the decrease in the prevalence of HCV infection in HD patients, OCI could be the culprit for the constant distribution of HCV among this population[3].

Furthermore, our review estimated a two-fold higher rate of OCI among Egyptian MTPs patients (11.4%, 95%CI: 6.5%-19.2%) than Iranian patients (5.9%, 95%CI: 3.1%-11.1%). These findings were consistent with the reported rates for anti-HCV prevalence among this population in both countries. In a systematic review and meta-analysis of data from 10 countries in the Middle East, the pooled HCV prevalence among HD patients was estimated to be 25.3% (95%CI: 20.2%-30.5%); a much higher rate was reported from Egypt (50%, 95%CI: 46%-55%) in comparison with Iran (12%, 95%CI: 10%-15%)[87]. Indeed, medical care appears to be the main route of both past and new HCV transmission in Egypt[10]. On the other hand, in another recent review, the overall anti-HCV prevalence was estimated at a considerably lower rate (20.0%, 95%CI: 16.4%-23.9%) across Iranian populations at high risk of healthcare-related exposures, such as HD, hemophilia, and thalassemia patients[13].

The rate of occult hepatitis C is considerably higher among populations with liver involvement who were seronegative for HCV RNA[2,61,64]. In a recent survey in Egypt, the rate of OCI among 112 post-HCV non-viremic cases, including 55 non-cirrhotic and 57 cirrhotic patients (34.8%) was significantly higher than 62 healthy control individuals (3.23%)[35]. Likewise, our review indicated a high frequency of OCI among patients with LDs, including individuals with unexplained elevated liver enzymes and cryptogenic hepatitis as well as those with a history of exposure to HCV in the past (12.04%, 95%CI: 5.87%-23.10%); the highest rate was observed in patients with cryptogenic LDs (20.81%; 95%CI: 6.87%-48.35%). High rates of OCI among LD patients

have been reported from countries with both low and high HCV endemicity in the community[21,22,41,48]. Consistently, our analysis revealed that the rate of OCI among LD patients in Egypt (16.08%; 95%CI: 5.81%-37.35%) did not significantly (P = 0.178) differ from Iranian patients (11.46%; 95%CI: 3.64%-30.73%). Regarding active HCV infection among populations with LDs, high rates have also been reported from both countries. A detailed analysis of HCV epidemiology in the Middle East found a pooled mean prevalence of 35.5% (95%CI: 31.7%-39.5%) in all patients with LDs; the highest rates were estimated for HCC (56.9%; 95%CI: 50.2%-63.5%) and hepatic cirrhosis (50.4%; 95%CI: 40.8%-60.0%). The pooled rate was 58.8% (95%CI: 51.5%-66.0%) in Egypt, 55.8% (95%CI: 49.1%-62.4%) in Pakistan, and 15.6% (95%CI: 12.4%-19.0%) in other countries[88]. The rate of HCV infection in each LD population of each country was strongly correlated with HCV prevalence among their GP. The authors concluded that their findings highlight how the role of this infection in liver diseases is a reflection of its background level in the GP[88]. Moreover, in countries like Egypt and Pakistan, high rates of infections among various populations with LDs may support the contribution of HCV to the occurrence of liver disease[9,10]. On the other hand, a significantly lower HCV rate has been reported for Iranian patients with liver-related conditions (7.5%, 95%CI: 4.3%-11.4%)[13]. Another systematic review underlined the different etiology of HCC in countries of the Eastern Mediterranean region; Four-fifths of HCC patients in Egypt and half of the patients in Pakistan were infected with HCV; however, this value was as low as 8.5% for Iranian patients[80].

Our study had several limitations. Almost all of OCI reports (34 of 37) were from Iran and Egypt, and we did not find any data from 21 countries in the M and E region. Our study is also limited by the number of available documents for both healthy subjects and specific at-risk populations, such as IDUs, HIV-positive persons, and thalassemia or hemophilia patients. Other limitations of our review were the quality of retrieved evidence as well as the representativeness of the target populations. The findings of the majority of studies were based on examination of less than 100 consecutive samples selected by a non-random convenience sampling method. There was a wide heterogeneity in OCI rates even within specific subpopulations; nonetheless, there was no evidence that study location, data collection date, the detection technique of HCV RNA, patients' HCV serostatus, and sex-group representation in the sample affected the prevalence rates. Despite these shortcomings, we found a large amount of data in two countries, which contributed to the lowest and highest rate of chronic HCV infections in the region (namely, Iran and Egypt, respectively) that allowed us to conduct an analysis among different population categories and settings.

CONCLUSION

Our systematic review and meta-analysis quantified high levels of OCI prevalence, especially across risk populations in M and E countries. Recommendations include more appropriate OCI screening programs to target individuals who are at high risk for HCV infection, especially the patients undergoing dialysis and those with cryptogenic liver diseases. Besides, further investigations are needed regarding OCI among other risk populations, such as HIV- and HBV-infected subjects, IDUs, and thalassemia and hemophilia patients.

ARTICLE HIGHLIGHTS

Research background

Occult hepatitis C virus (HCV) infection (OCI) is defined as the presence of HCV genome in the liver samples or peripheral blood mononuclear cells despite a negative test for serum viral RNA. OCI, a common condition worldwide, might be associated with significant morbidities such as liver cirrhosis or hepatocellular carcinoma. No review has yet been performed to provide a pooled estimate for the OCI prevalence rate in the Middle East and Eastern Mediterranean (M and E) countries, a region with the highest rates of HCV infection in the world.

Research motivation

In this systematic review and meta-analysis, we tried to characterize a clear feature of OCI epidemiology in 26 countries of the M and E region based on documents found by

searching international and regional electronic sources as well as some local grey literature. We hope our findings help researchers to perform more investigations on diagnosis, management, and control of OCI, particularly in high-risk populations such as patients with chronic liver disease, multi-transfused patients, those infected with HIV, injecting drug users, *etc.*

Research objectives

The main objective of this review is to provide pooled mean estimates of the OCI rate and assess the contribution of potential variables on the between-study heterogeneity in the M and E region. The results would help professionals, investigators and policy makers to organize suitable activities regarding OCI, particularly in high-risk patients.

Research methods

A systematic review and meta-analysis was performed following PRISMA guidelines. A comprehensive search of electronic databases was conducted up to June 2020 in the Web of Science, PubMed, SCOPUS, ScienceDirect, ProQuest, the Index Medicus for the Eastern Mediterranean Region, Scientific Information Database, Iranian Database of publication (Magiran), and Iranian Databank of Medical Literature. Also some conference abstracts and all references from bibliographies of retrieved articles were manually reviewed. Forest plots were applied to demonstrate the point prevalence rates and the 95% confidence intervals, and subgroup and meta-regression analyses were applied to identify the factors contributing to heterogeneity between surveys.

Research results

Thirty-seven studies involving 5200 participants from Egypt, Iran, Pakistan, Saudi Arabia, and Turkey were analyzed. The overall pooled prevalence rate of OCI was 10.04%. The pooled rate among healthy populations was 4.79%, but the rate was much higher among patients with hematologic disorders (19.57%), HIV-positive subjects (12.95%), patients with chronic liver diseases (12.04%), and multi-transfused patients (8.71%). The rate of OCI was not significantly related to the country, disease subpopulations, year of study, the method of HCV RNA detection, sample size, patients' HCV serostatus and sex, and no significant change was detected in the OCI rate over time ($P > 0.05$).

Research conclusions

This review and meta-analysis demonstrates high rates of OCI prevalence, especially across risk populations in the M and E region. Some appropriate OCI screening programs are recommended to target individuals who are at risk of HCV infection.

Research perspectives

According to this systematic review and meta-analysis, further investigations are required in order to collect more data on the OCI frequency in M and E countries other than Egypt and Iran, two nations with the highest and lowest rates of chronic HCV infection in the region, respectively. Moreover, large scale studies are needed to evaluate OCI prevalence among less studied populations such as injecting drug users, HBV-infected patients, and thalassemia and hemophilia patients.

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CASE REPORT

Two-stage hepatectomy with radioembolization for bilateral colorectal liver metastases: A case report

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Abstract

BACKGROUND

Two-stage hepatectomy (TSH) is a well-established surgical technique, used to treat bilateral colorectal liver metastases (CRLM) with a small future liver remnant (FLR). However, in classical TSH, drop-out is reported to be around 25%-40%, due to insufficient FLR increase or progression of disease. Trans-arterial radioembolization (TARE) has been described to control locally tumor growth of liver malignancies such as hepatocellular carcinoma, but it has been also reported to induce a certain degree of contralateral liver hypertrophy, even if at a lower rate compared to portal vein embolization or ligation.

CASE SUMMARY

Herein we report the case of a 75-year-old female patient, where TSH and TARE were combined to treat bilateral CRLM. According to computed tomography (CT)-scan, the patient had a hepatic lesion in segment VI-VII and two other confluent lesions in segment II-III. Therefore, one-stage posterior right sectionectomy plus left lateral sectionectomy (LLS) was planned. The liver volumetry estimated a FLR of 38% (segments I-IV-V-VIII). However, due to a more than initially planned, extended right resection, simultaneous LLS was not performed and the patient underwent selective TARE to segments II-III after the first surgery. The CT-scan performed after TARE showed a reduction of the treated lesion and a FLR increase of 55%. Carcinoembryonic antigen and CA 19.9 decreased significantly. Nearly three months later after the first surgery, LLS was performed

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Grade D (Fair): 0
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and the patient was discharged without any postoperative complications.

CONCLUSION
According to this specific experience, TARE was used to induce liver hypertrophy and simultaneously control cancer progression in TSH settings for bilateral CRLM.

Key Words: Trans-arterial; Radioembolization; Two-stage hepatectomy; Colorectal liver metastases; Selective internal radiation therapy; Yttrium90; Case report

Core Tip: Two-stage hepatectomy and trans-arterial radioembolization (TARE) are usually used in advanced stage primary liver malignancies. In this case report, two-stage hepatectomy and TARE were combined, for the first time, to treat a patient with bilateral colorectal liver metastases and a small future liver remnant. In particular, TARE was performed to induce liver hypertrophy and at the same time to control tumor growth between stages, thus reducing the risk of tumor progression.

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INTRODUCTION

Two-stage hepatectomy (TSH) has been traditionally advocated for bilateral colorectal liver metastases (CLRM) that could not be resected in a single operation[1]. In TSH, when the future liver remnant (FLR) is considered not enough, contralateral portal vein ligation (PVL) or embolization (PVE) can be performed in the first stage to increase FLR volume. However, 25%-40% of patients will not undergo the second stage due to insufficient liver hypertrophy and/or progression of disease[2]. Trans-arterial radioembolization (TARE) consists of the selective intra-arterial administration of microspheres loaded with a radioactive compound—usually yttrium90—and has been shown to control tumor growth and to induce liver hypertrophy especially in patients with primary liver cancer. More recently, it has been shown to be effective also in CLRM setting[3]. However, the combination of these two techniques has never been explored before.

CASE PRESENTATION

Chief complaints

A 75-year-old female patient presented herself with mild abdominal pain.

History of present illness

Ultrasonography and abdominal computed tomography (CT) detected three unknown hepatic lesions in segment VI-VII (n = 1) and segment II-III (n = 2), respectively. The lesion of the right lobe seemed to infiltrate the right hepatic vein whereas the other two confluent lesions in segment II-III showed a particular intrabiliary growth pattern (Figure 1).

History of past illness

The patient had undergone endoscopic removal of a sigmoid polyp cancer (T1NxMxR0) 3 years before.

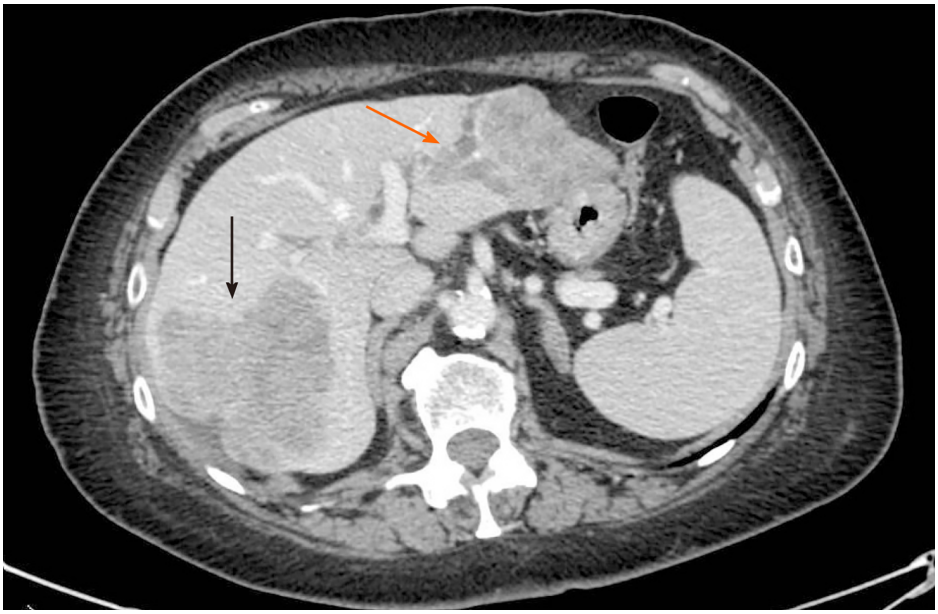

Figure 1 Pre-operative computed tomography-scan. The lesion occupying the right posterior segments of the liver (black arrow) and two other confluent lesions in the left lobe with intrabiliary growth pattern (orange arrow).

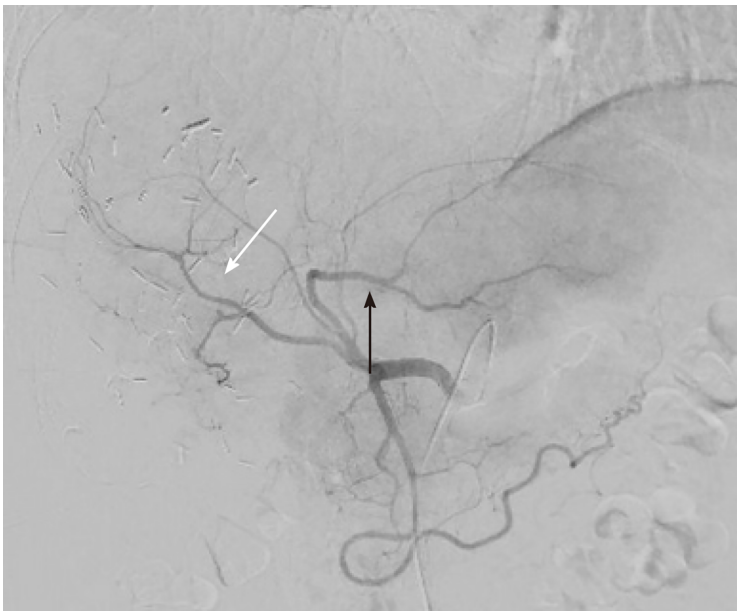

Figure 2 Arterial vascular anatomy during trans-arterial radioembolization procedure treating the tumor lesion in segments II-III (black arrow). The angiography also showed the remaining right anterior portal pedicle (white arrow).

Personal and family history

She suffered from hypertension and hypoparathyroidism. She had no family history of cancer.

Physical examination

The patient had a good performance status. Physical examination was unremarkable with vital signs within the normal range of values. No jaundice was observed.

Laboratory examinations

Liver function tests were normal and tumor markers were increased (carcinoembryonic antigen, CEA = 3284.9 ng/mL; CA 19-9 = 703.9 U/mL).

Imaging examinations

Esophagogastroduodenoscopy and colonoscopy were negative. According to liver volumetry, total functional liver volume (TFLV) measured 1635 mL and FLR (segments I-IV-V-VIII) 621 mL, with a resulting FLR/TFLV of 38%.

FINAL DIAGNOSIS

The final diagnosis of the presented case was suspected bilateral hepatic metastases from colorectal cancer.

TREATMENT

The operation started with a minimally invasive approach, but it was converted to open surgery due to diaphragm infiltration by the lesion located in the right liver. Intraoperative ultrasound showed that part of segment VIII was also involved. After detachment of the lesion from the diaphragm and its suture, a portal branch of segment V was ligated during parenchymal transection. Given the wider than initially planned hepatic surgery (segments V-VI-VII + part of segment VIII) and the difficulties encountered during the first resection, left lateral sectionectomy (LLS) was postponed. As a bridge treatment, TARE was chosen in order to control locally the disease while waiting for FLR increase. According to the CT-scan performed 10 d after surgery, FLR measured 632 mL. A small intrabdominal fluid collection was incidentally detected close to the surgical site as well as an ischemic area in segment V. The patient was discharged home on postoperative day 14, without major complications. The final diagnosis, based on histopathology of surgery specimen, was adenocarcinoma from colorectal cancer (KRAS and BRAF wild-type). TARE was carried out 11 d after discharge and realized with a single treatment (200 Gy) of Selective Internal Radiation (SIR) spheres (Sirtex Medical, Sydney, Australia) without any post-procedural complications (Figure 2). Forty-seven days after TARE, the patient underwent a new CT-scan showing a 32% reduction of the confluent lesion in segment II-III, with a surprisingly final FLR volume of 980 mL (FLR increase = 55%, FLR/TFLV = 77%) (Figure 3). CEA and CA 19.9 decreased to 93.7 ng/mL and 92.5 U/mL, respectively. After resolution of the abdominal collection by percutaneous drainage, we planned the second stage of surgery and 3 mo later after the first operation, the patient underwent LLS.

OUTCOME AND FOLLOW-UP

The postoperative course was uneventful. The patient did not receive any adjuvant chemotherapy and almost two years after the first surgery is still alive and free of disease.

DISCUSSION

According to this specific experience, TARE was used for the first time, combined with classical TSH, to control cancer progression between stages, waiting for adequate liver hypertrophy before the second resection.

TARE has been already shown to produce effective liver parenchyma hypertrophy in patients with primary hepatic malignancies treated with lobar 90Y radioembolization therapy[4]. After TARE, however, compared to PVE/PVL, the hypertrophy is radiation-induced rather than caused by embolization and is reached at a slower rate. According to a recent systematic review[5], the median kinetic growth rate of the controlateral lobe for patients underwent lobar TARE for CRLM was 0.8% per week compared to 6.1% of PVE. Despite a slower increase, however, a hyper-trophy of 26%-47% was obtained at time intervals ranging from 44 d to 9 mo[4], of similar magnitude to that observed after PVE. In addition, Birgin *et al*[5] found that up to 84% of patients affected by primary and secondary hepatic malignancies had a local tumor control following TARE and about 30% of unresectable tumors underwent hepatic resection.

Table 1 Review of the literature including patients submitted to preoperative trans-arterial radioembolization for colorectal liver metastases

Ref.	Year	Type of study	Pts included, *n*	CRLM[1], *n* (%)	Tumor location (*n*)	Bilobar *n* (%)	Prior resection, *n* (%)	Resectability, *n* (%)
Gray *et al*[18]	2001	HAI *vs* HAI + TARE in unresectable CRLM; RCT	74	36 (48.6)	Colon (29), rectum (7)	36/36 (100)	0	1/36 (2.8)
Lim *et al*[6]	2005	TARE after failure of FU in unresectable CRLM; prospective	30	30 (100)	NA	NA	0	1/30 (3.3)
Sharma *et al*[7]	2007	TARE + FOLFOX4 in unresectable CRLM; prospective (phase I)	20	20 (100)	Right colon (4), sigmoid (5), rectum (4), other colon sites (7)	NA	0	2/20 (10)
Cosimelli *et al*[19]	2010	TARE in unresectable CRLM; prospective (phase II)	50	50 (100)	Colon (41), rectum (9)	35/50 (70)	12/50 (24.0)	2/50 (4.0)
Hendlisz *et al*[8]	2010	FU *vs* TARE + FU in unresectable CRLM; RCT	44	21 (47.7)	NA	NA	NA	1/21 (4.8)
Brown *et al*[9]	2011	TARE *vs* CHT *vs* no therapy before hepatectomy; case-control	840	16 (1.9)	NA	NA	NA	16/16 (100)
Whitney *et al*[10]	2011	TARE in unresectable liver disease; retrospective	44	15 (34)	Rectum (15)	0	0	1/15 (6.7)
Vouche *et al*[11]	2013	TARE in unresectable liver disease; retrospective	83	8 (9.6)	NA	0	0	1 (12.5)
Wang *et al*[20]	2013	TARE before liver resection for CRLM; retrospective	24	24 (100)	Sigmoid (1), rectum (1), other colon sites (1), unknown (21)	1/3 (33.3)	0	3/24 (12.5)
Henry *et al*[12]	2015	TARE before liver resection for metastatic cancer; retrospective	9	4 (44.4)	NA	NA	0	4/4 (100)
Justinger *et al*[22]	2015	TARE in marginally resectable CRLM; retrospective	13	13 (100)	Right colon (2), sigmoid (4), rectum (7)	9/13 (69.2)	7/13 (53.8)[2]	11/13 (84.6)
Moir *et al*[13]	2015	TARE in unresectable liver disease; retrospective	44	22 (50)	NA	NA	NA	4/22 (18.2)
Maleux *et al*[14]	2016	TARE in unresectable CRLM; NA	88	71 (80.6)	NA	58/71 (81.6)	10/71 (14.0)	1/71 (1.4)
Lewandowski *et al*[23]	2016	TARE in unresectable right-sided liver disease; retrospective	13	1 (7.6)	NA	0	NA	1/1 (100)
Wright *et al*[15]	2017	TARE in unresectable liver disease; retrospective	465	6 (1.2)	NA	NA	NA	6/6 (100)
van Hazel *et al*[16]	2016	FOLFOX6 *vs* FOLFOX6 + TARE ± Bevacizumab; RCT	530	267 (50.3)	Left colon (141), right colon (72), rectum (45), other colon sites (7), unknown (2)	NA	NA	38/267 (14.2)
Pardo *et al*[21]	2017	TARE before liver resection or transplantation; retrospective	100	30 (30)	NA	44/100	7/30 (23.3)[2]	30/30 (100)
Wasan *et al*[17]	2017	FOLFOX *vs* FOLFOX + TARE; RCT	1103	554 (50.2)	Colon (421), rectum (116), unknown (17)	NA	NA	56/554 (10.1)

[1]Treated with transarterial radioembolization.

[2]Associating liver partition and portal vein ligation for staged hepatectomy. CHT: Chemotherapy; CRLM: Colorectal liver metastases; FU: Florouracil; HAI: Hepatic artery infusion; NA: Not available; RCT: Randomized controlled trial; TARE: Transarterial radioembolization.

Review of the literature, including only studies of patients submitted to hepatectomy for CRLM after preoperative TARE (n = 18)[6-18] (Tables 1 and 2), showed that even though many of them comprised bilateral distribution of CRLM[18-

Table 2 Review of the literature including patients submitted to preoperative trans-arterial radioembolization for colorectal liver metastases

Ref.	FLR increase %	Time TARE-surgery, median (range), mo	Type of hepatic resection (*n*)	Post-operative mortality %	Survival, median (range), mo	Disease free, median (range), mo	Recurrence after surgery, *n* (%)
Gray *et al*[18]	NA	NA	NA	0	96	NA	NA
Lim *et al*[6]	NA	NA	NA	NA	NA	22	1/1 (100)
Sharma *et al*[7]	NA	NA	LLS + S6 (1), RH + S3 (1)	NA	NA	NA	NA
Hendlisz *et al*[8]	NA	NA	RH (1)	0	NA	1.5	1/1 (100)
Brown *et al*[9]	NA	6.5 (4-13)	NA	NA	NA	NA	NA
Whitney *et al*[10]	NA	NA	RT (1)	0	NA	24	1/1 (100)
Vouche *et al*[11]	NA	NA	RT (1)	0	NA	NA	NA
Wang *et al*[20]	NA	NA (4-9)	RH (2), LH + S6 (1)	0	NA	NA	NA
Henry *et al*[12]	NA	5 (2-8)	LLS (1), multiple wedge, HAI pump (1), RT + RFA (1), RT (1)	50	13 (0-27)	6.2 (1.8-10.5)	3/4 (75)
Justinger *et al*[22]	32.9 (ALPPS), 27.1 (no ALPPS)	2 (1-5)	RT (4), RH (5), mesohepatectomy (1), LT (1)	7.6	25 (12-38)[1]	NA	NA
Moir *et al*[13]	NA	4 (2-11)	NA	0	15 (11-19)[1]	NA	NA
Maleux *et al*[14]	NA	NA	S + RFA (1)	0	NA	NA	NA
Lewandowski *et al*[23]	15	1.6 (1-7)	RT (1)	0	4.8	NA	NA
Wright *et al*[15]	NA	9 (3-20)	RT (2), S (1), RH (3)	16.6	25 (NA)	NA	NA
van Hazel *et al*[16]	NA	NA	NA	0	NA	NA	NA
Pardo *et al*[21]	NA	NA	NA	10	NA	NA	NA
Wasan *et al*[17]	NA	NA	NA	3.6	NA	NA	NA

[1]95% confidence interval. ALPPS: Associating liver partition and portal vein ligation for staged hepatectomy; HAI: Hepatic artery infusion; LH: Left hepatectomy; LLS: Left lateral sectionectomy; LT: Left trisectionectomy; RFA: Radiofrequency ablation; RH: Right hepatectomy; RT: Right trisectionectomy; S: Segmentectomy; NA: Not available.

20], only Pardo *et al*[21] reported a "two-stage resection" in 10 patients, 7 of whom underwent associating liver partition and portal vein ligation for staged hepatectomy (ALPPS), probably from the cohort of Justinger *et al*[22] In this latter study, resectability of ALPPS + TARE was 85.7%. Increase of FLR in CRLM patients was reported only in few studies[22,23]. In our report, TARE to segment II-III led to a FLR increase of 55%, probably induced by the combined regenerative effect produced by the first

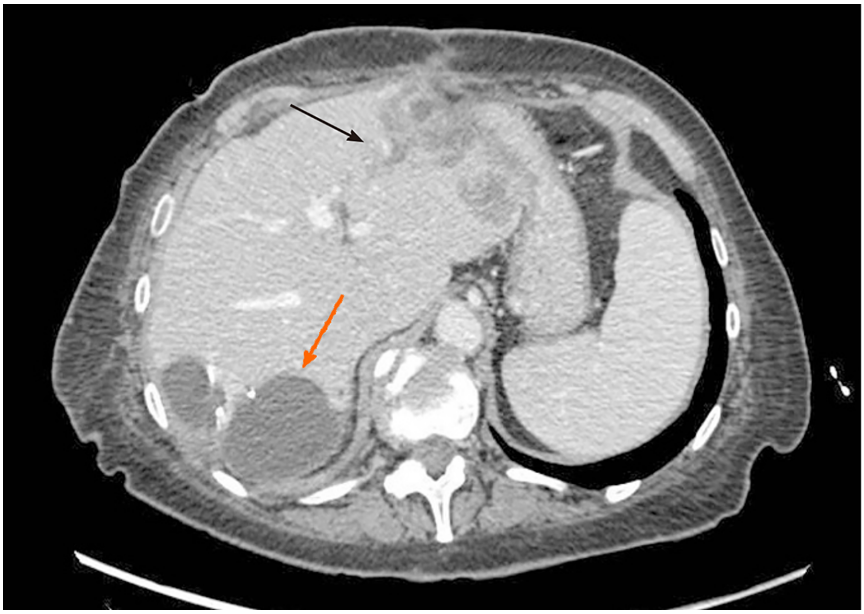

Figure 3 Computed tomography-scan performed one month after trans-arterial radioembolization. The reduction of the lesion of the left lobe and the intrabiliary growth pattern (black arrow). An intrabdominal fluid collection was found close to the surgical site (orange arrow).

liver resection, similar to what happens in ALPPS procedure, and by TARE itself. If a larger FLR hypertrophy was required, the role of TARE in combination also with classical portal vein occlusion techniques such as PVE/PVL or ALPPS, could have been explored. However, in this case, TARE was preferred over PVE or PVL since segment IV had to be preserved being part of the FLR. Furthermore metastasis in segment II-III could have progressed leading to the drop out of the patient. On the other side, the risk of proceeding with a second simultaneous hepatectomy or ALPPS was deemed too high. Last but not least, from the oncological point of view, this strategy may allow surgeons, without dealing with time issue, to select only patients with favorable tumor biology, according to radiological response after TARE[12].

CONCLUSION

TARE in TSH setting may represent a viable option to increase resectability in patients with bilateral CLRM by stimulating liver hypertrophy and controlling locally the disease. Future larger, comparative studies may help answer the questions above.

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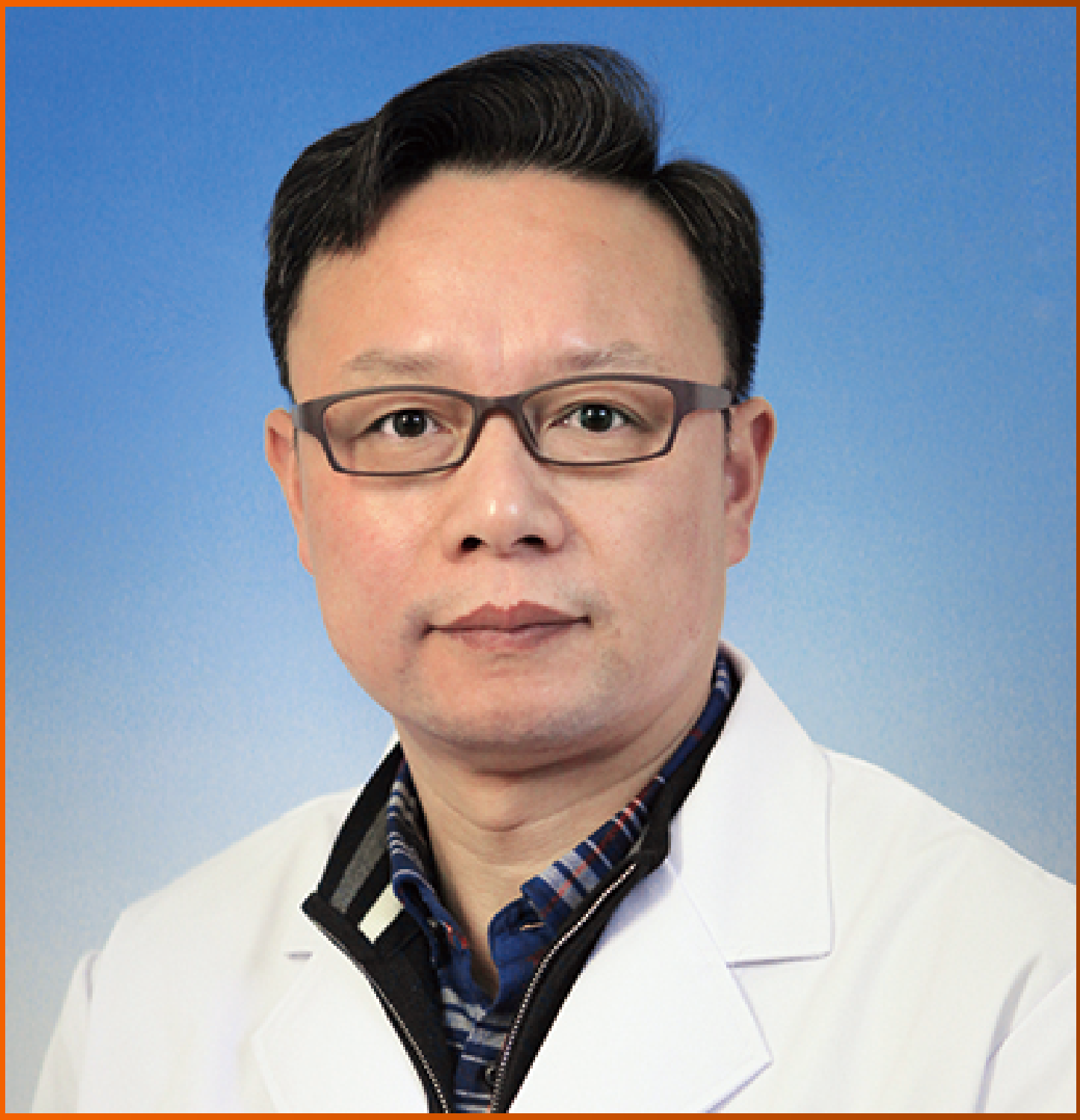

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REVIEW

Molecular pathways of liver regeneration: A comprehensive review

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Abstract

The liver is a unique parenchymal organ with a regenerative capacity allowing it to restore up to 70% of its volume. Although knowledge of this phenomenon dates back to Greek mythology (the story of Prometheus), many aspects of liver regeneration are still not understood. A variety of different factors, including inflammatory cytokines, growth factors, and bile acids, promote liver regeneration and control the final size of the organ during typical regeneration, which is performed by mature hepatocytes, and during alternative regeneration, which is performed by recently identified resident stem cells called "hepatic progenitor cells". Hepatic progenitor cells drive liver regeneration when hepatocytes are unable to restore the liver mass, such as in cases of chronic injury or excessive acute injury. In liver maintenance, the body mass ratio is essential for homeostasis because the liver has numerous functions; therefore, a greater understanding of this process will lead to better control of liver injuries, improved transplantation of small grafts and the discovery of new methods for the treatment of liver diseases. The current review sheds light on the key molecular pathways and cells involved in typical and progenitor-dependent liver mass regeneration after various acute or chronic injuries. Subsequent studies and a better understanding of liver regeneration will lead to the development of new therapeutic methods for liver diseases.

Key Words: Liver regeneration; Molecular pathways; Hepatic progenitor cells; Cytokines;

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Micro ribonucleic acid; Partial hepatectomy

Core Tip: The liver is a unique parenchymal organ with a regenerative capacity that can restore up to 70% of its volume. A variety of different factors and signaling pathways are involved in the process of liver mass regeneration during the priming, proliferative and termination phases. This review describes the types of liver regeneration, the phases of typical liver regeneration, the cell types involved in liver regeneration, the process of alternative liver regeneration, and the stem cells and micro ribonucleic acids that play roles in liver mass regeneration.

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INTRODUCTION

The capacity of the liver to regenerate has been well known since the myths of Prometheus, who was banished from Olympus by Zeus. Legend has it that eagles pecked out half of his liver every day, but because the liver regrew during the night, the hero endured never-ending torture[1]. Today, many centuries after these events, liver regeneration is a universally known phenomenon that has been studied at the molecular and cellular levels. However, many aspects remain unclear[2].

Liver regeneration is a complex process regulated by the interaction between growth factors and cytokines secreted near the site of injury or transferred to the liver by the blood. This strictly orchestrated process is divided into 3 phases: Priming, proliferation, and termination[3]. The sum of all signals that sense the physiologically necessary liver mass is called the "hepatostat", which can initiate and terminate liver regeneration[4]. This phenomenon reflects the correlation between the needs of organisms and the organ mass that is required for homeostasis[5].

A better understanding of liver regeneration mechanisms will help improve the methods used to treat various organ diseases, prevent hepatic failure in high-risk patients, control liver grafts for transplantation, and more[6]. Importantly, the term "liver regeneration" is used improperly because during actual regeneration, not only the function of the organ but also the morphology is restored whereas only compensatory hypertrophy occurs in the liver. Second, mature hepatocytes are the source of new liver cells, not stem cells; however, stem cells play an important role in some cases of liver regeneration[7]. However, the term "liver regeneration" is widely accepted and the most commonly used term[8].

TYPES OF LIVER REGENERATION

Until recently, it was believed that the liver mass after partial hepatectomy (PH) or injury recovers *via* hepatocyte proliferation for 1–2 cell cycles; however, recent studies have shown that different stimuli define the type of liver regeneration that occurs[9]. There are two known types of liver regeneration: The first is conducted through the hypertrophy and/or hyperplasia of hepatocytes and biliary epithelial cells (BECs) and is called typical regeneration. Typical regeneration is specific to a healthy liver that was exposed to resection or an acute liver injury; conversely, progenitor-dependent regeneration requires the reprogramming of specific hepatic cells, whose activation depends on the volume of the residual liver mass. Progenitor-dependent regeneration is specific to chronic liver diseases and massive acute liver injuries[10,11]. Thus, a 2/3 hepatectomy leads to the immediate hypertrophy of hepatocytes and further hyperplasia, whereas a 1/3 hepatectomy only triggers cell hypertrophy. Various chronic diseases and massive injuries initiate the activation of hepatic progenitor cells

(HPCs), which are responsible for liver regeneration[9]. Consequently, typical liver regeneration is driven by mature hepatocytes and BECs, whereas the alternative regeneration method is performed by HPCs[11].

TYPICAL LIVER REGENERATION

PH causes a hemodynamic disturbance, expressed as a portal pressure escalation, which serves as a regeneration stimulus. Consequently, hepatocytes, BECs, Ito cells, Kupffer cells (KC) and sinusoid endothelial cells (SECs) are proliferated. Interestingly, hepatocytes proliferate first, whereas BECs start to proliferate only 2-3 d after PH. After a 2/3 PH, the hepatocytes go through one cycle of DNA synthesis, which is required for the restoration of 60% of the liver mass. In the following stages, several but not all hepatocytes continue to proliferate to achieve complete liver recovery. Afterward, apoptotic activity increases with the purpose of correcting an excessive regenerative response[12].

Phases of typical liver regeneration

The beginning of each phase is initiated by a certain molecule set released in response to organ damage[13]. The earliest regeneration drivers are portal pressure changes and an increasing level of urokinase plasminogen activator (uPA)[8,14].

Priming phase: During the first phase of regeneration, hepatocytes, driven by various cytokines, simultaneously enter the G_1 phase of the cell cycle[10].

The increasing blood pressure in the hepatic sinusoids is conditioned by the incompatibility between the volume of the liver and the volume of inflowing venous blood[15], which results in a turbulent flow and mechanically stimulates SECs to secrete large amounts of uPA. uPA promotes plasminogen-plasmin transformation, leading to matrix metalloproteinase (MMP) activation and fibrinogen degradation. Plasmin and MMPs are involved in extracellular matrix (ECM) remodeling, resulting in the release of growth factors, such as hepatocyte growth factor (HGF)[8].

Two proinflammatory cytokines are the main mediators of the first phase: Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6); these cytokines are secreted primarily by liver macrophages under the influence of bacterial lipopolysaccharide and the C3a and C5a components of the complement system[16]. IL-6 drives the acute phase response and initiates cytoprotection and the proliferation of hepatocytes *via* the IL-6-IL-6R interaction and the activation of coreceptor glycoprotein 130 (gp130), which activates the Janus kinase (JAK)/signal transducer and activator of transcription (STAT), Mitogen-activated protein kinase (MAPK) and PI3K/AKT signaling pathways[5,17]. Although gp130 is present on the surface of most cells, IL-6R is primarily located on hepatocytes. However, there are also soluble IL-6Rs that initiate the trans-signaling pathway within cells lacking IL-6R and enhance the regenerative response of hepatocytes[3]. Fazel Modares *et al*[18] elicited the crucial role of the trans-signaling pathway in liver regeneration after PH because hepatocyte IL-6R activation alone was not sufficient to initiate cell proliferation. TNF-α has two main functions: It activates the NF-κβ signaling pathway through direct interaction with TNF-R1 on Kupffer cell surfaces and through the indirect induction of inhibitory KB kinase; it also stimulates hepatocyte c-Jun N-terminal kinase (JNK). JNK phosphorylates the c-Jun transcription factor in the nucleus to induce cyclin-dependent kinase 1 transcription, which activates hepatocyte proliferation[8].

The augmenter of liver regeneration (ALR) protein, which has three isoforms (15, 21 and 23 kDa) and is expressed primarily in the liver, testes, kidneys and brain, plays a crucial role in liver regeneration. Each isoform of ALR has a different location within the cell and thus plays a different role[19]. For example, mitochondrial long-form ALR translocates proteins and initiates MitoNEEt release, which leads to cell proliferation. Long-form ALR expression increases in cases of pathology and reduces liver damage, protects against oxidative stress and endoplasmic reticulum stress by decreasing Ca++ levels, and has an antimetastatic effect on hepatocellular carcinoma (HCC). Cytoplasmic short-form ALR enhances the hepatocyte response to IL-6 by inducing the phosphorylation of STAT3; it also has an antimetastatic effect on hepatoma by inhibiting the migrative and invasive capacity of cells[20]. After PH, the ALR concentration increases immediately and activates MAPK signaling; enhances IL-6, TNF-α and inducible nitricoxide synthase production by Kupffer cells; and inhibits NK cell activity. Short-form ALR protects hepatocytes by inhibiting apoptosis stimuli[21].

Proliferative phase

During the second phase of liver regeneration, the G1/M phase transition occurs, which is driven by two groups of mitogens: Complete mitogens, including HGF, TGF-α, epidermal growth factor (EGF), and HB-EGF; and the stimulation of DNA synthesis and cell proliferation *via* Ras-MAPK and PI3K/AKT signaling activation and auxiliary mitogens, including bile acids, vascular endothelial growth factor (VEGF), noradrenalin, insulin-like growth factors (IGFs), estrogen and serotonin[3].

HGF is produced by mesenchymal liver cells and interacts with the methionine (MET) receptor, leading to PI3K and MAPK signaling protein phosphorylation followed by PI3K/AKT and extracellular-signal-regulated kinase 1/2 signaling activation. This process results in the proliferation, migration, and differentiation of liver cells and antiapoptotic effects[22,23]. Epidermal growth factor receptor (EGFR)-transmembrane receptors with tyrosine kinase activity interact with EGF, TGFa, amphiregulin (AR), epigen, and HB-EGF, leading to MAPK, PI3K/AKT–mammalian target of rapamycin (mTOR) and STAT signaling activation, which drives hepatocyte proliferation[24]. Natarajan *et al*[25] identified impaired liver regenerative capacity and delayed cyclin D1 expression in mice lacking EGFR.

Nuclear factor erythroid 2-related factor 2 (NRF2) transcription factors, which regulate a wide range of genes including antioxidant proteins and detoxifying enzymes, are activated in response to increased reactive oxygen species levels. The expression of this molecule increases in the earliest stages of liver regeneration as a result of cellular damage[26]. Zou *et al*[27] discovered the important role of Nrf2 in the regulation of cell cycle progression in mice. Nfr2 is a transcriptional suppressor of Cyclin A2 and a regulator of the Wee1/Cdc2/Cyclin B1 pathway, which controls the beginning of the M phase[27]. Nfr2 also regulates hepatocyte proliferation by modulating the insulin/IGF-1 and Notch1 signaling activities and facilitates the capability of hepatocyte nuclear factor 4 alpha (HNF4α) to keep newly formed hepatocytes in a differentiated state[28].

Bile acids are the main end products of cholesterol metabolism and are synthesized exclusively in the liver, where they function as signaling molecules that activate membrane G-protein-coupled BA receptor 1 (or TGR5) and nuclear farnesoid X receptor (FXR)[29]. After the loss of liver mass due to PH, the bile acids concentration increases during the first minute, which leads to FXR activation, resulting in inhibited BA synthesis and induction of the *FOXM1B* gene[30]. *FOXM1B* is a transcription factor that regulates DNA synthesis and mitosis *via* cyclin-dependent kinase 2 (CDK2) activation, which is required for the G1/S transition and CDK1 activation and is responsible for the S/M transition[31]. FXR activation also appears in enterocytes and leads to the induction of fibroblast growth factor (FGF)15/FGF19 expression. The Fgfr4/β-Klotho receptor, which is located on the hepatocyte surface, inhibits BA synthesis and activates the cell cycle *via FOXM1B* induction when activated[32]. Fgfr4/β-Klotho activation also regulates the termination of liver regeneration and terminal organ size. Kong *et al*[33] showed that mice with enhanced Fgf15 expression have the most active Hippo signaling pathway, which induces cellular senescence and suppresses transcriptional activation. TGR5, which is located on KC, SEC and BEC surfaces, leads to cAMP induction and nuclear factor kappa B (NF-κB)-signaling inhibition[34]. As a result, decreased proinflammatory cytokine synthesis occurs in KCs and bone marrow macrophages *via* the protein kinase B-dependent activation of the mTOR[35]. TGR5 protects the liver from BA overload by increasing its excretion with urine; it also enhances the secretion of HCO_3^- and Cl^- and controls BA polarity because inordinately hydrophobic molecules can damage the regenerating liver[36].

Wnt ligands are glycoproteins secreted by nonparenchymal liver cells, mostly KCs and SECs, and are crucial molecules of liver regeneration[37]. Wnt ligands lead to the integration of Axin into the cytoplasmic membrane through interaction with the Frizzled receptor and the coreceptors LRP5/6, resulting in impaired function of the β-catenin degradation complex. Therefore, Wnt ligands lead to β-catenin accumulation, followed by its translocation to the nucleus and interaction with members of the transcriptional T cell factor family, resulting in target gene transcription, for example, of cyclin D1, leading to hepatocyte proliferation[38]. Preziosi *et al*[39] identified the constitutional secretion of Wnt2 and Wnt9b by central vein endotheliocytes and the essential role of these molecules in the basal activation of β-catenin and metabolic zonation of hepatocytes. PH leads to increased Wnt2 and Wnt4 expressions within all zones of the hepatic acinus and the additional secretion of Wnt9b and Wnt5b within the pericentral zone during the first 12 h. This leads to a 7–8-fold increase in cyclin D1 expression within the periportal and intermediate zones and 20-and 100-fold increases in glutamine synthetase expression within the intermediate zone and the pericentral zone, respectively. The role of increased glutamine synthetase expression remains

unknown but is thought to be an enhancer of pericentral detoxification since the other 2/3 of hepatocytes restore organ mass[39].

The Hedgehog (Hh) signaling pathway is a morphogenic pathway that regulates embryonic development and is implicated in homeostasis maintenance[40,41]. Among vertebrates, this pathway is activated within a special organelle, the primary cilium (PC), *via* the interaction of Hh ligands Sonic hedgehog, Indian hedgehog and Desert hedgehog and the Ptched receptor[42]. After that, phosphatidylinositol 4-phosphate[43], sumoylated molecules and cholesterol[44] form a complex with smoothened (Smo), which leads to its activation. Activated Smo dislocates to the apex of the PC and activates Glis (including Gli1, Gli2 and Gli3), which then translate to the nucleus and regulate gene transcription[45]. The said pathway is canonical, but there are also different types of noncanonical Hh signaling pathways; for example, the Smo-free activation of Glis or the Hh pathway arises beyond the PC[45-47]. Ochoa *et al*[48] identified a meaningful role of Hh signaling in liver regeneration. PH leads to Hip inhibition, thus activating the Hh pathway *via* an increase in the Indian hedgehog level in the replicative period and an increase in the Sonic hedgehog level in the postreplicative period[48]. Platelet-derived growth factor, TGF-β, and EGF are secreted in response to liver damage induced by JNK-dependent Hh ligand synthesis[49,50]. Hh signaling activation occurs within hepatocytes, Ito cells[51] and BECs[52], leading to ECM remodeling, progenitor cell expansion and liver epithelial cell proliferation[45]. Additionally, Hh signaling controls Yes-associated protein 1 (YAP) of activated Ito cells[53]. The Hh-YAP signaling pathway induces the glutaminolysis required for Ito cell activation to regulate liver regeneration[54]. Furthermore, Hh signaling facilitates cell survival *via* inhibiting hepatocytes, BECs, Ito cells and progenitor cell apoptosis[55].

Notch signaling is an important pathway in embryonic development, homeostasis maintenance, and liver regeneration[56]. Mammals have 4 types of receptors for this pathway (Notch1, Notch2, Notch3, and Notch4); Notch1 and Notch2 are located primarily on BECs and HPCs whereas Notch3 and Notch4 are expressed by the mesenchymal compartment of the liver and are poorly represented on epithelial liver cells. JAG-1 and DLL-4 are ligands of Notch signaling that are expressed in the liver[57]. The main role of this pathway in liver development is the JAG1-NOTCH2 interaction, which results in the differentiation of hepatoblasts to BECs and the development of the intrahepatic biliary tree[58]. Lu *et al*[59] showed that the role of the Notch–RBPJ interaction is to drive HPC differentiation to BECs *via* Yap inactivation after PH in mice. The direction of HPC differentiation is defined by the balance of NOTCH signaling and Wnt ligands[60]. Ortica *et al*[61] pointed out the important role of Notch3 in HPC differentiation to hepatocytes. Zhang *et al*[62] elicited the regulatory role of Notch signaling in hepatocyte proliferation *via* the NICD/Akt/Hif-1α pathway after PH, whereas its inhibition leads to delayed S phase entry, impaired S phase and M phase progression, and the loss of the hepatocyte mitotic rhythm due to cyclin E1, A2 and B1 dysregulation. Yang *et al*[63] demonstrated the involvement of Notch signaling in the regeneration of 8 types of liver cells, which is performed by the activity of 9 different pathways and regulates cellular proliferation, apoptosis, the cell cycle, *etc.*

Termination phase

When the needed liver mass: Body mass ratio is achieved, cellular proliferation stops due to inhibitory molecules that control the rapidity and direction of liver regeneration. Among the inhibitors of cell proliferation, IL-1, which is synthesized by nonparenchymal liver cells, inhibits the DNA synthesis induced by HGF, EGF and TGF-α. IL-6 is multifunctional and plays a role as both a liver regeneration inducer and inhibitor; its effect depends on the time and dose of the molecule. The IL-6-dependent inhibition of proliferation is likely to occur by increasing p21 expression[64]. The JAK/STAT signaling pathway is inhibited by 8 members of the SOCS family of proteins; hereafter, only SOCS1 and SOCS3 contain the extended SH2 and kinase inhibitory region. SOCS1 directly binds and inhibits JAK, whereas SOCS3 binds to cytokine receptors, forms a complex with JAK and inhibits the STAT3 signaling pathway. SOCS3 is the main suppressor of the signaling pathway activated by IL-6; it inhibits the phosphorylation of coreceptor gp130, JAK and STAT3. SOCS1 negatively regulates the hepatocyte proliferation induced by HGF *via* c-MET signaling inhibition and likely regulates the TNF-α levels because it interacts with toll-interleukin 1 receptor domain-containing adaptor protein, which drives the synthesis of a current mediator[65].

Some TGF-β family members function as inhibitors of proliferation. In particular, TGF-β1 plays a special role in binding to receptor types 1 and 2 and inducing cell apoptosis to correct an excessive liver mass. Outside of the liver, TGF-β1 is synthesized in platelets and the spleen. The spleen might be involved in the termination phase for

it inhibits HGF and its c-MET receptor expression. In this regard, splenectomy leads to increased hepatocyte proliferation in the first 48 h after PH. Other members of the TGF-β family are involved in the termination phase of liver regeneration, including activin A-hepatocyte proliferation inhibitors and bone morphogenetic proteins (BMPs)[3]. BMP9 is expressed exclusively by liver tissues and in part by hepatocytes. BMP9 regulates a variety of biological functions such as glucose and lipid metabolism, angiogenesis, oncogenesis, and fibrogenesis, and it affects liver regeneration after acute injuries[66]. Addante *et al*[67] reported a regulatory function of BMP9 over HPCs that is affected by anaplastic lymphoma kinase 2 type I receptor activation, resulting in SMAD 1, 5, and 8 induction, HPC apoptosis stimulation, and a reduction in HPCs. Apart from its negative influence on liver regeneration, BMP9 also has profibrogenic activity and promotes HCC proliferation and invasion. Additionally, BMP9 enhances the expression of TLR4 on the SEC surface, leading to inflammatory cell recruitment. Therapy with anti-BMP-9/ anaplastic lymphoma kinase 1 can potentially enhance hepatocyte proliferation among patients with chronic liver diseases and decrease the probability of fibrosis and HCC development[68].

HNF4-α regulates hepatocyte differentiation and, according to Huck *et al*[69], promotes the termination of liver regeneration. The expression of the current molecule significantly decreased during the priming phase and increased during the following phases, which is necessary for termination and hepatocyte function recovery after PH[69]. HNF4-α is a YAP and TGF-β/SMAD3 antagonist; therefore, decreased expression of this molecule stimulates promitogenic functions and activates connective tissue growth factor. Increased HNF4-α expression during the subsequent phases of regeneration prevents the excessive synthesis of connective tissue and therefore fibrosis[70]. Hnf4-α also leads to the inhibition of HPC proliferation and migration in rats[71].

Integrin-linked kinase is a suppressor of hepatocyte proliferation that is located under the cytoplasmic membrane and is associated with a3/b1 integrins of the ECM. Interruption of this connection results in hepatostat imbalance and excessive liver mass. Focal adhesion kinase is also associated with a3/b1 integrin and promotes hepatocyte proliferation[72].

The Hippo signaling pathway is a crucial regulator of the terminal organ size within mammals. The key component of the mammalian Hippo pathway is a kinase cascade in which the Ste20-like kinases 1/2 phosphorylate and activate large tumor suppressor 1/2, its adapter protein Mps one binder 1, and the transcriptional coactivators Yap and Taz[73]. Phosphorylated Yap and Taz emerge from the nucleus, where they are bound to transcription factors that control the proliferation and differentiation of cells, such as TEAD family members[74]. The Hippo/Yap signaling pathway is likely an integrator of a large number of alternative growth factor signaling pathways and regulates liver size by balancing negative and positive regulatory signals[72]. The Hippo signaling pathway does not have any specific receptors and is regulated by molecules that control cellular polarity and morphology, intercellular adhesion and other processes. The activity of this pathway is modulated in response to mechanical deformation and intercellular adhesion defects and cell adhesion to the intercellular matrix. Consequently, Hippo signaling senses cellular and tissue integrity[75]. Intranuclear Yap is located in periportal hepatocytes and BECs, whereas pericentral hepatocytes contain few current molecules, which is exactly the opposite of the constitutive Wnt ligand content; therefore, current pathways inhibit one another[76]. Table 1 summarizes the main molecular factors in liver regeneration.

Cells involved in liver regeneration

The hepatic acinus is the structural and functional unit of the liver. It consists of three zones. The hepatocytes in the first zone have a periportal location and are specialized in gluconeogenesis and the beta-oxidation of fatty acids; conversely, hepatocytes in the third zone lie pericentral and perform glycolysis, lipogenesis and detoxification. Therefore, hepatocytes are functionally heterogeneous and express various genes depending on their localization[77]. Experiments performed on mice have mainly investigated diploid hepatocyte populations within the third zone, which express the early progenitor cell markers Tbx3 and Axin2 and can proliferate twice as fast as other hepatocytes. This ability depends on Wnt ligand expression in nearby SECs[78]. Sun *et al*[79] reported that Axin2$^+$ pericentral hepatocytes are not confined to the liver stem cell compartment and do not have an enhanced capacity for proliferation. Cells of every zone participate in cellular homeostasis. The authors further controverted the opinion that Axin2$^+$ pericentral hepatocytes translocate to the periportal zone. Axin2$^+$ induction was identified in every zone of the acinus during regeneration[79].

Hybrid hepatocytes, which account for 5% of all hepatocytes, were found in the first

Table 1 The main factors driving liver regeneration

Factor of regeneration	Influence on LR
TNF-α	Induction of CDK-1
IL-6	Activation of the JAK/STAT, MAPK, and PI3K/AKT signaling pathways
Hh signaling pathway	ECM remodulation; induction of progenitor cell and liver epithelial cell expansion; induction of glutaminolysis; inhibition of hepatocyte, BEC, Ito cell and progenitor cell apoptosis
ALR	lfALR: Enhancement of the hepatocyte response to IL-6 and STAT3 phosphorylation induction. MAPK signaling pathway activation; NK cells inhibition; increase in IL-6, TNFα and iNOS production by Kupffer cells, sfALR: Inhibition of proapoptotic stimuli
NRF2	Regulation of M phase entry, hepatocyte proliferation, maintenance of newly formed hepatocytes in a differentiated state
Growth factors (HGF, TGF-α, EGF, HB-EGF)	Stimulation of DNA synthesis and cell proliferation *via* Ras-MAPK and PI3K/AKT signaling pathway activation
BAs	Activation of CDK2, cell cycle, regulation of termination phase and terminate liver size, decrease in the inflammatory cytokine production, enhancement of BA excretion and $HCO3^-$, Cl^- secretion, control of BA polarity
Wnt-β-catenin	Hepatocyte proliferation induction
Notch signaling pathway	Modulation of HPC differentiation toward BECs, regulation of hepatocyte proliferation, mitotic rhythms, cyclin E1, A2 and B1
IL-1	DNA synthesis inhibitor
SOCSs	c-MET and JAK-STAT signaling pathway inhibition
TGF-β1, activin A, BMPs	Induction of apoptosis to correct excessive liver mass
HNF4	Regulation of hepatocyte differentiation, initiation of the termination phase, antagonism YAP and TGF-β/SMAD3, prevention of excessive connective tissue synthesis, inhibition of HPC proliferation and migration
Hippo/YAP signaling pathway	Terminal liver size control

LR: Liver regeneration; TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; CDK-1: Cyclin-dependent kinase 1; JAK/STAT: Janus kinase/signal transducer and activator of transcription; Hh: Hedgehog; ECM: Extracellular matrix; MAPK: Mitogen-activated protein kinase; ALR: Augmenter of liver regeneration; lfALR: Long-form ALR; sfALR: Short-form ALR; iNOS: Inducible nitric oxide synthase; NRF2: Nuclear factor erythroid 2-related factor 2; BA: Bile acids; EGF: Epidermal growth factor; HGF: Hepatocyte growth factor; HPC: Hepatic progenitor cells; BEC: Biliary epithelial cells; MET: Methionine; BMP: Bone morphogenetic proteins; HNF4 : Hepatocyte nuclear factor 4 alpha; YAP: Yes-associated protein.

zone of acinus. These cells express the hepatic transcription factors Hnf4a and sulfur oxide (Sox) 9, which are active in BECs. Hybrid hepatocytes are capable of differentiation into BECs and hepatocytes and help recover liver mass after various chronic diseases[80].

In contrast with the regenerative pool of the third zone, cells of the first zone do not proliferate in the absence of functional damage. The specific marker of periportal hepatocytes is the major facilitator superfamily domain-containing 2a[9]. Liver regeneration in the homeostatic state was performed *via* hepatocyte self-renewal within each acinus zone. Pu *et al*[81] elicited the capacity of major facilitator superfamily domain-containing 2a+ hepatocytes to proliferate more actively after PH and to completely replace pericentral hepatocytes during $CCl4^-$ induced chronic injury. Therefore, depending on the type and duration of damage, liver regeneration occurs *via* different pools of cells[81].

Immune cells, including Kupffer cells, circulating monocytes and lymphocytes, play an important role in liver regeneration. Kupffer cells secrete mediators of proliferation, such as HGF, IL-6, TNF-α and Wnts, stimulating angiogenesis *via* VEGF-A secretion. Circulating monocytes play an important role in the first hours of liver regeneration, as indicated by the significantly increased number of adhesion molecules on the SEC surface. Monocytes play a role because it takes time for Kupffer cells to reach the sinusoids from the space of Disse[82]. Organ damage initiates the release of chemoattractants, such as osteopontin, monocyte chemoattractant protein-1, and intercellular adhesion molecule 1, resulting in macrophage recruitment to the liver where lipopolysaccharide and the C3a and C5a components of the complement system activate the NF-κB signaling pathway and the synthesis of IL-6 and TNF-α[83]. Apart from macrophage activation, components of the complement system directly influence liver regeneration. C5a binds to C5aR1, whose expression on hepatocyte surfaces

significantly increases during regeneration, inducing cellular growth. C3a and C3b might facilitate liver regeneration since organ recovery within C3-deficient mice (C3–/–) was disturbed in a previous experiment[84].

NK and natural killer T cells inhibit regenerative processes *via* interferon (IFN)-γ secretion, which stimulates the synthesis of antiproliferative proteins, such as STAT1, IRF-1, and p21CIP1/WAF1, by hepatocytes. The influence of natural killer T cells on liver regeneration is significantly lower[83,85]. The medium limitation of NK cell activation is required for normal liver regeneration, which provides the increased expression of the coinhibitory receptor T cell Ig and ITIM domain (TIGIT) on these cell surfaces. TIGIT binds to the poliovirus receptor (PVR), which is located on hepatocyte and Kupffer cell surfaces and results in the inhibition of INF-γ secretion by NK cells. Hepatocyte expression of PVR significantly increased because the current protein is not only a ligand of NK cell receptors but is also a mediator of cellular growth, adhesion, migration and immunomodulation. However, the main role of PVR in liver regeneration seems to be interaction with TIGIT[86]. Eosinophils are key cells that secrete IL-4, which stimulates the G1 phase entrance of hepatocytes by binding to IL4Rα[87].

Hepatic stellate cells (HSCs, Ito cells) are located in the space of Disse and function in retinoid storage during the inactive stage[88,89]. Ito cells have regenerative potential and, in addition to growth factor secretion, can exhibit stem cell properties. Thus, stellate cells have the capacity to differentiate into HPCs, hepatocytes and BECs based on the influence of certain cytokines[90,91]. Ito cells demonstrate this capacity, as described above, in the case of chronic liver diseases, including cirrhosis[92]. Swiderska-Syn *et al*[93] demonstrated that hepatocytes require modulation of the epithelial-mesenchymal transition in multipotent progenitors derived from HSCs. A crucial role in this process is canonical Hh signaling. Although Ito cells have characteristics of multipotent cells, they improve the supportive role of each progenitor pool rather than nullify the importance of other liver progenitor populations[93]. HPC expansion and infiltration are correlated with ECM remodeling. HSCs engage in the degradation of collagen, forming an HPC niche that is rich in laminin, hyaluronic acid (HA) and collagen III, which are necessary for the development of the undifferentiated HPC phenotype. Collagen type I and fibronectin promote cell cycle arrest and HPC differentiation into hepatocytes and BECs[94]. Several studies have contradicted the capacity of HSCs to give rise to an epithelial pool of liver cells in various models of liver injury and in isolated cell cultures. The sources of hepatocytes and BECs are mature hepatocytes and bipotential liver progenitor cells[95,96]. Kordes *et al*[97] showed that the pancreatic stellate cells of rats express stem cell markers, such as CD133 and nestin, and have the possibility to display the β-catenin-dependent Wnt and Notch signaling pathways, which are required for stem cell maintenance and expansion. Transplantation of these cells after the surgical removal of 70% of the liver mass and the inhibition of hepatocyte proliferation (2AAF/PHX) led to the transdifferentiation of current cells into Hnf4α$^+$ hepatocytes and panCK$^+$ BECs[97]. Further studies in the given field are required because the role of HSCs in liver regeneration is significant. Research by Mabuchi *et al*[98] defined the importance of HSC and hepatocyte interactions in the early phases of liver regeneration, resulting in HSC activation[98]. Activated stellate cells transdifferentiate into myofibroblasts, secreting ECM components and cytokines, which drive the proliferation and differentiation of liver cells[92]. Among the cytokines secreted by Ito cells, HGF, lymphotoxin-beta, FGF, IL-6, NOTCH, delta-like noncanonical Notch ligand 1 and TGF-β1 play important roles[92,99]. HSCs regulate HPC proliferation *via* the antiproliferative effect of TGF-β1, which controls the termination phase of liver regeneration. Ito cells regulate the cytokine profile, affecting various phases of liver mass restoration[100]. Konishi *et al*[73] demonstrated the intensification of hepatocyte proliferation *via* HSC activation after ischemia-reperfusion injury (IRI). Herein, activated YAP and TAZ served as the inductors of HSC proliferation in the postischemic liver[73].

In addition to playing a role in thrombogenesis, platelets are involved in the development of inflammation and several syndromes; they also lead to the metastasis of some tumors and are required for liver regeneration. Previous studies have elicited impaired regeneration after PH under conditions of thrombocytopenia, whereas an elevated level of platelets was associated with enhanced regeneration since platelets produce HGF, Platelet-derived growth factor and TGF-β[101]. Partial resection or chronic liver injury leads to platelet accumulation in the sinusoids and space of Disse, likely *via* von Willebrand factor (vWF) secretion by SECs[102]. vWF plays a crucial role in the early stages of liver regeneration by promoting platelet adhesion, which is significantly decreased when anti-vWF antibodies are present. After the initiation of regeneration, the secretion of vWF antigens increases. The postoperative level of vWF

antigens may be used to predict the survival prognosis[103]. Platelets secrete various growth factors that positively influence liver regeneration. The most important secreted cytokines are HGF, IGF and serotonin, which promote hepatocyte proliferation[104]. Human platelets do not secrete a considerable amount of HGF; therefore, the primary platelet mediator of liver regeneration is IGF-1. Apart from hepatocytes, platelets also interact with SECs and Kupffer cells and thus positively affect liver regeneration. Sphingosine-1-phosphate is secreted by platelets and stimulates SEC proliferation and IL-6 secretion, which drives DNA synthesis within hepatocytes. The interaction between platelets and Kupffer cells leads to the activation of both cells[105]. Platelets enhance the Kupffer cell secretion of mediators, *i.e.*, TNF-α and IL-6, that are required for liver regeneration[101]. Platelets can either activate angiogenesis or inhibit it, depending on the mediator secreted from α-granules. Thus, thrombospondin 1 is an antiangiogenic mediator, whereas VEGF has a proangiogenic function. As long as platelets secrete both of these mediators, the PH outcome depends on the pattern of α-granule secretion[106]. Since platelets secrete many mitogens, the transfusion of blood enriched with platelets promotes liver regeneration after PH; however, it may lead to complications, including fatality[3].

A general scheme of the molecular processes involved in the various phases of typical liver mass regeneration is shown in Figure 1.

PROGENITOR-DEPENDENT LIVER REGENERATION

The mechanisms described above are specific for healthy livers and occur among living liver donors. However, in most cases, liver resection occurs within patients with impaired liver function, and subsequent regeneration proceeds in a nonstandard way, which can lead to hepatic failure and death[107].

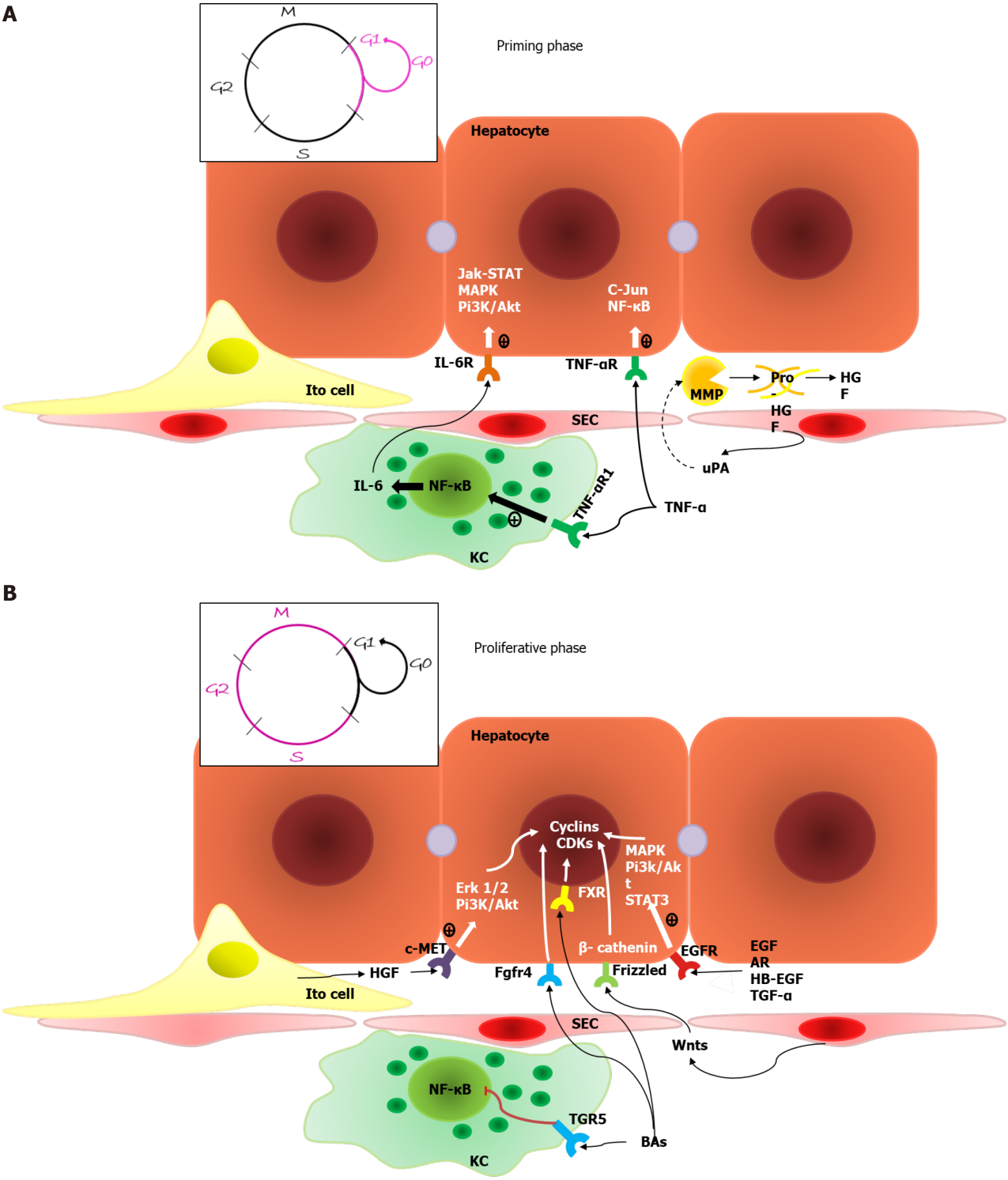
Acute liver failure caused by intoxication, viral hepatitis A, B or E, autoimmune liver disease, *etc.*, is often followed by widespread necrotic and apoptotic zones, and adequate liver regeneration becomes impossible[107]. During acute liver failure, the main regenerative role is given to HPCs, as indicated by the increased level of alpha-fetoprotein (AFP). Therefore, a high AFP level is correlated with a positive prognosis after acetaminophen-induced liver damage[12]. The immune system regulates liver regeneration *via* necrotic cell phagocytosis and controls inflammatory reactions in response to injury. The number of proliferative macrophages in the liver significantly increases after organ damage, and monocytes are recruited from the bloodstream and differentiate into macrophages in response to increasing the colony-stimulating factor 1 levels. Colony-stimulating factor 1 injection promotes liver regeneration after PH; conversely, a low level of the current factor is correlated with a negative patient prognosis[11].

Liver steatosis is associated with an impaired regenerative function, in which GADD34 plays an important role since its increased expression promotes liver regeneration within mice. IRI often complicates the posttransplantation period and impairs typical liver regeneration. The current complication is followed by increased receptor for advanced glycation end product levels, which might be a therapeutic target. Thus, receptor for advanced glycation end product inhibitor injection leads to a reduction in organ damage and the induction of liver regeneration. The excessive synthesis of ECM components by activated HSCs inhibits hepatocyte proliferation, and if macrophage MMPs do not promote connective tissue restitution, the angioarchitecture of hepatic lobules is impaired, resulting in cirrhosis[107]. In the liver, damage due to cirrhosis and hepatitis B or C often reveals hepatocytes with BEC markers, such as epithelial cell adhesion molecule (EpCAM), on their surface. The presence of these intermediate hepatobiliary cells is thought to be explained by their origin from biliary compartment progenitors[108].

Hepatocytes are the main cells driving typical liver regeneration, whereas alternative liver regeneration is performed by HPCs[92]. The process of progenitor-dependent liver regeneration is shown in Figure 2.

RESIDENT STEM CELLS OF THE LIVER

Therefore, the liver regenerative capacity is significantly impaired during chronic liver diseases due to the accumulation of senescent hepatocytes[109,110]. In this case, liver mass restoration is performed by HPCs[111]. HPCs are located in the canals of Hering and have a bipotential nature; in other words, they can differentiate into both hepatocytes


A
M
G1
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Priming phase
Hepatocyte
Jak-STAT
MAPK
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TNF-αR
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MMP
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F
SEC
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IL-6
NF-κB
TNF-αR1
TNF-α
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G1
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Proliferative phase
Hepatocyte
Cyclins
CDKs
MAPK
Pi3k/Ak
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Erk 1/2
Pi3K/Akt
FXR
c-MET
β- cathenin
EGFR
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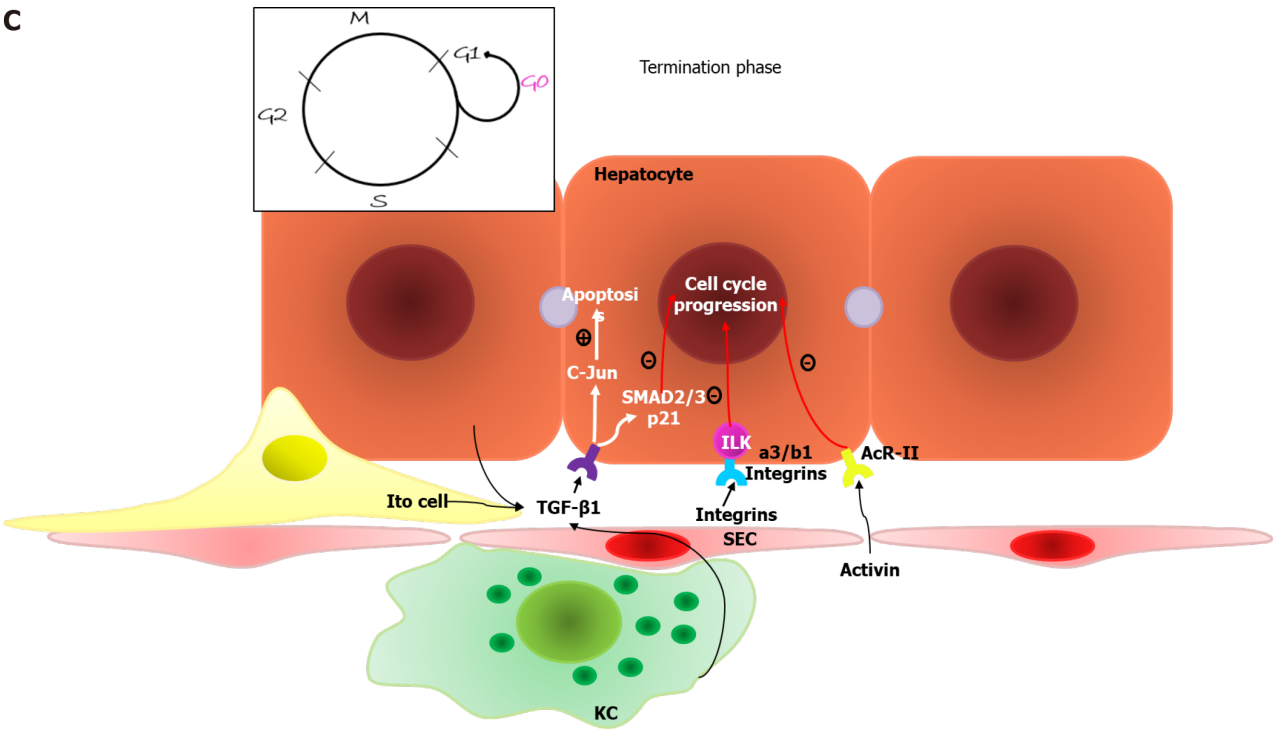

Figure 1 Typical liver regeneration. A: Priming phase. Mature hepatocytes undergo the G0-G1 transition driven by interleukin-6 and tumor necrosis factor-α. Sinusoid endothelial cells produce urokinase plasminogen activator in response to increased blood pressure. Urokinase plasminogen activator activates matrix metalloproteinase, resulting in extracellular matrix remodeling and the release of growth factors; B: Proliferative phase. Numerous factors, including Wnt-ligands, growth factors and bile acids, lead to the transcription of cyclin-dependent kinase and cyclins, resulting in the S-M transition and hepatocyte proliferation. Bile acids also suppress the synthesis of inflammatory cytokines by Kupffer cells; C: Termination phase. Different factors, primarily tumor necrosis factor-β family members, initiate the cell cycle arrest of hepatocytes and reversion to the G0 phase and cause the apoptosis of newly formed cells to correct the excessive regenerative response. TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; MMP: Matrix metalloproteinase; KC: Kupffer cells; SEC: Sinusoid endothelial cells; HGF: Hepatocyte growth factor; EGFR: Epidermal growth factor receptor; MAPK: Mitogen-activated protein kinase.

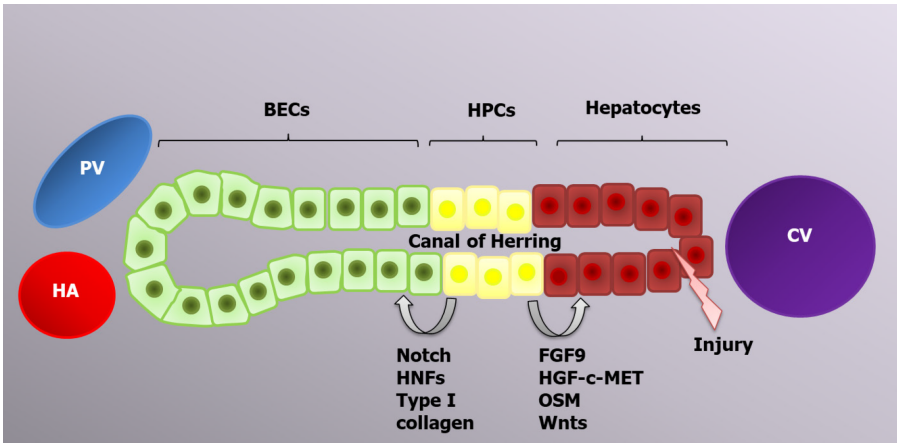

Figure 2 Progenitor-dependent liver regeneration. In case of excessive acute injury or chronic liver diseases, hepatic progenitor cell activation occurs in response to different inflammatory cytokines, including tumor necrosis factor-like weak inducer of apoptosis. Depending on the type of stimulus, hepatic progenitor cells can differentiate into biliary epithelial cells or hepatocytes to restore the liver mass. PV: Portal vein; HA: Hepatic artery; CV: Central vein; BECs: Biliary epithelial cells; HPCs: Hepatic progenitor cells; HNFs: Hepatocyte nuclear factors.

and BECs, the choice of which is determined by the activation of certain genes[12,108]. The canals of Hering connect the hepatocyte canalicular system and the biliary tree, and such a location of HPCs is consistent with their bipotential features[94]. Transplantation of current cells leads to liver regeneration enhancement *via* HPC proliferation and differentiation, which can be applicable for the treatment of certain liver diseases[112-115]. CK19, EpCAM and CD133 are markers common to both HPCs and BECs. Trop2 (Tacstd2) is a transmembrane molecule that is present on the HPC surface and absent on BECs; therefore, it can play a role as a specific marker, similar to

Foxl1[116].

The origin of HPCs is still being researched. Many scientists think that HPCs arise from mature differentiated BECs due to the presence of similar markers and cell localization. The expression of hepatocyte markers, such as albumin, AFP and HNF4α, appears earlier than HPC expansion. Newly formed HPCs have various markers on their surface, including the BEC markers HNF1b and CK19, which are maintained until the HPCs differentiate into mature hepatocytes[117]. Hepatocytes and BECs are formed from common cells, called hepatoblasts, during the second trimester of embryonic development. Consequently, the possibility of hepatocyte to BEC transdifferentiation and vice versa is genetically feasible and might be programmed to form a facultative pool of progenitors[12].

HPC compartment activation in the human liver is called ductular reaction because of the role of ductular epithelium activation. In the niche, HPCs are surrounded by epithelial and nonparenchymal cells, immune cells, and the components of the ECM, which transport activating signals[118]. As long as HPCs drive the regeneration of massive or chronic damage facilitated by immune cells, inflammatory cytokines, such as TNF-α, lymphotoxin-β, interferon-γ and IL-6, will play a crucial role in HPC activation. TNF-like weak inducer of apoptosis (TWEAK) is a TNF superfamily member and the main inducer of HPC activation[119]. Macrophages and NK cells are primary sources of TWEAK ligands. The interaction with target cells is realized by FGF-inducible 14 receptors. The TWEAK/ FGF-inducible 14 interaction leads to ductular reaction initiation *via* activation of the NF-κB signaling pathway[120]. HPC regulation is also performed by free oxygen radicals, which act as second messengers, realizing the balance between self-renewal and the differentiation of current cells. Low reactive oxygen species levels promote HPC proliferation *via* extracellular-signal-regulated kinase 1/2, Jun 1/2, Wnt and NF-κB signaling[121].

HPC differentiation into hepatocytes and BECs is regulated by a variety of signaling pathways. Thus, FGF9, the HGF-c-MET signaling pathway[122] and oncostatin M activate AKT and STAT3, which are required for HPC differentiation into hepatocytes, whereas HNF-6, HNF-1β and NOTCH signaling lead to BEC development[123,124]. All-trans retinoic acid is a significant active metabolite of vitamin A that is involved in HPC differentiation by increasing miR-200a expression, which regulates cell autophagy[125]. Ma *et al*[126] demonstrated the regulatory function of autophagy in HPC differentiation into hepatocytes *via* activation of the Wnt/β-catenin signaling pathway. Autophagy can also regulate HPC differentiation into BECs since it inhibits the Notch1 signaling pathway, which is required for the development of biliary duct cells. Therefore, autophagy is decreased during the early stages of liver regeneration[127].

Recently, a new pool of multipotential biliary progenitor cells, which can differentiate into hepatocytes, BECs and the islets of Langerhans cells, was identified in peribiliary glands, which are epithelial invaginations of extrahepatic and large intrahepatic biliary ducts[108]. This pool was named biliary tree stem/progenitor cells (BTSCs). BTSCs express stem cell markers such as Sox17, Pdx1, Sox9, EpCAM, Sall4 and Lgr5 on their surface. BTSCs are primarily involved in biliary epithelium regeneration in chronic diseases such as primary sclerosing cholangitis, cholangiocarcinoma, nonanastomotic strictures and biliary atresia[128].

MICRO RIBONUCLEIC ACIDS AND LIVER REGENERATION

Micro ribonucleic acids (MiRNAs) are short molecules of 19–25 nucleotides in length that regulate the posttranscriptional silencing of target genes. One miRNA molecule can regulate hundreds of mRNAs, thus controlling the expression of various genes[129,130]. After PH, miRNA expression is primarily decreased (miR-16, miR-22, miR-23, miR-24, miR-26a, miR-29, miR-30, miR-31, miR-33, miR-122a, miR-126, miR-127, miR-145, miR-150 and miR-378); however, the expression of certain miRNAs increases (miR-21, miR-26b, miR-192, miR-194, MiR34a, miR-122, miR-203 and miR-221), thus affecting the hepatocyte cell cycle[131]. Table 2 summarizes the significant miRNAs in liver regeneration after PH.

Castro *et al*[132] demonstrated the crucial role of miRNAs in liver regeneration after PH. Thus, it was demonstrated that the expression of 26 different miRNAs changes during regeneration, notably in both increasing and decreasing ways. The expressions of miR-19a, -21, and-214 were significantly increased. MiR-21 transcription is activated by activator protein 1 (AP-1), which is also required for the activation of the important Stat3 and TGF-β signaling pathways[132]. Ng *et al*[133] pointed out the regulatory role of miR-21 in hepatocyte cell cycle events preceding the S phase *via* the indirect induction

Table 2 Main micro ribonucleic acids influencing liver regeneration

miRNA	Expression change	Target genes	Influence on LR
miR-21	Increased	Rhob, Sox7, Crebl2, Bcl-2, Btg2, Timp3, Reck, Pdcd4, Tgfbi, Smad7, PTEN	Induction
miR -19a	Increased	PTEN	Induction
miR-214	Increased	PTEN	Induction
miR-203	Increased	SOCS3	Induction
miR-27a/b	Increased	Tmub1	Induction
miR-503	Decreased	Cyclin D1, Cyclin E2, CDC25A, CDKN1B, CHK1	Suppression
miR-23a	Decreased	TNF-α, c-Myc CCNL2, HNF4G MET	Suppression
miR-150	Decreased	TNF-α, survivin, FoxP1, c-Myb	Suppression
miR-663	Decreased	TGF-β1, AP-1, Jun-B, Jun-D	Suppression
miR-378	Decreased	Odc1	Suppression
miR-34a	Decreased	INHBB	Suppression
miR-33	Decreased	CDK6, EEF1A1, RAP2A	Suppression
miR-26a	Decreased	MAP3K2, MXI1, SENP5, CCND2, CCNE2	Suppression

miRNA: Micro ribonucleic acids; PTEN: Phosphatase and tensin homolog; LR: Liver regeneration; TNF-α: Tumor necrosis factor-α; CDK-6: Cyclin-dependent kinase 6.

of cyclin D1 translation, which occurs due to a reduction in cell cycle inhibitor expression. MiR-21 has a binding site on Ras homolog gene family member B, whose expression leads to the suppression of Akt1 activation, thus regulating cyclin D1 expression *via* mTORC1[133]. Additionally, miR-21 plays a significant role in decreasing phosphatase and tensin homolog expression, resulting in increased Akt and mTOR activities[134]. MiR-203 induces liver regeneration *via* IL-6/STAT3 signaling enhancement and SOCS3 expression inhibition[135]. MiR-27a/b regulates hepatocyte proliferation during regeneration because it suppresses Tmub1 expression[136], which suppresses the IL-6/STAT3 signaling pathway[137].

The decreased expression occurs within molecules such as miRs-503, -23a, -150, -663, -654 and is associated with their negative influence on liver regeneration. Thus, miR-150 inhibits TNF-α expression, which is essential for liver regeneration[138]. Increased miR-503 expression leads to the enhancement of essential cell cycle gene expression, including that of cyclin D1, E1, E2, F, Wee1, CDC25A and CHK1[139]. The AP-1 transcription factors, including the Jun and Fos family members, are the target genes of miR-663[140]. The c-Jun/AP-1 signaling pathway controls hepatocyte proliferation and has antiapoptotic activity *via* p-53-dependent pathway suppression[141]. An important negative regulator of hepatocyte epithelial-mesenchymal transition is miR-378, whose expression is decreased by Smo during liver regeneration, resulting in Hh-pathway activation and the transdifferentiation of hepatocytes and BECs into myofibroblasts[142]. MiR-34a expression is significantly decreased during the first days after PH, whereas the expression of its target genes (Notch1, Notch 4 and Hes1) is increased, leading to hepatocyte differentiation and growth enhancement[143]. MiRs inhibiting liver regeneration are also important because they prevent excessive regeneration. Among these molecules, for example, miR-33 suppresses CDK6 and CCND1[144], and miR-26a targets CCND2 and CCNE2[145].

A further understanding of the miRNAs involved in normal and progenitor-dependent liver regeneration can improve the use of miRNAs for the diagnosis of different liver diseases, control the adequacy of liver regeneration and act as a potential therapy for insufficient liver regeneration.

STIMULATION OF INSUFFICIENT LIVER REGENERATION

Therapeutic methods for insufficient liver regeneration treatment are lacking, although many studies have focused on the efficiency of various molecules in promoting liver regeneration. Shi *et al*[146] determined that baicalin can stimulate liver regeneration after

acetaminophen-induced acute liver injury in mice *via* inducing hepatocyte proliferating cell nuclear antigen, increasing cyclin D1 expression and Nrf2 cytosolic accumulation, and enhancing IL-18 Levels, leading to the upregulation of hepatocyte proliferation. So *et al*[147] showed the promotion of liver regeneration after the inhibition of EGFR or MEK/extracellular signal-regulated kinase (ERK) and the genetic suppression of the EGFR-ERK-SOX9 axis *via* inducing HPC-to-hepatocyte differentiation in zebrafish. The research of Xiang *et al*[148] noted the therapeutic effect of IL-22Fc in inducing liver regeneration in acute-on-chronic liver failure patients due to the shift from anti-regenerative IFN-γ/STAT1 to the pro-regenerative IL-6/STAT3 pathway. Li *et al*[149] reported that aldose reductase (AR) is a new potential therapeutic target for enhancing normal and fatty liver regeneration after surgery and IRI because the knockout of AR leads to enhanced oxisome proliferator activated receptor-α and oxisome proliferator activated receptor-γ expression, thus improving energy metabolism in the liver. The research of Loforese *et al*[150] revealed that the inhibition of MST1 and MST2 with si-RNA resulted in improved hepatocyte proliferation in aged mice after PH; therefore, Ste20-like kinases 1/2 may be a potential therapeutic target. Many other molecules and molecular pathways have been shown to enhance liver regeneration in experimental models. Further studies would help implicate the potential therapy in the clinic and improve the survival of patients with different liver diseases in the near future.

Mesenchymal stem cells (MSCs) have a self-renewal capacity and are derived from the bone marrow, adipose tissue, umbilical cord, *etc*. They are the subject of focus in regenerative medicine and serve as a potential therapy for different liver diseases[151,152]. MSCs were shown to improve liver regeneration in patients with cirrhosis by elevating anti-apoptotic factors, such as HGF and IGF-1, and angiogenic and mitogenic factors. In acute liver failure animal models, MSCs have been shown to promote liver regeneration mostly by suppressing the oxidative stress and inflammation *via* reducing TNF-α, IFN-γ and IL-4 Levels and stimulating liver regeneration with various released factors such as PGE_2 and delta-like 4[153,154]. MSCs can also stimulate liver regeneration after PH by upregulating hepatic cell proliferation and downregulating fat accumulation and HGF, IL-6, IL-10 and TNF-α serum levels[155].

Further studies in this field can help determine how to prevent hepatic failure after surgical interventions and acute and chronic injuries *via* improving liver regeneration.

CONCLUSION

Liver regeneration is driven by multiple molecular processes. Biomolecular factors permit the possibility of targeted therapy to prevent serious complications, such as liver failure due to a decreased cellular regenerative potential.

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MINIREVIEWS

Hepatitis D virus and liver transplantation: Indications and outcomes

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Abstract

Hepatitis D virus (HDV) is a dependent virus that relies on hepatitis B virus for its replication and transmission. Chronic hepatitis D is a severe form of viral hepatitis that can result in end stage liver disease. Currently, pegylated interferon alpha is the only approved therapy for chronic HDV infection and is associated with significant side effects. Liver transplantation (LT) is the only treatment option for patients with end-stage liver disease, hepatocellular carcinoma, or fulminant hepatitis due to coinfection with HDV. As LT for HDV and hepatitis B virus coinfection is uncommon in the United States, most data on the long-term impact of LT on HDV are from international centers. In this review, we discuss the indications and results of LT with treatment options in HDV patients.

Key Words: Hepatitis delta virus; Liver transplant; Hepatitis B immunoglobins; Hepatocellular carcinoma

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(HBV) to synthesize the pathogenic genomes. Therefore, it can only survive as a coinfection with HBV or as a superinfection. Chronic HDV infection results in rapid liver damage and can result in end stage liver disease. Currently, pegylated interferon alpha is the only approved therapy for chronic HDV infection and is associated with significant side effects. Thus, liver transplant remains the only option for patients with end-stage liver disease, hepatocellular carcinoma due to coinfection or superinfection with HDV and HBV, fulminant liver failure and those who cannot be treated with interferon-based therapies. Post transplantation reinfection with HDV/HBV is an undesirable outcome. Though, there is a consensus that hepatitis B immune globulin in combination with a potent nucleoside/nucleotide analogue have shown promising results. In addition, there is ongoing research for newer treatment drugs. This review article focuses on liver transplant in patients as a result of hepatitis D virus. We have discussed the epidemiology, pathogenesis, clinical presentation, indication of liver transplantation, treatment options and the outcomes. New therapy trials have been also discussed in the treatment section. We believe that this topic is an area of knowledge gap and this article will cover the basics.

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INTRODUCTION

Hepatitis D virus (HDV) was discovered in 1970s by Rizzetto *et al*[1]. It is formed by 1678 nucleotide single stranded ribonucleic acid (RNA) virus which is circular in shape and contains two viral proteins that are p24 and p27[2,3]. There is a total of 8 genotypes of HDV in the world[4]. HDV is not able to make its own proteins and relies on hepatitis B virus (HBV) to synthesize the pathogenic genomes. Therefore, it can only survive as a coinfection with HBV or as a superinfection. Around 5% of HBV carriers worldwide have been exposed to HDV and the prevalence of HDV coinfection in United States is reported to be 12%[5,6]. Chronic HDV infection results in rapid liver damage compared to patients infected with HBV alone. In addition, incidence of cirrhosis is almost three times with HBV/HDV chronic coinfection and associated with increased rate of early decompensation leading to hepatocellular carcinoma (HCC)[7]. As HDV uses host polymerase for replication, HBV polymerase inhibitors are not effective against it[8]. Therefore, the only widely accepted treatment is interferon at high doses which has a success rate of 25% to 30%, which is defined as virological response after one year of conventional or PEG-INFa treatment with most studies measuring virological response after 6 mo of treatment[9]. Thus, liver transplant (LT) remains the only option for patients with end-stage liver disease, HCC due to coinfection or superinfection with HDV and HBV, fulminant liver failure and those who cannot be treated with interferon-based therapies. In this review we discuss the indications of liver transplantation and its outcome in patients with HDV.

EPIDEMIOLOGY

There are about 240 million people worldwide who have positive hepatitis B surface antigen (HBsAg). Amongst them 2% to 8% are co-infected with HDV resulting in approximately 20 to 40 million suffering from HDV[10,11]. However, recent studies have estimated the coinfection number to be higher as up to 72 million[12]. HDV is endemic in the Middle East, Mediterranean Area, Amazon Region, and African countries[13]. In Europe, HDV is mainly a problem in Eastern European immigrant populations and amongst intravenous drug users (IVDU)[14,15]. There are 8 different HDV genotypes and genotype 1 is the most common in North America[16]. Testing for HDV has not been widespread in the United States and the prevalence has been underestimated. There has been a 3.4% HDV seropositive rate reported in the veteran population positive

with HBsAg[17]. In comparison, National Health and Nutrition Examination Survey data (1999-2012) showed a significantly lower rate of HDV prevalence (0.02%) in the civilian population[18]. It increased to 0.11% in a repeat National Health and Nutrition Examination Survey (2011-2016) study[19]. Both these studies are limited as they excluded homeless, incarcerated, and other high-risk individuals. However, a study done among patients with IVDU in Baltimore by Kucirka *et al*[20] reported 11% prevalence of HDV in 2005-2006. Similarly, Gish *et al*[21] conducted a study in California on chronic HBV patients reporting a coinfection rate of 8%. This variability warrants routine testing of HDV in HBV carriers with specific recommendations for screening, treatment and follow-up. This will aid risk stratification of patients and allow for early discovery of complications, which in turn may improve outcomes.

PATHOGENESIS, CLINICAL FEATURES AND DIAGNOSIS

HDV is parenterally transmitted and has variable clinical manifestations. There are two major patterns of infection that are described in literature. Notably, coinfection of HBV with HDV and superinfection of HDV in chronic HBV-infected patients. HDV develops innate and adaptive immunity and there are specific markers such as HDV RNA, hepatitis D antigen and anti-HDV antibodies, such as IgM and IgG, which help to detect and differentiate the chronicity of the disease[22].

As HDV's virulence is dependent on HBV, coinfection results from simultaneous acute HBV and HDV. It is usually transient and cannot be clinically distinguished from HBV infection[23]. HDV has incubation period of approximately 1 mo resulting in clinical symptoms of fatigue, loss of appetite and nausea. It is accompanied with a rise in liver enzymes, including serum alanine aminotransferase and aspartate aminotransferase. Then comes the jaundice phase with increase in bilirubin levels. As it is usually self-limiting and most patients recover completely with only 2% leading to chronic infection[7,24].

Superinfection with HDV can also result in acute hepatitis which is more severe than seen with co-infection. This is because HBV has already set the ground for more aggressive disease progression. It can lead to acute liver failure with clinical symptoms starting as nausea and progressing to coagulopathy, encephalopathy and coma[25]. About 80% to 90% of patients progress to chronic hepatitis. Amongst them, some dated studies have reported up to 70% to 80% progress to cirrhosis within 5 to 10 years[26]. However, newer studies suggested a 4% annual progression to cirrhosis[27]. This variability might be due to the different genotypes of HDV. Although there is controversy in the literature over whether HDV has oncogenic properties, cirrhosis from HDV does increase the risk of HCC, which is the second most common cause of cancer deaths in men worldwide[28].

HBsAg is necessary before other markers for HDV are investigated to establish the diagnosis. One important distinguishing test is IgM anti-HBc, which is only present in acute HDV/HBV coinfection and not in acute HDV superinfection. Likewise, HDV RNA is a sensitive marker for acute infection and reaches a very high quantitative value in chronic patients. Similarly, the presence of anti-HDV IgM or high anti-HDV IgG titer can differentiate between current and past infections. Therefore, knowing these markers helps to differentiate the disease pattern (Table 1)[23].

INDICATIONS FOR LIVER TRANSPLANTATION

The global disease burden of HBV/HDV coinfection is increasing with 10.6% of HBsAg carriers without high risk sexual behavior or IVDU are HDV[12]. HDV can lead to a more severe form of viral hepatitis than in HBV mono-infection[29]. Irrespective of whether being coinfected with HBV or as superinfection, HDV can cause fulminant hepatitis[30]. The clinical course of fulminant hepatitis D is 4 to 30 d and transplant free survival is as low as 20%[7,31].

Chronic HDV results in rapid liver fibrosis, earlier decompensation, higher risk of HCC development and annual mortality rate between 7% to 9%[32]. Mortality rates of greater than 50% at 15 years follow up have been reported in Taiwan[33]. Though direct oncogenic properties of HDV is not clearly described, higher rates of cirrhosis in HDV patients can lead to increased rates of HCC[34]. Rates of HCC are variable across the globe with studies showing anti-HDV antibodies ranging from 4% to 23% in HBsAg positive HCC patients[35,36]. Treatment option for HDV has limited success. Therefore, the only definitive therapy for patients with end-stage liver disease, HCC, or

Table 1 Summarizes markers specific for coinfection and superinfection

	Coinfection	Superinfection
HDV infection	Acute	Acute or chronic
IgM anti-HBc	Positive	Negative
Serum HDV RNA	Transient	Persistent and high
IgM anti-HDV	Transient	Persistent
IgG anti-HDV	Late appearance and low	Persistent and high

HDV: Hepatitis D virus; RNA: Ribonucleic acid; HBc: Hepatitis B core.

fulminant hepatitis due to HDV is liver transplant[13].

TREATMENT OPTIONS

Currently there is no United States Food and Drug Administration approved treatment for HDV[37]. However, PEG-IFNa is commonly used and is also recommended by major liver societies such as American Association for the Study of Liver Diseases (AASLD) and European Association for the Study of the Liver[38,39]. Pegylated form requires only weekly dosing and metanalysis has shown increased suppression (29%) of HDV RNA at 6 mo compared to standard IFN alpha (19%)[40]. In addition, PEG-IFNa is also associated with lower rates of side effects such as anorexia, nausea, weight loss, alopecia, leukopenia and thrombocytopenia[41,42]. Although there is no definite treatment duration, negative HDV RNA at 24 wk is being considered a reference for virological response and treatment for 48 wk is recommended[38,43]. Pegylated IFN has been studied in combination with nucleosides. The Hep-Net/International Delta Hepatitis Intervention Trial randomized 90 patients to adefovir, peginterferon, or the combination arm. Approximately 25% achieved virological response at 24 wk in the peginterferon and combination group and none in the adefovir group[44]. Similarly, the HIDIT-2 trial (which replaced adefovir with tenofovir) yielded similar results to the trial[21] and did not show a response in the nucleoside alone[45]. Thus, combination of interferon with nucleosides or increasing the duration of treatment has shown no additional benefits.

In addition, newer experimental treatments are currently underway. One such example is the use of oral prenylation inhibitor lonafarnib (LNF). Prenylation inhibitors have been shown to abolish HDV-like particle production *in vitro* and *in vivo* [46]. LNF interferes with the HDV cycle and targets the virion assembly step in the hepatocyte cytoplasm, where the nascent HDV nucleoprotein complex is enveloped by HBsAg[47]. To explore this, the LOWR HDV-1 [Lonafarnib with and without Ritonavir (RTV) in HDV-1] phase two clinical trial was conducted by Yurdaydin *et al*[47] with the intention to study optimal LNF dosing while assessing tolerability and viral response when combined with P450 3A4 inhibitor RTV or PEG-IFNa. Results showed that LNF, whether as monotherapy or as combination with PEG-IFNa, led to HDV-RNA viral load decline in all patients. All treated patients in different treatment regimens reported GI adverse effects consisting of anorexia and weight loss. Higher dose of LNF, 300 mg peroral BID, was associated with increased adverse effects. RTV helped to lower LNF dose (100 mg per oral BID dosing) while still achieving better antiviral results. Similarly, LNF 100 mg BID with PEG-IFNa helped in more substantial and rapid HDV-RNA reduction, compared to PEG-IFNa alone[47]. Such trials have opened the door to further explore newer treatment options for HDV.

PEG-IFNa is only used amongst patients with compensated liver disease. For those who undergo LT, long-term survival depends on the prevention of allograft reinfection. LT for HDV is not common in United States and studies in literature are mostly from other countries[48,49]. Due to antivirals and with hepatitis B immune globulin (HBIG), rates of HBV/HDV reinfection after LT has decreased[50]. Currently there is no specific prophylaxis for HDV. However, as its growth is dependent on HBV, the focus should be on preventing HBV infection.

Levels of HBV DNA (> 105 copies/mL) strongly predict HBV reinfection in HBsAg positive LT recipients[51]. As per AASLD recommendations, all HBsAg-positive recipients should receive prophylactic nucleoside/nucleotide analogs with or without

HBIG post-LT. In addition, pretransplant hepatitis B e-antigen/HBV-DNA levels should not be taken into consideration and HBIG monotherapy should not be used. They further suggest that entecavir, tenofovir disoproxil fumarate, and tenofovir alafenamide should be the preferred antivirals and continued indefinitely post-LT[38]. The use of HBIG depends on the recipient and virologic factors. In medically adherent HBV mono-infected recipients with undetectable or low-level viremia at the time of LT and no evidence of concurrent infection, no HBIG or a very short course (5 d) of HBIG post-LT combined with long-term antiviral therapy is highly effective in preventing HBV recurrence[38]. On the other hand, in HBV/HDV co-infected recipients, the combination of long-term HBIG and antiviral therapy may be the best approach in preventing HBV and HDV recurrence[38].

Data regarding the dosage of HBIG therapy varies across transplant centers. In previous studies HBIG has been given as either high (≥ 10000 IU/mL) or low (< 10000 IU/mL) dose for either a fixed duration (median of 6 mo) or indefinitely post-LT[50]. It is administered either intravenous or intramuscularly during an hepatic phase, followed by daily doses during the first week, with subsequent doses given monthly or by following anti-HBs titers based on the transplant center protocol[50]. A trough anti-HBs titer of at least 100 IU/L is thought to be protective and reinfection rate can be further reduced by maintaining anti-HBs titers consistently above 500 IU/L[52] (Figure 1)[48].

LIVER TRANSPLANT OUTCOMES

Long-term survival following LT for viral hepatitis depends on prevention of allograft reinfection[53]. This is a well-known concept for HBV as well as HCV and can be applied for HDV related LT as well. LT for HDV started in the late 1980s from Europe. One of the earliest reporting was from Rizzetto *et al*[54] from Italy, on 7 patients who underwent LT due to HDV cirrhosis. It resulted in reinfection rate of 70% with HDV and milder forms of hepatitis were reported in 40% of the cases. This encouraged others to believe that LT was a feasible option for ESLD from HDV. Ottobrelli *et al*[55] reported a larger series of 22 patients, which showed 80% reinfection rate and 73% survival rate at one year. Although the reinfection rate was high, the clinical course was mild, therefore giving hope to the patients that LT was the possible cure for HDV. At that time, it was unclear whether administration of HBIG will be beneficial in preventing reinfection. Therefore, a multicenter study was done in Europe and amongst 110 patients who underwent LT due to HDV cirrhosis, the three-year actuarial risk of HBV recurrence after transplantation was reported as 70% ± 14% in the group who received no HBIG and 17% ± 6% in patients who received HBIG for > 6 mo[56]. The actuarial three-year survival was reported as 83%. In that study, long-term administration of HBIG (RR: 2.22; 95% confidence interval: 1.13-4.33; $P < 0.001$) and HDV superinfection (RR: 6.25; 95% confidence interval: 3.13-12.42; $P < 0.001$) were reported as independent predictors of better survival[56]. With the passage of time and development of new antivirals which when used in combination with HBIG, post-LT HBV/HDV reinfection has significantly decreased. In a retrospective study Adil *et al*[57] reported HBV recurrence rate of 5.1% and no HDV recurrence among 255 patients, after a mean follow-up of 30 mo. Similarly, study by Idilman *et al*[58] endorsed this, showing that amongst 90 patients with delta co-infection-related cirrhosis who underwent LT, only one recipient (who received lamivudine and HBIG combination), had HBV recurrence upon follow up. Moreover, in an another study with 104 HDV patients, with a longer follow up of 82 mo, the survival and HBV recurrence rates were 97% and 13.4% respectively[59]. Thus, it was confirmed that it is very important for survival and viability of the graft that the patients remain HBsAg-negative after transplantation.

Interestingly, studies have shown that presence of HDV infection appears to provide a protective effect against HBV reinfection in LT patients, possibly *via* suppression of HBV replication resulting in longer survival rates[49]. Recently a study published on LT patients in Brazil showed significantly higher 4-year survival rate of 95% in HDV group ($n = 29$), compared to 75% in HBV group ($n = 40$)[60]. One of the largest series involving hepatic transplantation in patients with HDV ($n = 76$), identified 88% survival after 5 years[61]. This is likely because of low HBV recurrence rate in these series.

HDV leading to HCC has also been treated with LT. Romeo *et al*[27] performed a retrospective study where 29 of 299 patients diagnosed with HBV/HDV had liver transplant; amongst these 29 patients, 10 patients (34%) had HCC. After transplant, 5

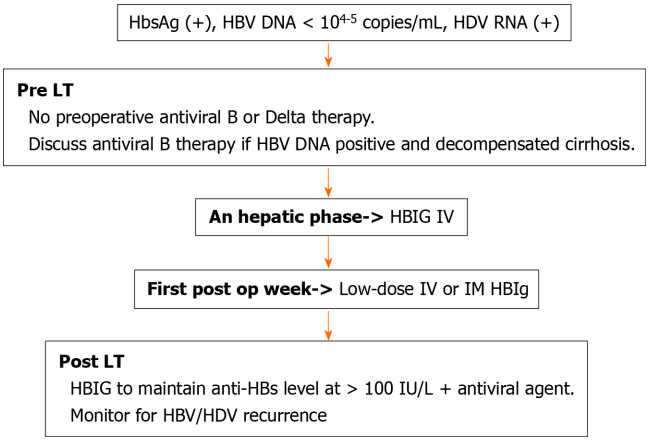

Figure 1 Possible treatment flowchart in hepatitis D virus liver transplant patients. HbsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; DNA: Deoxyribonucleic acid; RNA: Ribonucleic acid; HBIG: Hepatitis B immune globulin; LT: Liver transplantation.

patients died (3 with primary graft failure, 1 with tumor recurrence, and 1 with non-liver–cancer-related reasons). Similarly, a retrospective study was conducted in Turkey amongst 25 live donor LT recipients with chronic HBV/HDV, 11 of which had HCC. The cumulative 5-year survival was 74%. In the HCC group, 7 of 11 tumors matched the Milan criteria and 4 patients did not (in whom 2 patients had HCC recurrence after 2 years which was treated by ablation techniques)[32]. Thus, results from our review supports the AASLD guideline, that using HBIG in conjunction with oral antivirals post-transplantation, changes the natural history of the liver disease even among recipients with HCC.

CONCLUSION

HDV presents a severe health burden with liver transplantation as the only treatment for patients with End-stage Liver Disease, hepatocellular carcinoma, or fulminant hepatitis. Post transplantation reinfection with HDV/hepatitis B virus is an undesirable outcome as it affects survival. While transplant centers across the world have their own protocols, there is a consensus that hepatitis B immune globulin in combination with a potent nucleoside/nucleotide analogue have shown promising results. In the future, with the potential approval of the pipeline drugs for HDV treatment, their role in the post-transplant setting also needs to be explored. Currently, the data on liver transplant due to HDV is limited and more randomized controlled trials investigating the duration and frequency of hepatitis B immune globulin as well as the specific anti-HBs titer level are needed to optimize the pre- and post-transplant treatment plans.

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MINIREVIEWS

Lymphatic dysfunction in advanced cirrhosis: Contextual perspective and clinical implications

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Abstract

The lymphatic system plays a very important role in body fluid homeostasis, adaptive immunity, and the transportation of lipid and waste products. In patients with liver cirrhosis, capillary filtration markedly increases, primarily due to a rise in hydrostatic pressure, leading to enhanced production of lymph. Initially, lymphatic vasculature expansion helps to prevent fluid from accumulating by returning it back to the systemic circulation. However, the lymphatic functions become compromised with the progression of cirrhosis and, consequently, the lymphatic compensatory mechanism gets overwhelmed, contributing to the development and eventual worsening of ascites and edema. Neurohormonal changes, low-grade chronic inflammation, and compounding effects of predisposing factors such as old age, obesity, and metabolic syndrome appear to play a significant role in the lymphatic dysfunction of cirrhosis. Sustained portal hypertension can contribute to the development of intestinal lymphangiectasia, which may rupture into the intestinal lumen, resulting in the loss of protein, chylomicrons, and lymphocyte, with many clinical consequences. Rarely, due to high pressure, the rupture of the subserosal lymphatics into the abdomen results in the formation of chylous ascites. Despite being highly significant, lymphatic dysfunctions in cirrhosis have largely been ignored; its mechanistic pathogenesis and clinical implications have not been studied in depth. No recommendation exists for the diagnostic evaluation and therapeutic strategies, with respect to lymphatic dysfunction in patients with cirrhosis. This article discusses the perspectives and clinical implications, and provides insights into the management strategies for lymphatic dysfunction in patients with cirrhosis.

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Core Tip: Lymphatic dysfunction appears to play a significant role in the pathophysiology of advanced cirrhosis. Sustained portal hypertension, neurohormonal changes, and low-grade chronic inflammation have been implicated in causing lymphatic dysfunction in advanced cirrhosis, leading to worsening of ascites, lymphedema, and abnormal lipid transport; it also results in increased susceptibility to infections. Chylous ascites and intestinal lymphangiectasia are the rare manifestations of lymphatic dysfunction in cirrhosis, leading to loss of protein, fat, lymphocytes, and immunoglobins, with several clinical consequences. Lymphatic dysfunctions in cirrhosis have been ignored to date; hence, new exploratory research must be undertaken to gain insight into this important subject.

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INTRODUCTION

The lymphatic system consists of capillaries located inside the tissue that are highly permeable and are needed to transport lymph containing cellular proteins, lymphocytes, and lipoproteins[1-4]. It is essential for maintaining homeostasis of tissue *via* interstitial fluid reabsorption, immune cell trafficking, and the transport of lipids[3-5]. The lymphatic system removes interstitial fluid from tissues and returns it to the bloodstream. When this interstitial fluid gets into lymphatic capillaries, it is called lymph. The liver is the largest organ generating lymph, and liver lymphatics are believed to play a vital role in maintaining normal hepatic function by helping to eliminate protein, cholesterol, and immune infiltrates[5]. In the absence of normal lymphatic function, interstitial fluid accumulation may contribute to clinical manifestations such as lymphedema and ascites[6]. In patients with early cirrhosis, the lymphatic system helps to prevent development of ascites by reabsorbing excess fluid in the hepatic and splanchnic areas. As a result, lymph flow is enhanced, which promotes hepatic lymphangiogenesis[7,8]. However, in advanced cirrhosis patients, this compensatory mechanism is not adequate to prevent the development of ascites. Moreover, there appears to be an impaired lymphatic pump function in patients with an advanced liver disease[9]. Despite its significant clinical value, the literature on lymphatic dysfunction in cirrhosis is very limited, and the area remains open for new investigations. This article summarizes the current knowledge regarding dysfunctions of lymphatic system in patients diagnosed with liver cirrhosis, with special attention to pathophysiology, clinical implications, and insights into management strategies.

LYMPHATIC VASCULAR SYSTEM

The lymphatic system consists of a large network of lymphatic vessels, with lymphoid organs and tissues. Lymphatic vessels are classified anatomically into capillaries and collecting vessels. Further, the lymphatic capillaries are closed-ended and composed of a single layer of lymphatic endothelial cells (LECs). The initial lymphatics are highly permeable for transport of interstitial fluid macromolecules and immune cells. LECs have anchoring filaments that contract and relax, which enable them to "flap" open to allow interstitial fluid uptake[10,11]. The lymphatics capillaries merge into larger collecting lymphatic vessels, which possess a continuous basement membrane and have unidirectional bicuspid valves with contractile smooth muscle cells' (SMCs) covering for assisting the flow of lymph. Similar to lymphatic capillaries, the liver has

sinusoids, consisting of a single layer of liver sinusoidal endothelial cells (LSECs), without the basement membranes[12]. Hepatic lymph is produced by plasma components filtered through the LSECs into the space of Disse. In the gastrointestinal tract, lymphatics are present in mucosal, submucosal, and muscular layers; they merge with collecting lymphatic vessels near the mesenteric border. The lymphatics present in the center of each intestinal villus are referred to as lacteals, which have a structure similar to the lymphatic capillaries elsewhere, consisting of a single layer of LECs, without a basement membrane[13].

There is constant filtration of plasma into the interstitial space during the passage of blood through the capillaries. The rate of filtration is primarily dictated by the hydrostatic pressure and plasma oncotic pressure in the capillaries. Due to the change in interstitial pressure, interstitial fluid enters the lymphatic capillaries, as lymph, and moves towards larger lymphatic vessels[14]. The contractile activity of SMCs, of the collecting lymphatic vessels, is believed to be one of the major driving forces of lymphatic circulation[15]. The Ca^{2+} channels of SMCs and nitric oxide (NO) produced in LECs is thought to contribute to the regulation of lymphatic flows, by modulating the contractility of SMCs[16]. In liver, most of the lymph from space of Disse drains into lymphatic vessels in the area near portal triads. Some part of the lymph also circulates into the interstitium around the central vein or underneath the Glisson's capsule. Finally, all the liver lymphatic vessels converge into the hepatic hilum and flow into the lymph nodes arranged in the lesser omentum along the hepatic vessels and hepatic ducts[5,17]. The collecting lymphatic vessels, from all organs, connect to one or more lymph nodes and, finally, lymph trunks, which ultimately drain into the subclavian vein *via* thoracic duct or right lymph trunk (Figure 1). Thus, interstitial fluid, collected as lymph, is finally returned to the blood circulation through the lymphatic vessels. It is estimated that approximately 3 L to 5 L of lymph fluid travel through the thoracic duct each day, of which 50% to 90% comes from the intestines and liver[18]. Being capillary ultrafiltrate, all plasma proteins are present in lymph. However, several proteins derived from extracellular matrix, cellular metabolism, and cell death are enriched in lymph instead of the plasma[19]. Therefore, the composition of the lymph arising from various areas varies to a degree.

FUNCTIONS OF LYMPHATIC SYSTEM

The lymphatic system plays an important role in maintaining tissue homeostasis, by transporting interstitial fluid, serum protein, and lipids from tissues to the systemic circulation. After plasma filtration through the capillaries, the only way the fluid can be returned to blood circulation is *via* the lymphatic system[20]. When there is a mismatch between capillaries filtration and lymphatic removal, fluid accumulation occurs in the extravascular space. Lymphatic system plays a key role in adaptive immunity. It delivers antigen and antigen-presenting cells to the regional lymph nodes, where they evoke immune responses. Lymphatics also play a role in controlling the inflammatory response, by influencing the drainage of extravasated fluid and inflammatory mediators, and by facilitating the discharge of infiltrated immune cells from inflamed sites[21,22]. Moreover, lymphatic vessels are essential for the removal of cholesterol from peripheral tissues[23]. LECs are known to take up cholesterol carried by high-density lipoprotein, and dysfunctional LECs can lead to the development of hepatic steatosis[24]. Furthermore, intestinal lacteals play important role in the absorption of fat and fat-soluble vitamins as chylomicrons.

LYMPHATIC SYSTEM CHANGES IN CIRRHOSIS

In patients with cirrhosis, capillary filtration increases steadily and gradually, primarily due to an increase in hydrostatic pressure. This contributes to an enhanced lymph production, with consequent lymphatic compensatory responses, such as an increase in the number and size of lymphatic vasculature, to enhance the drainage of interstitial fluid[8,25,26]. Several structural and functional changes in the lymphatic system have been reported in patients with cirrhosis.

Increase in the lymph flow

An increased architectural distortion in cirrhosis causes resistance to sinusoidal blood flow, increased hydrostatic pressure in the sinusoid, and increased filtration of plasma.

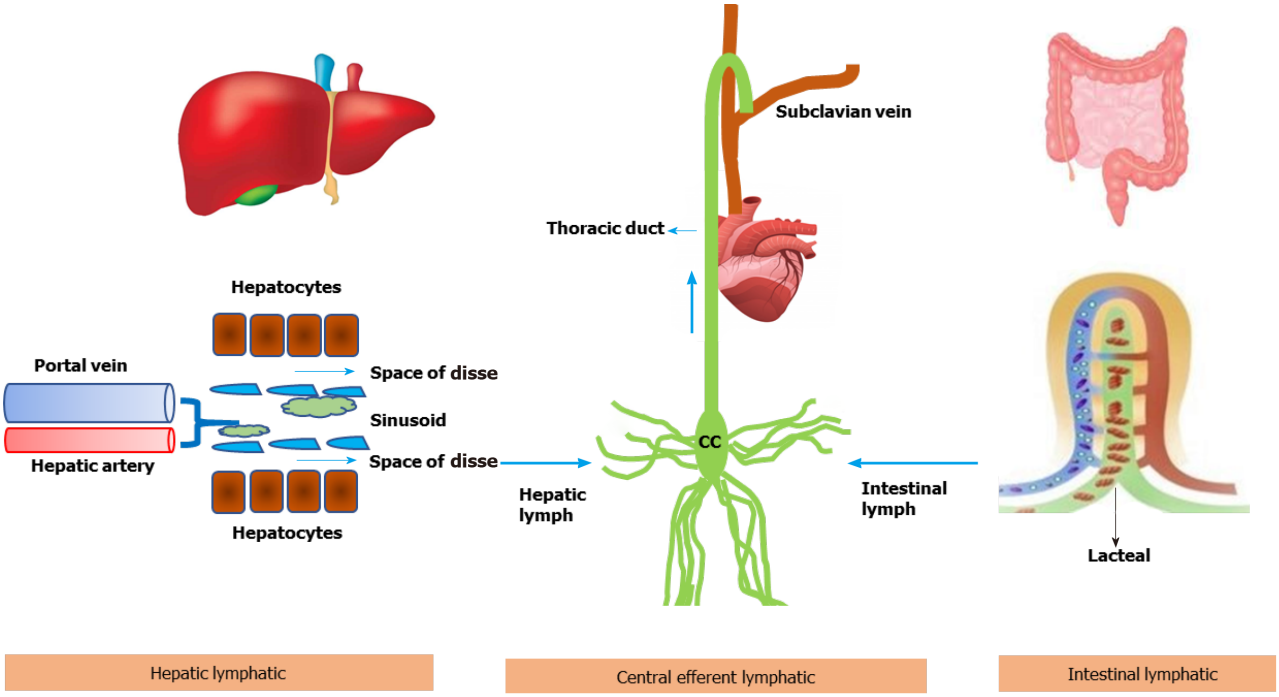

Figure 1 Schematic diagram showing lymph flow kinetics from liver and intestine to the systemic circulation. The capillary filtrate enters the lymphatic capillaries, as lymph, and moves towards larger lymphatic vessels. In liver, lymph is produced by filtration of plasma through the sinusoidal endothelial cells into the space of Disse. The collecting lymphatic vessels from all organs connect to one or more lymph nodes, and finally to the lymph trunks which ultimately drain into subclavian vein *via* cysterna chyli and thoracic duct. Approximately 80% of thoracic duct lymph comes from the intestines and liver.

This process may be further enhanced by concomitant hypoalbuminemia and increased capillary permeability under certain circumstances. Thus, lymph production and flow is greatly increased (up to 30 folds) in patients with cirrhosis[27,28]. Witte *et al*[7] demonstrated that lymph in the thoracic duct of cirrhosis patients had a high protein concentration. Because the protein concentration of hepatic lymph is higher (50%-80% of plasma), such overproduction of lymph in cirrhosis appears to come primarily from the liver. However, with advancement of cirrhosis, the protein content of hepatic lymph also decreases because of a dysfunctional lymphatic transport system. In an animal study of cirrhotic livers, a positive correlation between hepatic lymph flow and increasing portal pressures was found. Moreover, this study also demonstrated a compromised functional capacity of lymphatic vessels to absorb interstitial fluid[29].

Increase in the number and density of lymphatic vessels

Dumont and Mulholland[30] were the first to describe an increased diameter and lymph flow in the thoracic duct, in patients with cirrhosis. Such expansion of lymphatic vasculature has also been reported by Sadek *et al*[31] on computed tomography and Shimada[32] on laparoscopy. The expansion of lymphatic density correlates positively with the severity of fibrosis around the portal tracts of human liver. Yamauchi *et al*[26] found that the intrahepatic lymphatic vessels remain stable during the early stages of liver disease, but when it progresses to advanced cirrhosis, it increases significantly. In addition, Yokomori *et al*[33] recently calculated the density of lymph vessels by immunohistochemistry in patient specimens and found that the density increased with the progression of liver disease, peaking at the most advanced stages of cirrhosis. In cirrhotic livers, a substantial increase in vascular endothelial growth factors (VEGF)-D expression, an inducer of lymphangiogenesis, was observed and in addition, VEGF-D expression was found to be positively associated with liver fibrosis progression[8]. This lymphangiogenic response may help to enhance the drainage of increased interstitial fluid.

Lymphatic oversaturation and flow dysfunction

The lymphatic system keeps tissue edema free, by returning excess tissue fluid back to the bloodstream. In cirrhotic patients, when interstitial fluid is increased, expansion of lymphatics and increased lymphatic flow initially tries to prevent development of ascites and edema[7]. However, it is not clear as to what extent the lymphatic

vasculature may compensate for enhanced lymph production. In a sustained increase of the hydrostatic pressure, fall in plasma oncotic pressure, compounding effects of capillarization/defenestration of sinusoidal endothelium, and neurohormonal changes, the compensatory mechanism is gradually overwhelmed, resulting in fluid accumulation in the extravascular space[34,35]. In the splanchnic circulation of cirrhosis patients, arteriolar vasodilation occurs; it increases the production of splanchnic lymph beyond the ability of the lymphatic system to transport and, thus, triggers lymph leakage into the peritoneal cavity. Moreover, an increased splanchnic vascular permeability and chronic retention of renal sodium and water plays a major role in the sustained development of ascites[36,37]. Over time, increased pressure and flow stasis in the intestinal lymphatic channels may lead to lymphangiectasia, followed by the rupturing of dilated lacteals and intestinal loss of protein, chylomicrons, and lymphocyte[38]. Rarely, the rupture of subserosal lymphatic, secondary to a sustained high pressure, results in the development of CA[39].

Apart from lymphatic oversaturation, functional defect in the lymphatic transport system has also been reported in patients with cirrhosis. Henriksen[40] have described a model of lymphatic conductivity (flow rate per unit pressure difference), based on protein kinetic and hemodynamic measurement in patients with cirrhosis. They found that lymphatic conductance in the thoracic duct was three times higher than normal in patients without ascites, while in patients with tense ascites, these values were close to normal. Moreover, conductance in the right lymphatic duct system was ten times below that of thoracic duct of cirrhotic patients with ascites. The results of this study suggest that a relatively insufficient lymphatic drainage plays an important role in the accumulation of ascites in decompensated cirrhosis. Recently, the functionality of the splanchnic and peripheral lymphatic system was studied by fluorescent lymphangiography, in an experimental model of rats exposed to chemokine ligand 4 (CCL4). A substantial decrease in fluorescence-labeled lymphatics was observed in cirrhotic rats, in both peripheral and splanchnic regions, indicating a deficiency in lymphatic drainage[9].

PATHOPHYSIOLOGY OF LYMPHATIC DYSFUNCTION IN CIRRHOSIS

The pathophysiological mechanism behind lymphatic dysfunction in cirrhosis is an area yet to be explored at cellular and molecular level (Figure 2). In a study on cirrhotic rats with ascites, Ribera *et al*[9] found that an impaired lymphatic drainage in the splanchnic and peripheral regions was accompanied by increased activity of endothelial nitric oxide synthase (eNOS) and production of NO by LECs. In addition, SMC coverage of lymphatic vessels was found to be significantly decreased. Interestingly, when cirrhotic rats were treated with inhibitor of eNOS activity (L-NG-methyl-L-arginine, L-NMMA), a significant improvement of lymphatic drainage, reduction in ascetic fluid volume, and an increase in lymphatic smooth muscles were seen. Therefore, this study demonstrated a role of NO in the lymphatic dysfunction of cirrhotic rats. Whether the same applies for human cirrhosis remains to be seen. Lymphangiogenesis observed in cirrhosis appears to be due to increased expression of several induces of lymphogenesis, such as VEGF-D and VEGF-C. Their levels have been found to be significantly elevated during hepatic fibrosis and positively correlated with fibrosis progression[8,41]. Study on cirrhotic rat has found a four-fold increase in VEGF-D, in the endothelial cells. Additionally, the receptor of this VEGF (VEGR-3) was found to be overexpressed in the LECs of cirrhotic rats[42]. It has recently been shown that autonomic nervous system is a key modulator of the lymphatic vessels' function[43].

Lymphatic function, in general and in patients with cirrhosis, can be modulated by numerous factors including age, obesity, diabetes, dyslipidemia, neurohormonal alterations, and chronic inflammation. Neurohormonal changes are known to occur in advanced cirrhosis, and the levels of a number of vasoactive substances such as noradrenaline, histamine, substance P, prostaglandins, and endothelin are altered, which can affect contractility of lymphatic vessels[44-46]. Intestinal motility plays an important role in the propulsive motion of intestinal lymph, and by inducing VEGF-C, intestinal microbiota is an important regulator of intestinal lacteal integrity[13,47]. Therefore, the intestinal dysmotility and intestinal dysbiosis that are frequently seen in advanced cirrhosis may interfere with intestinal lymphatic function. Moreover, Cirrhosis and portal hypertension (PHT) is known to create a state of low-grade chronic inflammation[48]. Furthermore, gut dysbiosis, bacterial translocation, and release of Inflammatory cytokines such as tumor necrosis factor alpha, and

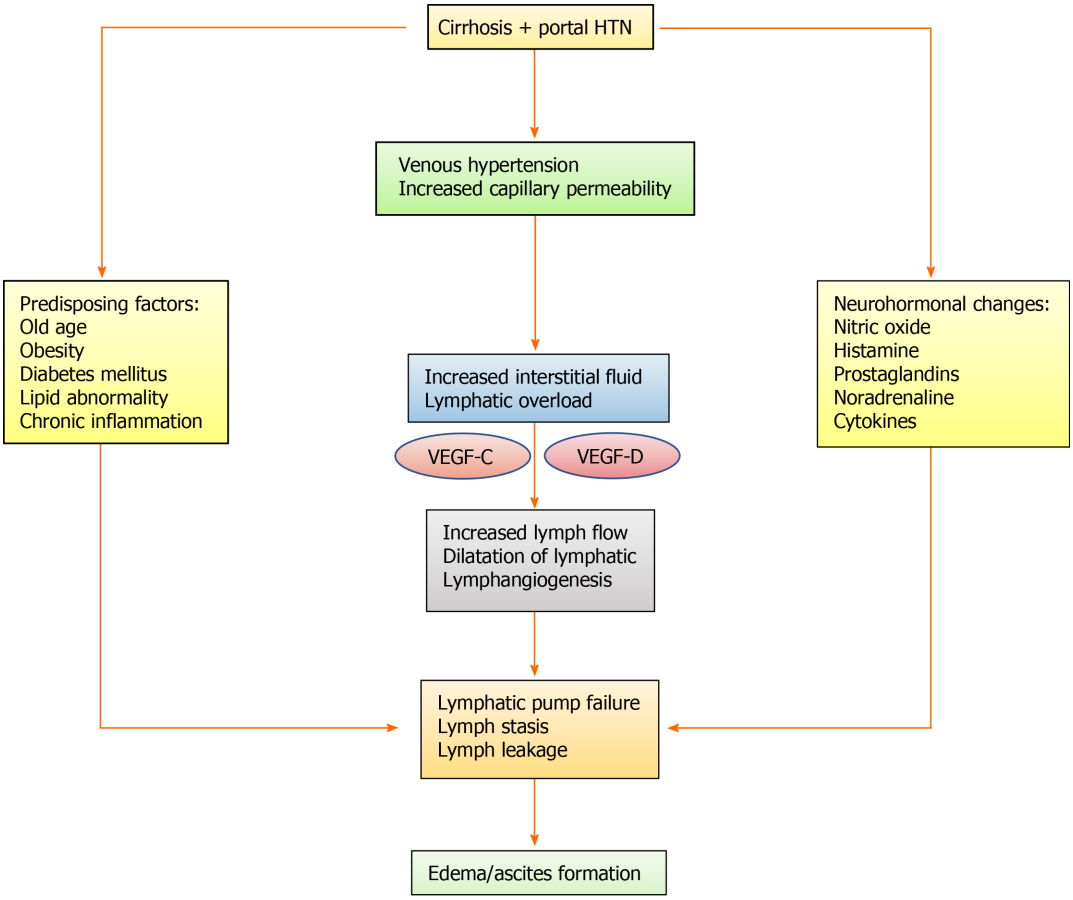

Figure 2 Flow diagram showing the possible pathophysiological mechanism behind lymphatic abnormalities in cirrhosis patients leading to fluid imbalance. The exact pathophysiological mechanism, at cellular and molecular level, is poorly understood in human cirrhosis. Some of the information has been derived from the experimental study on animal. VEGF: Vascular endothelial growth factor; HTN: Hypertension.

interleukin-1β occur in cirrhosis[49]. Consequently, chronic inflammation and neurohormonal disturbances, in advanced cirrhosis, can lead to structural and physiologic changes in the lymphatic system. Dysfunctional lymphatics, with lymph stasis, can impair lipid transport and stimulate adipogenesis in the affected area[50,51].

Old age and obesity also affect lymphatic functions. Aging induces structural changes in the lymphatic vessels, such as loss of extracellular matrix, reduced contractile protein expression, and changes in eNOS and histamine gradients, which tend to decrease the lymphatic transport of interstitial fluids[52,53]. Obesity results in several structural and physiological changes in the lymphatic system, including increased lymphatic leakiness, decreased contractility of the collecting vessel, and changes in the architecture of the lymph node, which significantly affect lymphatic transport functions[54,55]. Notably, most cirrhosis patients belong to the old age group, and obesity is presently a growing cause of non-alcoholic fatty liver disease (NAFLD)-related cirrhosis. Given that obesity is a growing cause of NAFLD-related cirrhosis and that most patients with cirrhosis are older, they may be at a higher risk of developing lymphatic dysfunction.

CLINICAL IMPLICATIONS OF LYMPHATIC DYSFUNCTION

Lymphatic dysfunctions have been aptly described in patients with cirrhosis; however, little has been described about the clinical consequences of such dysfunctions. Given the role of lymphatic vasculature in the body fluid homeostasis, adaptive immunity, and the transport of lipid and waste materials, it is tempting to speculate that lymphatic dysfunctions, in cirrhosis, may have several clinical implications, particularly with regard to the body fluid homeostasis.

Edema and ascites

In advanced cirrhosis, the activation of compensatory vasoconstrictor pathways compromises glomerular filtration, causing greater renal retention of sodium and water. This further increases the production of lymph, burdening the already inefficient lymphatic system with the responsibility for drainage. Moreover, inability of the lymphatic system to recirculate extravasated albumin may worsen pre-existing hypoalbuminemia, leading to a change in the transcapillary oncotic pressure gradient and worsening of fluid imbalance. Additionally, serum albumin is also required for furosemide to work properly[56]. Therefore, severe lymphatic dysfunction can lead to the development of refractory edema and ascites in patients with cirrhosis.

Lymphedema should be fairly common in patients with advanced cirrhosis for obvious reasons; however, its description is lacking in existing literature. Lymphedema is deposition of protein-rich lymph fluid within the tissues, as a consequence of lymphatic leak and an imbalance between the rate of lymph production and drainage. Recent evidences suggest that lymphedema can also occur as an immune response secondary to lymphatic injury or metabolic derangements, including adiposity and infection[57]. Furthermore, fat deposition is present in lymphedema due to failure of lipid transport and stimulation of adipogenesis[50,51]. Clinically, a diagnosis of lymphedema can be made by physical characteristics, including pitting edema, peau-d'orange appearance, and a positive Stemmer sign. Patients with lymphedema are often susceptible to various skin infections, such as cellulitis.

Intestinal lymphangiectasia

An increase in lymphatic pressure secondary to PHT may lead to dilatation of the intestinal lymphatics, known as intestinal lymphangiectasia[58]. A sustained rise in lymph pressure leads to the rupture of lymphangiectasia and lymph leakage into the lumen of the intestines, with many clinical consequences (Figure 3). As intestinal lymph contains many proteins, lipoproteins, and lymphocytes, its loss would result in hypoproteinemia, hypoalbuminemia, lymphocytopenia, and hypogammaglobulinemia[59,60]. Hence, in patients with advanced cirrhosis, lymphangiectasia can lead to worsening of ascites, by causing severe hypoalbuminemia. The disruption of lymphatic flow, in lymphangiectasia, leads to malabsorption of fats and fat-soluble vitamins (vitamins A, D, E, and K), which may cause steatorrhea, vision problems, muscles weakness, osteopenia, and coagulopathy in cirrhosis patients. In addition, loss of lymphocytes may contribute to an increased susceptibility to infection in cirrhosis[60].

Chylous ascites

Chylous ascites (CA) results from the leakage of lipid-containing lymph (chyle) into the peritoneal cavity[61]. Elevated lymphatic pressure secondary to PHT can rarely cause rupture of dilated subserosal intestinal lymphatics, leading to the formation of CA[39]. Intestinal lymph, which constitutes 50%-75% of intra-abdominal lymph, contains fat droplets rich in triglyceride and appears to be milky in color. CA is found in 0.5%-1% of patients with cirrhosis, and cirrhosis is responsible for 11% of cases of atraumatic CA[62,63]. In patients with cirrhosis, CA may also develop due to complications of shunt surgery, sclerotherapy-related thoracic duct injury, or hepatocellular carcinoma[62,64]. A diagnosis of CA is made when triglyceride concentration of fluid is ≥ 110 mg/dL. It is to be noted that a rupture of hepatic lymph, which drains 25%-50% of abdominal lymph, does not produce CA, as hepatic lymph is devoid of fat droplets.

Other clinical implications

Patients with lymphatic dysfunction often exhibit impaired immune function predisposing them to a variety of infections[65,66]. Recurrent cellulitis/erysipelas and interdigital fungal infections are common in presence of lymphedema. The lymphatic vasculature is preferential route for the spread of cancer cells. Therefore, lymphangiogenesis can promote tumor metastasis if patients with cirrhosis have hepatocellular carcinoma[67]. Moreover, lymphatic dysfunction may interfere with the removal of inorganic material, dying cells, and mutant cells from the body, but such adverse effects are unknown in patients with cirrhosis. Furthermore, lymphatic dysfunction can affect oral bioavailability of lipophilic drugs, which require functional intestinal lacteals for absorption.

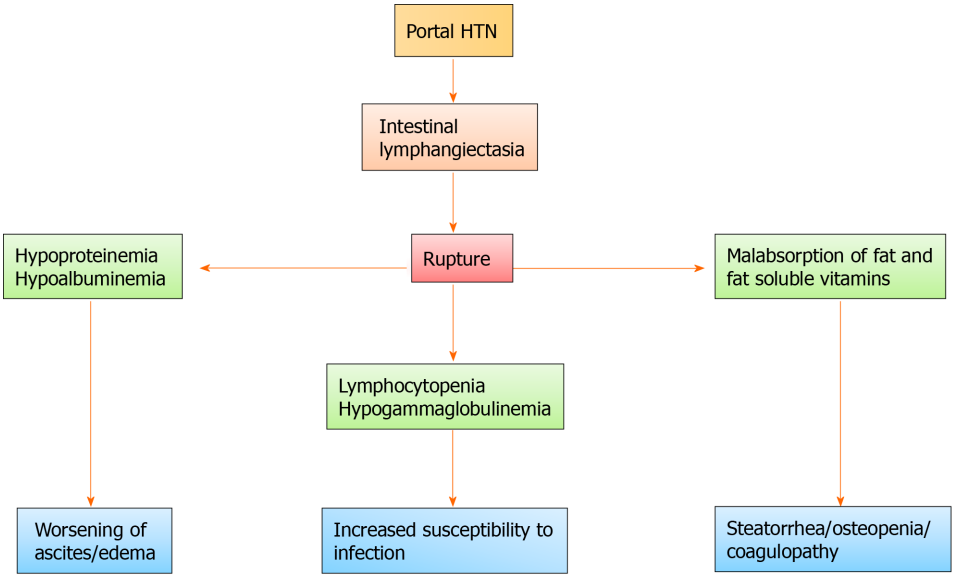

Figure 3 Flow diagram showing clinical consequences arising from the rupture of intestinal lymphangiectasia. HTN: Hypertension.

ASSESSMENT OF LYMPHATIC DYSFUNCTIONS IN CIRRHOSIS

No recommendation exists with regard to the diagnosis and assessment of lymphatic dysfunction in patients with cirrhosis. Table 1 provides a rational overview of the assessment of lymphatic dysfunction in cirrhosis patients. Techniques to evaluate the lymphatic system radiologically are still evolving[68]. There are various imaging techniques available, such as X-ray or magnetic resonance lymphography, lymphoscintigraphy, and duplex ultrasonography. The gold standard that offers insight into the lymphatic anatomy as well as lymph flow dynamics is lymphangioscintigraphy. However, these imaging modalities are often limited by sub-optimal resolution, lack of standardization, invasiveness, risk of radiation exposure, and low availability[69]. Therefore, as of now, no recommendation can be made with respect to the use of a radiological technique for assessment of lymphatic dysfunction in patients with cirrhosis.

Lymphatic dysfunction, especially in elderly cirrhosis with diabetes and dyslipidemia, should be considered when there is severe generalized edema, sctrotopenile swelling, diuretic-resistant ascites, and peripheral lymphedema. On blood investigation, the presence of disproportionate hypoproteinaemia, combined with severe lymphocytopenia, may also suggest lymphatic dysfunction. Intestinal lymphangiectasia is an endoscopic manifestation of lymphatic abnormality in cirrhosis. It is characterized by swollen mucosa with scattered white spots, white villi, and chyle-like substances covering the mucosa (Figure 4). This must be confirmed *via* histopathological examination, which should reveal dilated intestinal lacteals in the lamina propria region of the intestinal villi. Morphologically, it is often difficult to distinguish lymphatic vessels from blood vessels. Therefore, use of specific lymphatic endothelium markers may be necessary for accurate identification of lymphatic vessels on pathological specimens[25,70]. These markers include LYVE-1 (lymphatic vessel endothelial hyaluronan receptor), Prox-1 (a transcription factor), and podoplanin or D2-40 (lymphatic vessel endothelial hyaluronic acid receptor-1). However, even these markers may not be exclusive to lymphatic vessels. Mouta Carreira *et al*[25] found that LYVE-1 is also present in Kupffer cells and normal LSECs. Therefore, a combination of lymphatic markers should be used for accurate identification. Finally, presence of CA, as evident by milky appearance of ascitic fluid with triglyceride levels > 110 mg/dL, indicates lymphatic abnormality related to cirrhosis, after exclusion of alternative causes such as malignancy, tuberculosis, post-operative or post-radiation status, and cardiac diseases.

THERAPEUTIC PERSPECTIVE

From a pathophysiological point of view, a number of therapeutic options are available for lymphatic dysfunctions, but no adequate evidence is available for the use

Table 1 Assessment of risk factors, clinical markers and investigations for lymphatic dysfunction in cirrhosis

Parameters	Findings that support or indicate lymphatic dysfunction
Risk factors	(1) Old age; (2) metabolic syndrome (obesity, diabetes, dyslipidemia); and (3) concomitant inflammatory disorders
Clinical examination	(1) Diuretic-resistant ascites; (2) severe generalised edema, scrotal/penile swelling; (3) lymphedema: Peau-d'orange appearance and a positive stemmer sign; (4) frequent cellulitis/lymphangitis of affected limbs; and (5) hyperkeratotic skin lesions, yellow nail
Blood investigations	(1) Hypoproteinaemia and hypoalbuminemia; (2) lymphocytopenia; and (3) hypogammaglobulinemia
Ascitic fluid analysis	Chylous ascites: Milky appearance, fluid triglyceride level ≥ 110mg/dL
Upper endoscopy	Intestinal lymphangiectasia: Whitish congested villi in duodenum
Radiological imaging: (lymphography, lymphoscintigraphy)	Abnormal lymphatic structure and/or lymph flow dynamics: Dilated lymphatic vessels, lymph stasis, lymph leakage
Histopathological examination (liver/intestine)	(1) Increase in number and size of lymphatic structures; and (2) specific lymphatic endothelial markers for accurate identification: Prox-1, podoplanin, LYVE-1

LYVE-1: Lymphatic vessel endothelial hyaluronan receptor.

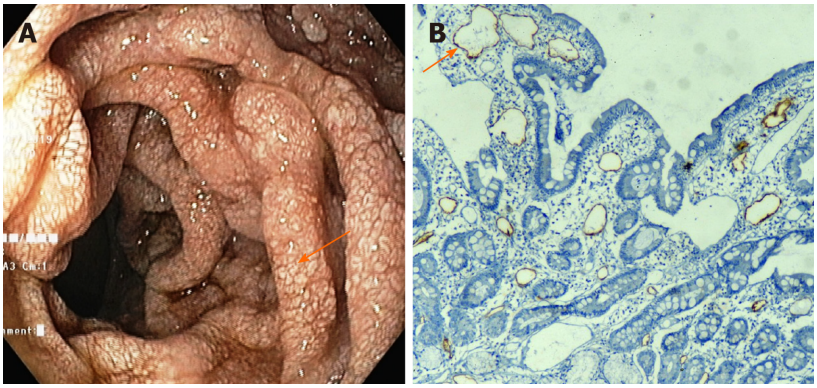

Figure 4 Intestinal lymphangiectasia in a patient with cirrhosis. A: Upper gastrointestinal endoscopy of a patient showing whitish swollen villi in the duodenum, suggestive of intestinal lymphangiectasia; B: On immunohistochemistry (× 10), markedly dilated vessels were seen in the lamina which showed strong D2-40 positivity indicating dilated lymphatics.

of several of them in patients with cirrhosis (Table 2). The mobilization of fluid is particularly difficult in cirrhosis patients with lymphatic dysfunction. An effort should be made to minimize capillary filtration into the interstitial space. Local skincare and compression therapy remains the cornerstone for lymphedema affecting limbs. Common infections, such as cellulitis, should be vigorously treated, as they can deteriorate lymphedema very rapidly. Limb elevation may facilitate lymphatic drainage and prevent the transfer of tissue fluid to an affected limb due to gravity. Pressure effect of compression therapy with elastic stockings/gloves or bandages may help to minimize capillary leakage, reduce lymph regurgitation, and avoid the movement of fluid related to gravity[71]. However, compression therapy should be avoided when cellulitis, venous thrombosis, and congestive heart failure are present. Obesity and salt consumption may worsen lymphedema; therefore, salt and calorie diet should be restricted. Role of conventional diuretic therapy in lymphatic edema, per se, is limited; however, it may be beneficial in mixed-origin edema which occurs in cirrhosis patients. In addition, diuretics may also render lymphedema worse by removing fluid and increasing lymph protein concentration, resulting in a reversed gradient of oncotic pressure and increased vulnerability to infection. The role of newer molecules with diuretic activity, such as V2-receptor antagonist and sodium-glucose cotransporter 2 (SGLT2) inhibitors, needs to be explored in cirrhosis patients with lymphatic dysfunction. Tolvaptan is an oral selective V2-receptor antagonist and a novel water diuretic. Unlike loop diuretics, tolvaptan has a different effect on fluid distribution, and it can ameliorate fluid retention with a low risk of a worsening renal function[72,73]. SGLT2 inhibitors are the new class of antihyperglycemic agents with a good safety profile in cirrhosis patients. SGLT2 inhibitors have been shown to have

Table 2 Possible therapeutic strategies for treatment of lymphatic dysfunction in cirrhosis

To decrease formation of lymph	
Decrease water retention	Low salt diet, diuretic therapy
Control of portal hypertension	Beta-blocker, octreotide, transjugular intrahepatic portosystemic shunt
Increase interstitial pressure	Compression therapy
To promote lymphatic drainage	
Facilitate fluid movement into the lymphatic vessels	Compression therapy, limb elevation, diuretic therapy (limited role)
Increase contractility of the lymphatic vessels	Nor-adrenaline, phenylephrine, nitric oxide-inhibitors (experimental)
Facilitate lysis of interstitial protein	Benzopyrones (coumarin and flavoids)
Promote lymphangiogenesis	Prostaglandins E2 (experimental), vascular endothelial growth factor-C (experimental)
To control aggravating factors for lymphatic dysfunction	
Care of lymphedema	Control of infection (aggressive use of antibiotics), avoidance of trauma, hot bath and other heat-producing treatment
Control risk factors	Control of diabetes, dyslipidemia and obesity
To decrease leakage of lymph	
Decrease stimulants of intestinal lymph flow	Low fat diet, octreotide
Decrease leakage of lymph by intervention	Compression therapy, antiplasmin (tranexamic acid); radiological intervention to obliterate the site of leak
To correct underlying condition	
Definitive therapy of cirrhosis	Liver transplantation

significant diuretic effects and, interestingly, without altering the intravascular volume, they can induce interstitial fluid clearance[74]. In addition to inducing glycosuria and natriuresis, these agents have beneficial effects on neurohormonal regulation and hepatorenal fibrosis[75]. Given that DM is also a risk factor for lymphatic dysfunction, SGLT2 inhibitors may be potentially helpful in diabetic patients with cirrhosis, with lymphatic dysfunction.

The contractile function of lymphatic vessels is very important for the reabsorption of extravascular fluid. While lymphatic vessels can modulate their contractile function in response to various neural, hormonal endothelial and humoral factors, no specific therapeutic agent has been approved for this purpose. In an animal study, intravenous adrenaline infusion has been found to increase the frequency of lymphatic contraction and lymph flow in efferent lymphatic vessels[76]. In an experimental study, significant improvements were observed in lymphatic vessels' contractility and lymphatic drainage, when treated with an eNOS inhibitor[9]. Inhibition of eNOS can, therefore, be a useful therapeutic target for lymphatic dysfunction in cirrhosis. However, any attempt to inhibit NO must take into account the fact that inhibition of intrahepatic NO may increase intrahepatic pressure, so that the resulting increased lymph production may negate its impact on improving the drainage of the lymph. As a result, to target only eNOS of extra-hepatic lymphatic vessels, a tissue-specific delivery strategy is required. Benzopyrones (flavonoids and coumarin) have been found to be effective in lymphatic edema treatment[77]. These drugs facilitate removal of accumulated interstitial proteins, by binding and causing phago-proteolysis by macrophages. However, there are some concerns regarding coumarin hepatoxicity, and there is a lack of evidence on the use of this medication in cirrhosis.

Low fat diets are currently recommended for the treatment of intestinal lymphangiectasia, as intestinal lymph flow is highly affected by oral fat intake[77]. For fat nutrition, medium-chain triglycerides supplementation should be used as they are directly absorbed through the portal venous system, without involvement of intestinal lacteal. Additionally, octreotide has been found helpful in patients with intestinal lymphangiectasia, by reducing splanchnic blood flow and the leakage of intestinal lymph[78]. Moreover, tranexamic acid has been found to cause significant reduction in protein loss in patients with intestinal lymphangiectasia, possibly due to the inhibition of tissue fibrinolytic activity that decreases the capillary permeability to protein[79]. Finally, transjugular intrahepatic porto-systemic shunt and liver transplantation have

been found to be effective therapy of PHT-induced protein-losing enteropathy, possibly caused by intestinal lymphangiectasia[80,81]. Regarding CA, a number of treatment options have been identified, including low-fat diet, medium-chain triglyceride, octreotide, total parenteral nutrition, embolization of leaking lymph vessel by radiological intervention, and surgical peritoneovenous shunt[39,82]. Nevertheless, there are no research reports comparing either of these treatment modalities. Initially, these patients should be managed with conservative approaches, and when they fail, repeated paracentesis should be used for symptomatic relief, and further invasive therapies may be considered.

It has been found that splenectomy effectively decreases portal pressure and corrects hypersplenism in patients with cirrhosis[83,84]. Since the progression of cirrhosis may result in a parallel increase in portal pressure, it would be worth investigating whether a reduction in portal pressure, after splenectomy, contributes to decreased lymph formation and decreased overload of the lymphatic system. However, in patients with advanced decompensated cirrhosis, where lymphatic dysfunction is maximal, splenectomy may not always be feasible[84]. Furthermore, caution is needed while contemplating albumin therapy in cirrhotic patients with lymphatic dysfunction. Henriksen *et al*[85] have recently found that in patients suffering from advanced cirrhosis, with diuretic-resistant ascites, the transport rate of albumin from plasma into the peritoneal cavity is highly elevated and exceeds the back transport rate of albumin into the plasma. Patients with advanced cirrhosis have accelerated trans-capillary escape rate of albumin, due to greater hydrostatic pressure and capillary permeability[86]. Hence, the molecules of albumin are more likely to extravasate rapidly into the interstitium. To recirculate the escaped albumin back to plasma, proper lymphatic functions are needed. However, in patients with advanced cirrhosis, the escaped albumin is less likely to be recirculated back into the plasma, due to deficient lymphatic function. This would not only fail to correct circulating hypovolemia, the reason for which it is given, but accumulation of albumin in the interstitium would facilitate development of reversed oncotic pressure gradient and extravascular movement of fluid, leading to worsening of edema and ascites[87]. Albumin, however, also has anti-inflammatory, immunomodulatory, and anti-oxidant properties[88]. It would be interesting to investigate these non-oncotic properties of albumin on lymphatic functions, as chronic inflammation and neurohormonal alterations play a significant role in lymphatic dysfunction of cirrhosis.

CONCLUSION

In conclusion, a greater understanding of the lymphatic vascular system has emerged over the last two decades, following the discovery of specific lymphatic endothelial markers and technical advances in lymphatic imaging. However, the role of lymphatic dysfunctions in the pathophysiology of advanced cirrhosis is still poorly understood. Given the major role of the lymphatic system in body fluid homeostasis, immunity, and metabolism, it is plausible to understand that in patients with cirrhosis, a defective lymphatic system may have several clinical consequences. This field is, therefore, largely open to new research. A better understanding of lymphatic pathophysiology in cirrhosis will significantly enhance our ability to manage such patients and design targeted therapy.

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ORIGINAL ARTICLE

Basic Study

Papaya improves non-alcoholic fatty liver disease in obese rats by attenuating oxidative stress, inflammation and lipogenic gene expression

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Abstract

BACKGROUND

Non-alcoholic fatty liver disease (NAFLD) is a global health issue that is correlated with obesity and oxidative stress.

AIM

To evaluate the anti-NAFLD effect of papaya in high fat diet induced obesity in rats.

METHODS

Four-week-old male Sprague-Dawley rats were divided into four groups after 1 wk of acclimatization: Group 1 was the rats fed a normal diet (C); group 2 was the rats fed a high fat diet (HFD); group 3 was the rats fed a HFD with 0.5 mL of papaya juice/100 g body weight (HFL), and group 4 was the rats fed a HFD with 1 mL of papaya juice/100 g body weight (HFH) for 12 wk. At the end of the treatment, blood and tissue samples were collected for biochemical analyses and histological assessment.

RESULTS

The results of the HFH group showed significantly reduced body weight (HFH *vs*

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HFD, $P < 0.01$), decreased NAFLD score (HFH *vs* HFD, $P < 0.05$), and reduced hepatic total cholesterol (HFL *vs* HFD, $P < 0.01$; HFH *vs* HFD, $P < 0.001$), hepatic triglyceride (HFH *vs* HFD, $P < 0.05$), malondialdehyde (HFL, HFH *vs* HFD, $P < 0.001$), tumour necrosis factor-α (HFH *vs* HFD, $P < 0.05$) and interleukin-6 (HFH *vs* HFD, $P < 0.05$) when compared to the HFD group. However, the liver weight showed no significant difference among the groups. The activities of catalase and superoxide dismutase significantly increased in HFH when compared with the HFD group ($P < 0.05$ and $P < 0.001$, respectively). The suppression of transcriptional factors of hepatic lipogenesis, including sterol regulatory element-binding protein 1c and fatty acid synthase, were observed in the papaya treated group (HFH *vs* HFD, $P < 0.05$). These beneficial effects of papaya against HFD-induced NAFLD are through lowering hepatic lipid accumulation, suppressing the lipogenic pathway, improving the balance of antioxidant status, and lowering systemic inflammation.

CONCLUSION

These current results provide experimental-based evidence suggesting papaya is an efficacious medicinal fruit for use in the prevention or treatment of NAFLD.

Key Words: High fat diet; Lipogenic gene expression; Non-alcoholic fatty liver disease; Obesity; Oxidative stress; Papaya

Core Tip: High fat diet consumption causes non-alcoholic fatty liver disease (NAFLD). This is one of the major liver diseases found worldwide. Liver fat accumulation leads to dysfunction of liver due to oxidative stress and inflammation. Papaya is an important export fruit from Asian and Latin America. It is a nutrient rich fruit with many medicinal properties. Our present study clearly demonstrated that the hepatoprotective mechanism of papaya against NAFLD was a result of the association of the hypolipidemic, anti-inflammatory, and antioxidant activities. This study provides evidence for the beneficial effects of papaya to reverse the progression of NAFLD in obese rats.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is of growing concern since its prevalence is increasing worldwide[1]. NAFLD is characterised by an accumulation of triglycerides and fatty acids in hepatocytes. The circulating pool of free fatty acids (FFAs) is increased in obese individuals and accounts for the majority of lipid accumulation in NAFLD. Excessive consumption of diets rich in fat is related to oxidative stress in various tissues including vessels, adipose tissues and liver and consequent to disease development[2]. Normally, oxidative stress such as reactive oxygen species (ROS) and reactive nitrogen species are continuously generated from inside the cells (*e.g.*, electron transfer, cellular metabolism), but there is the counterbalance by the antioxidant system to defend the body from cellular or tissue damage[3]. In NAFLD, an imbalance of oxidant synthesis and antioxidants is the major contributor to the pathogenesis of the disease, leading to liver injury and hepatocyte deterioration[4]. Antioxidants have been suggested to be beneficial for health promotion and disease prevention. Therefore, we hypothesised that fruit rich in antioxidants may have potential benefit against NAFLD.

Carica papaya known as pawpaw or papaya is in the family of Caricaceae[5]. It is widely cultivated in many regions of the world, including Central and South America,

Asia, and Africa, and its principal markets for consumption are the United States and Europe[6]. Papaya is a nutraceutical plant with many medicinal properties. Some studies have reported its health benefits including the treatment of gastrointestinal related disorders, diabetes, hypertension, hypercholesterolemia and hepatotoxicity, and its anti-microbial, anti-parasitic, and anti-viral properties[7,8]. Almost all parts of papaya can be used, especially the fruit of *C. papaya*. It is a nutritional source that is high in fibre, minerals and strong antioxidants including vitamin A, C and E. However, its health benefits in NAFLD are still the subject of research.

The purpose of this study was to evaluate the effect of papaya juice in the treatment of NAFLD. The doses of papaya juice used in this study can be practically applied to human use. Since papaya is low cost, easily available and widely marketed worldwide, the results from this study could be implemented in nutritional intervention that may be used in the prevention and treatment of NAFLD.

MATERIALS AND METHODS

Plant material and preparation of papaya

The Holland variety of papaya fruit (*Carica papaya* L.) was derived from a supermarket in Phitsanulok, Thailand. The fruit was harvested at a ripe stage, when papaya presents yellow areas on 50%-75% of the skin[9]. The juice was freshly prepared by extraction from the homogenised flesh of the Holland cultivar and separated from the pulp by squeezing it several times. The juice was then centrifuged at 1500 × *g* for 20 min. The papaya composition as shown in Table 1 was analysed by Food and Nutrition Laboratory, Institute of Nutrition, Mahidol University, Nakhon Pathom, Thailand.

Animals and experimental design

The NAFLD animal model was developed as described previously[10]. Four-week-old male Sprague-Dawley rats weighing between 100 and 120 g were purchased from the National Laboratory Animal Centre at Salaya campus, Mahidol University (Nakon Pratom, Thailand). All animal experiments were carried out after getting approval from the Animal Ethics Committee at the Centre for Animal Research at Naresuan University, Phitsanulok, Thailand (Approval number NU-AE 580714). All procedures were performed in accordance with Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press)[11]. The animals were acclimatised for 1 wk and then randomised into four groups (n = 6-7). Group 1 was the control rats fed a commercial normal diet for 8 wk (C), while the three remaining groups (2-4) were fed a high fat diet (HFD) for 8 wk and oral gavage for 1 mo as follows; Group 1 was fed a normal diet for 8 wk and then treated with distilled water for an additional 4 wk, animals were maintained on a normal diet. After the first 8 wk period on HFD, animals in group 2 were fed a HFD for 4 wk, while those of groups 3 and 4 were kept on HFD and received 0.5 mL and 1 mL/100 g body weight/day of papaya juice, respectively.

The doses of papaya used in 0.5 mL of papaya juice/100 g body weight (HFL) and 1 mL of papaya juice/100 g body weight (HFH) were the equivalent of approximately 125 and 250 g of papaya consumed by a person, respectively. Diet composition of control and high fat diets were formulated according to AIN-93G as previously described with a slight modification[12]. Briefly, the high fat diets were composed of 1.5% cholesterol, 20% palm oil and 0.25% cholic acid. Body weights of rats were recorded weekly. At the end of the 12th week, the animals were euthanised by pentobarbital injection. The blood was drawn through cardiac puncture. Blood and tissue samples were collected and kept at -80 °C for further analysis.

Biochemical analyses

The serum was used to measure aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) by Bio Lab Medical Centre (Phitsanulok, Thailand).

Analysis of hepatic TAG and cholesterol content

Hepatic lipid was extracted according to a modified Folch method, as previously described[13]. Briefly, lipids were extracted from 0.5 g of liver with a mixture of chloroform/methanol (2:1, v/v) and dried under N_2. The pellets were dissolved and used for the analysis of hepatic lipid contents. The hepatic contents of triglyceride and

Table 1 Composition of papaya

Nutrients	Value
Energy (kcal)	26.62 ± 0.26
Moisture (g)	92.86 ± 0.06
Protein (g)	0.65 ± 0.02
Total fat (g)	0.00 ± 0.00
Total carbohydrate (g)	6.01 ± 0.09
Soluble dietary fibre (g)	0.87 ± 0.03
Ash (g)	0.49 ± 0.01
Total sugar (g)	3.97 ± 0.02
Calcium (mg)	25.95 ± 0.98
Potassium (mg)	14.50 ± 0.28
Iron (mg)	0.56 ± 0.01
Total phenolic compounds (mg gallic acid/g papaya)	0.56 ± 0.01
Carotenoid profile:	
Beta-cryptoxanthin (μg)	596.04 ± 15.27
Lycopene (μg)	1166.88 ± 11.24
Beta-carotene (μg)	78.96 ± 1.45

total cholesterol were determined using a colorimetric assay kit according to the instructions of the manufacturer (HUMAN Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany).

Histopathological analyses

To analyse the histopathology of the liver, the tissue was fixed immediately after removal in 10% formalin. The liver tissue was then embedded in paraffin, sectioned, and stained with haematoxylin and eosin. The histopathological features were scored for the liver lesions using NAFLD activity score (NAS) according to Xu *et al*[14]. NAS component represents the sum of score ranging from 0-8 for three histological features: Hepatocyte ballooning (0-2), lobular inflammation (0-3) and steatosis (0-3). The total NAS score of 0-3 was defined as not nonalcoholic steatohepatitis (NASH). The score greater than 5-8 was considered as NASH. The hepatic lipid accumulation assessment was modified from Malakul *et al*[12]. In brief, the frozen liver samples with optimal cutting temperature-embedded were cryosectioned at 5 μm with a cryostat, fixed in 4% v/v formalin for 10 min and then stained with Oil Red O working solution for triglycerides and free fatty acid staining.

Hepatic lipid peroxidation

The isolated rat livers were homogenised in phosphate buffered saline (PBS), and the total protein content of liver tissues was measured using a Bradford assay kit (Sigma-Aldrich, St. Louis, MO, United States). The lipid peroxidation of the hepatic tissue homogenate was determined by a thiobarbituric acid assay. The solutions were prepared according to Liu *et al*[15]. Briefly, the mixture of 15% trichloroacetic acid, 0.25 N HCl (Sigma-Aldrich) and 0.37% 2-thiobarbitulic acid (POCH, Sowinskiego, Poland) with 1:1:1 ratio was prepared. Then 200 μL of these reagents were added in each eppendorf tube and incubated in heat block at 95 °C for 15 min. The solutions were centrifuged at 3500 × *g* for 25 min and the supernatant in each tube was pipetted to 96 well plates. The samples were then measured at absorbance 535 nm with malondialdehyde as a standard, and the unit was expressed as μmoL/mg protein.

Catalase and superoxide dismutase activities

The livers were homogenised in ice cold PBS. The homogenate was centrifuged, and the supernatant were taken to measure the activities of catalase (CAT) and superoxide dismutase (SOD) by using commercial assay kits (Cayman Chemical Company, Ann Arbor, MI, United States). The final units for enzyme activities were normalised with

protein concentration.

Determination of biomarkers of inflammation

The liver homogenates were used to determine the levels of tumour necrosis factor-α (TNF-α) and interleukin 6 (IL-6) by using commercial assay kits (Sigma-Aldrich). The final units for TNF-α and IL-6 were normalised with protein concentration.

Analysis of gene expression

Total ribonucleic acid (RNA) of the liver was isolated using RiboZol (Amresco, Dallas, TX, United States) according to the protocol provided by the manufacturer. The complementary deoxyribonucleic acid synthesis was performed in a reaction mixture containing 4 μL of reaction buffer, 2 μL of deoxyribonucleotide triphosphate, 1 μL of random primer, 1 μL of RNAse inhibitor, 1 μL of reverse transcriptase and 500 ng of total RNA. Polymerase chain reaction (PCR) was performed with PCR thermocycling. The PCR products were measured by agarose gel electrophoresis technique with 2% agarose gel and 1 × TBE running buffer (1M Tris, 0.9M boric acid and 1 mmoL/L EDTA). Deoxyribonucleic acid was stained with a fluorescent colour (Biotechnology, Daejeon, Korea). Each sample was assayed in triplicate, and β-actin was amplified in parallel to serve as an internal control for reverse transcription-PCR quantification. All mRNA gene expression data were normalised to the expression level of β-actin.

The sequences of the primers for genes used in this study were indicated as follows; *SREBP-1c*: forward 5′-TGGATTGCACATTTGAAGACAT-3′, reverse 5′-GCTCCTCTTTGATTCCAGGC-3′; *ACC:* forward 5′-GCCTCTTCCTGACAAACGAG-3′, reverse 5′-TCCATACGCCTGAAACATGA-3′; *FAS*: forward 5′-GGACAT GGTCACAGACGATGAC-3′, reverse 5′-GTCGAACTTGGACAGATCCTTCA-3′. *ACTB*: forward5′-TGTCCACCTTCCAGCAGATGT-3′, reverse 5′-AGCTCAGTAACAGTCGA -3′.

Statistical analysis

Results are presented as the mean ± SE of the mean. Statistical analyses were performed using IBM SPSS version 23 (Armonk, NY, United States). Group difference was assessed by a one-way analysis of variance, followed by Tukey's test for multiple comparisons. A P value < 0.05 was considered statistically significant.

RESULTS

Effects of papaya on liver weight, lipid contents and serum components in rats

The initial body weight and body weight at week 8 of all the experimental groups were not significantly different. However, at the end of treatment the HFD group showed significantly increased body weight when compared with the C group, while those parameters decreased in the HFH group. The result also showed that papaya improved hepatic lipid contents in HFD-fed rats. The HFD group showed significantly increased hepatic triglycerides (TG) and cholesterol levels when compared with the C. The TG levels were significantly decreased in the HFH (P < 0.05), while total cholesterol (TC) was significantly decreased in both the HFL (P < 0.01) and HFH (P < 0.001) when compared with the HFD group. This result indicated that papaya markedly reduced the hepatic TG and TC contents. The serum levels of AST, ALT and ALP were significantly increased in rats fed a HFD. Higher levels of those enzymes suggest that a HFD can induce liver inflammation or liver damage. Moreover, the liver damage indices also significantly decreased in the papaya treated group when compared to the HFD group (Table 2). This result suggests that papaya administration may improve liver injury found in NAFLD.

Effects of papaya on lipid accumulation

Oil Red O staining showed that hepatic lipid accumulation of HFD was significantly higher than that in the C group. The oral administration of papaya to HFD rats reduced steatosis and lipid droplet size as shown in Figure 1A. In addition, it showed that the liver samples from the HFD group showed significant fat deposition with the highest scores in steatosis, lobular inflammation and hepatocyte ballooning. The HFD group scores were significantly higher than those of the control group (P < 0.001), which strongly indicated the development of NAFLD. Interestingly, the significant reduction of steatosis, lobular inflammation and hepatocyte ballooning was observed after 4 wk of treatment with papaya (Figure 1B).

Table 2 Effects of papaya on body weight, liver weight, hepatic lipid contents and liver damage indices in high fat diet induced obesity in rats

	C	HFD	HFL	HFH
Initial weight (g)	218.0 ± 12.18	236.5 ± 13.58	215.5 ± 14.01	221.8 ± 14.29
Body weight at week 8 (g)	398.6 ± 9.462	457.6 ± 18.93[a]	462.9 ± 17.11[a]	454.9 ± 7.584[a]
Body weight at week 12 (g)	465.83 ± 11.13	536 ± 33.24[c]	509.33 ± 33.57[a]	471.33 ± 15.04[e]
Liver weight (% of body weight)	2.66 ± 0.13	4.46 ± 0.3[c]	4.49 ± 0.51[c]	4.33 ± 0.26[c]
Hepatic triglycerides (mg/dL)	140.60 ± 13.95	211.00 ± 26.25[a]	172.50 ± 7.89	152.20 ± 12.68[d]
Hepatic cholesterol (mg/dL)	74.30 ± 5.58	152.60 ± 9.44[c]	112.40 ± 7.96[b,e]	92.38 ± 6.66[f]
Serum ALT (U/mL)	38.80 ± 2.29	214.00 ± 48.95[c]	109.30 ± 20.85[d]	86.00 ± 7.57[d]
Serum AST (U/mL)	126.40 ± 4.72	302.70 ± 51.72[b]	211.30 ± 7.88	137.70 ± 21.42[e]
Serum ALP (U/mL)	60.60 ± 1.80	86.20 ± 4.60[b]	91.00 ± 4.16[b]	88.00 ± 6.08[b]

Data are expressed as the mean ± SE of the mean (n = 6-7).
[a]$P < 0.05$.
[b]$P < 0.01$.
[c]$P < 0.001$ *vs* C.
[d]$P < 0.05$.
[e]$P < 0.01$.
[f]$P < 0.001$ *vs* high fat diet group. ALP: Alkaline phosphatase; ALT: Alanine transaminase; AST: Aspartate transaminase; C: Control; HFD: High fat diet; HFH: High fat diet treated with 1 mL of papaya juice/100 g body weight; HFL: High fat diet treated with 0.5 mL of papaya juice/100 g body weight.

Effects of papaya on the oxidative status and antioxidant activities

Papaya improved lipid peroxidation in HFD-fed rats. The HFD group showed significantly increased lipid peroxidation when compared with the C ($P < 0.001$). Furthermore, lipid peroxidation was significantly decreased in the HFD treated with papaya 0.5 and 1 mL/100 g body weight ($P < 0.001$) when compared with the HFD group (Figure 2A). In contrast, the CAT and SOD activities were found to decrease in the HFD group, whereas those significantly increased in HFH group (Figure 2B and C).

Effects of papaya on proinflammatory cytokines in liver tissue

The results showed that HFD in rats significantly increased the serum levels of TNF-α (Figure 3A) and IL-6 (Figure 3B), while these two cytokine levels significantly decreased in the HFD treated with papaya 1 mL/100 g body weight ($P < 0.05$). Taken together, papaya administration can counterbalance lipid peroxidation and inflammation, which is normally found in NAFLD. Improvements in antioxidant activity were also observed.

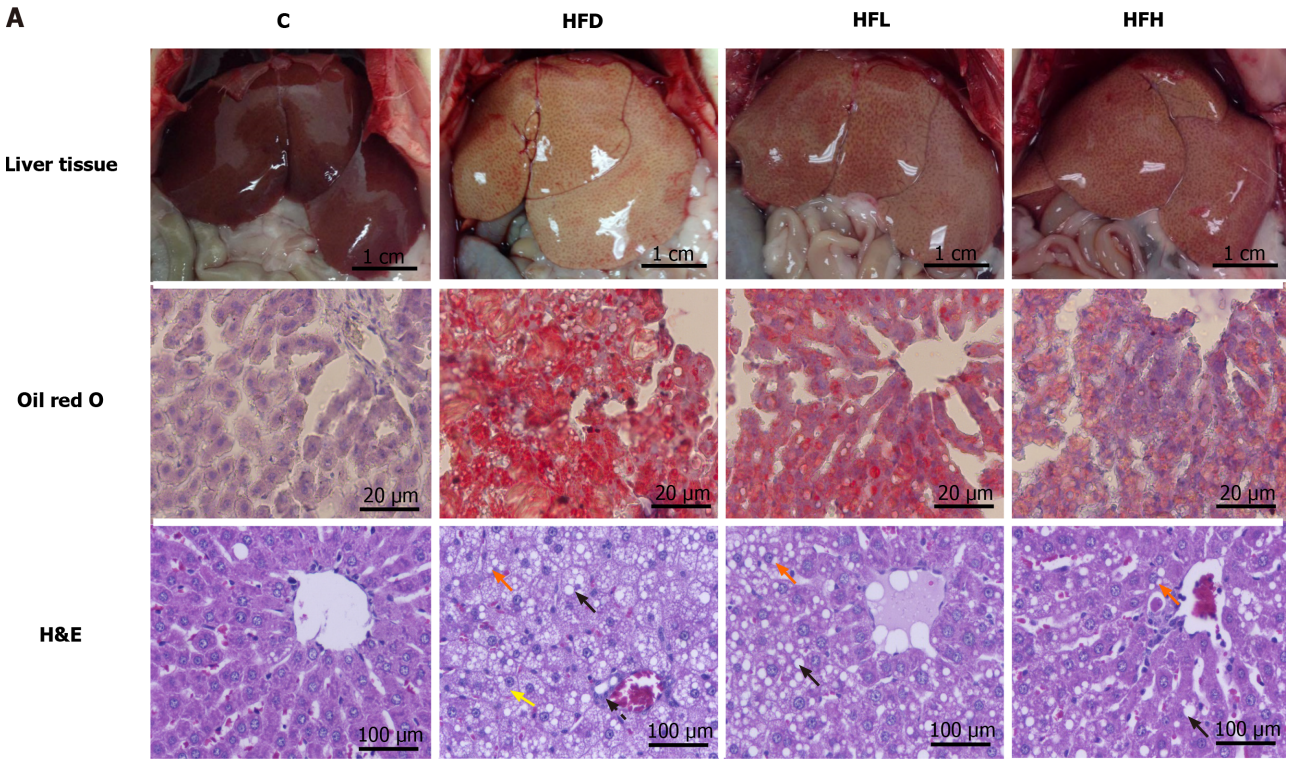
Effects of papaya on the de novo lipogenic gene in liver tissue

The mRNA expression of *SREBP-1c* and *FAS* had a tendency to increase in HFD rats as compared with the control. A significantly decreased expression of those genes were observed in HFH rats ($P < 0.05$) as shown in Figure 4A, B and D, respectively. In contrast, the expression of *ACC* was not different among the groups (Figure 4C). The data indicated that a possible involvement of lipogenesis in the papaya treated group is partially mediated through *SREBP-1c*, which down-regulates the expression of *FAS*. This event may account for the decreased fatty acid metabolism in the liver of rats treated with high doses of papaya juice.

DISCUSSION

Oxidative stress and inflammation are the main components that contributed to the pathogenesis of NAFLD. Many natural products rich in polyphenols, and strong antioxidant activity have been studied for their positive benefits in the treatment of NAFLD[16]. The presence of these bioactive compounds as well as the significant antioxidant activity *in vitro* has been observed in the pulp and fruit peel of papaya[9].

Our present study demonstrated that papaya attenuated lipid accumulation in



B

Steatosis	0.00 ± 0.00	2.17 ± 0.75[c]	1.67 ± 1.21[b]	1.17 ± 0.75
Lobular inflammation	0.00 ± 0.00	2.00 ± 0.63[c]	1.33 ± 1.03[a]	1.00 ± 0.89
Hepatocyte ballooning	0.00 ± 0.00	1.83 ± 0.41[c]	1.33 ± 0.52[c]	1.50 ± 0.55[c]
NAS	0.00 ± 0.00	6 ± 1.79[c]	4.33 ± 2.76[c]	3.67 ± 2.19[c,d]

Figure 1 Effect of papaya on non-alcoholic fatty liver disease. A: Macroscopic and microscopic appearance in rat hepatocytes. Macrovesicular steatosis (black arrow) are large lipid droplets that are present in the hepatocytes. Microvesicular steatosis (red arrow) are small lipid droplets that are present in the hepatocytes. Hepatocyte ballooning is recognised as cell swelling and enlargement within the cytoplasm (yellow arrow). Lobular inflammation in non-alcoholic steatohepatitis foci (dotted line arrow) are scattered in the hepatic lobule; B: Comparative analysis of non-alcoholic fatty liver disease activity score for all treatment groups. Data are expressed as mean ± SE of the mean (n = 6-7). [a]P < 0.05, [b]P < 0.01, [c]P < 0.001 *vs* C, and [d]P < 0.05 *vs* high fat diet group. C: Control; H&E: Hematoxylin and eosin; HFD: High fat diet; HFH: High fat diet treated with 1 mL of papaya juice/100 g body weight; HFL: High fat diet treated with 0.5 mL of papaya juice/100 g body weight; NAS: Non-alcoholic fatty liver disease activity score.

HFD-induced obesity in rats. In this study the *in vivo* model of NAFLD was successfully established and developed to lipid accumulation in liver after feeding the rats an HFD. Those rats fed a HFD exhibited an increase in the weight of the liver and lipid contents, which is a feature of NAFLD[14]. The reverse alterations in hepatic lipid accumulation can be explained by the effects of papaya on lipid metabolism. The mechanism may be, in part, by the inhibition of pancreatic lipase by papaya[17]. Pancreatic lipase is an enzyme secreted from the pancreas and works in the small intestine to hydrolyse TG from diet to glycerol and free fatty acids. In this case, papaya juice hinders the digestion of TG, resulting in the reduction of lipid absorption and then promotion of the excretion of lipids outside the body. From previous studies, it has been shown that the excessive hepatic accumulation of TG and FFA induced hepatic steatosis[18]. From our study, it was demonstrated that the treatment with papaya ameliorates lipid accumulation in liver in HFD rats *via* the modulation of lipid metabolism-related molecules.

In NAFLD pathogenesis, imbalanced lipid metabolism leads to simple steatosis, oxidative damage and secretion of proinflammatory mediators. The liver serves as the major regulator for lipid metabolism that involves in several steps[19]. Hepatic lipid content is regulated by the cellular molecules that control the input and the output. The regulation depends on the metabolic status, the facilitation of hepatic fatty acid uptake, synthesis and storage in the liver, or the rapid metabolism to hepatic fatty acid oxidation as a source of energy may occur[20].

SREBP-1c exerts a significant control over the *de novo* synthesis of *FAS*[20]. It was further found that papaya eliminated hepatic steatosis in HFD rats. The latter effect

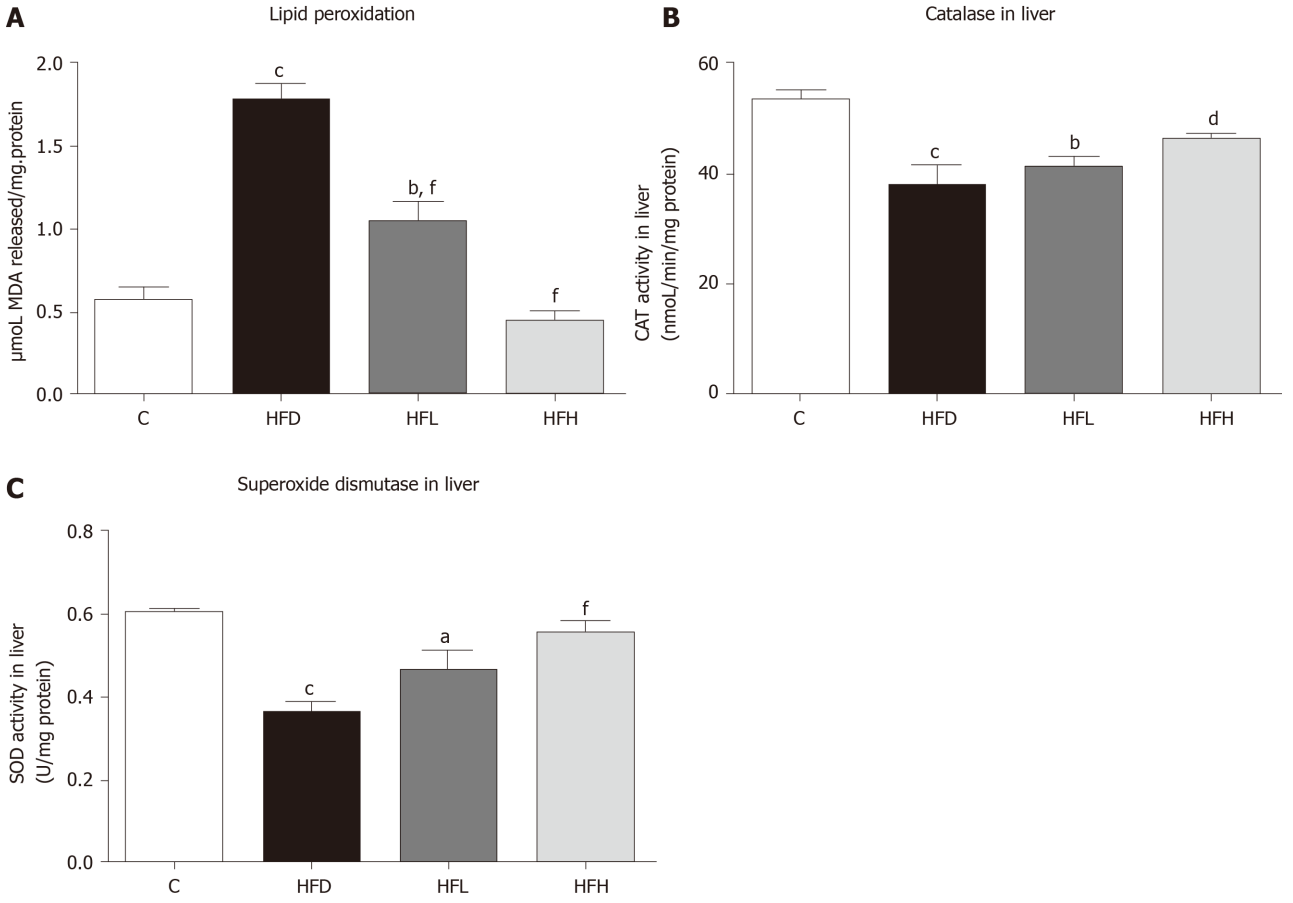


Figure 2 Effects of papaya on antioxidant activities in liver tissue. A: Lipid peroxidation in the liver; B: Activity of catalase (CAT) in the liver; C: Activity of superoxide dismutase (SOD) in the liver. Data are expressed as mean ± SE of the mean (n = 6-7). $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* control (C), and $^{d}P < 0.05$, $^{f}P < 0.001$ *vs* high fat diet (HFD) group. HFH: High fat diet treated with 1 mL of papaya juice/100 g body weight; HFL: High fat diet treated with 0.5 mL of papaya juice/100 g body weight; MDA: Malondialdehyde.

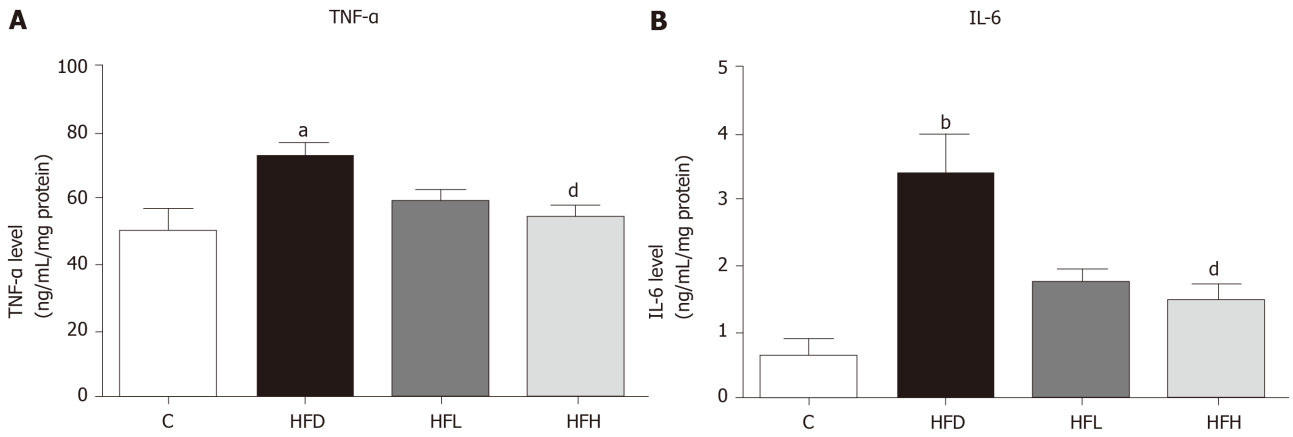


Figure 3 Effects of papaya on proinflammatory cytokines in liver tissue. A: Tumour necrosis factor-α (TNF-α) in the liver; B: Interleukin 6 (IL-6) in the liver. Data are expressed as mean ± SE of the mean (n = 6-7). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control (C), and $^{d}P < 0.05$ *vs* high fat diet (HFD) group. HFH: High fat diet treated with 1 mL of papaya juice/100 g body weight; HFL: High fat diet treated with 0.5 mL of papaya juice/100 g body weight.

might be partially mediated by the regulation of *SREBP-1c*. *SREBP-1c* is an important transcription factor of *de novo* lipogenesis in the liver, while its downstream gene-*FAS* is responsible for fatty acid catabolism[21]. In the livers of obese rats treated with papaya, *SREBP-1c* and *FAS* were remarkably decreased. This implies that papaya exerts its anti-lipogenic effect in consequence of the suppressed regulation of *SREBP-1c* and *FAS*, leading to decreased hepatic lipid accumulation.

Several studies have also demonstrated the antioxidant capacity of β-carotene and

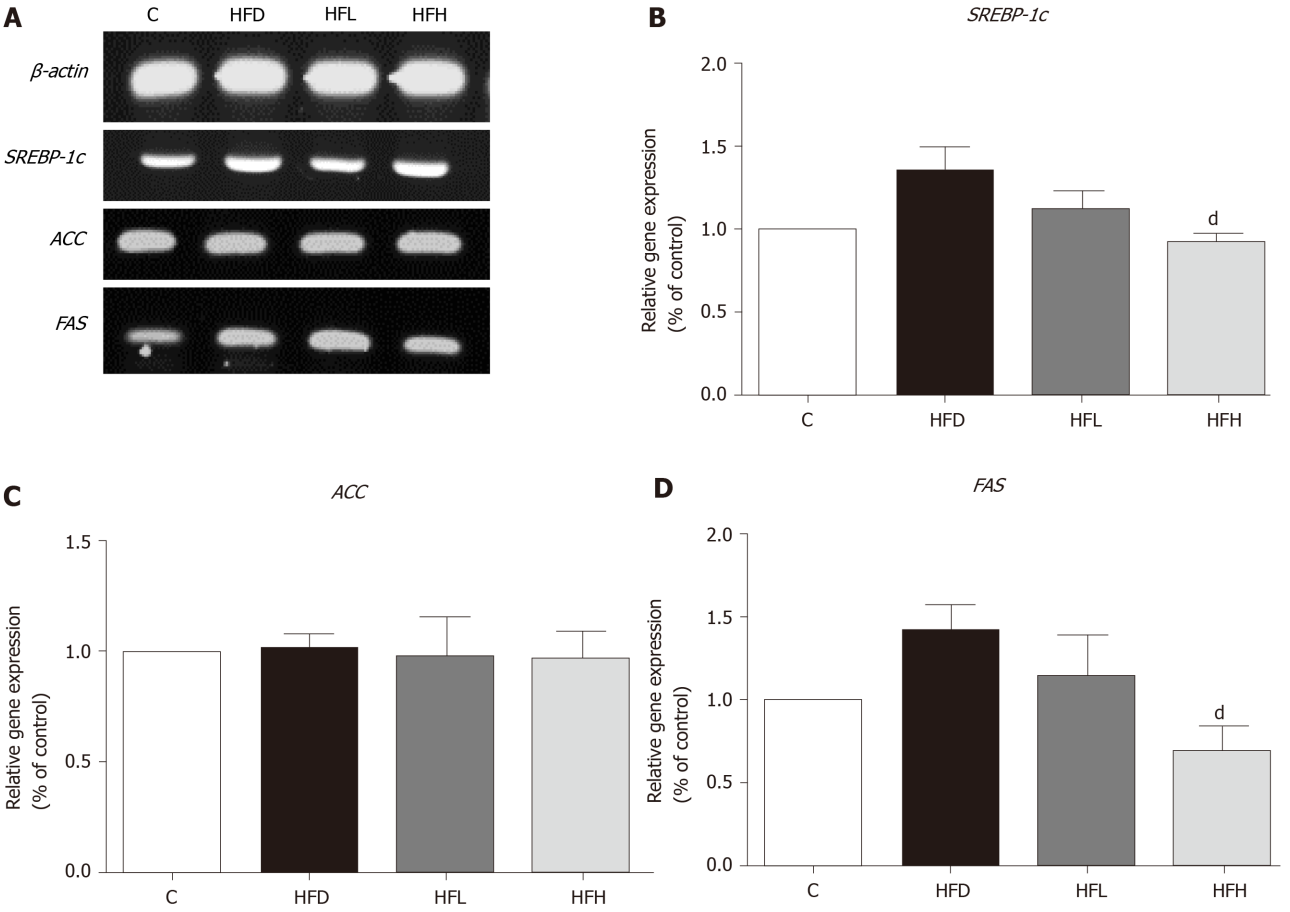


Figure 4 Effects of papaya on *de novo* lipogenic gene expression in liver tissue. A: Immunoblotting analysis of *SREBP-1c*, *ACC*, *FAS* and *ACTB*. *ACTB* was used as a normalization gene; B: Relative gene expression of *SREBP-1*; C: Relative gene expression of *ACC*; D: Relative gene expression of *FAS*. Data are expressed as mean ± SE of the mean (n = 5). $^{d}P < 0.05$ *vs* high fat diet (HFD) group. C: Control; HFH: High fat diet treated with 1 mL of papaya juice/100 g body weight; HFL: High fat diet treated with 0.5 mL of papaya juice/100 g body weight.

its act against oxidative stress in different models[22,23]. The significantly elevated hepatic content of TG, TC and malondialdehyde in NAFLD rats is a strong indicator of liver damage and oxidative stress[24]. The pathogenesis of NAFLD is widely accepted by the two-hit hypothesis; the first hit presents increasing levels of FAs and is a key part in the development of hepatic steatosis. Prolonging of hepatocellular damage and sensitised liver leads to the presence of oxidative stress and the release of cytokine or adipokine mediators, this situation is called a second hit[25]. More specifically, high fat consumption leads to increased FAs in liver and either enter β-oxidation or are stored as TG. The mitochondrial β-oxidation serves as energy sources and can generate numerous free radicals including ROS and lipid peroxidation from the electron transport chain though the mitochondrial respiration pathway[26]. Normally, the antioxidant defensive systems help to protect the organs against the deleterious substances[27]. Among these, SOD is a key antioxidant enzyme for the first defence reaction with the ROS-mediated cellular damage. SOD participates in the conversion of superoxide anions into less harmful H_2O_2 and oxygen. CAT is another antioxidant enzyme that can catalyse H_2O_2 into water and oxygen[28]. From our results, it clearly shows that SOD and CAT activities in the liver were significantly increased after papaya treatment. The mechanism is still unknown, but it might be because of the carotenoid compounds in papaya. Papaya is one of the important dietary sources for carotenoids including β-carotene and lycopene[29]. The liver is the main place for storage carotenoids, the powerful antioxidants from food, and this compound may help scavenge the results of oxidative stress produced in the liver[16].

High fat accumulation in the liver causes impairment of cellular homeostasis. ROS and lipid peroxidation generated in NAFLD are potent inducers of cytokine production and trigger the release of cytokine proinflammatory mediators such as TNF-α and IL-6[30]. TNF-α plays a crucial role in exert in a variety of biological effects including systemic inflammation and takes part in many stages of liver disease[31]. In contrast, IL-6 is secreted from various kinds of cells and is necessary to leukocyte

recruitment and tissue homeostasis[32]. Recent studies have been reported that IL-6 enhances liver inflammation and related to insulin resistance in NAFLD[29]. Proinflammatory cytokine overproduction causes hepatocyte dysfunction and develops fibrosis later on.

We demonstrated from our results that papaya can reduce liver inflammation by the inhibiting the overproduction and activity of proinflammatory cytokines generated in high fat induced hepatic inflammation tissue. The mechanism may be from indirect action of papaya to reduce ROS and can modulate the overwhelming production of cytokines. In addition, papaya itself may play a direct role in the inflammation processes. As reported earlier, papaya possesses anti-inflammatory and immuno-modulatory properties as stated both *in vitro* and i*n vivo* studies[33]. Liver inflammation can aggravate liver damage, resulting in the progression of fibrosis, cirrhosis or liver failure. Reduced inflammatory secretion from cytokines may prevent steatosis and alleviate the progression of the disease[34].

CONCLUSION

This study demonstrates for the first time the hepatoprotective capacity of the papaya fruit on the damage caused by HFD induced hepatic steatosis. From the obtained results, it can be suggested that the mechanism of action of the hepatoprotective effect of the papaya against the hepatic lipid accumulation in NAFLD was the combined result of the association of the anti-lipogenic, anti-inflammatory and antioxidant activities of papaya (Figure 5).

Moreover, the doses of papaya used in this study can be of practical use in human medicine. The results of this study provide experimental-based evidence suggesting papaya is an efficacious nutritional strategy for use in the prevention or treatment of NAFLD. However, future research should be performed using human trials to elucidate the intervention of papaya in clinical and public health implications.

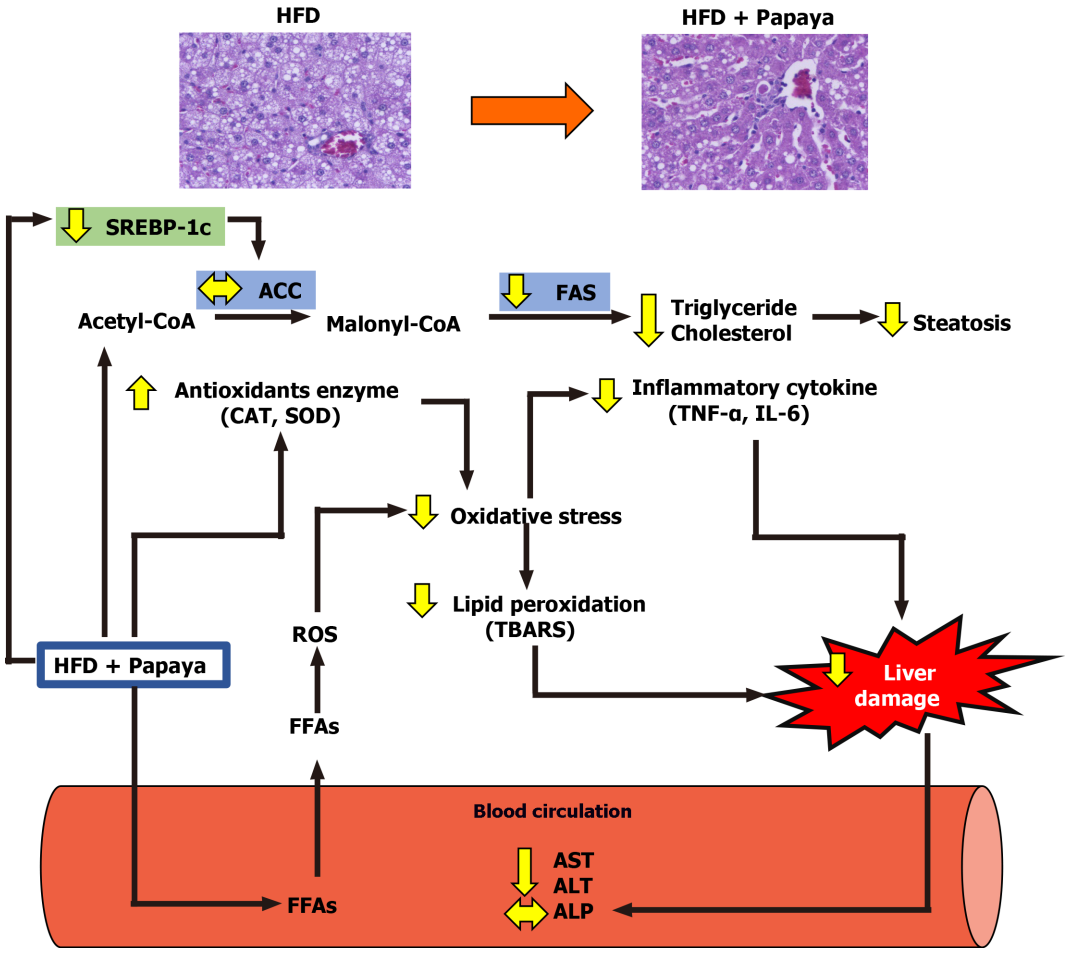


Figure 5 Schematic diagram of possible mechanism of papaya juice on non-alcoholic fatty liver disease. The beneficial effect of papaya against hepatic steatosis in obese rats may occur through the inhibition of lipogenic pathways by reducing *SREBP-1c* and *FAS* gene expression, causing the reduction of hepatic fat accumulation. Papaya can improve enzymatic antioxidants [catalase (CAT) and superoxide dismutase (SOD)] and decrease lipid peroxidation in the liver. The administration of papaya significantly decreased proinflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukin 6 (IL-6) to modulate liver damage. Papaya is therefore able to reduce the activities of aspartate transaminase (AST) and alanine transaminase (ALT) in serum. Overall, this study provides evidence for the beneficial effects of papaya to reverse the progression of non-alcoholic fatty liver disease in obese rats. ALP: Alkaline phosphatase; FFAs: Free fatty acids; HFD: High fat diet; ROS: Reactive oxygen species; TBARS: 2-Thiobarbituric acid reactive substances.

ARTICLE HIGHLIGHTS

Research background

High fat diet consumption causes fat accumulation in liver [nonalcoholic fatty liver disease (NAFLD)], which leads to liver dysfunction due to oxidative stress and inflammation

Research motivation

Papaya is a nutritional, healthy and affordable fruit. It is available in all regions of the world and can be found year-round. Additional scientific evidence on the health and nutritional benefits of papaya are needed to promote health and papaya consumption.

Research objectives

To evaluate papaya's health benefit against NAFLD in obese rats.

Research methods

Rats were fed with a high fat diet for 12 wk to induce obesity. Papaya juice at the implement doses were administered to the rats. Hepatic lipid contents, oxidative stress, inflammatory cytokines, lipogenic genes and liver pathology were assessed.

Research results

The hepatoprotective action of papaya against the accumulation of hepatic fat was a

result of the association of the hypolipidemic effect partially through a suppression of *SREBP-1c* and *FAS*, anti-inflammatory and antioxidant activities.

Research conclusions

The results of this study provide experimental-based evidence that can contribute to the implement of papaya in the prevention and treatment of obesity and associated metabolic disorders.

Research perspectives

Our study offers an optimistic view of an anti-NAFLD effect of papaya; however, further evidence from human clinical studies is necessary.

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ORIGINAL ARTICLE

Basic Study

Promotive action of 2-acetylaminofluorene on hepatic precancerous lesions initiated by diethylnitrosamine in rats: Molecular study

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Author contributions: Hasanin AH and El Gayar N conceived the idea and designed the animal model and statistical analysis; Habib EK performed histochemical examinations; Matboli M conducted bioinformatics analysis, biochemical and molecular assay; all authors drafted the manuscript and critically reviewed the manuscript and approved the final version of the manuscript for publication.

Institutional animal care and use committee statement: All animal procedures were carried out in accordance with the National Institute of Health guide for the care and use of laboratory animals (NIH Publication No. 85-23, revised 1996) and were approved by the Institutional Animal Ethics Committee for Ain Shams University, Faculty of Medicine (approval No. 17585).

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

Data sharing statement: No

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Abstract

BACKGROUND

Diethylnitrosamine (DEN) induces hepatic neoplastic lesions over a prolonged period.

AIM

To investigate the promotive action of 2-acetylaminofluorene (2-AAF) when combined with DEN in order to develop a rat model for induction of pre-cancerous lesion and investigate the molecular mechanism underlying the activity of 2-AAF.

METHODS

The pre-precancerous lesions were initiated by intraperitoneal injection of DEN for three weeks consecutively, followed by one intraperitoneal injection of 2-AAF at three different doses (100, 200 and 300 mg/kg). Rats were separated into naïve, DEN, DEN + 100 mg 2-AAF, DEN + 200 mg 2-AAF, and DEN + 300 mg 2-AAF groups. Rats were sacrificed after 10 wk and 16 wk. Liver functions, level of alpha-fetoprotein, glutathione S-transferase-P and proliferating cell nuclear antigen staining of liver tissues were performed. The mRNA level of RAB11A, BAX, p53, and Cyclin E and epigenetic regulation by long-noncoding RNA (lncRNA) RP11-513I15.6, miR-1262 (microRNA), and miR-1298 were assessed in the sera and liver tissues of the rats.

RESULTS

2-AAF administration significantly increased the percent area of the precancerous

additional data are available.

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foci and cell proliferation along with a significant decrease in RAB11A, BAX, and p53 mRNA, and the increase in Cyclin E mRNA was associated with a marked decrease in lncRNA RP11-513I15.6 expression with a significant increase in both miR-1262 and miR-1298.

CONCLUSION

2-AFF promoted hepatic precancerous lesions initiated through DEN by decreasing autophagy, apoptosis, and tumor suppression genes, along with increased cell proliferation, in a time- and dose-dependent manner. These actions were mediated under the epigenetic regulation of lncRNA RP11-513I15.6/miR-1262/miR-1298.

Key Words: Acetylaminofluorene; Hepatic precancerous lesion; Diethylnitrosamine; Autophagy; Apoptosis; MicroRNA

Core Tip: 2-Acetylaminofluorene epigenetically regulated the expression of long-noncoding RNA RP11-513I15.6/miRNA-1262/miR-1298 (microRNA, miRNA) resulted in decrease in RAB11A, BAX, and p53 mRNA, and the increase in Cyclin E mRNA leading to increased hepatocyte proliferation and decreased apoptosis promoting hepatocellular promoted precancerous lesion in rat models.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the 6th common cancer and the 2nd leading cause of cancer mortality all over the world[1]. Its incidence is elevated, which is attributed to the rising proportion of individuals infected with hepatitis C virus[2]. The molecular pathogenesis of cancer and the underlying tumor biology has been progressing. Spontaneous animal models, induced models, transplantable models, transgenic models, and viral models were used to investigate the biological mechanism of HCC with respect to the liver-targeted key pathways[3]. Rodent has a short life span due to which the cellular transformation is observed from initiation to malignancy, thereby rendering it as a preferred model system[4]. However, modeling a malignant liver disease is challenging due to the urgent need for optimal models for preclinical studies.

Several hepatotoxic agents, such as carcinogen diethylnitrosamine (DEN), have been repeatedly administered to induce general liver disease and HCC over a prolonged period. DEN produces small foci of dysplastic hepatocytes *via* ethylation of various nucleophilic sites in deoxyribonucleic acid[5], resulting in cirrhosis and liver cancer within 18 wk as presented by mutations in β-catenin[6] and p53[7]. HCC induced by DEN activates the H-ras proto-oncogene[8]. Interestingly, variable time intervals, tumor promoters, DEN doses, and application routes were applied by various groups to induce hepatic precancerous lesions in a dose- and time-dependent manner. A two-stage model was established using DEN as a genotoxic compound and phenobarbital to induce HCC[9]. Another two-step HCC model was established according to the Solt-Farber protocol; herein, the initiation by DEN was followed by partial hepatectomy, leading to an elevated number of initiated cells[10].

2-Acetylaminofluorene (2-AAF) serves as a model carcinogen with genotoxic and epigenetic properties[11]. The present study proposed that genotoxic 2-AAF metabolites produce G to T transversion-initiated cells along with cirrhotic alteration due to chronic toxic effect on mitochondrial respiration[12]. Also, electron drainage by 2-AAF causes an uncoupling effect on oxidative phosphorylation[13].

Malik *et al*[14] reported a protocol for HCC induction in the liver without hepatectomy, wherein male Wistar rats were injected with DEN intraperitoneally, and then, 2-AAF repeatedly. This model showed oxidative stress, cell damage, and advanced HCC.

The present study aimed to investigate the development of precancerous lesions by DEN injection intraperitoneally (100 mg/kg body weight), followed by a single intraperitoneal (i.p.) injection of promoter 2-AAF at three different doses (100, 200 and 300 mg/kg) at two intervals of 10 wk and 16 wk, respectively.

MATERIALS AND METHODS

Chemicals

DEN with ≥ 99% purity (CAT number 55-18-5) and 2-AAF with ≥ 98% purity (CAT number 53-96-3) were purchased from (Sigma-Aldrich, St. Louis, United States).

Experimental protocol

A total of 60 adult male Wistar rats (200-250 g) were used. The animals were maintained at 22-24 °C and twelve hours light/dark cycles and received standard rat chow and tap water. All animal experiments were carried out according to the National Institute of Health guide for dealing with laboratory animals (National Research Council (US) Institute for Laboratory Animal Research. No. 85-23, revised 1996). The study was approved by Ain Shams University, Faculty of Medicine Institutional Animal Ethics Committee (approval No. 17585). The animals were acclimatized for 1 wk and weighed before each injection for accurate determination of the drug dosage.

Wister rats were randomly and equally divided into naïve, DEN, DEN + 100 mg 2-AAF, DEN + 200 mg 2-AAF, and DEN + 300 mg 2-AAF groups. The four DEN groups were injected i.p. with 100 mg/kg per week for 3 wk, followed by 1 wk interval. Then, 2-AAF was injected once intraperitoneally at 3 different doses for the 2-AAF three groups (100, 200 and 300 mg/kg). The naïve group was injected with 0.9% NaCl as described above. In each group, half of the animals were sacrificed at the end of week 10 and the remaining at week 16 (Figure 1).

Specimen collection

Rats were anesthetized before withdrawing the retro-orbital blood samples; sera were collected by centrifugation at 1200 × *g* for 10 min. Subsequently, the rats were sacrificed, and liver samples collected. All the samples were maintained at -80 °C for further tests of liver function, and the level of alpha-fetoprotein (AFP) and RNA extraction in the liver samples were examined.

Tissue preparation for histological and immunohistochemical examinations

The liver specimens were collected from all animals in each group, with fixation in 10% neutral formaldehyde for 24 h, followed by dehydration, then embedded in paraffin blocks. Then, 5 μm sections were subjected to hematoxylin-eosin (HE) staining to detect any histopathological changes. Images were captured using an Olympus BX50 Light microscope (Olympus, Japan).

Glutathione S transferase-placental immunohistochemistry

The sections were dewaxed using xylene, followed by hydration using ethanol gradient. The endogenous peroxidase activity was inhibited by hydrogen peroxide. Subsequently, the sections were washed with water and rinsed with phosphate-buffered saline (PBS) before probing with glutathione S-transferase-P (GST-P) primary antibody (1:250; Abcam, cat.# AB106268, San Francesco, CA, United States) at 4 °C overnight. The GST-P-positive area stained brown. The morphometric analysis was carried out using Leica Q win V.3 software after capturing the images using a Leica DM2500 microscope (Leica, Wetzlar, Germany).

Proliferating cell nuclear antigen immunohistochemistry staining

The sections were prepared for proliferating cell nuclear antigen (PCNA) staining (1:400; Santa Cruz Biotechnology, Santa Cruz, CA, United States) for 2 h as described above. Irrespective of the location within the hepatic lobule of the staining intensity, the nuclei were scored as positive or negative. The PCNA labeling indices are represented as the expression of positively stained nuclei (10 fields/slide at × 400).

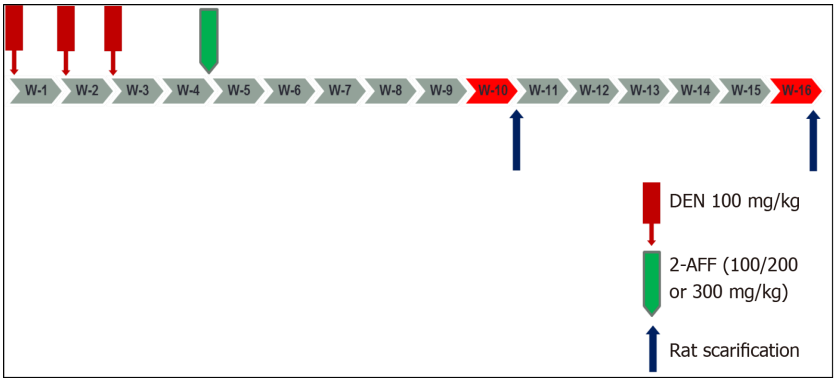


Figure 1 Schematic of the study design. DEN: Diethylnitrosamine; 2-AAF: 2-Acetylaminofluorene.

AFP and liver function

The levels of alanine aminotransferase (ALT), AFP, total bilirubin, and direct bilirubin were analyzed quantitatively using a commercial ELISA kit on sera samples.

Molecular assay

Bioinformatics-based selection of molecular parameters to investigate the oncogenic mechanism of the chemicals used in the HCC model: The molecular biomarker panel was obtained in two steps: (1) A panel of key genes, such as Ras-related in brain11gene (RAB11A), p53, BAX and cell cycle-related gene Cyclin E1 according to Gene Atlas Data Base (https://www.ebi.ac.uk/gxa/home) and protein Atlas Data Base (https://www.proteinatlas.org/) that play a major role in hepatic carcinogenesis, including autophagy, apoptotic genes, and cell cycle; and (2) lncRNA-RP11-513I15.6 was selected using a database of long-noncoding RNA (lncRNA) that act as competitive endogenous RNA (ceRNAs) (http://gyanxet-beta.com/Lncedb/index.php). This lncRNA acts as a master regulator of the target mRNAs by competing with miR (microRNA, miRNA)-1262 and miR-1298 binding with the genes mentioned above. The selected lncRNA and miRNA were based on the specificity to HCC, competing endogenous RNA score, and the number of target sites of mRNA. Finally, the pathway enrichment analysis by Diana database (http://www.microrna.gr/miRPathv2) for both miR-1262 and miR-1298 revealed that these were linked to autophagy, cell cycle regulation, cell adhesion, and other pathways associated to carcinogenesis.

Total RNA extraction

Total RNA was extracted from sera samples by miRNEasy® RNA isolation kit (Qiagen, Düsseldorf, Germany). The RNA integrity and concentration were determined on an Ultraspec 1000 UV/visible spectrophotometer (Amersham Pharmacia Biotech, Cambridge, United Kingdom). The RNA purity was 1.8-2. Subsequently, the total RNA was reverse transcribed into complementary DNA by miScript II RT Kit (Qiagen, Düsseldorf, Germany) on a Hybaid thermal cycler (Thermo Electron, Waltham, MA, United States).

Real-time quantitative Polymerase Chain Reaction of the RNA panel

The expression of mRNA and lncRNA in the rat sera and liver tissues was measured by RT² SYBR Green ROX real-time quantitative polymerase chain reaction (qPCR) Mastermix and Quantitect SYBR Green Mastermix Kit (Qiagen, Düsseldorf, Germany), respectively. The specific primers were provided (Qiagen, Düsseldorf, Germany), using Step One Plus™ System (Applied Biosystems Inc., Foster City, CA, United States). B-actin (accession NM_001101) served as the endogenous control.

The miRNA expression in the sera and liver tissue was investigated according to the protocol of miScript SYBR Green kit Qiagen (Düsseldorf, Germany). *RNU-6* served as the endogenous control. The specific PCR primers were synthesized by Qiagen (Düsseldorf, Germay).

The PCR program was according to the following cycles: Denaturation at 95 °C for 15 min followed by forty cycles of denaturation for 10 s at 94 °C, then annealing for 30 s at 55 °C, and finally extension for 34 s at 70 °C. Each reaction was done in duplicate.

The threshold cycle (Ct) value of each sample was calculated using the StepOnePlus™ software v2.2.2 (Applied Biosystems). *Ct* value > 36 was considered

negative. The specificities of the amplicons were confirmed using the melting curve analysis software of Applied Biosystems. The expression of the target molecules was measured using the $2^{-\Delta\Delta Ct}$ method[15]. The expression of the target gene was normalized against that of the housekeeping gene for the samples and compared to the reference sample.

Statistical analysis

The values are expressed as means ± SD. The statistical differences among all groups were assessed using one-way ANOVA, and Tukey's test. $P < 0.05$ was considered to be statistically significant. The statistical analyses were done using Graphpad Prism, version 5.0. (2007: San Diego, United States).

RESULTS

The naïve groups at weeks 10 and 16 that did not show significant differences were pooled as a single group.

Histological and immunohistochemical examination

The liver sections of the naïve control group stained with HE revealed normal architecture of hepatic lobules, central veins, and portal triads. Neither localized lesion nor alternating pre precancerous foci or dysplastic nodules were observed throughout the experimental period (Figure 2A-D).

The histopathology of the liver sections of different groups with DEN either alone or when combined with 2-AAF showed the development of multistage hepatocellular pre precancerous lesions. An apparent increase in the incidence, number, and size of the lesions was observed as a result of increased dose and duration of the usage of DEN and 2-AAF. The liver specimens of rats sacrificed at week 10 showed small early and well-differentiated foci of cellular alteration after injection of DEN solely (Figure 2E), while varying numbers of multiple aggregations of small nodules were present after administration of both DEN + 2-AAF (Figure 2F-H). The simultaneous occurrence of multiple nodules reflected either the dissemination of hepatocytes with cellular atypia from a single primary lesion to form satellite nodules or the synchronous development of several other independent lesions. The localized lesions of foci of cellular alteration did not compress the surrounding hepatic parenchyma but merged with it imperceptibly. However, lack of or minimal disruption of hepatic lobular architecture was observed.

The histological analysis of these pre- precancerous lesions varied greatly from week 10-16 with respect to different stages of differentiation and growth patterns. The lesions observed by the end of week 16 were large and less differentiated (Figure 2I-P). Multiple dysplastic nodules were scattered, compressing the surrounding liver parenchyma and occupying most of the examined fields. These dysplastic nodules were uniform lesions and discriminated from the surrounding liver tissue based on their morphology, cytoplasmic staining, size of the nucleus, and presence of cellular atypia. The nodular cells did not show sinusoidal spaces and were large with clear cytoplasm.

The immunohistochemically-stained liver sections with the GST-P antibody revealed the presence of multiple GST-P-positive areas in all groups after administration of DEN + 2-AAF. Moreover, small positive areas of cellular foci were noted in the group treated with DEN and sacrificed at week 10 (Figure 3A). Multiple GST-P-positive areas, variable in size, were scattered in-between negatively stained hepatocytes among groups treated with DEN + 2-AFF and sacrificed at week 10 (Figure 3B-D). The number and size of the GST-P-positive areas were markedly increased in groups that received DEN + 2-AFF and sacrificed at week 16, especially those that received high doses showed large positive hyperplastic nodules occupying most of the examined fields (Figure 3E-H). The % surface area of GST-P-positive hepatic lesions was measured among different groups and statistically analyzed (Figure 3I).

The immunohistochemical analysis showed an elevated expression of PCNA in groups that received DEN + 2-AAF as compared to those treated with DEN alone. The higher the dose of 2-AAF combined with DEN and longer the duration, higher the expression rate. Strikingly, significant differences were detected between DEN/2-AAF 200 and 300 as compared to DEN/2-AAF 100 at weeks 10 and 16, respectively (Figure 4 and Table 1).

Table 1 Expression rate of hepatocytes positive for proliferating cell nuclear antigen was calculated as number of positive field expression in 10 fields per rat liver tissue

Group	10 wk duration	16 wk duration
DEN	+	++
DEN + 100 AAF	+	++
DEN + 200 AAF	++	+++
DEN + 300 AAF	++	+++

+: Positive expression found in 1-3 fields; ++: Positive expression found in 4-6 fields; +++: Positive expression found in 7-10 field. DEN: Diethylnitrosamine; AAF: Acetylaminofluorene.

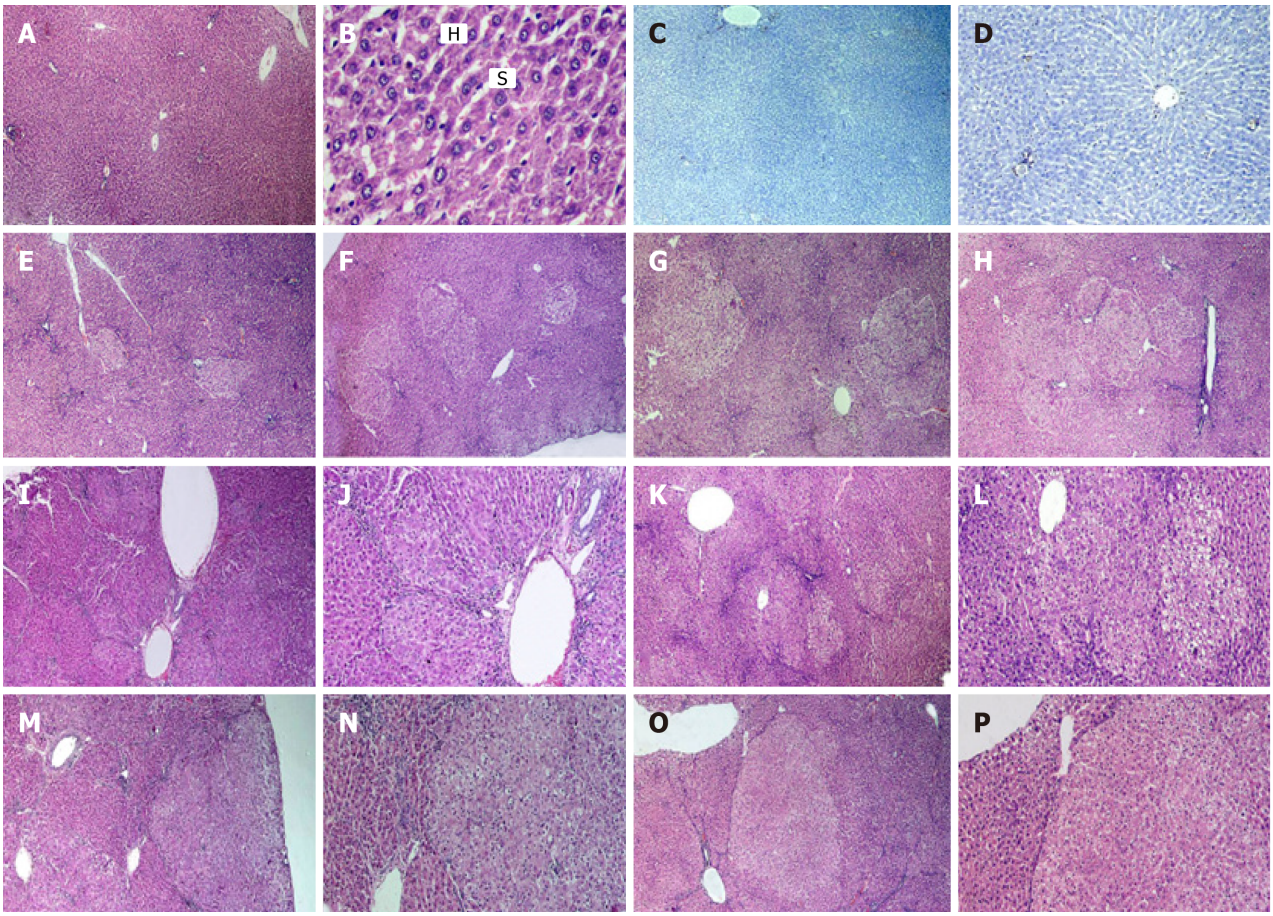


Figure 2 Histological and immunohistochemical examination. A-D: Images of liver sections of naive group. Hematoxylin-eosin (HE) stained sections show normal hepatic architecture, portal triad, central vein and radiating cords of hepatocytes (H) with blood sinusoids (S) present in between (A and B). Immunohistochemically-stained section with anti-glutathione S transferase-P demonstrating negative reaction (C). Immunohistochemically-stained section with proliferating cell nuclear antigen antibodies (D); E-H: HE images of liver sections of rats that received diethylnitrosamine (DEN) and different doses of 2-acetylaminofluorene (2-AAF) and were sacrificed at week 10. Show multiple foci of cellular alteration of different sizes (dotted shapes), not compressing the surrounding hepatic parenchyma. DEN group (E), DEN+ 2-AAF 100 mg group (F), DEN + 2-AAF 200 mg group (G) and DEN + 2-AAF 300 mg group (H); I-P: HE liver sections of rats that received DEN and different doses of 2-AAF sacrificed at week 16, show larger, well discriminated, less differentiated dysplastic nodules compressing the surrounding liver tissue with disruption of hepatic lobular architecture were observed. DEN group (I and J), DEN + 100 mg 2AAF group (K and L), DEN + 200 mg 2AAF group (M and N), DEN + 300 mg 2AAF group (O and P). A, C, E-H × 40; D, J, L, N and P × 100; I, K, M and O × 40, B × 400.

Effect on liver function and AFP

Table 2 showed that by the end of weeks 10 and 16, liver function tests (ALT, albumin, T-bilirubin, D-bilirubin) and AFP had a significant decline after DEN and 2-AAF were administered at three doses as compared to the naïve group. 2-AAF addition to DEN significantly increased the level of AFP as compared to DEN alone with significant differences between 2-AAF doses at the two time points in a dose-dependent manner.

Table 2 Effect of diethylnitrosamine and 2-acetylaminofluorene on alpha-fetoprotein and liver function

	AFP	ALT	Total bilirubin	Direct bilirubin	Albumin
Naïve	22.8 ± 1.13	33.3 ± 6.83	0.30 ± 0.18	0.27 ± 0.14	3.77 ± 0.23
Week 10					
DEN	89.2 ± 28.8[d]	63.0 ± 27.5[d]	1.44 ± 0.45[d]	1.03 ± 0.14[d]	2.49 ± 0.15[d]
DEN + 100 AAF	116 ± 52.1[d]	78.3 ± 17.8[d]	2.07 ± 0.44[d,e]	1.40 ± 0.39[d]	2.83 ± 0.19[d]
DEN + 200 AAF	223 ± 124[b,d]	82.7 ± 12.7[d]	2.73 ± 0.23[a,d,e]	1.67 ± 0.19[a,d,e]	2.73 ± 0.14[d]
DEN + 300 AAF	305 ± 126[d,e]	98.0 ± 10.7[d,e]	3.13 ± 0.36[b,d,e]	2.13 ± 0.61[d,e]	3.15 ± 0.38[c,d,e]
Week 16					
DEN	159 ± 32.2[d]	94.1 ± 6.4[d]	2.13 ± 0.55[d]	1.60 ± 0.39[d]	2.0 ± 0.62[d]
DEN + 100 AAF	290 ± 241[d]	104 ± 31.9[d]	2.23 ± 0.36[d]	2.25 ± 0.63[d]	2.57 ± 0.37[d]
DEN + 200 AAF	815 ± 143[a,d,f]	128 ± 36.9[d]	4.10 ± 0.39[a,d,f]	2.53 ± 0.63[a,d,f]	2.17 ± 0.29[d]
DEN + 300 AAF	1059 ± 360[b,d,f]	210 ± 63.2[b,c,d,f]	4.47 ± 0.99[b,d,f]	3.10 ± 0.39[d,f]	2.13 ± 0.67[b,c,d,f]

Values are mean ± SD; number of animals = 6 rats/each group.
[a]$P < 0.05$ when DEN + 200 acetylaminofluorene (AAF) is compared to the DEN + 100 AAF.
[b]$P < 0.05$ when DEN + 300 AAF is compared to the DEN + 100 AAF.
[c]$P < 0.05$ when DEN + 300 AAF is compared to the DEN + 200 AAF.
[d]$P < 0.05$ compared to the naïve group.
[e]$P < 0.05$ compared to the diethylnitrosamine (DEN) group at week 10 group.
[f]$P < 0.05$ compared to the DEN at week 16 group. One-way ANOVA followed by Tukey's multiple comparison test. DEN: Diethylnitrosamine; 2-AAF: 2-Acetylaminofluorene; AFP: Alpha-fetoprotein; ALT: Alanine aminotransferase.

Effect of DEN/2-AAF on the expression of RAB11A, BAX, p53, Cyclin E mRNA among the rat groups

The fold-changes in the relative quantification (RQ) of RAB11A mRNA in rats' liver tissues and sera showed a significant decrease as compared to naïve rats in all groups at both weeks 10 and 16. Compared to DEN alone, a significant decrease was noted in the RQ of RAB11A mRNA in DEN/2-AAF 200 and 300 in sera and tissues at weeks 10 and 16 as compared to the significant change in DEN/2-AAF 100 in tissue at week 10. Moreover, only a significant decrease was detected in DEN/2-AAF 300 as compared to DEN/2-AAF 100 in serum at week 10 (Figure 5A).

Compared to the naïve group, rats that received DEN solely or when combined to 2-AAF for 10 wk or 16 wk showed a significant decrease in the level of *BAX* mRNA in both liver tissues and sera. 2-AAF addition to DEN significantly decreased the expression of BAX mRNA as compared to DEN alone, except for 2-AAF at a dose of 100mg, in the serum at week 10. Only DEN/2-AAF 300 showed a significant decrease as compared to DEN/2-AAF 100 at week 10 in the liver tissues. The serum BAX mRNA level exhibited insignificant differences among the three DEN/2-AAF groups at both weeks 10 and 16 (Figure 5B).

Furthermore, compared to the naïve group, all groups that received DEN alone or combined with 2-AAF, a significant decrease was detected in the rat liver tissue and sera p53 mRNA. All 2-AAF groups showed a significant decrease over DEN alone except for 2-AAF 100 in the liver tissues at week 10. However, insignificant differences were noted among the three groups DEN/2-AAF 100, 200 and 300 at both weeks 10 and 16 in both liver tissues and sera (Figure 5C).

The Cyclin E mRNA in the rat liver tissues showed a significant increase between DEN/2-AAF 200 and 300 as compared to DEN/2-AAF 100 at week 10. In addition, a significant increase was noted between DEN/2-AAF 300 and DEN/2-AAF100, 200. Furthermore, rats that received DEN either alone or combined with 2-AAF showed a significant increase in the serum Cyclin E mRNA level as compared to the naïve group. All rats that received 2-AAF exhibited a significant increase in Cyclin E mRNA over DEN alone, except for 2-AAF 100, in the rat sera at week 10. Also, a significant increase was observed in DEN/2-AAF 200 and 300 over DEN/2-AAF 100 in the liver tissues at week 10. In addition, a significant increase occurred in 2-AAF 300 over 2-AAF 100 and 200 in the tissues at week 16. A significant increase was noted in 2-AAF 300 over both 2-AAF 100 and 200 at week 10 and in 2-AAF 300 over 2-AAF 100 at week 16 in rat sera (Figure 5D).

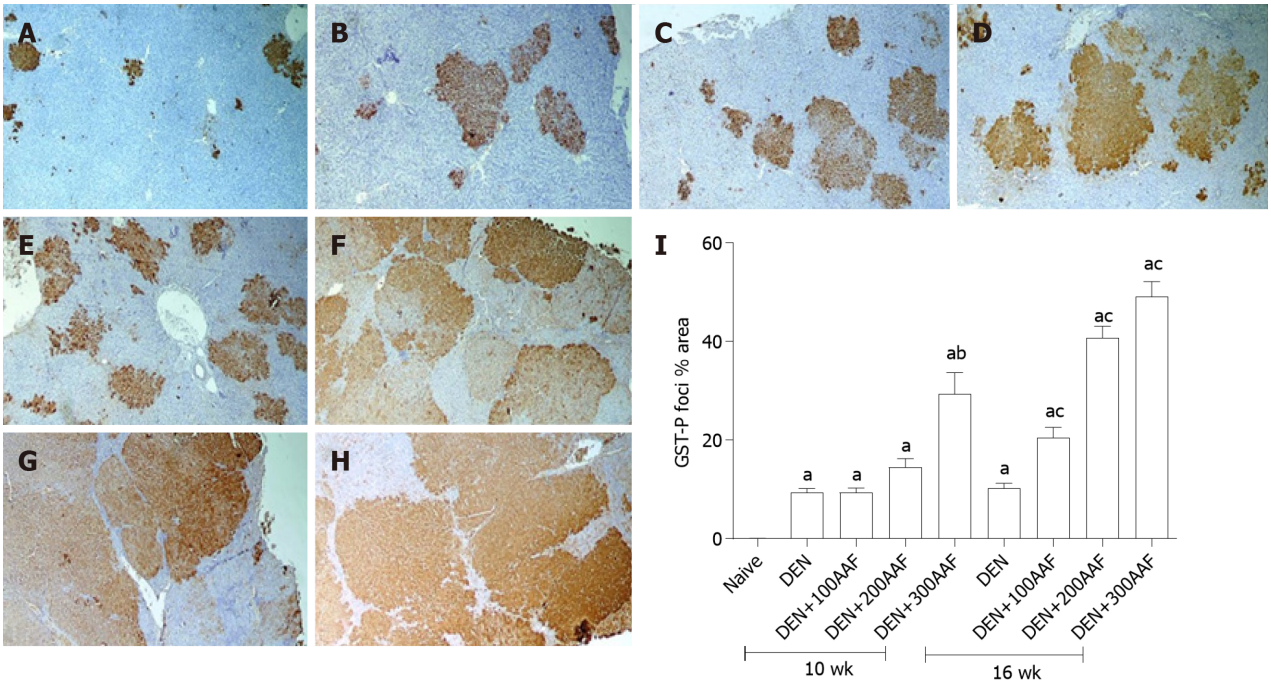


Figure 3 Histological and immunohistochemical examination. A-H: Images of rats' liver sections immunohistochemically-stained with glutathione S transferase-P (GST-P) antibody, show multiple GST-P-positive hepatic foci and nodules (brown stained collection of cells) of different sizes scatter in-between negatively stained hepatic parenchyma. Rats sacrificed at week 10 (A-D), rats sacrificed at week 16 (E-H) [A and E: diethylnitrosamine (DEN) group; B and F: DEN + 100mg 2-acetylaminofluorene (2-AAF) group; C and G: DEN + 200 mg 2-AAF group; D and H: DEN + 300 mg 2-AAF (× 40)]; I: shows the effect of DEN and 2-AAF at different doses on GSTP foci % area in the liver. Values are mean ± SE; number of animals = 6 rats/each group. $^{a}P < 0.05$ compared to naïve group; $^{b}P < 0.05$ compared to DEN group at week 10, $^{c}P < 0.05$ compared to DEN at week 16 group. One-way ANOVA followed by Tukey's multiple comparison test. DEN: Diethylnitrosamine; 2-AAF: 2-Acetylaminofluorene; GST-P: Glutathione S transferase-P.

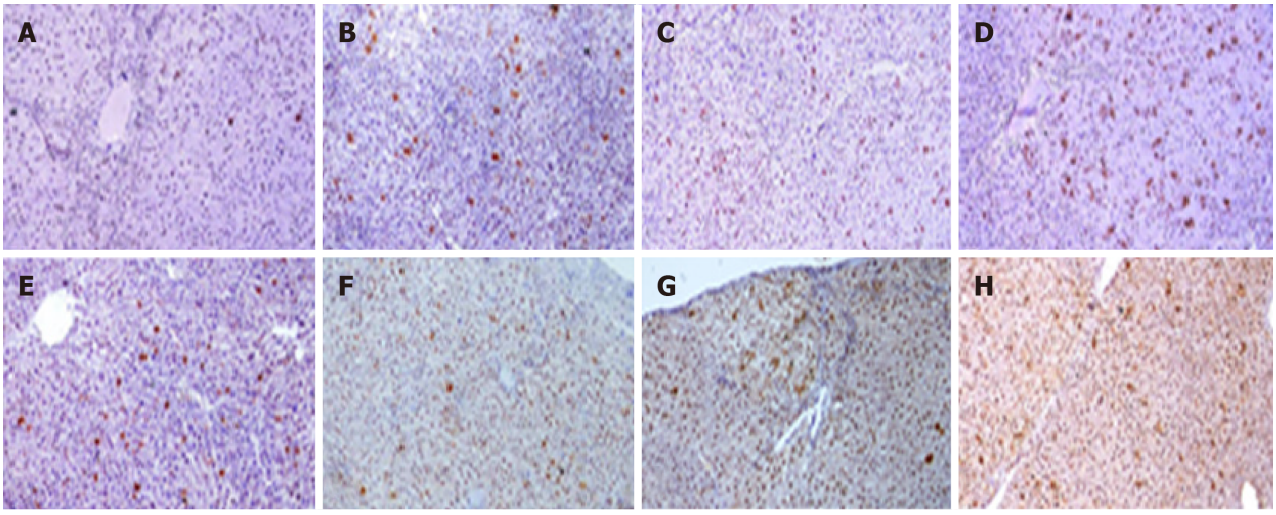
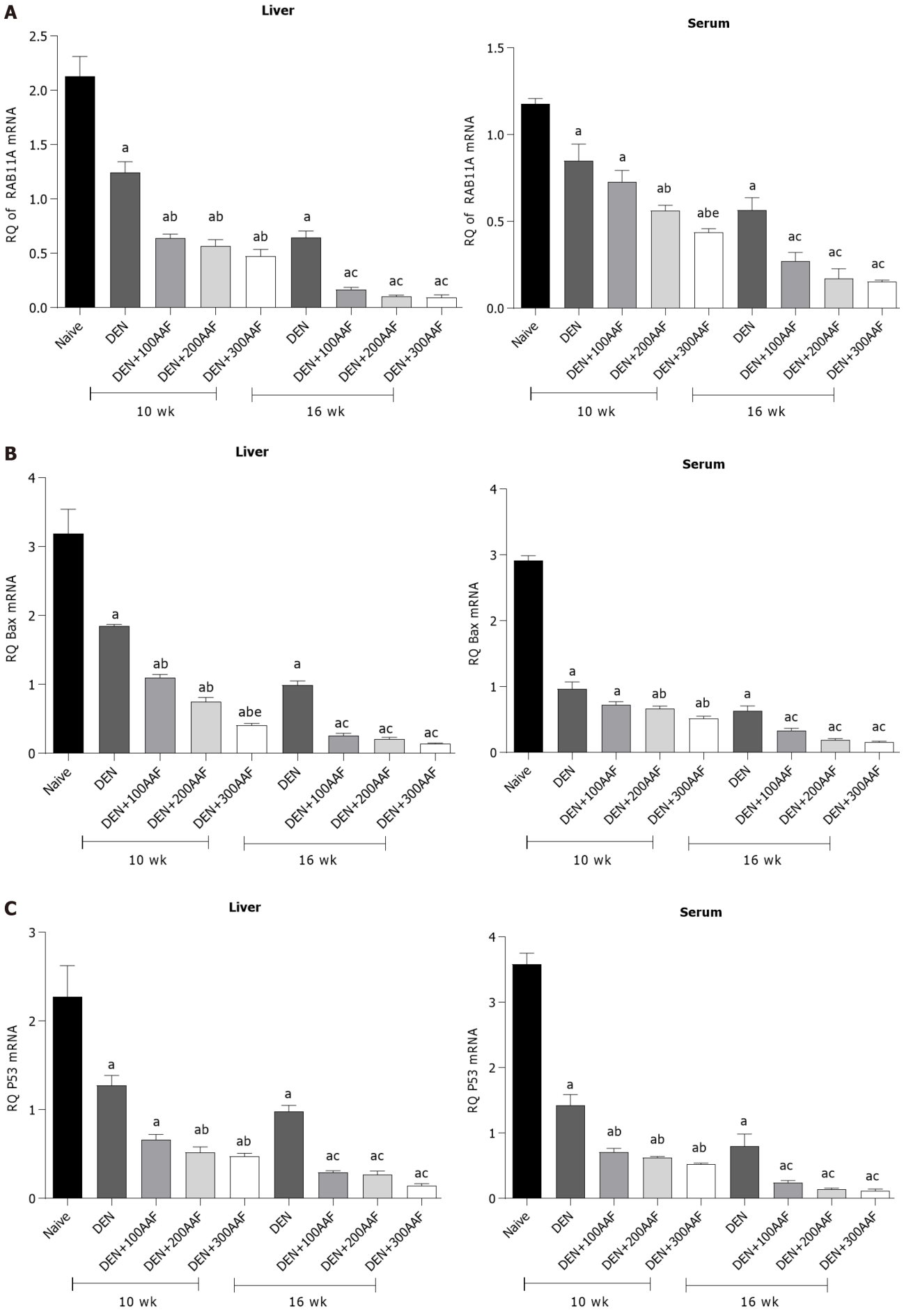


Figure 4 Images of rats' liver sections stained immunohistochemically with proliferating cell nuclear antigen. Positive immune-reactive nucleus (brown dots) scatter in-between negatively stained liver tissue of rats who received diethylnitrosamine (DEN) and different doses of 2-acetylaminofluorene (2-AAF). A-D: Rats sacrificed at week 10; E-H: Rats sacrificed at week 16 (A and E: DEN group; B and F: DEN + 100 mg 2AAF group; C and G: DEN + 200 mg 2AAF group; D and H: DEN + 300 mg 2AAF; magnification × 100).

Finally, 2-AAF administration resulted in a significant increase in the level of Cyclin E mRNA with a concomitant decrease in RAB11A, p53, and BAX mRNA expression in the liver tissues and sera as compared to DEN alone. Also, significant differences were reported for 2-AAF 300 as compared to the other 2 doses, especially in the level of Cyclin E mRNA.


A
Liver
RQ of RAB11A mRNA
2.5
2.0
1.5
1.0
0.5
0.0
a
ab
ab
ab
a
ac
ac
ac
Naive
DEN
DEN+100AAF
DEN+200AAF
DEN+300AAF
DEN
DEN+100AAF
DEN+200AAF
DEN+300AAF
10 wk
16 wk
Serum
RQ of RAB11A mRNA
1.5
1.0
0.5
0.0
a
a
ab
abe
a
ac
ac
ac
Naive
DEN
DEN+100AAF
DEN+200AAF
DEN+300AAF
DEN
DEN+100AAF
DEN+200AAF
DEN+300AAF
10 wk
16 wk
B
Liver
RQ Bax mRNA
4
3
2
1
0
a
ab
ab
abe
a
ac
ac
ac
Naive
DEN
DEN+100AAF
DEN+200AAF
DEN+300AAF
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10 wk
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Serum
RQ Bax mRNA
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10 wk
16 wk

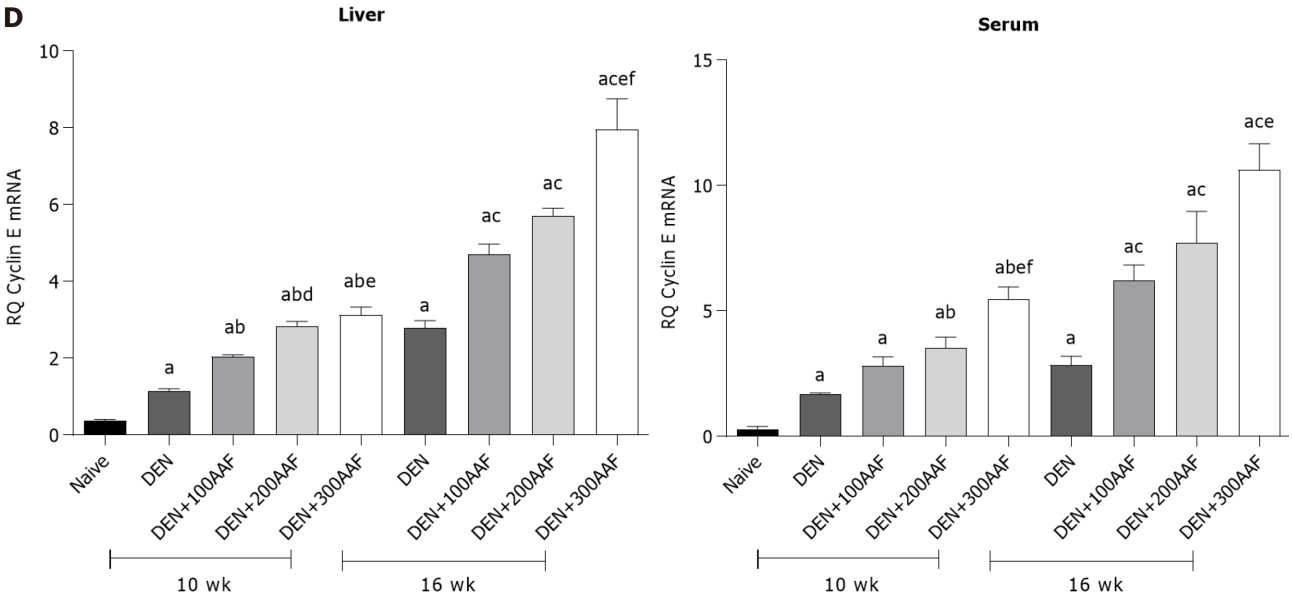

Figure 5 Effect of diethylnitrosamine and 2-acetylaminofluorene at different doses. A: Relative quantification (RQ) of RAB11A mRNA; B: RQ of BAX mRNA; C: RQ of p53 mRNA; D: RQ of Cyclin E mRNA in the liver and serum in rats. Values are mean ± SE; number of animals = 6 rats/each group. [a]*P* < 0.05 compared to the naïve group; [b]*P* < 0.05 compared to the diethylnitrosamine (DEN) group at week 10 group; [c]*P* < 0.05 compared to the DEN at week 16 group; [d]*P* < 0.05 when DEN 200 + acetylaminofluorene (AAF) is compared to the DEN 100 + AAF; [e]*P* < 0.05 when DEN 300 + AAF is compared to the DEN 100 + AAF; [f]*P* < 0.05 when DEN 300 + AAF is compared to the DEN 200 + AAF. One-way ANOVA followed by Tukey's multiple comparison test. DEN: Diethylnitrosamine; 2-AAF: 2-Acetylaminofluorene.

Effect of DEN/2-AAF on the expression of lncRNA-RP11-513I15.6, miR-1262, and miR-1298 among the rat groups

The levels of lncRNA-RP11-513I15.6, miRNA-1262, and miR-1298 were assessed in the liver tissues and sera of all groups at the end of weeks 10 and 16. One-way ANOVA and Tukey's multiple comparison test showed significant differential expression in RQ among the studied groups.

Compared to the naïve group, the RQ of lncRNA-RP11-513I15.6 in rat liver tissues and sera in DEN and DEN/2-AAF groups showed a significant decrease at both weeks 10 and 16. A significant decrease was noted in the 2-AAF groups as compared to DEN alone, except for 2-AAF 100 mg, in the liver in week 10 and 2-AAF 100 mg in the sera at week 16. At week 10, a significant difference was observed between DEN/2-AAF 200 and DEN/2-AAF 300 than DEN/2-AAF100 mg in liver tissues. At week 16, a significant difference was noted in DEN/2-AAF 300 over DEN/2-AAF 100 mg in liver tissues, while the differences between the three groups either on week 10 or 16 were insignificant (Table 3).

miR-1262 exhibited a significant increase in the rats who received either DEN alone or in combination with 2-AFF as compared to the naïve group. Compared to DEN alone, all 2-AAF groups showed a significant increase except for 2-AAF 100 at week 10 in both liver tissues and sera. At week 10, 2-AAF 300 mg showed a significant difference over 2-AAF 100 mg and 200 mg in liver tissues. Moreover, at week 16, a considerable difference was observed between 2-AAF 200 mg and 300 mg over DEN/2-AAF 100 mg. At the serum level, significant differences were detected in DEN/2-AAF 300 mg over DEN/2-AAF 100 mg in both weeks 10 or 16 (Table 3).

Compared to the naïve group, all groups that received DEN or DEN in combination with 2-AAF showed a remarkable increase in the level of miR-1298. Compared to DEN alone, all groups that received 2-AFF showed a significant increase in the level of miR-1298, except for 2-AFF 100 mg, at week 10 in both liver tissues and sera. At week 10 in liver tissues, DEN/2-AAF 200 mg and 300 mg showed a significant increase over DEN/2-AAF 100 mg, while at week 16, a significant difference was detected in DEN/2-AAF 300 mg over both DEN/2-AAF 100 mg and 200 mg. At week 10, significant differences were noted in DEN/2-AAF 300 over DEN/2-AAF 100 mg and 200 mg in the sera, and at week 16, a significant increase was observed in DEN/2-AAF 300 mg over DEN/2-AAF 100 mg (Table 3).

Finally, 2-AAF administration exhibited a significant increase in miR-1298 and miR-1262 with a concomitant decrease in lncRNA-RP11-513I15.6 expression in the liver tissues and sera over DEN alone; also, significant differences were observed in 2-AFF

Table 3 Effect of diethylnitrosamine and 2-acetylaminofluorene on relative quantification of lncRNA-RP11-513I15.6 (long-noncoding RNA), relative quantification of miR-1262 and relative quantification of miR-1298 (microRNA)

	RQ of lncRNA-RP11-513I15.6		RQ of miR-1262		RQ of miR-1298	
	Liver	Serum	Liver	Serum	Liver	Serum
Naïve	2.33 ± 0.31	1.86 ± 0.41	0.38 ± 0.09	0.26 ± 0.34	0.77 ± 0.26	0.1 ± 0.04
Week 10						
DEN	1.31 ± 0.36[d]	0.99 ± 0.15[d]	1.63 ± 0.28[d]	1.40 ± 0.13[d]	1.55 ± 0.37[d]	1.08 ± 0.11[d]
DEN + 100 AAF	1.03 ± 0.2[d]	0.63 ± 0.13[d,e]	2.26 ± 0.54[d]	2.23 ± 0.19[d]	1.85 ± 0.12[d]	1.48 ± 0.56[d]
DEN + 200 AAF	0.58 ± 0.12[a,d,e]	0.44 ± 0.05[d,e]	2.52 ± 0.44[d,e]	2.81 ± 0.49[d,e]	2.46 ± 0.37[d,e]	1.82 ± 0.47[b,c,d,e]
DEN + 300 AAF	0.47 ± 0.037[b,d,e]	0.39 ± 0.005[d,e]	3.9 ± 0.36[d,e]	3.59 ± 1.10[b,d,e]	2.88 ± 0.11[d,e]	3.30 ± 0.18[d,e]
Week 16						
DEN	0.76 ± 0.1[d]	0.52 ± 0.13[d]	3.12 ± 0.62[d]	2.15 ± 0.08[d]	2.52 ± 0.56[d]	2.23 ± 0.26[d]
DEN + 100 AAF	0.46 ± 0.04[d,f]	0.22 ± 0.04[d]	4.48 ± 0.63[d,f]	4.08 ± 0.32[d]	3.92 ± 0.61[d,f]	4.56 ± 0.61[d,f]
DEN + 200 AAF	0.26 ± 0.07[d,f]	0.14 ± 0.07[d,f]	5.71 ± 0.76[a,d,f]	7.38 ± 2.24[d,f]	4.58 ± 0.56[d,f]	5.78 ± 1.72[d,f]
DEN + 300 AAF	0.14 ± 0.04[b,d,f]	0.12 ± 0.06[d,f]	6.45 ± 1.04[b,d,f]	9.78 ± 4.32[b,d,f]	5.89 ± 1.27[d,f]	7.38 ± 2.05[b,d,f]

Values are mean ± SD; number of animals = 6 rats/each group.
[a]$P < 0.05$ when DEN + 200 acetylaminofluorene (AAF) is compared to the DEN + 100 AAF.
[b]$P < 0.05$ when DEN + 300 AAF is compared to the DEN + 100 AAF.
[c]$P < 0.05$ when DEN + 300 AAF is compared to the DEN + 200 AAF.
[d]$P < 0.05$ compared to the naïve group.
[e]$P < 0.05$ compared to the diethylnitrosamine (DEN) group at week 10 group.
[f]$P < 0.05$ compared to the DEN at week 16 group. One-way ANOVA followed by Tukey's multiple comparison test. DEN: Diethylnitrosamine; 2-AAF: 2-Acetylaminofluorene; RQ: Relative quantification; lncRNA: Long-noncoding RNA; miR: MicroRNA.

300 mg over the other two doses.

DISCUSSION

The nodules and cancer progression has been analyzed using animal models of carcinogenesis[16]. The present study aimed to develop a model of chemically-induced pre precancerous nodules in rat liver using DEN + 2-AAF and explore the putative molecular mechanism at the genetic and epigenetic levels. The conformation of premalignant epithelial tissues was disrupted by pre- and neoplastic liver nodules in experimental animals before the onset of cancer[17]. DEN is used to induce precancerous and cancerous lesions. It is metabolically activated by the liver cytochrome cytochrome P450 (CYP450) system, followed by induced DNA damage and oxidative stress in hepatocytes during cancer initiation[18]. The drawback of this model is the duration required for appropriate tumor development[19]. The initiated cells can be stimulated to proliferate and form hepatocyte foci and nodules by the administration of promotor agent, such as 2-AAF that causes toxicity, cell death, and carcinogenesis[20]. Carcinogens exert their carcinogenicity through either epigenetic effects without direct interaction with DNA or genotoxic effects[21].

GST-P immunohistochemistry served as an optimal marker of hepatic pre precancerous in rats[22]. In addition, PCNA is an essential cell cycle regulator; its expression serves as a tool for studying cell proliferation and identifying the replicating cells[23]. The nuclei of hepatocytes with positive PCNA immunostaining indicate hepatic regeneration. Also, a large number of cells circulating in GST-P-positive areas were observed. Furthermore, liver regeneration induced by massive hepatic necrosis was associated with the proliferation of hepatocytes.

Accumulating evidence suggested that oncogenic transformation is associated with resistance or impeded apoptotic pathway. The cancer therapy targets such autophagic imbalance[24]. RAB proteins are members of the Ras superfamily consisting of small monomeric GTPases that regulate the intracellular trafficking of several cell types. RAB11 GTPases are involved in the recycling of endosomes as well as controlling trafficking and autophagy process[25]. Previous studies demonstrated a significant role

of RAB11A in pancreatic cancer[26] and non-small cell lung cancer[27].

A majority of the tumors present defects in the cell cycle, especially the loss of tumor suppressor p53, which prevents cell proliferation in response to DNA damage or dysregulation of oncogenes, inducing apoptosis or cellular senescence. p53 heterozygous mutant is susceptible to the occurrence of HCC[28,29]. Cyclin was overexpressed in many human cancers, including ovarian and breast cancers. AKT acts as a cytoplasmic central regulator of cell cycle signaling (Cyclin D1 and E) and cell survival (Mdm^2/p53)[30,31]. Cyclin E1 is a regulatory subunit of Cyclin-dependent kinase 2 (CDK2). Cyclin E1 is upregulated in human HCCs and associated with poor prognosis[32,33]. Notably, the dysfunction of apoptosis with dysregulation of BCL-2 and BAX has been reported in many cancers, including bladder cancer[34]. BAX is a central regulator of cell death, leading to mitochondrial dysfunction. Also, it is one of the proapoptotic Bcl-2 family proteins that regulate apoptosis in normal and cancer cells[35].

Interestingly, previous studies reported the role of tumor suppressor miR-1262 in cancers. The expression of miR-1262 was dysregulated in the lung[36] and colon cancers[37]. On the other hand, hsa-miR-1298 is a microRNA gene, correlated to undefined RNA class and localized on the X chromosome (Xq23), (114715233-114715344 bp), 112 bases in length. Calvisi *et al*[6] demonstrated the secretion of circulating miR-21, miR-221a, miR-519d and miR-1228 in HCC patients. The high mobility group "A" family consisted of lncRNA RP11-513I15.6, which encoded the small nuclear proteins. Moreover, it play a significant role as an oncogene and is frequently overexpressed in different malignancies, such as HCC out[38], breast cancer[39], and ovarian cancer[40].

CONCLUSION

Administration of DEN to rats produced changes in hepatocytes with increased GST-P and PCNA expression and development of precancerous hepatic foci. The transformed cells proliferated when challenged with another carcinogen (2-AAF) as a promoter. These changes increased with the elevated dose of 2-AFF and duration of the experiment. DEN and 2-AAF affected the mRNA-biomarkers, including RAB11A, BAX, p53, and Cyclin E. Thus, the oncogenic properties of DEN and 2-AAF were observed in induced HCC model, which might be attributed to the suppression of p53, autophagy, and apoptosis along with the activation of the cell cycle. Moreover, it significantly increased the level of miR-1262 and miR-1298 with a concomitant decrease in the expression of lncRNA-RP11-513I15.6. This phenomenon led to the hypothesis that lncRNA-RP11-513I15.6 is a part of competing endogenous RNA, decreasing the level of miR-1262 and miR-1298, which, in turn, regulates the selected target mRNAs.

ARTICLE HIGHLIGHTS

Research background

2-Acetylaminofluorene (2-AAF) dose dependently promoted hepatic precancerous lesion. Over diethylnitrosamine (DEN), 2-AAF decreased autophagy. Over DEN, 2-AAF decreased apoptosis and tumor suppression gene. Over DEN, 2-AAF increased hepatic cell proliferation. 2-AAF epigenetically regulated long-noncoding RNA (lncRNA) RP11-513I15.6/miRNA-1262/miR-1298 (microRNA = miRNA = miR).

Research motivation

Urgent need for hepatocellular carcinoma (HCC) rat model for preclinical trials.

Research objectives

The present study aimed to develop a model of chemically-induced pre precancerous nodules in rat liver using DEN + 2-AAF and explore the putative molecular mechanism at the genetic and epigenetic levels.

Research methods

Bioinformatics-based selection of molecular parameters to investigate the oncogenic mechanism of the chemicals used in the HCC model followed by induction of animal

model by. intraperitoneal injection of DEN for three weeks consecutively, followed by one intraperitoneal injection of 2-AAF at three different doses (100, 200 and 300 mg/kg. Rats were sacrificed after 10 wk and 16 wk. Liver functions, level of alpha-fetoprotein, glutathione S-transferase-P and proliferating cell nuclear antigen staining of liver tissues were performed. The mRNA level of RAB11A, BAX, p53, and Cyclin E and epigenetic regulation by lncRNA RP11-513I15.6, miR-1262, and miR-1298 were assessed in the sera and liver tissues of the rats.

Research results

2-AAF administration significantly increased the percent area of the precancerous foci and cell proliferation along with a significant decrease in RAB11A, BAX, and p53 mRNA, and the increase in Cyclin E mRNA was associated with a marked decrease in lncRNA RP11-513I15.6 expression with a significant increase in both miR-1262 and miR-1298.

Research conclusions

Administration of DEN to rats produced changes in hepatocytes with increased glutathione S-transferase-P and proliferating cell nuclear antigen expression and development of precancerous hepatic foci. The transformed cells proliferated when challenged with another carcinogen (2-AAF) as a promoter. These changes increased with the elevated dose of 2-AFF and duration of the experiment. DEN and 2-AAF affected the mRNA-biomarkers, including RAB11A, BAX, p53, and Cyclin E. Thus, the oncogenic properties of DEN and 2-AAF were observed in induced HCC model, which might be attributed to the suppression of p53, autophagy, and apoptosis along with the activation of the cell cycle. Moreover, it significantly increased the level of miR-1262 and miR-1298 with a concomitant decrease in the expression of lncRNA-RP11-513I15.6. This phenomenon led to the hypothesis that lncRNA-RP11-513I15.6 is a part of competing endogenous RNA, decreasing the level of miR-1262 and miR-1298, which, in turn, regulates the selected target mRNAs.

Research perspectives

More *in vitro* functional studies are urgently need to explore the competing endogenur role of lncRNA in HCC pathogenesis.

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ORIGINAL ARTICLE

Basic Study

BIR repeat-containing ubiquitin conjugating enzyme (BRUCE) regulation of β-catenin signaling in the progression of drug-induced hepatic fibrosis and carcinogenesis

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Abstract

BACKGROUND

BIR repeat-containing ubiquitin conjugating enzyme (BRUCE) is a liver tumor suppressor, which is downregulated in a large number of patients with liver diseases. BRUCE facilitates DNA damage repair to protect the mouse liver against the hepatocarcinogen diethylnitrosamine (DEN)-dependent acute liver injury and carcinogenesis. While there exists an established pathologic connection between fibrosis and hepatocellular carcinoma (HCC), DEN exposure alone does not induce robust hepatic fibrosis. Further studies are warranted to identify new suppressive mechanisms contributing to DEN-induced fibrosis and HCC.

AIM

To investigate the suppressive mechanisms of BRUCE in hepatic fibrosis and HCC development.

METHODS

mentorship to CLV; Du CY performed critical revision of the manuscript, obtained funding, study supervision and manuscript revisions.

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Male C57/BL6/J control mice [loxp/Loxp; albumin-cre (Alb-cre)$^-$] and BRUCE Alb-Cre KO mice (loxp/Loxp; Alb-Cre$^+$) were injected with a single dose of DEN at postnatal day 15 and sacrificed at different time points to examine liver disease progression.

RESULTS

By using a liver-specific BRUCE knockout (LKO) mouse model, we found that BRUCE deficiency, in conjunction with DEN exposure, induced hepatic fibrosis in both premalignant as well as malignant stages, thus recapitulating the chronic fibrosis background often observed in HCC patients. Activated in fibrosis and HCC, β-catenin activity depends on its stabilization and subsequent translocation to the nucleus. Interestingly, we observed that livers from BRUCE KO mice demonstrated an increased nuclear accumulation and elevated activity of β-catenin in the three stages of carcinogenesis: Pre-malignancy, tumor initiation, and HCC. This suggests that BRUCE negatively regulates β-catenin activity during liver disease progression. β-catenin can be activated by phosphorylation by protein kinases, such as protein kinase A (PKA), which phosphorylates it at Ser-675 (pSer-675-β-catenin). Mechanistically, BRUCE and PKA were colocalized in the cytoplasm of hepatocytes where PKA activity is maintained at the basal level. However, in BRUCE deficient mouse livers or a human liver cancer cell line, both PKA activity and pSer-675-β-catenin levels were observed to be elevated.

CONCLUSION

Our data support a "BRUCE-PKA-β-catenin" signaling axis in the mouse liver. The BRUCE interaction with PKA in hepatocytes suppresses PKA-dependent phosphorylation and activation of β-catenin. This study implicates BRUCE as a novel negative regulator of both PKA and β-catenin in chronic liver disease progression. Furthermore, BRUCE-liver specific KO mice serve as a promising model for understanding hepatic fibrosis and HCC in patients with aberrant activation of PKA and β-catenin.

Key Words: BIR repeat-containing ubiquitin conjugating enzyme; Diethylnitrosamine; Mouse model; Liver fibrosis; Liver cancer; Hepatocellular carcinoma

Core Tip: Upon diethylnitrosamine (DEN) exposure, BIR repeat-containing ubiquitin conjugating enzyme (BRUCE) liver-deficiency accelerates chronic liver diseases such as fibrosis and hepatocellular carcinoma (HCC) in mice. Our previous study established the role of BRUCE in the protection of the liver against DEN-induced liver injury and subsequent disease progression. Here we report a chronic fibrosis background induced by hepatic BRUCE knockout in mice that recapitulates the fibrosis background in HCC patients. We also report a BRUCE-dependent suppression of β-catenin activity through the suppression of protein kinase A (PKA) activity. This study provides a therapeutic potential involving the inhibition of PKA and β-catenin activities in patients with liver disease that carry BRUCE inactivating mutations.

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INTRODUCTION

The liver is constantly exposed to a variety of viral and bacterial products, environmental toxins, as well as alcohol intake and food antigens. The liver can

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rapidly detect damaging agents and protects itself against the damage without generating widespread inflammation and fibrosis, which are leading causes for liver cancers[1]. Diethylnitrosamine (DEN) is one of the most potent hepatocarcinogens that induces carcinogenic liver injury and development of hepatocellular carcinoma (HCC) in rodents. The application of DEN in rodents has become an attractive experimental model to study the pathogenetic alterations underlying hepato-genotoxic injury and the formation of HCC[2,3]. HCC represents the primary form of liver malignancy and the fourth most common cause of cancer-related deaths worldwide[4]. It has been well documented that the time and incidence of HCCs initiated by DEN differ greatly among mouse strains. DEN-induced HCC development is delayed in the tumor-resistant C57/BL6/J strain as compared to the more sensitive C3H/HE strain[5]. In addition, to induce robust hepatic fibrosis, a hallmark of human HCC development[6,7], administration of a single agent of DEN is insufficient in the C57/BL6/J strain, but when coupled with additional chemicals such as carbon tetrachloride, a fibrogenic agent, hepatic fibrosis is accelerated[8].

BIR repeat-containing ubiquitin-conjugating enzyme (BRUCE) is a hybrid ubiquitin conjugase and ligase[9]. BRUCE has two major pro-survival functions *in vitro*: Promotion of DNA damage repair and suppression of apoptosis. In the cell nucleus, BRUCE promotes DNA damage repair by homologous recombination (HR) to preserve genomic stability[10-12]. To achieve this function, BRUCE is recruited to damaged chromatin adjacent to DNA breaks, where it facilitates chromatin relaxation and accessibility, allowing for HR factors to be loaded onto DNA breaks to facilitate HR repair[11,12]. In the cytoplasm, BRUCE acts as a member of the inhibitor of apoptosis protein (IAP) family of proteins[13-15]. It inhibits the intrinsic mitochondrial pathway of apoptosis by post-translational ubiquitination of pro-apoptotic proteins to promote their degradation by the ubiquitin-proteasome system (UPS)[13,15-18]. In addition to these *in vitro* functions, we and others have demonstrated an *in vivo* anti-apoptosis function of BRUCE in mice, where it suppresses the mitochondrial pathway of apoptosis[16,17]. Furthermore, we have reported a DNA repair function of BRUCE in the protection of the mouse liver[3]. Utilizing an albumin-cre (Alb-cre) mediated liver-specific BRUCE knockout (LKO) mouse model, we have demonstrated for the first time that the BRUCE-ATR (Ataxia Telangiectasia and Rad3-related) signaling axis protects against DEN-induced liver injury and DEN-initiated HCC. BRUCE LKO mice had increased hepatocellular DNA damage accumulation induced by DEN, downregulated ATR-mediated DNA damage response, and an exacerbated HCC development with a fibrotic background[3]. However, the mechanisms underlying the liver fibrosis and HCC have not yet been characterized in this model.

Liver fibrosis is characterized by an excessive accumulation of the extracellular matrix (ECM) resulting in scar tissue formation[19]. Hepatocyte damage and death is an initial consequence of liver injury that initiates several events leading to the recruitment of inflammatory cells and the activation of hepatic stellate cells (HSCs)[20]. Upon liver damage, apoptotic hepatocytes release damage associated molecular patterns (DAMPs) and proinflammatory factors to activate neighboring Kupffer cells and HSCs, thereby inducing persistent inflammatory responses and fibrosis, respectively[19-21]. Activated HSCs are the principal source for deposition of ECM as activated HSCs are responsible for producing an excessive amount of ECM components, mainly collagens[20]. Although liver fibrosis occurs as a wound healing response to chronic toxin-mediated liver injury, chronic liver fibrosis can eventually lead to cirrhosis and HCC[2,22,23]. It is highly likely that the prognosis of liver fibrosis and HCC depend on genetic variations among multiple genes and the interactions of these genes with environmental factors and each other[24].

β-catenin is expressed throughout the adult liver. It is well documented that Wnt/β-catenin signaling regulates liver homeostasis, injury and tumorigenesis[25]. The nuclear expression and accumulation of β-catenin is an indication of its activation. As a leading contributor to chronic liver disease progression, aberrant β-catenin activation is detected during the early stages of chronic inflammation, fibrosis, steatosis, steatohepatitis, and hepatoblastoma as well as late stage of HCC. Aberrant activation of the Wnt/β-catenin signaling pathway (overexpression, mutations, increased nuclear expression of β-catenin) is found in up to 50% of human HCCs and correlated with tumor progression and poor prognosis[26,27].

While a hepatic fibrosis background is a hallmark of human HCC, the regulators of this pathology remain largely unclear. The HCC developed in our BRUCE LKO mouse model is associated with fibrosis[3], suggesting that BRUCE is a regulator of this pathology. Therefore, the BRUCE liver-KO mouse model allows us to examine how BRUCE regulates fibrosis and HCC. In this study, we used our mouse model to examine the pro-fibrotic and pro-tumorigenic signaling pathways. Furthermore, we

also investigated the stages of chronic liver disease to determine the point of fibrosis induction, as well as tumor initiation in the BRUCE liver-KO mice.

MATERIALS AND METHODS

Generation of genetically modified conditional LKO mice

The BRUCE Alb-Cre KO mice (C57/BL6 mice) were previously described[3]. Genotypes were confirmed by PCR and ablation of BRUCE protein expression in mouse liver tissues confirmed by Western blot.

DEN induction

To initiate chronic liver disease pathogenesis, DEN (Sigma, #N0756) was delivered intraperitoneally (i.p.) into control and BRUCE liver KO mice of 14-day old male mice at 25 mg/kg of body weight. Control and KO mice were sacrificed at the following time points: 3-, 6-, 8-, and 14-mo post exposure to DEN and livers were collected for further studies.

Hematoxylin and eosin staining

Slides were first dewaxed by three xylene washes for 6 min each. Slides were placed into two washes of 100% ethanol for 15 s each followed by a single 95% and 70% ethanol wash, for 15 s each. Slides were then washed with tap water for 1 min then dipped into filtered hematoxylin for 10-12 min. Slides were then rinsed in several washes of tap water until the water was clear. Slides were then dipped twice into a 0.3% Acid Solution (made with ethanol and HCl) then rinsed with tap water for 2 min. Slides were placed into 0.3% ammonia water (made with ammonium hydroxide and distilled water) until the tissue acquired a blue jean color. Slides were rinsed for 2 min in tap water then incubated in 95% ethanol for 20 s. Slides were then placed into Eosin-Y solution for 30 s-1 min, then dehydrated. The dehydration process included: 95% ethanol incubation for 20 s, three 100% ethanol incubations for 20 s, and three xylene incubators for 15 s each. Finally, slides were mounted.

Sirius red staining

Dewaxed slides were hydrated in an ethanol series: 100% for 5 min, 100% for 5 min, 95% for 3 min, and 70% for 3 min. Slides were then incubated in pico-sirius red for one hour followed by two times of washes in acidified water (made with glacial acetic acid). Slides were dried using filter paper then dehydrated in 3 changes of 100% ethanol for 3 min each. Finally, slides were incubated in xylene for 3 min each then mounted.

Sirius red image analysis

To quantify Sirius red images, the scale bar was first measured using the straight-line tool, creating a line along the length of the scale bar. Following this, the scale bar was measured by selecting Analyze > Set Scale to set the scale to micrometers. The scale bar length in micrometers was entered into the "known distance" space and the "um" was entered into "unit of length." To split the image into three channels, we selected Image > Type > RGB stack then select Image > Stacks > Make Montage to view all three channels at once. The green channel (middle) was selected using the square tool then we selected Image > Adjust > Threshold. The slider was moved lower until the collagen is highlighted in red, then "Set" was selected. The square tool was then used to delete the scale bar area, which was then painted white to prevent its inclusion from the calculated area. Finally, to set measurements, we selected Analyze > Set Measurements and selected "area", "area fraction", "limit to threshold", and "display label". Finally, to measure we selected Analyze > Measure. The average of the measurements was taken as well as the standard deviation and graphed to represent the quantification analysis.

Liver RNA isolation and RNA sequencing

Liver samples were placed in RNAlater® Solution (Ambion, #AM7020) and kept at 4 °C. Liver RNA isolation was performed using the mirVana™ miRNA Isolation Kit (Ambion, #AM1560) according to the manufacturer's protocol. RNA was sent to University of Cincinnati Genomics, Epigenomics and Sequencing Core for sequencing analysis.

Immunohistochemistry protocol

Paraffin-embedded (formalin-fixed) liver tissue was sectioned to 5-8 μm thickness. Slides were deparaffinized in a series of xylene treatments (5 min). Slides were then rehydrated in an ethanol series (100% for 5 min, 100% for 5 min, 95% for 3 min, and 70% for 3 min). Slides were rinsed with 1 × PBS for 5 min. Antigen retrieval was performed using a solution of 0.1 M Citric Acid and 0.1 M Sodium Citrate. Antigen retrieval solution was boiled for 10 min, then slides were placed in the solution in Coplin jars and boiled in the microwave, 5 min at 100% power, then 5 min at 60% power twice. During each boil, top off the antigen retrieval solution with distilled water. Endogenous peroxidase was blocked by incubating the slides in 30% H_2O_2 in Methanol. Slides were washed twice in PBS for 5 min. Slides are blocked in 5% normal Goat Serum (Vector Labs, #S-1000) in PBST (made with 0.1% Triton X-100) for one hour at room-temperature. Primary antibody incubation was done overnight at 4 °C. Slides are washed twice with PBS for 5 min. Then slides were incubated with a secondary antibody for one hour at room-temperature. Slides were then washed twice in PBS for 10 min and incubated for 30 min with a Vectastain Elite ABC solution according to the manufacturer's instructions (Vector Labs, #PK-6100). Slides were then washed twice in PBST for 5 min. Slides were developed by DAB (Sigma, #D3939). Slides were rinsed in tap water followed by a counterstain with hematoxylin. Slides were rinsed with tap water until water is clear then incubated in an acid rinse for 1 min. Slides were rinsed again and incubated with a bluing solution for 1 min. Slides were rinsed then dehydrated in an ethanol series, followed by xylene washes. Slides were mounted and analyzed. Primary Antibodies used in this study include alpha-smooth muscle actin (α-SMA) (CST 19245T), β-catenin (CST 9582), and Ki67 (CST 12202). The secondary antibody used was a biotinylated goat anti-rabbit immunoglobulin G antibody (Vector Labs, #BA-1000).

Image analysis α-SMA data

Images were analyzed using the "Fiji" version of ImageJ software. Image was opened. Color Deconvolution was selected for images stained specifically in the nuclei. To decrease the interference of cytoplasmic staining, images that had nuclear and cytoplasmic staining, under the image pull down, RGB stack was selected under type. To decrease cytoplasmic signal, go to Image > Type > RGB stack. Once the RGB window appears, select Image > Stacks > Make Montagne then perform color deconvolution. For both nuclear-specific and other images, select the Vectors pulldown > "HDAB". The "Colour_2" image window was selected and measured. The units of intensity derived in the results window were transferred to an excel spreadsheet. The optimal density (O.D.) was calculated using the formula, O.D. = log (max intensity/mean intensity), where the max intensity should be 255. The average optimal density and standard deviations were calculated and graphed.

Ki67 scoring

Slides were examined and percent nuclear positive hepatocytes *per* field (under 20 × magnification) were counted *per* 100 cells.

β-catenin scoring

Slides were examined under 20 × magnification and percent nuclear positive cells were calculated per field using the cell counter feature in Fiji.

Preparation of mouse liver subcellular fractions

Control and LKO livers of mice 3- and 8-mo post-DEN exposure were harvested. Cytoplasmic and nuclear fractions were prepared from these livers using the Thermo Scientific Subcellular Protein Fractionation Kit for Tissues (Cat. No. 87790), according to the manufacturer's recommendations.

Immunoblotting

Protein extracts (40-100 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose filter. The filter was blocked with 5% dry milk in PBST for 1 h at room temperature, followed by incubation with primary antibody overnight at 4 °C or 3 h at room temperature. The filter was then washed in PBST 3 times for 5 min each, followed by incubation with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After washing with PBST, the filter was developed with ECL for 1 min and exposed to X-ray film. Quantification of the polypeptide bands was performed with the Fiji software.

Liver cDNA preparation and RT-PCR analysis

Liver cDNA was isolated from RNA templates as described previously[3]. The PCR mix was made using the 1/10 cDNA solution as the template, primers, and the DreamTaq PCR master mix (2 ×) (Thermo Fischer Scientific, #K1071). The PCR products were separated by electrophoresis on a 2% agarose gel. RT-PCR was setup according to the iQ™ Sybr® Green Supermix (BioRad, #170-8882) with the 1/10 cDNA as the template. PCR conditions for the semi-quantitative and RT-PCR are as follows: Initial denaturation-95 °C for 1 cycle; for 40 cycles: Denaturing-95 °C, annealing-Tm as indicated below, extension-72 °C; and an optional hold at 4 °C. Gene primers were obtained from Integrated DNA Technologies.

RNA sequencing

RNA from Control and LKO livers of mice exposed to DEN for 8 mo was isolated as described previously[3]. RNA samples were submitted to the Genomics, Epigenomics and Sequencing Core at the University of Cincinnati.

Cell culture and transfection

HepG2 and THLE2 cells were purchased from ATCC. HepG2 cells were cultured in DMEM high glucose medium with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a CO_2(5%) incubator. THLE2 cells were cultured in special medium as suggested by ATCC. The siRNA transfection of cells was mediated by lipofectamine RNAiMAX (Thermo, Cat. No. 13778030) following manufacturer's instruction.

Immunofluorescence analysis

THLE2 cells were fixed and stained with primary antibodies against BRUCE and protein kinase A (PKA). After washes, cells were incubated with secondary antibodies coupled with Alexa Fluor 594 and Alexa Fluor 488, respectively. Samples were analyzed and photos acquired under Zeiss Fluorescence Microscope.

Preparation of whole cell lysates of human cancer cells

Cell pellets were lysed and sonicated to elute whole cell lysates in RIPA buffer with protease inhibitor tablets (Roche) and phosphatase inhibitors of 10 mmol/L NaF and 50 mmol/L β-glycerophosphate. The lysates were centrifuged at 15000 g for 20 min and the supernatant was collected.

Antibodies: The antibodies used in this study were: BRUCE from Novus (NB300-264); α-SMA (CST 19245T); Total β-catenin (CST 9582); Ki67 (CST 12202); phospho-β-catenin Ser-675 (CST 4176)**;** Lamin A/C (CST 4777); Actin (CST 3700); Glyceraldehyde-3-phosphate dehydrogenase (CST 2118); phospho-PKA substrate (RRXS*/T*) (100G7E) (CST 9624)

Reagents and siRNAs: DEN (#N0756) from Sigma; BRUCE siRNA and control siRNA were synthesized by Dharmacon[16]. Control siRNA sequence is UUCUCCGAACG-UGUCACGUdTdT. The BRUCE siRNA sequence is GGCACAGCAGCTCTTATCA.

Data analysis: The results are expressed as the means ± SD of the determinations. The statistical significance of the difference was determined by a two-tailed Student's *t*-test.

RESULTS

Liver-specific KO of BRUCE promotes early tumor onset and an exacerbated HCC mimicking patient-like histological features in DEN-exposed mice

DEN administration in mice promotes chronic liver injury and HCC development[5]. Control (loxp/Loxp; Alb-Cre$^-$) and LKO (loxp/Loxp; Alb-cre$^+$) mice were exposed to DEN at postnatal day 15 to induce liver injury and malignant transformation to HCC. Mice with and without BRUCE expression in the liver were sacrificed at various time points for studies of liver disease progression (Figure 1A). Fifty percent of the LKO mice developed tumors 8 mo after DEN administration, while the control littermates did not begin to develop tumors until 10 mo post DEN administration (Figure 1B and C). By 14 mo, 100% of the LKO mice developed HCC (n = 17) whereas 80% of the control developed HCC (n = 10) (Figure 1B). More of the LKO mice developed an exacerbated HCC phenotype (Figure 1B and D). Histology of the HCC tumors revealed a trabecular architecture identical to the histologic patterns of HCC patients

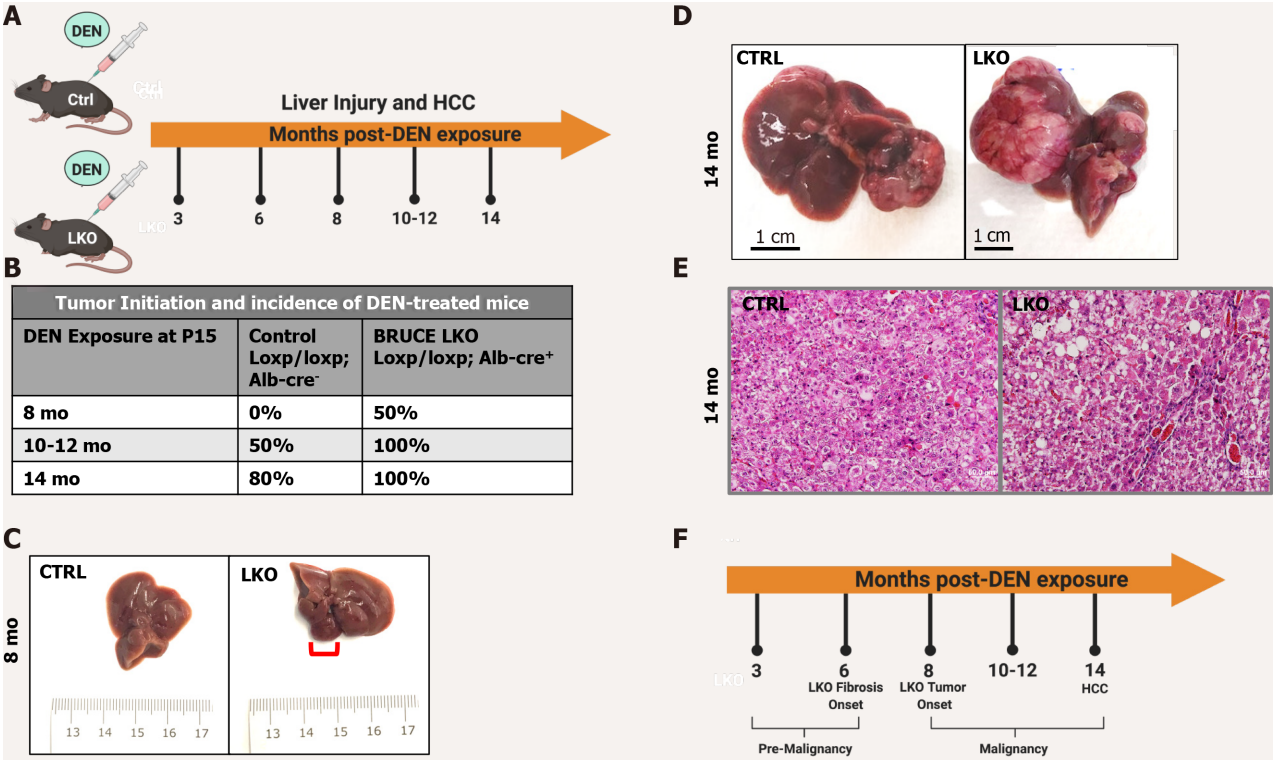


Figure 1 Diethylnitrosamine-induced hepatic malignancy leads to earlier tumor initiation and an exacerbated patient hepatocellular carcinoma-like phenotype in BRUCE LKO mice. A: Diethylnitrosamine (DEN) model. Control and BRUCE LKO mice were treated with DEN over a time course schedule as indicated; B: Tumor onset in LKO mice happened after 8 mo post-DEN exposure, while tumor onset did not begin in control mice until 10-12 mo; C: Tumor in LKO mouse traced in red; D: After 14 mo of DEN exposure, control and LKO mice develop hepatocellular carcinoma; however, the LKO mice have a more exacerbated phenotype; E: Hematoxylin and Eosin staining reveals a trabecular histologic feature in LKO but not control; F: Timeline of key events of DEN-induced hepatic malignancy model. DEN: Diethylnitrosamine; LKO: Liver-specific knockout; HCC: Hepatocellular carcinoma; CTRL: Control; BRUCE: BIR repeat-containing ubiquitin conjugating enzyme.

with poor prognosis (Figure 1E). The pathogenic highlights of this study were summarized into a pre-malignant and a malignant stage (Figure 1F). Together the data suggests that hepatic BRUCE deficiency accelerates and exacerbates DEN-induced HCC development in C57/BL6/J mice.

Hepatic BRUCE deficiency promotes hepatocyte damage and compensatory proliferation

We have reported that DEN administration to BRUCE LKO mice induces more DNA damage accumulation in hepatocytes than that in control mice. We have also demonstrated that the repair of DEN induced hepatocellular DNA damage requires the BRUCE-ATR DNA repair axis[3]. Recently it has been reported that excessive hepatocyte apoptosis plays a tumor-promoting role in nonalcoholic steatohepatitis (NASH)-associated liver cancer in mice[28]. We and others have reported on the importance of BRUCE in the regulation of DNA damage response as well as inhibiting apoptosis[11,12,16], yet the connection of the aforementioned roles of BRUCE have not yet been examined for whether sustained DNA damage correlates with or triggers apoptosis in hepatocytes[29,30]. In addition, DEN exposure induces hepatocellular DNA damage and gene mutations in mice which is a carcinogenic mechanism underlying DEN-initiated liver injury and development of HCC[31]. However, the apoptotic regulators that are critical in the regulation of hepatic apoptosis induced by exposure to DEN have not been well established. We reasoned that BRUCE LKO mice have lost the IAP function of BRUCE in the liver, thus DEN exposure of LKO mice could induce more prominent hepatocyte apoptosis than in the control. Indeed, our RNA-seq analysis found that DEN administration results in a higher level of apoptotic gene expressions (Figure 2A), suggesting that hepatic BRUCE protects against DEN-induced hepatic apoptosis.

One of the pathological outcomes of hepatocyte apoptosis is the facilitation of hepatic inflammation and fibrosis through two major mechanisms: (1) Replenishment of lost hepatocytes *via* compensatory proliferation of quiescent hepatocytes through

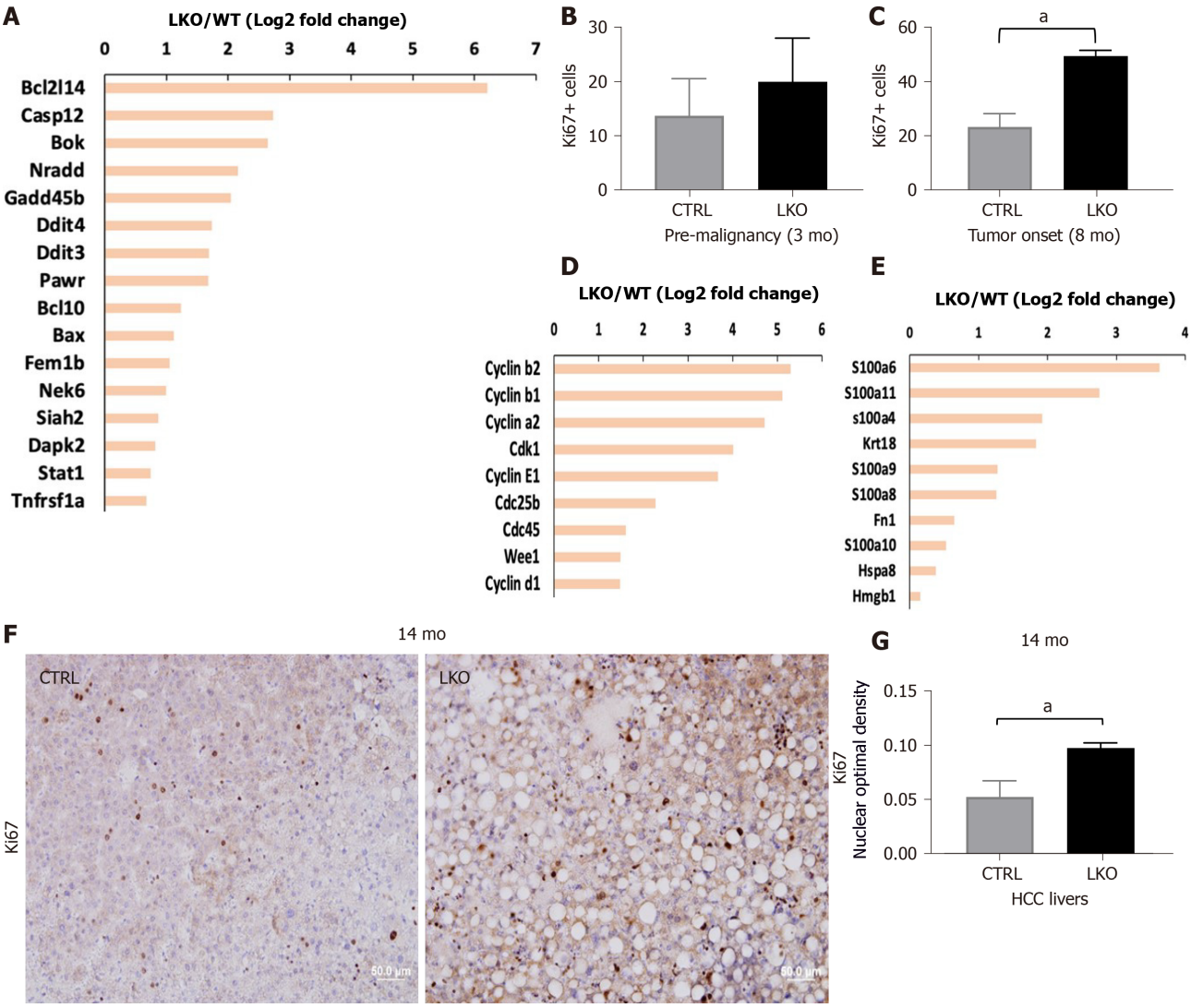


Figure 2 BRUCE deficiency increases diethylnitrosamine-induced liver injury and hepatic proliferation. A: Apoptotic gene expression is increased in liver-specific BRUCE KO livers at the time of tumor onset; B: Hepatic proliferation was measured by immunohistochemistry staining of the livers against Ki67. Livers exposed to diethylnitrosamine for 3 mo have an increase of Ki67+ cells; C: At the time of tumor onset in LKO livers there is an increase of Ki67+ positive cells; D: At the time of tumor onset, RNA-seq analysis reveals an increase of known cell cycle markers; E: Damage associated molecular patterns in the LKO livers; F and G: Ki67 staining by immunohistochemistry in 14 mo hepatocellular carcinoma livers, as well as quantification, show an increase of proliferation in LKO livers. $^{a}P<0.05$. HCC: Hepatocellular carcinoma; LKO: Liver-specific knockout; CTRL: Control.

feed-forward apoptosis-proliferation circles[26,32]; and (2) Release of proinflammatory DAMPs which result in liver inflammation and fibrosis[33]. Our immunohistochemical staining of a proliferation marker, Ki67, showed enhanced hepatocyte compensatory proliferation in BRUCE-deficient mouse livers compared to the control, during the pre-malignant stage (3 mo post DEN administration) (Figure 2B). This proliferation continued to the time of tumor onset (8 mo post DEN exposure) (Figure 2C). This elevated hepatic proliferation is supported by increased expression of multiple critical cell-cycle regulatory genes in BRUCE-deficient livers by RNA-seq analysis (Figure 2D). In addition, gene expression of DAMP molecules including HMGB1 and S100 are also increased in KO livers (Figure 2E). Moreover, increased cellular proliferation in human HCC has been correlated with tumor progression and poor prognosis[34,35]. During the malignant stage of 14 mo post DEN exposure, the HCC tissues from LKO mice exhibited increased Ki67 expression compared to the HCCs from control mice (Figure 2F and G). Together our data demonstrate that hepatic BRUCE deficiency results in elevated hepatic cell apoptosis and proliferation. Moreover, the release of DAMPs would exacerbate liver inflammation.

Hepatic BRUCE deficiency accelerates fibrosis in mice exposed to DEN

Hepatic fibrosis is characterized by an excessive accumulation of ECM in which collagen fibers are the major component produced by activated HSCs. A unique

feature of liver cancer is its close association to liver fibrosis. More than 80% of HCCs develop in fibrotic or cirrhotic livers, suggesting an important role of liver fibrosis in the pre-malignant environment of the liver[6]. Although fibrosis is a feature of human HCC, it is not necessarily recapitulated in various murine liver disease models, which makes these models inferior in modeling the progression of fibrosis to HCC. Interestingly, the LKO mice developed significant fibrosis in the pre-malignant stage at 6 mo following DEN administration, as shown by the Sirius red staining of collagen fibers (Figure 3A and B). Notably the collagen fibers in BRUCE KO livers showed signs of the advanced stage of "bridging fibrosis"[36], as evidenced by the fibrotic spreading that extends between portal and central vein areas (Figure 3A). This bridging fibrosis is in sharp contrast to the control mice in which fibrosis was limited to portal or venular areas (Figure 3A). Upon chronic liver damage, injured hepatocytes undergo cell death which releases DAMP molecules and activate the normally quiescent HSCs[33]. To directly examine HSC activation, we analyzed the expression of α-SMA, which is expressed by HSCs and reflects their activation into a myofibroblast-like phenotype. The results revealed an increased α-SMA expression in HSCs which suggests an elevated activation of HSCs in LKO mice as compared to the control (Figure 3C and D). To validate these pro-fibrotic events at the gene expression level, RNA-seq analysis was conducted and multiple pro-fibrotic or fibrotic genes were found to have higher levels of expression in the LKO liver than that of the control (Figure 3E). Furthermore, elevated inflammatory gene expression was evident in LKO mice compared to control (Figure 3F). As liver fibrosis is characterized by the deposition of fibrillar collagens, the elevated gene expression of multiple types of collagens (Figure 3E) support the elevated fibrosis phenotype. In addition, HSCs contribute to the accumulation of ECM by producing excessive amounts of pro-fibrotic factors such as tissue inhibitor of metalloproteinases (TIMPs)[37]. Indeed, we observed an elevated level of *TIMP1* gene expression in LKO mice (Figure 3F). Moreover, the turnover of ECM, controlled by matrix metalloproteinases (MMPs), also promotes fibrosis and therefore a number of MMPs are highly expressed in liver fibrosis[19]. We also found that the gene expression of a number of MMPs were increased in LKO mouse livers (Figure 3F). Altogether, the data indicate that DEN exposure of BRUCE LKO mice aggravates hepatic fibrosis at both the histological and gene expression levels.

To ascertain whether the hepatic fibrosis is sustained during the malignancy stage, HCC tissues from 14-mo exposed mice were analyzed for both Sirius red staining and α-SMA expression. The results from both analyses demonstrated an exacerbated fibrosis that concur with HCCs (Figure 3G-J), demonstrating that sustained and chronic fibrosis coexists with HCCs, which is a hallmark of human HCCs. Altogether, hepatic BRUCE deficiency in the DEN-induced HCC model is sufficient to drive fibrosis in both pre-malignant and malignant stages.

Hepatic BRUCE deficiency promotes β-catenin signaling in the premalignant stage

Activated Wnt/β-catenin pathway is indicative of the stabilization of β-catenin in the cytoplasm and its subsequent translocation to the cell nucleus, where it achieves its gene transcription function by activating gene expressions for promotion of hepatic inflammation and fibrosis[38-41]. Remarkably, there was a pronounced increase of nuclear localization of β-catenin in the liver sections from BRUCE LKO mice in the pre-malignant stage of 3 mo (Figure 4A and B) as well as at tumor onset of 8 mo post DEN exposure (Figure 4C and D). The nuclear localization of β-catenin in liver tissue sections assessed by immunohistochemistry (IHC) was further validated by a biochemical approach. Specifically, liver protein extracts from mice at the pre-malignant and tumor-onset stages were further fractionated into cytoplasmic and nuclear fractions and immunoblotted for β-catenin. There was a much higher level of total β-catenin in the nuclear fraction than in the cytosol of both control and LKO samples (Figure 4E). Promoted by the increase of nuclear β-catenin in liver tissue sections, we postulated that there could be an increase in β-catenin activity in the nuclear fraction of LKO liver samples. To test this possibility, we compared the levels of β-catenin phosphorylation at Ser-675, an activated form of β-catenin phosphorylated by cAMP-dependent PKA[42,43] in the control and LKO samples. Indeed, there was a dramatic increase of phospho-β-catenin at Ser-675 in the nuclear fractions of the LKO livers in both stages of pre-malignancy and tumor onset (Figure 4E), suggesting that β-catenin plays an important role in the promotion of hepatic inflammation and fibrosis in the early, pre-malignant stage. The RNA-seq analysis confirmed an upregulation in the expression of multiple Wnt ligands and regulators (Figure 4F), and β-catenin target genes (Figure 4G). Together the data demonstrate an aberrant activation of the Wnt/β-catenin pathway, which plays a pro-

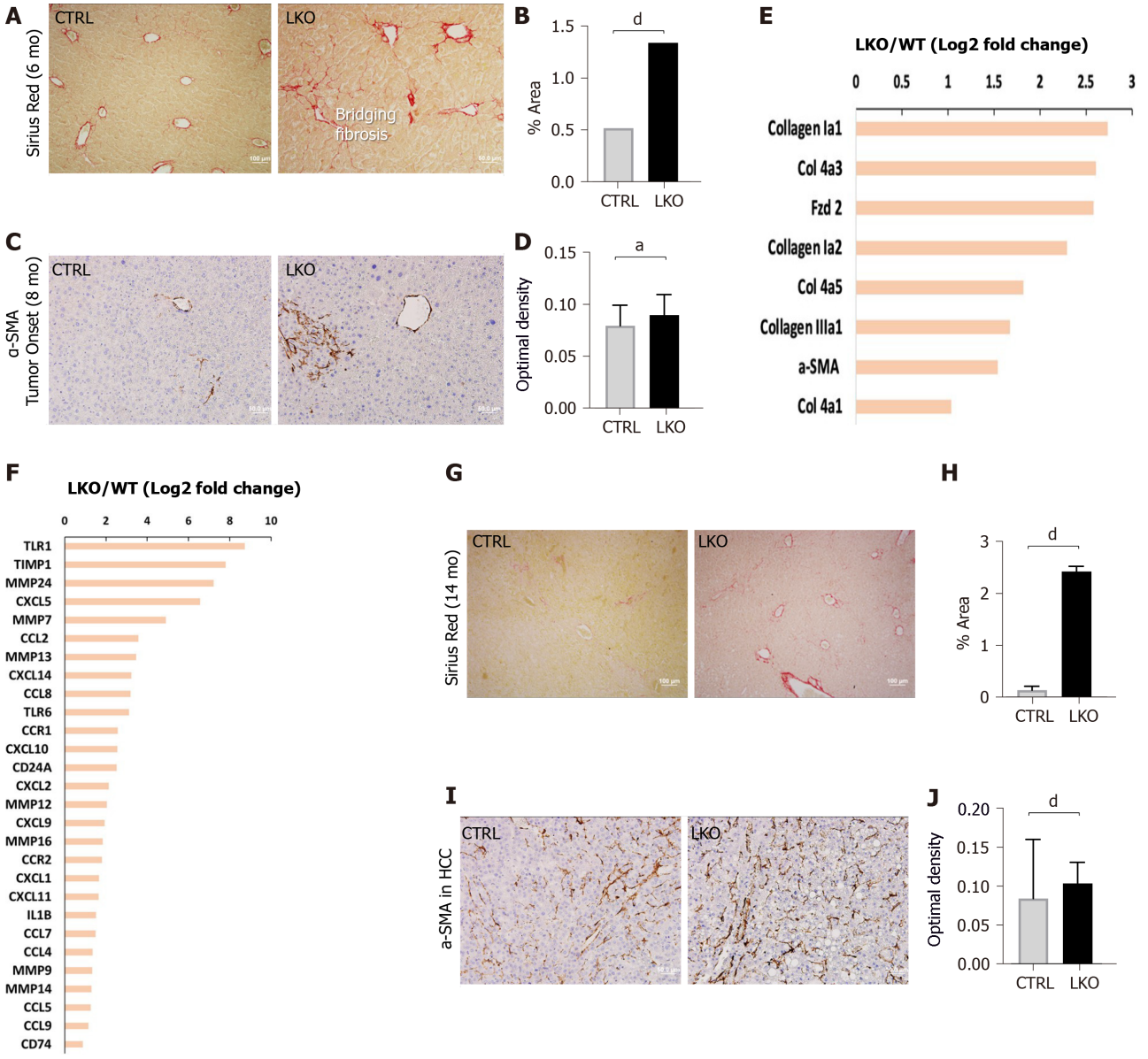


Figure 3 BRUCE deficiency accelerates and increases diethylnitrosamine-induced fibrosis. A and B: Sirius red staining of control and liver-specific BRUCE KO livers 6 mo post-diethylnitrosamine (DEN) exposure show an increase of Sirius red staining in the LKO livers, verified by quantification to the right; C and D: α-smooth muscle actin (SMA) immunohistochemistry at LKO tumor onset show an increase in α-SMA in LKO livers which is quantified in; E and F: RNA-seq analysis at the time of LKO tumor onset reveal that LKO livers demonstrate key patterns of human hepatic fibrosis, such as increased collagens and increased α-SMA as well as increased inflammation-related markers, such as CCL2; G and H: Sirius red staining of 14 mo post-DEN exposed HCC livers reveal an increase of collagen deposition, which was quantified; I and J: α-SMA immunohistochemistry of HCC livers, including quantification demonstrate an increase of activated hepatic stellate cells in 14 mo DEN-exposed LKO livers. $^{a}P < 0.05$; $^{d}P < 0.001$. HCC: Hepatocellular carcinoma; LKO: Liver-specific knockout; α-SMA: α-smooth muscle actin; CTRL: Control.

inflammatory and fibrotic role in LKO livers as shown in Figure 3. Collectively, these results indicate a new mechanism for an upregulated Wnt/β-catenin pathway resulting from hepatic BRUCE deficiency during the pre-malignant stage of hepatic inflammation and fibrosis in mice.

Hepatic BRUCE deficiency upregulates β-catenin signaling in malignant HCC livers

At age 14 mo, BRUCE LKO mice had a more exacerbated DEN-induced HCC phenotype (Figure 1D) consistent with a human HCC-like trabecular histological feature (Figure 1E). Livers from LKO mice maintained the nuclear localization of β-catenin (Figure 5A and B). Additionally, mRNA levels of β-catenin were measured by qRT-PCR and found to be increased in the LKO livers (Figure 5C). To confirm the IHC analysis of increased β-catenin, we analyzed β-catenin protein levels by Western blot analysis (Figure 5D) and noticed a concomitant increase in protein expression in LKO HCC livers. Additionally, cyclin D1, a downstream target of β-catenin, was increased

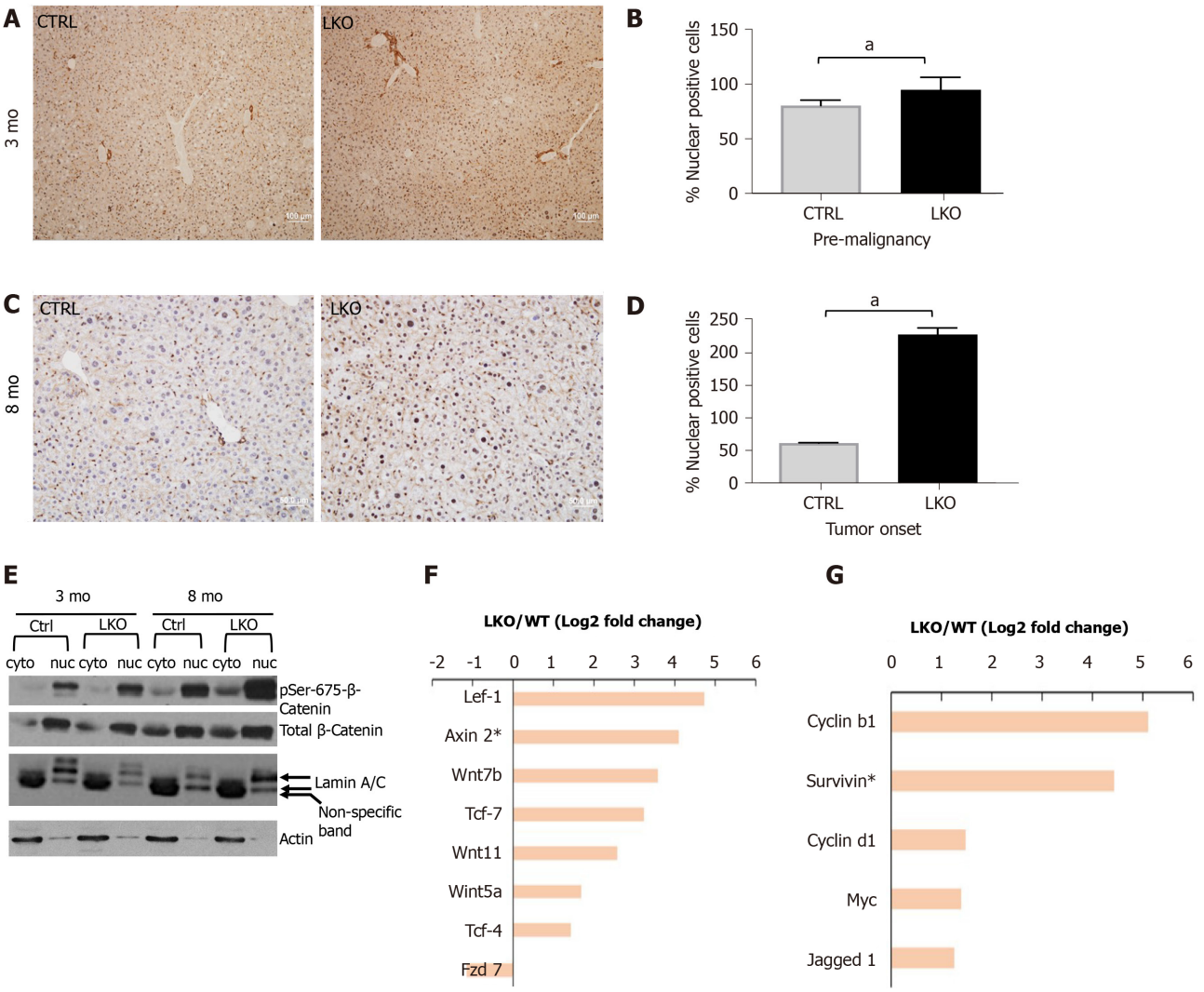


Figure 4 BRUCE deficiency promotes β-catenin activation in mice. A and B: After three months post-diethylnitrosamine (DEN) exposure, there is an increase in nuclear β-catenin staining by immunohistochemistry in the BRUCE KO livers. See quantification to the right; C and D: At tumor onset in LKO livers, nuclear β-catenin, shown by immunohistochemical staining, is increased in the LKO livers and quantified to the right; E: Phosphorylation of β-catenin at Ser-675 is increased in both the cytoplasmic and nuclear fractions of the LKO livers both pre-malignancy (3 mo post-DEN) and at tumor onset (8 mo post-DEN); F and G: At the time of tumor onset in LKO livers (8 mo post-DEN exposure), RNA sequencing analysis determined an increase in several canonical Wnt/β-catenin pathway members and target genes. [a]*P*< 0.05. LKO: Liver-specific knockout; CTRL: Control.

at the protein level in LKO HCC tissues (Figure 5E). Together the data demonstrate that BRUCE deficiency increases β-catenin nuclear accumulation in DEN-induced HCC. As β-catenin activity plays a critical oncogenic role in the development of HCC, these data suggest that upregulated β-catenin activity induced by BRUCE deficiency contributes to the accelerated HCC development in mice.

Loss of BRUCE stabilizes β-catenin through regulation of PKA activity in vitro and in vivo

An increase of nuclear β-catenin in LKO mice at the stages of pre-malignant (3 mo), tumor onset (8 mo) and malignant (14 mo) (Figure 4) suggests that hepatic BRUCE regulates β-catenin activation. To investigate the underlying mechanisms, we utilized an *in vitro* cell culture system of the human liver cancer cell line HepG2 which allows for knockdown (KD) experiments. We first determined if an increase of phospho-β-catenin at Ser-675 can be induced by KD of BRUCE expression in HepG2 cells. HepG2 cells were transfected with either a control or a BRUCE siRNA followed by preparation of the whole cell protein lysates for Western blot analysis. Knockdown of BRUCE in HepG2 cells resulted in increased levels of both the total β-catenin protein and phospho-β-catenin at Ser-675 (Figure 6A), demonstrating that BRUCE negatively regulates β-catenin activation. Since phosphorylation of β-catenin at Ser-675 is PKA-dependent, we reasoned that loss of BRUCE expression might be linked to the

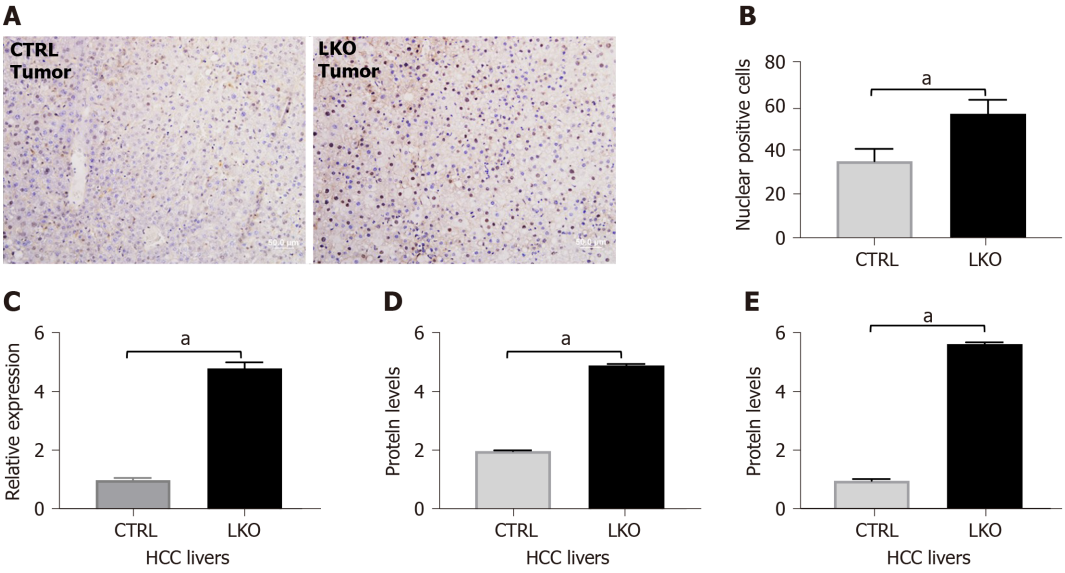


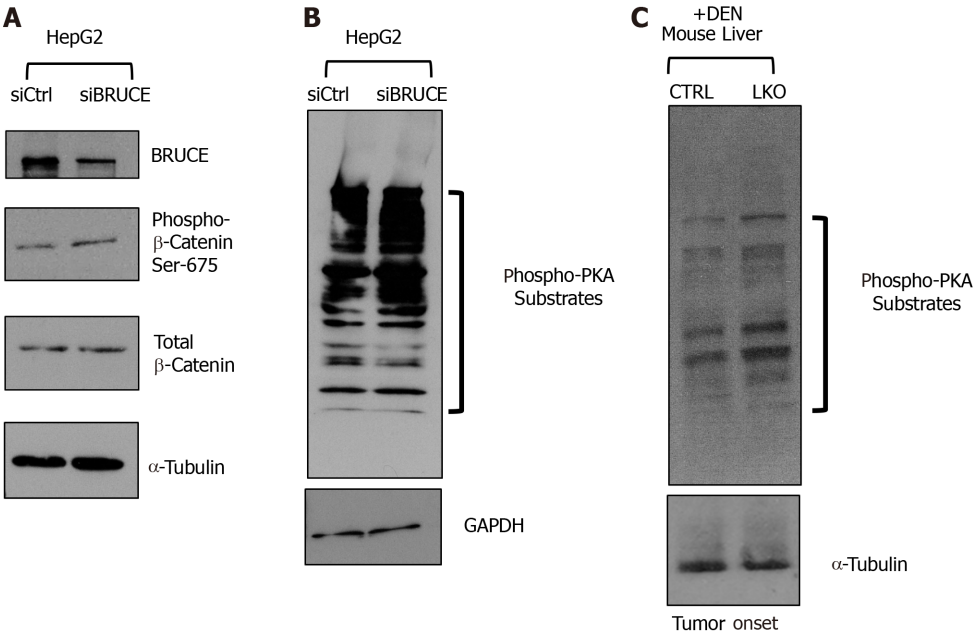
Figure 5 BRUCE deficiency increases nuclear β-catenin and activity in hepatocellular carcinoma livers. A and B: Immunohistochemistry of β-catenin in liver tumors after 14 mo of DEN-exposure which is quantified in; C: RT-PCR analysis of β-catenin in liver tumors after 14 mo post-DEN revealed an increase in mRNA levels in BRUCE knockout livers compared to control; D and E: Graphical representation of western blot analysis of β-catenin in liver tumors after 14 mo of DEN-exposure and cyclin D1, a downstream target of β-catenin. $^{a}P < 0.05$. HCC: Hepatocellular carcinoma; LKO: Liver-specific knockout; CTRL: Control.

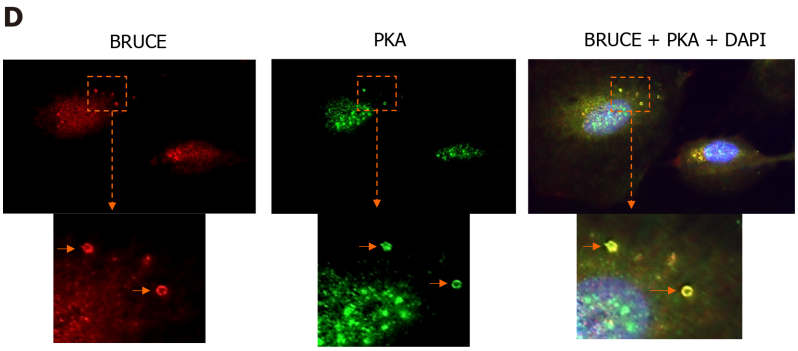
activation of PKA activity. To test this possibility, we compared PKA activity in cells with and without BRUCE knockdown by examination of the levels of PKA phosphorylated substrates using an antibody specific to PKA substrates. The Western blotting results showed an increase in phospho-PKA substrates in BRUCE KD cells (Figure 6B), demonstrating that the loss of BRUCE expression induces activation of PKA, thereby resulting in a higher PKA activity. This link of BRUCE loss to PKA activity elevation was also reproduced *in vivo* in DEN-exposed mice, demonstrated by the increase of phospho-PKA substrates by Western blot analysis of liver protein extracts at the time of tumor onset in the LKO mice (Figure 6C). To further delineate the correlation of BRUCE loss with the upregulation of PKA activity, we performed co-immunofluorescence analysis of both proteins with a human normal hepatocyte line, THLE2. We found that BRUCE and PKA were co-localized in endosomes (Figure 6D). BRUCE is reported to be on endosomes in non-hepatocytes[44]; however, this is the initial report of BRUCE and PKA colocalization on endosomes in human hepatocytes. This colocalization suggests that in endosomes of hepatocytes, BRUCE interacts with PKA to restrain hyperactivation of PKA, whereas loss of BRUCE releases the restriction of PKA activation and thus PKA activity is elevated.

With the observation of PKA-dependent phospho-β-catenin at Ser-675 upon liver injury with DEN, we propose a new signaling axis of BRUCE-PKA-β-catenin in the regulation of liver function. In this axis, hepatic BRUCE suppresses hyperactivation of PKA activity, thereby preventing aberrant phosphorylation and activation of β-catenin as well as its subsequent profibrogenic and oncogenic functions. In livers devoid of BRUCE, there is a loss of the BRUCE-dependent negative regulation of PKA activation; therefore, PKA phosphorylates and activates β-catenin to aggravate hepatic fibrosis and accelerate HCC (schematic, Figure 6E).

DISCUSSION

We have previously reported the clinical relevance of BRUCE in liver diseases in which BRUCE downregulation is found in a large portion of liver disease patients, including fibrosis, hepatitis, NASH, and HCC[3]. Upon assessment of BRUCE protein expression levels in liver specimens (male and female patients), we found that BRUCE levels were reduced in 54.5% of hepatitis samples (n = 22), 46.7% of cirrhosis samples (n = 30), and 84% of HCC samples (n = 25)[3]. These findings suggest a correlation between BRUCE expression levels and various liver disease stages. Additionally, we previously reported a 6% rate of deleterious BRUCE mutations in HCC patients, as deduced through the Cancer Genome Atlas. This rate was comparable to the mutation rate of other key DNA damage response (DDR) genes such as, *ATR*, *BRCA1* and





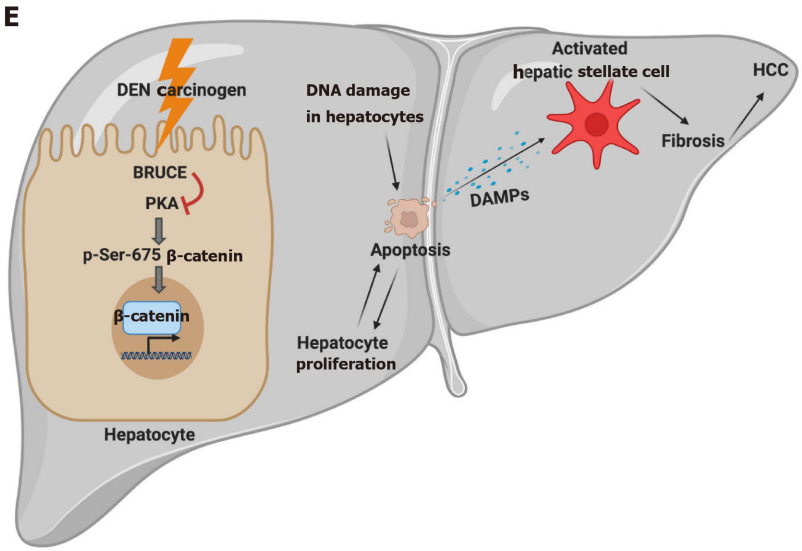


Figure 6 BRUCE-dependent regulation of β-catenin links to protein kinase activity. A: Whole cell lysates of HepG2 cells transfected with either an siCtrl or siBRUCE were blotted for BRUCE, phospho- and total-β-catenin, as well as a tubulin control; B: Lysates described in (A) were blotted for phospho-protein kinase A (PKA) substrates to measure PKA activity, as well as a glyceraldehyde-3-phosphate dehydrogenase control; C: Western blot analysis of mouse liver tissue lysates from control and liver-specific BRUCE knockout (LKO) exposed to diethylnitrosamine (DEN) for phospho-PKA substrates showing an increase in PKA activity in LKO livers at the time of tumor onset (8 mo); D: Immunofluorescence staining showing colocalization of BRUCE (red) and PKA (green) in endosomes (arrows) in normal human THLE2 hepatocyte line with cell nucleus counterstained with DAPI. The cellular areas outlined in dashed squares are enlarged and shown below; scale bar 20 μm; E: A working model showing a new BRUCE-PKA-β-catenin signaling axis involved in the regulation of fibrosis and HCC. BRUCE regulates β-catenin activation by inhibiting PKA-dependent phosphorylation-activation of β-catenin for hepatic proliferation and carcinogenesis. Mechanistically, BRUCE interacts with PKA in the hepatocyte cytoplasm to restrain PKA activity. When this interaction is disrupted by KO of BRUCE in the mouse liver, or by KD of BRUCE expression in

liver cancer cell line, the repression of PKA is derepressed and PKA-dependent phosphorylation-activation of β-catenin at Ser-675 occurs which results in hepatic proliferation. Meanwhile hepatocytes undergo apoptosis induced by DEN-DNA damage and these apoptotic hepatocytes release damage associated molecular patterns to activate hepatic stellate cells. The BRUCE-PKA-β-catenin signaling axis, together with DEN induced DNA damage, hepatic cell death, and oxidative stress, result in an early onset of fibrosis and accelerated HCC. DEN: Diethylnitrosamine; LKO: Liver-specific knockout; BRUCE: BIR repeat-containing ubiquitin conjugating enzyme; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; PKA: Protein kinase A; DAPI: 4',6-diamidino-2-phenylindole; HCC: Hepatocellular carcinoma; DAMPs: Damage associated molecular patterns; CTRL: Control.

BRCA2 in HCC patients. Furthermore, we delineated frameshift and nonsense mutations of BRUCE, particularly in BRUCE's ubiquitin conjugating (UBC) domain[3]. Our group has previously established that the UBC domain of BRUCE is necessary for its DDR function[11,12]. Therefore, deleterious BRUCE mutations would inactivate BRUCE's DDR function and could contribute to overall genomic instability leading to HCC development[3,12].

BRUCE has two major functions. It facilitates DDR to maintain genomic stability and as an IAP-family member, it suppresses apoptosis to maintain cell viability[15,16]. Liver KO of BRUCE abolishes both of these functions in the liver. While DDR inactivation and genomic instability promote HCC development in BRUCE-deficient settings[3], inflammation and fibrosis are well characterized risk factors in HCC pathogenesis[45,46]. Therefore, we focused on the progression of fibrosis in this study. The loss of hepatic BRUCE together with DEN administration contributes to an increase in DAMPs which lay the foundation for fibrosis as well as contribute to the progression of HCC. Loss of BRUCE's anti-apoptotic function results in elevated hepatocyte apoptosis and the release of DAMPs, which promote compensatory hepatocyte proliferation (Figure 2). Upon release of DAMPs, the quiescent HSCs will become activated which will progressively trigger the onset of fibrosis (Figure 3). As previously reported, increased hepatic fibrosis and compensatory proliferation are contributors to both HCC and poor prognosis[6,33-35,47].

Nonetheless, there are a number of risk factors that predominate the development of HCC in humans. Infection with hepatitis B or C viruses, alcohol consumption and metabolic syndrome are also major risk factors. Since DNA damage and apoptosis are likely common to liver fibrosis and HCC induced by these risk factors, BRUCE is anticipated to also protect against liver diseases induced by these risk factors, which is our future direction for this research. In addition to DNA repair and anti-apoptosis, BRUCE also regulates autophagy and cellular energy levels as we previously published[48]. As autophagy is involved in the regulation of liver homeostasis and liver injury and because of the robust autophagic activity found in liver tissue[49-51], it is likely that BRUCE also regulates liver injury, fibrosis and carcinogenesis through autophagy. BRUCE likely coordinates multiple signaling pathways including autophagy, DNA repair and apoptosis to preserve liver homeostasis.

Liver diseases present a huge health threat and are on the rise. However, the molecular pathways leading to fibrosis and HCC are not fully defined, which have hampered the development of mechanism-based therapeutic intervention. Aberrant β-catenin activation and its nuclear localization in the promotion of liver disease is found in up to 50% of human HCCs[22,27]. It is believed that aberrant β-catenin activation is a key contributor to chronic liver disease progression. Finding upstream regulators of β-catenin pathogenic activation is necessary for identification of the right sub-group of chronic liver disease patients for considering mechanism-based therapeutic targeting.

This study provides new insights into the molecular pathways that contribute to liver fibrosis and HCC. We revealed a previously unknown hepatocellular "BRUCE-PKA-β-catenin signaling axis" involved in the regulation of fibrosis and HCC (Figure 6E). In this signaling axis, we have identified a novel role of hepatocellular BRUCE in the suppression of aberrant activation of β-catenin through preventing PKA-mediated phosphorylation and activation of β-catenin both *in vivo* and *in vitro*. Mechanistically, we have revealed a novel interaction between BRUCE and PKA in the hepatocyte cytoplasm at endosomes, which provides the support for a functional interaction of these two proteins in the regulation of liver functions. This is further supported by our observations that upon disruption of BRUCE function either by liver KO (animal) or KD (HepG2 cell line), the repression of PKA is derepressed and PKA-dependent phosphorylation of β-catenin at Ser-675 occurs. This β-catenin phosphorylation is associated with the early onset of fibrosis and accelerated HCC in our mouse model (Figure 6E).

How might BRUCE regulate PKA protein levels and its activation? BRUCE itself is a hybrid protein harboring ubiquitin conjugase and ligase activities[15]. During the intrinsic mitochondrial pathway of apoptosis, BRUCE catalyzes ubiquitination of pro-

apoptotic proteins SMAC, Caspase-9 and others to reduce cellular apoptotic capacity to tip the balance of life and death towards cell death[17,51]. In addition, during DNA damage response induced by DNA double-strand and single-strand breaks, BRUCE ubiquitin ligase activity cooperates with the deubiquitinase USP8 to regulate ATM and ATR DNA damage responses to facilitate HR repair of DNA breaks[3,11,12]. Therefore, we propose that hepatic BRUCE-regulated protein ubiquitination signaling controls liver functions and conversely, lack of BRUCE expression results in dysregulation of ubiquitin signaling and accelerates liver disease development. BRUCE repression of PKA hyperactivation suggests a possible ubiquitination mechanism, in which BRUCE normally represses PKA activity through promotion of PKA ubiquitination and subsequent degradation through the UPS, thereby preventing β-catenin phosphorylation and activation. In the absence of BRUCE through genetic ablation of BRUCE in the mouse liver or gene knockdown in liver cancer cell lines, PKA becomes stabilized and β-catenin is activated. This possibility is currently under investigation in the lab.

This study has opened new avenues for focusing on BRUCE protection against liver injury, fibrosis and liver cancer. In addition to the "BRUCE-PKA-β-catenin signaling axis", other functions of BRUCE can also impact liver disease progression. In this regard, we have shown that BRUCE's function in the promotion of DNA damage repair is implicated in HCC development initiated by DEN[3]. Being an IAP in the suppression of mitochondrial pathway of apoptosis, BRUCE deficiency can make hepatocytes more susceptible to apoptosis under hepatic oxidative stress and detoxication, which are physiological processes inherent to livers. BRUCE also impacts autophagy and loss of BRUCE reduces cellular energy and increases autophagy flux[47]. Since autophagy regulates liver functions, the impact of BRUCE on liver autophagy and its connection with liver disease progression is under investigation in our lab. Future studies will focus on the interplay among these pathways in the maintenance of liver homeostasis and suppression of liver diseases in genetically modified murine models, including humanized murine models as it has shown promises to better understand human liver fibrosis.

The Wnt/β-catenin pathway has been regarded as a crucial mechanism involved in fibrosis and hepatocarcinogenesis. However, only a limited number of efficient targeted therapies are available for aberrant activation of this pathway in inhibiting chronic liver disease progression. Findings from this study provide the rationale to stratify the subset of liver disease patients with BRUCE mutant or deficiency and to test the therapeutic potential of targeting aberrant activation of the cAMP-PKA and Wnt/β-catenin pathways.

CONCLUSION

We previously reported the clinical relevance of somatic deleterious mutations in BRUCE or its downregulation in a large patient population with hepatitis, fibrosis and HCC[3]. In conclusion, this study identifies BRUCE as a suppressor of liver fibrosis in the premalignant and malignant stages in a DEN-induced hepatocarcinogenic murine model. Mechanistically, this study elucidates a previously unrecognized "BRUCE-PKA-β-catenin" signaling pathway contributing to hepatic proliferation, fibrosis and malignancy. Specifically, by using *in vitro* and *in vivo* approaches, we showed that hepatic BRUCE-deficiency releases its suppression of PKA kinase activity, leading to PKA-dependent phosphorylation and activation of β-catenin. In contrast to DEN exposure alone, which does not induce robust fibrosis, DEN treatment in a BRUCE null background accelerates fibrosis, which likely drives the early HCC development in BRUCE LKO mice. Considering the significant clinical relevance of BRUCE in patients with liver diseases, this study has demonstrated that our BRUCE LKO mouse model is a promising model for recapitulating human liver disease progression for dissecting the complicated pathological mechanisms underlying liver disease progression.

ARTICLE HIGHLIGHTS

Research background

BIR repeat-containing ubiquitin-conjugating enzyme (BRUCE) is a known ubiquitin conjugase/Ligase hybrid that has been shown to inhibit apoptosis, regulate efficient

DNA repair, and most recently promote tumor suppression in the liver. Our group previously showed that upon liver injury with diethylnitrosamine (DEN), loss of hepatic BRUCE promoted fibrosis and exacerbated hepatocellular carcinoma (HCC) development in mice.

Research motivation

About 80% of HCCs develop in fibrotic or cirrhotic livers, demonstrating the importance of understanding liver fibrosis as a factor contributing to hepatic malignancy. Identifying mechanisms that can regulate both fibrosis and HCC development simultaneously provides the possibility of opening therapeutic windows for treating fibrosis and HCC. Considering that over 50% of human HCCs have aberrant β-catenin mutations, targeting the Wnt/β-catenin has shown much promise. The key upstream regulators of this pathway that suppress fibrosis and HCC development remain elusive.

Research objectives

The objective of this study was to evaluate the mechanisms of BRUCE in inhibiting hepatic fibrosis and HCC upon liver injury induction.

Research methods

Male C57/BL6/J control mice [loxp/Loxp; albumin-cre (Alb-cre)$^-$] and BRUCE Alb-Cre KO mice (loxp/Loxp; Alb-Cre$^+$) were injected with a single dose of DEN at postnatal day 15. Mice were sacrificed at various time points to examine liver disease progression and liver biopsies were used in the analyses of the proposed mechanism.

Research results

Based on the exacerbation of fibrosis and HCC phenotypes observed in the liver-specific BRUCE knockout (LKO) mice that we previously reported, we hypothesized that, "the onset of fibrosis and tumorigenesis are likely earlier events in LKO mice". In the present study, we found that upon DEN-induction, BRUCE LKO livers developed fibrosis as early as after 6 mo of exposure. Additionally, the LKO mice developed tumors as early as 8-months after exposure compared to the WT tumor onset after 10 mo of DEN exposure. Furthermore, we observed increased accumulation of β-catenin, including its activity in LKO liver samples. The phosphorylation of β-catenin was determined by measuring nuclear levels of total β-catenin, and Ser-675 phosphorylated β-catenin. Additionally, the activity of protein kinase A (PKA), one of the upstream kinases that phosphorylates β-catenin at Ser-675, was found to be increased in both BRUCE-deficient mouse livers and a human liver cancer cell line. More importantly, BRUCE and PKA were found to be colocalized in the cytoplasm of hepatocytes.

Research conclusions

In conclusion, this study further demonstrated BRUCE's liver tumor suppressive function, by identifying the early onset of tumorigenesis in LKO mice. Furthermore, the current study elucidated a novel role of BRUCE in the negative regulation of PKA activity in order to negatively regulate β-catenin stabilization and activity. Together, BRUCE's regulation of β-catenin through PKA, is a likely mechanism used to suppress hepatic diseases, such as fibrosis and HCC.

Research perspectives

While further investigation is warranted, this study revealed the novel role of BRUCE in hepatic regulation of β-catenin upon liver injury. Further establishing BRUCE's regulation of PKA activity can possibly provide more promising therapeutic approaches for treating liver disease patients with aberrant expression of BRUCE and β-catenin.

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ORIGINAL ARTICLE

Retrospective Study

Early tacrolimus exposure does not impact long-term outcomes after liver transplantation

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Abstract

BACKGROUND

Tacrolimus trough levels (TTL) during the first weeks after liver transplantation (LT) have been related with long-term renal function and hepatocellular carcinoma recurrence. Nevertheless, the significance of trough levels of tacrolimus during the early post-transplant period for the long-term outcome is under debate

AIM

To evaluate the effect of TTL during the first month on the long-term outcomes after LT.

METHODS

One hundred fifty-five LT recipients treated *de novo* with once-daily tacrolimus were retrospectively studied. Patients with repeated LT or combined transplantation were excluded as well as those who presented renal dysfunction prior to transplantation and/or those who needed induction therapy. Patients were classified into 2 groups according to their mean TTL within the first month after transplantation: ≤ 10 (n = 98) and > 10 ng/mL (n = 57). Multivariate analyses were performed to assess risk factors for patient mortality.

RESULTS

CEIC E13/08.

Informed consent statement: Patients gave written consent to be included in the liver transplantation prospective data base. The requirement for specific informed consent for this study was waived because of the retrospective nature of the study.

Conflict-of-interest statement: MG is a member of advisory boards and has received honoraria from Astellas, Novartis and Chiesi. JB has received honoraria from Astellas and Novartis. AV has received honoraria from Astellas and Novartis. All other authors have no conflicts to declare.

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Mean levels within the first month post-transplant were 7.4 ± 1.7 and 12.6 ± 2.2 ng/mL in the ≤ 10 and > 10 groups, respectively. Donor age was higher in the high TTL group 62.9 ± 16.8 years *vs* 45.7 ± 17.5 years (P = 0.002) whilst mycophenolate-mofetil was more frequently used in the low TTL group 32.7% *vs* 15.8% (P = 0.02). Recipient features were generally similar across groups. After a median follow-up of 52.8 mo (range 2.8-81.1), no significant differences were observed in: Mean estimated glomerular filtration rate (P = 0.69), hepatocellular carcinoma recurrence (P = 0.44), *de novo* tumors (P = 0.77), new-onset diabetes (P = 0.13), or biopsy-proven acute rejection rate (12.2% and 8.8%, respectively; P = 0.50). Eighteen patients died during the follow-up and were evenly distributed across groups (P = 0.83). Five-year patient survival was 90.5% and 84.9%, respectively (P = 0.44), while 5-year graft survival was 88.2% and 80.8%, respectively (P = 0.42). Early TTL was not an independent factor for patient mortality in multivariate analyses.

CONCLUSION

Differences in tacrolimus levels restricted to the first month after transplant did not result in significant differences in long-term outcomes of LT recipients.

Key Words: Liver transplantation; Tacrolimus levels; Prolonged released tacrolimus; Once-daily tacrolimus; Renal function; Survival; Outcomes

Core Tip: This is a retrospective study to evaluate the effect of early tacrolimus trough levels (TTL) on the long-term outcomes after liver transplantation. Patients were classified into 2 groups according to mean TTL within the first month: ≤ 10 (n = 98) and > 10 ng/mL (n = 57). After a median follow-up of 52.8 mo (range 2.8-81.1), no significant differences were observed in: Mean estimated glomerular filtration rate, hepatocellular carcinoma recurrence, *de novo* tumors, biopsy-proven acute rejection rate and five-year patient and graft survival. Differences in tacrolimus levels within the first month after liver transplant did not result in significant differences in long-term outcomes.

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INTRODUCTION

Tacrolimus represents the keystone of current immunosuppressive regimens after liver transplantation (LT)[1]. Monitoring of trough drug levels is required to maintain them within the therapeutic range[2]. In the case of LT, there is some debate regarding the significance of trough levels of tacrolimus in the early post-transplant period for the long-term outcome. Initial recommendations were extrapolated from kidney transplantation, but LT does not require the high doses needed to prevent acute cellular rejection (ACR) in other allografts[3]. In this regard, various studies have explored the idea of minimizing initial tacrolimus trough levels (TTL)[4-6].

Mean TTL < 10 ng/mL within the first month after LT was associated with less renal impairment within 1 year in a recent meta-analysis[7]. In this study, tacrolimus concentration between 6 and 10 ng/mL were recommended as more appropriate after LT. Mean TTL > 10 ng/mL within the first month after LT but not thereafter has been also associated with increased risk of hepatocellular carcinoma (HCC) recurrence[8]. High exposure to calcineurin inhibitors was an independent predictor of HCC recurrence by multivariate analysis in this study (RR: 2.82; P = 0.005). Moreover, Rodríguez-Perálvarez *et al*[3] reported that mean TTL of 7-10 ng/mL during the first

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two weeks after LT was effective in preventing ACR, and was related with significantly superior results in graft survival than TTL above or below this range. More recently, the survival time of patients with mean TTL < 5 ng/mL during the first four weeks after LT was observed to be significantly shorter than that of patients with higher mean TTL[9]. Despite these studies, the actual role of initial TTL on long-term outcomes after LT is difficult to assess. Retrospective studies did not report TTL during the follow-up period[3,9], and therefore the influence of potential differences among groups in tacrolimus exposure throughout the follow-up cannot be ruled out. In addition, in some reports TTL were maintained different in the study groups not only during the first month but throughout the whole follow-up, though not significantly, with the consequent difference of long-term tacrolimus exposure and the potential influence on the outcomes[4,6,8].

Our experience with the use of once-daily tacrolimus (Tac-QD) *de novo* after LT has been published[10]. Outstanding long-term patient and graft survival was achieved with the use of *de novo* Tac-QD in a minimizing immunosuppression protocol in LT recipients. With the aim of assessing the significance of the early post-transplant period in the outcomes of LT, we conducted this study to determine the real role of early TTL within the first month on long-term outcomes after LT.

MATERIALS AND METHODS

Design and patients

We conducted a retrospective analysis of a prospectively collected database of patients transplanted between April 2008 and May 2013. A total of 237 consecutive LTs were performed during the study period. Patients in the database with repeated LT (n = 13) or combined transplantation (n = 8) were excluded from this analysis, as were those who died within the first week after LT (n = 5) and those who did not receive Tac-QD for various reasons (n = 11). Patients who presented renal dysfunction prior to transplantation, defined as estimated glomerular filtration rate (eGFR) < 60 mL/min/1.73 m^2, and/or those who needed induction therapy (n = 45 overall) were also excluded to avoid bias in the early TTL measurements due to their particular immunosuppressive protocol with induction therapy and delayed initiation of tacrolimus. Finally, 155 adult LT recipients, whose immunosuppressive therapy was based on Tac-QD *de novo*, were eligible for this study and were followed up until December 31, 2015. Patients with HCC met the preoperative Milan criteria. To determine the effect of early exposure to tacrolimus on long-term outcomes and renal function, patients were classified into two groups according to their mean TTL during the first month after LT: ≤ 10 ng/mL or > 10 ng/mL. All TTL obtained during the first month were used to define the mean values.

The study was performed in accordance with relevant guidelines and regulation. No organs were procured from prisoners. The prospective database received the approval of the Research Ethics Committee of the Hospital Universitario Cruces, No. CEIC E13/08. All patients gave informed consent to be included in the prospective database; the requirement for specific informed consent was waived because of the retrospective nature of the study.

Early post-transplantation immunosuppressive therapy

Initial immunosuppression included Tac-QD and steroids 20 mg/day, except in those patients with diabetes mellitus who were treated with Tac-QD and mycophenolate-mofetil (MMF), avoiding the use of steroids. Tac-QD was administered within the first 24 h after LT, either orally or *via* a nasogastric tube. Patients considered at risk of renal dysfunction received MMF at a daily dose of 1000-2000 mg. Initial Tac-QD dose was 0.15 mg/kg *per* day (or 0.1 mg/kg *per* day if combined with MMF). Subsequent doses were adjusted according to trough levels. Serum tacrolimus levels were monitored regularly every 48 h until discharge. Target TTL were 5-10 ng/mL during the first 3 mo; however, if trough levels were lower but liver function tests were normal, the TacQD dose was not preventively increased. Azathioprine was not used in our patients.

Clinical follow-up and long-term immunosuppressive therapy

Biliary reconstruction in our patients is performed with end-to-end choledocho-choledochostomy with T-tube. When the patient progresses well, T-tube is closed on postoperative day 3 to avoid the potential effect that biliary diversion might have on TTL. Cholangiography is performed on day 7 and in the third postoperative month

before T-tube removal. During these three months, patients are monitored weekly at home after hospital discharge, and also seen every two weeks at the outpatient clinic. Patients are monitored with liver function tests and TTL monthly afterwards until completion of the first year, and every 2-3 mo for a further two years. Stable patients with no relevant comorbidities are seen every 4 to 6 mo from the third year on.

The treating physicians adjusted immunosuppressive treatment according to their clinical judgment. Target TTL were progressively reduced: 4-9 ng/mL from month 3 to 6, 3-8 ng/mL from month 6 to 12, < 7 ng/mL after the first year and < 5 ng/mL after the second year onwards. Immunosuppressive protocol included steroids withdrawal 3-4 mo after transplantation, except in case of autoimmune disease (in which low-dose prednisone 5 mg/day was maintained), and in patients with hepatitis C virus (HCV), in whom withdrawal was delayed until months 12-18. Duration of treatment with MMF depended on side effects and/or clinical requirements. Adherence to treatment was assessed at each visit by asking the patients regarding any deviations from the prescribed regimen.

Endpoints and definitions

Outcome variables were: (1) Long-term renal function; (2) Immunosuppression-related morbidity; (3) Patient survival; and (4) Graft survival.

Long-term renal function was assessed by eGFR based on the modification of diet in renal disease formula. K/DIGO guidelines were used to define and classify chronic kidney disease[11]. Metabolic syndrome was defined according to already stablished definitions[12]. Fasting plasma glucose repeatedly > 126 mg/dL was used to define *de novo* diabetes whilst dyslipidemia was considered when treatment was prescribed for elevated blood cholesterol or triglycerides, and arterial hypertension when antihypertensive treatment was initiated. Patients with HCC met the Milan criteria. ACR was biopsy-proven acute rejection (BPAR) in all cases. BPAR were graded according to the Banff International Consensus Document[13]. Liver biopsy was not performed *per* protocol but indicated according to clinical evolution. In case of BPAR, tacrolimus exposure was further increased as the initial step. In case of severe rejection or if the graft dysfunction persisted after Tac-QD adjustments, three consecutive daily 500 mg corticosteroid boluses were used. Early graft dysfunction was defined according to previous specifications[14].

Statistical analysis

Qualitative variables are summarized as percentages and quantitative variables using means and standard deviations or median and interquartile range. Comparisons between frequencies of characteristics among trough-level groups were performed using the Chi-squared test or Fisher test, and continuous variables were compared using the Kruskall-Wallis test. Patient and graft survival were analyzed using the Kaplan-Meier method, in which patients lost to follow-up were censored at their last recorded visit. Graft loss was defined as retransplantation or death with non-functioning graft. Death with functioning graft was censored for the analysis of graft survival. The log-rank test was used to compare survival among the three groups. A univariate Cox regression analysis was performed to identify clinical and treatment factors related with patient survival including all patients in the cohort. Those variables with a $P < 0.200$ were included in a multivariate Cox regression model. Variables with the higher P value were excluded one by one until all variables had a $P < 0.05$. The proportional hazard assumption was tested. The statistical methods of this study were reviewed by Lorea Martinez-Indart from Bioinformatics and Statistics Platform, Biocruces Bizkaia Health Research Institute. Statistical analysis was performed using SPSS version 23.0.

RESULTS

All patients were Caucasian and received whole grafts from donation after brain-death. Ninety-eight were included in the ≤ 10 ng/mL group and 57 in the > 10 ng/mL group. A median of 7 samples of TTL (range 5-12) were used to obtain the mean TTL during the first month after transplant. Donor and recipient characteristics of the two groups are summarized in Table 1. Recipient features were generally similar across groups, including age, cause of liver disease, model for end-stage liver disease (MELD) score, baseline kidney function and pre-transplant comorbidities. The only significant difference between groups was the age of the graft donor (older for recipients whose early TTL were > 10 ng/mL); consequently, stroke as the cause of death was more

Table 1 Donors and recipients characteristics

	≤ 10 ng/mL, *n* = 98	> 10 ng/mL, *n* = 57	*P* value
Donors			
Age, year (mean ± SD)	54.7 ± 17.5	54.7 ± 17.5	0.002
Male	58 (59.2%)	35 (61.4%)	0.786
Cause of death			0.004
Stroke	57 (58.2%)	48 (84.2%)	
Trauma	27 (27.6%)	6 (10.5%)	
Other	14 (14.3%)	3 (5.3%)	
Graft steatosis	19 (19.6%)	12 (21.1%)	0.827
Recipients			
Age, years (mean ± SD)	55.3 ± 8.4	53.2 ± 9.8	0.227
Male	81 (82.7%)	48 (84.2%)	0.802
MELD (mean ± SD)	13.1 ± 5.6	12.7 ± 5.3	0.618
Hepatocellular carcinoma	45 (45.9%)	29 (50.9%)	0.551
Cause of liver disease			0.283
Alcohol abuse	45 (45.9%)	24 (42.1%)	
HCV	40 (40.8%)	18 (31.6%)	
HBV	3 (3.1%)	5 (8.8%)	
Cho/estatic liver disease	3 (3.1%)	4 (7%)	
Other	7 (7.1%)	6 (10.5%)	
Medical history (*pre* LT)			
MDRD-4 (mean ± SD)	107.8 ± 35.7	16.7 ± 33.7	0.223
Diabetes mellitus	18 (18.4%)	9 (15.8%)	0.683
Arterial hypertension	12 (12.2%)	10 (17.5%)	0.362
Mean tacrolimus trough levels 1 mo (ng/mL)	7.38 ± 1.68	12.62 ± 2.25	NA
Corticosteroids	80 (82.5%)	49 (86.0%)	0.571
Mycophenolate mofetil	32 (32.7%)	9 (15.8%)	0.024

MELD: Model for end-stage liver disease; MDRD-4: Modification of diet in renal disease; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; LT: Liver transplantation.

frequent among those donors. Corticosteroids were similarly used in all groups; however, MMF use was significantly more common in the group with TTL ≤ 10 ng/mL.

Evolution of mean TTL during the follow-up of the two groups is shown in Figure 1. Mean levels within the first month post-transplant were 7.4 ± 1.7 and 12.6 ± 2.2 ng/mL in the ≤ 10 and > 10 groups, respectively (Table 1). Levels decreased in the > 10 mg/mL group within the first three months and were similar in both groups by the third month. From the third month on, a steady decrease in TTL was observed in both groups. Of note, for the purpose of this study, TTL were significantly different among groups only during the first month after LT, but not during the rest of the follow-up (P = 0.65).

Median follow-up was 52.8 mo (range 2.8-81.1) for those patients with early levels ≤ 10 ng/mL and 52.6 mo (10.8-79.1) for patients with tacrolimus mean levels > 10 ng/mL. Patient outcomes after transplantation are summarized in Table 2. There were no statistically or clinically relevant differences among groups. Mean TTL during the early post-transplant period did not affect renal function. Creatinine clearance fell in parallel in both groups (P = 0.67), decreasing similarly during the first 6 mo to remain steady thereafter until the end of follow-up, at mean levels of approximately 80

Table 2 Recipients outcomes after liver transplantation

	≤ 10 ng/mL, *n* = 98	> 10 ng/mL, *n* = 57	*P* value
Biopsy-proven acute rejection	12 (12.2%)	5(8.8%)	0.505
Arterial complications	12 (12.2%)	7(12.3%)	0.995
Biliary complications	13 (13.3%)	8 (14%)	0.893
Infection (any)	49(50.0%)	26 (45.6%)	0.598
Cytomegalovirus infection	26 (26.5%)	12 (21.1%)	0.445
Retransplantation	5 (5.1%)	5 (8.8%)	0.500
HCC recurrence[1]	1 (2.3%)	0	0.999
HCV recurrence[2]	35 (87.5%)	14 (77.8%)	0.438
De novo tumor	10 (10.2%)	5 (8.8%)	0.771
New-onset arterial hypertension	35(36.1%)	19 (36.5%)	0.827
New-onset diabetes	21 (21.6%)	6 (12.7%)	0.127
Tacrolimus withdrawal. Causes:	18 (18.4%)	8 (14.0%)	0.486
Kidney failure	7	1	
Neurotoxicity	1	2	
Metabolic syndrome	6	4	
Metabolic synd + kidney failure	1	-	
Other	3	1	
MDRD-4 at 5 yr (mean ± SD)	82.5 ± 19.4	80.32 ± 14.7	0.686
Deaths. Causes:	10 (10.2%)	8 (14.0%)	0.827
HCV recurrence	5	3	
De novo tumor	1	2	
Sepsis	2	1	
Stroke	0	1	
Other	2	1	

[1]Including variables with $P < 0.2$ in univariate analysis, highlighted in bold.
[2]Renal dysfunction during hospitalization. HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; MDRD-4 stands for: Modification of Diet in Renal Disease.

mL/min/1.73 m^2 in all groups (Figure 2). Patients with higher levels within the first month after LT did not present more immunosuppression-related toxicity including new-onset diabetes, hypertension, HCC recurrence or *de novo* tumors. BPAR occurred with low and similar frequency in all groups (12.2%, and 8.8% in ≤ 10 and > 10 mg/mL, respectively; $P = 0.50$). Only 10 patients were treated with corticosteroid boluses (8 (66.7%) and 2 (40.0%), respectively; $P = 0.99$), and the rest responded to tacrolimus dose escalation. There was no relationship between the decision to withdraw tacrolimus during follow-up and the initial trough level.

Eighteen patients died during the follow-up and were evenly distributed across groups ($P = 0.83$) (Table 2). The most common cause of death was HCV recurrence. Five-year patient survival in the study groups was 90.5% and 84.9%, respectively ($P = 0.44$) (Figure 3A), while 5-year graft survival was 88.2% and 85.8%, respectively ($P = 0.42$) (Figure 3B).

Univariate and multivariate analysis

All patients were included in a univariate and multivariate Cox regression analysis to study factors associated with patient mortality. Multiple variables from donor and recipients were considered in the univariate analysis, as well as various outcomes and adverse events. This analysis was performed considering the two mean TTL groups described in methods, and also dividing the sample into two groups using the cut-off

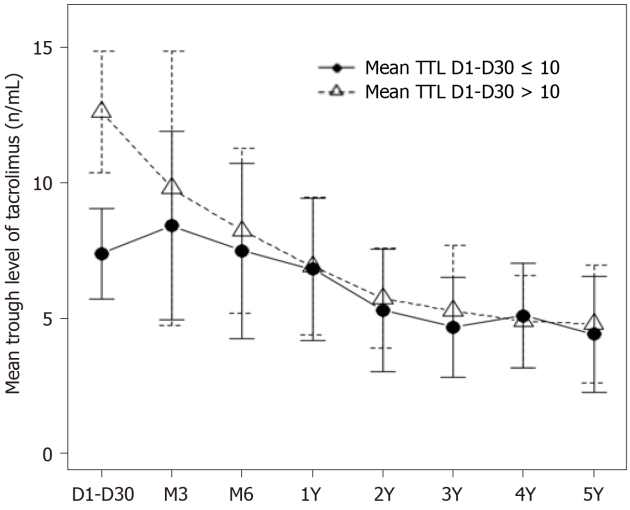

Figure 1 Mean serum tacrolimus levels according to the mean tacrolimus trough levels for each group within 1 mo after transplantation (mean ± SD). *P* = 0.65 comparing means from month 3 (M3) to year 5 (5Y). TTL: Tacrolimus trough levels.

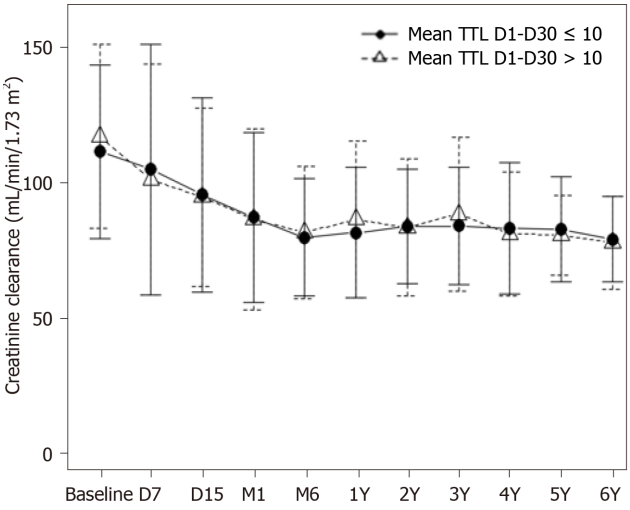

Figure 2 Mean creatinine clearance according to the mean tacrolimus trough levels for each group within 1 mo after transplantation (mean ± SD) (*P* = 0.67). TTL: Tacrolimus trough levels.

level 8 ng/mL or three groups using cut-off levels of < 7 ng/mL, 7-10 ng/mL and > 10 ng/mL. Multivariate analysis revealed that factors independently related with patient mortality were *de novo* tumor (HR = 13.8; 95%CI: 4.1-46.9; *P* < 0.001), MELD score ≥ 20 (HR = 6.1; 95%CI: 1.9-19.6; *P* = 0.003), HCV infection as the cause of liver disease (HR = 4.9; 95%CI: 1.7-14.1; *P* = 0.003) and arterial complications (HR = 3.7; 95%CI: 1.1-12.6; *P* = 0.03) (Table 3). Early TTL was not an independent factor for patient mortality.

DISCUSSION

This analysis aimed to further explore factors related to long-term clinical outcomes in our LT patients treated *de novo* with Tac-QD, with particular interest in the effect of mean TTL during the early post-transplant period. In order to have an adequate follow-up time to study long-term outcomes, patients transplanted between 2008 and 2013 were included in the study. Considering the time when LTs were performed, we followed a policy of immunosuppression minimization with target TTL of 5-10 ng/mL during the first 3 mo; however, a significant number of patients in this cohort were outside our target levels during the first month after LT, although this was corrected afterwards, as shown in Figure 1. We divided our cohort into two groups of early TTL (within 1 mo) as previously done by Rodríguez-Perálvarez *et al*[7,8] who found a

Table 3 Univariate and multivariate analysis of factors associated with patient mortality

	Univariate analysis		Multivariate analysis[1]	
	P value	HR (95%CI)	*P* value	HR (95%CI)
Age of donor ≥ 70 years	0.55	0.73 (0.26-2.05)		
Recipient				
Liver steatosis	0.1	0.408 (0.09-1.79)		
Age ≥ 60 years	0.94	1.04 (0.39-2.78)		
HCV infection as cause of liver disease	0.02	3.02 (1.17-7.81)	0.003	4.94 (1.72-14.17)
Presence of hepatocellular carcinoma	0.57	1.31 (0.52-3.34)		
MELD score ≥ 20	0.02	3.16 (1.12-8.91)	0.003	6.06 (1.88-19.56)
Diabetes before transplantation	0.63	1.32(0.43-4.01)		
Hypertension before transplantation	0.05	2.78 (0.98-7.90)	-	-
MDRD-4 at baseline	0.35	0.99 (0.97-1.01)		
Mycophenolate mofetil at initial therapy	0.89	1.07 (0.38-3.02)		
Outcomes and complications				
BPAR	0.20	2.08 (0.67-6.43)		
Arterial complications	0.06	2.91 (0.94-9.06)	0.03	3.76 (1.12-12.62)
Biliary complications	0.59	1.41 (0.40-4.92)		
Renal dysfunction early after transplant[2]	0.08	2.40 (0.90-6.38)	-	-
Renal hypertension	0.82	1.12 (0.42-3.02)		
De novo diabetes	0.02	3.25 (1.24-8.55)	-	-
Cardiovascular	0.14			
Arterial hypertension	0.08	0.32 (0.09-1.15)	-	-
Heart failure	0.26	0.31 (0.04-2.37)		
De novo tumor	0.005	4.20 (1.56-11.32)	< 0.001	13.82 (4.06-46.98)
HCV recurrence	0.22	1.79 (0.70-4.53)		
HCC recurrence	0.008	16.61 (2.10-131.07)	-	-
Any infection	0.71	1.12 (0.47-3.03)		
Bacterial infection	0.04	2.71 (1.04-7.07)	-	-
Viral infection	0.39	0.61 (0.20-1.87)		
Fungal infection	0.87	1.19 (0.16-9.03)		
Cytomegalovirus infection	0.79	0.86 (0.28-2.62)		
Normal renal function at last visit (MDRD-4 ≥ 60 mL/min/1.73 m^2)	0.92	1.08 (0.23-5.08)		
Mean tacrolimus levels at days 1-30 after LT				
> 10 ng/mL *vs* ≤ 10 ng/mL	0.44	1.44 (0.57-3.65)		
< 7 ng/mL (reference)[3]	0.32			
7-10 ng/mL	0.31	0.49 (0.12-1.96)		
> 10 ng/mL	0.59	1.33 (0.47-3.73)		
> 8 ng/mL *vs* < 8 ng/mL[3]	0.78	1.14 (0.44-2.95)		
Early graft dysfunction	0.08	2.44 (0.890-6.63)	< 0.001	6.02 (2.34-15.49)

[1]Including variables with $P < 0.2$ in univariate analysis, highlighted in bold.
[2]Renal dysfunction during hospitalization.

[3]Additional analysis modifying cut-off values. MELD: Model for end-stage liver disease; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; BPAR: Biopsy-proven acute rejection; MDRD-4 stands for: Modification of Diet in Renal Disease; LT: Liver transplantation.

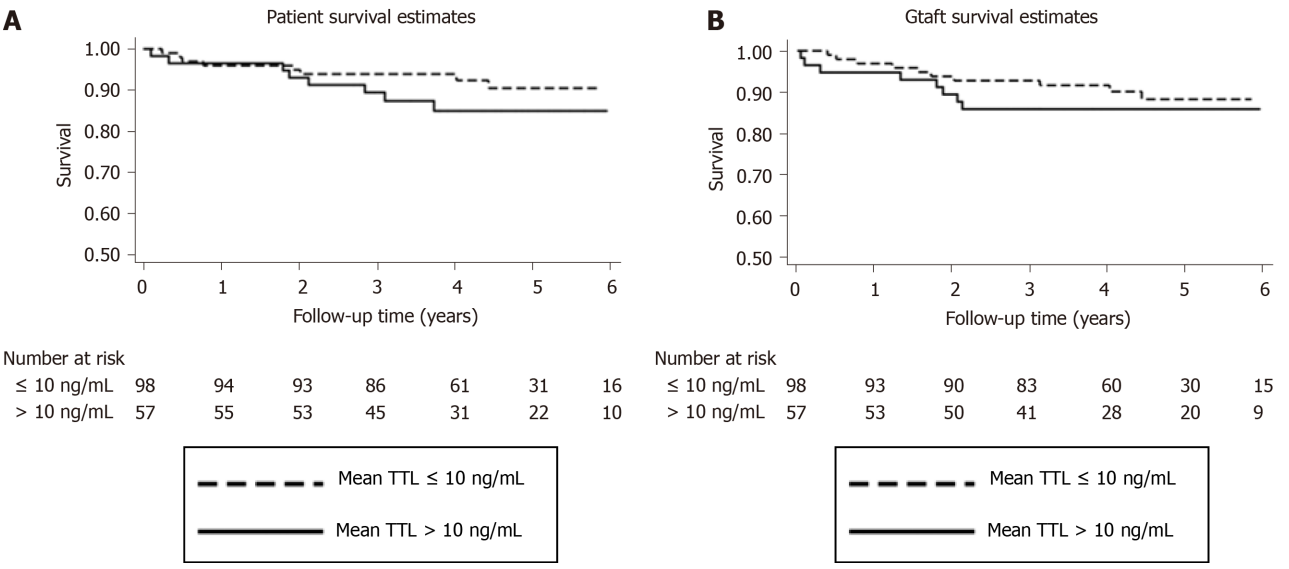

Figure 3 Kaplan-Meier survival curves after liver transplantation according to the mean tacrolimus trough levels for each group within 1 mo after transplantation. A: Patient survival (P = 0.44); B: Graft survival (P = 0.42). TTL: Tacrolimus trough levels.

significant improvement of outcomes when mean TTL within the first month post-LT were ≤ 10 ng /mL, compared with patients with > 10 ng/mL[7,8]. Of note, patients treated with induction therapy and delayed introduction of low-dose tacrolimus, namely those with pretransplant renal dysfunction, were excluded in our study to avoid bias as most of these patients would have probably ended in the low mean TTL group. In contrast to the published studies, we did not find significant differences in long-term renal function, HCC recurrence, immunosuppression-related toxicity or patient and graft survival in both groups of early TTL. In addition, multivariate analysis in our study, performed three times with different cut-off values for early TTL, demonstrated the lack of influence of early TTL on long-term patient survival.

In our study, donor age was significantly higher in the group with high TTL. Aging is characterized by a decline of liver cellular function that could determine alterations in immunosuppressants liver metabolism and pharmacokinetic. In this sense, it has been suggested that aged donor livers might exhibit lower drug clearance with consequently higher TTL[15]. Nevertheless, this circumstance was not detrimental in our experience as both TTL groups achieved comparable long-term outcomes.

According to the literature, the relative risk of death more than 1 year after LT suffers a 4-fold to 5-fold increase when renal dysfunction is present[16,17]. In our study, renal function evolved similarly in the two groups, with an expected 20% decrease in eGFR during the initial period after LT-as already described by other authors[18]-and maintenance of renal function from month 6 onwards. This contrasts with the progressive decline in renal function in the Mid/long-term repeatedly reported in literature[19-21]. Although, some authors have found no relationship between TTL within 15 d after LT and chronic renal impairment[3,9], high TTL within the first month after LT has been associated with worse renal function in different studies[7,20]. Karie-Guigues *et al*[20] found that the introduction of MMF significantly reduced the TTL at the end of the first month after LT, and this was associated with a significantly less marked reduction of the eGFR at 12 and 60 mo. Rodríguez-Perálvarez *et al*[7] also observed in a meta-analysis that reduced TTL (< 10 ng/mL) within the first month after LT were associated with less renal impairment at 1 year[7]. Nevertheless, both studies can be discussed. In the former study, TTL were shown at months 1, 12 and 60 after LT; however, no data were shown on the evolution of TTL between those time points and so, results could be biased due to different exposition to tacrolimus in both groups[20]. In the latter study, only two clinical trials were used in the meta-analysis and TTL were maintained higher in both study groups along the whole follow-up although differences did not achieve significance[7].

We can hypothesize that TTL early after LT have little effect on the evolution of long-term renal function when a tacrolimus minimization policy is implemented during long-term follow-up, as in our case. A longer period of high TTL in the post-transplant period might be needed to negatively affect the mid/long-term renal function. In accordance with this idea, the role of cumulative exposure to tacrolimus in eGFR decline after LT has been recently addressed[22]. In this study, conventional/high exposure to tacrolimus within the first 3 mo resulted in a more pronounced eGRF decline as compared with minimization (23.3 mL/min *vs* 9.5 mL/min; $P \leq 0.001$).

The role of tacrolimus exposure in HCC recurrence has been also addressed in different studies. High TTL (> 10 ng/mL) within the first month after LT but not thereafter was associated with increased risk of HCC recurrence at 5 years by Rodríguez-Perálvarez *et al*[8] (RR = 2.8; $P = 0.005$). Of note, in this study, tacrolimus levels were consistently lower during the 3-year follow-up in the non-recurrence group, although differences did not achieve significance. In another study, high exposure to tacrolimus was followed by a 50% recurrence rate *vs* 9.1% in patients with low exposure ($P = 0.001$)[23]. In this study, high exposure was described as > 10 ng/mL during the first year and not only during the first month reflecting a significant higher exposure to tacrolimus along the follow-up. In our study, overall HCC recurrence rate was extremely low and no differences were found between groups. Low exposure to tacrolimus not only during the early post-transplant period but in the long term, and our strict selection policy, all patients fulfilled Milan criteria prior to transplantation, might have positively influenced these remarkable results in our study. Recently, other authors have also reported the lack of effect of the first fifteen days of calcineurin inhibitor exposure in the development of HCC recurrence or *de novo* tumors after LT[24]. Again, it seems that longer periods of high exposure to tacrolimus-and not only during the first month after transplant-are needed to influence the development of *de novo* tumors or HCC recurrence.

Early TTL were not related with an increase in BPAR rates in our study. Reduction in early TTL was associated with the use of MMF and this could explain why the BPAR rate was not higher in patients with lower early TTL. Immunosuppression therapy with tacrolimus, MMF and steroids is currently the most common combination following LT[1], and has been demonstrated to be effective in reducing TTL while maintaining or even reducing the acute rejection rate[4,6].

We observed a relatively low rate of immunosuppression-related toxicity in terms of *de novo* diabetes or arterial hypertension and no differences were seen according to early TTL. In addition, development of *de novo* tumors was not influenced by TTL during the first month in our study.

In our study, factors associated with patient survival in multivariate analysis were *de novo* tumor, higher severity of liver disease (MELD score > 20), baseline HCV infection and arterial complications after LT. These factors have been repeatedly reported to be related to patient and graft survival after LT in the pre-direct-acting antivirals era[16,25,26]. Of note, early TTL were not an independent risk factors for patient survival in our study.

We recognize some limitations in our study. It is retrospective, although the data were retrieved from a prospective database. Indeed, the number of patients included in the different groups are limited and hence the number of patients who experienced adverse events of interest such as impairment of renal function or HCC recurrence are also limited. In addition, MMF was more frequently used in the lower TTL group although immunosuppression-related morbidity is more likely related with tacrolimus exposure rather than to the use of MMF. Nevertheless, our study has several strengths: (1) Median follow-up was more than 4 years in both groups, which seems sufficient to assess the long-term outcomes and draw meaningful conclusions; and (2) Regarding TTL, our study groups were significantly different only within the first month after LT, which was the target period of time in the study, but not during the rest of the follow-up, what reinforces the adequacy of the study for our purpose and avoids the significant potential bias of having not only different early TTL but different TTL during the study period.

CONCLUSION

In summary, TTL within the first month after LT had no significant effect on long-term renal function, immunosuppression-related morbidity and 5-year patient or graft survival in our study. Early post-transplant tacrolimus level was not an independent factor for long-term patient in multivariate analysis. We conclude that relatively small

differences in mean tacrolimus levels restricted to the first month after LT do not determine differences in long-term immunosuppression-related morbidity and patient survival and therefore, larger exposure to tacrolimus seems to be needed to influence long-term outcomes. Larger studies should be advisable to confirm our results; however, these studies should be done on the basis of different TTL only during the early post-transplant period and not along the follow-up to avoid potential biases.

ARTICLE HIGHLIGHTS

Research background

Immunosuppression is a cornerstone in liver transplantation (LT) and current immunosuppressive regimens are mostly based on tacrolimus. At present, side effects relating anticalcineurin inhibitors are one of the main concerns for long-term outcomes after LT. Side effects are commonly related with drug dose and trough levels.

Research motivation

Tacrolimus trough levels (TTL) above 10 ng/mL during the first weeks after liver transplant have been related with mid and long-term outcomes including impairment of renal function and an increased rate of hepatocellular recurrence, *de novo* tumors and new-onset diabetes.

Research objectives

The aim of this study was to assess the influence of the TTL during the early post-transplant period in the long-term outcomes of LT.

Research methods

This was a retrospective study of 155 consecutive liver transplants treated with an immunosuppressive regimen based on *de novo* once-daily tacrolimus. Patients were classified into 2 groups according to their mean TTL within the first month after transplantation: ≤ 10 ng/mL (n = 98) and > 10 ng/mL (n = 57). All TTL obtained during the first month were used to define the mean values. Multivariate analyses were performed to assess risk factors for patient mortality.

Research results

TTL were significantly different among groups only during the first month after transplantation, but not during the rest of the follow-up After a median follow-up of 52.8 mo (range 2.8-81.1), no significant differences were observed in the evolution of the mean estimated glomerular filtration rate, hepatocellular carcinoma recurrence, development of *de novo* tumors, new-onset diabetes, new-onset arterial hypertension or biopsy-proven acute rejection rate. Five-year patient and graft survival were comparable. Early tacrolimus trough level was not an independent factor for patient mortality in multivariate analyses.

Research conclusions

Differences in tacrolimus levels restricted to the first month after transplantation did not result in significant differences in long-term outcomes of liver transplant recipients.

Research perspectives

Mid and long-term calcineurin inhibitors-related side effects after LT should be studied considering the cumulative exposure to tacrolimus along the follow-up and not only the trough levels observed during the early post-transplant period.

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META-ANALYSIS

Efficacy and safety of once daily tacrolimus compared to twice daily tacrolimus after liver transplantation

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Abstract

BACKGROUND

Once daily tacrolimus regimen was found to exhibits similar bioavailability, safety and efficacy properties compared to twice-daily tacrolimus in kidney transplantation patients.

AIM

To compare the efficacy and safety of once-daily prolonged release tacrolimus compared to twice-daily tacrolimus in liver transplantation patients.

METHODS

MEDLINE, EMBASE, CENTRAL databases were searched for clinical trials until December 2020. Efficacy outcome measured as the rate of treatment failure indicated by biopsy-proven acute rejection, Serum creatinine, graft loss, or death. Two reviewers independently selected studies, collected data and assessed risk of bias. The results are reported as risk ratio with 95% confidence interval (CI) for dichotomous data.

RESULTS

Seven studies included with 965 patients. All the included studies were of moderate quality according to the risk of bias assessment using Cochrane Risk of

any other conflict of interest is associated with this work.

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Bias tool. Biopsy-proven acute rejection was reported in four studies, and pooled analysis of those studies indicated similar rejections in both twice daily and once daily tacrolimus groups (risk ratio: 1.06, 95%CI: 0.84-1.34, n = 758, I^2 = 0%) and also we found no significant difference between both groups for renal outcome (serum creatinine; mean difference, 0.001 mg/dL, 95%CI: -0.042 to 0.043, n = 846, I^2 = 18.6%). Similarly, there was similar number of adverse events such as hypertension, headache, back pain, blood related disorders, infections and nausea observed in both groups.

CONCLUSION
The analysis findings confirm that both once daily and twice daily tacrolimus formulations are comparable in terms of efficacy and safety outcomes.

Key Words: Prolonged release; Tacrolimus; Liver transplantation; Graft rejection; Renal impairment; FK level

Core Tip: Tacrolimus, a calcineurin inhibitor is an important component of the immunosuppressive regimens post liver transplantation. Compliance to immunosuppression treatment generally is important and non-adherence is a major risk factor of graft rejection and loss. Compliance to medication declines over the course of time in patients after liver transplantation due to several factors and this contributes to about 20% of late acute rejection. The efficacy of once daily tacrolimus regimens has been reported in many studies and this systematic review/meta-analysis confirmed the evidence of comparable efficacy and safety of prolonged release tacrolimus to the twice daily immediate release formulation.

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INTRODUCTION

Advances in immunosuppression regimens after solid-organ transplantation have significantly improved patient and graft survival. Tacrolimus, a calcineurin inhibitor is an important component of the immunosuppressive regimens widely used following liver transplantation (LT). Compliance to immunosuppression treatment however is important and non-adherence is a recognized contributing factor in rejection and graft loss[1,2].

Compliance to medication declines over the course of time in patients after LT due to several factors including the number of drugs to consume and the rate of rejection/infections increases. Previous reviews directed at recipients transplanted between the late 1980s and mid-2000s showed that the prevalence of non-adherence to immunosuppressive medications averaged about 25%. This non-adherence to medications was felt to contribute to about 20% of late acute rejection episodes and 16%-36% of graft losses[3]. To maintain good adherence, less frequently administering regimen were proved to be effective[4].

Recently, tacrolimus once-daily prolonged-release (PR) formulation was developed. Based on the previous literature, it was evident that conversion from the twice-daily, immediate release (IR) to PR tacrolimus was well tolerated, safe and conveniently used in stable patients after LT[5,6]. However, there is no systematic review that has been conducted till date to confirm the efficacy and safety of PR tacrolimus compared to IR tacrolimus.

MATERIALS AND METHODS

Database search

This systematic review and meta-analysis was performed according to Cochrane Collaboration[7] and Preferred Reporting Items for Systematic Reviews and Meta-Analyses Statement[8].

We searched MEDLINE, EMBASE, CENTRAL databases since inception to December 2020 using an extensive search strategy to identify relevant literature. We used the following terms: Tacrolimus, liver transplantation and dosage forms (Supplementary file) while searching databases with human and English language restrictions. In addition, we also searched clinicaltrials.gov.in and Google Scholar and references of previously published relevant papers to find more relevant trials.

Eligibility criteria

Clinical trials conducted on adult (> 18 years) patients who received a primary LT from a deceased or living donor, having an average serum tacrolimus level of 1-10 ng/mL for more than 6 wk, that compared once daily tacrolimus to twice daily tacrolimus in LT patients were included.

Exclusion criteria

Studies were excluded if they had patients with a previous organ transplant other than liver and multiple organ transplantations. Studies also conducted on paediatric population and lack of a control group (the study had only included patients who received once daily tacrolimus. We also excluded studies only assessed pharmacokinetics of tacrolimus. Finally, studies without full-text such as conference proceedings, editorials, reviews, secondary analyses and letters excluded.

Outcomes: Efficacy was measured as the rate of treatment failure indicated by biopsy-proven acute rejection (BPAR), liver graft loss, or death while safety was assessed by the incidence of adverse events.

Study selection and data extraction

Two reviewers independently (KB and RT) screened the identified studies according to the aforementioned criteria and excluded studies that were found to be clearly irrelevant. We obtained the full text of the remaining studies and the same two reviewers screened full texts and selected trials for inclusion. The same two reviewers independently extracted data from included trials into the predesigned and validated data collection form. Disagreements were resolved by arbitration, and consensus was reached after discussion. We collected study characteristics (type of design with duration of intervention and methods), baseline demographics, and efficacy and safety outcome data from each included trial.

Quality assessment

Two reviewers (KB and RT) independently assessed quality of included studies using Cochrane Risk of Bias tool[9], and disagreements were resolved by discussion. If a consensus could not be reached, any discrepancy was resolved by a senior author. Seven domains of quality assessment included random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting, and other sources of bias.

Statistical analysis

We performed statistical analysis using Comprehensive Meta-analysis Version 3.0[10]. We reported the results as risk ratio with 95% confidence interval (CI) for dichotomous data and continuous data as mean difference. We used a random-effects model to combine individual results regardless whether there was significant heterogeneity or not. We tested heterogeneity among trial results using the I^2 statistic[7]. We considered a value greater than 50% as substantial heterogeneity. Publication bias was not assessed due to limited number of included studies in this review.

RESULTS

A total of 701 articles from databases search and 15 from additional searches identified. After removing duplicates 543 studies remained for screening. Upon

screening titles and abstracts, 490 clearly irrelevant articles removed. The remaining 48 articles subjected to full text screening. Finally, seven clinical trials met the inclusion criteria[11-17]. The flow of the randomised controlled trial included in our analysis is shown in Figure 1.

Study characteristics

A total of seven clinical trials were included with 965 patients. Study characteristics were summarized in Table 1. Studies included are conducted in various countries including United States, Japan, United Kingdom, and one study in another 16 countries.

The mean age of included patients was 52.8 years and majority (71%) of them were males. Four studies had follow-up for one year while the other two had follow up for 3 and 6 mo. In four studies, concomitant treatment with mycophenolate mofetil and steroids was allowed. All the included studies were of moderate quality according to the risk of bias assessment using Cochrane Risk of Bias tool (Figure 2).

Efficacy outcomes

Acute rejection confirmed by biopsy was reported in four studies[12,15-17], and pooled analysis of those studies indicated similar rejection rate in both twice daily and once daily tacrolimus groups (risk ratio 1.06, 95%CI: 0.84-1.34, n = 758, I^2 = 0%; Figure 3).

DISCUSSION

This systematic review and meta-analysis compared PR tacrolimus to IR tacrolimus in LT recipients. The efficacy and safety outcomes were found to be similar for both regimens.

Adherence to the immunosuppressant regimen post-LT is important for preventing rejection and graft loss. The reported rate of non-adherence to immunosuppressant regimens is 15%-40%, which could lead to significantly higher rate of graft rejection, graft loss and severe impact on long-term survival[18]. It was observed that once daily tacrolimus is safe and is associated with better adherence and low variability of liver function tests[18,19].

A study by Muduma *et al*[20] looked at the cost effectiveness of PR tacrolimus in LT recipients. Based on a United Kingdom specific analysis of the projected cost-utility of PR tacrolimus relative to IR tacrolimus and cyclosporin, once daily tacrolimus was cost-effective, improved life expectancy and quality adjusted life year and incremental cost effectiveness ratio below £20000 per a quality adjusted life year gained. Over a 3-year time horizon, one graft would be saved for every 14 patients treated with PR tacrolimus with minimal impact on cost when compared to IR tacrolimus.

The results of recently published systematic review showed that PR tacrolimus when compared to the IR tacrolimus resulted in no significant difference in the glomerular filtration rate, BPAR and the safety outcomes among the kidney transplant recipients[21]. The findings of our review are also in congruent with the previous review. In contrast, another meta-analysis based on combination of two clinical trials and four observational studies found that once daily tacrolimus is effective for the first year after liver transplantation, however, there was no significant difference in 1-year mortality and adverse events between once daily and twice daily tacrolimus groups[22].

PR tacrolimus has been introduced as helpful therapeutic option to increase the patient adherence to immunosuppressive treatment. Studies with short follow-up and pharmacokinetic evaluation were not included in this review, however one study which evaluated pharmacokinetic outcomes along with efficacy outcomes showed similar BPAR, graft losses and safety outcomes such as hypertension, infections and blood related disorders[16] between groups. Of the included studies in our systematic review, four reported concomitant immunosuppressant therapies administration such as corticosteroids, and mycophenolate mofetil. It was evident that those concomitant drugs have negative association with occurred adverse events with tacrolimus[23]. An eight years long-term follow up study based on European Liver Transplant Registry has recently been published study and the findings were in favour of PR in terms of graft losses and acute rejections. This very large population study also reported better outcome in those converted from IR to PR tacrolimus after 1 mo compared to those maintained on IR tacrolimus-based immunosuppression. They concluded that patients on PR tacrolimus continues to provide ongoing benefits for graft and patient survival beyond 3 years post transplantation[24]. The major limitation of the study, were the lack of data on the dosages and the trough levels of tacrolimus were not captured. In

Table 1 Characteristics of the included studies

Ref.	Year	Country	Study design	Follow-up period	Sample size	Donor type	Mean age	Concurrent therapy
Alloway *et al*[11]	2014	United States	Phase-II, 3-sequence, open-label, multicenter, prospective study	1 yr	59	NR	49.8	Mycophenolate mofetil
Kim *et al*[13]	2016	South Korea	2-armed, parallel group, prospective, randomized, open-label, phase IV	1 yr	79	Deceased	54	NR
Sańko-Resmer *et al*[14]	2012	United Kingdom	Multicentre, open-label, single-sequence, crossover, phase IIIb	3 mo	98	NR	55	None
Shin *et al*[17]	2018	South Korea	Phase IV, randomized, open-label, comparative, single-center study	6 mo	100	NR	52	Corticosteroid, mycophenolate mofetil, and basiliximab.
Trunečka *et al*[15]	2010	16 countries	1:1-randomized, double-blind, double-dummy, two-arm, parallel-group phase III, comparative study	1 yr	471	NR	52	Mycophenolate sodium
DuBay *et al*[12]	2019	United States	Phase II, open label, multicenter, randomized trial	1 yr	29	Deceased	54.4	Mycophenolate mofetil, mycophenolic acid sodium, prednisone, or azathioprine
Fischer *et al*[16]	2010	Germany	Randomized, phase II, multicenter, open-label, prospective trial	6 wk	129	NR	47	Anti-fungal, antibiotics, anti-hypertensives, antiepileptics and rifampicin)

NR: Not reported.

addition, the lack of clarity on the IR tacrolimus preparations the cohort received. The retrospective design of the study was the main reason for its exclusion in our analyses as it did not meet the eligibility criteria.

Strengths and limitations

The major strength of our study is that, we have only included clinical trials of long-term follow-up to address efficacy and safety of PR tacrolimus. There was no heterogeneity found for all the outcomes assessed, except for any adverse events. One of the limitations of our review is that, we have only included studies published in English language, which means some of the studies published in other language might have been missed. Publication bias assessment was also not assessed due to less than ten studies included in the analysis, however due to our intense search effort it was evident that we did not miss any study meeting this review's eligibility criteria. Majority of the studies were of open-label design, that could have introduced bias, however this could not be avoided due to the nature of administration. In addition, the paucity of studies of PR tacrolimus in Asian patients renders data from this review of high interest to the transplant community.

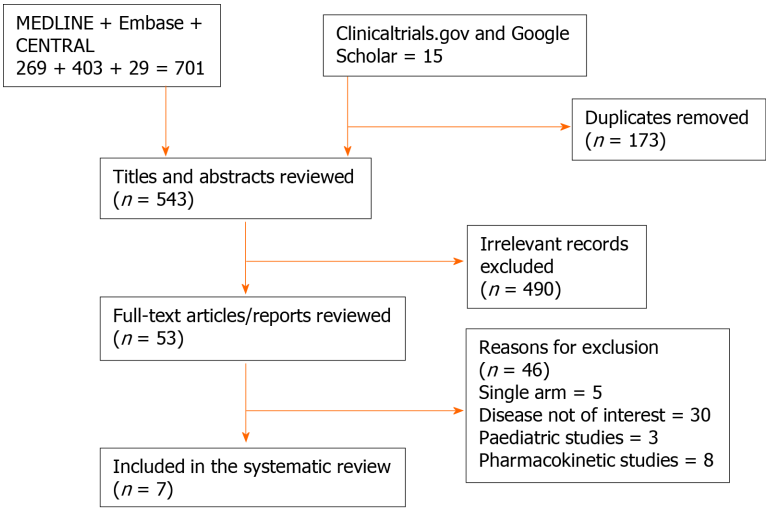

Figure 1 Preferred reporting items for systematic reviews and meta-analyses flow chart.

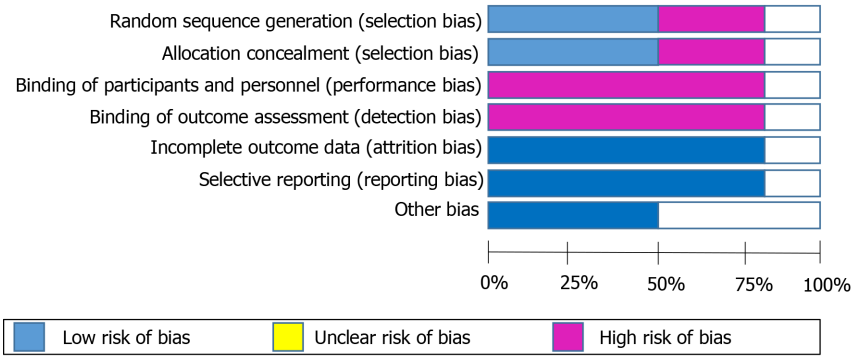

Figure 2 Risk of bias assessment according to Cochrane risk of bias tool.

CONCLUSION

Our systematic review and meta-analysis indicate that both PR and IR tacrolimus formulations are comparable in terms of efficacy and safety outcomes. However, to confirm these findings, long-term follow-up randomized controlled trials with large sample sizes are required. Also, to assess acceptability by patients, quality of life and economic evaluations should be conducted.

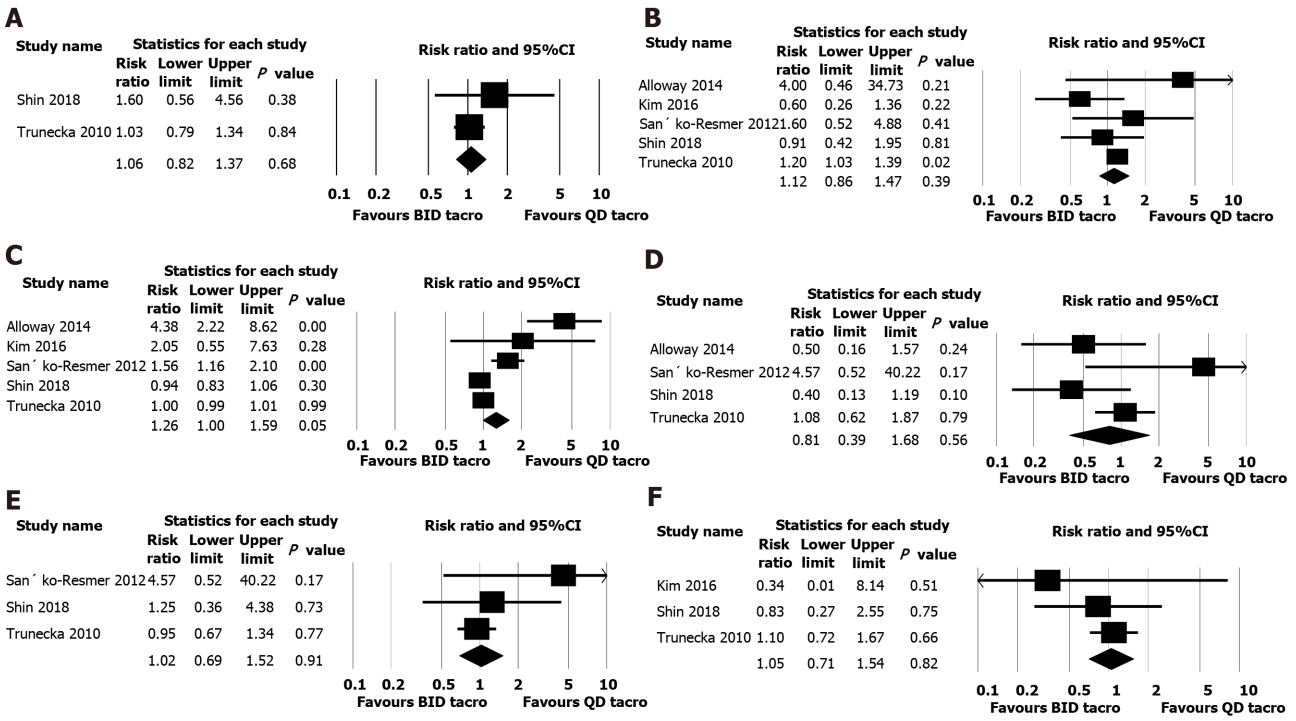

Figure 3 Efficacy outcomes. A: Acute graft rejection; B: Infection; C: Any adverse drug reaction; D: Headache; E: Back pain; and F: Blood disorders. CI: Confidence interval.

ARTICLE HIGHLIGHTS

Research background

Tacrolimus, a calcineurin inhibitor is an important immunosuppressive medication post liver transplantation. Compliance to immunosuppression is important and non-adherence can lead to rejection and graft loss. To maintain good adherence, less frequently administering regimen were proved to be effective.

Research motivation

Recently, tacrolimus once-daily prolonged-release (PR) formulation was developed. Several studies have shown evidence that conversion from the twice-daily, immediate release to PR tacrolimus was well tolerated, safe and conveniently used in stable patients after liver transplantation.

Research objectives

Our objective was to conduct a metanalysis and systematic review of the published clinical trials that studied the safety and efficacy of PR tacrolimus compared to immediate release tacrolimus.

Research methods

MEDLINE, EMBASE, CENTRAL databases were searched for clinical trials until December 2020. Efficacy outcome measured as the rate of treatment failure indicated by biopsy-proven acute rejection, Serum creatinine, graft loss, or death. Two reviewers independently selected studies, collected data and assessed risk of bias. The results are reported as risk ratio with 95%CI for dichotomous data.

Research results

Seven studies included with 965 patients. All the included studies were of moderate quality according to the risk of bias assessment using Cochrane Risk of Bias tool. Biopsy-proven acute rejection was reported in four studies, and pooled analysis of those studies indicated similar rejections in both twice daily and once daily tacrolimus groups. We also found no significant difference between both groups for renal outcome (serum creatinine; mean difference, 0.001 mg/dL, 95%CI: -0.042 to 0.043, n = 846, I^2 = 18.6%). Similarly, there was similar number of adverse events such as hypertension, headache, back pain, blood related disorders, infections and nausea

observed in both groups.

Research conclusions

The analysis findings confirm that both once daily and twice daily tacrolimus formulations are comparable in terms of efficacy and safety outcomes.

Research perspectives

Long-term follow-up randomized controlled trials with large sample sizes are required. Also, to assess acceptability by patients, quality of life and economic evaluations should be conducted.

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CASE REPORT

Conversion hepatectomy for hepatocellular carcinoma with main portal vein tumour thrombus after lenvatinib treatment: A case report

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Abstract

BACKGROUND

Hepatocellular carcinoma (HCC) accompanied by portal vein tumour thrombus (PVTT) presents an aggressive disease course, worsening liver function reserve, and a high recurrence rate. Clinical practice guidelines recommend systemic therapy as the first-line option for HCC with portal invasion. However, to achieve longer survival in these patients, the treatment strategy should be concluded with removal of the tumour by locoregional therapy. We experienced a case of initially unresectable HCC with main PVTT converted to radical hepatectomy after lenvatinib treatment.

CASE SUMMARY

A 59-year-old male with chronic hepatitis C infection visited our clinic as a regular post-surgery follow-up. Contrast-enhanced abdominal computed tomography revealed a liver mass diffusely located at the lateral segment with a massive PVTT extending from the umbilical portion to the main and contralateral third-order portal branches. With the diagnosis of unresectable HCC with Vp4 (main trunk/contralateral branch) PVTT, lenvatinib was started at 12 mg/d. The computed tomography taken 3 mo after starting lenvatinib showed regression of the PVTT, which had retreated to the contralateral first-order portal branch. He tolerated the full dose without major adverse effects. With cessation of lenvatinib

for 7 d, radical left lobectomy and PVTT thrombectomy were conducted. The patient's postoperative course was uneventful. Microscopically, the primary lesion showed fibrotic changes, with moderately to poorly differentiated tumour cells surrounded by granulation tissues in some areas. The majority of the PVTT showed necrosis. He was alive without recurrence for 8 mo.

CONCLUSION
This is the first case of HCC with Vp4 PVTT in which radical conversion hepatectomy was succeeded after lenvatinib treatment.

Key Words: Hepatocellular carcinoma; Lenvatinib; Portal vein tumour thrombus; Conversion hepatectomy; Case report

Core Tip: Patients with hepatocellular carcinoma (HCC) with portal vein tumour thrombus demonstrate an aggressive disease course, decreased liver function reserve, and higher recurrence rates after treatment. Clinical practice guidelines recommend systemic therapy as the first-line option for HCC with portal invasion. However, to achieve longer survival in these patients, the treatment strategy should be concluded with removal of the tumour. We report the first case of HCC with main portal vein tumour thrombus, in which radical conversion hepatectomy was successfully performed after lenvatinib treatment. Lenvatinib has several strengths that validate its use for targeting conversion hepatectomy for unresectable HCC.

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INTRODUCTION

Portal vein tumour thrombus (PVTT) is a condition of hepatocellular carcinoma (HCC) that leads to the wide dissemination of tumours throughout the liver and causes a deterioration of liver function, leading to poor prognosis. PVTT is classified as Vp1 (segmentary), Vp2 (secondary order branch), Vp3 (first order branch), and Vp4 (main trunk/contralateral branch)[1], and clinical practice guidelines recommend systemic therapy as the first-line option for HCC with portal invasion[2,3]. Current systemic therapy for HCC consists of receptor tyrosine kinase inhibitors (TKIs) and checkpoint inhibitors[4]. As a newly introduced TKI, lenvatinib is a multitargeted TKI that inhibits vascular endothelial growth factor receptor 1-3, platelet-derived growth factor receptor-alpha, rearranged during transfection, and stem cell factor receptor. Lenvatinib is characterized by high tumour regression and tumour necrosis effects[4,5]. However, post progression survival is recognized as being short[6], and the post hoc exploratory analysis disclosed severe morbidities related to lenvatinib treatment in patients with HCC with Vp4 PVTT (data not shown). To achieve longer survival in patients with advanced HCC, the treatment strategy should be concluded with removal of the tumour by locoregional therapy (LRT) because of the limitation of systemic therapy alone[7-9]. Here, we present a case of initially unresectable HCC with Vp4 PVTT converted to radical hepatectomy after lenvatinib treatment.

CASE PRESENTATION

Chief complaints

A 59-year-old male presented to our clinic as a regular post-surgery follow-up for

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HCC.

History of present illness

The patient received segmentectomy 5 and cholecystectomy for a single HCC 2 years prior.

History of past illness

He had hepatitis C virus infection with genotypes 1a which was treated with 24 wk of ledipasvir/sofosbuvir 5 years prior, and sustained virologic response rate was achieved. He received the radiofrequency ablation a year before the first hepatectomy.

Personal and family history

The patient had a history of alcohol use with 200 mL daily intake for 35 years. Since HCC was diagnosed, the patient had quitted alcohol drinking. He had no family history of cancer.

Physical examination

The patient's temperature was 36.5 °C, heart rate was 74 bpm, respiratory rate was 14 breath/min, blood pressure was 128/81 mmHg and oxygen saturation in room air was 98%. There was an operative scar for a J-shaped incision on the abdomen from the previous liver resection. No ascites and encephalopathy were detected.

Laboratory examinations

Laboratory exams were normal except for a slight increase in aspartate aminotransferase levels of 52 U/L and protein induced by des-γ-carboxy prothrombin of 107 mAU/mL. Electrocardiogram, chest X-ray and arterial blood gas were also normal.

Imaging examinations

Contrast-enhanced (CE) abdominal computed tomography (CT) revealed a liver mass diffusely located at the lateral segment with a massive PVTT extending from the umbilical portion to the main portal and the contralateral third portal branches (Vp4) (Figure 1A and B).

MULTIDISCIPLINARY EXPERT CONSULTATION

Amane Takahashi, MD, PhD, Chief Surgeon, Department of Gastroenterological Surgery, Saitama Cancer Center

As a treatment strategy, we should administer lenvatinib at a dose of 12 mg, following the clinical guidelines. The reason for choosing lenvatinib, not sorafenib was that lenvatinib demonstrated higher response rate compared with sorafenib in an open-label, phase III, multicentre, non-inferiority trial involving patients with advanced HCC (the REFLECT trial). If the PVTT exhibited shrinkage to the contralateral first portal branch, we would be able to remove the tumour surgically. We should be careful to follow the liver function during lenvatinib treatment, since the post hoc exploratory analysis revealed severe morbidities including liver failure in cases with Vp4 PVTT.

FINAL DIAGNOSIS

With the diagnosis of unresectable HCC with Vp4 PVTT, lenvatinib was started at 12 mg/d. CT taken two weeks after starting lenvatinib showed regression of PVTT (by 11%) with partial disappearance of contrast enhancement, retreating to the contralateral second-order PV (Figure 2A). At 3 mo, the PVTT regressed further to the contralateral first-order branch with more loss of contrast enhancement (by 58%), meeting the definition of partial response according to the modified Response Evaluation Criteria in Solid Tumours criteria (Figure 2B and C). During lenvatinib treatment, liver function was maintained within Child-Pugh A (5 points), and the albumin-bilirubin (ALBI) score was -3.45 to -2.93 (Grade 1). He tolerated the full dose without treatment-related adverse effects (TRAEs).

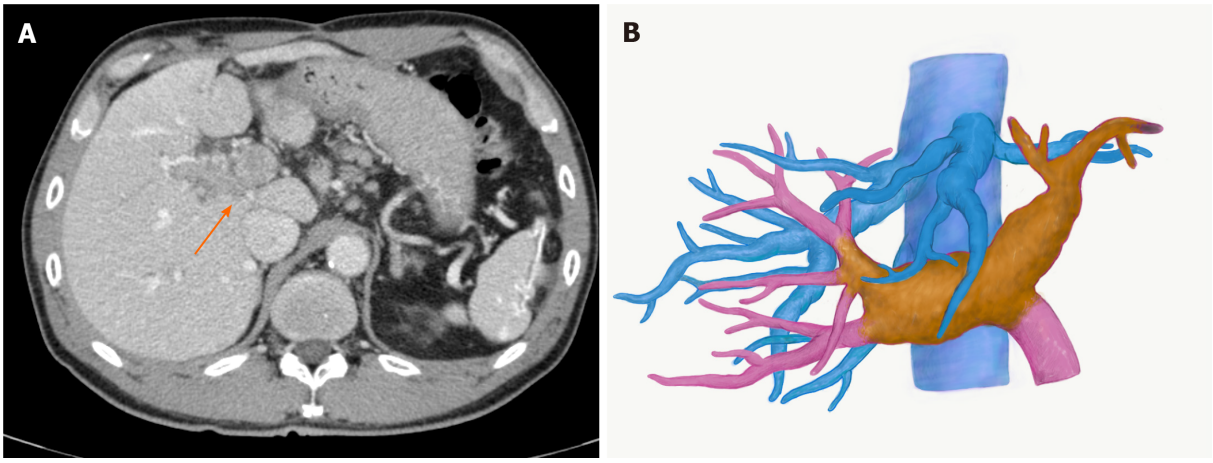

Figure 1 Images of hepatocellular carcinoma with portal vein tumour thrombus before lenvatinib treatment. A: Computed tomography image. An arrow indicates portal vein tumour thrombus; B: Three-dimensional image. The yellow mass demonstrates a viable portal vein tumour thrombus, extending to the contralateral third portal branch.

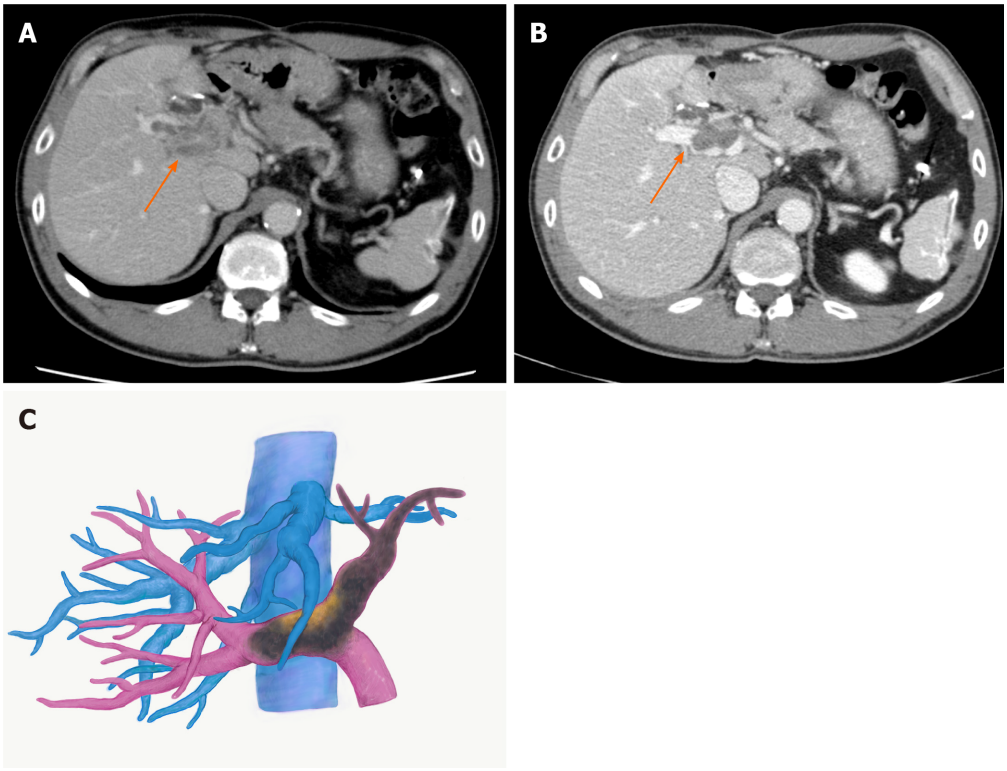

Figure 2 Images of hepatocellular carcinoma with portal vein tumour thrombus after lenvatinib treatment. A: Computed tomography image two weeks after the treatment. The portal vein tumour thrombus (PVTT) showed regression with partial disappearance of contrast enhancement; B: Computed tomography image three months after the treatment; C: Three-dimensional image three months after the treatment. The PVTT regressed to the contralateral first-order branch with loss of contrast enhancement. Arrows indicate PVTT.

TREATMENT

After cessation of lenvatinib for 7 d, left lobectomy with PVTT thrombectomy was performed. Intraoperatively, no intrahepatic satellite lesions, ascites or disseminated nodules were identified. The left hepatic artery, left PV and left hepatic duct were isolated at the hilum. After ligating and disconnecting the left hepatic artery, the right, left and main PVs were exposed. After checking the PVTT by ultrasound, the PVs were clamped by Satinsky forceps (Figure 3A). Venotomy was placed at the bifurcation of the left PV, and the PVTT was thrombectomized (Figure 3B). After flushing the PV with normal saline and confirming that no PVTT remained, the left PV stump was closed by 6-0 proline (Figure 3C). Liver dissection was completed along the

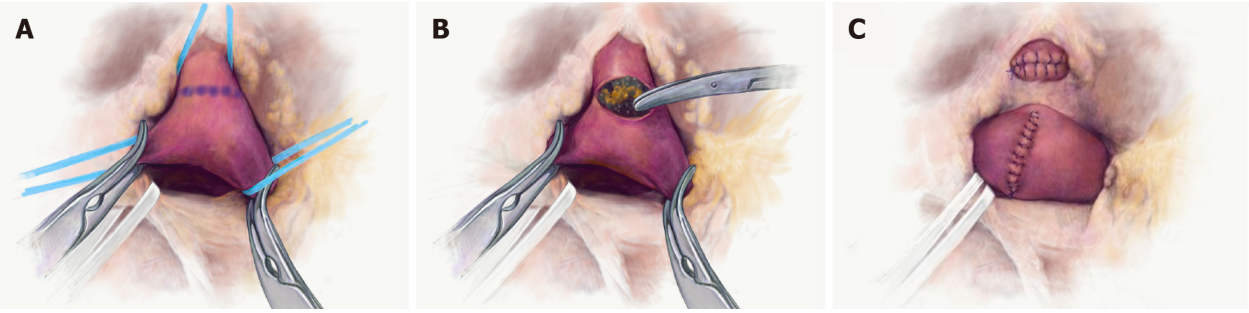

Figure 3 Portal vein tumour thrombus thrombectomy. A: The right and the main portal veins were clamped by Satinsky forceps. Venotomy was placed at the bifurcation of the left portal vein; B: The portal vein tumour thrombus was thrombectomized; C: The left portal vein stump was closed by 6-0 proline.

middle hepatic vein. The left hepatic duct and hepatic vein were cut and closed with 6-0 proline. The specimen was finally removed.

OUTCOME AND FOLLOW-UP

The patient's postoperative course was uneventful. Macroscopically, the primary tumour at the parenchyma was obscure. The PVTT demonstrated a white to brownish nodule with a size of 60 mm × 30 mm × 25 mm (Figure 4A). Microscopically, the primary lesion demonstrated fibrotic changes with haemosiderin deposition. In some areas, moderately to poorly differentiated tumour cells and tumour cells with necrotic changes were surrounded by granulation tissues and fibrosis (Figure 4B and C). The majority of the PVTT showed necrosis (Figure 4D). According to the Union for International Cancer Control classification, the tumour was finally staged as T3 N0 M0 Stage IIIB. He is alive with no evidence of recurrence 8 mo post-surgery.

DISCUSSION

Patients with HCC with PVTT usually have an aggressive disease course, decreased liver function reserve, limited treatment options, higher recurrence rates after treatment and poor overall survival (OS). The median OS is reported to be as poor as 2-4 mo with best supporting care[10]. The Barcelona Clinic Liver Cancer (BCLC) staging classifies patients with PVTT with Child-Pugh A or B liver function reserve as advanced HCC with BCLC stage C. The recommended treatment option for this group is systemic therapy with sorafenib or lenvatinib as the first-line treatment[4]. LRT including hepatectomy was not recommended over systemic therapy since there was inadequate evidence to inform the balance of benefit *vs* harm[2]. LRT could only be considered for HCC with Vp1/2, only as an option within research settings[2]. In a Japanese nationwide surveillance study consisting of more than 6000 patients with HCC with PVTT, propensity score matching analysis demonstrated a longer median OS in the surgical group than in the non-surgery group (2.87 years *vs* 1.10 years, $P < 0.001$)[11]. However, surgical benefit was acknowledged when PVTT was limited to the first-order branch (Vp3), and no surgical benefit was observed among patients with Vp4 PVTT. The problem in this study was that more than half of the patients with Vp3/4 underwent non-curative resection, and the impact of curative resection was not clarified in this cohort. Several retrospective studies have demonstrated survival benefits of curative hepatectomies with aggressive PV thrombectomy or *en block* resection for Vp4 PVTT[12-14]. Hatano *et al*[14] conducted a retrospective multi-institutional study regarding the outcome of macroscopically curative hepatic resection in 400 patients with HCC with Vp3/4 PVTT. The results demonstrated a median survival time and 5-year OS rate of 21.5 mo and 25.7%, respectively. OS time showed no statistically significant difference between Vp3 and Vp4.

Lenvatinib was initially approved as the first-line therapy for advanced HCC in Japan in 2018. The REFLECT trial met its primary endpoint of non-inferiority to sorafenib in OS[6]. Lenvatinib was superior to sorafenib in progression-free survival (PFS) and time to tumour progression (TTP). Although the complete response rate was low, the objective response rate (ORR) in the lenvatinib group was significantly higher than that in the sorafenib group (40.6% *vs* 12.4%, $P < 0.001$). TRAEs, including hand-

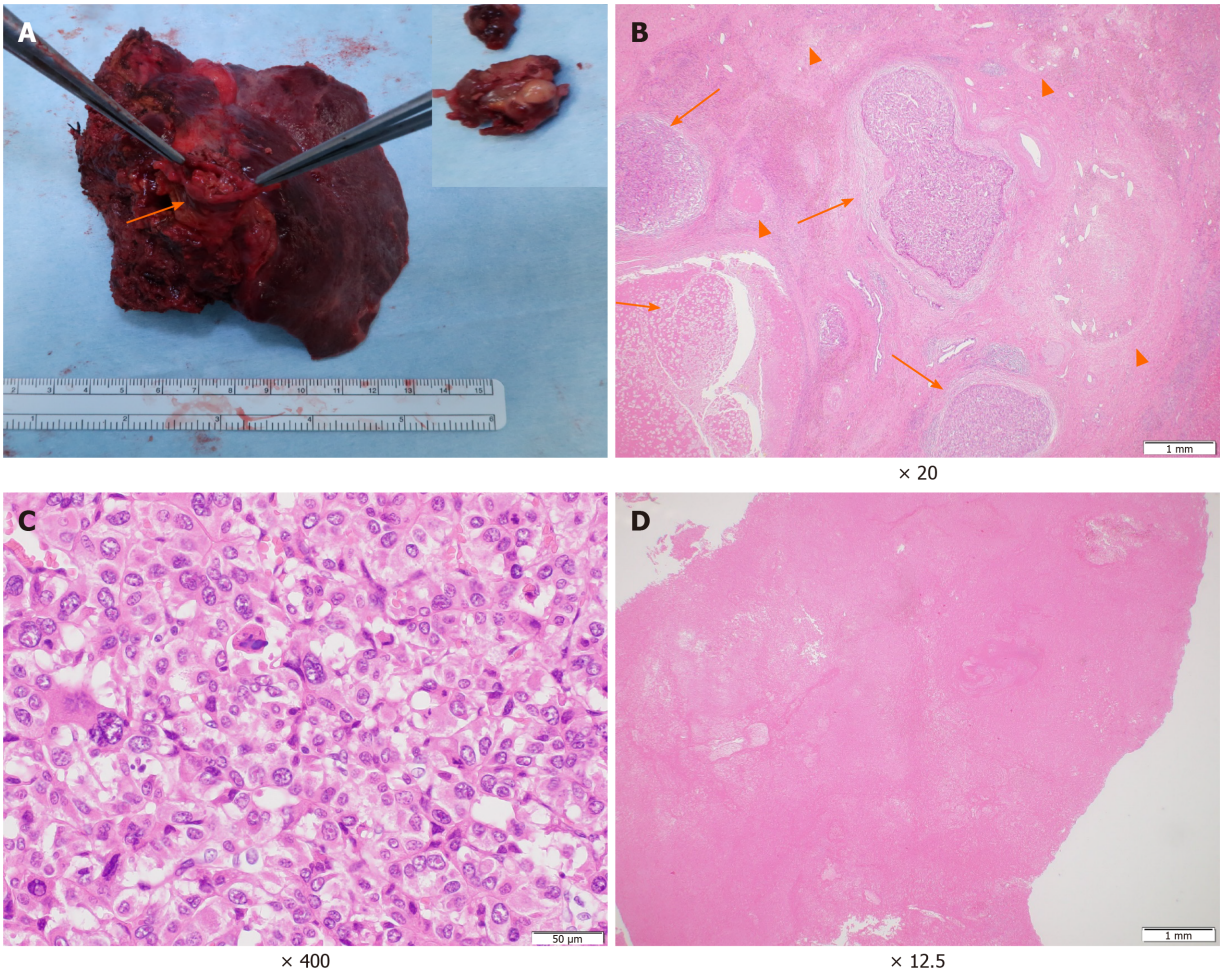

Figure 4 Macroscopic and microscopic findings of the main tumour and the portal vein tumour thrombus. A: A white to brownish nodule was found in the left portal vein (arrows). Inlet: close-up picture of the removed portal vein tumour thrombus; B: The primary lesion showed severe fibrotic change with haemosiderin deposition. In the fibrosis, the viable tumour cell nests (arrow) and the necrotic tumour lesions (arrowhead) were scattered; C: High magnification demonstrated moderately to poorly differentiated tumour cells; D: Most of the portal vein tumour thrombus showed necrotic changes.

foot syndrome, hypertension, proteinuria, and anorexia, were comparable between lenvatinib and sorafenib. These side effects are not life-threatening, and they can usually be controlled by supportive medical treatments. Subsequent studies have reported a relatively high ORR with lenvatinib of 29.4%-45.0%[15-17]. On the other hand, the REFLECT trial excluded HCC cases that had main PV invasion, and the outcomes of this cohort were unclear. The efficacy of lenvatinib treatment for unresectable HCC with major PVTT has been reported in some case reports and retrospective studies[18-20]. Kuzuya *et al*[20] compared the outcomes of advanced HCC with Vp3/4 PVTT between sorafenib and lenvatinib as the first-line systemic therapy. The ORR was significantly higher using lenvatinib (53.8% *vs* 14.3% $P = 0.0193$), and the median OS and TTP were significantly longer in the lenvatinib group than in the sorafenib group. No patient discontinued lenvatinib treatment secondary to TRAEs. These reports may characterize lenvatinib as having a relatively strong antitumour effect against HCC including PVTT, with less emergence of serious side effects.

Other characteristics of lenvatinib treatment are the rapid antitumour effects and preservation and fast recovery of liver function[20]. The antitumour effects of lenvatinib have been described as quick, which could be confirmed in 2 wk, and these early radiologic changes could be biomarkers to predict clinical outcomes, including OS[16]. Another group similarly stated that the changes in arterial tumour perfusion on CE-ultrasound at 1 wk were associated with the radiological antitumour response on CE-CT at 8 wk[21]. Regarding the preservation of liver function, patients treated with lenvatinib maintained liver functional reserves better than those treated with sorafenib[22]. Furthermore, ALBI scores in the lenvatinib group improved faster than those in the sorafenib group[20]. In our case, the patient tolerated the full dose while maintaining liver function without major side effects. The tumour including the PVTT showed early necrotic changes 2 wk after lenvatinib treatment.

Based on these reports, lenvatinib is characterized by the following strengths: (1) Relatively strong antitumour effect not only on the main tumour but also on PVTT; (2) Quick antitumour effects that could be noted in 1-2 wk; and (3) Preservation and early recovery of liver function with less incidence of life-threatening TRAEs. Because of these strengths, lenvatinib can be considered an optimal chemotherapeutic agent targeting radical conversion hepatectomy for unresectable HCC. The good indication might be unresectable HCC with a large size or with PV invasion. Multiple intra-extra hepatic HCC can be considered as long as curative resection is feasible, since pathological complete response is usually difficult to attain by lenvatinib alone, and the tumours can quickly regrow during the drug cessation period[19,23]. Lenvatinib demonstrates quick antitumour effects, and it deteriorates liver function temporally[17]. The treatment effects on the tumour and liver function reserve should be evaluated in a short period to avoid missing the best timing for conversion. Since severe morbidities related to lenvatinib treatment were reported in advanced HCC with PVTT (data not shown), physicians should be reminded to perform careful observation during the treatment period, especially in cases with Vp3/4 PVTT, since liver function could deteriorate quickly.

Identification of serum biomarkers for the prediction of lenvatinib response would be of significant benefit for the proper selection of patients for treatment. The post hoc exploratory analysis of the REFLECT trial revealed that the occurrence of hypertension, diarrhoea, proteinuria, or hypothyroidism was generally associated with longer OS in patients with unresectable HCC treated with lenvatinib[24]. Another group stated that maintaining a higher relative dose intensity (RDI) in the early period after starting lenvatinib was associated with a higher ORR and longer PFS[25]. In our case, the patient did not complain of any TRAEs that deteriorated his quality of life, and he could continue lenvatinib with RDI of 100% without decreasing the lenvatinib dose. It is reasonable to think that the high RDI might be the main reason for this significant antitumour effect, leading to PR and conversion hepatectomy.

Four conversion cases with lenvatinib treatment, including ours, were reported in the previous literature (Table 1)[23,25,26]. Three cases were treated with lenvatinib monotherapy, and one case was treated with a combination of lenvatinib and nivolmab. Unresectable factors in these cases were large tumour size with inadequate residual liver volume, lung metastasis, and Vp4 PVTT. The duration of lenvatinib treatment in cases of large tumours and PVTT cases was short, 3-6 mo, and RDIs before conversion were all high (over 70%) in these cases. All cases demonstrated good postoperative courses with no evidence of tumour recurrence. Since the length of its market use is still short, it is necessary to gain experience and cases to clarify which cohort is suitable for targeting conversion.

Recently, there have been reports regarding the efficacy of proton beam therapy for advanced HCC with PVTT[27,28]. Proton beam therapy has advantages in that it is less invasive to patients; however, it requires high medical expenses and a large-scale facility that is not widely available worldwide. Because of its strong and quick antitumour effects with fewer TRAEs, conversion hepatectomy using lenvatinib could be an ideal strategy. A clinical trial is currently underway in Japan regarding conversion surgery during lenvatinib administration for unresectable HCC. Several molecular targeting agents and checkpoint inhibitors are being developed and will be coming to the market soon. These sequential flows could explore a new strategy against unresectable HCC.

CONCLUSION

In conclusion, we experienced the first case of HCC with Vp4 PVTT in which radical conversion hepatectomy was successfully performed after lenvatinib treatment. Lenvatinib has several strengths that validate its use for targeting radical conversion hepatectomy for unresectable HCC. A multicentre prospective trial is needed to clarify its clinical utility.

Table 1 Case reports of conversion hepatectomy after lenvatinib treatment

Ref.	Age/sex	Background disease	Regimen	Reason for unresectivity	Former treatment	Child-Pugh classification	Duration	RDI (%)	Type of hepatectomy	Prognosis
Sato *et al*[23] (2019)	66/F	HCV	Lenvatinib	Large size	TACE	8 (B)	6 mo	70	Extended right hepatectomy	3 mo alive with no recurrence
Chen *et al*[26] (2019)	69/F	HBV	Lenvatinib, nivolmab	Large size	Sorafenib TACE	8 (B)	3.5 mo	100	Extended right hepatectomy	3 mo alive with no recurrence
Takahashi *et al*[25] (2019)	82/F	Non B/C	Lenvatinib	Lung metastasis	None	5 (A)	13 mo	38	Extended posterior segmentectomy	5 mo alive with no recurrence
Present study	59/M	HCV	Lenvatinib	PVTT (Vp4)	None	5 (A)	3 mo	100	Left hepatectomy	8 mo alive with no recurrence

F: Female; M: Male; HCV: Hepatitis C virus; HBV: Hepatitis B virus; PVTT: Portal vein tumour thrombus; TACE: Transarterial chemoembolization; RDI: Relative dose intensity; Vp4: Main trunk/contralateral branch.

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MINIREVIEWS

Pathologic and molecular features of hepatocellular carcinoma: An update

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Abstract

Morphological diversity and several new distinct pathologic subtypes of hepatocellular carcinoma (HCC) are now well-recognized. Recent advances in tumor genomics and transcriptomics have identified several recurrent somatic/genetic alterations that are closely related with histomorphological subtypes and have therefore, greatly improved our understanding of HCC pathogenesis. Pathologic subtyping allows for a diagnosis which is clinically helpful and can have important implication in patient prognostication as some of these subtypes are extremely aggressive with vascular invasion, early recurrence, and worst outcomes. Several targeted treatments are now being considered in HCC, and the reporting of subtypes may be quite useful for personalized therapeutic purpose. This manuscript reviews the recently identified histomorphological subtypes and molecular alterations in HCC.

Key Words: Pathology; Hepatocellular carcinoma subtypes; Macrotrabecular massive; Steatohepatitic; Fibrolamellar; Molecular alterations

Core Tip: We summarize several new distinct histologic subtypes of hepatocellular carcinoma (HCC) and recurrent molecular alterations in HCC. Major histologic subtypes like macrotrabecular massive, fibrolamellar HCC, steatohepatitic HCC, scirrhous HCC, lymphoepithelioma-like HCC, and combined hepatocellaular-cholangiocarcinoma are discussed in detail. Rare and provisional histological variants are also discussed.

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INTRODUCTION

The incidence of hepatocellular carcinoma (HCC) has been increasing steadily over the past two decades and currently ranks as the fifth most common cancer in men and seventh in women[1,2]. HCC is now the fourth-most common cause of cancer-related deaths and the most frequent primary liver neoplasia, causing more than 80%-85% of liver cancer cases globally[3]. Major risk factors associated with HCC are chronic infection with hepatitis B virus and hepatitis C virus, chronic alcohol consumption, and non-alcoholic fatty liver disease associated with metabolic syndrome, diabetes and obesity. Prognosis of patients with HCC remains poor with 5-year survival rate of 18%, as the majority of these tumors are detected at a clinically advanced stage[3]. Hepatocarcinogenesis is a multistep process of malignant transformation of hepatocytes through the sequential accumulation of multiple genomic and epigenomic alterations. HCC is a histologically and genetically diverse cancer[4]. Indeed, several new pathologic subtypes of HCC have been reported recently and new underlying genetic alterations have been described. HCC histological growth patterns are closely related to molecular alterations and oncogenic pathways.

PATHOLOGY OF PRECANCEROUS LESIONS AND CONVENTIONAL HCC

It is now well-established that HCC evolves from precancerous lesions (dysplastic foci/dysplastic nodules). By consensus, the sequence of hepatocarcinogenesis includes low-grade dysplastic nodule (LGDN), high-grade dysplastic nodule (HGDN), early HCC, and small progressed HCC[5,6] (Figure 1). This classification is also supported by molecular studies on increasing accumulation of clonal molecular alterations[7]. Dysplastic foci (< 1 mm in size) are identified incidentally in chronic liver disease (CLD) and are microscopic lesions composed of dysplastic hepatocytes (Figure 2A). The nature of dysplasia is similar to that observed in dysplastic nodules: Large cell change, small cell change, or focal iron free area. Large cell change is characterized by cellular enlargement with enlarged pleomorphic nuclei, abundant cytoplasm, and frequent multinucleation of hepatocytes. The nuclear-cytoplasmic ratio is preserved in large cell change. Small cell change is characterized by decreased cell volume, increased nuclear-cytoplasmic ratio, cytoplasmic basophilia, mild nuclear pleomorphism, and hyperchromasia. Iron-free foci in patients with marked hepatic iron overload show immunohistochemical evidence of proliferative activity and are associated with a high incidence of HCC. Dysplastic nodules are usually identified in livers with cirrhosis but are also occasionally found along with CLD without cirrhosis. These are around 5-15 mm in diameter and can be single or multiple lesions. A LGDN is a distinctly nodular lesion displaying a monotonous cell population with a mild increase in cellular density, a clear trabecular arrangement, and no architectural atypia in comparison to the neighbouring cirrhotic liver. HGDNs are characterized by hepatocyte proliferation with atypical cytological and/or architectural features that are not sufficient for a diagnosis of HCC. HGDN show higher cellular density and frequently demonstrates small cell change.

Macroscopically, lesions with foci of malignant transformation may demonstrate variable features like vaguely nodular, expansile nodular, multinodular, multicentric, cirrhotomimetic, nodular with perinodular extension, and infiltrative types (Figure 2B-D). Small HCCs, ≤ 2 cm, are divided into two groups. Early HCC are vaguely nodular with indistinct margins and usually show higher cellular density than the surrounding cirrhotic tissue with increased nuclear to cytoplasmic ratio, irregular trabeculae, pseudoacini formation, and unpaired arterioles (Figure 3A). Stromal invasion is one of the most important characteristics to differentiate early HCC from HGDN, but is however difficult to identify. Progressed HCC are distinctly nodular with distinguishable margins, frequently capsulated, and show infiltrative or expansile growth pattern. Morphologically, conventional HCC show 4 major architectural growth patterns: Trabecular, solid, pseudoglandular/acinar, and macrotrabecular

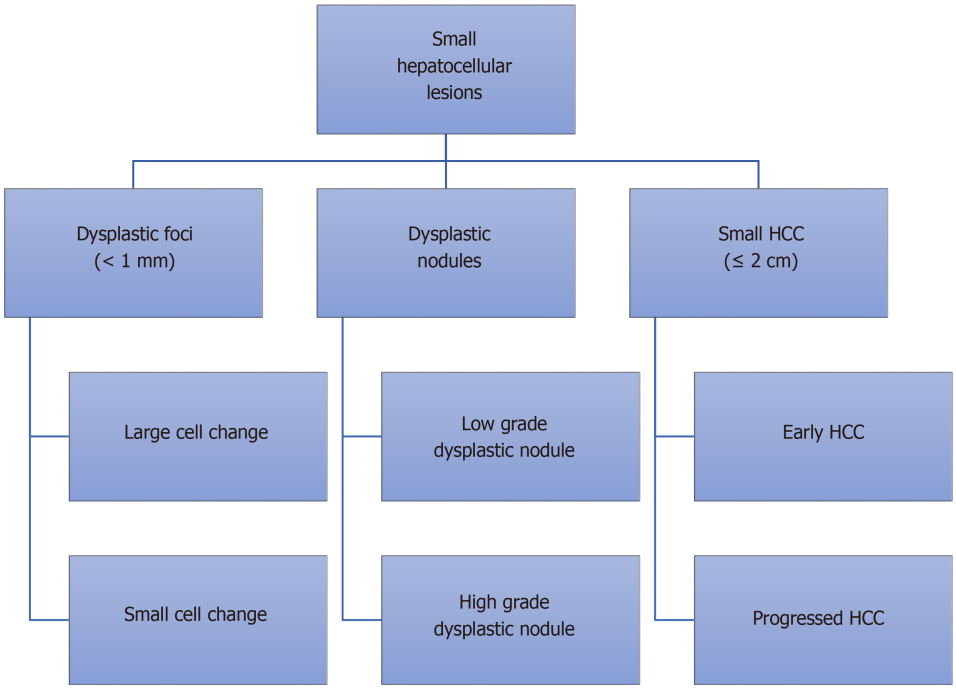

Figure 1 International consensus group for hepatocellular neoplasia classification of small hepatocellular lesions. HCC: Hepatocellular carcinoma.

(Figure 3B-D) and several cytological features (clear cell, steatosis, pleomorphism, multinucleation, foamy cells, oncocytic cells, spindle cells), with frequent co-existence of several features (Figure 4)[8,9]. Various intra-hepatocytic inclusions may be seen like hyaline globules (Figure 5A), Mallory-Denk bodies, bile, and pale bodies. Two histological grading systems for HCC are available. The WHO three-tiered grading system is based on a combination of cytological features and differentiation, and further grades the tumor into well, moderately, and poorly differentiated types[10]. Primary hepatic undifferentiated carcinoma is not included in the WHO grading system as it shows no evidence of either hepatic or biliary differentiation. It is the system most commonly used by pathologists[10,11]. Edmondson and Steiner grading system divides HCC into four grades based on histological differentiation with grade 1 being very well differentiated[12]. A correlation between the histological grade and patient prognosis has been reported[13]. Poorly differentiated HCC are associated with higher recurrence after surgery[14].

ANCILLARY STUDIES FOR THE PATHOLOGIC DIAGNOSIS OF HCC

Differentiation of HCC from other malignancies can be difficult; immunostaining can be helpful to differentiate between these lesions. Arginase-1 is a binuclear manganese metalloenzyme and is the most sensitive and specific marker of hepatocytic differentiation[15]. It shows diffused nuclear and cytoplasmic staining. Carcinoma with hepatoid differentiation and rare cases of adenocarcinoma (including colorectal, pancreatic, breast, and prostatic primaries), cholangiocarcinoma, and may however, show focal or weak Arginase-1 positivity[16]. Hepatocyte paraffin 1 (Hep-Par 1) is a monoclonal antibody that reacts with the urea cycle enzyme carbamoyl phosphate synthetase 1 of liver mitochondria. It shows diffuse granular cytoplasmic staining in normal and neoplastic hepatocytes[17]. Hep-Par 1 is unfortunately frequently negative for poorly differentiated HCCs. Few cholangiocarcinoma and metastatic adenocarcinoma may show Hep-Par 1 immunopositivity. Glypican 3 is excellent marker for neoplastic hepatocytes with cytoplasmic, membranous, or golgi-zone pattern of immunopositivity[18]. Other immunostains like polyclonal carcinoembryonic antigen (pCEA), CD10 and villin shows a distinct canalicular immunostaining pattern in HCC[16]. Alpha-fetoprotein (AFP) immunohistochemistry is not very useful in diagnosis of HCC as it has low sensitivity and is often only focally positive. Albumin RNA *in situ* hybridization has been shown to be a highly sensitive maker for hepatocellular differentiation[19]. Its specificity is however suboptimal and it can be

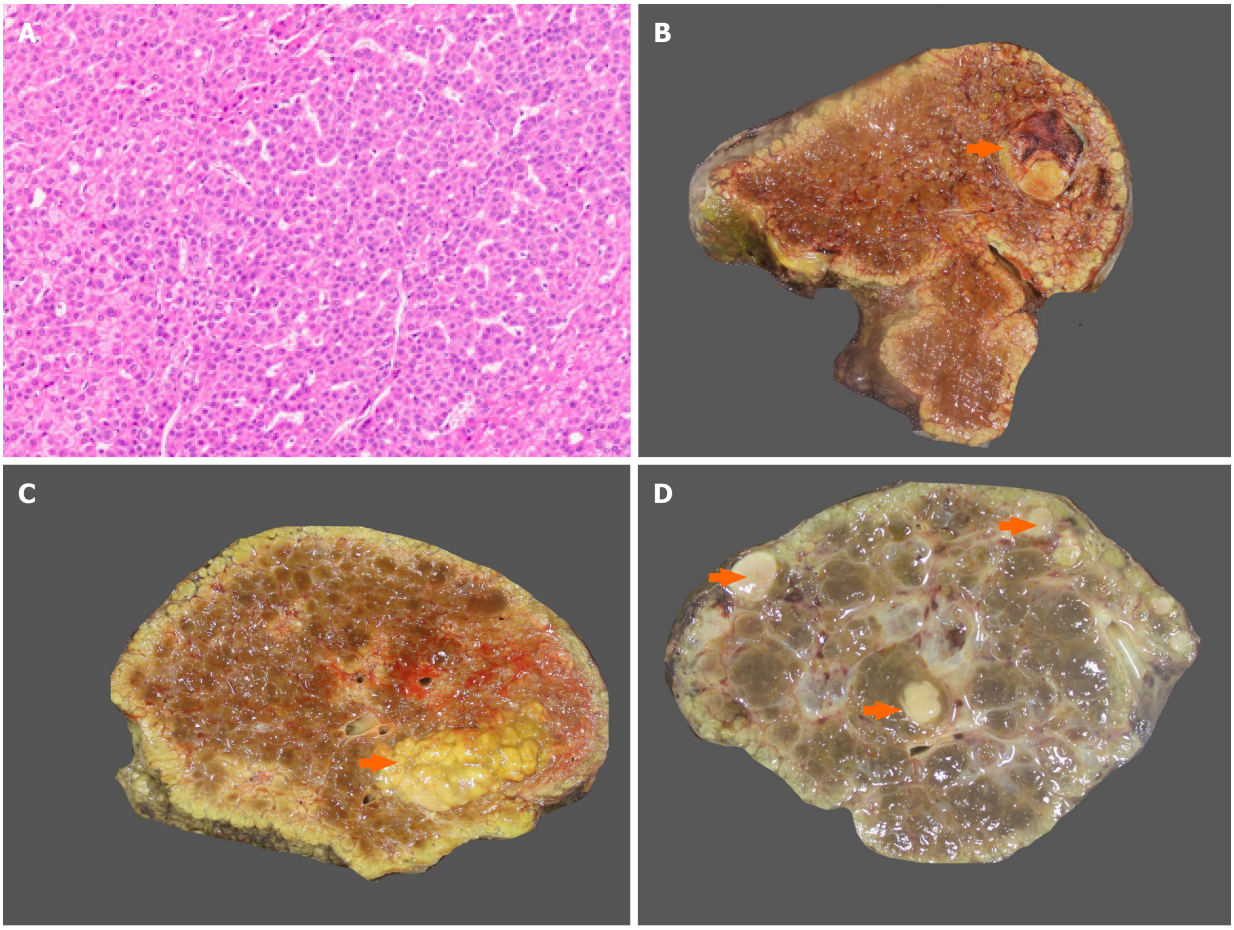

Figure 2 Dysplasia and gross morphology of hepatocellular carcinoma. A: Dysplastic foci with small cell change (hematoxylin and eosin); B: Nodular hepatocellular carcinoma (HCC) in a cirrhotic liver (arrow); C: Multinodular HCC in a cirrhotic liver (arrow); D: Multicentric HCC (arrow).

positive in tumors demonstrating hepatocytic differentiation, such as hepatoid carcinomas of various sites, intrahepatic cholangiocarcinoma (iCCA), gall bladder adenocarcinoma, and yolk sac tumour[20]. Well-differentiated HCCs may also be difficult to distinguish from dysplastic nodules. Loss of reticulin, stromal invasion, and neoarteriolization are particularly useful in these cases. The combination of 3 immunomarkers-glypican 3, glutamine synthetase (GS), and heat shock protein 70-can be used to differentiate early HCC from HGDN[12].

DISTINCT PATHOLOGICAL SUBTYPES WITH MOLECULAR FEATURES

Table 1 summarizes distinct pathological subtypes and their molecular features.

MACROTRABECULAR MASSIVE HCC

The Macrotrabecular-Massive HCC (MTM-HCC) subtype represents a novel histomorphological subtype of HCC. It represents 10%–20% of all cases of HCC. Histologically, it is defined by a macrotrabecular (> 6 cells thick) architectural pattern involving > 50% of the entire tumour, regardless of the associated cytological features (Figure 5B)[4]. Most trabeculae in MTM-HCC are ≥ 10 cells thick[10]. On trucut biopsy analysis, MTM-HCC case is classified if at least 1 focus of macrotrabecular pattern is observed, and the percentage of the macrotrabecular pattern is not taken into account. Pathologists robustly identify MTM-HCC with good inter-observer agreements. MTM-HCC is also characterized by an association with tumor protein 53 (TP53) mutations and fibroblast growth factor 19 (FGF19) amplifications[21]. Being an aggressive form of HCC, it is associated with poor prognostic factors, such as higher Barcelona Clinic Liver Cancer (BCLC) stage B or C, higher AFP levels (> 100 ng/dL), larger tumor size,

Table 1 Hepatocellular carcinoma distinct subtypes with pathological and molecular features

Distinct subtypes	Pathological features	Molecular features
Macrotrabecular massive	Macrotrabeculae > 50% of the tumor, staellite nodules, vascular invasion	TP53 mutations and FGF19 amplifications
Steatohepatitic	Steatohepatitis in the tumor	IL6/JAK/STAT pathway activation
Scirrhous	Dense fibrosis in > 50% of the tumor	Activation of (TGF-β) pathway, with overexpression of VIM, SNAIL (SNAI1), SMAD4 and TWIST
Fibrolamellar	Large polygonal tumor cells with abundant eosinophilic granular cytoplasm and dense bands of intratumoral fibrosis	Recurrent chimeric *DNAJB1-PRKACA* gene fusion
Lymphoepithelioma-like	Neoplastic epithelial cells with a prominent lymphoid infiltrate	Marked focal amplification of chromosome 11q13.3
Progenitor	Immunohistochemical expression of biliary marker CK19 in > 5% of tumor cells	TP53 mutations
Combined hepatocellular-cholangiocarcinoma	Unequivocal presence of both hepatocytic and cholangiocytic differentiation	TP53, TERT, IDH mutations

DNAJB1-PRKACA: DnaJ heat shock protein family member B1 (DNAJB1) and protein kinase 3'-5'-cyclic adenosine monophosphate-activated catalytic subunit alpha; IL6: Interleukin-6; JAK: Janus kinas; STAT: Signal transducer and activator of transcription; FGF19: Fibroblast growth factor 19; TERT: Telomerase reverse transcriptase; IDH: Isocitrate dehydrogenase; SNAIL (SNAI1): SNAIL family transcriptional repressor 1; SMAD4: SMAD family member 4; TWIST: *Twist*-related protein; TGF-β: Transforming growth factor beta.

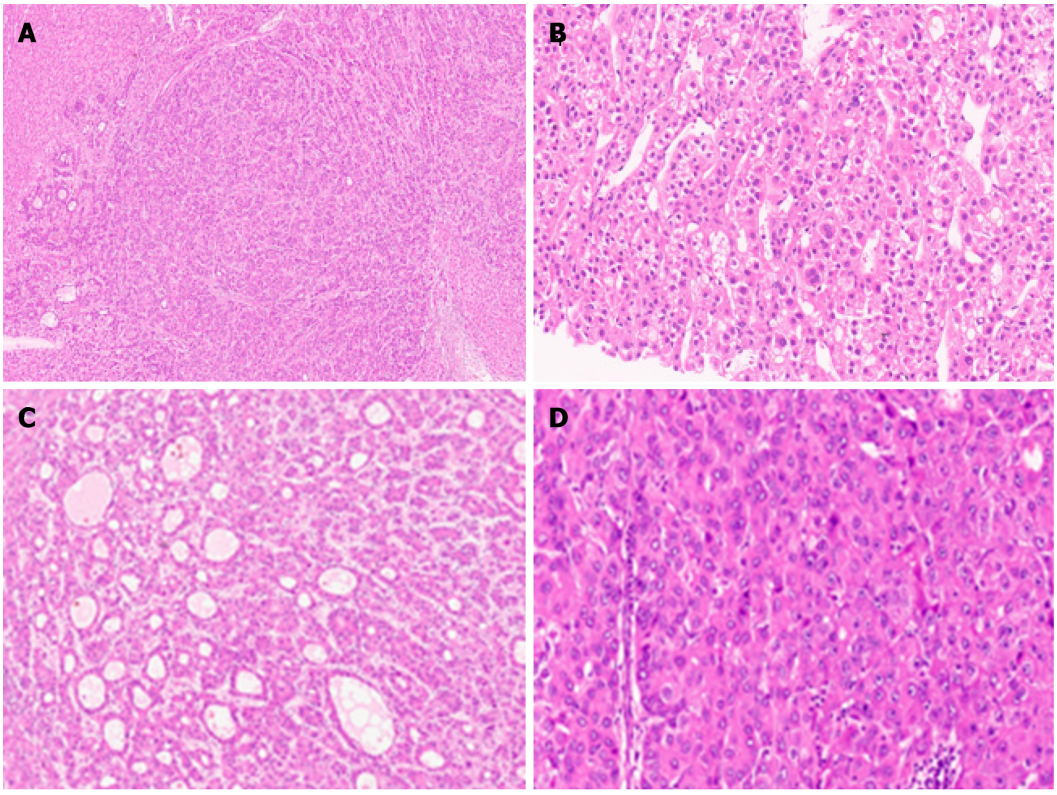

Figure 3 Well differentiated hepatocellular carcinoma. A: Early hepatocellular carcinoma (HCC) with pseudoacinar pattern [hematoxylin and eosin (H&E)]; B: Well differentiated HCC with thin trabeculae (H&E); C: Well differentiated HCC with pseudoacini (H&E); D: HCC with solid sheet growth pattern (H&E).

frequent satellite nodules, substantial necrosis, and macro or microvascular invasion; hence, there is a higher risk of early tumor recurrence and poor disease-free and overall survival rate (Figures 5C and D)[22]. These findings have been further validated by several groups. The other characteristics are its association with viral hepatitis B infection and profound activation of angiogenesis[23]. Presence of the satellite nodule on the multiphase liver magnetic resonance imaging (MRI) has been described as independent factor associated with both early and overall tumor recurrence[24]. Rhee

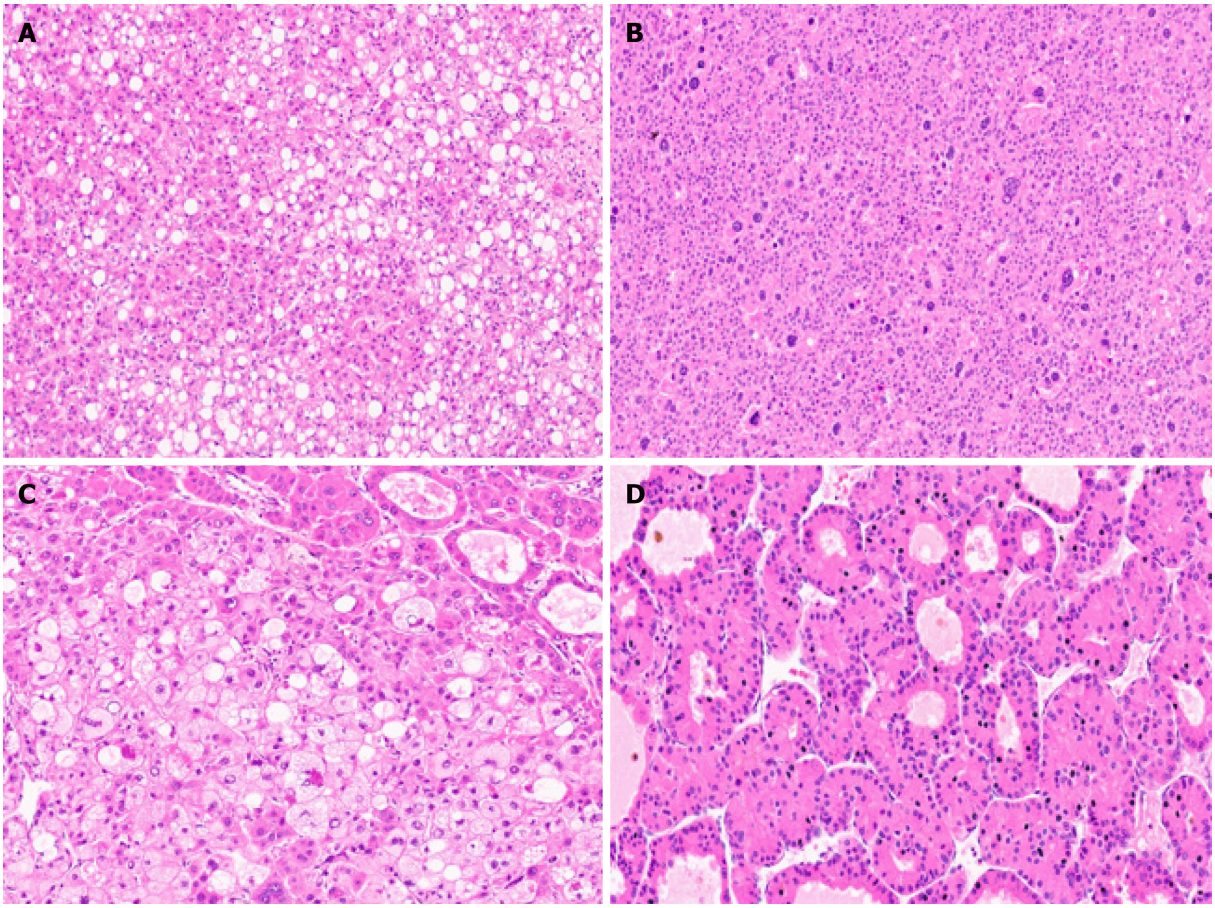

Figure 4 Hepatocellular carcinoma cytological features. A: Hepatocellular carcinoma (HCC) with fatty change [hematoxylin and eosin (H&E)]; B: Marked pleomorphism in an HCC (H&E); C: Foamy cell cytoplasm in an HCC (H&E); D: HCC with oncocytic cells (H&E).

et al[25] reported imaging findings of MTM-HCC by gadoxetic acid-enhanced MRI. With gadoxetic acid-enhanced MRI findings, including arterial phase hypovascular component, they were able to stratify the probability of MTM-HCC and obtain prognostic information[25]. The gene expression profile associated with the MTM-HCC subtype is characterized by the activation of neoangiogenesis, with overexpression of angiopoietin 2 and Vascular Endothelial Growth Factor A (VEGFA). Angiopoietin 2 is responsible for the destabilization of established vasculature and subsequent neoangiogenesis, and also disturbs interactions between endothelial and periendothelial cells, which results in an increased receptiveness to VEGFA[4]. These tumors have high expression of neoangiogenesis-related genes, which led to the discovery of Endothelial-Specific Molecule 1 as a reliable immunostaining marker[26]. Immune assessment of MTM-HCC using expression of the programmed death ligand 1 (PD-L1) and Chemokine-like factor MARVEL transmembrane domain containing 6 (CMTM6) protein coded immune-checkpoint inhibitors showed higher tumoral PD-L1 expression, higher density of inflammatory cells, and higher CMTM6 expression. Therefore, combined expression of PD-L1 and CMTM6 were associated with shorter overall and disease-free survival[27].

STEATOHEPATITIC HCC

Steatohepatitic HCC (SH-HCC) first described in 2010 by Salomao *et al*[28], and is a distinct histological subtype strongly associated with underlying steatosis and/or steatohepatitis and metabolic syndrome[28]. SH-HCC demonstrates morphological features similar to steatohepatitis with macrovesicular steatosis, hepatocellular ballooning with cytoplasmic clarification, Mallory-Denk bodies, pericellular fibrosis, and patchy inflammation (Figure 6A)[29]. The steatohepatitis should be a dominant part of the tumor morphology, and at least 50% of the tumor should show this pattern. Fibrosis can be best demonstrated on histochemical stain like Masson trichrome. The

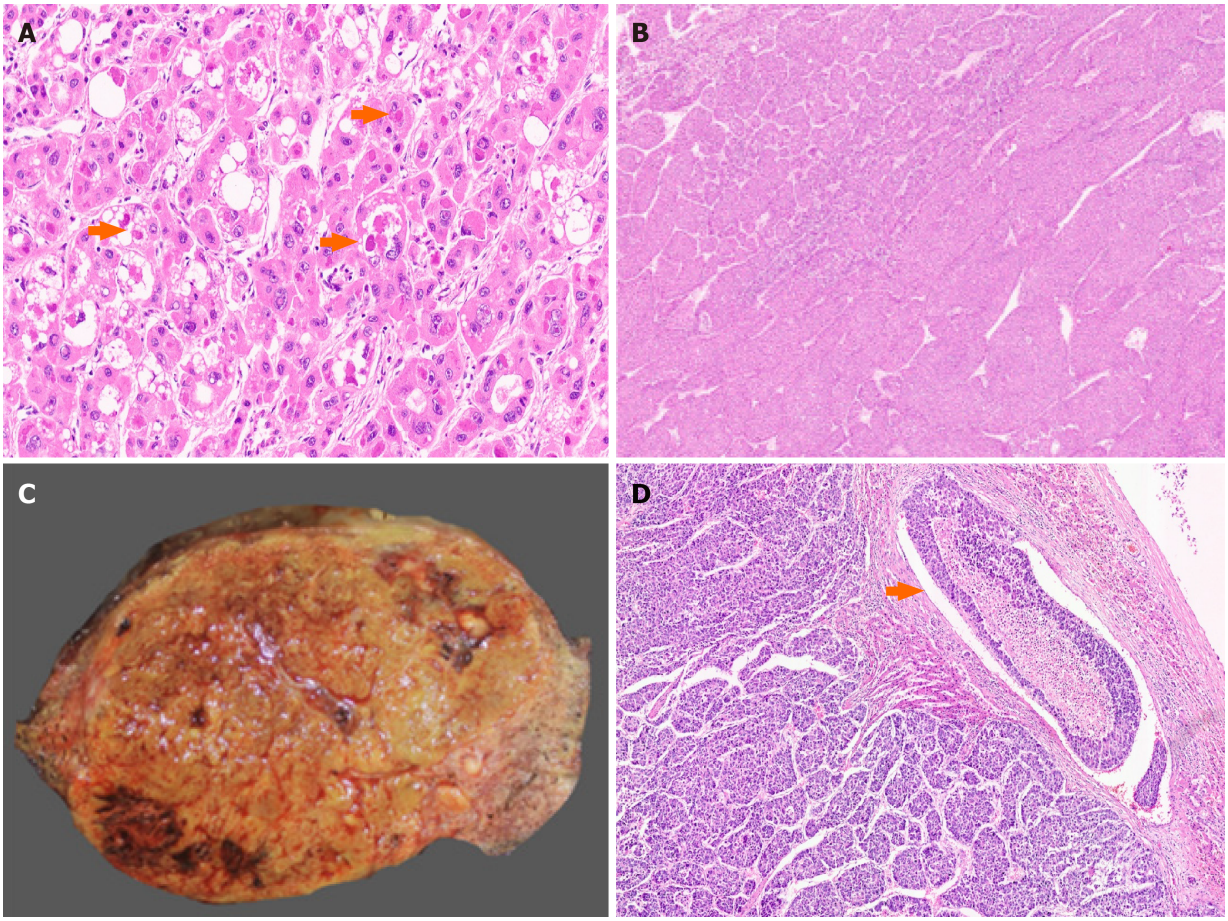

Figure 5 Conventional and macrotrabecular massive hepatocellular carcinoma. A: Hyaline globules in a conventional hepatocellular carcinoma (HCC) [arrow, hematoxylin and eosin (H&E)]; B: Macrotrabecular massive HCC (H&E); C: Large macrotrabecular massive HCC with satellite nodule; D: Macrotrabecular massive HCC with vascular invasion (arrow, H&E).

immunophenotyping of SH-HCC is similar to conventional HCC; however, it shows increased immunostaining with markers of inflammation like C-reactive protein due to interleukin (IL)-6/Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway activation[21]. SH-HCC are well-differentiated to moderately differentiated tumors and are associated G4 transcriptomics subclass. In a recent transcriptomic analysis by Van Treeck *et al*[30] SH-HCC demonstrated a distinctive differential gene expression profile with upregulation of the sonic hedgehog signal transduction pathway based on GLI1 family zinc finger 1 (GLI1) overexpression. *GLI1* gene encodes a protein that functions as a transcription factor protein and plays a role in the regulation of stem cell proliferation. There was reduced expression of carnitine palmitoyltransferase 2 (CPT2) transcripts. CPT2 is a mitochondrial enzyme with an essential role in fatty acid β-oxidation and carnitine metabolism. In a mouse model of obesity-driven and non-alcoholic steatohepatitis-driven HCC, metabolic reprogramming mediated by the downregulation of CPT2 enables protection of neoplastic hepatocytes from lipotoxicity[31]. Therefore; reduced level of CPT2 is believed to facilitate survival of malignancy in obesity-associated HCC. Lee *et al*[32] recently suggested that alteration of the tumor stroma might play an important role in SH-HCC development, and as compared to classical HCC, cancer-associated fibroblasts in SH-HCC and non-tumoral stellate cells were characterized by increased expression of IL-6, a key governor of the JAK/STAT pathway[32]. SH-HCC appears to have similar overall and disease-free survival, development of metastasis, or local recurrence compared with conventional HCC[29].

SCIRRHOUS HCC

Scirrhous HCC represent approximately 5% of all cases[33]. Radiologic findings are atypical and often show arterial phase peripheral enhancement and venous phase

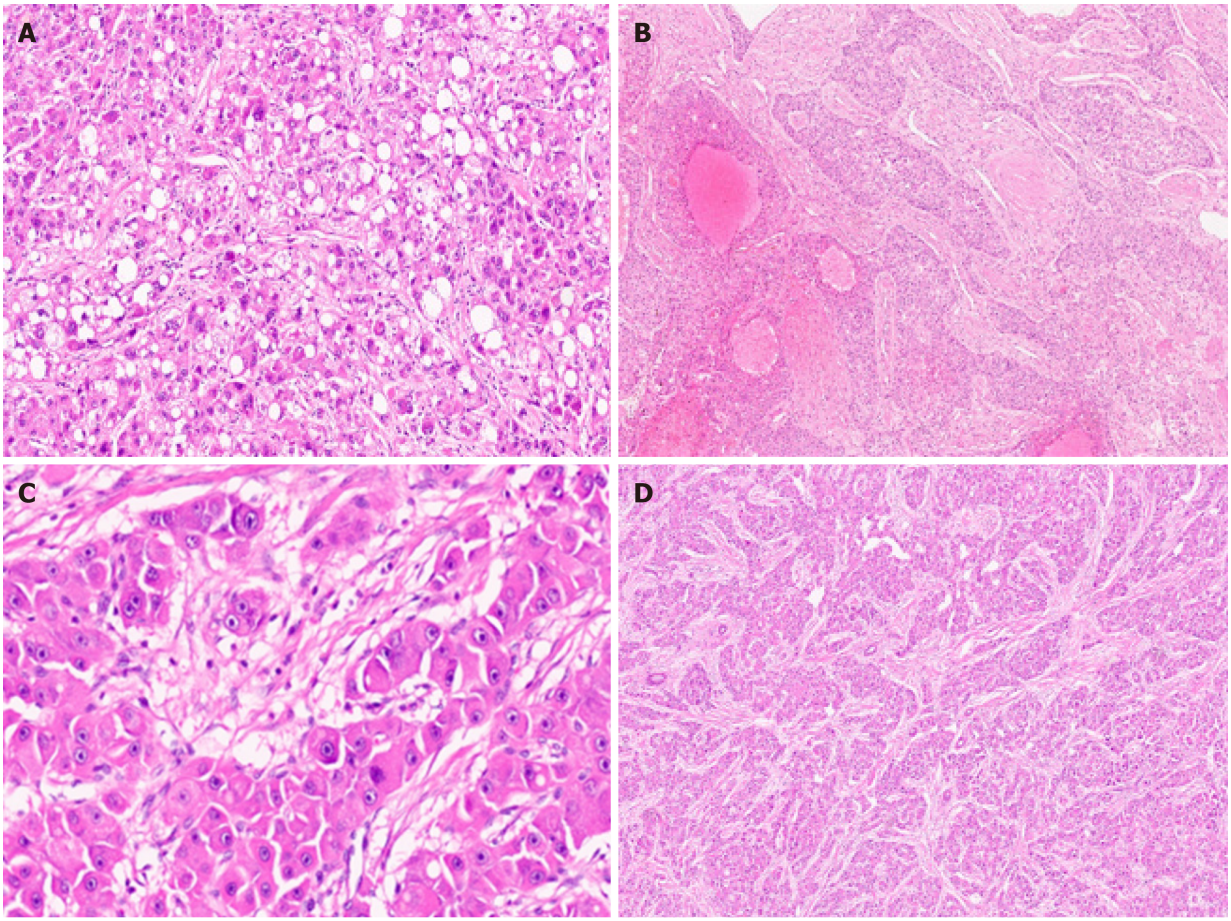

Figure 6 Hepatocellular carcinoma subtypes. A: Hepatocellular carcinoma (HCC) with steatohepatitic pattern [hematoxylin and eosin (H&E)]; B: Sclerotic HCC (H&E); C: Fibrolamellar HCC with large cells and prominent nucleoli (H&E); D: Fibrolamellar HCC with lamellar fibrosis (H&E).

persistent enhancement[34]. Scirrhous HCC is characterized by tumor cell clusters surrounded by abundant fibrous stroma which should constitute at least 50% of the tumor (Figure 6B)[11]. The presence of marked intratumoural fibrosis may lead to a faulty impression of intrahepatic CCA on radiology and macroscopic examination. Scirrhous HCC are mostly well to moderately differentiated HCC. Steatosis, clear cell change, pale bodies, and hyaline bodies have also been reported. Immunohistochemically, there is lack of positive staining for primary hepatocellular stains like HepPar-1 and pCEA in more than 60% of scirrhous HCC, with arginase and glypican 3 positivity in around 80% of cases[35]. Immunostains used for adenocarcinoma, like cytokeratin (CK) 7, CK19, and epithelial cell adhesion molecule, are positive in more than 60% of cases and can lead to erroneous diagnosis of adenocarcinoma[36]. Scirrhous HCC may resemble fibrolamellar HCC histologically, and molecular testing for DNAJ heat shock protein family member B1 (DNAJB1) and protein kinase 3'-5'-cyclic adenosine monophosphate (cAMP)-activated catalytic subunit alpha (PRKACA) fusion can be performed in histologically difficult cases[37]. There is no significant difference in prognosis in Scirrhous HCC compared with conventional HCC[38]. Expression of various cholangiocarcinoma-like and stem-cell-like genomic traits, including CK7 (KRT7), CK19 (KRT19), THY1, and CD133/Prominin-1, have been reported in scirrhous-HCC, and it has therefore been suggested that scirrhous HCC harbour intermediate molecular features, between HCC and cholangiocarcinoma[4,39]. Scirrhous HCC genomic profile also shows activation of transforming growth factor beta pathway/epithelial-to-mesenchymal transition related genes, with overexpression of Vimentin, SNAIL family transcriptional repressor 1, SMAD family member 4, and Twist-related protein[21].

FIBROLAMELLAR HCC

Fibrolamellar HCC (FL-HCC) is a rare and unique histologic subtype of liver cancer

with a predilection for adolescent and young adults (male:female, 1:1) without underlying liver disease, a characteristic morphological pattern with large neoplastic cells, distinct immunostaining, and recurrent genomic abnormalities typically involving PRKACA[40]. FL-HCC comprises approximately only 1% of primary liver cancer[41]. FL-HCC commonly presents as an abdominal mass with enlargement of liver, pain in abdomen, and features of biliary obstruction secondary to external compression by the mass lesion[42]. Rarely FL-HCC can present with paraneoplastic manifestations. These tumors are mostly solitary, large, and well circumscribed grossly with a yellow tan colored cut surface, and areas of central scarring are identified in almost 70% of cases[43,44]. Importantly, FL-HCC are much more likely to invade regional lymph nodes. Histologically, the tumor cells are large, polygonal with abundant eosinophilic granular cytoplasm (because of numerous mitochondria), centrally located nuclei with vesicular chromatin, and prominent nucleoli (Figure 6C). Focal bi-or multi-nucleation are also reported. Dense bands of intratumoural fibrosis arranged in lamellar (parallel arrangement) pattern separates the trabeculae and clusters of tumor cells (Figure 6D). FL-HCC also show presence of pale or hyaline bodies; however, these are not specific and may be observed in conventional HCC. Immunophenotyping shows neoplastic cells are positive of CD68 and CK-7 (biliary lineage) apart from markers of hepatic differentiation (Arginase 1, Hep-Par 1 and albumin mRNA as detected by *in situ* hybridization). Honeyman *et al*[37] first reported a specific 400-kilobase deletion on chromosome 19 in FL-HCC leading to recurrent chimeric *DNAJB1-PRKACA* gene fusion, genetic footprint of FL-HCC. DNAJB1 encodes a member of heat shock protein 40 which is involved in protein folding within cells, while PRKACA codes for the cAMP-dependent protein kinase catalytic subunit alpha; the molecular alteration results in upregulation of PRKACA activity by a promoter switch mechanism[45,46]. Both fluorescence *in situ* hybridization (FISH) or reverse transcription polymerase chain reaction are available now to detect DNAJB1-PRKACA fusion for confirming the diagnosis of FL-HCC. Recently, the genetic alteration (*DNAJB1-PRKACA* gene fusion) has also been identified in a set of oncocytic pancreaticobiliary neoplasm; however, DNAJB1-PRKACA fusion is still the most accurate test when the diagnosis of FL-HCC is doubtful[47,48]. FL-HCC has a unique gene expression profile, with Erb-b2 receptor tyrosine kinase (ERBB) 2 overexpression and glycolysis upregulation leading to compensatory mitochondrial hyperplasia, and various neuroendocrine genes, including Proprotein Convertase Subtilisin/Kexin Type 1, Neurotensin, Delta/Notch Like EGF Repeat Containing and Calcitonin Related Polypeptide Alpha[49].

LYMPHOEPITHELIOMA-LIKE HCC

Lymphoepithelioma-like HCC (LEL-HCC) also known as lymphocyte-rich-HCC is an uncommon variant of HCC and comprises < 1% of primary liver cancer[11]. LEL-HCC are associated with lower rates of recurrence after surgery and has an overall favorable survival rate when compared with conventional HCC[50]. LEL-HCC morphologically resembles lymphoepithelioma-like carcinomas, a poorly differentiated epithelial tumor first described in nasopharynx, characterized by a prominent immune stroma/microenvironment[4]. Subsequently it has been diagnosed in various organs such as stomach, colon, salivary glands, lungs, thymus, uterus, and ovaries[51]. These liver tumors are composed of poorly or undifferentiated neoplastic epithelial cells with a prominent lymphoid infiltrate[52]. A study of 11 cases of LEL-HCC by Wada *et al*[53] proposed quantitative criteria > 100 tumor infiltrating lymphocytes in 10 high power microscopic filed to define significant lymphocytic infiltration[53]. WHO defines LEL-HCC subtype as the condition in which lymphocytes outnumber pleomorphic neoplastic cells in most microscopic fields, but no clear cutoffs for lymphocyte number has been provided[10]. In contrast to LEL cholangiocarcinoma, which are frequently associated with EBV infection and are well described in literature, LEL-HCC are not associated with EBV infection and are not well characterized in literature[52,54,55]. Grossly, these are well circumscribed tumors with variable encapsulation. Histologically, the tumors are composed of atypical cells with syncytial cytoplasm and nuclei with prominent nucleoli and infiltrated by abundant lymphocytes (Figure 7A). Tumor cells show positivity for markers like Hep-Par 1 and Glypican 3 indicating hepatocellular origin. Immunohistochemical profile of the infiltrating immune cells shows a predominance of cytotoxic CD8+ lymphocytes[52]. Rare molecular studies are available on LEL-HCC. A recent study by Chan *et al*[56] showed marked focal amplification of chromosome 11q13.3 in LEL-HCC. Calderaro *et al*[57] showed high level

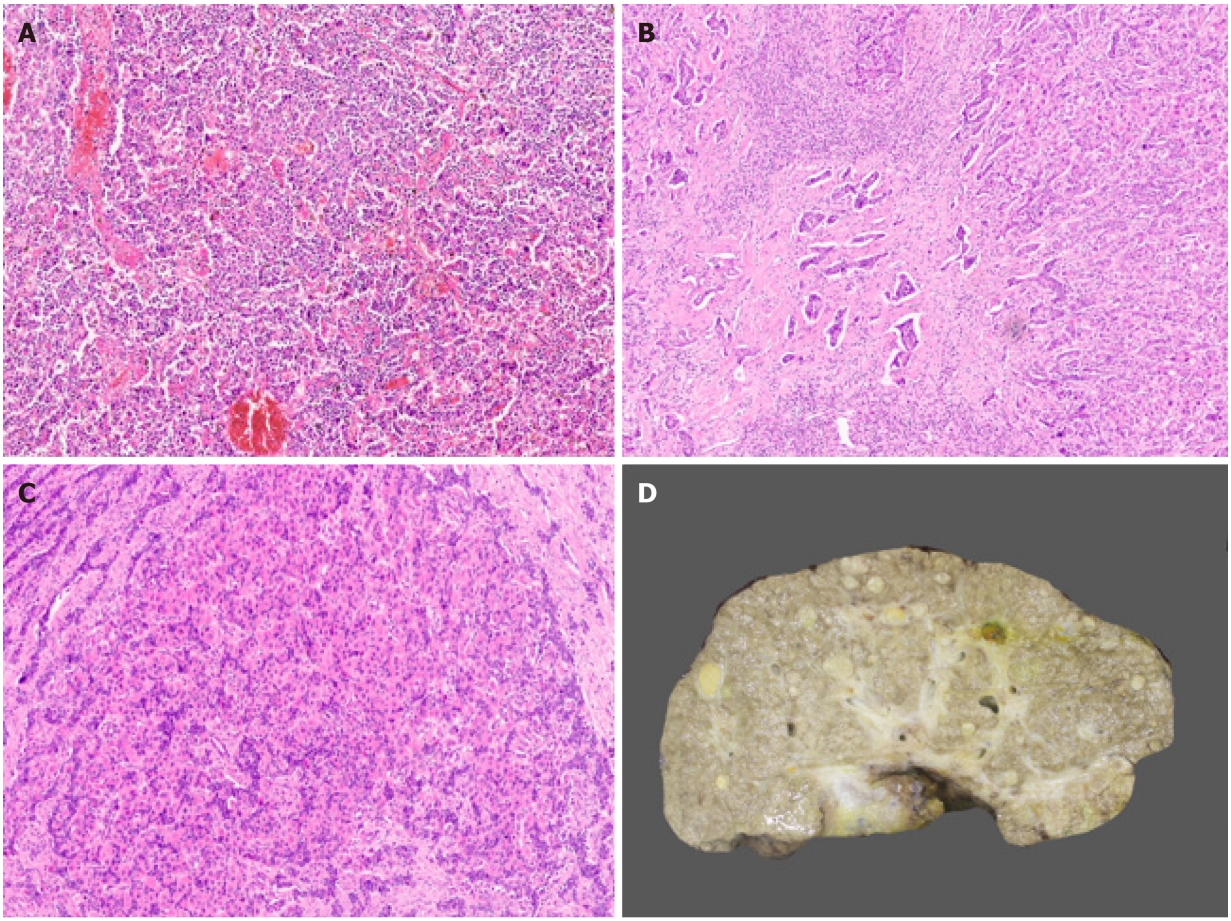

Figure 7 Hepatocellular carcinoma subtypes. A: Lymphoepithelioma like hepatocellular carcinoma (HCC) [hematoxylin and eosin H&E)]; B: Combined hepatocellular-cholangiocarcinoma (cHCC-CCA) with hepatocytic and cholangiocytic component (H&E); C: cHCC-CCA with stem/progenitor cell features (H&E); D: Cirrhotomimetic HCC with numerous tumor nodules.

of PD-L1 and programmed cell death 1 expression in intratumoural inflammatory cells in LEL-HCC. These findings indicate LEL-HCC might be sensitive to drugs targeting immune checkpoint inhibitors. No association of LEL-HCC with a transcriptomic subclass has been identified. Immune class of HCC reported by Sia *et al*[58] characterized by markers of an adaptive T-cell response or exhausted immune response was also not associated with increased number of somatic mutations[58].

PROGENITOR HCC

The progenitor subtype of HCC is defined by the immunohistochemical expression of biliary marker CK19, in more than 5% of neoplastic cells[59,60]. Dedifferentiation of malignant hepatocytes or malignant transformation of hepatic progenitor/stem cells may give rise to this histological subtype[4]. There is growing evidence that progenitor cells, activated during acute and CLD, can directly give rise to HCC. This phenotype is associated with mutation in TP53 and particular genomic subclasses (GI-G3, S2) of HCC[21]. CK19 expression is also reported in HCC after transarterial chemoembolization[61].

COMBINED HEPATOCELLULAR-CHOLANGIOCARCINOMA

Combined hepatocellular-cholangiocarcinoma (cHCC-CCA) is a rare primary liver cancer. Diagnosis of cHCC-CCA is challenging because of its pathological heterogeneity, unique molecular alterations, poorly defined radiological features, and non-specific clinical features. The WHO 2010 Classification defined a classical type of cHCC-CCA (tumor containing unequivocal, intimately mixed elements of both HCC

and intrahepatic CCA), and 3 subtypes of cHCC-CCA with stem/progenitor cell features: Typical, intermediate cell, and cholangiocellular[62]. The WHO consensus classification published in 2019 removed the 3 different stem/progenitor cell subtypes and defined cHCC-CCA as a primary liver carcinoma with unequivocal presence of both hepatocytic and cholangiocytic differentiation (Figure 7B) within the same tumor[63]. This change was implemented because "stem/progenitor cells" identified as small cells with scant cytoplasm, a high nuclear/cytoplasmic ratio, and hyperchromatic nuclei may potentially be seen in all forms of cHCC-CCA; cholangiocellular carcinoma is not always associated with hepatocellular component and subtyping has no prognostic or clinical relevance.

The hepatocellular and cholangiocarcinoma components in cHCC-CCA may be intimately mixed or lie in separate regions of a tumor. Collision of HCC and iCCA arising separately in the same liver should not be included under cHCC-CCA. The diagnosis of cHCC-CCA should be based on hematoxylin and eosin staining only and immunophenotyping can be performed to confirm histologic components. However, IHC alone should not define the diagnosis of cHCC-CCA[64]. Stem/progenitor cell features (Figure 7C) can be mentioned in the comment section of the histology report. Intermediate cell carcinoma is a unique form of cHCC-CCA comprising of monomorphic tumor cells, smaller than hepatocytes but larger than stem/progenitor cells, and has features intermediate between hepatocytes and cholangiocytes. These malignant cells are arranged in strands or trabeculae in an abundant fibrous stroma. Molecular studies of cHCC-CCA are limited and the earlier reported literature suggested that these tumors have a distinct mutational profile with isocitrate dehydrogenase (IDH) mutations usually observed in intrahepatic CCA[4,65]. However this remains debated as a recent study performed by Joseph *et al*[66] demonstrated that the genetics of cHCC-CCA classical type, are distinct from intrahepatic CCA but similar to conventional HCC with alteration in telomerase reverse transcriptase (TERT), p53, and cell cycle genes[66]. Few studies have also reported enrichment in stem/progenitor-like signatures, supporting the concept of a stem/progenitor cell origin of cHCC-CCA[67]. cHCC-CCA has a dismal prognosis, worse than that of either HCC or iCCA, and currently, there are no accepted international management guidelines for cHCC-CCA.

RARE AND PROVISIONAL PATHOLOGICAL SUBTYPES OF HCC

These pathological subtypes are rare and provisional because limited published literature is available.

FIBRONODULAR HCC

Fibronodular HCC (FN-HCC) is a recently described candidate variant[68]. FN-HCC histology is characterized by extensive fibrosis dividing a single tumor into multiple well circumscribed distinct nodules with no significant intranodular fibrosis between single or clusters of neoplastic cells[54]. These tumors show well to moderate differentiation with trabecular or solid growth pattern. Scattered pseudoacini are also described. FN-HCC are reported to be more likely to arise in liver with lower fibrosis stage and lower advanced BCLC stage. They have lower rates of tumor progression. Imaging analysis of FN-HCCs revealed higher rates of non-peripheral washout and a new distinct pattern of enhancement which is characterized by the presence of multiple rounded nodules within a lesion embedded in fibrotic-appearing parenchyma, called as 'popcorn' appearance of the lesion[68].

CHROMOPHOBE HCC WITH ABRUPT ANAPLASIA

This histological subtype is characterized by a unique set of morphological features: smooth chromophobic cytoplasm which can be either slightly eosinophilc or basophilic, abrupt focal nuclear anaplasia (small tumor cell clusters with marked nuclear anaplasia in a background of tumor cells with bland round nuclei and inconspicuous nucleoli), and scattered microscopic pseudocysts[9,69]. This subtype is associated with distinct molecular features with respect to telomere maintenance resulting in alternative lengthening of telomeres (ALT), which can be detected by

telomere FISH. ALT is a telomerase-independent mechanism of telomere maintenance and is found in > 90% of chromophobe HCC with abrupt anaplasia and < 10% of unselected HCCs. Wood *et al*[69] also investigated somatic mutations of alpha-thalassemia/mental retardation, X-linked, Histone H3, and Death Domain Associated Protein identified in various ALT positive tumors reported at other sites in two cases of chromophobe HCC with abrupt anaplasia; however, no mutations were identified[69-71].

GRANULOCYTE COLONY-STIMULATING FACTOR PRODUCING HCC/NEUTROPHIL-RICH HCC

This rare subtype is characterized by production of granulocyte colony-stimulating factor (G-CSF), leading to diffuse infiltrates by neutrophils[72-74]. There is no clear histological definition for this variant. Morphologically, these tumors are poorly differentiated HCC, usually with areas of sarcomatous differentiation and numerous neutrophils. These generally occur in older individuals, grow rapidly, have a high probability of distant metastases, and the overall prognosis seems to be poor as compared with conventional HCC. The mechanism of the production of G-CSF in HCC remains unclear; a close relationship between G-CSF production in malignant cells and their dedifferentiation has been reported[74].

LIPID-RICH HCC

Lipid-rich HCCs have a foamy cytoplasm resulting from lipid accumulation, with numerous very tiny droplets of fat[75-77]. These can be associated with few larger fat droplets. The differential includes lipid-rich variants of metastatic carcinoma. Immunostaining with Hep-Par 1 and Arginase is helpful in doubtful cases.

CIRRHOTOMIMETIC OR DIFFUSE CIRRHOSIS LIKE-HCC

Cirrhotomimetic (CM) or diffuse cirrhosis like-HCC is a rare variant of liver cancer characterized by small cirrhosis-like tumor nodules that are intimately admixed within the cirrhotic liver parenchyma[78-81]. This tumor pattern is often diagnosed incidentally on the native liver explanted at the time of transplantation or autopsy liver specimen, as most of the times, it is clinically and radiologically undetectable (Figure 7D). These tumors are well to moderately differentiated and majority of patients show no significant elevation in serum AFP values[79]. Pseudoacinar architectural growth pattern with bile production and numerous Mallory-Denk bodies have been demonstrated in these tumors. Few studies have investigated tumor nodules in CM-HCC and suggested that these are synchronous multiclonal HCCs[82,83]. One recent study evaluated the liver explants post transcatheter arterial chemoembolization in CM-HCC and non-CM-HCC and reported lower rates of complete pathologic necrosis and poorer overall survival in CM-HCC after liver transplantation as compared with non-CM-HCCs[84].

CLEAR CELL HCC

Clear cell HCC is an uncommon histological variant of HCC. WHO defines this tumor as the condition when > 80% of the neoplastic cells show clear cell morphology[10]. Glycogen accumulation leads to clearing of the cytoplasm; admixed minor steatosis is also acceptable. These are well to moderately differentiated tumors with similar or better prognosis than conventional HCC[85-87]. There is, however, no distinct definition of this subtype and clear cells may be observed in other subtypes.

HEPATIC CARCINOSARCOMA

Hepatic carcinosarcomas are composed of both malignant epithelial component and

mesenchymal components[9]. These neoplasms are extremely rare. The carcinomatous component is moderately to poorly differentiated HCC. The sarcomatous component shows morphologic or immunohistochemical evidence of mesenchymal differentiation, such as leiomyosarcoma, rhabdomyosarcoma, chondrosarcoma, fibrosarcoma, or rarely osteosarcoma. There is scant data on molecular alterations[88,89]. One earlier study revealed mutation in TP53, Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha and FGFR3 genes[88]. One recent study using targeted next-generation sequencing with a panel of 329 cancer-related genes identified TP53, Neurofibrin 1/2 mutations, and VEGFA amplification in both carcinomatous and sarcomatous components[89]. Amplifications involving MET and platelet-derived growth factor receptor A were identified only in the sarcomatous components, whereas mutation affecting ERBB4 and amplifications of Cyclin D1 and FGF 3/4/19 were present only in the carcinomatous components.

MYXOID HCC

This rare morphological subtype of HCC shows well to moderately differentiated neoplastic cells with a trabecular growth pattern, separated by abundant extracellular myxoid/mucin material[9,90]. The neoplastic cells stain strongly with HepPar1 and Arginase-1, and are negative for biliary marker CK19. These tumors typically show loss of liver fatty acid binding protein and also immunostaining with strong and diffuse positivity for GS.

CONCLUSION

Pathology of HCC has evolved significantly in the last two decades. We are now well versed with various dysplastic liver lesions and multiple distinct pathologic subtypes of HCC. There is also remarkable improvement in our understanding of HCC pathogenesis as tumor genome sequencing has identified recurrent molecular alterations and oncogenic pathways and how this correlates with various morphological findings. Identification of genetic alterations also gives us an opportunity to develop targeted therapies that can prevent recurrence and improve patient survival.

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MINIREVIEWS

Infantile giant cell hepatitis with autoimmune hemolytic anemia

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Abstract

Giant cell hepatitis (GCH) is characterized by large and multinucleated (syncytial) hepatocytes in the context of liver inflammation. Infantile GCH is typically associated with autoimmune hemolytic anemia in the absence of any other systemic or organ-specific autoimmune comorbidity. The etiology is unknown; concomitant viral infections (as potential trigger factors) have been identified in a few patients. The pathogenesis reportedly relies upon immune-mediated/autoimmune mechanisms. This condition should be considered in any infant developing Coombs-positive anemia; indeed, anemia usually precedes the development of hepatitis. The clinical course is usually aggressive without the appropriate immunosuppressive therapy, which may include steroids, conventional immunosuppressors (*e.g.*, azathioprine and cyclophosphamide as first-line treatments), intravenous immunoglobulin, and biologics (rituximab). Improvements in medical management (including the availability of rituximab) have significantly reduced the mortality of this condition in the last decade.

Key Words: Giant cell hepatitis; Autoimmune hemolytic anemia; Rituximab; Infantile hepatitis; Jaundice; Hyperbilirubinemia

Core Tip: This review discusses the main characteristics of giant cell hepatitis associated with autoimmune hemolytic anemia including etiology, pathogenesis, pathophysiology, clinical aspects, prognosis, and therapy. All of the available case reports and case series have been considered to provide an overall picture of this disease and its general clinical management.

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INTRODUCTION

Giant cell hepatitis (GCH) refers to a histopathological picture of liver inflammation characterized by large and multinucleated (syncytial) hepatocytes; ≥4-5 nuclei can be seen in the affected cells of the liver parenchyma, along with other features of hepatitis such as lobular fibrotic rearrangements, Kupffer cell hypertrophy, and spotty necrosis[1,2].

In adults, GCH is rare; indeed, it is mainly observed and described in children, mostly in the first years of life. The giant cell transformation of hepatocytes is considered as an altered/dysfunctional regenerative response of hepatocytes in the context of different underlying liver diseases, such as chronic autoimmune hepatitis (AIH), and/or exposure to various noxious agents including drugs and viral infections[2-4].

The histopathological finding of partial or diffuse giant cell transformation of hepatocytes is more frequent in infantile, and in particular, neonatal hepatitis. Indeed, GCH is considered in the differential diagnosis of neonatal cholestasis, where biliary atresia and idiopathic/GCH account for 70% to 80% of all cases; the diagnostic work-up usually includes liver biopsy to achieve a complete and final diagnosis[1,5,6]. GCH is also associated with congenital atresia, and thus, both conditions may coexist. However, neonatal GCH has been described in patients with pathological non-obstructive neonatal jaundice (*e.g.*, blood group incompatibility, hereditary spherocytosis), congenital syphilis, perinatal hemochromatosis, viral infections (*e.g.*, cytomegalovirus, rubella) and metabolic diseases[7,8]. Torbenson *et al*[1] analyzed the etiology of GCH in 62 newborns: 49% of cases were idiopathic, whereas the remaining patients were variably affected with hypopituitarism (15%), biliary atresia (8%), Alagille syndrome (6%), progressive familial intrahepatic cholestasis or other bile salts defects (6%), neonatal hemochromatosis (5%), viral infections (4%), and other diseases (8%, *i.e.* cystic fibrosis, alpha-1-antitrypsin deficiency, severe combined immuno-deficiency, AIH)[1].

Infantile GCH is rarely described in patients with post-neonatal hepatitis, and interestingly, is typically associated with autoimmune hemolytic anemia (AHA); this condition is mostly diagnosed in children aged 1 mo to 2 years[9]. Such a pathological association is unusual in post-infantile (childhood and adult) GCH. Indeed, Coombs-positive anemia is found in < 10%-15% cases[10]. Infantile GCH + AHA, as a specific disease pattern, was first recognized in 1981 by Bernard *et al*[11], who described 4 children developing chronic AHA combined with liver disease, which was histologically characterized by severe hepatitis with "diffuse giant cell transformation and extensive fibrosis"[11].

ETIOLOGY AND PATHOGENESIS

The etiology of GCH + AHA is unknown and specific and/or clear trigger factors have not been identified. Indeed, no individual etiological clues have been identified in most patients, except for some cases in whom viral infections (*e.g.*, paramyxoviruses, varicella-zoster virus, cytomegalovirus) have been reported[4,12-14].

The pathogenesis of GCH + AHA reportedly relies on immune mediated/autoimmune mechanisms, even though this was not included in the classification of pediatric autoimmune liver diseases, according to a recent European Society for Paediatric Gastroenterology Hepatology and Nutrition hepatology committee position statement, which considered three liver disorders: AIH, autoimmune sclerosing cholangitis, and *de novo* AIH after liver transplant[15]. However, several clinical and pathological findings suggest the involvement of immunological mechanisms in infantile GCH, in addition to the AHA comorbidity by itself. Indeed, Nastasio *et al*[16] summarized these aspects, including the response to immunosuppressive therapies, the evidence of complement-mediated (C3a- and C5a-driven) hepatocyte injury and liver inflammation, and the sporadic association with autoimmune diseases other than

AHA[16]. Importantly, the typical histological features of GCH + AHA differ from those described in the aforementioned "classical AIH," and in fact, autoimmune liver disease-related autoantibodies are absent. However, a "strong immune/autoimmune component" characterizes the pathogenesis of GCH + AHA[17].

Interestingly, Whitington *et al*[18] emphasized that the histopathology of GCH + AHA is similar to that of Gestational Alloimmune Liver Disease (GALD), which accounts for most cases of neonatal hemochromatosis, characterized by a prominent liver giant cell transformation as well[18,19]. The authors showed that, unlike AIH patients, children with GCH + AHA had diffuse and intense C5b-9 complex deposition in the liver, suggesting that the giant cell transformation in these patients was the result of complement-mediated hepatocyte injury, similar to GALD fetuses and newborns, in whom immunoglobulin G-induced complement-mediated hepatocyte injury has been demonstrated[20,21].

These observations support the fact that GCH + AHA is an autoimmune disease in which giant cell transformation is an "unspecific" reactive response to antibody- and complement-mediated hepatocyte injury. Both hepatitis and Coombs-positive anemia may be consequences of a common systemic B cell immune dysregulation leading to autoantibody production.

PATHOPHYSIOLOGY AND CLINICAL ASPECTS

In general, GCH + AHA should be suspected in any child aged 1 mo to 2 years, who presents with severe liver disease and anemia. The median age of the onset is about 8 mo, and thus, most cases manifest before 1 year of age[22]. Both males and females can be affected, without a clear gender preponderance; in the largest cases series published by Maggiore *et al*[9], there were 9 male and 7 female patients[9]. If all other case reports and small case series are considered (Table 1)[9,11-14,18,22-41], among the 51 reported patients with infantile GCH + AHA, 25 were female and 19 were male (no gender specification was available for 7 patients).

In detail, GCH should be considered in any infant developing Coombs-positive AHA, especially if jaundice is direct, namely characterized by a component of conjugated bilirubin > 20% of total bilirubin. Indeed, AHA and, in general, all hemolytic anemia cases usually show jaundice deriving from the accumulation of indirect bilirubin, because its excessive production (due to the increased heme catabolism) cannot be readily cleared from the bloodstream and metabolized by the liver[42]. In summary, whereas isolated AHA (which may also show mild-moderate increase of liver enzymes) is characterized by indirect jaundice, GCH is accompanied by clear signs of cholestasis, and thus direct jaundice, in addition to the fact that the increase in liver enzymes is usually very pronounced.

Indeed, the increase in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) is usually at least 15-20 times higher than the upper normal limit of the respective age-related reference range[9], but cases with milder liver enzyme elevation (< 5-10 times the upper normal limit) have been described, especially in the initial phases of hemolytic disease[31,37,39]. In this regard, the development of anemia usually precedes the onset of liver disease by a variable period of time, ranging from 1 mo to > 1 year. Therefore, the diagnosis of GCH + AHA often follows a previous diagnosis of isolated AHA[31].

Moreover, the increase in gamma-glutamyl transferase (GGT) is not usually very pronounced and is often not greater than 2-3 times the age-related upper normal limit[9,38]. Such a GGT increase, especially when associated with mild to moderate AST/ALT abnormalities, could be consistent with several common infectious illnesses (*e.g.*, cytomegalovirus, Epstein-Barr virus, mycoplasma pneumoniae)[43-45], which may also trigger immune-mediated hemolytic diseases, and thus, should be appropriately excluded through diagnostic work-up[46,47].

Therefore, the measurement of ALT, AST, and GGT is recommended in all young children diagnosed with AHA at the onset and during follow-up of the disease. If liver enzymes are highly and/or persistently elevated without any clear (infectious) explanation, these children should undergo liver biopsy to assess the liver histopathological features, and in detail, whether GCH is present[9]. In addition to a histopathological picture inconsistent with AIH, these patients are serologically negative for significant titers of anti-mitochondrial, anti-smooth muscle, anti-liver kidney microsomal autoantibodies, and anti-nuclear antibodies[31].

Table 1 Overview of the demographic features and outcome in patients with infantile giant cell hepatitis and autoimmune hemolytic anemia

Ref.	Clinical cases, n	Age in mo	Gender	Outcome	Rituximab	Follow-up in mo	Cause of death
Before 2011 (case reports/series providing individual data on infantile GCH + AHA)							
Bernard *et al*[11], France, 1981	1	10	F	Fatal	N	-	Liver failure
	2	9	M	Fatal	N	-	Liver failure
	3	24	M	Fatal	N	-	Liver failure
	4	6.5	M	Alive	N	N/A	-
Imgrueth *et al*[23], Switzerland, 1986	5	5	F	Alive	N	9	-
	6	8	M	Alive	N	24	-
Brichard *et al*[24], Belgium, 1991	7	7	F	Fatal	N	-	Encephalopathy
Weinstein *et al*[25], United States, 1993	8	5	F	Alive	N	30	-
Perez-Atayde *et al*[26], United States, 1994	9	23	F	Fatal	N	-	Sepsis
	10	9	M	Alive	N	8	-
Choulot *et al*[27], France, 1996	11	15	M	Alive	N	144	-
Melendez *et al*[28], United Kingdom, 1997	12	8	M	Fatal	N	-	Liver and renal failure
Hartman *et al*[29], Israel, 2001	13	6	M	Fatal	N	-	Liver failure
Gorelik *et al*[30], United States, 2004	14	4	F	Alive	Y	36	-
Kashyap *et al*[31], India, 2006	15	4	F	Alive	N	2	-
Vajro *et al*[12], Italy, 2006	16	10	F	Alive	N	36	-
Miloh *et al*[32], United States, 2007	17	2	M	Alive	Y	24	-
Rovelli *et al*[33], Italy, 2007	18	14	M	Alive	Y	48	-
Baran *et al*[13], Turkey, 2010	19	3	F	Fatal	Y	-	Sepsis and renal failure
Ünal *et al*[14], Turkey, 2010	20	2	F	Fatal	Y	-	Sepsis
	21	6	M	Fatal	N	-	N/A
	22	11	M	Alive	Y	18	-
Maggiore *et al*[9](largest case series providing aggregate data on infantile GCH + AHA)							
Maggiore *et al*[9], Italy, 2011	16 cases	2.5-17	M (*n* = 9); F (*n* = 7)	Alive (*n* = 12); Fatal (*n* = 4)	Y (*n* = 2); N (*n* = 10)	2-28 yr	OLT (*n* = 1); Sepsis (*n* = 3)

After 2011 (case reports/series providing individual data on infantile GCH + AHA)							
Raj *et al*[22], United States, 2011	1	6	F	Alive	N	30	-
Lega *et al*[34], Italy, 2013	2	8	M	Alive	N	6	-
Bouguila *et al*[35], Tunisia, 2013	3	9	N/A	Fatal	N	-	Sepsis
Whitington *et al*[18], Canada & United States, 2014	4	22	F	Alive	Y	48	-
	5	14	F	Alive	Y	48	-
	6	6	F	Alive	N	48	-
	7	4	F	Fatal	N	-	N/A
	8	6	M	Alive	Y	36	-
Bakula *et al*[36], Poland, 2014	9	7	N/A	Alive	Y	30	-
	10	8	N/A	Alive	Y	26	-
	11	2	N/A	Alive	Y	5	-
	12	12	N/A	Alive	Y	76	-
	13	7	N/A	Fatal	N	-	Hemophagocytosis (after HSCT)
Paganelli *et al*[37], Italy, 2014	14	3	F	Alive	Y	N/A[1]	-
	15	14	F	Alive	Y	N/A[1]	-
	16	12	F	Alive	Y	N/A[1]	-
	17	16	M	Alive	Y	N/A[1]	-
Marsalli *et al*[38], Italy, 2016	18	5	F	Alive	N	N/A[1]	-
	19	8	M	Alive	N	N/A[1]	-
	20	10	F	Alive	N	N/A[1]	-
	21	10	F	Alive	N	N/A[1]	-
	22	6	F	Alive	Y	N/A[1]	-
	23	7	F	Alive	N	N/A[1]	-
	24	8	M	Alive	N	N/A[1]	-
Cho *et al*[39], South Korea, 2016	25	2	N/A	Alive	N	36	-
Matarazzo *et al*[40], Italy, 2020	26	5	F	Alive	Y	141	-

	27	9	F	Alive	Y	91	-
	28	8	M	Alive	Y	76	-
Kim *et al*[41], South Korea, 2020	29	7	M	Alive	Y	19	-

[1]The authors did not provide the follow-up length for individual patients; however, they provided general information on follow-up in their respective case series (Paganelli *et al*[37]: "At last follow-up visit, all patients were alive with their native liver 2 to 16 year after disease presentation"; Marsalli *et al*[38]: "Follow-up (median 17.4 mo, range 7-24 mo)." F: Female; HSCT: Hematopoietic stem cell transplantation; M: Male; N/A: Not available; N: No; OLT: Orthotopic liver transplantation; Y: Yes.

PROGNOSIS AND THERAPY

The clinical course of GCH + AHA is usually aggressive. According to the analysis of 22 cases reported to 2006, the mortality rate was about 45%. The Italian-French multicentric analysis including 16 pediatric patients (evaluated over a 28-year period and published by Maggiore *et al*[9]) reported a lower mortality rate (25%), probably due to a better therapeutic (*i.e.* immunosuppressive) approach over time. Indeed, if the cases reported after 2011 are specifically considered, only 3 of 29 patients died, which corresponds to an overall mortality rate as low as 10% (Table 1)[9,11-14,18,22-41]. The therapeutic regimens described in these case reports and small case series were widely heterogeneous. Such a discussion goes beyond the scope of this review, but it is worth mentioning that the biological therapy with rituximab was part of the treatment of many more patients after 2011 (rituximab used in 16 of 29 cases) compared to the previous period (rituximab used in 8 of 48 cases), which may have contributed to the reduced mortality in the cases described in the last decade. Indeed, despite an initial response to immunosuppressive therapy, relapses occur in many cases, and liver disease/failure is the main pathological component accounting for a negative prognosis. The hematological component is usually better controlled with immunosuppressive therapy. In fact, persistent and clinically relevant hemolysis has been described in a few patients, who required splenectomy and/or plasmapheresis to control a severe and resistant hematological condition[27,29].

In general, liver disease can be controlled in half of patients with initial immunosuppressive therapy, which may be withdrawn in very few patients. The remaining patients develop more severe disease, which is only partially responsive (or not responsive at all) to immunosuppressive therapy; in some of these cases, the clinical course is rapid and fatal, because of the liver failure by itself and/or its complications, such as severe seizures disorder/encephalopathy and/or concomitant infections[12,29]. Indeed, these children may also develop hemophagocytosis leading to a clinical picture of macrophage activation syndrome, as first described by Hartman *et al*[29].

In those clinical cases with the most severe prognosis, orthotopic liver transplantation (OLT) was also considered. However, the prognosis remained poor. In 1997, Melendez *et al*[28] revised 4 cases undergoing this procedure and 3 of them ultimately

died. Importantly, all of these patients showed recurrence of GCH in the transplanted liver within the first few months[28]. A positive transplantation outcome without relapse was described by Kerkar *et al*[48] in a patient developing progressive hepatic encephalopathy. However, despite the association with Coombs-positive anemia, this patient may have not been a case of infantile GCH + AHA, since he had positive anti-liver kidney microsome antibodies, and only partial/patchy giant cell transformation was observed in the liver. Moreover, anemia was associated with thrombocytopenia, suggesting the possibility of type 2 AIH or systemic autoimmune dysfunction leading to several organ immune-mediated disorders, as further supported by the appearance of bullous pemphigoid after liver transplant[48]. Due to constant disease relapses after OLT, such a therapeutic approach has been basically abandoned in the clinical setting of GCH + AHA[12].

Without rapid and appropriate immunosuppressive treatment, the liver function rapidly deteriorates in these patients with infantile GCH + AHA, leading to a progressive and fatal course, as already mentioned. The early institution of an aggressive steroids therapy usually has beneficial effects on both liver function and autoimmune hemolytic anemia. Combination therapy with steroids and azathioprine/cyclophosphamide is often the first-line therapy, which is able to significantly reduce mortality in the early phases of disease activity[31]. However, due to the frequent steroid-resistant cases and/or relapses after immunosuppression step-down/withdrawal, several and additional immunosuppressive agents have been variably used (based upon all the available case reports and series), including cyclosporine, tacrolimus, 6-mercaptopurine, mycophenolate and vincristine[12].

In addition to these immunosuppressive drugs, some immunomodulatory therapies have also been used[23,26,29]. In this regard, the first experiences included the use of intravenous immunoglobulins (IVIGs), which were administered according to variable therapeutic schemes, as reviewed by Lega *et al*[34]. Actually, these authors used a high-dose regimen (2 g/kg) that was repeated on a monthly basis for more than 6 mo, in association with immunosuppressive therapy[34]. Marsalli *et al*[38] focused their study on IVIG use and concluded that this treatment can help to significantly and rapidly reduce the activity of the liver disease, in combination with prednisone and other immunosuppressive therapies[38]. Some authors also reported the use of plasmapheresis[23,29,30].

However, as mentioned above, the most important advances in infantile GCH + AHA derived from the use of rituximab. In 2004, Gorelik *et al*[30] reported its use to treat the hematological component, but Miloh *et al*[32] first reported a GCH + AHA infant affected with severe liver disease resistant to steroids, azathioprine, sirolimus, and IVIG, who significantly improved after the therapy with rituximab[30,32]. Eventually, several authors reported the successful use of rituximab. For instance, Bakula *et al*[36] reported 4 GCH + AHA infants, who achieved complete remission with rituximab after the failure of the first-line therapy with steroids and azathioprine. Therefore, these authors and others proposed rituximab as the treatment of choice for the early stages of the disease[36,40]. Indeed, unresponsiveness to rituximab is suggested to be more likely when its use is delayed[13]. Additional experiences confirmed the safety and effectiveness of rituximab, even in association with other immunosuppressive agents (*e.g.*, cyclosporine). Moreover, the early treatment could reduce the use of steroids and, thus, prevent several side effects[37]. In some patients, rituximab induced a complete and long-lasting remission and allowed the discontinuation of all immunosuppressive drugs[40]. To conclude, Rovelli *et al*[33] reported a positive result by using alemtuzumab, which is a humanized monoclonal antibody directed against CD52 (cluster of differentiation 52, a glycoprotein expressed on circulating T and B lymphocytes and natural-killer cells). Even though long-term remission of the liver disease was reported in this case of GCH + AHA, to date, this is the only experience with alemtuzumab.

CONCLUSION

Infantile GCH is a clinical condition that should be considered in any infant developing Coombs-positive anemia, in the presence of significant abnormalities of liver function tests and direct hyperbilirubinemia. Indeed, anemia usually precedes the development of hepatitis. This clinical condition requires timely and appropriate immunosuppressive therapy, which may include steroids, conventional immunosuppressors, intravenous immunoglobulin, and biologics (rituximab). Improvements in the medical management (including the availability of rituximab)

have significantly reduced the mortality of this condition in the last decade.

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MINIREVIEWS

Long-term albumin infusion in decompensated cirrhosis: A review of current literature

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Abstract

Decompensated cirrhosis is characterized by chronic inflammation and severe portal hypertension leading to systemic circulatory dysfunction. Albumin infusion has been widely used in decompensated cirrhosis in patients with spontaneous bacterial peritonitis, large-volume paracentesis and hepatorenal syndrome. Emerging data suggest long-term albumin infusion has both oncotic and non-oncotic properties which may improve the clinical outcomes in decompensated cirrhosis patients. We review the current literature on both the established and potential role of albumin, and specifically address the controversies of long-term albumin infusion in decompensated cirrhosis patients.

Key Words: Albumin; Cirrhosis; Hepatic encephalopathy; Hepatorenal syndrome; Acute-on-chronic liver failure; Spontaneous bacterial peritonitis; Large-volume paracentesis

Core Tip: Decompensated cirrhosis is characterized by chronic inflammation and severe portal hypertension leading to systemic circulatory dysfunction. Albumin infusion has been widely used in decompensated cirrhosis in patients with spontaneous bacterial peritonitis, large-volume paracentesis and hepatorenal syndrome. Emerging data suggest long-term albumin infusion has both oncotic and non-oncotic properties which may improve the clinical outcomes in decompensated cirrhosis patients.

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INTRODUCTION

Long-term albumin infusion in decompensated cirrhosis: A critical review of current literature

Concept of compensated and decompensated cirrhosis: Cirrhosis represents the common pathway of all chronic liver disease resulting in over a million deaths every year[1]. The natural history of liver cirrhosis includes an asymptomatic compensated stage and a decompensated cirrhosis stage with clinically overt complications as ascites, jaundice, variceal bleeding and hepatic encephalopathy (HE)[2]. The median survival reduces significantly from 12 to 2 years as patients progress from the compensated to decompensated cirrhosis at an annual rate of 5%-7%[2,3].

Decompensated cirrhosis is characterized by chronic inflammation and severe portal hypertension leading to systemic circulatory dysfunction[4]. As a corrective response to portal hypertension, excessive nitric oxide secretion results in both splanchnic and arterial vasodilatation, which thus impairs organ perfusion[5,6]. To ensure adequate organ perfusion, the arterial pressure is maintained by increased activity of the renin-aldosterone-angiotensin system[7]. The understanding of circulatory dysfunction in patients with decompensated cirrhosis has led to the use of albumin and vasoconstrictors to improve circulatory dysfunction and prevent kidney injury[8]. Such an approach is paramount because the presence of acute kidney injury (AKI) is associated with significantly longer hospitalization stay and higher mortality in patients with decompensated cirrhosis[9].

Human albumin has widely been used in decompensated cirrhosis patients for varying indications. While the established indications of albumin infusion as endorsed by the current societal guidance include spontaneous bacterial peritonitis (SBP), large-volume paracentesis (LVP) and hepatorenal syndrome (HRS)[10], albumin infusion is often used beyond these indications in the daily clinical practice. Although some of the recently published studies have reported the beneficial effect of regular long-term albumin infusion in patients with decompensated cirrhosis[11-13], regular long term albumin infusion is not completely innocuous. Not only is albumin more expensive than crystalloids as volume expander, serious adverse events as pulmonary oedema and even death have also been reported[14].

With this background, we aim to critically review the current literature on both the established and potential role of albumin, and specifically addressed the controversies of long-term albumin infusion in decompensated cirrhosis patients.

WHAT IS ALBUMIN?

Albumin is the main circulating protein in healthy adults. Structurally it is a small (66500 Dalton), negatively-charged protein that consists of 2 sub-domains[15,16]. Albumin is exclusively synthesized within the liver. It is up-regulated by hormones (insulin, cortisol and growth hormone)[17-19] and down-regulated by inflammatory mediators (tumor necrosis factor and interleukin-6)[20]. Once produced, up to 40% of albumin is released into the bloodstream. The half-life of albumin ranges from 12 to 19 d[21]. The degradation of albumin occurs mostly within the liver, kidney and muscle[22].

Function of albumin

Albumin has both oncotic and non-oncotic properties[15,23]. The potent oncotic property of albumin is primarily derived from the direct oncotic effect from high plasma concentration, which accounts for about two-thirds of its osmotic effect. The Gibbs-Donnan effect, where the negatively-charged albumin molecule also attracts positively charged molecules such as sodium within the bloodstream, is responsible for the remaining one-third of the osmotic effect of albumin[23].

Albumin transports hydrophobic molecules (such as bilirubin, bile acid, long-chain fatty acids) to hepatocytes for detoxification and elimination[24]. Recent evidence suggests that the effect of albumin goes beyond the oncotic functions and transport, but also include immunomodulatory and antioxidant functions as well. Albumin is shown to attenuate prostaglandin E2 mediated immune-dysfunction in patients with

decompensated cirrhosis[25]. It also exerts immunomodulatory effect by down-regulating the expression of tumor necrosis factor-alpha and pro-inflammatory nuclear factor-kappa B[26]. Another property attributed to albumin is that it also functions as an antioxidant to scavenge reactive oxygen and nitrogen species in our body[27,28].

Albumin in decompensated cirrhosis

Hypoalbuminemia is a known predictor of poor survival in decompensated cirrhosis and serves well as a constituent of Child-Turcotte-Pugh score. What is less well appreciated is the fact that abnormalities with serum albumin in decompensated cirrhosis patients are both quantitative and qualitative[29]. The quantitative reduction of serum albumin concentration is a result of dilution from sodium and water retention, reduced synthesis from hepatocytes as well as increased trans-capillary leak, particularly amongst patients with refractory ascites[30,31]. The quality of albumin is further compromised in decompensated cirrhosis due to a higher proportion of oxidized albumin[32]. The oxidized albumin differs from native albumin because it has a lower binding capacity, impaired antioxidant properties and a shortened half-life[31]. Oxidized albumin not only correlates with the severity and complication of cirrhosis but also with short and long-term mortality[29,32]. This understanding on both the quantitative and qualitative alterations of albumin has resulted in the concept of "effective albumin concentration" in decompensated cirrhosis, which takes into account both the amount of albumin and its structural integrity[33].

ESTABLISHED INDICATION OF ALBUMIN IN DECOMPENSATED CIRRHOSIS

SBP

SBP is defined based on the presence of > 250 polymorphonuclear cells/mm^3 or positive ascitic fluid cultures, in the absence of an intraabdominal source of infection or malignancy[34]. Renal impairment is reported in up to 33% patients following SBP and is associated with inpatient mortality, despite resolution of infection[34,35]. In the first randomized trial which investigated the role of intravenous albumin infusion in SBP, Sort *et al*[36] demonstrated that albumin infusion and cefotaxime significantly reduced the risk of renal impairment (33% *vs* 10%), inpatient mortality (29% *vs* 10%) and 3-month mortality (41% *vs* 22%)[36]. The benefits of albumin especially in patients at high risk of developing renal impairment (baseline serum bilirubin ≥ 4 mg/dL or creatinine ≥ 1 mg/dL) were subsequently confirmed in a meta-analysis of randomized trials[37].

Is albumin mandatory in SBP patients with low risk of renal impairment, particularly those who did not fulfil the above criteria? A meta-analysis reported a low pooled incidence of renal impairment and death (2.8% and 3.8%, respectively) among the patients with low risk of renal impairment[37]. The number needed-to-treat to prevent one case of renal impairment and death is 45 and 27, respectively. Given the limited data in low-risk SBP patients, further prospective randomized trials are required to confirm the benefit of albumin infusion in SBP patients with low risk of renal impairment.

Post-paracentesis circulatory dysfunction

Paracentesis-induced circulatory dysfunction (PICD) is a known complication of LVP in patients with decompensated cirrhosis. The reported incidence varies widely between 17.1% to as high as 72.7%, depending on whether albumin infusion was given during LVP[38]. PICD classically has been defined as at-least 50% or more rise in serum renin levels up to 6 d following a large volume paracentesis[39]. PICD can lead to arterial hypotension and the resultant renal impairment has been associated with readmissions and mortality[39].

Several studies have evaluated the role of albumin infusion in large volume paracentesis. Albumin infusion (given at 6-8 g/L of ascitic fluid drained) has shown to prevent PICD in paracentesis beyond 5 L[39,40]. In a meta-analysis of randomized trials, albumin infusion is associated with a lower risk of PICD (OR = 0.39, 95%CI: 0.27-0.55) and mortality (OR = 0.64, 95%CI: 0.41-0.98) following paracentesis[38]. Specifically, all the included trials removed beyond 5 L of ascitic fluid; the majority of the studies administered 6-8 g of albumin 20% *per* L of ascitic fluid removed. With this understanding, the current guidelines recommending albumin replacement in

paracentesis beyond 5 L to prevent PICD[38].

HRS

HRS is the functional renal failure secondary to intrarenal vasoconstriction in patients with decompensated cirrhosis or acute liver failure[10]. Emerging data suggest HRS to be driven by both renal hypoperfusion from systemic circulatory dysfunction as well as increased circulating pro-inflammatory cytokines[41].

Currently, most of the evidence for albumin infusion in HRS is derived from HRS type 1 (also known as HRS-AKI). In a prospective, non-randomized study to investigate the role of albumin infusion, with and without terlipressin, in patients with HRS-AKI, Ortega *et al*[42] demonstrated that albumin infusion significantly improves HRS-AKI in addition to terlipressin alone (albumin: 77% *vs* 25%)[42]. Ever since then, albumin has become an integral part of HRS treatment with vasoactive drugs such as terlipressin, noradrenaline or octreotide[42-53]. Most studies administer 20-40 g of albumin *per* day and titrate according to fluid status to avoid fluid overload. Combination of albumin and terlipressin reverse HRS-AKI in up to 56% of patients in randomized clinical trials[43-45]. However, treatment-related adverse events leading to treatment discontinuation still occur in up to 43% of patients during the clinical trials. These complications (namely acute coronary syndromes and peripheral vascular ischemia) are mostly caused by intense systemic vasoconstriction attributable to terlipressin and can be partially mitigated by continuous terlipressin infusion (complication rates of 35% *vs* 62%), without compromising the treatment efficacy[46].

Even though albumin and terlipressin infusion achieves reversal of HRS-AKI in up to 60% of patients, it may not eventually result in reduced mortality. Several notable studies have evaluated the mortality benefit of albumin and vasoconstrictor in HRS-AKI with conflicting results[43-45,47-53]. Based on two of the recent meta-analyses, there is no conclusive survival benefit of albumin and vasoconstrictor infusion in HRS-AKI when compared to placebo[54,55].

Type-2 HRS is different from type-1 as it has a more subtle course and lower short-term mortality. Albumin and terlipressin infusion has also been shown to improve renal function in HRS type 2. However, the recurrence rate of HRS type 2 after treatment discontinuation is high and there is no clear benefit on mortality of these patients[56-58].

THE ROLE OF ALBUMIN IN DECOMPENSTAED CIRRHOSIS: BEYOND GUIDANCE

Non-SBP infection

As the circulating human albumin is less than optimal both quantitatively and qualitatively in decompensated cirrhosis. Theoretically, the benefit of albumin infusion may be expanded to non-SBP infection, especially those with renal impairment. It is also widely accepted that while renal impairment is often reversible in patients with decompensated cirrhosis with non-SBP infection, the 3-mo mortality is significantly higher compared to patients without renal impairment (55% *vs* 13%)[59]. Some notable literatures have tried to answer this quandary with help of randomised clinical trials (RCT). In a single-center RCT, Guevara *et al*[60] randomized 110 patients with non-SBP infections to receive standard antibiotics with or without albumin[60]. The dose of albumin administered was similar to SBP (1.5 g/kg on day 1 and 1 g/kg on day 3) regimen. Despite a reduction in serum creatinine, renin and aldosterone (which indicates an improvement in renal and circulatory function), the 3-mo survival rates were similar between the two groups[60]. In another RCT, Thévenot *et al*[61] randomized 191 patients with decompensated cirrhosis (Child-Pugh score > 8) with sepsis to receive albumin in addition to antibiotic. The rate of renal failure and mortality at three months were similar in both groups (albumin: 14.3% *vs* 13.5%, and, albumin: 70.2% *vs* 78.3%, respectively)[61]. However, 8.3% of patients developed pulmonary oedema following albumin infusion, and two patients died as a result of pulmonary oedema. These findings were confirmed in a recent meta-analysis of randomized trials, which showed that albumin infusion did not reduce the risk of renal impairment or death in non-SBP infection[14]. As albumin infusion did not improve renal function or survival, yet may result in adverse events such as pulmonary oedema or even death, the current guideline does not recommend albumin infusion for patients with non-SBP infection[10].

HE

HE is a neuropsychiatric manifestation associated with poor prognosis in decompensated cirrhosis resulting from the complex interplay between effective circulatory volume, ammonia, systemic inflammation and portosystemic shunting. As albumin is known to improve systemic circulatory dysfunction and oxidative stress-mediated tissue injury, there has been growing interest in using albumin to treat or prevent HE.

The preventive role of albumin infusion was investigated in a single center cohort study by Riggio *et al*[62] The author enrolled 23 patients following Transjugular intrahepatic portal-systemic shunt (TIPSS) to receive albumin infusion for three weeks. The risk of developing new HE was similar to a historical cohort which did not receive albumin infusion[62], suggesting that infusion of albumin may not have any role in preventing TIPSS or systemic shunting-related HE.

The role of albumin for the treatment of HE was first studied in 15 alcoholic cirrhosis patients with diuretic-induced HE. Patients were randomized to receive albumin or colloid infusion titrated accordingly to the central venous pressure[63]. Despite having a similar reduction in serum ammonia in both groups, the albumin group has a greater improvement in HE grade. Similar beneficial effects were observed in a prospective, open-labelled randomized study, Sharma *et al*[64] enrolled 120 patients with overt HE (graded based on the West Haven criteria) to receive either lactulose, with and without albumin[64]. Albumin was administered at 1.5 g/kg/d until the resolution of HE or day 10 of admission. Albumin group was more likely to achieve complete resolution of HE (albumin: 75% *vs* 53%), shorted hospitalization stays (albumin: 6.4 d *vs* 8.6 d) and lower mortality (albumin: 18% *vs* 32%). Furthermore, the albumin group had a greater decline in the serum tumor necrosis factor alfa, interleukin-6 and endotoxin level when compared to lactulose alone. However, this beneficial effect of albumin is not consistently demonstrated across studies. In a multicenter, double-blind, randomized controlled study, 56 patients with HE were randomized to receive albumin infusion (1.5 g/kg on day 1 and 1 g/kg on day 3) *vs* 0.9% saline[65]. This study remarkably did not find any significant difference in HE resolution by day 4, even though albumin infusion was associated with better transplant-free survival in patients with HE [hazard ratio (HR) 0.27, 95%CI: 0.11-0.74]. The current societal guidelines do not endorse the use of long-term albumin infusion for either the treatment or prevention of HE in patients with decompensated cirrhosis[10].

Acute-on-chronic liver failure

Acute-on-chronic liver failure (ACLF) is a distinct clinical entity characterized by systemic inflammation associated with multiorgan failure and high short-term mortality among decompensated cirrhosis patients[66]. As systemic inflammation is the hallmark of ACLF, the pleiotropic properties of albumin to rapidly expand the intravascular volume and ameliorate systemic inflammation makes albumin a promising treatment option in ACLF. Although clinical studies in past investigating the role of extracorporeal devices[67,68] provide the proof of concept that albumin infusion could play an effective role in the management of patients with ACLF, only a few studies have been carried out to specifically investigate the effect of albumin infusion in patients with ACLF.

In a recent multicenter randomized study (INFECIR-2 trial), Fernández *et al*[69] randomized 108 patients with decompensated cirrhosis and non-SBP infection resulting in ACLF to receive albumin or placebo in addition to antibiotic[69]. More patients in the albumin group experienced resolution of ACLF (82.3% *vs* 33.3%), even though the overall mortality were similar to patients receiving antibiotics alone[69]. Though promising, more robust data is required to support the use of albumin in ACLF.

LONG-TERM ALBUMIN IN DECOMPENSATED CIRRHOSIS

There have been growing interests in long-term albumin use among decompensated cirrhosis patients. We summarize all the relevant studies describing the use of long-term albumin in decompensated cirrhosis in Table 1. Wilkinson and Sherlock[70] first studied the role of long-term albumin infusion in the 1960s. They randomized 16 patients with diuretic refractory ascites to receive albumin infusion *vs* standard medical therapy (SMT)[70]. Albumin infusion was titrated based on serum oncotic pressure and maintained up to 19 mo. Apart from improving general "well-being",

Table 1 Characteristics of studies on long-term albumin infusion in decompensated cirrhosis patients

No	Ref.	Country	Study design	Follow-up duration[1]	Study population	Exclusion criteria	Duration of albumin infusion (d)	Sample size	Child-Pugh Score (A/B/C)	MELD score (albumin *vs* SMT)[1]	Intervention	Control
1	Wilkinson and Sherlock[70], 1962	England	Single centre, non-randomized	22 mo	Cirrhosis patients with ascites despite 6 wk of dietary and diuretic therapy	HCC	616	16	NA	NA	Albumin 25-100 g until serum colloid oncotic pressure 38-40 cm of water	SMT
2	Gentilini *et al*[71], 1999	Italy	Single centre, randomised controlled trial	3 yr	Adult cirrhosis patients with ascites after 1 wk of bed rest and low sodium diet	Renal or cardiac failure, HCC or other malignancies, HE (grades 2-4), infections, gastrointestinal bleeding or DIVC	1095	126	0/67/59	NA	Albumin 12.5 g/d	SMT
3	Romanelli *et al*[72], 2006	Italy	Single centre, randomised controlled trial	84 mo (2-120)	Adult cirrhosis patients with ascites	Active alcohol abuse; previous ascites (grades 2 and 3) or HE; cardiac, respiratory or renal impairment; diabetes; refractory ascites; HCC or other malignancies; gastrointestinal bleeding; infections or DIVC	1440	100	0/46/54	NA	Albumin 25 g weekly in the first year, 25 g every two weeks thereafter	SMT
4	Caraceni *et al*[11], 2018	Italy	Multicentre, randomised controlled trial	18 mo	Adult cirrhosis patients with medically controlled uncomplicated ascites	Refractory ascites, recent decompensation, TIPS, HCC, liver transplantation, ongoing alcohol abuse, extrahepatic organ failure and albumin use for the treatment of ascites within one month	540	431	64/282/85	12 (10–15), 13 (10–16)	Albumin 40 g twice weekly for 2 wk, and 40 g weekly up to 18 mo	SMT
5	Sola-Vera *et al*[40], 2003	Spain	Multicentre, randomised controlled trial	1 yr	Cirrhotic patients with ascites on the liver transplantation waiting list	Arterial hypertension; treatment with psychotropic drugs or antibiotic; TIPS; cardiac or respiratory failure; previous or currently listed for liver transplant; HIV or HCV infection, contraindications to midodrine	365	196	NIL	16 ± 6.2, 17 ± 6.0,	Midodrine 15-30 mg/d and Albumin 40 g/15 d for 1 yr	SMT
6	Di Pascoli *et al*[13], 2019	Italy	Non-randomised, prospective study	Mean 408 +/- 394 d	Adult cirrhosis patients with refractory ascites	HCC beyond Milan criteria or severe extrahepatic diseases	720	70	CTP 9.3 ± 1.7; 9.5 ± 1.6	15.2 ± 5.4, 14.9 ± 5	Human albumin 20 grams twice *per* week	SMT, LVP when indicated
7	China *et al*[75], 2018	United Kingdom	Multicentre randomised controlled trial	6 mo	Adult cirrhosis patients hospitalised with acute decompensation and hypoalbuminemia (serum albumin < 30 g/L)	Advanced HCC; heart failure	14	828	NA	NA	Albumin 20-80 g/d until serum albumin ≥ 35 g/L	SMT

[1]Presented in mean (± SD) or median (interquartile range).

SMT: Standard medical therapy, HCC: Hepatocellular carcinoma; HE: Hepatic encephalopathy; DIVC: Disseminated intravascular coagulopathy; TIPS: Transjugular intrahepatic portosystemic shunt; LVP: Large-volume paracentesis; NA: Not available; NIL: Nanoimprint lithography; HIV: Human immunodeficiency virus; HCV: Hepatitis C virus; CTP: Cytoplasmic transduction peptide.

long-term albumin infusion did not improve overall survival or reduce the need for diuretics.

In another single center randomized study, Gentilini *et al*[71] enrolled 126 patients with refractory ascites to receive either albumin infusion or SMT[71]. Patients received weekly albumin infusion of 25 g in the first year, followed by 25 g every two weeks up to 3 years. Long-term albumin infusion reduced ascites recurrence and ascites-related readmission without improving the overall survival. Subsequently, the same group performed a follow-up study 7 years later in 2006, evaluating the long-term outcomes of long-term albumin infusion with an extension of the follow-up period to a median of 84 mo[72]. They recruited 100 patients with new-onset, clinically significant ascites and randomized them to receive either albumin or SMT. The effect of long-term albumin in ascites management was again demonstrated, with less ascites recurrence (39% *vs* 85%) in the albumin group. More importantly, long-term albumin infusion improved 5-year transplant-free survival (albumin: 62% *vs* 26%) for the first time, even though the sample size was relatively small.

The ANSWER study (the human Albumin for the TreatmeNt of aScites in patients With hEpatic cirRhosis) enrolled 431 patients of decompensated cirrhosis with medically controlled ascites and compared the clinical outcomes in patients receiving long term albumin infusion *vs* SMT[11]. In this study, long term albumin infusion (40 g twice weekly for two weeks, followed by 40 g weekly) in addition to SMT was associated with significantly lower mortality (HR 0.62, 95%CI: 0.40-0.95). The ascites control were better in albumin group with a lower risk for paracentesis (HR 0.48, 95%CI: 0.35-0.54) and refractory ascites (HR 0.43, 95%CI: 0.29-0.62). Also, long-term albumin infusion was associated with a lower risk of both SBP and non-SBP related bacterial infection, grade III and IV HE, HRS, renal dysfunction and hyponatremia. Long-term albumin infusion was well-tolerated. Finally, long-term albumin infusion was also shown to be cost-effective, primarily by a reduction in hospital admission, risk of paracentesis and HRS.

In another prospective but non-randomized study, Di Pascoli *et al*[13] enrolled 70 patients with cirrhosis and refractory ascites to receive either long-term albumin infusion *vs* SMT[13], with the primary endpoint of 24-mo survival. Subjects in the albumin group received 20 g of albumin twice weekly. The study demonstrated a significant improvement in 24-mo survival in the albumin group when compared to the SMT (58% *vs* 35% in SMT) over a mean follow up of 408 d. Furthermore, the albumin group had a lower risk of emergency hospitalizations from SBP, non-SBP infection and HE. While the liver transplantation rate was similar in both groups (11% *vs* 8% in SMT), it should be highlighted that none of the patients with refractory ascites received Transjugular intrahepatic portosystemic shunt (TIPS). More data is required to evaluate the comparative efficacy of long-term albumin and TIPS for refractory ascites.

The MACHT trial (midodrine and albumin for cirrhotic patients in the waiting list for liver transplantation) however offered a contrasting view on the survival benefit of long-term albumin in decompensated cirrhosis patients[12]. In this multicenter, randomized, double-blind, placebo-controlled trial, 196 patients on the transplant waiting list were enrolled to receive either SMT or albumin infusion (40 g every 15 d for one year) plus midodrine with cirrhosis-related complications being the primary end-point. In contrast to the ANSWER trial, the cirrhosis-related complications, ascites control and overall survival were similar between albumin and SMT group. However, 3 important features of the MACHT trial must be considered and the results interpreted in accordingly. First, a relatively higher proportion of patients in both groups received transplantation, (68% in albumin *vs* 55% in SMT group). Second, the duration of albumin therapy was relatively short (median duration of 80 d). Thirdly, the dose of albumin therapy used was also lower than that used in all the other studies. A dosage of 40 g every 15 d was used, as compared to higher dosages in all the other trials. The failure of albumin therapy group to show a better outcome may potentially be attributed to these three factors.

IS LONG-TERM ALBUMIN READY FOR PRIME TIME?

The ANSWER study has provided valuable insights on using long-term albumin infusion as a pathophysiological approach to prevent cirrhosis related complications and death in stable cirrhosis patients with medically-controlled ascites. Nevertheless, it is worth noting that the ANSWER study excluded more advanced-cirrhosis patient with refractory ascites and recent decompensation (variceal bleeding, bacterial

infection). In patients with refractory ascites, the comparative efficacy between long-term albumin infusions *vs* TIPS, which is a one-off procedure with good efficacy, remains unanswered. Besides, only 3.2% (14/431) of patients with hepatitis C related cirrhosis received direct-acting antiviral therapy in the ANSWER study. As the treatment with direct-acting viral therapy is expected to improve the clinical outcomes in these patients[73,74], whether this specific subset of decompensated patients would benefit from long-term albumin infusion following sustained virological response remains unanswered.

The most recent published data, although in abstract form, evaluating the benefits of albumin infusion comes from the ATTIRE (Albumin to prevent infection in chronic liver failure) study which included patients with cirrhosis hospitalized for acute decompensation and hypoalbuminemia (serum albumin < 30 g/L)[75]. In this multicenter randomized trial which enrolled 778 patients to receive albumin infusion *vs* SMT, the primary endpoint was having a new infection, renal dysfunction or mortality from day 3 to 15 of treatment. The results of this study show that the risk of renal dysfunction and death were similar between albumin and SMT group and thus albumin infusion may not be beneficial in these patients. The PRECOISA (Effect of long-term administration of albumin in subjects with decompensated cirrhosis and ascites) study which aims to investigate the impact of long-term albumin on the 1-year mortality and ACLF, is currently ongoing (NCT03451282). The results of PRECOISA will hopefully provide robust evidence for the use of long-term albumin infusion in decompensated cirrhosis patients.

CONCLUSION

Decompensated cirrhosis is characterized by systemic circulatory dysfunction from portal hypertension and systemic inflammation. In decompensated cirrhosis, albumin dysfunction both in terms of quantity and quality. The established therapeutic role of albumin infusion in decompensated cirrhosis includes SBP, HRS and in patients undergoing LVP. Although long-term albumin seemed promising to prevent ascites-related complications in decompensated cirrhosis, the existing studies were heterogeneous in terms of their study population, follow-up duration, and the dose of albumin infusion, thus making the interpretation on the survival benefit particularly challenging. The positive results of long-term albumin infusion will likely increase the global demand for intravenous albumin, particularly among decompensated cirrhosis patients. Meanwhile, the cell-free concentrated ascites reinfusion therapy (CART) may be a novel alternative to intravenous albumin infusion in patients with ascites[76], however more data is required to evaluate the efficacy and safety of CART, particularly among cirrhosis patients with refectory ascites.

Future upcoming studies evaluating the role of long-term albumin infusion to ameliorate systemic inflammation and cirrhosis-related complications are expected in the next few years. Till then, the use of albumin beyond the established indication should be individualized. Future studies should focus on refining the dosages, schedule of long-term albumin infusion and on the specific population groups which would benefit the most.

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Bile acid indices as biomarkers for liver diseases I: Diagnostic markers

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Abstract

BACKGROUND

Hepatobiliary diseases result in the accumulation of toxic bile acids (BA) in the liver, blood, and other tissues which may contribute to an unfavorable prognosis.

AIM

To discover and validate diagnostic biomarkers of cholestatic liver diseases based on the urinary BA profile.

METHODS

We analyzed urine samples by liquid chromatography-tandem mass spectrometry and compared the urinary BA profile between 300 patients with hepatobiliary diseases *vs* 103 healthy controls by statistical analysis. The BA profile was characterized using BA indices, which quantifies the composition, metabolism, hydrophilicity, and toxicity of the BA profile. BA indices have much lower inter- and intra-individual variability compared to absolute concentrations of BA. In addition, BA indices demonstrate high area under the receiver operating

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characteristic curves, and changes of BA indices are associated with the risk of having a liver disease, which demonstrates their use as diagnostic biomarkers for cholestatic liver diseases.

RESULTS

Total and individual BA concentrations were higher in all patients. The percentage of secondary BA (lithocholic acid and deoxycholic acid) was significantly lower, while the percentage of primary BA (chenodeoxycholic acid, cholic acid, and hyocholic acid) was markedly higher in patients compared to controls. In addition, the percentage of taurine-amidation was higher in patients than controls. The increase in the non-12α-OH BA was more profound than 12α-OH BA (cholic acid and deoxycholic acid) causing a decrease in the 12α-OH/ non-12α-OH ratio in patients. This trend was stronger in patients with more advanced liver diseases as reflected by the model for end-stage liver disease score and the presence of hepatic decompensation. The percentage of sulfation was also higher in patients with more severe forms of liver diseases.

CONCLUSION

BA indices have much lower inter- and intra-individual variability compared to absolute BA concentrations and changes of BA indices are associated with the risk of developing liver diseases.

Key Words: Hepatobiliary diseases; Bile acids; Bile acid indices; Diagnosis; Biomarker; Liver diseases

Core Tip: We have developed the concept of "bile acids (BA) indices" based on the detailed quantitative analysis of the urinary BA profile in patients with cholestatic liver diseases. We demonstrated the use of BA indices as diagnostic biomarkers for cholestatic liver diseases. BA indices had much lower inter- and intra-individual variability compared to absolute concentrations of the total and individual BA. In addition, BA indices demonstrated high area under the receiver operating characteristic curves, and changes of BA indices were associated with the risk of having a liver disease as determined by the logistic regression analysis.

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INTRODUCTION

Bile acids (BA) have many physiological functions such as cholesterol absorption and elimination, fat absorption, and maintenance of healthy microbiome[1,2]. BA are also signaling molecules/hormones, which are involved in the regulation of their own homeostasis, thyroid hormone signaling, glucose and lipid metabolism, energy expenditure, and cellular immunity[2-5]. Conversely, certain BA are also cytotoxic at high concentrations and have deleterious effects on hepatocytes and cholangiocytes, which play a major role in liver injury during various liver diseases[5-8].

Cholestatic liver diseases are associated with a reduction in bile flow due to impairment of bile flow or defects in bile production[9]. This causes accumulation of BA in the liver, which spills out into the systemic circulation, extrahepatic tissues, and eventually into urine. Numerous clinical and preclinical studies have shown up to a 100-fold increase in BA concentrations in the blood and urine during various liver diseases[8,10-13]. Elevated BA concentrations were shown to correlate with the progression of damages to the liver and bile duct in cholestatic rats, rabbits, and in humans[14-18].

quality classification
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Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

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Biomarkers currently used in the clinic for the diagnosis and prognosis of liver diseases are primarily serum liver enzymes such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) as well as bilirubin[19,20]. However, they are not specific to the liver or bile duct injuries, may increase in non-hepatobiliary diseases, and require severe cell injury at advanced disease stages before their blood levels increase[19,20]. BA were extensively investigated for decades as biomarkers for numerous hepatobiliary diseases[13,21-23]. However, these efforts never translated into the clinic, with the few exception of limited use in the diagnosis of intrahepatic cholestasis of pregnancy and biliary atresia in infants. This could be attributed to the marked differences in the physiological and pathological properties of the different individual BA. For example, detailed profiling of the more toxic and relevant individual BA rather than total BA concentration may better correlate with the liver condition during hepatobiliary diseases[10,12,24]. Also, the extreme inter-and intra-individual variability of total and individual BA concentrations due to many factors such as food ingestion and diurnal variation, makes it challenging to determine the normal baseline ranges[25,26].

We have developed the concept of "BA indices", which are ratios calculated from the absolute concentrations of individual BA and their metabolites (Table 1). These ratios provide comprehensive quantification of the composition, metabolism, hydrophilicity, formation of secondary BA, and toxicity of the BA profile[9,26]. BA indices have much lower variability than the absolute BA concentrations used to calculate them. Indeed, we have demonstrated that BA indices offered numerous advantages over absolute total and individual BA concentrations including low inter- and intra-individual variability and were resistant to covariate influences such as age, gender, body mass index (BMI), food consumption, and moderate alcohol consumption[9,26].

We have expanded on our previous pilot study, where we have recruited 300 patients with liver diseases and 103 control subjects over a period of 7 years. This study includes a series of two papers. In this article, we have shown the utility of BA indices as diagnosing markers for liver diseases by compared the urinary BA profile between healthy controls and patients and between patients with different severity levels of liver disease. In the 2nd article, we have built a survival model, the Bile Acid Score (BAS), to predict the prognosis of liver diseases using significant BA indices identified in this article.

MATERIALS AND METHODS

Study participants

For controls, 103 healthy subjects (32 male and 71 female) without liver diseases between the ages of 19 and 65 years were recruited by the Clinical Research Center at the University of Nebraska Medical Center (UNMC) (Omaha, NE, United States). The registry URL was (https://www.clinicaltrials.gov/ct2/show/NCT01200082?term=alnouti&draw=2&rank=1). The clinical trial number was NCT01200082. Inclusion criteria for the healthy controls included normal liver functions, as verified by ALT < 50 U/L, AST < 56 U/L, gamma-glutamyl transferase < 78 U/L, absence of diabetes, and no- or moderate alcohol drinking[27] The study was approved by Institutional Review Board at UNMC and written informed consents were provided for all participating subjects. Thirty milliliters urine samples were collected from controls at fasting conditions in the first visit, and 1, 2, and 4 wk thereafter.

Patients diagnosed with one or multi-hepatobiliary conditions due to chronic hepatitis C (n = 71) , hepatitis B (n = 15), alcoholic liver disease/alcoholic cirrhosis (n = 117), primary biliary cholangitis (PBC) (n = 12), primary sclerosing cholangitis (n = 17), autoimmune hepatitis (n = 27), alpha-1-antitrypsin deficiency (n = 6), nonalcoholic fatty liver disease/nonalcoholic steatohepatitis (n = 56), carcinoma (n = 26), cryptogenic cirrhosis (n = 11), polycystic liver disease (n = 5), elevated liver function test (LFT) (n = 22), and unknown etiology (n = 5), were enrolled in the hepatology clinic in UNMC. A total of 300 patients (157 male and 143 female) between the ages of 19 years and 83 years were recruited. Thirty milliliters of urine samples were collected on their first and follow-up visits to the hepatology clinic. All urine samples were stored in -80 °C until analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Patients were divided into three disease-severity groups based on their model for end-stage liver disease (MELD) score: low-MELD (6-15 score), medium-MELD (16-25), and high-MELD (26-40). High MELD group was not included while performing the statistical analysis, because there were only four subjects in that group.

Table 1 List of bile acid indices

Composition	Hepatic metabolism	Hydrophilicity	CYP8B1 activity	Intestinal contribution
Concentration of individual BA	Total sulfated	Total mono-OH	Total 12α-OH	Total primary
% of individual BA	Total G-amidated	Total di-OH	Total non-12α-OH	Total secondary
	Total T-amidated	Total tri-OH	12α-OH/non-12α-OH	Primary/secondary
	% Sulfation	% Mono-OH	CA/CDCA	% Primary
	% Amidation	% Di-OH	% 12α-OH	% Secondary
	% G-amidation	% Tri-OH	% Non-12α-OH	
	% T-amidation			

BA: Bile acids; G: Glycine; T: Taurine; CDCA: Chenodeoxycholic acid; CA: Cholic acid.

In addition, patients were also categorized according to hepatic decompensation (presence or history of encephalopathy, bleeding varices, ascites, or jaundice)[28].

Non-BA parameters

AST, ALT, albumin, and serum creatinine were measured using the Beckman Coulter reagents (Beckman Coulter, Inc, Brea, California). Protime and international normalized ratio (INR) were measured using STANeoplastine "CI PLUS 10" reagent kit (Diagnostica Stago Inc, Parsippany, New Jersey). Total bilirubin in serum was analyzed using QuantiChromTM Bilirubin assay kit (BioAssay Systems, Hayward, CA, United States). AST/ALT ratio and AST/platelet ratio index (APRI) were calculated.

BA quantification by LC-MS/MS

Urine samples were extracted using solid phase extraction as described previously[9,26,29,30]. BA concentrations were quantified by LC-MS/MS, as we described previously[31].

Calculation of BA indices

In addition to the absolute concentration of individual and total BA, the BA profile in urine was characterized using "BA indices" (Table 1), and as we have described previously[9,26,30,31]. BA indices describe the composition, hydrophilicity, formation of 12α-OH BA by CYP8B1, metabolism, and formation of secondary BA by intestinal bacteria. The composition indices were calculated as the ratio of the concentration of individual BA in all of their forms (sulfated, unsulfated amidated, and unamidated) to the total concentration of BA. The percentages of mono-OH BA: [lithocholic acid (LCA)], di-OH BA: [ursodeoxycholic acid (UDCA), murideoxycholic acid (MDCA), chenodeoxycholic acid (CDCA), hyodeoxycholic acid (HDCA), and deoxycholic acid (DCA)], and tri-OH BA: [cholic acid (CA), muricholic acid (MCA), and hyocholic acid (HCA)] were calculated as the ratio of the concentration of the sum of the respective BA in all their forms to the total concentration of BA.

The 12α-OH BA are formed by CYP8B1 in the liver and include DCA, CA, nor-DCA, and 3-dehydroCA. Therefore, CYP8B1 activity can be measured by the ratio of 12α-OH BA to the remaining of all other BA (non-12α-OH BA). Another marker for CYP8B1 is the ratio of CA to CDCA because CA is formed by the 12α hydroxylation of CDCA. In the same way, the ratio of 12α-OH (DCA, CA, nor-DCA, and 3-dehydroCA in all of their forms) to non-12α-OH (CDCA, HDCA, LCA, UDCA, MDCA, HCA, MCA, 12-oxo-CDCA, 6-oxo-LCA, 7-oxo-LCA, 12-oxo-LCA, isoLCA, isoDCA in all of their forms) was calculated.

BA are metabolized primarily by sulfation, glycine (G), and taurine (T) amidation in the liver. The percentage of individual BA sulfation was calculated as a ratio of the concentration of sulfated BA, in both the amidated and unamidated forms, to the total individual BA concentration in all their forms (amidated, unamidated, sulfated, and unsulfated). In both the sulfated and unsulfated forms, the percentage of individual BA amidation have been calculated as the ratio of the concentration of amidated BA, to the total concentration of individual BA in all of their forms (amidated, unamidated, sulfated, and unsulfated). Additionally, percentages of amidation were divided into the percentages of BA existing as G or as T amidates.

The ratio of primary (CA, CDCA, MCA and HCA in all of their forms) to secondary BA (DCA, LCA, UDCA, HDCA, MDCA, Nor-DCA, 12-oxo-CDCA, 3-dehydroCA, 6-oxo-LCA, 7-oxo-LCA, 12-oxo-LCA, isoLCA, and isoDCA in all of their forms) was calculated.

Statistical analysis

Independent sample-*t*-test and Mann-Whitney test were used to study the demographic differences between controls and patients because the sample size was > 30[32]. Independent sample-*t*-test was used for continuous variables and Mann-Whitney test was used for categorical variables. The demographic variables were (age, BMI, gender, and race). Subjects were divided into four age groups (19-29, 30-41, 42-53, 54-83 years), and the variable age was studied as both a continuous and a categorical variable. Subjects were also divided into three BMI groups (normal: BMI < 25, overweight: BMI 25-29.9, and obese: BMI ≥ 30) and the effect of BMI was studied as both a continuous and a categorical variable. Also, subjects were divided into five race groups (White, Black, Asian, Hispanic, others), and the variable race was studied as a categorical variable.

Urine samples were collected from controls and patients on their first visit and follow-up visits. Mixed effects models were used to compare patients *vs* controls and the demographic variables were included as covariates. Statistically significant covariates were returned to the mixed effects models as interaction terms with the primary group, *i.e.*, patients *vs* control.

BA indices were compared between controls, low-MELD (patients), and medium-MELD (patients) groups using mixed effects models followed by pairwise comparisons using Bonferroni's adjustment if the *P* value was < 0.05. BA indices were compared between compensated and decompensated patients using mixed effects models. Mixed effects models were also used to determine the association between non-BA parameters including (AST, ALT, bilirubin, MELD score, AST/ALT, creatinine, INR, APRI, protime, and albumin) and BA indices. Receiver operating characteristic curve (ROC) analyses were used to determine cut-off values of BA as markers for the diagnosis of liver diseases with optimum sensitivity and specificity. The areas under the ROC curve (AUC) values were compared between urinary BA profiles and non-BA parameters. The mixed effects models were used to compare BA indices with AUC > 0.7 between controls and the patients with specific disease subtypes described in the "Study Participants" section (same patients can belong to different disease groups). Polycystic liver disease and unknown etiology subtypes were not included in the comparison between the disease subtypes because they had < six subjects.

Univariate logistic regression analysis was used to determine the association between BA concentrations and indices and the likelihood of developing a liver disease. From logistic regression analysis, the odds ratios (ORs) were calculated for a 10% and 20% change from the mean value of BA indices in the healthy controls.

P value of 0.05 was considered significant for all the statistical tests described above. All statistical analysis was performed using the Statistical Product and Service Solutions (SPSS) software, version 25 (IBM corporation, Armonk, NY, United States).

RESULTS

Demographics

Table 2 shows a summary of the demographics of both patients and controls participants. We enrolled 103 controls (32 males and 71 females) and 300 patients (157 males and 143 females), who were treated for cholestatic liver diseases in UNMC, over the period from November of 2011 to December of 2018. To compare the demographics between the two groups, age and BMI covariates were compared as both continuous and categorical variables using *t*-test, and Mann-Whitney test, respectively. While gender and race were compared as categorical variables using Mann-Whitney test. Age, gender, and BMI were significantly different between control and patients (*P* value < 0.05), while race was not different. Therefore, the statistically significant demographic variables (age, BMI, and gender) were included as covariates in the mixed effects models to compare BA indices between patients and controls.

Table 2 Demographics

	Controls	Patients
n	103	300
Gender[1]		
Male, female	32, 71	157, 143
Age (yr)[1]		
mean ± SE	44.3 ± 0.64	52.1± 0.54
19-29	17	11
30-41	28	40
42-53	30	92
54-83	28	157
BMI[1]		
mean ± SE	27.5 ± 0.28	30.9 ± 0.32
Normal BMI < 25	30	69
Overweight BMI = 25-29.9	45	104
Obese BMI ≥ 30	28	127
Race		
White	88	247
Black	7	14
Asian	7	13
Hispanic	1	8
Others	0	18

[1]Significant difference between controls and patients ($P < 0.05$).
BMI: Body mass index.

Differences in BA between patients vs controls are not due to differences in demographics

Because some of the covariates (age, BMI, and gender) were significantly different between the two groups (Table 2), we reran the univariate mixed effect analysis with these covariates (multivariate analysis). First, association between these covariates and BA indices was identified, and then the covariates with significant association with BA indices were incorporated in the multivariate mixed effect analyses as interaction terms with the group (patients and controls). We did not find any difference in the association between covariates and BA indices between the two groups except for the % primary and % secondary BA with gender (Supplementary Table 1).

BA profiles in controls vs patients

Table 3 shows the absolute concentrations of major urinary BA in controls and patients. Table 4 compares representative absolute BA concentrations and indices between controls and patients. Supplementary Table 2 shows the full list of BA concentrations and indices. Total BA was 5.9-fold higher in patients compared with controls. All individual BA concentrations were also higher in patients, except MDCA, but to different extents. The highest increase was in UDCA (11.9-fold), while the lowest increase was for DCA and HDCA (1.6-fold). The percentage of UDCA, CDCA, MCA, CA, and HCA were higher (1.2-1.6-fold), while the percentage of LCA, DCA, HDCA, and MDCA were lower (0.5-0.8-fold) in patients *vs* controls.

Unamidated, G-amidated, and T-amidated BA which were 3.3-, 5.9-, and 9.4-fold higher in patients than controls. Therefore, the overall % amidation and % G-amidation did not change or slightly decreased in patients, whereas % T-amidation increased from 8.0% in controls to 10.8% in patients. Similarly, the concentrations of both sulfated and unsulfated were approximately 6-fold higher in patient; so that the

Table 3 Absolute concentrations of major bile acids in controls and patients

BA	Unamidated	G-BA	T-BA	Total
	mean ± SE, μmol/L			
Controls				
Unsulfated BA				
LCA	0.000 ± 0.00	0.000 ± 0.00	0.000 ± 0.00	0.001 ± 0.00
UDCA	0.004 ± 0.00	0.033 ± 0.00	0.002 ± 0.00	0.038 ± 0.00
CDCA	0.003 ± 0.00	0.008 ± 0.00	0.002 ± 0.00	0.013 ± 0.00
DCA	0.022 ± 0.00	0.011 ± 0.00	0.002 ± 0.00	0.035 ± 0.00
HDCA	0.01 ± 0.00	0.00 ± 0.00	ND	0.007 ± 0.00
MDCA	0.060 ± 0.01	ND	ND	0.058 ± 0.01
CA	0.179 ± 0.03	0.067 ± 0.00	0.009 ± 0.00	0.255 ± 0.03
MCA	0.028 ± 0.00	0.287 ± 0.02	0.041 ± 0.00	0.356 ± 0.02
HCA	0.008 ± 0.00	0.016 ± 0.00	0.001 ± 0.00	0.026 ± 0.00
Other BA[1]	0.160 ± 0.01	-	-	0.160 ± 0.01
Total unsulfated	0.464 ± 0.04	0.422 ± 0.02	0.057 ± 0.00	0.943 ± 0.05
Sulfated BA				
LCA	0.010 ± 0.00	0.780 ± 0.04	0.220 ± 0.01	1.010 ± 0.05
UDCA	0.450 ± 0.02	1.040 ± 0.05	0.030 ± 0.00	1.520 ± 0.07
CDCA	0.070 ± 0.01	2.380 ± 0.13	0.060 ± 0.00	2.510 ± 0.13
DCA	0.010 ± 0.00	2.900 ± 0.14	0.220 ± 0.02	3.130 ± 0.16
CA	0.004 ± 0.00	0.056 ± 0.01	0.126 ± 0.01	0.190 ± 0.01
Total sulfated	0.535 ± 0.03	7.170 ± 0.28	0.650 ± 0.03	8.350 ± 0.31
Overall total	1.000 ± 0.05	7.590 ± 0.29	0.710 ± 0.03	9.300 ± 0.33
Patients				
Unsulfated BA				
LCA	0.004 ± 0.00	0.001 ± 0.00	0.0001 ± 0.00	0.005 ± 0.00
UDCA	0.079 ± 0.03	0.410 ± 0.17	0.012 ± 0.00	0.500 ± 0.21
CDCA	0.020 ± 0.00	0.090 ± 0.01	0.100 ± 0.02	0.210 ± 0.03
DCA	0.040 ± 0.00	0.040 ± 0.00	0.010 ± 0.00	0.090 ± 0.01
HDCA	0.010 ± 0.00	0.00 ± 0.00	ND	0.010 ± 0.00
MDCA	0.050 ± 0.01	ND	ND	0.050 ± 0.01
CA	0.240 ± 0.03	0.550 ± 0.07	0.320 ± 0.08	1.120 ± 0.14
MCA	0.120 ± 0.02	1.940 ± 0.29	0.730 ± 0.09	2.790 ± 0.34
HCA	0.010 ± 0.00	0.170 ± 0.02	0.090 ± 0.02	0.270 ± 0.04
Other BA[1]	0.860 ± 0.13	-	-	0.860 ± 0.13
Total	0.460 ± 0.04	0.42 ± 0.02	0.06 ± 0.00	5.910 ± 0.57
Sulfated BA				
LCA	0.030 ± 0.01	2.230 ± 0.20	0.650 ± 0.06	2.910 ± 0.24
UDCA	1.560 ± 0.23	15.30 ± 2.68	1.230 ± 0.27	18.10 ± 3.08
CDCA	0.190 ± 0.03	18.70 ± 1.79	1.910 ± 0.38	20.80 ± 2.07
DCA	0.040 ± 0.01	4.280 ± 0.54	0.520 ± 0.07	4.840 ± 0.58
CA	0.080 ± 0.01	0.910 ± 0.13	1.030 ± 0.21	2.010 ± 0.31

Total	1.900 ± 0.24	41.40 ± 4.12	5.340 ± 0.74	48.70 ± 4.77
Overall total	3.330 ± 0.33	44.60 ± 4.46	6.610 ± 0.85	54.60 ± 5.20

[1]Other bile acids: Nor-deoxycholic acid, 12-oxo-chenodeoxycholic acid, 3-dehydrocholic acid, 6-oxo-lithocholic acid, 7-oxo-lithocholic acid, 12-oxo-lithocholic acid, isolithocholic acid, and isodeoxycholic acid.
ND: Not detected; -: Not quantified; BA: Bile acids; G: Glycine; T: Taurine; CDCA: Chenodeoxycholic acid; CA: Cholic acid; LCA: Lithocholic acid; UDCA: Ursodeoxycholic acid; DCA: Deoxycholic acid; HDCA: Hyodeoxycholic acid; MDCA: Murideoxycholic acid; MCA: Muricholic acid; HCA: Hyocholic acid.

% sulfation of BA was unchanged.

The absolute concentrations of mono-, di-, and tri-OH BA were also higher in patients compared with controls, but the % mono-OH decreased (0.8-fold), di-OH remained unchanged, and % tri-OH increased (1.4-fold) due to increasing % CA (1.2-fold), % MCA (1.6-fold), and % HCA (1.5-fold).

Total 12α-OH and non-12α-OH BA were 2.3-fold and 8.2-fold higher in patients, so that the ratio of 12α-OH/ non-12α-OH and the % 12α-OH decreased (approximately 0.5-fold), while % non-12α-OH BA increased (1.2-fold).

Total primary and secondary BA were 8.1-fold and 4.6-fold higher in patients, so that the ratio of primary/secondary BA was 3.6-fold higher. Therefore, % primary BA was 1.4-fold higher, while % secondary BA was 0.80-fold lower in patients *vs* controls.

BA profile in low vs medium-MELD patients

Table 5 compares representative urinary BA concentrations and indices between low- and medium-MELD patients. Total BA concentrations was twice and individual BA concentrations were (1.15-fold to 3.9-fold) higher in medium *vs* low-MELD patients (Table 5).

Unamidated BA concentration was lower, while G-amidated and T-amidated BA were higher in the medium-MELD patients. Therefore, % T-amidation was 1.5-fold higher, while there was minimal difference in the % amidation and % G-amidation between medium and low-MELD patients. Similarly, the concentrations of both sulfated and unsulfated were 1.3- and 2-fold higher in medium *vs* low-MELD. On the other hand, the % sulfation of BA was only 1.07-fold higher, but it was statistically significant.

The absolute concentrations of mono-, di-, and tri-OH BA were also (1.8-2-fold) higher in medium-MELD patients, but the % mono-OH decreased (0.86-fold); while % di- and % tri-OH remained unchanged.

Total 12α-OH and non-12α-OH BA were both higher in medium *vs* low-MELD patients, but to different extents so that % non-12α-OH BA remained unchanged, while % 12α-OH decreased and the ratio of 12α-OH/ non-12α-OH was approximately 0.7-fold lower.

Total primary BA were 3.4-fold higher, while total secondary BA were slightly (0.9-fold) lower in medium-MELD patients, so that the ratio of primary/secondary BA was 2.3-fold higher. Similarly, % primary BA was 1.4-fold higher, while % secondary BA was 0.6-fold lower in medium- MELD patients.

BA profile in compensated vs decompensated patients

Table 6 compares representative urinary BA concentrations and indices between decompensated and compensated patients. In general, the same trend in the higher *vs* lower MELD patients comparison was observed in the decompensated *vs* compensated patients. Total BA was 1.3-fold higher, all individual BA were higher, but to variable extents. The percentage of CDCA, HDCA, CA, and HCA were higher (1.3-2.1-fold), while the percentage of LCA, UDCA, DCA, and MDCA were lower (0.3-0.7-fold) in decompensated *vs* compensated patients.

The % T-amidation was 1.3-fold higher in decompensated vs. compensated patients, while there was no difference in the % amidation, % G-amidation, or % sulfation. The % mono-OH decreased (0.73-fold), % di-OH remained unchanged, and % tri-OH slightly increased (1.13-fold) due to increasing % CA and % HCA. The ratio of 12α-OH/ non-12α-OH lower, the % 12α-OH, and CA/CDCA ratio decreased (0.7-0.8-fold), while % non-12α-OH BA remained unchanged.

Total primary BA were two-fold higher, while total secondary BA were 0.8-fold lower, so that the ratio of primary/secondary BA was 2.6-fold higher in decompensated patients. Therefore, % primary BA was 1.5-fold higher, while % secondary BA was 0.56-fold lower in decompensated patients.

Table 4 Representative bile acids concentrations and indices in controls *vs* patients

BA (μmol/L) or BA indices	Controls		Patients		Patients *vs* controls	
	mean	SE	mean	SE	Ratio	*P* value
Total BA	9.30	0.33	54.6	5.20	5.87	0.000
Total LCA	1.01	0.05	2.92	0.24	2.88	0.000
Total UDCA	1.56	0.07	18.6	3.23	11.9	0.001
Total CDCA	2.52	0.13	21.0	2.09	8.35	0.000
Total DCA	3.16	0.16	4.92	0.58	1.56	0.072
Total HDCA	0.01	0.00	0.01	0.00	1.57	0.051
Total MDCA	0.06	0.01	0.05	0.01	0.90	0.992
Total CA	0.44	0.03	3.13	0.44	7.09	0.003
Total MCA	0.36	0.02	2.79	0.34	7.83	0.000
Total HCA	0.03	0.00	0.27	0.04	10.6	0.001
Other BA[1]	0.16	0.01	0.86	0.13	5.54	NA
% LCA	11.5	0.38	9.20	0.39	0.79	0.002
% UDCA	17.7	0.49	21.3	0.88	1.21	0.138
% CDCA	27.1	0.65	36.3	0.94	1.34	0.000
% DCA	31.1	0.68	14.6	0.53	0.47	0.000
% HDCA	0.07	0.01	0.04	0.00	0.54	0.052
% MDCA	0.64	0.04	0.36	0.05	0.56	0.135
% CA	5.25	0.27	6.27	0.25	1.19	0.064
% MCA	4.03	0.16	6.39	0.34	1.58	0.003
% HCA	0.30	0.02	0.45	0.04	1.52	0.018
Total Unamidated	1.00	0.05	3.33	0.33	3.34	0.000
Total G-amidated	7.59	0.29	44.6	4.46	5.88	0.000
Total T-amidated	0.71	0.03	6.61	0.85	9.37	0.001
% Amidation	87.7	0.47	86.9	0.65	0.99	0.053
% G-amidation	79.7	0.49	76.0	0.71	0.95	0.000
% T-amidation	7.98	0.26	10.8	0.46	1.35	0.005
Total Unsulfated	0.94	0.05	5.91	0.57	6.26	0.000
Total Sulfated	8.35	0.31	48.7	4.77	5.83	0.000
% Sulfation	88.5	0.46	82.9	0.60	0.94	0.000
Total Mono-OH	1.01	0.05	2.92	0.24	2.88	0.000
Total Di-OH	7.30	0.29	44.6	4.58	6.11	0.000
Total Tri-OH	0.82	0.04	6.19	0.65	7.52	0.000
% Mono-OH	11.5	0.38	9.16	0.39	0.79	0.002
% Di-OH	76.6	0.50	72.7	0.65	0.95	0.001
% Tri-OH	9.58	0.33	13.1	0.43	1.37	0.000
Total 12α-OH	3.62	0.17	8.35	0.83	2.30	0.001
Total non-12α-OH	5.67	0.20	46.2	4.68	8.15	0.000
12α-OH/non12α-OH	0.65	0.02	0.33	0.01	0.51	0.000
CA/CDCA	0.24	0.01	0.24	0.02	1.00	0.625
% 12α-OH	36.7	0.62	22.1	0.54	0.60	0.000

% non-12α-OH	63.3	0.62	77.9	0.54	1.23	0.000
Total Primary	3.34	0.15	27.2	2.59	8.15	0.000
Total Secondary	5.95	0.23	27.4	3.52	4.59	0.000
Primary/ Secondary	0.69	0.03	2.52	0.22	3.63	0.000
% Primary	36.7	0.70	49.4	1.06	1.35	0.000
% Secondary	63.3	0.70	50.6	1.06	0.80	0.000

[1]Other bile acids: Nor-deoxycholic acid, 12-oxo-chenodeoxycholic acid, 3-dehydrocholic acid, 6-oxo-lithocholic acid, 7-oxo-lithocholic acid, 12-oxo-lithocholic acid, isolithocholic acid, and isodeoxycholic acid.

NA: Not available; BA: Bile acids; G: Glycine; T: Taurine; CDCA: Chenodeoxycholic acid; CA: Cholic acid; LCA: Lithocholic acid; UDCA: Ursodeoxycholic acid; DCA: Deoxycholic acid; HDCA: Hyodeoxycholic acid; MDCA: Murideoxycholic acid; MCA: Muricholic acid; HCA: Hyocholic acid.

ROC curve analysis

Supplementary Table 3 lists the AUC for BA concentrations and indices. Supplementary Table 4 shows the full list of BA concentrations and indices. Total BA, CDCA, CA, % DCA, % HDCA, % MDCA, total G-Amidated, total unsulfated, total sulfated, total di-OH, total tri-OH, total non-12α-OH, 12α-OH/non12α-OH, % 12α-OH, % non-12α-OH, total primary, primary/secondary, % primary, and % secondary produced AUC > 0.7. Figure 1 shows ROC curves of BA indices with AUC > 0.7. Potential cut-off values selected based on the optimum specificity and sensitivity for BA indices with AUC > 0.7 are listed in Supplementary Table 5.

Risk analysis: Logistic regression analysis

Table 7 shows the results of logistic regression analyzes for BA indices with ROC (AUC) > 0.7. Logistic regression analysis detects whether there is a risk of liver disease associated with changes in BA indices. The risk of liver disease increased with changing levels of all BA indices ($P < 0.05$) except (% HDCA and % MDCA). Additionally, the OR from the logistic regression analysis quantifies the magnitude of the risk of developing liver diseases per unit (10% and 20% of the normal value) changes in BA indices. For example, for every 20% increase in the % non-12α-OH BA, the likelihood of having a liver disease increases 2.72-folds (OR: 2.72; $P < 0.05$). In contrast for every 20% increase in the % 12α-OH BA, the likelihood of having a liver disease decreases 0.56-folds (OR: 0.56; $P < 0.05$).

BA profile in different liver disease subtypes

Table 8 compare BA indices with ROC-AUC > 0.7 between controls *vs* patients with specific liver disease subtype. Mixed effects models were used to compare disease subtypes individually *vs* controls. The goal was to identify BA indices that can serve as diagnostic biomarkers for specific liver disease subtypes.

We have found that most BA indices were significantly different between controls *vs* all individual liver disease subtypes. Total BA, total CDCA, total CA, total G-amidated, total unsulfated, total sulfated, total di-OH, total tri-OH, Total non-12α-OH, % non-12α-OH and total primary were higher (1.1- to 39.5-fold) in every liver disease group compared with controls. % Primary and primary/secondary were higher (1.1-fold to 9.27-fold) in all liver disease group compared with controls except in PBC. % DCA, % HDCA, % 12α-OH, and 12α-OH/non-12α-OH were lower (0.07-fold to 0.85-fold) in every liver disease group compared with controls. % MDCA and % secondary was lower in all liver disease group compared with controls except in elevated LFT and PBC, respectively.

Non-BA parameters

In addition to BA indices, we have also examined other biomarkers currently used in the clinic to evaluate liver functions. These non-BA parameters include AST, ALT, AST/ALT, bilirubin, albumin, INR, protime, creatinine, APRI, and MELD. Table 9 compares the non-BA parameters in controls and patients using mixed effects models. All the non-BA parameters were higher in patients compared to controls except albumin and protime, which were lower in patients. Within the patient population, all non-BA parameters were higher in medium compared to low- MELD patients except albumin, and ALT. The same results also applied to decompensated *vs* compensated patients.

Table 5 Representative bile acids concentrations and indices in medium- *vs* low- model for end-stage liver disease patients

BA (μmol/L) or BA indices	Low-MELD		Medium-MELD		Medium- *vs* low-MELD	
	mean	SE	mean	SE	Ratio	*P* value
Total BA	59.2	7.94	116	24.8	1.96	1.000
Total LCA	3.40	0.35	6.01	1.72	1.77	0.175
Total UDCA	24.4	5.34	18.6	6.30	0.76	0.172
Total CDCA	18.3	2.31	71.4	16.3	3.90	0.000
Total DCA	5.30	0.96	6.08	1.47	1.15	1.000
Total HDCA	0.01	0.00	0.01	0.00	0.61	1.000
Total MDCA	0.05	0.01	0.06	0.01	1.28	1.000
Total CA	2.80	0.48	10.6	4.45	3.79	0.000
Total MCA	3.58	0.57	2.15	0.46	0.60	0.210
Total HCA	0.25	0.04	0.86	0.36	3.48	0.002
% LCA	9.31	0.53	7.97	1.47	0.86	1.000
% UDCA	23.1	1.29	14.3	2.52	0.62	1.000
% CDCA	34.7	1.21	55.6	3.17	1.60	0.000
% DCA	13.8	0.65	7.18	1.33	0.52	0.005
% HDCA	0.04	0.01	0.01	0.00	0.32	0.661
% MDCA	0.29	0.04	0.13	0.03	0.43	1.000
% CA	5.75	0.30	8.70	1.25	1.51	0.145
% MCA	7.15	0.48	3.70	0.91	0.52	0.000
% HCA	0.46	0.07	0.75	0.15	1.61	0.148
Total Unamidated	4.24	0.55	2.87	0.72	0.68	0.062
Total G-amidated	48.4	6.89	92.8	19.7	1.92	1.000
Total T-amidated	6.58	1.04	20.7	7.30	3.15	0.040
% Amidation	86.7	0.87	94.4	1.28	1.09	0.005
% G-amidation	75.5	0.96	77.2	2.73	1.02	1.000
% T-amidation	11.2	0.64	17.1	2.15	1.53	0.002
Total unsulfated	6.99	0.93	9.04	2.42	1.29	1.000
Total sulfated	52.3	7.21	107	23.2	2.05	1.000
% Sulfation	82.4	0.81	88.3	1.34	1.07	0.009
Total mono-OH	3.40	0.35	6.01	1.72	1.77	0.175
Total di-OH	48.1	7.01	96.2	20.9	2.00	1.000
Total tri-OH	6.63	0.90	13.6	4.90	2.06	0.301
% Mono-OH	9.31	0.53	7.97	1.47	0.86	1.000
% Di-OH	72.0	0.90	77.2	2.14	1.07	0.058
% Tri-OH	13.4	0.59	13.1	1.40	0.98	0.274
Total 12α-OH	8.55	1.23	16.8	4.86	1.96	0.053
Total non-12α-OH	50.7	7.21	99.6	21.5	1.96	1.000
12α-OH/non12α-OH	0.30	0.01	0.20	0.02	0.68	0.135
CA/CDCA	0.21	0.01	0.17	0.03	0.81	1.000
% 12α-OH	21.0	0.69	16.1	1.44	0.77	0.008
% non-12α-OH	79.0	0.69	83.9	1.44	1.06	0.008

Total primary	25.0	3.08	85.1	19.5	3.41	0.000
Total secondary	34.3	5.78	31.3	8.05	0.91	0.316
Primary/secondary	2.19	0.24	5.02	1.16	2.29	1.000
% Primary	48.1	1.40	68.7	3.10	1.43	0.014
% Secondary	51.9	1.40	31.3	3.10	0.60	0.014

BA: Bile acids; MELD: Model for end-stage liver disease; G: Glycine; T: Taurine; CDCA: Chenodeoxycholic acid; CA: Cholic acid; LCA: Lithocholic acid; UDCA: Ursodeoxycholic acid; DCA: Deoxycholic acid; HDCA: Hyodeoxycholic acid; MDCA: Murideoxycholic acid; MCA: Muricholic acid; HCA: Hyocholic acid.

The AUC for non-BA parameters was > 0.7 for all of them except creatinine, protime, and AST/ALT ratio. Also, per logistic regression analysis, the risk of being diagnosed with a liver disease increased to various extents with changing levels of all non-BA parameters ($P < 0.05$) except creatinine and AST/ALT. For example, for every 20% increase in the albumin and protime, the likelihood of having a liver disease decreases 0.28-fold and 0.85-fold, respectively. In contrast for every 20% increase in the other non-BA parameters, the likelihood of having a liver disease increases 1.13-fold to 3-fold (Supplementary Table 6).

In addition, we have found that most non-BA parameters were significantly different between controls *vs*. all individual liver disease subtypes (Supplementary Table 7). Creatinine, INR, AST, ALT, bilirubin, AST/ALT, and MELD were higher in most liver disease group compared with controls. In contrast, albumin and protime were lower in most liver disease group compared with controls.

Association between non-BA parameters and BA indices

Supplementary Table 8 shows the association between non-BA parameters and BA indices using mixed effects models. We have found that all non-BA parameters were significantly associated with most BA concentrations/indices, except creatinine ($P > 0.05$).

DISCUSSION

To ensure that the difference in the BA profiles between patients and controls are not due to the differences in the demographics we showed that: (1) Most of BA were not associated with demographic covariates, and (2) The ones that were associated had the same extent of association in the patient and control groups (Supplementary Table 1).

Patients were categorized based on the severity of the liver disease using MELD[33-37] and the compensation status[28]. Accordingly, we have compared the BA profiles between entire patient *vs* control populations as well as among the patients with different levels of disease severity. Most BA (except MDCA) were higher, but to different extents, in patients *vs* controls (Table 4) and in the more-severe patient groups, *i.e.*, medium *vs* low-MELD (Table 5) as well as decompensated *vs* compensated (Table 6). In particular, the percentages of the primary BA (CDCA, CA, and HCA) were higher, while the percentage of the secondary BA (DCA) was lower. The % primary BA was 1.4-fold higher, while % secondary BA was 0.8-fold lower and the ratio of primary/secondary BA was 3.6-fold higher in patients *vs* controls (Table 4). The same trend was also observed in the patients with more severe form of the disease, where the % of primary BA also increased with the severity of the liver disease (medium-MELD > low-MELD > controls) and (decompensated > compensated > controls), whereas % secondary BA decreased with the severity of the disease. (Tables 5 and 6).

Cholestatic diseases are associated with impaired bile flow to the intestine, which translates into reduced transformation of primary into secondary BA by intestinal bacteria[9,25,38-40]. Therefore, while all BA concentrations were higher in patients due to the impairment of bile flow, the proportion of secondary BA (formed in the intestine) decreased with the severity of the cholestatic disease, which may reflect the extent of bile flow impairment.

The conjugation of BA with G and T decreases their pKa, increases their ionization and solubility, enhances their urinary elimination, and decreases their toxicity[30,41-44]. However, T-amidated BA are generally less cytotoxic and more ionized than G-

Table 6 Representative bile acids concentrations and indices in compensated *vs* decompensated patients

BA (μmol/L) or BA indices	Compensated		Decompensated		Decompensated *vs* compensated	
	mean	SE	mean	SE	Ratio	*P* value
Total BA	66.6	10.8	86.9	14.9	1.31	0.160
Total LCA	3.73	0.54	4.26	0.70	1.14	0.547
Total UDCA	27.0	6.49	21.0	9.82	0.78	0.687
Total CDCA	20.4	3.42	45.0	6.28	2.20	0.001
Total DCA	6.85	1.76	4.93	0.73	0.72	0.394
Total HDCA	0.01	0.00	0.02	0.01	1.61	0.430
Total MDCA	0.06	0.01	0.05	0.01	0.86	0.619
Total CA	2.62	0.40	6.28	1.51	2.39	0.024
Total MCA	4.48	0.93	4.07	0.83	0.91	0.864
Total HCA	0.20	0.04	0.64	0.14	3.23	0.002
% LCA	9.00	0.64	6.61	0.64	0.73	0.020
% UDCA	24.9	1.97	12.0	1.32	0.48	0.007
% CDCA	33.2	1.62	54.74	2.05	1.65	0.000
% DCA	14.3	0.98	9.17	1.00	0.64	0.000
% HDCA	0.02	0.01	0.03	0.01	1.42	0.532
% MDCA	0.34	0.14	0.11	0.01	0.33	0.264
% CA	6.07	0.54	7.58	0.48	1.25	0.262
% MCA	7.26	0.72	7.21	0.82	0.99	0.542
% HCA	0.35	0.05	0.74	0.08	2.09	0.005
Total unamidated	4.35	0.69	3.88	1.04	0.89	0.876
Total G-amidated	56.2	9.81	70.5	12.5	1.25	0.240
Total T-amidated	5.97	0.79	12.6	2.58	2.11	0.010
% Amidation	87.9	1.15	93.6	0.75	1.06	0.003
% G-amidation	76.5	1.30	78.8	1.23	1.03	0.161
% T-amidation	11.5	0.93	14.8	1.02	1.29	0.161
Total unsulfated	7.84	1.19	9.53	1.85	1.22	0.310
Total sulfated	58.7	9.97	77.4	13.4	1.32	0.156
% Sulfation	82.7	1.12	85.2	0.99	1.03	0.054
Total mono-OH	3.73	0.54	4.26	0.70	1.14	0.547
Total di-OH	54.4	9.55	70.9	13.1	1.31	0.174
Total tri-OH	7.30	1.25	11.0	1.96	1.51	0.085
% Mono-OH	9.00	0.64	6.61	0.64	0.73	0.020
% Di-OH	72.7	1.14	76.0	1.31	1.05	0.016
% Tri-OH	13.7	0.92	15.5	0.95	1.13	0.674
Total 12α-OH	10.1	2.08	11.44	1.75	1.14	0.554
Total non-12α-OH	56.5	9.36	75.51	14.0	1.34	0.137
12α-OH/non12α-OH	0.33	0.02	0.24	0.02	0.71	0.002
CA/CDCA	0.21	0.02	0.17	0.01	0.79	0.043
% 12α-OH	22.0	1.07	17.3	0.99	0.79	0.001
% non-12α-OH	78.0	1.07	82.7	0.99	1.06	0.001

Total primary	27.7	4.46	56.0	7.59	2.02	0.001
Total secondary	38.8	7.43	31.0	10.3	0.80	0.874
Primary/secondary	2.27	0.44	5.98	0.69	2.64	0.001
% Primary	46.9	2.05	70.3	1.88	1.50	0.000
% Secondary	53.1	2.05	29.7	1.88	0.56	0.000

BA: Bile acids; G: Glycine; T: Taurine; CDCA: Chenodeoxycholic acid; CA: Cholic acid; LCA: Lithocholic acid; UDCA: Ursodeoxycholic acid; DCA: Deoxycholic acid; HDCA: Hyodeoxycholic acid; MDCA: Murideoxycholic acid; MCA: Muricholic acid; HCA: Hyocholic acid.

Table 7 Univariate logistic regression analysis of bile acids concentrations and indices[1]

BA (μmol/L) or BA indices	*B* value (regression coefficient)	*P* value	Exp(B)-odds ratio		
			1-unit change	10% change	20% change
Total BA	0.080	0.000	1.08	1.08	1.16
Total CDCA	0.226	0.000	1.25	1.06	1.12
Total CA	1.181	0.000	3.26	1.05	1.11
% DCA	-0.080	0.000	0.92	0.78	0.61
% HDCA	-1.898	0.069	0.15	0.99	0.97
% MDCA	-0.174	0.162	0.84	0.99	0.98
Total G-amidated	0.084	0.000	1.09	1.07	1.14
Total unsulfated	0.784	0.000	2.19	1.08	1.16
Total sulfated	0.080	0.000	1.08	1.07	1.14
Total di-OH	0.094	0.000	1.10	1.07	1.15
Total tri-OH	0.731	0.000	2.08	1.06	1.13
Total non-12α-OH	0.146	0.000	1.16	1.09	1.18
12α-OH/non12α-OH	-2.349	0.000	0.10	0.86	0.74
% 12α-OH	-0.079	0.000	0.92	0.75	0.56
% non-12α-OH	0.079	0.000	1.08	1.65	2.72
Total primary	0.190	0.000	1.21	1.07	1.14
Primary/secondary	0.834	0.000	2.30	1.06	1.12
% Primary	0.033	0.000	1.03	1.13	1.27
% Secondary	-0.033	0.000	0.97	0.81	0.66

[1]Bile acids with receiver operating characteristic (ROC)-areas under the ROC curve > 0.7 were included in this table.
BA: Bile acids; G: Glycine; CDCA: Chenodeoxycholic acid; CA: Cholic acid; DCA: Deoxycholic acid; HDCA: Hyodeoxycholic acid; MDCA: Murideoxycholic acid.

amidated BA[43,45,46]. Even though unamidated as well as T-and G-amidated BAs were higher in patients, the increase in T-amidated BA was the most profound. Therefore, % T-amidation increased, while % G-amidation decreased in patients *vs* controls (Table 4) as well as in medium-MELD *vs* low-MELD (Table 5) and decompensated *vs* compensated patients (Table 6). The preferential accumulation of T-amidated BA can be interpreted as an adaptive compensating response to protect the liver from BA toxicity by increasing elimination of the more toxic G-amidated and unamidated compared to the less toxic T-amidated BA[9,26,47]. In addition, T-amidated BA has the highest affinity as substrates for the canalicular transporter, Bile Salt Export Pump (BSEP) (T-amidated > G-amidated > unamidated BA)[48-50]. Therefore, an impairment of the BA transport by BSEP, as documented in some cholestatic diseases[51-53], is expected to preferential accumulation T-amidated BA.

Table 8 Bile acids concentrations and indices in controls and patients with specific liver disease subtype[1]

BA (μmol/L) or BA indices	Controls		Hepatitis C		Hepatitis B		Laennec cirrhosis		Primary biliary cholangitis		Primary sclerosing cholangitis		Autoimmune Hepatitis		α-1 antitrypsin deficiency		NASH		Carcinoma		Cryptogenic cirrhosis		Elevated LFT	
	n = 103		*n* = 71		*n* = 15		*n* = 117		*n* = 12		*n* = 17		*n* = 27		*n* = 6		*n* = 56		*n* = 26		*n* = 11		*n* = 22	
	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
Total BA	9.30	0.33	53.3[a]	9.96	13.7[a]	5.23	62.0[a]	9.44	237[a]	69.8	124[a]	27.4	71.9[a]	15.2	25.4[a]	6.79	29.8[a]	4.31	90.9[a]	26.7	56.0[a]	16.3	106[a]	69.3
Total CDCA	2.52	0.13	27.0[a]	4.89	6.76[a]	4.16	30.0[a]	4.25	28.6[a]	9.99	39.4[a]	10.7	29.2[a]	8.46	9.14[a]	3.12	13.6[a]	2.33	31.9[a]	8.76	27.7[a]	7.39	31.3[a]	18.8
Total CA	0.44	0.03	3.05[a]	0.54	1.16[a]	0.70	4.00[a]	1.02	5.07[a]	2.27	6.47[a]	2.25	1.96[a]	0.39	2.44[a]	0.85	1.65[a]	0.23	3.54[a]	1.11	2.70[a]	0.59	5.55[a]	3.09
% DCA	31.1	0.68	16.2[a]	1.27	19.9[a]	3.26	13.1[a]	0.95	7.99[a]	2.22	9.01[a]	1.86	15.6[a]	1.43	18.9	4.23	15.7[a]	1.22	14.9[a]	1.43	7.93[a]	2.68	17.4[a]	3.07
% HDCA	0.07	0.01	0.02[a]	0.00	0.06	0.02	0.02[a]	0.01	0.03	0.02	0.01	0.00	0.02	0.00	0.03	1.21	0.05	0.01	0.06	0.02	0.04	0.03	0.05	0.04
% MDCA	0.64	0.04	0.19[a]	0.04	0.38	0.08	0.16[a]	0.03	0.15[a]	0.07	0.18[a]	0.06	0.22[a]	0.05	0.38	0.16	0.49	0.21	0.16[a]	0.05	0.07[a]	0.01	1.34	0.98
Total G-amidated	7.59	0.29	44.8[a]	9.11	11.6[a]	4.52	50.8[a]	8.26	210[a]	60.4	106[a]	24.0	61.8[a]	13.5	16.3[a]	4.58	26.0[a]	4.01	78.1[a]	24.5	49.2[a]	14.9	86.8[a]	58.2
Total unsulfated	0.94	0.05	7.69[a]	1.43	1.62[a]	0.38	7.94[a]	1.21	17.6[a]	8.66	6.21[a]	1.13	6.06[a]	1.44	3.82[a]	1.21	4.64[a]	0.65	13.0[a]	3.16	3.58[a]	0.58	13.0[a]	9.22
Total sulfated	8.35	0.31	45.6[a]	8.73	12.1[a]	4.96	54.1[a]	8.59	219[a]	62.0	117[a]	26.5	65.9[a]	14.7	21.6[a]	6.11	25.2[a]	4.04	77.9[a]	24.2	52.4[a]	16.0	92.9[a]	60.2
Total di-OH	7.30	0.29	41.0[a]	7.98	10.6[a]	4.41	49.05[a]	7.97	214[a]	62.50	111[a]	25.9	60.9[a]	13.7	15.9[a]	4.72	23.2[a]	3.87	71.9[a]	23.0	50.3[a]	16.0	91.0[a]	61.0
Total tri-OH	0.82	0.04	8.61[a]	1.63	1.82[a]	0.80	8.59[a]	1.54	12.48[a]	6.02	9.28[a]	2.44	4.83[a]	0.76	5.24[a]	1.83	4.27[a]	0.66	13.8[a]	3.62	3.92[a]	0.65	10.8[a]	6.50
Total non-12α-OH	5.67	0.20	41.8[a]	7.46	10.3[a]	4.52	50.8[a]	7.85	224[a]	66.9	113[a]	26.5	62.3[a]	14.2	19.4[a]	5.59	23.8[a]	3.74	75.7[a]	22.1	51.1[a]	16.1	94.7[a]	66.1
12α-OH/non12α-OH	0.65	0.02	0.37[a]	0.05	0.51	0.08	0.31[a]	0.02	0.15[a]	0.05	0.29[a]	0.05	0.33[a]	0.04	0.40	0.07	0.34[a]	0.02	0.28[a]	0.03	0.22[a]	0.05	0.37[a]	0.06
% 12α-OH	36.7	0.62	22.8[a]	1.28	31.1	2.9	21.3[a]	1.0	10.8[a]	2.69%	18.8[a]	2.6	22[a]	1.69	27.1	3.18	23.5[a]	1.14	20.6[a]	1.55	16.2[a]	2.88	24.8[a]	2.8
% non-12α-OH	63.3	0.62	77.2[a]	1.28	68.9	2.9	78.7[a]	1.0	89.2[a]	2.69	81.2[a]	2.6	78.0[a]	1.69	72.9	3.18	76.5[a]	1.14	79.4[a]	155	83.8[a]	2.88	75.2[a]	2.8
Total primary	3.34	0.15	35.6[a]	6.23	8.58[a]	4.93	38.6[a]	5.47	41.1[a]	15.6	48.6[a]	12.6	34.1[a]	8.91	14.4[a]	4.33	17.9[a]	2.70	45.7[a]	12.1	31.6[a]	7.89	42.1[a]	23.9
Primary/secondary	0.69	0.03	3.70[a]	0.55	1.52[a]	0.59	4.33[a]	0.60	0.30	0.10	2.88[a]	0.68	2.09[a]	0.60	1.26	0.26	2.28[a]	0.30	2.32[a]	0.40	6.43[a]	2.09	1.70[a]	0.43
% Primary	36.7	0.70	60.2[a]	1.99	47.0[a]	3.93	60.9[a]	1.7	18.0[a]	2.97	51.6[a]	5.09	50.1[a]	2.77	50.3	5.43	52.8[a]	2.21	56.1[a]	3.25	68.2[a]	5.80	49.3[a]	4.6
% Secondary	63.3	0.70	39.8[a]	1.99	53.0[a]	3.93	39.1[a]	1.7	82.0[a]	2.97	48.4[a]	5.09	49.9[a]	2.77	49.7	5.43	47.2[a]	2.21	43.9[a]	3.25	31.8[a]	5.80	50.7[a]	4.6

[1]Bile acids with receiver operating characteristic (ROC)-areas under the ROC curve > 0.7 were included in this table.

[a]Significant difference between each specific liver disease subtype *vs* controls ($P < 0.05$).

BA: Bile acids; G: Glycine; CDCA: Chenodeoxycholic acid; CA: Cholic acid; DCA: Deoxycholic acid; HDCA: Hyodeoxycholic acid; MDCA: Murideoxycholic acid.

Both sulfated and unsulfated BA were higher in patients (Table 4), but % sulfation was slightly higher in medium- compared with low-MELD and in decompensated compared with compensated patients (Tables 5 and 6). The upregulation of sulfation of BA by SULT2A1 in patients with liver diseases is thought as a compensatory response to eliminate and detoxify the accumulated toxic BA[8-13,54,55]. However, it is also possible that sulfation activity in these patients may eventually decrease due to exhaustion or defects of these recovery mechanisms. Therefore, while liver insults can be remediated by upregulating BA sulfation under normal conditions and in milder forms of liver diseases, but subjects who fail to upregulate this defensive mechanism or exhaust it under more severe forms of the diseases are at higher risk of developing the disease and/or may have worse prognosis[26]. Another explanation for the preferential accumulation of BA-sulfates could be related to the inhibition of their canalicular transport into bile by efflux transporters, mainly the multidrug resistance-associated proteins 2-4 (MRP 2-4). These transporters preferentially transport divalent amidated and conjugated (sulfated and glucuronidated) BA[56-59]. MRPs including MRP2 activity is known to be compromised in various cholestatic liver diseases due downregulation of their expression and/or membrane localization[60-62], which may lead to the preferential retention of their substrates including BA-sulfates in the liver and systemic circulation.

CYP8B1 catalyzes 12α-hydroxylation of the di-OH CDCA to the tri-OH CA. The CA/CDCA or the 12α-OH/non-12α ratios are used as probes to measure CYP8B1 activity[63-65]. The 12α-OH/non-12α-OH ratio was 50% lower in patients compared with controls (Table 4). Also, both ratios were lower in medium-MELD *vs* low-MELD as well as decompensated *vs* compensated patients (Tables 5 and 6). This indicates that CYP8B1 activity, which exclusively takes place in the liver[66,67], may be compromised during liver diseases in general and is further compromised with disease severity. Also, CDCA has a much higher affinity to BSEP than CA and other 12α-OH BA[49,68]. Therefore, when BSEP activity is compromised in the more severe liver diseases, it is expected to lead to the preferential accumulation of its high-affinity substrates including CDCA, which will also decrease the CA/CDCA and 12α-OH/non-12α ratios.

Many BA concentrations and indices demonstrated AUC > 0.7 supporting their potential as biomarkers for the diagnosis of liver diseases (Supplementary Table 3). We identified three potential cut-off values, which achieve a good balance between specificity and sensitivity (Supplementary Table 5). BA indices have higher AUC values than the absolute BA concentrations, which indicates that BA indices are more accurate in distinguishing between controls and patients.

We found correlation between the risk of developing a liver disease and many BA indices using logistic regression analysis ($P < 0.05$). The univariate logistic regression associated with a 20% change from the mean value for the absolute BA concentrations ranged from 1.11 to 1.18, whereas it was as high as 2.72 for BA indices (Table 7). This suggests that BA indices are more sensitive than absolute BA concentrations in terms of predicting larger magnitudes of the risk of developing a liver disease.

All the above analyses demonstrate that BA indices can serve as a global marker to differentiate the pooled cholestatic liver disease population from controls in this study. In addition, we have divided the patients into different individual disease groups and performed similar analyses in these groups vs. controls, for the individual diseases. Most BA indices with ROC-AUC > 0.7 were significantly different between controls *vs* most of the individual liver disease subtypes (Table 8). In particular, hepatitis C and cirrhosis were the largest subpopulations in our study, and all global diagnostic BA indices from the pooled patients vs. control analyses ($P < 0.05$ and ROC-AUC > 0.7) were also specific diagnostic markers for these two particular liver diseases vs. controls ($P < 0.05$).

We have found a significant correlation between BA indices and non-BA parameters, except creatinine ($P > 0.05$) (Supplementary Table 8). However, BA indices, in general, outperformed non-BA parameters as biomarkers for liver diseases on many levels. Non-BA parameters were 0.76-fold to 2.5-fold higher (Table 9), whereas BA indices were as high as approximately 12-fold higher (total UDCA) in patients compared to controls (Table 4). Similarly, the magnitude of change within the MELD groups, compensation status, and among individual diseases were all much higher in BA *vs* non-BA.

This study has the following limitations: (1) Severity of the liver diseases were assessed using MELD score, compensation status, and a panel of liver enzymes. However, liver histological evaluation was not included because it is not a routine practice to perform liver histology on all patients, but rather for specific patients as required by the hepatologists. And (2) we have enough subjects in this study to

Table 9 Summary of non-bile acids parameters

Non-BA parameters	Controls		Patients										ROC[1]
			Pooled		Low-MELD		Medium-MELD		Compensated		Decompensated		AUC
	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	
Creatinine (mg/dL)	0.87	0.01	0.99	0.05	0.93	0.07	1.33[b]	0.16	1.05	0.15	1.05	0.06	0.539
Albumin (g/dL)	3.96	0.02	3.61[a]	0.03	3.61	0.03	2.82[b]	0.10	3.69	0.04	3.03[c]	0.06	0.713
INR	0.99	0.01	1.18[a]	0.02	1.11	0.01	1.63[b]	0.10	1.15	0.03	1.36[c]	0.03	0.758
Protime (s)	13.4	0.10	10.2[a]	0.33	13.6	0.13	19.4[b]	0.98	11.2	0.52	13.7[c]	0.64	0.591
AST (U/L)	22.8	0.34	53.2[a]	2.31	52.1	2.59	79.2[b]	10.4	52.6	3.97	61.7	4.85	0.876
ALT (U/L)	21.0	0.46	51.0[a]	2.60	51.0	3.24	46.0	5.54	49.0	4.09	40.6	3.55	0.825
Bilirubin (mg/dL)	0.62	0.03	1.58[a]	0.09	1.31	0.05	5.02[b]	0.68	1.42	0.12	3.04[c]	0.29	0.804
AST/ALT	1.15	0.01	1.22	0.02	1.21	0.03	1.79[b]	0.09	1.21	0.04	1.61[c]	0.05	0.500
MELD	7.13	0.10	10.3[a]	0.24	9.07	0.16	18.9	0.42	9.54	0.37	14.0[c]	0.46	0.747
APRI	NA	NA	0.93	0.06	1.05	0.07	2.44[b]	0.42	0.94	0.08	1.63[c]	0.18	NA

[1]Areas under the receiver operating characteristic curve from receiver operating characteristic analysis of pooled patients *vs* controls.
[a]Significant difference between patients *vs* controls ($P < 0.05$).
[b]Significant difference between medium-model for end-stage liver disease *vs* low-model for end-stage liver disease groups ($P < 0.05$).
[c]Significant difference between decompensated *vs* compensated patients ($P < 0.05$).
NA: Not available; BA: Bile acids; MELD: Model for end-stage liver disease; INR: International normalized ratio; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; APRI: Aspartate aminotransferase/platelet ratio index; ROC: Receiver operating characteristic curve; AUC: Areas under the receiver operating characteristic curve.

perform solid statistics, but smaller number of subjects in many individual disease subgroups. Also, distribution of subjects between disease groups was unbalanced.

CONCLUSION

In summary, the results of this study demonstrated that total and all individual BA increased in patients with 11 different cholestatic diseases. However, the high inter-individual variability of BA absolute concentrations makes most of them statistically insignificant and prevent their utilization as diagnostic markers. In contrast, BA indices had much lower inter- and intra-individual variability, which allowed their use as diagnostic and prognostic markers for liver diseases. Furthermore, we have shown that several BA indices outperformed non-BA markers, currently used in the clinic, as

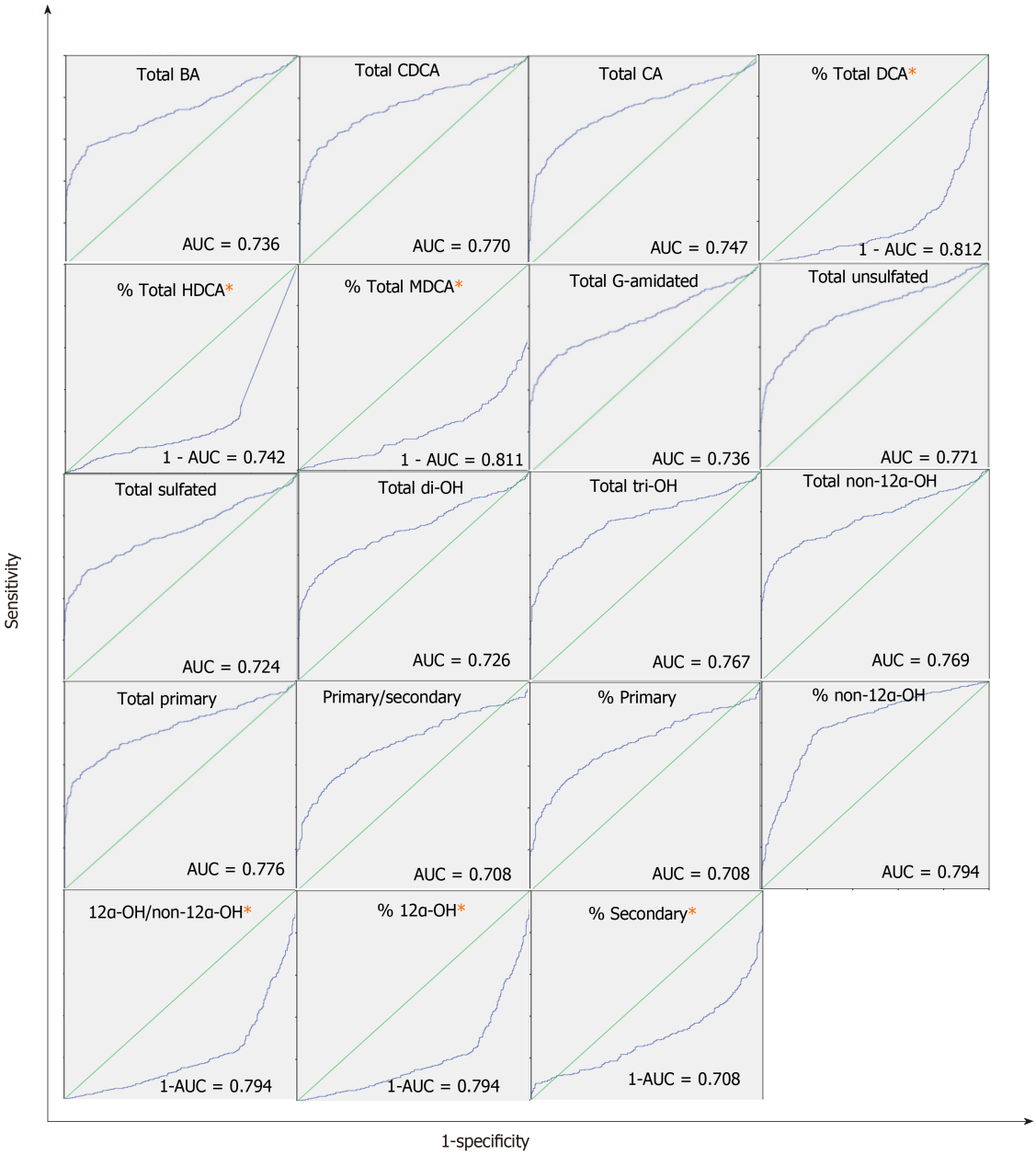

Figure 1 Receiver operating characteristics curves of bile acids concentrations and indices with area under the receiver operating characteristics curve > 0.7. The area under the receiver operating characteristics curve (AUC) for differentiating patients from healthy controls. The scale of both the Y-axis (sensitivity) and the X-axis (1-specificity) is 0-1. Bile acids (BA) indices are higher in patients vs. controls, and the positive actual state was patients except the ones annotated with "*", where BA indices were lower in patients compared to controls. For these BA indices, "1-AUC" instead of "AUC" was calculated. AUC: Area under the receiver operating characteristics curve; BA: Bile acids; CDCA: Chenodeoxycholic acid; CA: Cholic acid; DCA: Deoxycholic acid; HDCA: Hyodeoxycholic acid; MDCA: Murideoxycholic acid; G: Glycine.

diagnostic markers to differentiate our patient pool as well as individual cholestatic diseases against healthy controls.

The increase in the total BA concentration in patients can be attributed to specific changes in the BA pool composition. This increase primarily resulted from primary BA (CDCA, CA, and HCA), while the % of the secondary BA (LCA and DCA) were lower. This lead to about 4-fold increase in the primary/secondary BA ratio. Consequently, the BA pool has drastically shifted in patients from being 37% primary to approximately 50% primary BA. The increase in T-amidated BA was more profound than that of G-amidated BA, which lead to a marked increase in the % T-amidation. Furthermore, this trend of elevated primary and amidated BA was exacerbated with disease severity. This pattern can be a sign of less transformation of primary into secondary and less deconjugation of amidated BA by intestinal bacteria associated with more impairment of bile flow associated with more severe cholestatic diseases. % Sulfation was higher in patients with more severe forms of liver diseases indicating the

upregulation of sulfation in these patients as a compensatory response to detoxify BA accumulation. Finally, the increase in non-12α-OH was more profound than that of 12α-OH BA, which indicates that hepatic CYP8B1 activity is compromised in liver diseases in general and is further compromised with disease severity.

In the 2nd paper of this series, we have utilized BA indexes to build a survival model called "The Bile Acid Score", which we showed was able to predict the prognosis into adverse events including death and liver transplant in liver patients.

ARTICLE HIGHLIGHTS

Research background

Bile acids (BA) have been extensively investigated for decades as biomarkers for numerous hepatobiliary diseases. However, these efforts never translated into a widespread in the clinic, due to the extreme inter-and intra-individual variability of total and individual BA concentrations and the marked differences in the physiological and pathological properties of the different individual BA. To this end, we have developed the concept of "BA indices", which demonstrated their use as diagnostic biomarkers for cholestatic liver diseases.

Research motivation

Biomarkers currently used in the clinic are not specific to the liver or bile duct injurie. BA were extensively investigated for decades as biomarkers for numerous hepatobiliary diseases. This could be attributed to the marked differences in the physiological and pathological properties of the different individual BA. BA indices have much lower variability than the absolute BA concentrations used to calculate them. Indeed, we have demonstrated that BA indices offered numerous advantages over absolute total and individual BA concentrations including low inter- and intra-individual variability and were resistant to covariate influences such as age, gender, body mass index, food consumption, and moderate alcohol consumption.

Research objectives

The objective of this project was to discover and validate diagnostic biomarkers of cholestatic liver diseases based on the urinary BA profile. We have developed the concept of "BA indices", which are ratios calculated from the absolute concentrations of individual BA and their metabolites. BA indices have much lower variability than the absolute BA concentrations used to calculate them, which enabled their use as diagnostic biomarkers for cholestatic liver diseases.

Research methods

We analyzed urine samples by liquid chromatography-tandem mass spectrometry and compared the urinary BA profile between patients with hepatobiliary diseases *vs* healthy controls by statistical analysis (independent sample-*t*-test, Mann-Whitney test, Mixed effects models, by pairwise comparisons using Bonferroni's adjustment, receiver operating characteristic curve analyses, Univariate and multivariate logistic regression analysis).

Research results

The results of this study demonstrated that total and all individual BA increased in patients with 11 different cholestatic diseases. However, the high inter-individual variability of BA absolute concentrations makes most of them statistically insignificant and prevent their utilization as diagnostic markers. In contrast, BA indices had much lower inter- and intra-individual variability, which allowed their use as diagnostic and prognostic markers for liver diseases. Furthermore, we have shown that several BA indices outperformed non-BA markers, currently used in the clinic, as diagnostic markers to differentiate our patient pool as well as individual cholestatic diseases against healthy controls.

Research conclusions

BA indices demonstrated high area under the receiver operating characteristic curves, and changes of BA indices were associated with the risk of having a liver disease as determined by the logistic regression analysis, which demonstrated their use as diagnostic biomarkers for cholestatic liver diseases.

Research perspectives

We have developed survival models based on BA indices to predict the prognosis of hepatobiliary diseases which is illustrated in the second paper of this series.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Elderly patients (≥ 80 years) with acute calculous cholangitis have similar outcomes as non-elderly patients (< 80 years): Propensity score-matched analysis

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Abstract

BACKGROUND

Acute cholangitis (AC) is a disease spectrum with varying extent of severity. Age ≥ 75 years forms part of the criteria for moderate (Grade II) severity in both the Tokyo Guidelines (TG13 and TG18). Aging is associated with reduced physiological reserves, frailty, and sarcopenia. However, there is evidence that age itself is not the determinant of inferior outcomes in elective and emergency biliary diseases. There is a paucity of reports comparing clinical outcomes amongst elderly patients *vs* non-elderly patients with AC.

AIM

To investigate the effect of age (≥ 80 years) on AC's morbidity and mortality using propensity score matching (PSM).

METHODS

This is a single-center retrospective cohort study of all patients diagnosed with calculous AC (January 2016 to December 2016) and ≥ 80 years old (January 2012 to December 2016) at a tertiary university-affiliated teaching hospital. Inclusion criteria were patients who were treated for suspected or confirmed AC secondary to biliary stones. Patients with AC on a background of hepatobiliary malignancy, indwelling permanent metallic biliary stents, or concomitant pancreatitis were excluded. Elderly patients were defined as ≥ 80 years old in our study. A 1:1 PSM analysis was performed to reduce selection bias and address confounding factors.

Informed consent statement: This study was conducted using data collected from an institutional board approved standing database (National Healthcare Group Domain Specific Review Board, Ref No.: 2017/00200). Informed consent was hence not obtained from the included patients. Collected data were de-identified and were only accessible to members of the study team with no subsequent patient contact for data collection purposes. The study team made no attempts to access patients' medical records *via* the national electronic health record system.

Conflict-of-interest statement: All of the authors declare no conflicts of interest.

Data sharing statement: The data used in this study is not publicly available due to institutional policies. However, requests may be made to the corresponding author for access to de-identified data.

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Study variables include comorbidities, vital parameters, laboratory and radiological investigations, and type of biliary decompression, including the time for endoscopic retrograde cholangiopancreatography (ERCP). Primary outcomes include in-hospital mortality, 30-d and 90-d mortality. Length of hospital stay (LOS) was the secondary outcome.

RESULTS

Four hundred fifty-seven patients with AC were included in this study (318 elderly, 139 non-elderly). PSM analysis resulted in a total of 224 patients (112 elderly, 112 non-elderly). The adoption of ERCP between elderly and non-elderly was similar in both the unmatched (elderly 64.8%, non-elderly 61.9%, P = 0.551) and matched cohorts (elderly 68.8% and non-elderly 58%, P = 0.096). The overall in-hospital mortality, 30-d mortality and 90-d mortality was 4.6%, 7.4% and 8.5% respectively, with no statistically significant differences between the elderly and non-elderly in both the unmatched and matched cohorts. LOS was longer in the unmatched cohort [elderly 8 d, interquartile range (IQR) 6-13, *vs* non-elderly 8 d, IQR 5-11, P = 0.040], but was comparable in the matched cohort (elderly 7.5 d, IQR 5-11, *vs* non-elderly 8 d, IQR 5-11, P = 0.982). Subgroup analysis of patients who underwent ERCP demonstrated the majority of the patients (n = 159/292, 54.5%) had delayed ERCP (> 72 h from presentation). There was no significant difference in LOS, 30-d mortality, 90-d mortality, and in-hospital mortality in patients who had delayed ERCP in both the unmatched and matched cohort (matched cohort: in-hospital mortality [n = 1/42 (2.4%) *vs* 1/26 (3.8%), P = 0.728], 30-d mortality [n = 2/42 (4.8%) *vs* 2/26 (7.7%), P = 0.618], 90-d mortality [n = 2/42 (4.8%) *vs* 2/26 (7.7%), P = 0.618], and LOS (median 8.5 d, IQR 6-11.3, *vs* 8.5 d, IQR 6-15.3, P = 0.929).

CONCLUSION

Mortality is indifferent in the elderly (≥ 80 years old) and non-elderly patients (< 80 years old) with AC.

Key Words: Cholangitis; Choledocholithiasis; Cholelithiasis; Aged 80 and over; Geriatrics; Cholangiopancreatography; Endoscopic retrograde

Core Tip: There is a paucity of data on mortality outcomes amongst elderly *vs* non-elderly patients with acute cholangitis. The overall in-hospital mortality, 30-d mortality and 90-d mortality was 4.6%, 7.4% and 8.5% respectively, with no significant differences in both the unmatched and matched cohorts. Mortality was comparable in patients with delayed endoscopic retrograde cholangiopancreatography.

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INTRODUCTION

Gallstones are widely prevalent in the community, and patients with gallstones are at risk of complications like acute cholecystitis, acute pancreatitis, and acute cholangitis (AC). AC results from an obstructed biliary system with sepsis, and resulting endotoxic shock is associated with a mortality risk of up to 20%[1]. AC is a disease spectrum ranging from mild AC, which may respond to conservative management with medical therapy, to severe AC, which requires urgent biliary decompression in addition to fluid resuscitation and antibiotics[2]. Tokyo Guidelines (TG13 and TG18) are widely accepted internationally and form the basis for diagnosis, severity

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stratification, and management of patients with AC[3]. In AC, age determines the severity stratification, and age ≥ 75 years is a criterion for moderate (Grade II) severity in both the TG13 and TG18 guidelines[3]. Aging is associated with reduced cardiac output, impaired gas exchange, reduction in vital capacity, decline in lean body mass, creatinine clearance reduction, hepatic drug metabolism impairment, frailty, and sarcopenia[4]. Due to functional metabolic decline, multiple comorbidities, and atypical presentation with potential diagnostic delays, age contributes to inferior outcomes[5]. Age is an independent predictor of mortality in lower respiratory tract infections, urinary tract infections, gastrointestinal infections and biliary infections[5-8]. Age is also a predictor of disease severity with higher morbidity and mortality risk[9].

However, there is evidence that age itself is not the determinant of inferior outcomes in elective and emergency biliary diseases[10,11]. Endoscopic retrograde cholangiopancreatography (ERCP) have been demonstrated to be safe with good outcomes in elderly patients[11,12]. In a study including 149 acute cholecystitis patients treated with emergency laparoscopic cholecystectomy (LC), Amirthalingam *et al*[13] showed that patient comorbidities and not age determine outcomes. In a study reporting 85 patients with a median age of 83 years (interquartile range 80-89) and admitted to intensive care unit (ICU) with a diagnosis of AC, Novy *et al*[14] reported malnutrition [odds ratio (OR) = 34.5, 95% confidence interval (CI): 1.4-817.9] and sequential organ failure assessment (SOFA) score at 48 h (OR by unit 0.7, 95%CI: 0.5-0.9) were associated with higher 6-mo mortality. Further, aging may impact other clinically relevant non-mortality outcomes such as length of hospital stay (LOS). In a prospective study including 124 patients with acute hepatobiliary sepsis and a median age of 64.5 years, Mak *et al*[15] have reported that age predicts LOS. There is a paucity of comparative data reporting mortality and LOS amongst elderly and non-elderly patients with AC. Also, aging is associated with the confounding effect of comorbidity. This, along with heterogeneity of evidence reporting outcomes in patients with diverse etiology of AC, leaves a lacuna in the scientific literature on the real impact of age on patients with AC due to stone disease. Our hypothesis is, age ≥ 80 years old is associated with higher mortality in patients with AC. This propensity score-matched study aims to investigate if mortality is higher in the elderly (≥ 80 years old) patients with AC as compared to non-elderly (< 80 years old).

MATERIALS AND METHODS

This is a single-center retrospective cohort study of all patients diagnosed with calculous AC (January 2016 to December 2016) and ≥ 80 years old AC patients (January 2012 to December 2016) at a tertiary university-affiliated teaching hospital. We included patients treated for a suspected or confirmed AC diagnosis due to biliary stones[16]. Patients with AC on a background of hepatobiliary malignancy, indwelling permanent metallic biliary stents, or concomitant pancreatitis were excluded. The severity grading of AC in the TG13 included age greater than 75 years as a risk factor, which was retained in TG18[17]. Due to a higher sample of elderly patients, the overall cohort's median age was > 80 years, so we defined elderly as ≥ 80 years old. Non-elderly was defined as patients < 80 years old. Our local institutional review board (National Healthcare Group Domain Specific Review Board, No. 2017/00200) approved this study. This study's conduct is per the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement for retrospective cohort studies[18].

Study variables and outcomes

The patient demographics and clinical outcomes were studied. Patient demographics included age, gender, and comorbidities. Comorbidities included diabetes mellitus, ischemic heart disease, chronic obstructive pulmonary disease, asthma, chronic renal failure, and biliary disease history. Previous history of biliary colic, acute cholecystitis, AC, and acute biliary pancreatitis were collectively defined as history of biliary disease. Presenting symptoms at admission included abdominal pain, fever, vomiting, jaundice, and hypotension. Hypotension was defined as admission systolic blood pressure < 90 mmHg. Laboratory data included white blood cell count, platelet count, creatinine, prothrombin time, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, albumin, gamma-glutamyl transferase, and total bilirubin levels. The shock index (SI) was defined as heart rate divided by the respective systolic blood pressure on arrival in triage[19,20]. Abnormal SI was defined as SI < 0.5 or > 0.7). In patients undergoing ERCP and cholecystectomy, procedure-related data and outcomes

were collected. Delayed ERCP was defined as ERCP > 72 h from admission. The primary outcomes of this study were in-hospital mortality, 30-d mortality and 90-d mortality. In-hospital mortality was defined as any deaths which occurred during the same hospital admission, regardless of the duration from admission. The 30-d and 90-d mortality were defined as any deaths (including both patients who were still inpatient and those who were discharged) within 30 d and 90 d from admission. The secondary clinical outcome was LOS.

Treatment protocol

Patients who presented with septic shock were managed according to the Surviving Sepsis Campaign Guidelines for Management of Severe Sepsis and Septic Shock, 2012[21]. The definite diagnosis of AC was based on the TG13 Guidelines, namely, evidence of systemic inflammation (fever, chills, or laboratory data), cholestasis (jaundice or laboratory data), and imaging of the biliary tree (dilatation, stricture, stone, or stent)[16]. The severity was graded as mild, moderate, or severe as per TG13 guidelines[16]. Out unit was involved in TG07 classification, and we were early adopters of the TG13 system. Thus, the majority of patients had TG13 stratification done prospectively. Patients that were included before the TG13 publication were retrospectively assigned TG13 diagnosis and severity stratification. Blood cultures were taken for all patients included in our study. Broad-spectrum empiric intravenous antibiotics were administered based on local antibiogram and in compliance with the World Society of Emergency Surgery guidelines for optimal and rational use of antibiotics in intra-abdominal sepsis[22,23]. Patients with mild AC, patients who declined invasive intervention, and patients who were responsive to antibiotics alone were managed conservatively. Urgent biliary drainage was performed for patients with moderate and severe AC. The endoscopists' discretion and resources determined the timing of biliary drainage. ERCP was the first-line modality for biliary drainage. A diclofenac suppository is inserted routinely for post-ERCP acute pancreatitis prophylaxis. Percutaneous transhepatic biliary drainage (PTBD) was offered when ERCP was not feasible or contraindicated. Complete stone removal or temporary placement of biliary stents was performed at the endoscopists' discretion. Index admission cholecystectomy was reserved for patients with mild AC and subject to surgeon preference.

Statistical analysis

A 1:1 propensity score matching (PSM)[24] was performed by the first author (Chan KS). PSM was performed at a ratio of 1:1 using a caliper width of 0.2 of the standard deviation of the logit of the propensity score[25]. Patients were adjusted for 15 factors. Seven factors: clinical presentation (fever and hypotension) and laboratory investigations (white blood cell count, platelets, bilirubin, international normalized ratio, and albumin) impact clinical outcomes and thus were adjusted[16,26]. Eight factors were statistically significant ($P < 0.1$) during comparison of the initial demographics between the elderly and non-elderly: gender, comorbidities (ischemic heart disease, chronic renal impairment, and history of biliary disease), clinical presentation (abdominal pain, jaundice), and laboratory investigations (gamma-glutamyl transferase and creatinine), and thus were adjusted. Standardized mean difference (SMD) and Hansen and Bowers were used to assess for covariate and global imbalance, respectively[27].

Categorical values were described as percentages and analyzed by the chi-square test. Continuous variables were expressed as median (interquartile range, IQR) and analyzed by the Mann-Whitney *U* test, respectively. Statistical significance was determined by $P < 0.05$. All statistical analyses were performed with SPSS version 25.0 (SPSS Inc., Chicago, III., United States) and R software (R-3.3.3). The statistical review was performed by one of the co-authors qualified in biomedical statistics (Shelat VG).

RESULTS

Patient demographics and clinical profile

Five hundred fifty-six patients were managed for AC during the study period. Ninety-nine AC patients were excluded due to underlying malignancy. Four hundred fifty-seven patients met the inclusion: 318 (69.6%) elderly *vs* 139 (30.4%) non-elderly. The overall cohort's median age was 82.4 years (IQR 77.6-85.3), with female predominance (n = 252/457, 55.1%). About half (n = 240/457, 52.5%) of patients had a biliary disease history. One hundred and eighty (39.4%) patients had positive blood cultures, and

Escherichia coli was the most common pathogen (n = 129/180, 71.7%). Figure 1 summarizes the microbiology of patients who had positive blood cultures. One hundred and ninety-eight (43.3%) and 126 (27.6%) patients had Grade II and Grade III AC, respectively. When the data of overall cohort was analyzed according to the timing of ERCP (≤ 72 h *vs* > 72 h from admission), there was no difference in the ERCP timing for patients with at least Grade II AC [≤ 72 h, n = 88/201 (43.8%) *vs* > 72 h, n = 113/201 (52.2%), P = 0.368].

PSM with a 1:1 ratio resulted in 224 patients (elderly 112, non-elderly 112). Before PSM, 5 of 15 unmatched variables had SMD > 0.25; following PSM, all of the variables reached an SMD < 0.25 (Table 1 and Figure 2), suggesting an adequate and improved balance. Hansen and Bowers test for global significance also did not demonstrate statistical significance in the matched cohort (matched cohort: χ^2: 4.73, P = 0.994; unmatched cohort: χ^2: 67.4, P < 0.001). Baseline demographics in both the unmatched and matched cohorts are summarized in Table 1. The adoption of biliary drainage procedures was similar between elderly and non-elderly patients in the unmatched cohort. Eleven (3.5%) and 2 (1.4%) elderly and non-elderly respectively received urgent biliary drainage. However, in the matched cohort, elderly patients were more likely to undergo PTBD than non-elderly patients (11.6% *vs* 4.5%, OR 2.81, P = 0.049). Incidence of index admission cholecystectomy and interval cholecystectomy was also comparable between elderly and non-elderly patients in the unmatched cohort. However, in the matched cohort, elderly patients were less likely to undergo index admission cholecystectomy (1.8% *vs* 10.7%, OR 0.15, P = 0.006).

Clinical outcomes

The overall in-hospital mortality, 30-d mortality and 90-d mortality was 4.6%, 7.4% and 8.5% respectively; this was comparable between elderly *vs* non-elderly in both unmatched and matched cohorts. Peri-operative outcomes are summarized in Table 2. In the unmatched cohort, elderly patients had a statistically significant longer LOS (median 8 d, IQR 6-13 *vs* 8 d, IQR 5-11, P = 0.040). However, after matching, LOS was similar (median 7.5 d, IQR 5-11 *vs* 8 d, IQR 5-11, P = 0.982).

Table 3 summarizes the outcomes of patients who underwent ERCP. In the unmatched subgroup of patients who underwent ERCP and had delayed ERCP (> 72 h from admission) (elderly n = 121, non-elderly n = 38), the primary and secondary outcomes were indifferent between elderly and non-elderly patients respectively: in-hospital mortality [n = 2/121 (1.7%) *vs* 1/38 (2.6%), P = 0.699], 30-d mortality [n = 9/121 (7.4%) *vs* 2/38 (5.3%), P = 0.645], 90-d mortality [n = 11/121 (9.1%) *vs* 2/38 (5.3%), P = 0.453], and LOS (median 10 d, IQR 7-15 *vs* 8 d, IQR 6-12, P = 0.103). These outcomes remain indifferent after PSM matching: in-hospital mortality [n = 1/42 (2.4%) *vs* 1/26 (3.8%), P = 0.728], 30-d mortality [n = 2/42 (4.8%) *vs* 2/26 (7.7%), P = 0.618], 90-d mortality [n = 2/42 (4.8%) *vs* 2/26 (7.7%), P = 0.618], and LOS (median 8.5 d, IQR 6-11.3 *vs* 8.5 d, IQR 6-15.3, P = 0.929).

In the unmatched cohort, an abnormal SI was not associated with ERCP [abnormal SI: 178/282 (63.1%) *vs* normal SI: 114/175 (65.1%), P = 0.662]. This was observed in both the elderly [abnormal SI: 125/194 (64.4%) *vs* normal SI: 81/124 (65.3%), P = 0.871] and the non-elderly [abnormal SI: 53/88 (60.2%) *vs* normal SI: 33/51 (64.7%), P = 0.600]. There was no difference after PSM matching on the association of abnormal SI with ERCP: abnormal SI: 90/139 (64.7%) *vs* normal SI: 52/85 (61.2%), P = 0.590. This was true in both the elderly [abnormal SI: 48/66 (72.7%) *vs* normal SI: 29/46 (63%), P = 0.277] and the non-elderly [abnormal SI: 42/73 (57.5%) *vs* normal SI: 23/39 (59%), P = 0.883]. Subgroup analysis of patients with an abnormal SI on triage did not show any significant differences in outcomes between elderly and non-elderly patients (Table 4).

DISCUSSION

In this single-center propensity score-matched study, patients ≥ 80 years old with AC due to biliary stone disease had similar mortality compared to patients < 80 years old. With an increase in life expectancy globally, the elderly population is also increasing. In the elderly population where there is an increased prevalence of gallstones in the elderly population, biliary events including AC are also more common. The elderly poses a unique challenge due to underlying comorbidity, frailty, sarcopenia, functional decline, cognitive decline, and diminished reserves to withstand stress[4]. With diminished physiological reserves, sepsis resulting from AC poses a mortality risk, and our mortality outcomes are acceptable, considering mortality risk of up to 20% in patients with AC[1]. Our reported mortality is comparable to mortality of less than 11%

Table 1 Patient demographics and clinical profile

	Overall cohort, *n* = 457				PSM cohort, *n* = 224			
	Elderly, *n* = 318	Non-elderly, *n* = 139	*P* value	SMD	Elderly, *n* = 112	Non-elderly, *n* = 112	*P* value	SMD
Age, yr	84.0 (82.1, 86.6)	67.9 (57.1, 77.2)	< 0.001		84.3 (82.1, 87.3)	66.6 (55.6, 76.4)	< 0.001	
Gender[1], male (%)	132 (41.5)	73 (52.5)	0.029	0.221	57 (50.9)	53 (47.3)	0.593	0.071
Co-morbidities, *n* (%)								
Diabetes mellitus	124 (39)	55 (39.6)	0.908		44 (39.3)	47 (42)	0.683	
Ischemic heart disease[1]	87 (27.4)	27 (19.4)	0.071	0.188	22 (19.6)	23 (20.5)	0.868	0.022
Chronic renal impairment[1]	61 (19.2)	17 (12.2)	0.069	0.191	17 (15.2)	14 (12.5)	0.562	0.077
COPD and/or asthma	18 (5.7)	4 (2.9)	0.201		8 (7.1)	3 (2.7)	0.122	
History of biliary disease[1]	182 (57.2)	58 (41.7)	0.002	0.313	50 (44.6)	53 (47.3)	0.688	0.054
Clinical presentation								
Abdominal pain[1]	197 (61.9)	109 (78.4)	0.001	0.365	85 (75.9)	85 (75.9)	1.000	< 0.001
Fever[1]	141 (44.3)	67 (48.2)	0.446	0.077	50 (44.6)	52 (46.4)	0.788	0.036
Vomiting	142 (44.7)	63 (45.3)	0.895		51 (45.5)	50 (44.6)	0.893	
Jaundice[1]	48 (15.1)	38 (27.3)	0.002	0.302	27 (24.1)	23 (20.5)	0.521	0.085
Hypotension[1,2]	18 (5.7)	12 (8.6)	0.238	0.115	10 (8.9)	6 (5.4)	0.299	0.138
Laboratory investigations								
WBC[1] (10^9/L)	12.4 (8.9, 16.1)	12.1 (8.3, 15.9)	0.551	0.113	12.2 (8.6, 15.4)	12.1 (8.0, 16.2)	0.745	0.100
Platelets[1] (10^9/L)	192 (150, 250)	216 (166, 280)	0.047	0.131	193 (160, 252)	209 (162, 280)	0.308	0.101
Creatinine[1] (μmol/L)	103 (81, 138)	89 (68, 116)	< 0.001	0.094	103 (80, 136)	86 (67, 119)	0.003	0.088
Albumin[1] (g/L)	32 (28, 35)	35 (29, 38)	< 0.001	0.396	33 (29, 36)	34 (29, 38)	0.186	0.150
Bilirubin[1] (μmol/L)	54 (33, 84)	60 (34, 96)	0.226	0.096	65 (42, 93)	58 (33, 93)	0.287	0.104
ALT (IU/L)	133 (61, 247)	143 (68, 295)	0.330		142 (82, 244)	123 (59, 263)	0.294	
AST (IU/L)	160 (78, 366)	140 (72, 314)	0.165		176 (93, 365)	150 (74, 345)	0.149	
ALP (IU/L)	209 (130, 346)	188 (117, 314)	0.149		208 (137, 346)	184 (111, 291)	0.136	
GGT[1] (IU/L)	242 (129, 435)	327 (158, 562)	0.006	0.292	286 (165, 504)	286 (133, 523)	0.591	0.022
INR[1]	1.13 (1.02, 1.30)	1.20 (1.10, 1.30)	0.890	0.112	1.15 (1.00, 1.30)	1.20 (1.10, 1.30)	0.506	0.038
Microbiology, positive (%)	132 (41.5)	48 (34.5)	0.160		44 (39.3)	36 (32.1)	0.265	
Escherichia coli	99 (75)	30 (62.5)	0.100		33 (75)	24 (66.7)	0.413	
Klebsiella pneumoniae	32 (24.2)	14 (29.2)	0.503		16 (36.4)	12 (33.3)	0.777	
Enterobacter spp	3 (2.3)	1 (2.1)	0.939		0 (0)	1 (2.8)	0.266	
Pseudomonas aeruginosa	1 (0.8)	0 (0)	0.550		0 (0)	0 (0)	-	
Enterococcus spp	1 (0.8)	1 (2.1)	0.453		1 (2.3)	1 (2.8)	0.886	
Citrobacter spp	1 (0.8)	0 (0)	0.545		1 (2.3)	0 (0)	0.357	
Aeromonas spp	1 (0.8)	0 (0)	0.545		0 (0)	0 (0)	-	
CT scan, *n* (%)	108 (34)	52 (37.4)	0.477		43 (38.4)	45 (40.2)	0.784	
Cholelithiasis	75 (69.4)	31 (59.6)	0.218		21 (48.8)	14 (31.1)	0.089	
Biliary dilation	47 (43.5)	18 (34.6)	0.283		30 (69.8)	26 (57.8)	0.243	
Choledocholithiasis	63 (58.3)	18 (34.6)	0.005		27 (62.8)	14 (31.1)	0.003	
MRCP, *n* (%)	157 (49.4)	73 (52.5)	0.536		61 (54.5)	55 (49.1)	0.422	

Cholelithiasis	113 (72)	38 (52.1)	0.003	37 (60.7)	35 (63.6)	0.741
Biliary dilation	93 (59.2)	50 (68.5)	0.178	41 (67.2)	26 (47.3)	0.030
Choledocholithiasis	103 (65.6)	39 (53.4)	0.077	41 (67.2)	23 (41.8)	0.006
Shock Index, abnormal[3]	194 (61)	88 (63.3)	0.641	66 (58.9)	73 (65.2)	0.335
TG13 severity grading	2 (2, 3)	2 (1, 2)	< 0.001	2 (1, 3)	2 (1, 2)	0.016
Grade I	67 (21.1)	66 (47.5)		31 (27.7)	46 (41.1)	
Grade II	152 (47.8)	46 (33.1)		49 (43.8)	46 (41.1)	
Grade III	99 (31.1)	27 (19.4)		32 (28.6)	20 (17.9)	

All continuous variables were expressed as median (interquartile range) unless specified. All categorical variables were expressed as *n* (%) unless otherwise specified.

[1]Propensity score matching was performed for these variables due to potential and/or significant effects on clinical outcomes, or due to significant differences in demographics between the two study groups.

[2]Hypotension was defined as systolic blood pressure of < 90 mmHg.

[3]Shock index was defined as heart rate divided by the respective systolic blood pressure during triage, where the normal range is 0.5 to 0.7 (inclusive).

ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; COPD: Chronic obstructive pulmonary disease; CT: Computed tomography; GGT: Gamma-glutamyl transferase; INR: International normalized ratio; MRCP: Magnetic resonance cholangiopancreatography; PSM: Propensity score matching; SMD: Standardized mean difference; TG13: Tokyo Guidelines 2013; WBC: White blood cell.

Table 2 Clinical outcomes between elderly *vs* non-elderly patients

	Overall cohort, *n* = 457				PSM cohort, *n* = 224			
	Elderly, *n* = 318	Non-elderly, *n* = 139	OR, 95%CI	*P* value	Elderly, *n* = 112	Non-elderly, *n* = 112	OR, 95%CI	*P* value
Initial management								
ERCP	206 (64.8)	86 (61.9)	1.13 (0.75, 1.71)	0.551	77 (68.8)	65 (58)	1.59 (0.02, 2.75)	0.096
Percutaneous transhepatic biliary drainage	25 (7.9)	6 (4.3)	1.89 (0.76, 4.72)	0.166	13 (11.6)	5 (4.5)	2.81 (0.97, 8.17)	0.049
Conservative	98 (30.8)	49 (35.3)	0.82 (0.54, 1.25)	0.351	29 (25.9)	43 (38.4)	0.56 (0.32, 0.99)	0.045
Subsequent management								
Index admission cholecystectomy	16 (5.0)	13 (9.4)	0.51 (0.24, 1.10)	0.081	2 (1.8)	12 (10.7)	0.15 (0.03, 0.69)	0.006
Interval cholecystectomy	20 (6.3)	11 (7.9)	0.78 (0.36, 1.68)	0.525	7 (6.3)	10 (8.9)	0.68 (0.25, 1.86)	0.449
Length of hospital stay, days	8 (6, 13)	8 (5, 11)	-	0.040	7.5 (5, 11)	8 (5, 11)	-	0.982
In-hospital mortality	16 (5.0)	5 (3.6)	1.42 (0.51, 3.96)	0.500	6 (5.4)	5 (4.5)	1.21 (0.36, 4.09)	0.757
30-d mortality	27 (8.5)	7 (5)	1.75 (0.74, 4.12)	0.195	8 (7.1)	7 (6.3)	1.15 (0.40, 3.30)	0.789
90-d mortality	31 (9.7)	8 (5.8)	1.77 (0.79, 3.95)	0.160	8 (7.1)	8 (7.1)	1.00 (0.36, 2.77)	1.000

All continuous variables were expressed as median (interquartile range) unless specified. All categorical variables were expressed as *n* (%) unless otherwise specified. ERCP: Endoscopic retrograde cholangiopancreatography; CI: Confidence interval; OR: Odds ratio; PSM: Propensity score matching.

cited in more recent studies[28,29]. The higher mortality compared to some reports may be due to advanced age or co-morbidity associated with ageing[30]. With regards to the exact cause of mortality, we did not collect separate data, and this remains a limitation of our study. However, locally, our institution tracks procedure-related mortality separately; ERCP-related mortality is < 1% locally. Further, it is difficult to distinguish ERCP-related complications such as post-ERCP cholangitis from the index-admission sepsis. Due to the retrospective nature of our study, it is difficult to establish a cause-

Table 3 Subgroup analysis of patients who had endoscopic retrograde cholangiopancreatography on outcomes in elderly *vs* and non-elderly patients

	Overall cohort, *n* = 292				PSM cohort, *n* = 142			
	Elderly, *n* = 206	Non-elderly, *n* = 86	OR, 95%CI	*P* value	Elderly, *n* = 77	Non-elderly, *n* = 65	OR, 95%CI	*P* value
Timing of ERCP from presentation				**0.012**			-	0.247
Within 24 h	15 (7.3)	16 (18.6)			9 (11.7)	12 (18.5)		
24-48 h	36 (17.5)	13 (15.1)			14 (18.2)	11 (16.9)		
48-72 h	34 (16.5)	19 (22.1)			12 (15.6)	16 (24.6)		
>72 h	121 (58.7)	38 (44.2)			42 (54.6)	26 (40)		
Stone(s) removed	102 (49.5)	44 (51.2)	0.94 (0.57, 1.55)	0.797	36 (46.8)	30 (46.2)	1.02 (0.53, 1.99)	0.943
Stent placed	89 (43.2)	38 (44.2)	0.96 (0.58, 1.60)	0.877	35 (45.5)	30 (46.2)	0.97 (0.50, 1.89)	0.934
Length of hospital stay, d	9 (7, 13)	8 (5, 11)	-	0.016	8 (5, 12)	8 (5, 12)	-	0.546
In-hospital mortality	2 (1)	1 (1.2)	0.83 (0.08, 9.31)	0.882	1 (1.3)	1 (1.5)	0.84 (0.05, 13.73)	0.904
30-d mortality	13 (6.3)	4 (4.7)	1.38 (0.44, 4.36)	0.581	3 (3.9)	4 (6.2)	0.62 (0.13, 2.87)	0.536
90-d mortality	16 (7.8)	4 (4.7)	1.73 (0.56, 5.32)	0.337	3 (3.9)	4 (6.2)	0.62 (0.13, 2.87)	0.536

All continuous variables were expressed as median (interquartile range) unless specified. All categorical variables were expressed as *n* (%) unless otherwise specified. ERCP: Endoscopic retrograde cholangiopancreatography; CI: Confidence interval; OR: Odds ratio; PSM: Propensity score matching.

Table 4 Subgroup analysis of patients who had abnormal shock index on triage on outcomes in elderly *vs* non-elderly patients

	Overall cohort, *n* = 282				PSM cohort, *n* = 139			
	Elderly, *n* = 194	Non-elderly, *n* = 88	OR, 95%CI	*P* value	Elderly, *n* = 66	Non-elderly, *n* = 73	OR, 95%CI	*P* value
Length of hospital stay, d	8 (6-13)	8 (6-10.8)		0.379	8 (5-12)	6 (5-10)		0.217
In-hospital mortality	10 (5.2)	3 (3.4)	1.54 (0.41, 5.74)	0.517	3 (4.5)	3 (4.1)	1.11 (0.22, 5.71)	0.900
30-d mortality	19 (9.8)	4 (4.5)	2.28 (0.75, 6.91)	0.136	5 (7.6)	4 (5.5)	1.41 (0.36, 5.50)	0.616
90-d mortality	20 (10.3)	5 (5.7)	1.91 (0.62, 5.26)	0.205	5 (7.6)	5 (6.8)	1.12 (0.31, 4.04)	0.869

All continuous variables were expressed as median (interquartile range) unless specified. All categorical variables were expressed as *n* (%) unless otherwise specified. CI: Confidence interval; OR: Odds ratio; PSM: Propensity score matching.

effect relationship.

The principles of management of AC are early diagnosis, resuscitation, risk stratification, compliance to sepsis bundle, and source control[21]. Risk stratification is essential for resource allocation, patient and caregiver counselling, and timely proactive interventions. Source control is best achieved with endoscopic biliary decompression, *i.e.*, ERCP. The traditional systemic inflammatory response criteria lack specificity in hepatobiliary sepsis, and thus alternative indices are for risk stratification and prognostication of outcomes[15]. The SI (heart rate/systolic blood pressure) is a validated tool[19,31]. Yussof *et al*[31] demonstrated abnormal SI predicted mortality of severe sepsis in the emergency department. Our study however demonstrated that patients who had abnormal SI were equally likely to undergo

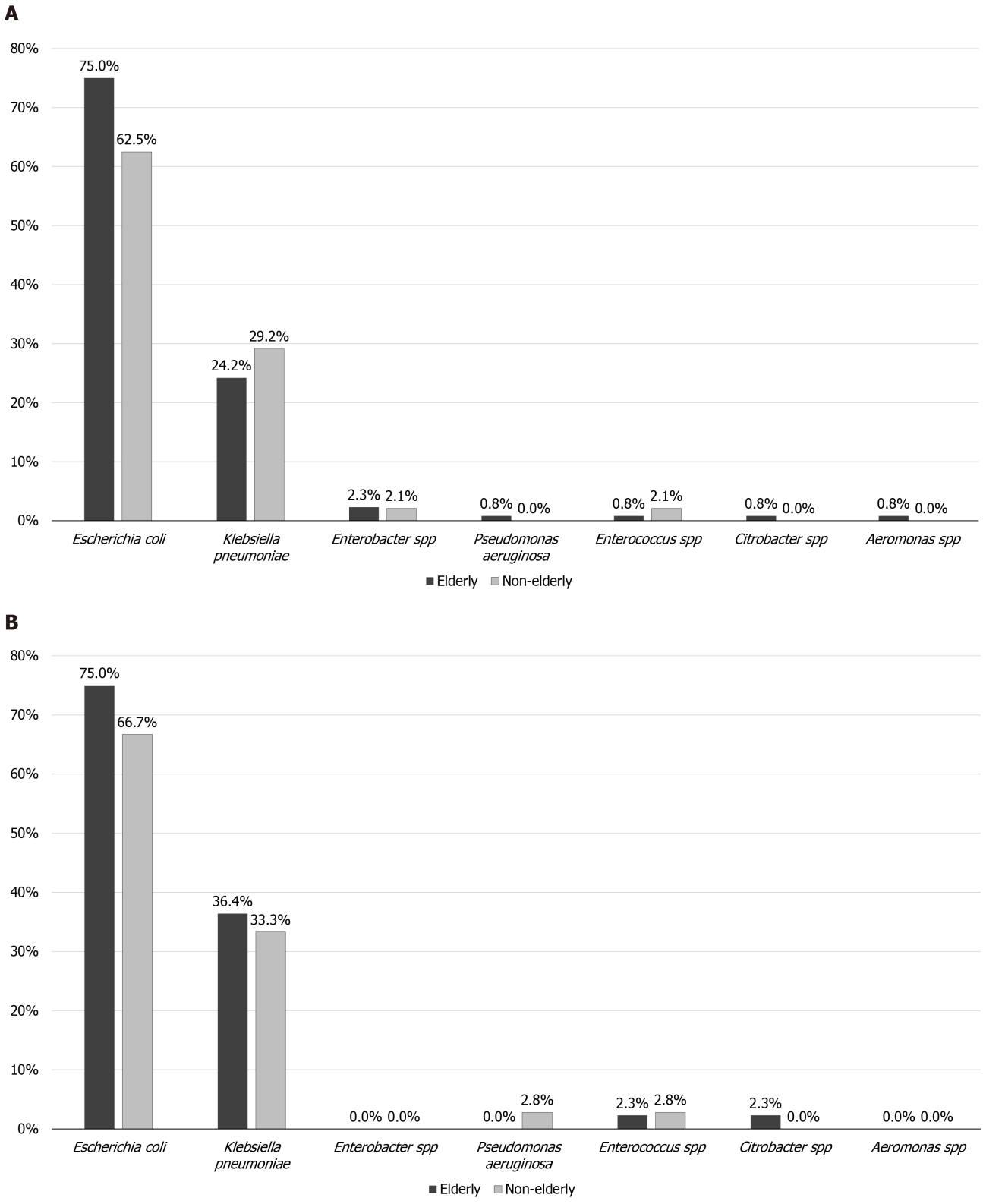

Figure 1 Microbiology of elderly and non-elderly patients who had positive blood cultures. A: Unmatched cohort; B: Matched cohort.

ERCP, and outcomes were comparable between elderly and non-elderly patients. SI is not reflective of the severity of sepsis as it does not take into account tissue perfusion indices and altered mental state. The decision for ERCP at the time of admission was based on the severity of AC and resources. Thus, SI does not predict the need for ERCP. Also, ERCP may occasionally be delayed in patients with abnormal SI in an attempt to resuscitate first. ERCP is an invasive procedure with approximately 10% risk of complications. Elderly patients undergoing ERCP are at higher risk of complications such as pancreatitis, hemorrhage, perforation, cardiorespiratory

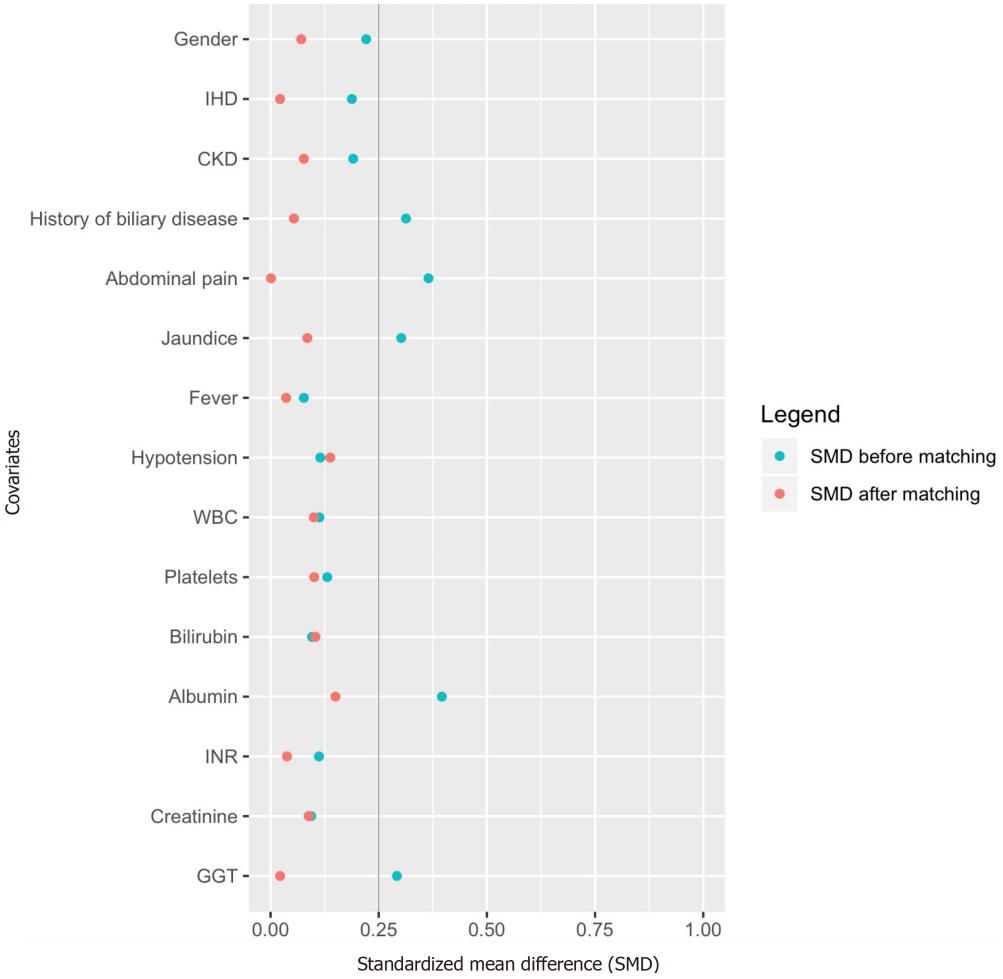

Figure 2 Plot of standardized mean difference in covariates: before propensity score matching (blue) and after propensity score matching (red). Standardized mean difference of < 0.25 indicates adequate balance. IHD: Ischemic heart disease; CKD: Chronic kidney disease; WBC: White blood cell; INR: International normalized ratio; GGT: Gamma-glutamyl transferase.

complications, and mortality[32]. This increased morbidity and mortality are attributed to underlying comorbidity and lower physiological reserves of the elderly[33].

However, several studies have shown no relationship between comorbidities and ERCP-related complications, except liver cirrhosis[34]. Many authors have demonstrated the safety and efficacy of ERCP in elderly patients[35,36]. In a single-center retrospective study reporting on efficacy and safety of ERCP in elderly patients with AC, Tohda *et al*[37] reported that patients ≥ 80 years old were more likely to have periampullary diverticulum (24.5% *vs* 13.3%), but equal technical success rates (95.1% *vs* 95.2%) and frequency of ERCP-related complications (6.9% *vs* 6.7%) as compared to patients < 80 years age. The authors reported a lower rate of post-ERCP pancreatitis in the elderly than non-elderly (1.0% *vs* 3.8%). We used PSM analysis to reduce the confounding effect of comorbidities on mortality outcomes, thus reducing the selection bias. We did not specifically compare procedure-related morbidity between elderly *vs* non-elderly and showed comparable LOS and mortality in both the unmatched and matched cohorts between elderly and non-elderly patients. Our experience shows that both stent insertion for biliary decompression and definitive stone removal can be safely performed. In particular, patient physiology, coagulopathy, and endoscopist experience are determinants of ERCP outcomes. Regarding the timing of ERCP, most authors agree that urgent ERCP should be done at the next available opportunity, and in clinical practice, timing is determined by local resources as well as clinical status. The majority of authors recommend ERCP within 24-72 h of admission[38]. Delay in ERCP in AC could influence patients' outcomes, and many authors define delay variably as the time to ERCP of more than 48-72 h since admission. Khashab *et al*[39] defined delay in ERCP as > 72 h after admission and reported that it was associated with prolonged LOS (OR 19.8, 95%CI: 2.18-178, $P = 0.008$). Navaneethan *et al*[40] defined delay in ERCP as > 48 h after admission and reported that it was associated with an increased risk of 30-d readmission. We defined delay as > 72 h after admission and did

not find any difference in clinical outcomes between elderly and non-elderly patients in both the unmatched and matched cohort. Khashab *et al*[39] demonstrated that delayed ERCP and age are associated with worse composite clinical outcomes (death, persistent organ failure and admission to ICU). However, as our 90-d mortality only had thirteen patients with delayed ERCP, it was not possible to perform subgroup analysis of age on clinical outcomes. It is possible that worse outcomes are associated with delay in ERCP but independent of age.

In addition, it is essential for patients with haemodynamic instability to be adequately resuscitated with airway management, prompt administration of vasopressor after volume replacement, and early engagement of critical care specialist or anesthetist, followed by prompt and early biliary decompression[41]. A recent study by Novy *et al*[14] in 2020, which analyzed the outcomes of 85 patients ≥ 75 years old with severe AC and admitted to ICU, showed that the majority (76%) of the ICU patients had ERCP within 24 h, which was attributed to the ease of access to facilities. Institutions with availability of ERCP services should consider early ERCP synchronized with resuscitation measures as delaying ERCP is associated with poor clinical outcomes[39]. Despite a policy for early ERCP, Novy *et al*[14] reported ICU mortality of 18%. This highlights that there are other determinants of mortality in critically ill patients. It is important to note that there is an inherent selection bias for elderly patients included in the study; patients not eligible for ICU admission may have more inferior pre-morbid status and deemed not suitable based on medical futility, or may have had advanced care planning performed and decided that ICU admission is unlikely to provide benefit for the patient[42]. Moreover, ICU admission implies the need for vasopressor therapy or intubation, which reflects the severity of the disease. We did not differentiate our patients based on their need for ICU admission or otherwise; or the use of vasopressor therapy. There is a paucity of data related to causative organisms and their impact on AC's clinical outcomes compared to other hepatobiliary diseases, such as acute cholecystitis or pyogenic liver abscesses[43]. Microbiology of patients with AC was also consistent with existing studies, where *Escherichia coli* and *Klebsiella pneumoniae* were the most typical organisms[44].

An alternative to biliary decompression is the use of PTBD. Our study demonstrated a significantly higher number of elderly patients who underwent PTBD compared to non-elderly patients [n = 13 (11.6%) *vs* n = 5 (4.5%), OR 2.81, P = 0.049] in the matched cohort. ERCP is traditionally the gold standard management for AC and has been proven to be safe and effective in the elderly population[36,37]. PTBD is regarded as a second-line treatment for patients who failed ERCP, with altered biliary anatomy, or were contra-indicated for ERCP. However, unlike ERCP which requires the use of moderate sedation or general anaesthesia, PTBD only requires the use of local anaesthesia. Despite the safety of ERCP in elderly patients, elderly patients are at higher risk of complications from the use of sedation[45]. Weighing the risks and benefits of endoscopic biliary decompression *vs* the use of sedation is also essential in the management of AC. Patient and/or family members may opt for PTBD which is deemed to be "less invasive" without the need for moderate sedation/general anaesthesia.

Following the acute management of AC, cholecystectomy should be offered to patients to prevent future recurrences. In our experience, non-elderly patients are more likely to undergo index admission LC (Matched cohort: P = 0.006). Five out of 12 patients in the non-elderly group who underwent index admission LC in the matched cohort did not receive ERCP. It is likely that in addition to age, underlying comorbidity and personal choices impact the decision for surgery. These findings are similar to a single-center retrospective study of Discolo *et al*[46]. In an eight-year study including 151 cholecystectomies for AC, Discolo *et al*[46] reported a more than 61% rate of index admission cholecystectomy, and patients with age > 75 years were more likely to receive delayed cholecystectomy (41.4% *vs* 21.5%, P = 0.01). The authors also showed that TG severity grading did not impact the decision for index admission cholecystectomy (P = 0.46). Furthermore, there was no difference in average operative time (P = 0.36), open conversion (P = 0.34), and intra-operative complications (P = 0.28) based on the timing of cholecystectomy. We did not perform subgroup analysis on postoperative outcomes in patients who underwent index admission cholecystectomy given the small sample size. In general, index admission cholecystectomy could reduce the risk of recurrent biliary events; however, more evidence is needed in patients with AC. We have previously reported our views on a policy of 'universal cholecystectomy', *i.e.*, patients with a diagnosis that requires cholecystectomy (*e.g.*, acute cholecystitis, AC, or acute biliary pancreatitis) procedure should receive index admission surgery unless contraindicated for general anesthesia or patient refusal[47].

The important issue that surfaces from our study is, if age should be considered as part of a risk stratification tool for the severity of AC. Age is usually included in severity classifications as a surrogate marker for functional capacity and extent of comorbidities. The use of other surrogate markers such as the clinical frailty scale or Charlson co-morbidity index may be a better predictor of disease severity in AC[48]. In reality however, age serves as a useful tool in view of its ease of use as well as age-associated reduced functional reserves that are not associated with any co-morbidity. While clinical outcomes are not determined by age in patients with AC in our study; based on available literature, we advocate that age should continue to remain as one of the component variables that determines disease severity in patients with AC.

There are several limitations of our study. A retrospective study is inherently prone to selection bias, and thus cause-effect cannot be established. PSM helps to reduce this bias, and such analysis ranks higher than traditional observational studies[24]. To the best of our knowledge, this is the first study using PSM to compare outcomes of AC secondary to biliary stones between elderly and non-elderly patients. PSM analysis cannot account for unknown confounding variables, and only a randomized controlled trial can overcome this bias. Our study included patients treated in 2012, *i.e.*, before the TG13 guidelines, and we retrospectively assigned TG13 criteria with possible reporting bias. We did not study the effect of polypharmacy, frailty, and Charlson's comorbidity index on AC outcomes. In a large population study over a decade in the Korean general population, Min *et al*[49] have reported that the use of proton pump inhibitor is associated with increased AC risk (hazard ratio 5.75, 95%CI: 4.39-7.54). We also did not evaluate comorbidities like cerebrovascular accident and liver cirrhosis, as data was not available for all the patients. Our study used the age of 80 years old as a cut-off compared to 75 years, used in TG13/18 guidelines. Existing studies evaluating the safety of ERCP in elderly patients have used a variety of cut-offs for age, ranging from 80 years old to 90 years old[35-37]. In addition, use of 75 years as a cut-off will reduce our sample size and impact the statistical power of study (96 patients < 75 years and 361 patients ≥ 75 years compared to 139 patients < 80 years and 318 patients ≥ 80 years respectively). Nevertheless, this difference in age cut-off reduces our study's generalizability from being considered an accurate validation study of TG13/18 guidelines. We also did not categorize which patients with history of biliary disease had prior ERCP and papillotomy. It is possible that elderly patients were more likely to have prior ERCP and papillotomy, and this could impact results of our study. We also did not collect data on disease or procedure-related morbidity and causes of mortality.

CONCLUSION

Elderly patients (≥ 80 years old) with AC have similar outcomes as compared to non-elderly patients (< 80 years old). In a subgroup of patients who underwent ERCP or with delayed ERCP, clinical outcomes are comparable between the elderly and non-elderly. Age alone may not predict the outcomes of AC and its use in the Tokyo Guidelines should be re-evaluated.

ARTICLE HIGHLIGHTS

Research background

Acute cholangitis (AC) is a disease spectrum with varying extent of severity. Age ≥ 75 years forms part of the criteria for moderate (Grade II) severity in the Tokyo Guidelines (TG13 and TG18). Aging is associated with reduced physiological reserves, frailty, and sarcopenia. However, there is evidence that age itself is not the determinant of inferior outcomes in elective and emergency biliary diseases.

Research motivation

Endoscopic retrograde cholangiopancreatography is deemed to be safe in elderly patients with AC. There is paucity of data on outcome determinants in elderly patients with AC. This era of ageing population prompted our interest to study the impact of age alone on outcomes of AC through the use of propensity score matching.

Research objectives

Our primary outcomes are in-hospital mortality, 30-d mortality and 90-d mortality. Secondary outcome is morbidity (length of hospital stay).

Research methods

This is a single-center retrospective cohort study of all patients diagnosed with calculous AC (January 2016 to December 2016) and ≥ 80 years old (January 2012 to December 2016) at a tertiary university-affiliated teaching hospital. Elderly was defined as ≥ 80 years old while non-elderly was defined as < 80 years old.

Research results

Four hundred fifty-seven patients with AC were included in this study (318 elderly, 139 non-elderly). Propensity score matching analysis resulted in a total of 224 patients (112 elderly, 112 non-elderly). The overall in-hospital mortality, 30-d mortality and 90-d mortality were 4.6%, 7.4% and 8.5% respectively, with no statistically significant differences between the elderly and non-elderly in both the unmatched and matched cohorts. Length of hospital stay was longer in the unmatched cohort [elderly 8 d, interquartile range (IQR) 6-13 *vs* non-elderly 8 d, IQR 5-11, P = 0.040], but was comparable in the matched cohort (elderly 7.5 d, IQR 5-11 *vs* non-elderly 8 d, IQR 5-11, P = 0.982).

Research conclusions

Mortality is indifferent in the elderly (≥ 80 years old) and non-elderly patients (< 80 years old) with AC.

Research perspectives

Age alone may not predict the outcomes of AC and its use in the Tokyo Guidelines should be re-evaluated.

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ORIGINAL ARTICLE

Retrospective Study

Retrospective analysis of complications related to endoscopic retrograde cholangio-pancreatography in patients with cirrhosis *vs* patients without cirrhosis

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Abstract

BACKGROUND

There is minimal objective data regarding adverse events related to endoscopic retrograde cholangio-pancreatography (ERCP) in patients with cirrhosis compared to those without cirrhosis and even fewer data comparing complications among cirrhosis patients based on severity of cirrhosis.

AIM

To determine if patients with cirrhosis are at increased risk of adverse events related to ERCP: mainly pancreatitis, bleeding, perforation, cholangitis, and mortality; And to see if higher Child-Pugh (CP) score and Model for End-Stage Liver Disease (MELD) score are associated with higher post-ERCP complications.

METHODS

We performed a retrospective analysis of 692 patients who underwent ERCP and analyzed the impact of cirrhosis etiology, gender, type of sedation used during procedure, interventions performed, and co-morbidities on the rate of complications in cirrhosis patients as compared to non-cirrhosis patients.

RESULTS

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Overall complications were higher in those with cirrhosis as compared to those without cirrhosis (P = 0.015 at significance level of 0.05). CP class, especially CP class C, was shown to be associated with a significantly higher rate of ERCP complications as compared to CP class A and CP class B (P = 0.010 at significance level of 0.05).

CONCLUSION
The results of our study reaffirm that liver cirrhosis has an impact on the occurrence of complications during ERCP. Our study shows that CP class seems to be more reliable as compared to MELD score in predicting complications of ERCP in cirrhosis patients.

Key Words: Cirrhosis; Complications; Advanced endoscopy; Endoscopic retrograde cholangio-pancreatography

Core Tip: What is previously known is that endoscopic retrograde cholangio-pancreatography is associated with a risk of adverse events. What is new in this manuscript is that complications are increased in patients with cirrhosis as compared to patients without cirrhosis. Statistical significance was demonstrated in patients classified as Child-Pugh (CP) Class C as compared to CP Classes A and B.

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INTRODUCTION

Endoscopic retrograde cholangio-pancreatography (ERCP) is a procedure utilized for the management of pancreatobiliary disorders, including but not limited to choledocolithiasis, biliary strictures, pancreatitis, and cholangitis[1]. However, like all procedures, there is an associated risk of adverse events, such as post-ERCP pancreatitis, bleeding, infection, perforation, and even death[2].

Patients with chronic liver disease and cirrhosis often require ERCP. However, because of hepatic synthetic dysfunction and portal hypertension, patients with cirrhosis have a much higher risk of developing adverse events and complications after invasive procedures[3]. Despite this, there remains a scarce amount of data investigating complications associated with ERCP in patients with cirrhosis as compared to patients without cirrhosis. There is even less information regarding adverse effects among patient with cirrhosis based upon cirrhosis severity.

Thus, our study aims to add to the limited body of knowledge regarding complications of ERCP in patients with cirrhosis. We hypothesized that patients with an underlying diagnosis of cirrhosis are at elevated risk of complications associated to ERCP, including mortality, pancreatitis, bleeding, perforation, and cholangitis. A secondary objective was to examine our hypothesis that a higher Child-Pugh (CP) score and/or Model for End-Stage Liver Disease (MELD) score is related to a greater number of post-ERCP complications in cirrhosis.

MATERIALS AND METHODS

This study was a retrospective review of all patients who underwent ERCP at a University hospital in Syracuse, NY, United States from 2012-2019. The project was presented to the Institutional Review Board and approved prior to its initiation. Chart review of 692 patients who underwent ERCP between January 1, 2012 and December 31, 2019 was conducted. Of the 692 patients, 174 patients had a diagnosis of cirrhosis at

the time of ERCP, and 518 patients did not. Demographics, co-morbidities [including chronic obstructive pulmonary disease (COPD), congestive heart failure (CHF), hypertension (HTN), diabetes mellitus (DM), chronic kidney disease (CKD), and hyperlipidemia (HLD)], indication for procedure, type of sedation used, type of intervention(s) performed, and complications within a 30-d period were analyzed for all subjects. Of the 174 patients with cirrhosis, we also recorded cirrhosis etiology and calculated their MELD score and CP class.

Statistical analysis of the complication rates in the groups with and without cirrhosis was performed using a chi-squared test, and fishers exact test when there were < 5 individuals in a category. Pearson's chi square test is sufficient when testing the impact of a single factor on binary outcome. Of those with cirrhosis, the complication rates in subjects grouped by Child score A, B, and C, as well as MELD score, were also compared using a chi-squared or fishers exact test. A P value of < 0.05 was considered statistically significant. Odds ratios with 95% confidence intervals were derived from logistic regression as a supportive method in confirming the findings of Child score significance.

RESULTS

A total number of 692 patients were included in our study. Of the 692, 174 had an underlying diagnosis of cirrhosis while 518 did not. Mean patient age was 58.6 years. Overall, there was a higher rate of complications in those with cirrhosis as compared to those without cirrhosis (P = 0.015) (Table 1). There was no statistical significance comparing the specific types of complications across the two groups (P = 0.897), including bleeding, pancreatitis, cholangitis, perforation, mortality, or other.

CP and MELD score

Complications in subjects with cirrhosis grouped by CP class are shown in Table 2. CP class, especially CP class C, was shown to be associated with a significantly higher rate of ERCP complications as compared to CP class A and CP class B (P = 0.010). In other words, a statistically significant proportion of cirrhosis patients with CP class A or class B are less likely to develop complications than those in CP class C (Figure 1). The odds ratios 0.342 with (0.132, 0.882) as 95% confidence interval for group A *vs* group C and 0.251 with (0.096, 0.6253) as 95% confidence interval for group B *vs* group C, as derived from logistic regression support the above conclusion (Table 3).

Complications in subjects with cirrhosis grouped by MELD score are shown in Table 4. There was no statistical significance when comparing complications in patients with cirrhosis with a MELD score of < 15 *vs* > 15 (P = 0.949). Thus, CP class was more reliable than MELD score in terms of predicating complications in cirrhosis.

Etiology of cirrhosis

We also analyzed the complication occurrence in cirrhosis patients based on underlying etiology. This included: Alcohol, hepatitis C, and non-alcoholic fatty liver disease. Etiology of cirrhosis did not have a significant difference in respect to complications related to ERCP (Table 5).

Gender

Gender did not have a statistically significant effect on complications between cirrhosis and non-cirrhosis patients (Table 5 and 6).

Anesthesia type

Type of anesthesia used during the ERCP did not have any statistically significant difference regarding complications between both cirrhosis and non-cirrhosis patients (Table 5 and 6).

Type of intervention

We collected data on whether the ERCP was for diagnostic or therapeutic purposes, as well as the types of intervention performed during the ERCP (Table 7 and 8). In non-cirrhosis patients, a "Diagnostic ERCP" showed a higher risk for complications (P = 0.039). Otherwise, type of intervention done did not have any statistically significant effect on complication occurrence between cirrhosis and non-cirrhosis patients.

Table 1 Complication status and different types of complications in group of subjects with/without cirrhosis, *n* (%)

	With cirrhosis (*n* = 174)	Without cirrhosis (*n* = 518)	*P* value
Any complication?			0.015[a]
No	133 (78.70)	448 (86.49)	
Yes	36 (21.30)	70 (13.51)	
Complications			0.897[1]
Bleeding	2 (5.56)	7 (10.00)	
Pancreatitis	11 (30.56)	25 (35.71)	
Cholangitis	2 (5.56)	5 (7.14)	
Perforation	1 (2.78)	2 (2.86)	
Mortality attributed to ERCP	0 (0.00)	1 (1.43)	
Other mortality	5 (13.89)	12 (17.14)	
Other	15 (41.67)	18 (25.71)	

[a]$P < 0.05$.
[1]Fishers exact test (used when < 5 individuals in a category). ERCP: Endoscopic retrograde cholangio-pancreatography.

Table 2 Child score of cirrhosis patients (*n* = 174) with or without any complication, *n* (%)

	A	B	C	*P* value
Any complication?				0.010[a]
No	46 (80.70)	56 (84.85)	20 (58.82)	
Yes	11 (19.30)	10 (15.15)	14 (41.18)	

[a]$P < 0.05$.

Table 3 Odds ratio estimates for Child-Pugh classes and Wald confidence intervals

Odds ratio	Estimate	95%CI	Limits
Child A *vs* B	1.363	0.532	3.491
Child A *vs* C	0.342	0.132	0.882
Child B *vs* C	0.251	0.096	0.653

Comorbidities

It was noted whether the patient had any of these comorbidities at the time of ERCP: COPD, CHF, HTN, DM, CKD, and HLD. In cirrhosis patients, COPD and HTN demonstrated significantly higher rates of complications (P = 0.009 and 0.003 correspondingly) (Table 9). In patients without cirrhosis, statistically significant complication rates were only demonstrated in those with an underlying diagnosis of COPD (P = 0.003) (Table 10).

DISCUSSION

In this retrospective cohort study of 692 patients, 174 with cirrhosis and 518 without cirrhosis, we found that the overall occurrence of complications was increased in those with cirrhosis to a statistically significant level. In subgroup analysis of CP class and MELD score, we found that CP class C was associated with higher risk of complications, and that CP class was a more reliable predictor of complications than MELD score. The years of experience amongst the advanced endoscopists ranged from approximately five to thirty years, with each performing approximately one-hundred

Table 4 Model for End-Stage Liver Disease score of cirrhosis patients (*n* = 174) with or without any complication, *n* (%)

< 10	10-15		> 15	*P* value
Any complication?				0.626
No	56 (74.67)	41 (82.00)	25 (78.13)	
Yes	19 (25.33)	9 (18.00)	7 (21.88)	
	< 10		≥ 10	
Any complication?				0.381
No	56 (74.67)		66 (80.49)	
Yes	19 (25.33)		16 (19.51)	
	≤ 15		> 15	
Any complication?				0.949
No	97 (77.60)		25 (78.13)	
Yes	28 (22.40)		7 (21.88)	

Table 5 Cirrhosis etiology, gender, and type of anesthesia effects on complication occurrence in the group of subjects with cirrhosis (*n* = 174), *n* (%)

Any complication?	No	Yes	*P* value
Alcohol etiology			0.192
No	65 (74.1)	22 (25.29)	
Yes	68 (82.93)	14 (17.07)	
HEPC etiology			
No	112 (78.87)	30 (21.13)	0.899
Yes	21 (77.78)	6 (22.22)	
NAFLD etiology			
No	123 (77.85)	35 (22.15)	0.461[1]
Yes	10 (90.91)	1 (9.09)	
Gender			
Female	51 (76.12)	16 (23.88)	0.507
Male	82 (80.39)	20 (19.61)	
Type of Anesthesia			
General Anesthesia	105 (78.36)	29 (21.64)	0.271
MAC	4 (57.14)	3 (42.86)	
Moderate conscious sedation	23 (85.19)	4 (14.81)	

[1]Fishers exact test (used when < 5 individuals in a category). HEPC: Hepatitis C; NAFLD: Non-alcoholic fatty liver disease; MAC: Monitored anesthesia care.

procedures per year.

There remains a scarcity in the literature regarding complications and adverse events after ERCP in cirrhosis patients, particularly those incorporating CP class and MELD score or type of intervention as predictors. A retrospective matched case-control study by Navaneethan *et al*[4] showed a higher risk of ERCP-associated hemorrhage in cirrhosis patients *vs* non-cirrhosis patients[4]. Similarly, Inamdar *et al*[5] found a higher rate of post-ERCP pancreatitis and bleeding in cirrhosis patients compared to non-cirrhosis patients. Furthermore, in subgroup analysis, compensated cirrhosis patients and non-cirrhosis patients had a similar complication profile as compared to decompensated cirrhosis patients except for a 2.2% higher rate of

Table 6 Gender and type of anesthesia effects on complication occurrence in the group of non-cirrhosis subjects (*n* = 518), *n* (%)

Any complication?	No	Yes	*P* value
Gender			0.692
Female	264 (85.99)	43 (14.01)	
Male	184 (87.20)	27 (12.80)	
Type of Anesthesia			
General Anesthesia	308 (85.56)	52 (14.44)	0.511
MAC	131 (89.12)	16 (10.88)	
Moderate conscious sedation	9 (81.82)	2 (18.18)	

MAC: Monitored anesthesia care.

Table 7 Type of Intervention in cirrhosis patients (*n* = 174) with or without any complication, *n* (%)

Any complication?	No	Yes	*P* value
Diagnostic ERCP			0.737[1]
No	122 (78.21)	34 (21.79)	
Yes	11 (84.62)	2 (15.38)	0.192
Sphincterotomy/sphincteroplasty			
No	86 (81.90)	19 (18.10)	
Yes	47 (73.44)	17 (26.56)	
Biliary intervention (stent, sweeping, dilatation, brushing)			1.000[1]
No	17 (80.95)	4 (19.05)	
Yes	116 (78.38)	32 (21.62)	
Spyglass			0.098[1]
No	128 (80.00)	32 (20.00)	
Yes	5 (55.56)	4 (44.44)	
Pancreatic intervention			1.000[1]
No	17 (80.95)	4 (19.05)	
Yes	116 (78.38)	32 (21.62)	
Manometry			
No	133 (78.7)	36 (21.3)	
Yes	0 (0)	0 (0)	

[1]Fishers exact test (used when < 5 individuals in a category). ERCP: Endoscopic retrograde cholangio-pancreatography.

pancreatitis[5]. More recently, Leal *et al*[6] reaffirmed a higher rate of adverse events after ERCP in cirrhosis *vs* non-cirrhosis patients[6]. In our study, no statistical significance was calculated when comparing the specific types of adverse events across the two groups, including bleeding, pancreatitis, cholangitis, perforation, mortality, or other. There have been other studies, such as ours, that demonstrated similar outcomes between groups[7]. Importantly, there remains a lack of conclusive evidence warranting further studies.

Data regarding the relationship of ERCP complications and CP class or MELD score are even more limited and contradictory. For instance, Adler *et al*[8] demonstrated that CP class A was associated with a lower risk of ERCP adverse events compared to class B and C combined[8]. Jagtap *et al*[9] found that overall post-ERCP adverse events were increased in patients with CP class C and MELD score > 18[9]. Li *et al*[10] demonstrated that CP class C was associated with a statistically significant higher risk of post-ERCP

Table 8 Type of Intervention in non-cirrhosis patients (*n* = 518) with or without any complication, *n* (%)

Any complication?	No	Yes	*P* value
Diagnostic ERCP			0.039[1]
No	422 (87.37)	61 (12.63)	
Yes	26 (74.29)	9 (25.71)	
Sphincterotomy/sphincteroplasty			0.252
No	262 (85.06)	46 (14.94)	
Yes	186 (88.57)	24 (11.43)	
Biliary intervention (stent, sweeping, dilatation, brushing)			0.133
No	70 (81.40)	16 (18.60)	
Yes	377 (87.47)	54 (12.53)	
Spyglass			0.118[1]
No	430 (87.04)	64 (12.96)	
Yes	18 (75.00)	6 (25.00)	
Pancreatic intervention			0.133
No	70 (81.40)	16 (18.60)	
Yes	377 (87.47)	54 (12.53)	
Manometry			0.252[1]
No	447 (86.63)	69 (13.37)	
Yes	1 (50.00)	1 (50.00)	

[1]Fishers exact test (used when < 5 individuals in a category). ERCP: Endoscopic retrograde cholangio-pancreatography.

bleeding, however showed no difference in bleeding between cirrhosis and non-cirrhosis patients[10]. Similarly, multiple studies have found higher rates of post-ERCP bleeding in CP class C compared to class A and B[11,12]. Our analysis correlates with these findings. However, Zhang *et al*[13] found no association of rates of adverse events with respect to CP class, and instead demonstrated MELD score as a more reliable predictor of higher rates of complications[13]. Interestingly, our study demonstrated a statistically significant proportion of cirrhosis patients with CP class A or class B were less likely to develop complications than those in CP class C. Our study demonstrated that MELD score was not reliable in predicting complications. Whereas our findings correlate with some of the already published studies, it takes research a step further by investigating the impact of cirrhosis etiology, gender, type of sedation used during procedure, interventions performed, and co-morbidities on the rate of complications of ERCP in cirrhosis patients as compared to non-cirrhosis patients.

Our study had several limitations. This includes its retrospective design and moderate sample size. Several patients did not have all the necessary lab values and information on the day of the documented ERCP. In these cases, we had to use the necessary data points obtained at the date closest to their ERCP to calculate MELD scores and CP class. Similarly, many of the data points we collected relied on accurate and complete physician documentation, which can have significant variance. In our data collection, we could not include all comorbidities of each patient, and therefore chose to include six common ones that can affect risk of procedural complications. We encourage that further studies include a broader scope of comorbidities, such as immunocompromising diseases, *etc*. Furthermore, we did not analyze specific pancreatic duct stenting, use of indomethacin, or coagulopathy in respect to outcome. Lastly, we only considered complications that occurred within the span of 30 d of ERCP. The clinical course of a cirrhosis patient who has undergone an invasive procedure may be more complex and indirect complications may occur further down the line.

Table 9 Comorbidities in cirrhosis patients (*n* = 174) with or without any complication, *n* (%)

Any complication?	No	Yes	*P* value
Chronic obstructive pulmonary disease			0.009[a]
No	114 (82.61)	24 (17.39)	
Yes	19 (61.29)	12 (38.71)	
Congestive heart failure			0.572[1]
No	116 (77.85)	33 (22.15)	
Yes	17 (85.00)	3 (15.00)	
Essential hypertension			0.003[a]
No	42 (66.67)	21 (33.33)	
Yes	91 (85.85)	15 (14.15)	
Diabetes mellitus			0.515
No	89 (80.18)	22 (19.82)	
Yes	44 (75.86)	14 (24.14)	
Chronic kidney disease			0.478[1]
No	124 (79.49)	32 (20.51)	
Yes	9 (69.23)	4 (30.77)	
Hyperlipidemia			
No	95 (76.00)	30 (24.00)	0.149
Yes	38 (86.36)	6 (13.64)	

[a]$P < 0.05$.
[1]Fishers exact test (used when < 5 individuals in a category).

CONCLUSION

In conclusion, the results of our study reaffirm that liver cirrhosis has an impact on the occurrence of complications during ERCP. Our study shows that CP class seems to be more reliable as compared to MELD score in predicting complications of ERCP in cirrhosis patients. However, we are also aware that CP and MELD scores are complementary to each other while evaluating outcomes of any surgery in patients with cirrhosis. These findings should encourage clinicians to be aware of the increased risk when referring for, or performing, an ERCP on a patient with cirrhosis. It is imperative to perform a thorough risk-benefit assessment taking into consideration the extent of liver disease and comorbidities prior to ERCP, as doing so may improve clinical outcomes. Further studies, particularly prospective studies, are required to confirm this risk and further delineate the relationship between cirrhosis and complication risk during ERCP.

Table 10 Comorbidities in non-cirrhosis patients (*n* = 518) with or without any complication, *n* (%)

Any complication?	No	Yes	*P* value
Chronic obstructive pulmonary disease			0.003[a]
No	421 (87.71)	59 (12.29)	
Yes	26 (70.27)	11 (29.73)	
Congestive heart failure			0.782[1]
No	424 (86.53)	24 (85.71)	
Yes	24 (85.71)	4 (14.29)	
Essential hypertension			0.071
No	237 (89.10)	29 (10.90)	
Yes	210 (83.67)	41 (16.33)	
Diabetes mellitus			0.652
No	350 (86.85)	53 (13.15)	
Yes	98 (85.22)	17 (14.78)	
Chronic kidney disease			0.827
No	413 (86.58)	64 (13.42)	
Yes	35 (85.37)	6 (14.63)	
Hyperlipidemia			0.531
No	350 (86.00)	57 (14.00)	
Yes	98 (88.29)	13 (11.71)	

[a]$P < 0.05$.
[1]Fishers exact test (used when < 5 individuals in a category).

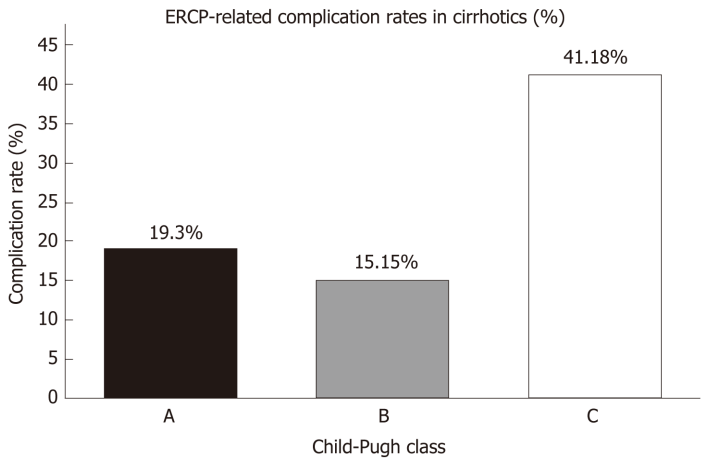

Figure 1 Endoscopic retrograde cholangio-pancreatography-related complications rates in cirrhotic patients based on Child-Pugh class.
ERCP: Endoscopic retrograde cholangio-pancreatography.

ARTICLE HIGHLIGHTS

Research background

Endoscopic retrograde cholangio-pancreatography (ERCP) is associated with a risk of adverse events. There remains a scarce amount of data investigating complications associated with ERCP in patients with cirrhosis as compared to patients without cirrhosis.

Research motivation

Our aim was to determine if patients with cirrhosis are at increased risk of complications associated with ERCP and if a higher Child-Pugh (CP) score and Model for End-Stage Liver Disease (MELD) score are linked to higher post-ERCP adverse events. Findings should encourage clinicians to be aware of the increased risk when referring for, or performing, an ERCP on a patient with cirrhosis.

Research objectives

Our primary aim was to determine if patients with an underlying diagnosis of cirrhosis are at elevated risk of complications compared to patients without cirrhosis, specifically pancreatitis, bleeding, perforation, cholangitis, and mortality. Our study takes previous research a step further by investigating the impact of cirrhosis etiology, gender, type of sedation used during procedure, interventions performed, and co-morbidities on the rate of complications of ERCP.

Research methods

This was a retrospective analysis in which a statistical analysis of the complication rates in the groups with and without cirrhosis was performed using a chi-squared test, and fishers exact test when there were < 5 individuals in a category. Odds ratios with 95% confidence intervals were derived from logistic regression as a supportive method in confirming the findings of Child score significance.

Research results

The results of our study reaffirm that liver cirrhosis has an impact on the occurrence of complications during ERCP. Our study demonstrated a statistically significant proportion of cirrhosis patients with CP class A or class B were less likely to develop complications than those in CP class C. Our study demonstrated that MELD score was not reliable in predicting complications.

Research conclusions

Complications are increased in patients with cirrhosis, especially those in CP Class C.

Research perspectives

Further studies, particularly prospective studies, are required to confirm the risk of performing an ERCP on a patient with cirrhosis, and further delineate the relationship between cirrhosis and complication risk during ERCP.

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ORIGINAL ARTICLE

Retrospective Study

Fatal arterial hemorrhage after pancreaticoduodenectomy: How do we simultaneously accomplish complete hemostasis and hepatic arterial flow?

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Abstract

BACKGROUND

Although arterial hemorrhage after pancreaticoduodenectomy (PD) is not frequent, it is fatal. Arterial hemorrhage is caused by pseudoaneurysm rupture, and the gastroduodenal artery stump and hepatic artery (HA) are frequent culprit vessels. Diagnostic procedures and imaging modalities are associated with certain difficulties. Simultaneous accomplishment of complete hemostasis and HA flow preservation is difficult after PD. Although complete hemostasis may be obtained by endovascular treatment (EVT) or surgery, liver infarction caused by hepatic ischemia and/or liver abscesses caused by biliary ischemia may occur. We herein discuss therapeutic options for fatal arterial hemorrhage after PD.

AIM

To present our data here along with a discussion of therapeutic strategies for fatal arterial hemorrhage after PD.

METHODS

We retrospectively investigated 16 patients who developed arterial hemorrhage after PD. The patients' clinical characteristics, diagnostic procedures, actual treatments [transcatheter arterial embolization (TAE), stent-graft placement, or surgery], clinical courses, and outcomes were evaluated.

Review Board of Shiga General Hospital, Moriyama, Japan.

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RESULTS

The frequency of arterial hemorrhage after PD was 5.5%. Pancreatic leakage was observed in 12 patients. The onset of hemorrhage occurred at a median of 18 d after PD. Sentinel bleeding was observed in five patients. The initial EVT procedures were stent-graft placement in seven patients, TAE in six patients, and combined therapy in two patients. The rate of technical success of the initial EVT was 75.0%, and additional EVTs were performed in four patients. Surgical approaches including arterioportal shunting were performed in eight patients. Liver infarction was observed in two patients after TAE. Two patients showed a poor outcome even after successful EVT. These four patients with poor clinical courses and outcomes had a poor clinical condition before EVT. Fourteen patients were successfully treated.

CONCLUSION

Transcatheter placement of a covered stent may be useful for simultaneous accomplishment of complete hemostasis and HA flow preservation.

Key Words: Pancreaticoduodenectomy; Endovascular treatment; Stent-graft; Covered stent; Transcatheter arterial embolization; Arterioportal shunting

Core Tip: Arterial hemorrhage after pancreaticoduodenectomy is fatal. This hemorrhage is caused by pseudoaneurysm rupture, and the gastroduodenal artery stump and hepatic artery are frequent culprit vessels. Simultaneous accomplishment of complete hemostasis and hepatic artery flow preservation is difficult after pancreaticoduodenectomy. Although complete hemostasis may be obtained by transcatheter arterial embolization or surgery, liver infarction and/or abscesses may occur. We here evaluate our experience including actual treatments (transcatheter arterial embolization, stent-graft placement, or surgery), and discuss therapeutic strategies. Transcatheter placement of a covered stent is useful for simultaneous accomplishment of complete hemostasis and hepatic arterial flow preservation.

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INTRODUCTION

The mortality rate after pancreaticoduodenectomy (PD) is currently < 5%[1-8] because surgical procedures and perioperative management techniques have been well established[1,9-11]. However, postoperative complications still remain a matter of concern[1,2,4,6,8,11-13]. Although arterial hemorrhage after PD is not frequent, it is fatal. Its mortality rate reportedly ranges from 10% to 60%[1,2,4,7,12,14-23], and it easily results in shock and coagulopathy[1,18,23]. Arterial hemorrhage is mainly caused by pseudoaneurysm rupture of a splanchnic artery[18,24], and the gastroduodenal artery (GDA) stump, common hepatic artery (CHA), and proper hepatic artery (PHA) are the most frequent culprit vessels[1-3,6,18,25-28]. Diagnostic and treatment strategies should be decided on a case-by-case basis[18,28,29].

Arterial flow, especially in the liver, is modified after PD (Figure 1). Briefly, the hepatopetal flow of the hepatic artery (HA) depends on the blood supply from the celiac artery (*e.g.*, the CHA and PHA), not from the superior mesenteric artery (SMA) [*e.g.*, the inferior pancreaticoduodenal artery (IPDA) and retrograde-flowing GDA] and collateral circulation (*e.g.*, hepatopetal collaterals *via* the inferior phrenic artery)[1,28,30,31]. This leads to a simple question: How do we simultaneously accomplish complete hemostasis and HA flow preservation? Endovascular treatment (EVT) [*e.g.*,

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transcatheter arterial embolization (TAE) and stent-graft placement] are currently available[1,6,13,16,18,19,32-40], and surgical arterioportal shunting has therapeutic potential for the arterial blood supply[41,42].

TAE provides complete hemostasis[1,2,13,19,20,23,39,43], although this approach increases the risk of severe complications associated with liver infarction caused by hepatic ischemia[1,13,18,19,23,30,32,33,39] and/or liver abscesses caused by biliary ischemia[6,34,35]. In contrast, transcatheter placement of a stent-graft (bare or covered stent) preserves HA flow[1,13,16,18,36-38,40], although technical failure of hemostasis may rarely occur[1]. From the viewpoint of cost-effectiveness, EVT is more advantageous than conventional surgery[29].

Complete hemostasis of fatal hemorrhage and preservation of HA flow should be simultaneously obtained; however, this may be difficult after PD because the hepatopetal arterial supply has been modified (Figure 1). In the present study, we retrospectively investigated our treatments for fatal arterial hemorrhage after PD and evaluated our own results. We also herein discuss the safety and feasibility of transcatheter stent-graft placement and especially validate the therapeutic potential of using a covered (not bare) stent for simultaneous accomplishment of complete hemostasis and HA flow preservation.

MATERIALS AND METHODS

Patients

This study focused on the postoperative state after PD (Figure 1); therefore, patients who underwent other surgeries (*e.g.*, distal pancreatectomy or gastrectomy) were excluded from further analysis. During a 14-year period (from January 2007 to December 2020), 291 PDs were performed in our institution. Fatal arterial hemorrhage occurred in 16 patients who underwent PD, and these patients were enrolled in this study. The patients' mean age at the time of PD was 73.4 ± 7.7 years, and the patients comprised 11 men and 5 women. The types of PD and postoperative complications are summarized in Table 1. The median follow-up duration after PD was 1.34 years [range, 14 d (death) to 9.55 years].

The clinical features, management strategy, and outcome of arterial hemorrhage were evaluated.

Surgical procedures of PD

The surgical procedures of PD have been described in detail elsewhere[9,44]. Lymphadenectomy and nerve dissection were performed in patients with malignancies in accordance with the Japanese guideline[45]. Briefly, the GDA from the celiac artery and IPDA from the SMA were cut after double ligation using a locking loop knot. Inherent reconstructions during subtotal stomach-preserving PD were performed by the modified Child's method with Braun's anastomosis. During pancreaticojejunostomy, an intraductal lost stent (pancreatic duct tube, 5 Fr, burled, MD41515; Sumitomo Bakelite Co., Ltd., Tokyo, Japan) was placed, and duct-to-jejunal anastomosis was performed with interrupted polydioxanone sutures (4-0 PDS II, violet, RB-1, Z712D; Ethicon, Inc., Cincinnati, OH, United States). Adequate approximation of the pancreatic stump and jejunal wall was ensured with interrupted polyvinylidene fluoride sutures (4-0 ASSP504-0IIN, ASFLEX, 75 cm; Kono Seisakusho Co., Ltd., Ichikawa, Chiba, Japan). choledochojejunostomy was performed with interrupted polydioxanone sutures. A linear stapler was employed for gastrojejunostomy, and the entry hole was closed by hand suturing in a layer-to-layer fashion. Braun's anastomosis was also performed by hand suturing in a layer-to-layer fashion.

Liver infarction caused by hepatic ischemia

Liver infarction was mainly diagnosed by imaging findings. A sudden increase in the serum aspartate aminotransferase concentration or a gradual increase in the total bilirubin concentration was used as supporting data[1].

Pancreatic leakage

Pancreatic leakage was diagnosed according to the criteria established by the International Study Group of Pancreatic Surgery[46].

TAE

TAE was performed as the EVT procedure in this study. We intend to arrest fatal

Table 1 Patients characteristics

Case number	Primary disease	Type of PD	Lymphadenectomy (categorization[1])	Nerve dissection	Associated pancreatitis	Pancreatic leakage	Postoperative complications	Hemorrhage oncet[2]	Sentinel bleeding	Symptoms	Sepsis	Shock	Liver ischemia
1	Insulinoma	SSpPD	No	No	No	Yes	-	7	No	Active bleeding from intraperitoneal drain	Yes	Yes	No
2	Gastric cancer	PD	Yes (D2)	No	No	Yes	-	20	No	Bleeding from wound	Yes	Yes	No
3	Gallbladder cancer	HPD	Yes (regional)	Yes	No	Yes	-	58	No	Hematemesis	No	No	No
4	Neuroendoicrine tumor	Lasparoscopic PD	No	No	No	Yes	-	18	Yes	Active bleeding from intraperitoneal drain	No	Yes	No
5	Bile duct cancer	PD	Yes (regional)	No	Yes	No	Digestive anastomotic failure	11	No	Active bleeding from intraperitoneal drain	No	No	No
6	Pancreatic cancer	SSpPD	Yes (D2)	Yes	Yes	Yes	-	22	No	Active bleeding from intraperitoneal drain	No	No	No
7	Bile duct cancer	SSpPD	Yes (regional)	Yes	No	Yes	-	14	No	Active bleeding from intraperitoneal drain	Yes	No	No
8	Gastric cancer	PD	Yes (D2+)	No	No	No	Ruptured suture (staple line)	32	Yes	Active bleeding fromintraperitoneal drain	Yes	Yes	No
9	Pancreatic cancer	SSpPD	Yes (D2)	Yes	Yes	No	-	6	Yes	Active bleeding from intraperitoneal drain	Yes	Yes	No
10	Pancreatic cancer	SSpPD	Yes (D2)	Yes	Yes	Yes	-	16	No	Melena	No	No	Yes
11	Pancreatic metastasis from renal cancer	SSpPD	No	No	No	Yes	-	30	Yes	Active bleeding from intraperitoneal drain	No	Yes	No
12	Ampullary cancer	SSpPD	Yes (D1)	No	No	Yes	-	6	Yes	Active bleeding from intraperitoneal drain	No	Yes	No
13	Pancreatic cancer	PD	Yes (D2)	Yes	Yes	Yes	-	14	No	Active bleeding from intraperitoneal drain	Yes	Yes	No
14	Intraductal papillary mucinous neoplasm	PpPD	No	No	No	Yes	-	22	No	Active bleeding from intraperitoneal drain	Yes	Yes	No
15	Pancreatic cancer	SSpPD	Yes (D1)	No	Yes	No	Biliary necrosis Ruptured cholangiojejunostomy	12	No	Active bleeding from intraperitoneal drain	Yes	Yes	No
16	Pancreatic cancer	SSpPD	Yes (D2)	Yes	Yes	Yes	-	28	No	Abdominal pain	Yes	Yes	Yes

[1]Intentional lymphadenectomy according to Japanese guidelines.
[2]Postoperative day after pancreaticoduodenectomy. HPD: Hepatopancreatoduodenectomy; PD: Pancreaticoduodenectomy; PpPD: Pylorus-preserving pancreaticoduodenectomy; SSpPD: Subtotal stomach-preserving pancreaticoduodenectomy.

hemorrhage by placement of microcoils (Deltaplush; Codman & Shurtleff, Inc., Raynham, MA, United States) in the pseudoaneurysm and/or culprit artery.

Stent-graft placement

The EVT procedures involved transcatheter placement of a stent-graft. In general, procedures of stent-graft placement were performed under local anesthesia. The target artery was dilated by a balloon catheter (Graftmaster; Abbott Laboratories, Chicago, IL, United States). Balloon catheter pressures was increased in manner of 2 atm per 5 s, and the maximum of intracatheter pressure was 15 atm (1520 kPa). A covered stent (Graftmaster; Abbott Laboratories), not a bare stent, was placed at the culprit artery. The size and length of covered stent was carefully decided on a case-by-case basis, based on angiographic findings after balloon dilation. We aimed to simultaneously obtain complete hemostasis of fatal hemorrhage and preservation of HA flow. The second overlapping stent-graft was implanted in an overlapping fashion, if needed. The actual procedure is shown in Figure 2.

Arterioportal shunting

An arterioportal shunt was surgically created (Figure 3). The ileocecal vein and artery were anastomosed in a side-to-side fashion using polypropylene suture. Thereafter, the hepatopetal flow of the portal vein (PV) was well oxygenated.

Ethical approval

This retrospective study was approved by the ethics review committee for clinical studies of our institution. The study was performed in accordance with the ethical guidelines of the Declaration of Helsinki. All patients involved in this study provided written informed consent authorizing the use and disclosure of their protected health information.

Statistical analysis

All results are shown as mean ± SD or median (range). Survival rates were calculated using the Kaplan-Meier method. All calculations were performed using statistical software (SPSS Inc., Chicago, IL, United States).

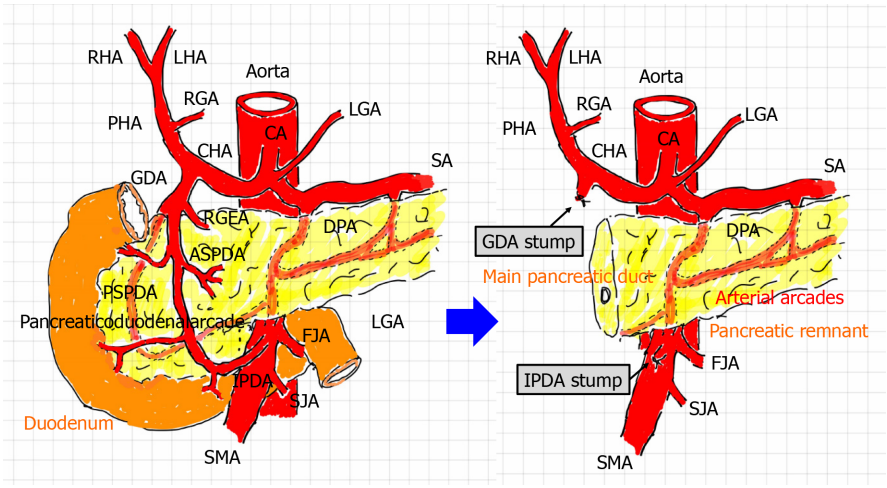

Figure 1 Arterial flow before and after pancreaticoduodenectomy. During pancreaticoduodenectomy (PD), the gastroduodenal artery (GDA) from the celiac artery and inferior pancreaticoduodenal artery (IPDA) from the superior mesenteric artery are ligated and then cut. Additionally, the pancreaticoduodenal arcade is resected. Hence, arterial flow to the liver is modified after PD. A hepatopetal blood supply from the GDA and IPDA *via* the pancreaticoduodenal arcade can no longer be expected. The hepatic artery flow depends on the celiac artery. Lymphadenectomy and nerve dissection for treatment of malignancies might render visceral arteries vulnerable to postoperative wall injuries. Arterial arcades still remain in the pancreatic remnant. ASPDA: Anterior superior pancreaticoduodenal artery; CA: Celiac artery; CHA: Common hepatic artery; DPA: Dorsal pancreatic artery; FJA: First jejunal artery; GDA: Gastroduodenal artery; HA: Hepatic artery; IPDA: Inferior pancreaticoduodenal artery; LGA: Left gastric artery; LHA: Left hepatic artery; PHA: Proper hepatic artery; RGA: Right gastric artery; RGEA: Right gastroepiploic artery; RHA: Right hepatic artery; PSPDA: Posterior superior pancreaticoduodenal artery; SA: Splenic artery; SJA: Second jejunal artery; SMA: Superior mesenteric artery.

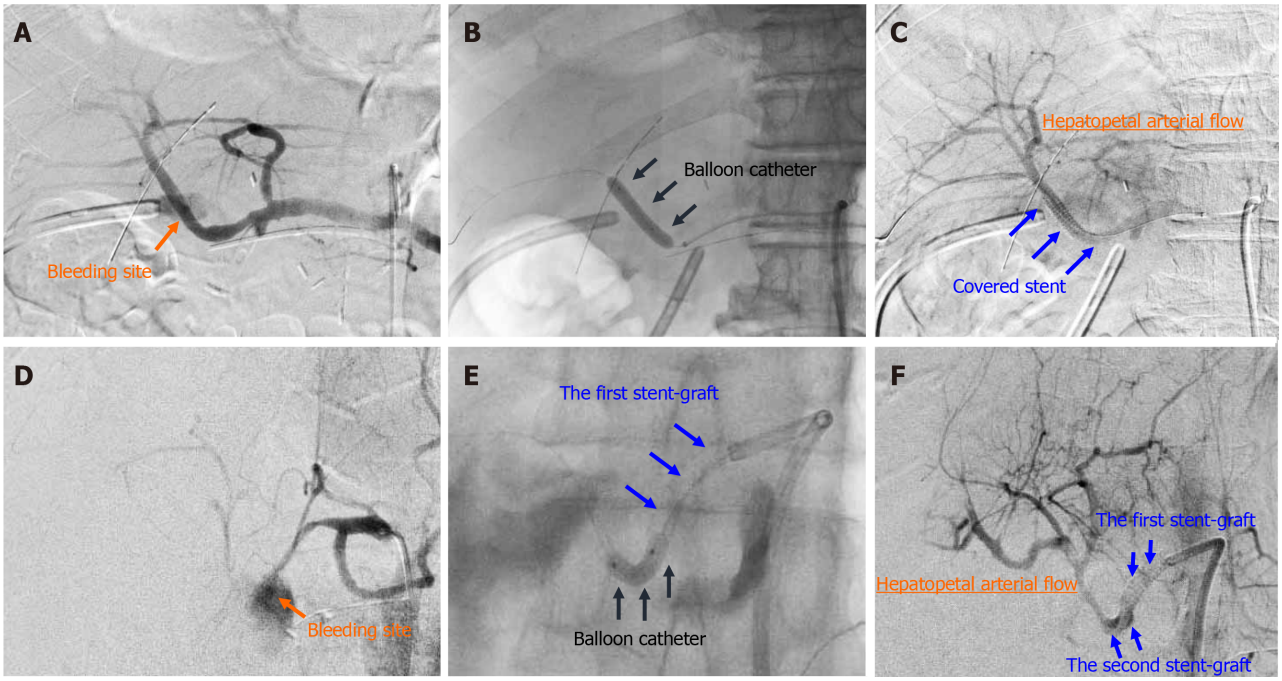

Figure 2 Actual procedures of transcatheter placement of covered stent. Actual procedures in patient 7 and patient 16 are shown. A: Patient 7, diagnostic angiography clearly detected the bleeding sites; B: Patient 7, the target artery was dilated by a balloon catheter; C: Patient 7, a covered stent was placed at the culprit artery; D: Patient 16, diagnostic angiography clearly detected the bleeding sites; E: Patient 16, the target artery was dilated by a balloon catheter; F: Patient 16, the second overlapping stent-graft was implanted in an overlapping fashion. The hepatopetal arterial flow resumed (C and F). Hence, complete hemostasis and preservation of hepatic artery flow were simultaneously obtained.

RESULTS

Institutional frequency of arterial hemorrhage after PD

The overall frequency of arterial hemorrhage after PD was 5.5% (16 of 291 patients who underwent PD in our institution).

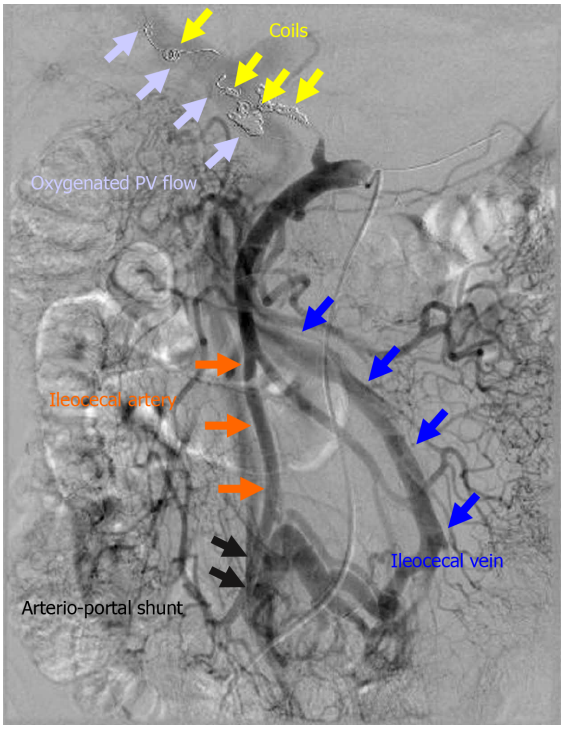

Figure 3 Actual finding of arterioportal shunting. The ileocecal vein and artery were anastomosed in a side-to-side fashion. In a patient in whom the initial endovascular treatment failed (patient 14), hemostasis was completed by additional transcatheter arterial embolization, and liver infarction subsequently occurred. Therefore, an arterioportal shunt was surgically created to oxygenate the portal vein flow. In this case, arterioportal shunting minimized progression to fatal liver infarction due to hepatic ischemia and refractory liver abscess due to biliary ischemia. PV: Portal vein.

Patients' characteristics and PD procedures

The primary diseases and surgical procedures are summarized in Table 1. Thirteen (81.3%) patients had malignancies. Lymphadenectomy and/or nerve dissection was performed in 12 (75.0%) patients.

Associated pancreatitis and pancreatic leakage

Associated pancreatitis occurred in seven patients, and nine (56.3%) patients had a soft pancreatic remnant (*i.e.*, pancreatic remnant without associated pancreatitis) (Table 1). Pancreatic leakage was observed in 12 (75.0%) patients (Table 1).

Hemorrhage onset, symptoms, and patients' conditions before EVT

The clinical characteristics at hemorrhage onset are summarized in Table 1. Hemorrhage onset occurred at a median of 18 d (range, 6-58 d) after PD. Sentinel bleeding was observed in 5 (31.3%) patients. Arterial hemorrhage was externalized through the intraperitoneal drain or wound in 13 (81.3%) patients and through the digestive tract in 2 (16.7%) patients. Sepsis, shock (including an unstable hemodynamic state), and liver infarction were observed in 9 (56.3%), 11 (68.8%), and 2 (16.7%) patients, respectively. Notably, four patients with poor clinical courses after EVT (Patients 13-16) had a poor clinical condition before EVT (Table 1).

Bleeding site, image findings, and definitive diagnosis

The most common and second most common sites of bleeding were the GDA stump (7 patients, 43.8%) and HA (4 patients, 25.0%), respectively (Table 2). Computed tomography (CT) angiography was the diagnostic modality in 13 (81.3%) patients. The imaging findings of CT angiography and angiography are summarized in Table 2. The median time from hemorrhage onset to definitive diagnosis and the median time from hemorrhage onset to EVT were 0 d (range, 0-1 d) and 0 d (range, 0-14 d), respectively (Table 2).

Actual EVT procedures, technical success of EVT, and long-term results after EVT

The treated arteries and ranges are summarized in Table 3. The initial EVT procedures were stent-graft placement in 7 (43.8%) patients, TAE in 6 (37.5%) patients, and combined therapy involving stent-graft placement and TAE in 2 (16.7%) patients

Table 2 Definitive diagnosis

Case number	Bleeding site	Diagnostic modality	CT angiographic findings	Angiographic findings	Time from hemorrhage onset to definitive diagnosis (d)	Time from hemorrhage onset to EVT (d)
1	RGA	CT angiography	Extravasation	Extravasation	0	0
2	SA	CT angiography	Extravasation	Extravasation	0	14[1]
3	RHA	CT angiography	Enlargement of pseudoaneurysm	Pseudoaneurysm; Extravasation	1	0
4	Cholangiojejunostomy	Clinical findings[2]	None	None	0	0
5	DPA	CT angiography	Extravasation	Extravasation	0	0
6	GDA stump	CT angiography	Extravasation	Extravasation	0	0
7	RHA	CT angiography	Extravasation	Extravasation	0	0
8	GDA stump	CT angiography	Pseudoaneurysm; Extravasation	Pseudoaneurysm	0	0
9	DPA	CT angiography	Extravasation	Extravasation; Pseudoaneurysm	0	0
10	PHA	CT angiography	Pseudoaneurysm	Obstruction of CHA; Pseudoaneurysm	0	1
11	RHA	CT angiography	Pseudoaneurysm	Pseudoaneurysm	0	0
12	GDA stump	Laparotomy[3]	None (hematoma only)	None (stenosis of CHA)	0	0
13	GDA stump	Angiography	Extravasation	Extravasation	0	0
14	GDA stump	CT angiography	Extravasation	Extravasation; Pseudoaneurysm	0	0
15	GDA stump	CT angiography	Minor extravasation	Extravasation; Pseudoaneurysm	0	1
16	GDA stump	CT angiography	Pseudoaneurysm; Extravasation	Pseudoaneurysm; Extravasation	0	0

[1]Hematemesis and endoscopic findings.
[2]Two surgical approaches were challenged during 14 d.
[3]Bleeding from the GDA was detected during laparotomy, even though computed tomography angiography and angiographic findings did not revealed extravasation. The endovascular treatment was done under the laparotomy. CHA: Common hepatic artery; CT: Computed tomography; DPA: Dorsal pancreatic artery; EVT: Endovascular treatment; GDA: Gastroduodenal artery; RGA: Right gastric artery; RHA: Right hepatic artery; PHA: Proper hepatic artery; SA: Splenic artery.

(Table 3).

The initial EVT failed and/or was incomplete in 4 (25.0%) patients, and the rate of technical success of the initial EVT was 75.0% (Table 3). The reasons for failed and/or incomplete EVT were stenosis in 2 patients, and subtle bleeding in one patient, and difficulty in packing in 1 patient (Table 3). Additional EVTs were performed in 4 (25.0%) patients (Table 3). Antiplatelet and/or anticoagulation agents were administered to 5 (31.3%) patients (Table 3), and these 5 patients continuously received medications even after discharge from our hospital.

Recanalization did not occur (0.0%) throughout the long-term follow-up after TAE (Table 3). Collateral circulation was observed in 2 (25.0%) of eight patients who underwent TAE (Table 3). Additionally, all implanted stent-grafts (100.0%) maintained their patency throughout the long-term follow-up after stent-graft placement (Table 3).

Surgical approaches including arterioportal shunting

Surgical approaches were utilized in eight patients and are summarized in Table 3. In one patient who underwent failed EVT (patient 10), hemostasis and ligation of the CHA were surgically performed under laparotomy. In one patient in whom the initial EVT failed (patient 14), hemostasis was completed by additional TAE, and liver infarction subsequently occurred. Therefore, an arterioportal shunt was surgically created to oxygenate the PV flow (Figure 3). In this case, arterioportal shunting

Table 3 Endovascular treatment

Case number	Treated artery (target and range)	TAE	Stent-graft placement			Technical success during EVT	Reasons for failed or incomplete EVT	Additional surgical approaches (day number[2])	Additional EVT (day number[2])	Antiplatelet and/or anticoagulation agents (number)	Long-term results of EVT		
											TAE		Stent-graft placement
			Stent type (number[1])	Size (mm)	Length (mm)						Collateral circulation (yr)[3]	Recanalization (yr)[3]	Patency (y)[3]
1	RGA	Coiling	-			Yes	-	No	No	No	No (4.39)	No (4.39)	-
2	CA; SA	-; Coiling	Covered stent (1); -	3.5; -	19; -	No	Stenosis	Hemostasis (-7 and -6)	Stent regrafting (+ 1); Coiling (+ 1)	No	-	-	-
3	PHA-LHA	-	Covered stent (1)	3.5	19	Yes	-	No	No	Yes (1)	-	-	Patent (0.72)
4	SMA branch; RHA	Coiling; -	-; Covered stent (1)	-; 3.5	-; 19	Yes	-	Lavage and cholangio-jejunal anastomosis (+ 7)	Stent regrafting (+ 28)	No	No (6.14); -	No (6.14); -	-; Patent (6.14)
5	SA branch	Coiling	-			Yes	-	No	No	Yes (1)	No (0.46)	No (0.46)	-
6	CHA-PHA	-	Covered stent (1)	3.5	19	Yes	-	No	No	No	-	-	Patent (0.93)
7	RHA	-	Covered stent (1)	3.5	19	Yes	-	Lavage and cholangio-jejunal anastomosis (+ 3)	No	No	-	-	Patent (1.27)
8	GDA	Coiling	-	-	-	No	Subtle bleeding[4]	No	No	No	No (0.24)	No (0.24)	-
9	DPA	Coiling	-	-	-	Yes	-	No	No	No	No (0.44)	No (0.44)	-
10	-	-	-	-	-	No	Stenosis	Hemostasis and ligation of CHA (± 0)	No	Yes (2)	-	-	Patent (1.95)
11	RHA	-	Covered stent (2)	3.0	19	Yes	-	Removal of hematoma (- 18)	Stent regrafting (+33)	Yes (2)	-	-	Patent (1.53)
12	CHA-PHA	-	Covered stent (1)	3.5	19	Yes	-	Removal of hematoma (± 0)	No	No	-	-	Patent (0.98)
13	CHA-PHA	Coiling	-	-	-	Yes	-	No	No	No	Yes (1.53)	No (1.53)	-
14	GDA	Coiling	-	-	-	No	Difficulty in packing	Arterio-portal shunting[5] (+ 4)	CHA coiling (+ 4)	Yes (1)	Yes (7.72)	No (7.72)	-
15	GDA	-	Covered stent (1)	3.5	19	Yes	-	No	No	No	-	-	Patent (0.00)

16	GDA	-	Covered stent (2)	2.6	19	Yes	-	Removal of hematoma (± 0)	No	No	-	-	Patent (0.01)

[1]The second stentgraft was implanted in overlapping fashion.
[2]The day number from the initial endovascular treatment (EVT).
[3]Findings in the latest dynamic image studies (time from the initial EVT).
[4]Subtle bleeding was observed at the end of EVT, but thereafter, complete hemostasis was fainally obtained.
[5]Shunt creation between the iliocecal artery and vein. CA: Celiac artery; CHA: Common hepatic artery; EVT: Endovascular treatment; GDA: Gastroduodenal artery; LHA: Left hepatic artery; RHA: Right hepatic artery; PHA: Proper hepatic artery; RGA: Right gastric artery; SA: Splenic artery; SMA: Superior mesenteric artery; TAE: Transcatheter arterial embolization.

minimized the patient's progression to fatal liver infarction due to hepatic ischemia and a refractory liver abscess caused by biliary ischemia.

Liver infarction due to hepatic ischemia

Liver infarction after EVT was observed in 2 (12.5%) patients (patients 13 and 14), and these patients underwent TAE (Tables 3 and 4). Complete hemostasis was obtained by TAE, but hepatopetal arterial flow was completely lost (Figure 4). Liver infarction due to hepatic ischemia subsequently occurred (Figure 4). In these patients, the serum aspartate aminotransferase concentration clearly increased after EVT (Figure 5). Fortunately, both patients successfully recovered from arterial hemorrhage after PD and liver infarction after TAE (Table 4).

Clinical course and outcome after EVT

The patients' clinical courses and outcomes after EVT are summarized in Table 4. Three patients (patients 2, 15, and 16) died during hospitalization, and the actual survival curves after PD and EVT are shown in Figure 6. The mean hospital stay after PD was 66.8 ± 27.7 d among 13 patients who achieved hospital discharge. Fourteen (87.5%) patients were successfully treated because the cause of death in 1 patient (patient 2) was unrelated to arterial hemorrhage (cancer-related death).

Two patients (patients 15 and 16) had a poor outcome even after successful EVT. These 2 patients had a poor clinical condition before EVT (Table 1). One patient (patient 15) had sepsis, shock, and disseminated intravascular coagulation before EVT and died of these conditions even after successful stent-graft placement (Tables 1 and 4). The other patient (patient 16) had sepsis, shock, and liver infarction before EVT (Table 1 and Figure 5) and finally died of liver failure even after successful stent-graft placement (Table 4).

DISCUSSION

In general, visceral artery pseudoaneurysms are rare but fatal[1,13,18,19,21,23,29,30]. The HA (

Table 4 Clinical course and outcome after endovascular treatment

Case number	Complication after EVT	Liver infarction after EVT	Hospital death (day number[1] and POD)	Clinical success[2]	Follow-up term (yr)	Cause of death	Prognosis (dead or alive)
1	-	No	No	Yes	5.57	Cancer-related death	Dead
2	-	No	Yes (+ 61 and 94)	Yes	0.26	Cancer-related death	Dead
3	-	No	No	Yes	0.72	Cancer-related death	Dead
4	-	No	No	Yes	8.36	-	Alive
5	-	No	No	Yes	0.56	Cancer-related death	Dead
6	Bleeding	No	No	Yes	1.04	Cancer-related death	Dead
7	-	No	No	Yes	1.34	Cancer-related death	Dead
8	-	No	No	Yes	0.52	Cancer-related death	Dead
9	-	No	No	Yes	0.47	Cancer-related death	Dead
10	-	No	No	Yes	1.95	-	Alive
11	-	No	No	Yes	1.69	-	Alive
12	-	No	No	Yes	1.46	-	Alive
13	-	Yes	No	Yes	1.74	Cancer-related death	Dead
14	Bleeding; Liver abscess	Yes	No	Yes	9.55	-	Alive
15	-	No	Yes (+ 1 and 14)	No	0.04	Bleeding, sepsis and DIC	Dead
16	-	No	Yes (+ 3 and 31)	No	0.08	Liver failure	Dead

[1]The day number from the initial EVT.
[2]Short-term clinical outcome. EVT: Endovascular treatment; DIC: Disseminated intravascular coagulation; POD: Postoperative day after pancreaticoduodenectomy.

i.e., the CHA, PHA, and lobular branches) is the second most frequent site of visceral pseudoaneurysms, and the splenic artery is generally the most common[47]. Pseudoaneurysms of the HA are usually iatrogenic[2,16,30,44] but may also be associated with localized infection or trauma[30]. Possible causes of intraoperative pseudoaneurysms include direct vascular injury during dissection or retraction, clamp injury to the vessel, or thermal injury *via* electrocautery[17,30]. Lymphadenectomy and/or nerve dissection for malignancy renders visceral arteries more vulnerable to further wall injuries[2,16,17,30,39,44,48]. Complications following PD commonly consist of localized infection, anastomotic failure, delayed gastric emptying, and gastrointestinal bleeding[2,6,8,9,29,30]. Although arterial hemorrhage after PD is not frequent, it is fatal[1,2,4,7,12,14-23]. The GDA stump is the most common site of arterial hemorrhage, and the CHA and PHA are the next most common sites[16,18,19,21,22,27]. Arterial hemorrhage of the SMA after PD has also been reported[49,50].

Pancreatic leakage compromises the arterial wall[2,6,16,17,19,22,24,29,30,39,44,51]. Pancreatic juice or localized infection gradually causes arterial wall erosions, resulting in pseudoaneurysms[2,6,16,17,22,24,29,39,44,51]. Pseudoaneurysm rupture causes sudden-onset, massive, and active hemorrhage[18]. Studies have shown a trend toward a higher prevalence of a soft pancreatic remnant in patients with arterial hemorrhage[2,6,44]. Leaving approximately 1 cm of the GDA stump, spreading an omental flap, and winding the HA by the round ligament of the liver have been suggested to minimize direct contact of pancreatic juice with adjacent vessels[18,22,51-53].

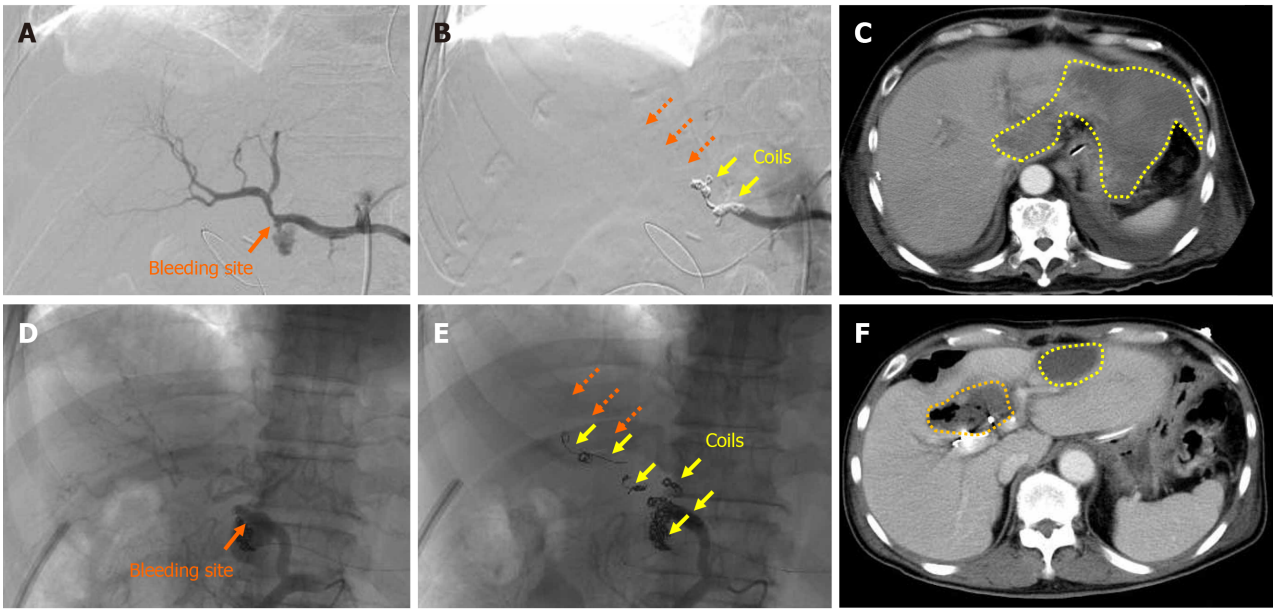

Figure 4 Liver infarction and abscess after transcatheter arterial embolization. Actual findings in patient 13 and patient 14 are shown. A: Patient 13, the bleeding sites were detected; B: Patient 13, complete hemostasis was obtained by transcatheter arterial embolization, but the hepatopetal arterial flow was completely lost (dotted orange arrows); C: Patient 13, the patient subsequently developed liver infarction due to hepatic ischemia (dotted yellow circles); D: Patient 14, the bleeding sites were detected; E: Patient 14, complete hemostasis was obtained by TAE, but the hepatopetal arterial flow was completely lost (dotted orange arrows); F: Patient 14, the patient subsequently developed liver infarction due to hepatic ischemia (dotted yellow circles) and a liver abscess due to biliary ischemia (dotted orange circle).

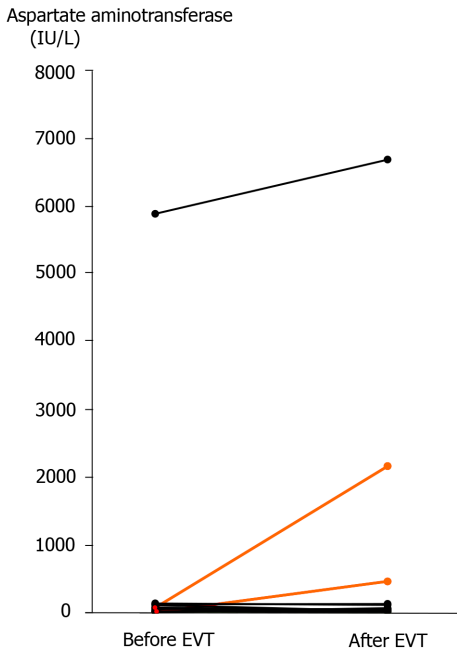

Figure 5 Serum aspartate aminotransferase concentration before and after endovascular treatment. Actual changes before and after endovascular treatment (EVT) are shown. In two patients who developed liver infarction after transcatheter arterial embolization (patients 13 and 14), the serum aspartate aminotransferase concentration was clearly elevated after EVT (orange lines). A high serum aspartate aminotransferase concentration was observed in a patient who had liver infarction before EVT (patient 16), and this patient finally died of liver failure even after successful stent-graft placement. EVT: Endovascular treatment.

Diagnostic procedures and imaging modalities are associated with certain difficulties[6,7,17,18,28,54]. Even based on laparotomy findings, definitive diagnosis may be difficult[54]. Bleeding from the digestive tract or intraperitoneal drain should be considered a warning because it is an important prelude to massive and active hemorrhage. The term "sentinel bleeding" was first coined in 1989 by Shankar and

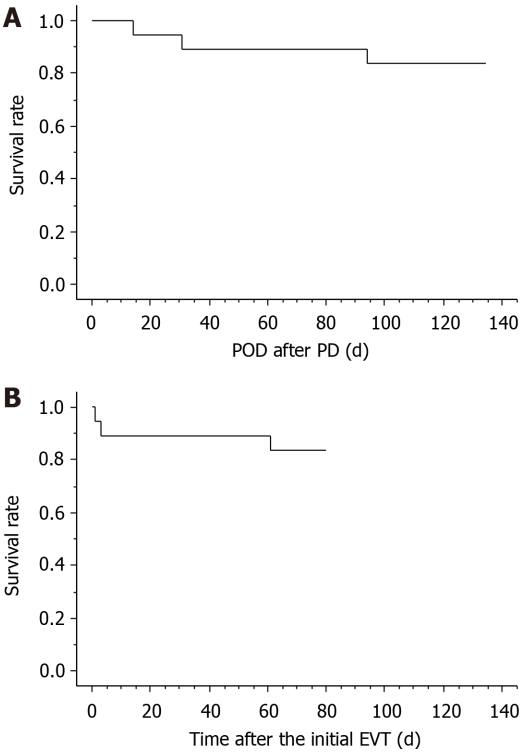

Figure 6 Short-term survival after pancreaticoduodenectomy and endovascular treatment. Three patients (patients 2, 15, and 16) died during hospitalization, and the actual survival curves after pancreaticoduodenectomy (PD) and endovascular treatment (EVT) are shown. Fourteen (87.5%) patients were successfully treated because the cause of death in one patient (patient 2) was unrelated to arterial hemorrhage (cancer-related death). A: PD; B: EVT. EVT: Endovascular treatment; PD: Pancreaticoduodenectomy; POD: Postoperative day.

Russell[55] and was further discussed by Brodsky and Turnbull in 1991[34]. The incidence of sentinel bleeding is approximately 30% to 80%[2,18]. Pseudoaneurysms can be detected by CT in patients with sentinel bleeding[6,7]. Sentinel bleeding should be regarded very seriously[2,4,6,18], even in asymptomatic patients with conservatively treated pancreatic leakage[6,56,57]. An accurate definitive diagnosis should be made immediately, before the patient's unstable hemodynamic state deteriorates[6,18,29]. Diagnostic digestive endoscopy delays adequate treatment in hemodynamically unstable patients because of pseudoaneurysm rupture[7,58]. The diagnostic potential of CT angiography[6,16,19,28,43] and diagnostic angiography[6,7,17,18,29,39,54] have been established. Diagnostic angiographic findings include extravasation of contrast medium, pseudoaneurysm formation, non-smooth arterial intima, local vascular spasm, stenosis, and distal arterial branch expansion[20,54]. Diagnostic angiography should be considered even in patients with suspected hemorrhage[6,7,17,18,29,39,54], and subsequent EVT should be adequately performed if necessary[1,7,16-18,30,54].

EVT represents the first-line treatment for arterial hemorrhage after PD[2,7,17,40,59-61]. Arterial hemorrhage easily results in unstable hemodynamic state[1,6,16,18,23,29,62], sepsis[2,6,7,13], and hepatic ischemia[6,28,39,63]. Prolonged hemorrhage leads to shock and coagulopathy[1,16,18,23], and further hemorrhage results in disseminated intravascular coagulation[1,16,18,23]. Complicated homeostasis is associated with a poor prognosis even after successful EVT[1,16,18], and the patient's condition before EVT is strongly associated with complications after EVT[1,16,29]. In fact, our two patients who had a poor clinical condition before EVT (*e.g.*, sepsis, shock, and liver infarction) finally died even after successful EVT (Tables 1 and 4). If a patient shows any signs of a suspected hemorrhage, EVT should be performed as soon as possible before the development of complicated homeostasis[1,2,16,18,23,30]. Concern exists regarding the placement of foreign bodies (*i.e.*, coils and stent-grafts) in the setting of infection or inflammation[40]. Intravascular stent infection can be a devastating complication, but it is very rare[40,64-66]. In fact, stent-grafts have been used to repair infected pseudoaneurysms[67,68]. Though pancreatic juice-related localized infection may associate with pseudoaneurysm and arterial wall erosion[2,6,16,17,22,24,29,39,44,51], we consider that stent-grafts can be placed even in suspicious infectious site.

Arterial hemorrhage after PD usually occurs after at least 1 d[24], and delayed hemorrhage generally occurs after 1 wk[6,58]. In one study, one-third of arterial

hemorrhages occurred 1 mo after PD[21]. Hence, delayed hemorrhage is common after PD[2,6,7-19,21,22,28,58,62], and the median or mean time point of hemorrhage onset ranges from 18 d to 21 d after PD[2,7,21,28]. Pancreatic leakage is a possible cause of delayed arterial hemorrhage[2,6,44], and delayed hemorrhage after PD carries a significantly higher mortality rate[2,6,7,21,28].

Because the GDA and IPDA were ligated and the pancreaticoduodenal arcade was resected during PD in the present study, hepatopetal blood supply *via* these arteries could no longer be expected (Figure 1). EVT may lead to severe complications (*e.g.*, hepatic ischemia, liver abscess formation, and PV stenosis)[1]. The EVT technique should be decided on a case-by-case basis[18,28,29]. Notably, the EVT procedure is strongly associated with complications after EVT[1,16]. Although TAE is technically easier than stent-graft placement[19], liver infarction secondary to hepatic ischemia frequently occurs[6,34,35]. Even a subtle ischemic change in the biliary tree results in intractable liver abscesses[6,34,35]. We also experienced a case of a refractory liver abscess due to biliary ischemia (patient 14) (Figure 4F). PV stenosis easily disturbs the hepatic parenchymal perfusion, resulting in liver infarction with a poor prognosis[23,69]. The rates of mortality and serious hepatic complications after EVT are approximately 20% to 50% and 20% to 80%, respectively[1,18,19,21,23,31,35,70-72].

TAE is advantageous for ensuring complete hemostasis[2,13,20,23,43,54,62,73-77], and the hemostatic rate is reportedly > 90%[17,20,23,54,71]. TAE is technically user-friendly at the most frequent site (*i.e.*, the GDA stump)[19,43], although both the proximal and distal sides of the GDA should be completely embolized[19]. To prevent recanalization and rebleeding, all arterial flows to the pseudoaneurysm should be completely interrupted[19,44,47,48]. Although the pancreaticoduodenal arcade is removed during PD, arterial arcades remain in the pancreatic remnant (Figure 1)[29,47]. Recanalization *via* the collateral circulation has been reported after TAE[29]; however, transcatheter techniques (*e.g.*, isolation, packing, and embolization) are available for various forms of pseudoaneurysms[29]. Notably, TAE is occasionally associated with serious hepatic complications caused by hepatic ischemia[1,16,23,30,32,33,69,70]. The liver has many potential collateral pathways that communicate with the adjacent arterial system[16,19,23,29,78,79], and a sudden complete block of HA flow immediately after surgery may induce an ischemic insult to the liver parenchyma[16,29,78,79]. Whether extrahepatic arteries (*e.g.*, the inferior phrenic artery and left gastric artery) provide sufficient hepatopetal collateral circulation to avoid fatal hepatic ischemia after TAE remains unclear[1,19,23,32]. Additionally, the liver can tolerate considerable TAE without significant liver infarction because it has a dual blood supply from the HA and PV[19,20,23,29]. TAE may cause liver infarction in patients with poor collateral circulation because of their postoperative status[29]. Approximately 30% to 80% of patients develop hepatic ischemia after TAE[69,71], and approximately 20% to 40% of patients progress to liver infarction[23,70,71]. The reported mortality rate ranges from 30% to 50%[19,31,35,72].

Simultaneous accomplishment of complete hemostasis and HA flow preservation is difficult[1,7,13,16,18,28,59]. Transcatheter placement of a covered stent may be of value in maintaining the patency of adjacent arteries, and stent-graft placement is an ideal technique to preserve HA flow[1,6,13,16,17,21,27-31,40,54,61,63,80-82]. If necessary, a second overlapping stent-graft can be implanted[40,83]. Actually, we placed a second stent in two patients (Table 3). Some researchers have described patients who underwent this EVT technique[30,31,63,80-82,84], and others have documented such cases in published case series[13,36,38,85]. The success rate of stent-graft placement reportedly ranges from 75% to 80% because the target arteries require a specialized stent size and/or exhibit narrowness and tortuosity[16,37,38,59]. The overall mortality and clinical outcomes are affected by the patients' conditions before stent-graft placement[16,29]. The use of antiplatelet agents or heparinization after stent-graft placement in the HA is still controversial[13,16,36,37,40,85]. Some clinicians do not use such agents in patients with an unstable hemodynamic state after arterial hemorrhage[37]. However, stent-graft placement in a patient with arterial hemorrhage after surgery carries a high risk of thrombosis because the damaged wall of the HA is surrounded by localized infection and/or massive hematoma, and the HA diameter is very small[16,40]. Hence, some clinicians use these agents after stent-graft placement[13,16,36,40,85].

Stent grafting is a technically difficult procedure and requires adaptation to vessels of various sizes[18,40]. However, this EVT technique is considered the most appropriate treatment method in patients with a favorable vascular anatomy. Stent-graft placement may fail for anatomical reasons (*e.g.*, tortuosity or variation)[13,17,40,59,84,86] or because of catheter-induced vasospasm or spontaneous thrombosis within the aneurysmal wall[17,87-89]. Actual reasons for failed or incomplete EVT in our institution were summarized in Table 3. Since stent-graft placement may technically failed due to tortuosity, variation, stenosis, vasospasm or thrombosis at the culprit

artery[13,17,40,59,84,86-89], preliminary dilatation by balloon catheter is indispensable even for self-expandable covered stent. The interventional radiologist who performs the procedure must have adequate experience and skill[59,62].

If EVT fails or is incomplete, the next management option for arterial hemorrhage is still a surgical approach[2,7,62]. Surgical exploration and complete hemostasis are difficult and hazardous because of postoperative adhesions and the patient's critical condition[17,18,48]. Surgical treatment is usually associated with a high mortality rate (29%-58%)[2,17-19,48]. Hepatopetal flow of the PV can be well oxygenated by creation of an arterioportal shunt, and some reports have described such cases[41,42]. The impact of a surgical approach involving arterioportal shunting on the prevention of liver infarction has been documented[90,91]. Although the clinical decision and optimal timing for the surgical approach of arterioportal shunting are still controversial, we consider that the presence of subtle clinical signs of progressive liver infarction after EVT is a clear indication for arterioportal shunting.

To our knowledge, most reports to date are limited to case reports or small series[13,30,31,36,38,63,80-82,84,85]. We acknowledge that this study has several limitations. The main limitation is that this was a retrospective study with a small number of patients from a single center. Of course, we have demonstrated our individual-tailored approach. Potential limitations due to bias and a small sample size are inherent to this type of study. This represents our experience in a single institution and our views may be affected by various biases. Hence, we understand that our conclusions must be drawn with extreme caution. However, we believe that transcatheter placement of a covered stent has therapeutic advantages for arterial hemorrhage after PD, with simultaneous accomplishment of complete hemostasis and HA flow preservation.

Actual therapeutic strategies for our patients who caused arterial hemorrhage after PD were summarized in Figure 7. We currently have an institutional therapeutic strategy for arterial hemorrhage after PD based on our own experiences: (1) CT angiography is performed if general condition is stable; (2) Diagnostic angiography is immediately performed even in a suspicious patient; and (3) Covered stent is subsequently placed at the culprit artery as the first line treatment.

CONCLUSION

In conclusion, transcatheter placement of a covered stent may be a powerful tool for simultaneous accomplishment of complete hemostasis and HA flow preservation, although arterial hemorrhage after PD is generally fatal.

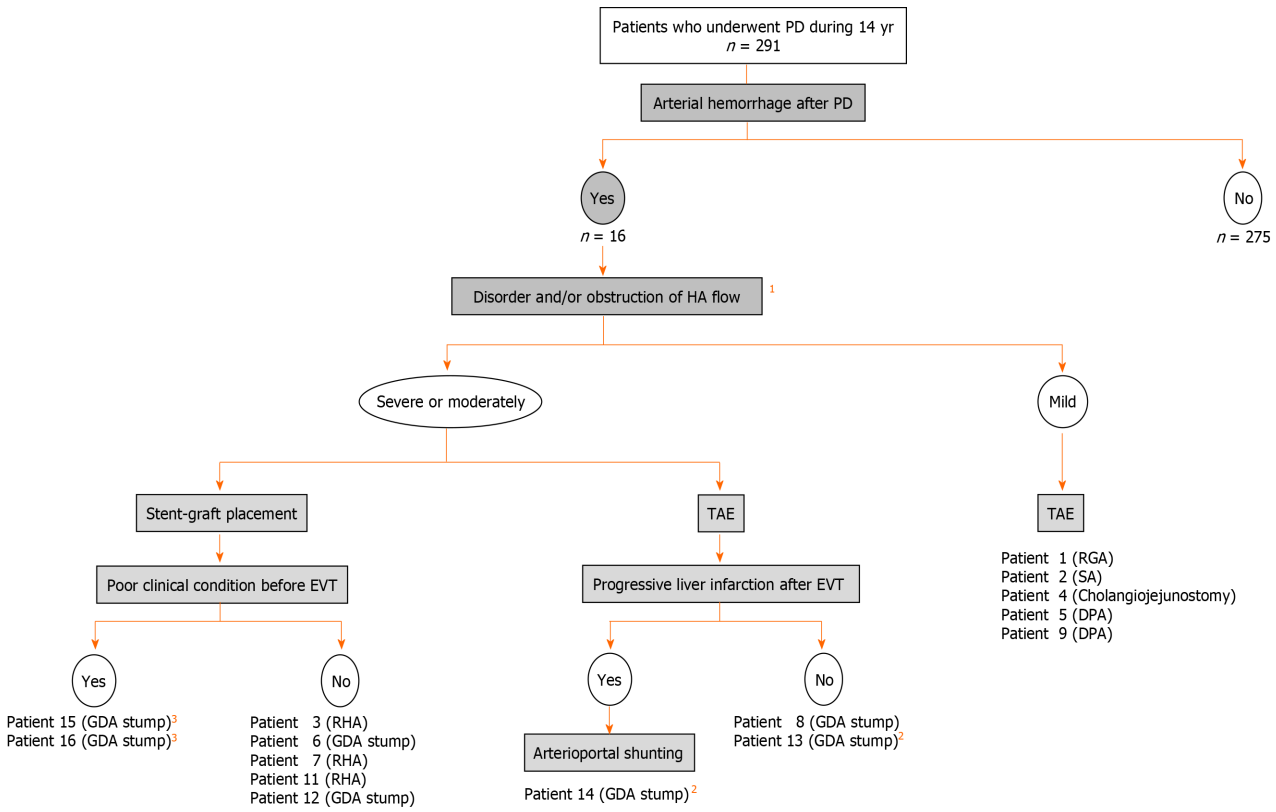

Figure 7 Flowchart of endovascular treatments and arterioportal shunting. Actual flowchart of our patients who caused arterial hemorrhage after pancreaticoduodenectomy was shown. [1]Image findings of computed tomography angiography and/or diagnostic angiography. [2]Patients with liver infarction after transcatheter arterial embolization. [3]Patients with poor outcome even after successful stent-graft placement. PD: Pancreaticoduodenectomy; HA: Hepatic artery; TAE: Transcatheter arterial embolization; EVT: Endovascular treatment; GDA: Gastroduodenal artery; RHA: Right hepatic artery; SA: Splenic artery; DPA: Dorsal pancreatic artery.

ARTICLE HIGHLIGHTS

Research background

Arterial hemorrhage after pancreaticoduodenectomy (PD) is fatal.

Research motivation

This hemorrhage is caused by pseudoaneurysm rupture, and the gastroduodenal artery stump and hepatic artery are frequent culprit vessels.

Research objectives

Simultaneous accomplishment of complete hemostasis and hepatic artery flow preservation is difficult after PD. Although complete hemostasis may be obtained by transcatheter arterial embolization or surgery, liver infarction and/or abscesses may occur.

Research methods

Arterial hemorrhage after PD is fatal. This hemorrhage is caused by pseudoaneurysm.

Research results

We here evaluate our experience including actual treatments (transcatheter arterial embolization, stent-graft placement, or surgery), and discuss therapeutic strategies.

Research conclusions

Transcatheter placement of a covered stent is useful for simultaneous accomplishment of complete hemostasis and hepatic arterial flow preservation.

Research perspectives

Therapeutic options for fatal arterial hemorrhage after PD is shown.

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ORIGINAL ARTICLE

Observational Study

Dried blood spot sampling as an alternative for the improvement of hepatitis B and C diagnosis in key populations

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Abstract

BACKGROUND

To achieve the elimination of hepatitis B and C, there is an urgent need to develop alternative strategies to increase the access of diagnosis, particularly among key populations such as people living with human immunodeficiency virus (HIV), individuals with coagulopathies and chronic kidney disease (CKD) patients.

AIM

Flores GL and Villar LM drafted the manuscript; Flores GL, Mota JC, Bastos FI and Villar LM critically revised the manuscript for intellectual content; All authors read and approved the final manuscript.

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To evaluate the use of dried blood spot (DBS) in the detection of hepatitis B virus (HBV) and hepatitis C virus (HCV) markers.

METHODS

A total of 430 individuals comprised of people living with HIV, coagulopathies and CKD provided paired serum and DBS samples. HBsAg, anti-HBc and anti-HCV were tested in those samples using a commercial electrochemiluminescence. Demographic and selected behavioral variables were evaluated to assess possible association with HBV and HCV positivity.

RESULTS

Using DBS, HBsAg prevalence varied from 3.9% to 22.1%, anti-HBc rates varied from 25.5% to 45.6% and anti-HCV positivity ranged from 15.9% to 41.2% in key populations. Specificities of HBV and HCV tests using DBS varied from 88.9% to 100%. The HBsAg assay demonstrated the best performance in CKD and coagulopathy individuals and the anti-HCV test had a sensitivity and specificity of 100% in people living with HIV. Accuracy of HBV and HCV detection in DBS varied from 90.2% to 100%. In the CKD group, HBsAg positivity was associated with infrequent use of condoms, and anti-HBc positivity was associated with sharing nail cutters/razors/toothbrushes. Anti-HCV reactivity was positively associated with a history of transplantation and length of time using hemodialysis in both specimens. In people living with HIV, only the male gender was associated with anti-HBc positivity in serum and DBS.

CONCLUSION

DBS with electrochemiluminescence are useful tools for the diagnosis and prevalence studies of hepatitis B and C among key populations and may increase the opportunity to foster prevention and treatment.

Key Words: Dried blood spot; Electrochemiluminescence; Hepatitis B; Hepatitis C; Key populations; Diagnosis

Core Tip: Dried blood spot (DBS) samples may be an alternative to serum to increase access and timeliness in the diagnosis of hepatitis B and C in key populations such as people living with human immunodeficiency virus, coagulopathies and chronic kidney disease. We found high accuracy for hepatitis B virus and hepatitis C virus detection using DBS. It was possible to observe similar hepatitis prevalence, demographic and clinical data related to hepatitis positivity in DBS and serum. DBS along with electrochemiluminescence could be used for diagnosis and prevalence studies of hepatitis B virus and hepatitis C virus among hard-to-reach populations.

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INTRODUCTION

Viral hepatitis is an important public health challenge with an estimated 257 million people living with chronic hepatitis B virus (HBV) and 71 million people living with chronic hepatitis C virus (HCV) worldwide[1,2]. HBV and HCV infection have a heterogeneous distribution in Latin America, where 7-12 million people have been infected with HBV and less than 2% are infected with HCV[3].

Some groups may be exposed more frequently to HBV and HCV infection mainly due to repeated exposure to contaminated blood that may occur during transfusions,

and hepatology

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hospitalizations, surgeries as well as other invasive procedures (including the management of chronic kidney disease (CKD) *via* hemodialysis) and last but not least coagulopathy individuals. In these groups, HBsAg prevalence varies from 3.9% to 7.0% and anti-HCV prevalence from 12.6% to 47.0%[4-8]. Another group at-risk for acquiring HBV and HCV is composed of people living with human immunodeficiency virus (HIV), as those viruses share common modes of transmission, such as sexual and parenteral transmission. Among people living with HIV, HBV prevalence varies from 2.8% to 10.3%, while HCV prevalence varies from 4.6% to 6.4%[3,7,9,10].

Diagnosis of infections with these viruses can be difficult in these at-risk groups, such as CKD individuals undergoing hemodialysis, coagulopathy individuals and people living with HIV, due to the difficulty of blood sample collection by venipuncture, their remote location and lack of health care.

In these real-life situations, biosecurity is an ever-present problem. In addition to difficulties affecting proper storage and transport of materials and samples, trained personnel are usually absent or scarce. Dried blood spot (DBS) samples could be a key alternative to serum obtained by venipuncture, which would increase access to diagnosis. These samples are easily collected using finger puncture and can be transported and stored at room temperature. Some studies have demonstrated the detection of HBV and HCV markers using DBS along with enzyme-linked immunosorbent assay in several groups, including monoinfected hepatitis patients and those coinfected with HIV[11-13].

Most studies aiming to detect hepatitis markers in DBS have employed enzyme immunoassays, but recently several laboratories have replaced manual or semimanual enzyme-linked immunosorbent assay with electrochemiluminescence (ECLIA). This technique is highly accurate, presents a low detection limit and delivers results quickly[14]. ECLIA has been used for detecting HBsAg and anti-HCV in DBS samples in monoinfected individuals with high sensitivity and specificity[15]. However, there is no information regarding the performance of ECLIA for the detection of HBV and HCV markers in DBS samples in key populations, such as individuals with coagulopathies, CKD patients and people living with HIV.

The main objective of this study was to investigate the putative influence of HIV infection as well as pathophysiological alterations in individuals with coagulopathies (hemophilia and von Willebrand disease) or CKD, vis-à-vis the performance of optimized ECLIA for the detection of HBsAg, anti-HBc and anti-HCV markers in DBS samples. This study also aimed to provide new data on the prevalence of these markers using DBS coupled with ECLIA.

MATERIALS AND METHODS

Study design and population

A cross-sectional study was conducted in different macro-regions of the country from June 2014 to March 2017. Basic sociodemographic data were collected using a standard questionnaire. While analyzing at-risk populations in Brazil presents inherent limitations in the sample-frames available, this study aimed to create a panel that was as broad as possible to maximize the use of individuals and samples obtained under the protocol.

Convenience samples include those as follows: Coagulopathy individuals, CKD patients and people living with HIV. Potential participants were recruited from hemodialysis clinics. Among them, coagulopathy individuals under follow-up in referral clinics as well as patients from HIV/AIDS services located in the northeastern and southeastern regions of Brazil were recruited.

These geographical domains correspond to an involuntary but insurmountable limitation. Although there are some data on the southernmost regions of Brazil from other research groups, data on the far north and central west locations represent a challenge in terms of budgetary constraints and logistics. To send research teams to such locations and to transport biological samples over such huge distances requires a sustained effort and costs comparable to travel across the whole territory of western Europe. Furthermore in Brazil, personnel (both technicians and ancillary personnel) and sample transportation remain a challenge due to a fractured and sometimes nonexistent aerial and terrestrial network.

Inclusion criteria for the selection of participants were as follows: Individuals of both sexes, aged 18 years or older, attending the healthcare centers involved in this study for their different medical conditions.

Three groups were included in this study: (1) Individuals with coagulopathies (hemophilia and von Willebrand disease) recruited from the coagulopathy outpatient clinic of the public Hematology and Hemotherapy Center of Ceará (Hemoce), located in Fortaleza city; (2) CKD patients on hemodialysis recruited from three private nephrology clinics that receive individuals from the public and private healthcare systems located in the states of Ceará and Rio de Janeiro; and (3) People living with HIV referred to the viral hepatitis ambulatory clinic (FIOCRUZ, RJ) from the gastroenterology outpatient clinic of the Gaffrée and Guinle Hospital (UNIRIO, RJ) infectious disease unit at Nova Iguaçu Hospital and the infectious disease outpatient clinic at the Clementino Fraga Filho Hospital (UFRJ).

Demographic characteristics and risk factors such as behavior, age, gender, marital status and education were defined using categories in the Brazilian Census and major national household surveys (*e.g.*, PNAD). All patients enrolled read and signed the informed consent form. The FIOCRUZ Ethics Committee approved this study (CAAE No. 34049514.7.3006.5258 e 34049514.7.3009.5051).

Laboratory tests

Paired serum and DBS samples were obtained by venipuncture. Whole blood (6 mL) was collected from each patient and 75 μL of this was applied to a 12 mm, preprinted circular disc on Whatman 903 protein protective card (Whatman, GE Healthcare, NJ, United States). To elute DBS samples, the 12 mm disc of filter paper was cut and transferred to a microtube containing 500 μL of 0.5% PBS/BSA for 18 h to 24 h[15]. The analysis of the serum samples was the gold standard for the detection of HBsAg, anti-HBc and anti-HCV. Serological markers were detected using a commercial ECLIA technique (Cobas E411, Roche, United States).

ECLIA in DBS samples

The ECLIA technique was used for the evaluation of HBsAg, anti-HBc and anti-HCV in DBS samples (Elecsys anti-HCV II, Elecsys HBsAg II and Elecsys anti-HBc II - Roche Diagnostics) following the manufacturer's instructions. In the anti-HCV and HBsAg assay, samples with sample/cutoff values < 1.0 were considered nonreactive, whereas for the anti-HBc assay, non-reactive samples should have an sample/cutoff value of > 1.0.

Statistical analysis

Absolute and estimated infection frequencies were calculated as well as mean and standard deviation of the patients' sociodemographic and clinical characteristics. For the association study, populations and markers were analyzed using the Chi-square test for homogeneity with a P value of 0.05. Variables with a proportion of missing values greater than 10.0% for each diagnostic test were excluded from the analysis. Unadjusted odds ratios (ORs) and respective 95% confidence intervals (95%CIs) were calculated for sociodemographic, behavioral and clinical variables as well as for each one of the diagnostic tests/seromarkers.

Associations were further analyzed using multiple logistic regression. Nonreactive samples were taken as the reference categories to which all other categories were cross-compared, yielding adjusted ORs and respective CIs. Only variables with statistical association at the level of 20% were entered into the multivariate models using a forward stepwise procedure. Maximum likelihood and the Wald test were used to assess the parsimony and fitness of intermediate models contemplating the exclusion or inclusion of different variables. Intermediate models were evaluated using the Hosmer-Lemeshow goodness-of-fit test using a 95%CI.

Taking ECLIA as the gold standard method for the sake of our analysis, sensitivity, specificity and positive and negative predictive values as well as accuracy were calculated for each biological outcome.

RESULTS

CKD patients

Among CKD patients (n = 284), HBsAg, anti-HBc and anti-HCV were detected in serum in 4.6%, 39.9% and 16.3% of individuals, respectively and were detected by DBS in 4.9%, 33.6% and 15.9% of individuals, respectively. Table 1 shows the sociodemographic and clinical characteristics of this population.

Table 1 Main sociodemographic and clinical characteristics of chronic kidney disease individuals, people living with human immunodeficiency virus and coagulopathy individuals

Variable		I_CKD	P_HIV	I_COAG
		n (%)	*n* (%)	*n* (%)
Gender	Female	101 (37.3)	37 (38.9)	1 (2.0)
	Male	170 (62.7)	58 (61.1)	50 (98.0)
Age	18-30	19 (7.0)	9 (9.5)	25 (50.0)
	30+	253 (93.0)	86 (90.5)	25 (50.0)
Marital status	Married	132 (49.3)	31 (35.2)	17 (33.3)
	Not married	136 (50.7)	57 (64.8)	34 (66.7)
Race	White	71 (27.6)	29 (46.0)	14 (29.2)
	Black	186 (72.4)	34 (54.0)	34 (70.8)
Length of education	Up to 8 yr	136 (51.1)	36 (38.7)	14 (27.5)
	9 or more	130 (48.9)	57 (61.3)	37 (72.5)
Acupuncture	Yes	21 (7.8)	9 (9.5)	15 (29.4)
	No	247 (92.2)	86 (90.5)	36 (70.6)
Tattoo	Yes	27 (10.1)	29 (31.2)	6 (12.0)
	No	240 (89.9)	64 (68.8)	44 (88.0)
Piercing	Yes	9 (3.4)	7 (7.5)	2 (4.1)
	No	259 (96.6)	86 (92.5)	47 (95.9)
Shared nail cutters/razor/toothbrush	Yes	190 (66.9)	70 (74.5)	22 (43.1)
	No	94 (33.1)	24 (25.5)	29 (56.9)
Blood or plasma transfusion	Yes	169 (63.3)	16 (17.2)	35 (70.0)
	No	98 (36.7)	77 (82.8)	15 (30.0)
Transfusion before 1994	Yes	34 (12.8)	7 (7.5)	25 (49.0)
	No	231 (87.2)	86 (92.5)	26 (51.0)
HBV vaccine	Yes	206 (72.5)	41 (43.2)	36 (70.6)
	No	78 (27.5)	54 (56.8)	15 (29.4)
Use of illicit drugs	Yes	18 (6.9)	22 (23.9)	6 (12.2)
	No	244 (93.1)	70 (76.1)	43 (87.8)
History of STI	Yes	61 (23.0)	44 (51.8)	10 (20.0)
	No	204 (77.0)	41 (48.2)	40 (80.0)
Alcohol consumption	Yes	48 (18.0)	31 (41.3)	30 (60.0)
	No	219 (82.0)	44 (58.7)	20 (40.0)
Condom use	Frequent	66 (25.6)	57 (64.0)	18 (39.1)
	Infrequent	192 (74.4)	32 (36.0)	28 (60.9)
Hemodialysis per week	3 times	236 (89.4)	-	-
	4 times or more	28 (10.6)	-	-
Hemodialysis time (mo)		76.1 (80.1)	-	-
Coagulopathy	Hemophilia	-	-	47 (92.2)
	von Willebrand 3	-	-	4 (7.8)
Type of hemophilia	Deficiency factor VIII	-	-	39 (84.8)
	Factor IV deficiency	-	-	7 (15.2)

Severity	Mild/moderate	-	-	13 (28.3)
	Serious	-	-	33 (71.7)
Inhibitory antibodies	Present	-	-	5 (11.4)
	Absent	-	-	39 (88.6)

I_CKD: Chronic kidney disease individuals; HBV: Hepatitis B virus; I_COAG: Coagulopathy individuals; P_HIV: People living with human immunodeficiency virus; STI: Sexually transmitted infection.

Most CKD patients were male (62.7%), over 30-years-old (93.0%), black (72.4%) and had up to 8 years of education (51.1%). The most risk behaviors were: Shared nail cutters/razors/toothbrushes (66.9%), previous transfusion of plasma or blood (63.3%), inconsistent use of condoms (74.4%) and the use of hemodialysis up to 3 times a week (89.5%).

HBV and HCV serological markers in DBS and serum were evaluated according to demographic and clinical data. Only statistically significant data are presented in Table 2. Infrequent use of condoms was associated with HBsAg positivity in serum and DBS (OR = 5.6 for serum and 4.4 for DBS). Sharing nail cutters/razors/toothbrushes was associated with anti-HBc positivity in serum and DBS (OR = 2.7 for serum and 2.6 for DBS). On the other hand, acupuncture and hemodialysis exposure was associated with anti-HBc detection in serum and a history of transplantation in DBS. Anti-HCV positivity was associated with a history of transplantation (OR = 2.8 for serum and DBS) and hemodialysis exposure (OR = 1.01 for both specimens).

People living with HIV

Among people living with HIV (n = 95), the mean age was 44.1 ± 11.4 years. Most individuals were male (61.1%), unmarried (64.8%), over 30-years-old (90.4%) and sharing nail cutters/razors/toothbrushes (74.5%) (Table 1). The prevalence of $HBsAg^+$ in serum/DBS was 21.0%/22.1%, of anti-HBc^+ was 40.0%/45.6% and anti-of HCV^+ was 25.5%/25.5%.

Table 3 shows the factors associated with the detection of HBV and HCV serological markers using DBS and serum in this group. Male gender (OR = 4.9) and blood transfusion (OR = 4.6) were associated with HBsAg reactivity in serum, while male gender was associated with anti-HBc positivity in serum (OR = 3.2) and DBS (OR = 2.9). No variable was associated with anti-HCV in this group.

Individuals with coagulopathy

Among coagulopathy patients (n = 51), the mean age was 31.3 ± 9.4 years and the main characteristics were: Male gender (98.0%), unmarried (66.7%), black (70.8%), had undergone blood or plasma transfusion (70.0%) and had severe hemophilia (71.7%) (Table 1).

The prevalence for each seromarker in serum was 3.9% for HBsAg, 31.4% for anti-HBc and 47.1% for anti-HCV. The prevalence for each seromarker from DBS was 3.9% for HBsAg, 25.5% for anti-HBc and 41.2% for anti-HCV. It was not possible to make a statistical analysis of this group due to the small size of the sample population.

Performance of ECLIA for HBV and HCV detection using DBS samples in high-risk groups

Among coagulopathy patients, HBsAg assay demonstrated the best performance (100% sensitivity and specificity) followed by anti-HBc (81.3% sensitivity and 100% specificity) and anti-HCV (83.3% sensitivity and 96.3% specificity). Among CKD patients, the best performance was observed for HBsAg (100% sensitivity and 99.6% specificity) followed by anti-HCV (93.5% sensitivity and 99.2% specificity) and anti-HBc (79.6% sensitivity and 97.1% specificity). Among people living with HIV, the best performance was observed for anti-HCV (100% sensitivity and specificity) followed by anti-HBc (97.2% sensitivity and 88.9% specificity) and HBsAg (85.0% sensitivity and 94.7% specificity).

Accuracy varied from 90.2% to 100% and incorrect classification was below 10% in all markers. Estimated prevalence varied between serum and DBS in coagulopathy patients, and CKD individuals showed low values of prevalence using DBS for anti-HBc and anti-HCV. In people living with HIV, estimated prevalence for HBsAg and anti-HBc were higher using DBS (Table 4).

Table 2 Bivariate analysis of sociodemographic and clinical characteristics according to hepatitis B virus and hepatitis C virus markers in chronic kidney disease individuals

Variable	Adjustment	HBsAg		Anti-HBc		Anti-HCV	
		DBS	Serum	DBS	Serum	DBS	Serum
Acupuncture	OR crude (95%CI)	-	-	-	4.0 (1.5-10.6)	-	-
	OR adjusted (95%CI)	-	-	-	5.1 (1.8-14.5)	-	-
Shared nail cutters/razor /toothbrush	OR crude (95%CI)	-	-	2.6 (1.4-4.6)	1.9 (1.1-3.2)	-	-
	OR adjusted (95%CI)	-	-	2.7 (1.5-4.8)	2.6 (1.5-4.7)	-	-
History of transplant	OR crude (95%CI)	-	-	2.9 (1.3-6.4)	-	5.8 (2.5-13.6)	5.8 (2.5-13.6)
	OR adjusted (95%CI)	-	-	2.7 (1.2-6.1)	-	2.8 (1.1-7.4)	2.8 (1.1-7.7)
Infrequent condom use	OR crude (95%CI)	5.6 (1.6-16.4)	4.4 (1.4-14.5)	-	-	-	-
	OR adjusted (95%CI)	5.6 (1.6-16.4)	4.4 (1.4-14.5)	-	-	-	-
Hemodialysis time (mo)	OR crude (95%CI)	-	-	-	1.01 (1.01-1.01)	1.01 (1.01-1.02)	1.01 (1.01-1.01)
	OR adjusted (95%CI)	-	-	-	1.01 (1.01-1.01)	1.01 (1.01-1.02)	1.01 (1.01-1.02)
Hemodialysis 4 times per week or more	OR crude (95%CI)	-	-	-	-	-	2.8 (1.1-6.9)
	OR adjusted (95%CI)	-	-	-	-	-	2.7 (1.1-7.4)

CI: Confidence interval; DBS: Dried blood spot; HBc: Hepatitis B core; HCV: Hepatitis C virus; OR: Odds ratio.

Table 3 Bivariate analysis of sociodemographic and clinical characteristics according to hepatitis B virus and hepatitis C virus markers in people living with human immunodeficiency virus

Variable	Adjustment	HBsAg		Anti-HBc		Anti-HCV	
		DBS	Serum	DBS	Serum	DBS	Serum
Male gender	OR crude (95%CI)	-	4.7 (1.3-17.4)	3.2 (1.3-7.8)	2.9 (1.1-7.3)	-	-
	OR adjusted (95%CI)	-	4.9 (1.2-19.2)	3.2 (1.3-7.8)	2.9 (1.1-7.3)	-	-
Blood or plasma transfusion	OR crude (95%CI)	-	4.2 (1.3-13.5)	-	-	-	-
	OR adjusted (95%CI)	-	4.6 (1.3-16.0)	-	-	-	-

CI: Confidence interval; DBS: Dried blood spot; HBc: Hepatitis B core; HCV: Hepatitis C virus; OR: Odds ratio.

DISCUSSION

To date, there are several studies reporting the importance of diagnosing hepatitis B and C in DBS samples[16-18]. However, the majority have focused only on HBsAg[13,16] and anti-HCV[11] along with manual assays. In the present study, an automated assay was evaluated for the detection of HBsAg, anti-HBc and anti-HCV in DBS samples from key populations demonstrating high sensitivities and specificities comparable to those observed in the general population[15]. These findings reinforce the importance of using DBS samples to reach these key populations in the diagnosis of viral hepatitis, which can be further facilitated using ECLIA.

Among CKD patients, HBsAg positivity in DBS or serum was associated with infrequent condom use, which was also found among young men enlisted in the Brazilian Army, demonstrating the importance of health campaigns with a focus on condom use[19]. Anti-HBc positivity in serum and DBS was associated with shared nail cutters/razor/toothbrush and highlights the discussion of the role of manicurists in the transmission of HBV. Villar *et al*[20] found a prevalence of 5.9% of anti-HBc in beauty professionals in southeast Brazil.

Anti-HCV positivity in serum and DBS was associated with a previous history of transplantation in CKD patients. A study that assessed the risk of transplant recipient infections showed that this will depend on the prevalence and incidence of HCV in a given population of donors and other risk exposures such as injecting drug use, men

Table 4 Test parameter values according to individuals with coagulopathies, chronic kidney disease and people living with human immunodeficiency virus

Diagnostic test parameters	I_COAG, *n* = 51			I_CKD, *n* = 284			P_HIV, *n* = 95		
	HBsAg	Anti-HBc	Anti-HCV	HBsAg	Anti-HBc	Anti-HCV	HBsAg	Anti-HBc	Anti-HCV
True positive (*n*)	2	13	20	13	90	43	17	35	24
True negative (*n*)	49	35	26	270	165	235	71	48	70
False positive (*n*)	0	0	1	1	5	2	4	6	0
False negative (*n*)	0	3	4	0	23	3	3	1	0
Sensitivity (%)	100	81.3	83.3	100	79.6	93.5	85.0	97.2	100
Specificity (%)	100	100	96.3	99.6	97.1	99.2	94.7	88.9	100
PPV (%)	100	100	95.2	92.9	94.7	95.6	81.0	85.4	100
NPV (%)	100	92.1	86.7	100	87.8	98.7	95.9	98.0	100
Correct classification (accuracy) (%)	100	94.1	90.2	99.6	90.1	98.2	92.6	92.2	100
Incorrect classification (%)	0	5.9	9.8	0.4	9.9	1.8	7.4	7.8	0
Estimated prevalence/serum (%)	3.9	31.4	47.1	4.6	39.9	16.3	21.1	40.0	25.5
Estimated prevalence/DBS (%)	3.9	25.5	41.2	4.9	33.6	15.9	22.1	45.6	25.5

DBS: Dried blood spot; HBc: Hepatitis B core; HCV: Hepatitis C virus; I_CKD: Chronic kidney disease individuals; I_COAG: Coagulopathy individuals; NPV: Negative predictive value; P_HIV: People living with human immunodeficiency virus; PPV: Positive predictive value.

who have sex with men, piercings and tattoos. These, among other risk factors, are already associated with transmission for the general population[21].

Among people living with HIV, HBsAg positivity was associated with male gender and blood and plasma transfusion using serum results, and anti-HBc positivity was associated with male gender using the results of both fluids. In Brazil, most HBV infected individuals were male (54.5%)[22], probably due to higher exposure to risk factors, such as promiscuity and drug use[23,24]. Although blood is screened for HBsAg and anti-HBc in blood banks in Brazil, molecular assays were only included in 2015. While rare, occult hepatitis B infection, mutations that escape vaccination and infected individuals occupying a certain immunological window could be potential donors of contaminated blood samples allowing HBV transmission[25,26].

DBS testing for HBsAg, anti-HBc and anti-HCV using ECLIA demonstrated high sensitivity and specificity in all groups. HBsAg testing demonstrated the best performance in coagulopathy individuals and CKD patients. Anti-HCV testing demonstrated higher efficiency in CKD individuals and people living with HIV and anti-HBc detection was more accurate in people living with HIV. The differences observed could be the result of different prevalences and risk behavior, such as multiple exposure to blood products that could interfere in the efficiency of the assay.

HBsAg and anti-HCV prevalence estimated by serum and DBS were similar in demonstrating that ECLIA along with DBS could be a potential tool for diagnosis of infected individuals in key populations. In contrast, anti-HBc prevalence varied by more than 5% between serum and DBS in all groups evaluated. In the present study, anti-HBc sensitivity varies from 79.6% to 97.2%, which is similar to findings in other studies that reported sensitivities from 76.9% to 97.6% using ECLIA or enzyme-linked immunosorbent assay for anti-HBc in the general population and people living with HIV[12,13,16]. Although there are differences found in anti-HBc prevalence in serum and DBS, there is an overlapping CI value for those specimens showing that DBS could be used for prevalence studies in key populations.

CONCLUSION

This study demonstrated the utility of HBsAg, anti-HBc and anti-HCV detection in DBS using ECLIA in high-risk populations. The use of DBS samples is much less

invasive, easier than venipuncture and could increase the access of diagnosis in people with limited social access as well as in people where it is difficult to draw blood. Automated assays such as ECLIA using DBS increases diagnostic speed, generating the diagnosis of many samples at once, which can be important during potential outbreaks in hemotherapy clinics for example. However, the anti-HBc marker should be used with due care, especially in the population of coagulopathy individuals and CKD patients, which due to multiple exposures may not show agreement with gold standard samples and therefore requires further study.

ARTICLE HIGHLIGHTS

Research background

Diagnosis of hepatitis B virus and hepatitis C virus (HCV) can be difficult in chronic kidney disease (CKD) individuals undergoing hemodialysis, coagulopathy individuals and people living with human immunodeficiency virus (HIV) due to the difficulty of blood sample collection by venipuncture, remote location and lack of health care.

Research motivation

There is no information regarding the performance of electrochemiluminescence (ECLIA) for the detection of hepatitis B virus and HCV markers in dried blood spot (DBS) samples in key populations, such as individuals with coagulopathies, CKD patients and people living with HIV.

Research objectives

To investigate the putative influence of HIV infection as well as pathophysiological alterations in individuals with coagulopathies (hemophilia and von Willebrand disease) or CKD in the performance of optimized ECLIA for the detection of HBsAg, anti-HBc and anti-HCV markers in DBS samples.

Research methods

The ECLIA technique was used for the evaluation of HBsAg, anti-HBc, and anti-HCV tests in DBS samples of CKD individuals undergoing hemodialysis, coagulopathy individuals and people living with HIV.

Research results

HBsAg detection presented sensitivities of 100% among coagulopathy and CKD patients and low sensitivity (85.0%) in people living with HIV. Anti-HBc detection had the best performance in people living with HIV followed by coagulopathy and CKD patients. Anti-HCV detection showed sensitivities above 83.0% in all groups. Specificities of these assays varied from 88.9% to 100%. Estimated prevalence was similar among serum and DBS except for the anti-HBc marker.

Research conclusions

This study demonstrated the utility of HBsAg, anti-HBc and anti-HCV detection in DBS using ECLIA in high-risk populations.

Research perspectives

Automated assays such as ECLIA using DBS increases diagnostic speed, generating the diagnosis of many samples at once, which can be important during potential outbreaks in hemotherapy clinics.

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CASE REPORT

Asymptomatic portal vein aneurysm: Three case reports

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Author contributions: De Sio I, Vitale LM and Niosi M performed the ultrasonography and interpreted the imaging findings; Priadko K reviewed the literature and drafted the manuscript; De Sio I contributed to manuscript drafting; Romano M was responsible for the revision and final approval of the manuscript; all authors issued final approval for the version to be submitted.

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Abstract

BACKGROUND

Portal vein aneurysm (PVA) is an uncommon vascular dilatation, showing no clear trend in sex or age predominance. Due to the low number of published cases and the lack of management guidelines, treatment of this condition remains a clinical challenge.

CASE SUMMARY

We present three cases of asymptomatic PVA; the first and second involve an extrahepatic manifestation, of 48 mm and 42.3 mm diameter respectively, and the third involves an intrahepatic PVA of 27 mm. All were diagnosed incidentally during routine check-up, upon ultrasonography scan. Since all patients were asymptomatic, a conservative treatment strategy was chosen. Follow-up imaging demonstrated no progression in the aneurysm dimension for any case.

CONCLUSION

As PVA remains asymptomatic in many cases, recognition of its imaging features is key to favourable outcomes.

Key Words: Extrahepatic portal vein aneurysm; Intrahepatic portal vein aneurysm; Asymptomatic; Ultrasonography imaging; Colour Doppler; Case report

Core Tip: Portal vein aneurysm (PVA) can be a congenital or acquired vascular malformation but in most cases is asymptomatic; as such, it remains underdiagnosed. We report on the features of PVA detected by ultrasonography, computed tomography and magnetic resonance imaging in three asymptomatic patients. Only one of our patients had a known predisposing factor (*i.e.*, liver cirrhosis). Throughout the

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surveillance period, our patients remained asymptomatic, with no dimensional changes in their PVAs. In reporting this case study, we highlight the need for PVA recognition and instituting a personalized management approach that takes into consideration factors predisposing to complications of this condition.

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INTRODUCTION

Portal vein aneurysm (PVA) is a vascular malformation that is rarely diagnosed but, if complications occur, can be life-threatening. The estimated number of reported cases is low, at approximately 200[1]. PVA is defined as a portal vein diameter exceeding 19 mm in cirrhotic patients and 15 mm in normal liver[1] and can be either congenital (due to vascular anomalies) or acquired (mostly due to cirrhosis and/or portal hypertension, that are present in approximately 28.0%-30.8% of cases)[2,3]. Several systematic reviews did not identify any sex-related predisposition[1,2]. Notably, among portal venous system aneurysms, those in the main extrahepatic portal vein appear to be the commonest[2]. The average mortality rate is 10% and this mostly involves patients who have undergone liver transplantation[2,4]. Incidental discovery of asymptomatic aneurysms normally occurs through abdominal imaging, such as with computed tomography (CT), magnetic resonance imaging (MRI), or ultrasonography.

The clinical management of PVA ranges from conservative follow-up, that lasts years, to surgical intervention, depending on the presence or absence of symptoms and complications, such as rupture, thrombosis and compression of adjacent organs[1,2].

In this article, we present clinical cases of three asymptomatic patients in whom PVA was an incidental finding. The clinical cases are accompanied by ultrasonography, CT and MRI images of asymptomatic extrahepatic PVAs and ultrasonographic images of intrahepatic PVA. We also provide a review of the relevant literature to advance the knowledge on this underdiagnosed condition.

CASE PRESENTATION

Chief complaints

Three patients, 81-year-old male, 52-year-old female and 73-year-old male respectively, presented to the outpatient clinic of our Unit of Diagnostic and Interventional Ultrasonography (Medical Center of the University Vanvitelli in Naples, Italy) for routine check-up for various pre-existing health issues.

History of past illness

Case 1: The 81-year-old patient's medical history included hepatitis B virus-associated well-compensated (*i.e.*, Child-Pugh classification stage A5) liver cirrhosis with portal hypertension and F1 oesophageal varices.

Case 2: Patient 2 had a previous history of dysmotility-like dyspepsia, for which the routine abdominal ultrasonography had been requested.

Case 3: Patient 3 was recovering from sepsis caused by infection of an aortal prosthesis and had no history of past illnesses relevant to the subsequent PVA finding.

Physical examination

Physical examination did not reveal any relevant signs in any of the patients.

Laboratory examinations

Blood testing of Case 2 affected by liver cirrhosis showed leucopenia, thrombocytopenia, and increased level of gamma globulins (2.3 g/dL; normal range: 0.7-1.6

g/dL) while blood testing of the other two patients yielded no abnormal findings.

Imaging examinations

Case 1: Ultrasound examination of patient 1 showed an extrahepatic aneurysmal dilatation of the portal vein (Figure 1A), with a maximal diameter of 48 mm. Colour Doppler examination showed the lesion to have the typical "Korean flag" appearance (Figure 1B), and a Doppler recording revealed flat venous flow (Figure 1C).

Case 2: Ultrasonographic examination of patient 2 detected extrahepatic aneurysmal dilatation of the portal vein (Figure 2A), with a maximal diameter of 42.3 mm (Figure 2B). Colour Doppler control examination showed a hepatopetal venous flow (Figure 2C) and a pulsating flow of venous type (Figure 2D). Considering the young age of the patient, second-level imaging techniques were performed. Abdominal CT (Figure 3) as well as contrast-enhanced MRI (Figure 4) confirmed the diagnosis of extrahepatic aneurysmal dilatation of portal vein.

Case 3: Abdominal ultrasonography of patient 3 showed an aneurysmal dilatation of the right branch of the portal vein (Figure 5A), with a maximal diameter of 27 mm (Figure 5B) and a typical "Korean flag" appearance (Figure 5C). No further diagnostic procedures were considered necessary.

FINAL DIAGNOSIS

Cases 1 and 2 were diagnosed with an acquired asymptomatic extrahepatic PVA while Case 3 was diagnosed with an acquired asymptomatic intrahepatic PVA.

TREATMENT

Due to lack of symptoms, ultrasonography surveillance every 6 mo was recommended. No specific treatment was prescribed.

OUTCOME AND FOLLOW-UP

For the 3-, 5- and 1-year of follow-up respectively, all the patients remained asymptomatic and no changes had been detected in the aneurysm measures.

DISCUSSION

PVA is a saccular or fusiform portal vein dilatation that was first described in 1956[5]. The commonest classifications divide PVAs into congenital or acquired, symptomatic or asymptomatic, and complicated or uncomplicated[2,6]. To date, the PVA reports in the literature are relatively scarce. A systematic review of 96 reports by Laurenzi *et al*[1] showed that the median age at diagnosis among 190 subjects was 52-year-old (0-89) with portal hypertension and liver cirrhosis discovered in 62 (32%) and 50 (26%) patients respectively with males and females being equally affected. Interestingly, the more recent studies describing PVA cases have shown weaker associations of the condition with chronic liver diseases or portal hypertension[7,8]. This is probably due to implementation and advancement of imaging techniques and of specific knowledge of specialists in the field. While chronic liver diseases remain the commonest acquired causal factors of PVA, other acquired cases are considered to originate from malignant invasion of the vein, inflammatory process due to pancreatitis, or trauma[6]. Most commonly, symptoms occur in patients with large extrahepatic aneurysmal dilatations while small aneurysms often remain asymptomatic[8,9]. Once thrombosed, PVA causes symptoms such as abdominal pain in 91%, fever in 53% and ascites in 38% of patients[10]. Authors noted that in symptomatic patients with or without portal hypertension, symptoms do not differ, except for gastrointestinal bleeding in patients suffering from elevated pressure in portal vein[1]. Unfortunately, no clear evidence exists helping to prospectively distinguish between aneurysms which will have a stable course *vs* those that are potentially complicated but it seems that unfavourable precursors of symptomatic and/or complicated disease are large size (> 3 cm), liver

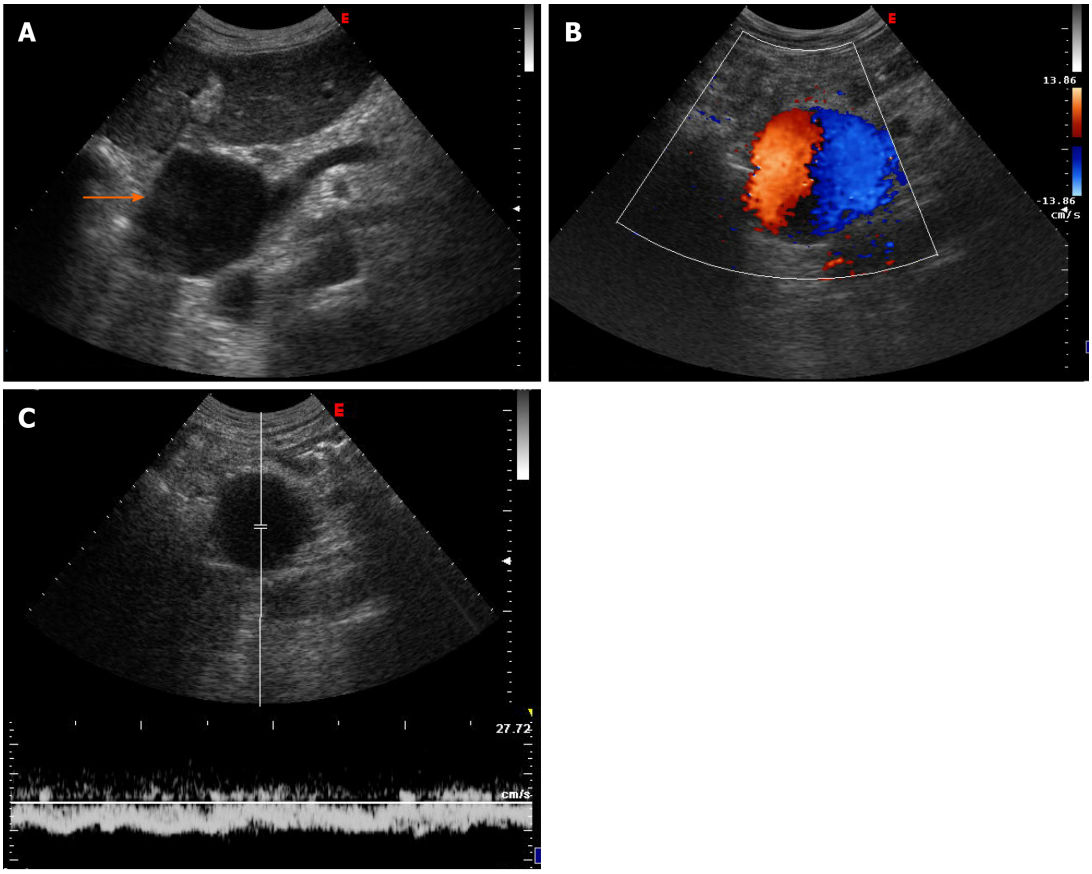

Figure 1 Abdominal ultrasonographic imaging of Case 1. A: Anechoic lesion corresponding to a notably dilated extrahepatic portal vein (arrow); B: Colour Doppler showed the "Korean flag" pathological sign in the dilated portal vein; C: Doppler recording showed flat venous flow.

and/or pancreatic diseases and thrombophilic risks. Koc *et al*[11] reported an incidence of thrombophilia in 4 out of 7 patients with thrombosed PVA, hence, pointing on an importance of thrombophilic screening in all the subjects with diagnosed PVA, even if asymptomatic at the beginning. Even though 18 cases of non-thrombosed PVAs exceeding 5 cm in their largest diameter were reported in the literature, with no anticoagulation taken before their diagnosis, many authors support a thrombophilic risk assessment[12]. While all of our patients were asymptomatic, only one (*i.e.*, the 81-year-old male) had predisposing factors to the formation of the portal aneurysm, namely hepatitis B virus-associated liver cirrhosis complicated with portal hypertension and oesophageal varices. A thorough examination of the other two patients did not reveal any predisposing risk factors. None of our patients had complications at the time of the first visit nor during the follow-up period. This is at odds with previous reports showing that abdominal pain occurs in approximately 50% of patients and upper gastrointestinal bleeding in less than 10%[2].

In general, complications of PVA vary depending on the location of the aneurysm and predisposing factors and include aneurysm thrombosis, rupture of the aneurysm, and compression of adjacent anatomical structures, such as the common bile duct, duodenum, or inferior vena cava[1,2]. Hence, complication risk assessment is a crucial management step that could help to avoid life-threatening outcomes of this condition. For patients with no risk factors for complications, a conservative strategy and follow-up surveillance using abdominal ultrasonography can be recommended, for up to 6 years or until resolution of the aneurysm[1,2,13]. While, in some studies, CT scan every 12 mo was the preferred surveillance strategy[14], the majority of published studies agree with ultrasonography being the preferred imaging technique for surveillance and monitoring of PVA growth, as it is relatively inexpensive and does not involve radiation exposure[15,16]. Ma *et al*[17] suggested a surveillance colour Doppler scanning as the method of choice for diagnosis and surveillance of aneurysms that are asymptomatic and do not increase in size over time while CT scan to be reserved for symptomatic lesions or indeterminate abdominal ultrasound scanning results. Moreover, ultrasonography is capable of differentiating a PVA from a hypervascular pancreatic mass[16], while contrast-enhanced CT and MRI are helpful in cases of

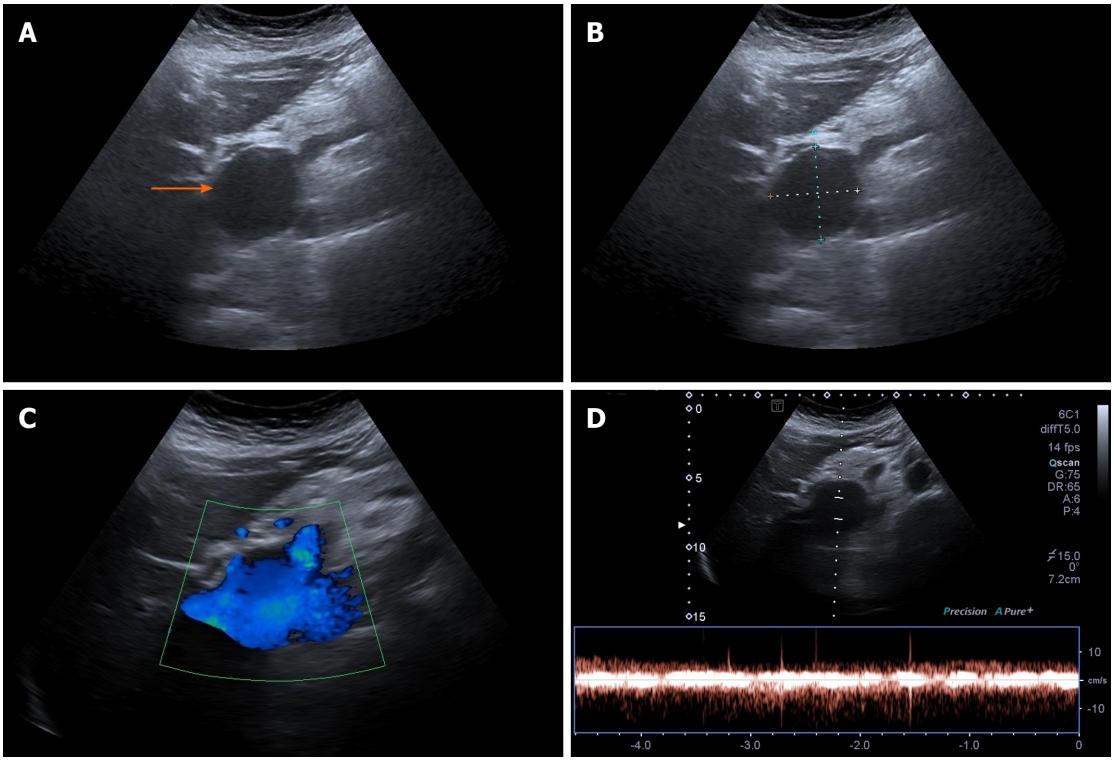

Figure 2 Abdominal ultrasonographic imaging of Case 2. A: Extrahepatic anechoic saccular lesion, indicating an aneurysmal dilation of the extrahepatic portal vein (arrow); B: The anechoic lesion was 42.3 mm at its maximal diameter; C: Colour Doppler showed hepatopetal venous flow in the extrahepatic aneurysmal dilated vessel; D: Doppler recording showed pulsating flow of venous type.

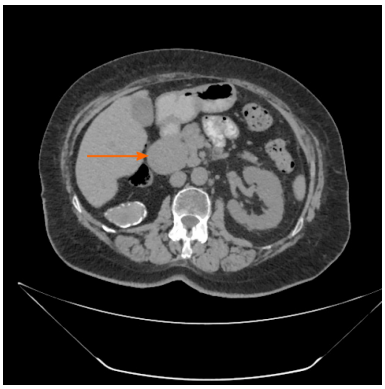

Figure 3 Abdominal computed tomographic scanning of Case 2. The axial image showed saccular extrahepatic aneurysmal dilatation of the portal vein (arrow).

diagnostic uncertainty between thrombosis and slow venous flow[18]. Second-level imaging techniques might also be helpful in differentiating compression of the surrounding viscera or rupture[15,18]. CT or MRI are, however, essential when planning surgical intervention[15,17].

After evaluation of our patients' health status, the conservative management strategy was chosen for each. In the long-term follow-up, none presented any change in aneurysmal dimension. Thus, it was decided to continue regular ultrasonographic examination. This decision was also supposed by studies that have shown partial or total regression of large PVAs over longer periods[13,14].

While the management strategy of large asymptomatic PVAs is still under debate, indications for active management are abdominal pain and occurrence of complications[1,2]. Surgical management depends on the aneurysm location and the presence of thrombi and portal hypertension. Aneurysmorrhaphy and aneurysmectomy are recommended in the absence of portal hypertension, while shunt procedures or liver transplantation are performed in case of portal hypertension[1,4,16]. Thrombolysis or thrombectomy are indicated in case of PVA thrombotic obstruction[2],

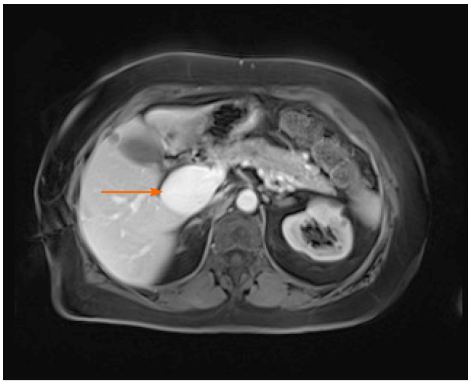

Figure 4 Contrast-enhanced magnetic resonance imaging of the abdomen of Case 2. The axial image showed saccular extrahepatic aneurysmal dilatation of the portal vein (arrow).

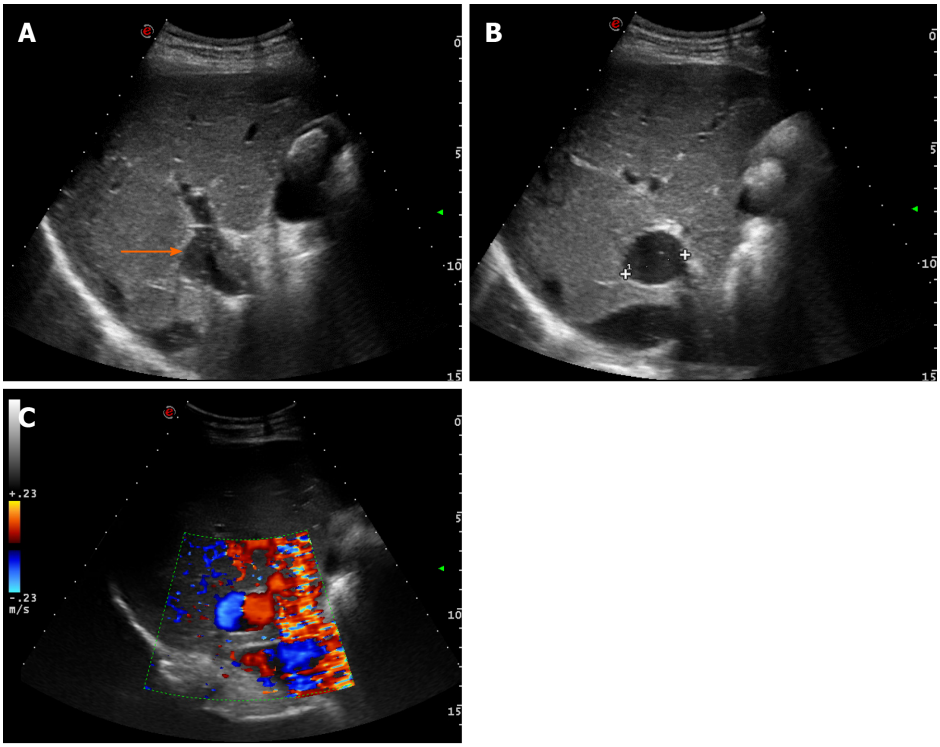

Figure 5 Abdominal ultrasonographic imaging of Case 3. A: Anechoic lesion of the right branch of the portal vein (arrow); B: The intrahepatic anechoic lesion was 27 mm at its maximal diameter; C: Colour Doppler showed the "Korean flag" pathological sign.

even though a case of conservative treatment was reported for a patient with PVA measuring 88 mm × 65 mm and complete thrombosis extending to the superior mesenteric and splenic veins[19].

CONCLUSION

Our cases, together with the review of the literature, support the concept that the management approach to PVA should be individualized, taking into account aneurysm size, complication risks, medical history, and presence of symptoms. Furthermore, our study highlights the need for gastroenterologists and radiologists to be familiar with PVA imaging features and those that facilitate differential diagnosis between PVA and other lesions, such as hypervascular abdominal masses[16].

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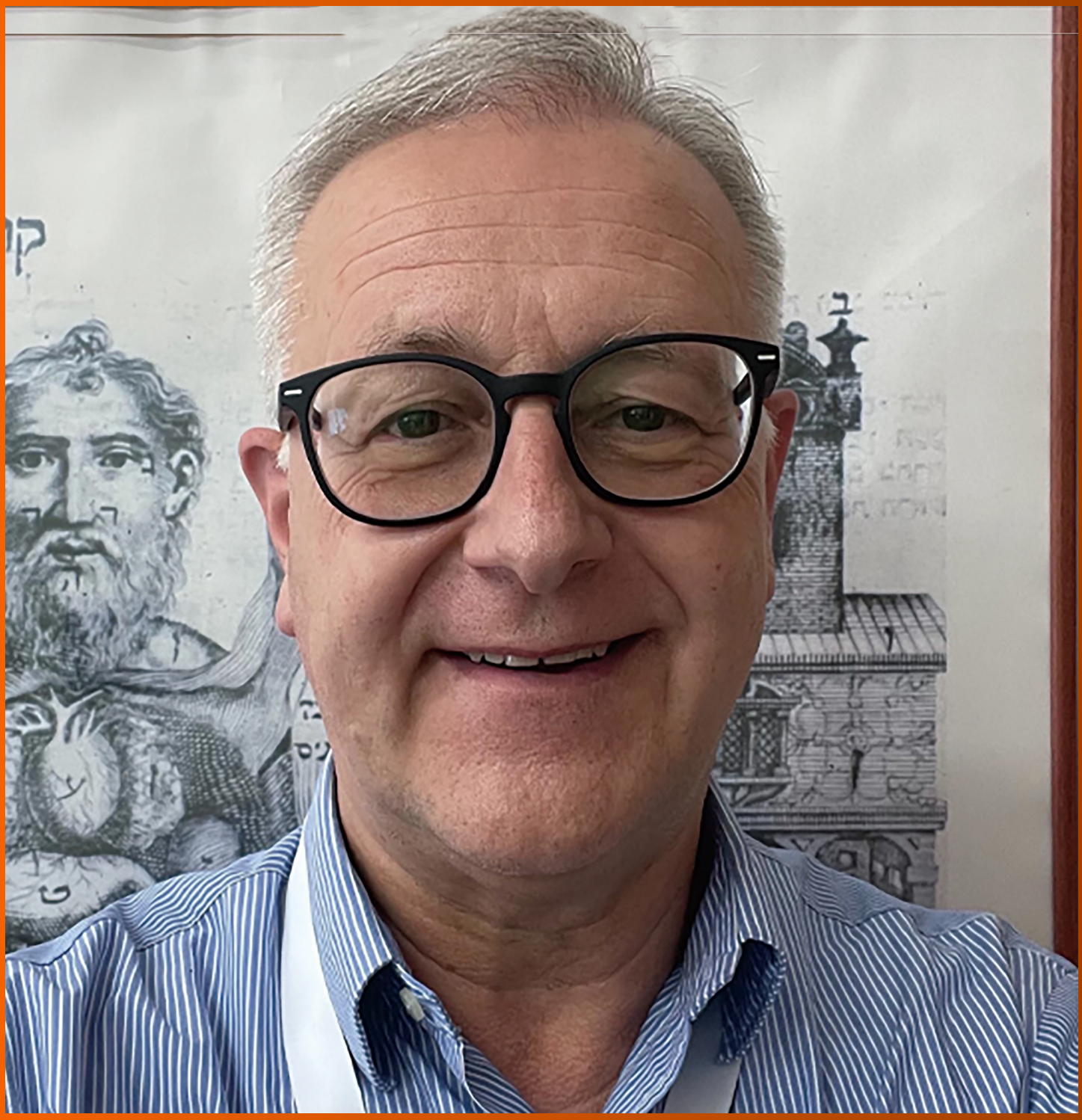

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The primary aim of *World Journal of Hepatology* (*WJH*, *World J Hepatol*) is to provide scholars and readers from various fields of hepatology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

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OPINION REVIEW

COVID-19 and the liver: What do we know so far?

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Abstract

The coronavirus disease 2019 (COVID-19) pandemic has caused unprecedented pressure on public health and healthcare. The pandemic surge and resultant lockdown have affected the standard-of-care of many medical conditions and diseases. The initial uncertainty and fear of cross transmission of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) have changed the routine management of patients with pre-existing liver diseases, hepatocellular carcinoma, and patients either listed for or received a liver transplant. COVID-19 is best described as a multisystem disease caused by SARS-CoV-2, and it can cause acute liver injury or decompensation of the pre-existing liver disease. There has been considerable research on the pathophysiology, infection transmission, and treatment of COVID-19 in the last few months. The pathogenesis of liver involvement in COVID-19 includes viral cytotoxicity, the secondary effect of immune dysregulation, hypoxia resulting from respiratory failure, ischemic damage caused by vascular endotheliitis, congestion because of right heart failure, or drug-induced liver injury. Patients with chronic liver diseases, cirrhosis, and hepatocellular carcinoma are at high risk for severe COVID-19 and mortality. The phase III trials of recently approved vaccines for SARS-CoV-2 did not include enough patients with pre-existing liver diseases and excluded immunocompromised patients or those on immunomodulators. This article reviews the currently published research on the effect of COVID-19 on the liver and the management of patients with pre-existing liver disease, including SARS-CoV-2 vaccines.

Key Words: COVID-19; Chronic liver disease; SARS-CoV-2; Severe acute respiratory syndrome coronavirus; Liver transplant; Liver and SARS-CoV-2 vaccines; SARS-CoV-2 induced liver injury

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Core Tip: Liver involvement in coronavirus disease 2019 (COVID-19) is caused by either viral cytotoxicity or secondary to systemic immune dysregulation. Patients with pre-existing liver disease are at high risk of disease progression, morbidity, and mortality. Chronic liver disease with COVID-19 should be managed as per the standard guidelines, with education on hand hygiene, social distancing, and face masks to reduce hospital admissions. There is no evidence that currently available vaccines for severe acute respiratory syndrome coronavirus 2 will have any complications different from other inactivated vaccines and are recommended for patients with pre-existing liver disease, hepatocellular carcinoma, or liver transplant recipients.

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INTRODUCTION

Coronavirus is an enveloped single-stranded RNA virus belonging to the Coronaviridae family and Orthocoronavirinae subfamily. Severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) respectively caused epidemics in 2003 and 2012. The pandemic of coronavirus disease 2019 (COVID-19) caused by the SARS-CoV-2 was first reported from Wuhan, China on December 31, 2019 in patients with atypical pneumonia[1]. While symptoms are mild in most patients, severe and critical symptoms (in 10%-15% of patients) like hypoxemia (SpO_2 < 94%), acute respiratory distress syndrome, multiorgan failure, or shock; may need hospitalization and respiratory support[2,3]. Older patients, especially those with comorbidities like hypertension, diabetes, chronic liver disease (CLD), and heart disease, are at risk of severe disease and mortality[2,3]. With the rapid spread of COVID-19, there has been significant concern regarding the safe management of patients with pre-existing liver disease (CLD), hepatocellular carcinoma (HCC), and candidates for a liver transplant. This review discusses the current evidence on liver involvement in COVID-19 and its impact on managing patients with CLD, including current recommendations for SARS-CoV-2 vaccines.

LIVER DYSFUNCTION IN COVID-19

Based on the published literature, 14%-53% of patients with COVID-19 developed hepatic dysfunction, and 2%-11% of the patients were reported to have underlying CLD[4-9]. Hepatic dysfunction characterized by elevated liver enzymes was significantly higher in severe and critical COVID-19 and was associated with poor outcomes[4]. In a meta-analysis of 45 studies, the most common biochemical abnormality of the liver in COVID-19 was hypoalbuminemia (39.8%), followed by elevation of gamma-glutamyl transferase (GGT 35.8%), or aminotransferases [aspartate aminotransferase (AST 21.8%) and alanine aminotransferase (ALT 20.4%)][10]. The incidence of elevated hepatic enzymes was also higher in COVID-19 patients requiring intensive care unit (ICU) admission as compared to non-ICU patients (62% *vs* 23%)[4]. In another meta-analysis of 128 studies, the most common hepatic abnormality was hypoalbuminemia (61.3%), followed by elevation of GGT (27.9%), ALT (23.3%), and AST (23.4%) in the patients with COVID-19. The degree of the hepatic abnormalities was directly proportional to the severity of the disease[11].

MECHANISM OF LIVER INJURY

The pathogenic properties of SARS-CoV-2 depend on the binding of viral spike proteins to the host angiotensin-converting enzyme 2 (ACE-2) receptors, which allows the virus to enter the target cells along with priming by the host transmembrane serine protease 2 (TMPRSS2)[12-14]. The ACE-2-TMPRSS2 is expressed in the ileum, liver, lung, nasal mucosa, bladder, testis, prostate, and kidney (in that order)[14-17]. SARS-CoV-2 binding to ACE-2 receptors in the upper respiratory tract is the primary site of replication and entry to the body[14]. ACE-2-TMPRSS2-positive cells in the gastrointestinal tract include enterocytes in the biliary duct or pancreatic duct epithelium and hepatocytes[14,17].

The mechanism of liver injury in COVID-19 is possibly multifactorial. SARS-CoV-2 might induce direct hepatoxicity (SARS-CoV-2 enters into the liver *via* cholangiocytes or translocation from gut to the liver) or indirect hepatic injury (from systemic inflammation with immune dysregulation, hypoxia from respiratory failure, ischemic damage due to coagulopathy or endotheliitis, right heart failure due to myocarditis, deterioration of pre-existing liver diseases, or drug-induced liver injury)[15] (Figure 1). The liver function abnormalities like increased GGT are consistent with a direct cytotoxic effect of SARS-CoV-2 on cholangiocytes[15,18]. However, the expression of ACE-2 receptors is minimal on hepatocytes suggesting a significant contribution of indirect causes of liver damage rather than direct hepatoxicity[16,18]. The treatment of severe COVID-19 with antiviral agents, immunomodulators, antibiotics, or supportive agents, may also cause hepatotoxicity. Among those agents, remdesivir, favipiravir, lopinavir/ritonavir combination, corticosteroids, and tocilizumab could increase liver enzyme levels[18-20]. Adjuvant drugs like acetaminophen and antibiotics may also cause hepatoxicity[20] (Table 1).

IMPACT OF COVID-19 ON PRE-EXISTING LIVER DISEASE

COVID-19 with CLD

In a systematic review and meta-analysis of 73 studies, the prevalence of CLD was 3% in 24299 COVID-19 patients[21]. Other studies reported a 3%-11% prevalence of underlying CLD with COVID-19[4-9,22]. The patients with CLD may also be more susceptible to contract SARS-CoV-2 infection[23]. Besides, the presence of CLD increased the risk of severe COVID-19 [pooled odds ratio (OR) 1.48] and overall mortality (pooled OR 1.78)[21,24]. Two other meta-analyses found that pre-existing liver diseases increase the risk of severe COVID-19, decompensation, and mortality[24,25]. From an extensive registry of over 17 million patients from the United Kingdom, COVID-19 was associated with a 2.34 (95% confidence interval: 1.94-2.83) times increased risk of mortality with liver disease[26]. The evidence is conflicting on the increased risk of severe COVID-19 in patients with chronic viral hepatitis[4,27]. However, SARS-CoV-2 infection in patients with chronic hepatitis B could have an increased risk of reactivation. A study of 21 patients with known chronic hepatitis B, SARS-CoV-2 infection was associated with hepatitis B reactivation in three patients[28].

Fatty liver disease with COVID-19

In a multicenter retrospective study from the United States, CLD and nonalcoholic fatty liver disease (NAFLD) were independent risk factors for ICU admission and invasive mechanical ventilation[29]. NAFLD was also associated with the progression of COVID-19 to severe disease in other studies[30-32]. The Asian Pacific Association for the Study of the Liver COVID-19 Liver Injury Spectrum Study (APCOLIS) study included 228 confirmed COVID-19 patients from 13 Asian countries with pre-existing liver disease. Metabolism associated fatty liver disease (MAFLD) was the commonest (61%) etiology[33]. In a retrospective study, a history of NAFLD/MAFLD was associated with increased odds of admission for COVID-19[34]. Obesity is common in patients with NAFLD and is an independent risk factor for severe COVID-19, invasive mechanical ventilation, and increased mortality[31,35]. However, in a study by Hashemi *et al*[29], the clinical severity of COVID-19 in patients of NAFLD was observed to be independent of obesity. The deleterious interplay of chronic inflammation observed in NAFLD with an acute inflammatory response to SARS-CoV-2 could explain the higher hepatic injury and a worse outcome in metabolically compromised NAFLD patients[36]. In another study, the extent of liver fat was correlated with serum markers of inflammation and oxidative stress[37]. It explains

Table 1 Impact of drugs currently used for the management of coronavirus disease 2019 on the liver

Drug	Mechanism of action	Impact on CLD management
Remdesivir	Viral RNA-dependent RNA polymerase inhibitor	Liver toxicity possible; No liver relevant drug-drug interactions
Lopinavir/ritonavir	Protease inhibitors	mTOR inhibitors (sirolimus, everolimus) should not be co-administered; Close monitoring of drug level is required for calcineurin inhibitors (cyclosporine, tacrolimus); The risk of lopinavir-associated hepatotoxicity in patients with very advanced liver disease is low; Patients with decompensated cirrhosis should not be treated
Tocilizumab	Humanized monoclonal antibody targeting interleukin-6 receptor	Patients with decompensated cirrhosis should not be treatedConsider risk of HBV reactivation
Methylprednisolone (steroids)	Bind nuclear receptors todampen proinflammatory cytokines	The risk of other infections (*e.g.*, spontaneous bacterial peritonitis) and viral shedding may increase in patents with decompensated liver cirrhosis; Consider antimicrobial prophylaxis; Consider risk of HBV reactivation
Favipiravir	Guanine analogue, RNA-dependent RNA polymerase	Elevation of ALT and AST possible; No data in cirrhosis available

ACE-2: Angiotensin-converting enzyme; CLD: Chronic liver disease; G6PD: Glucose-6-phosphate dehydrogenase; HBV: Hepatitis B virus; mTOR: Mammalian target of rapamycin; SBP: Spontaneous bacterial peritonitis.

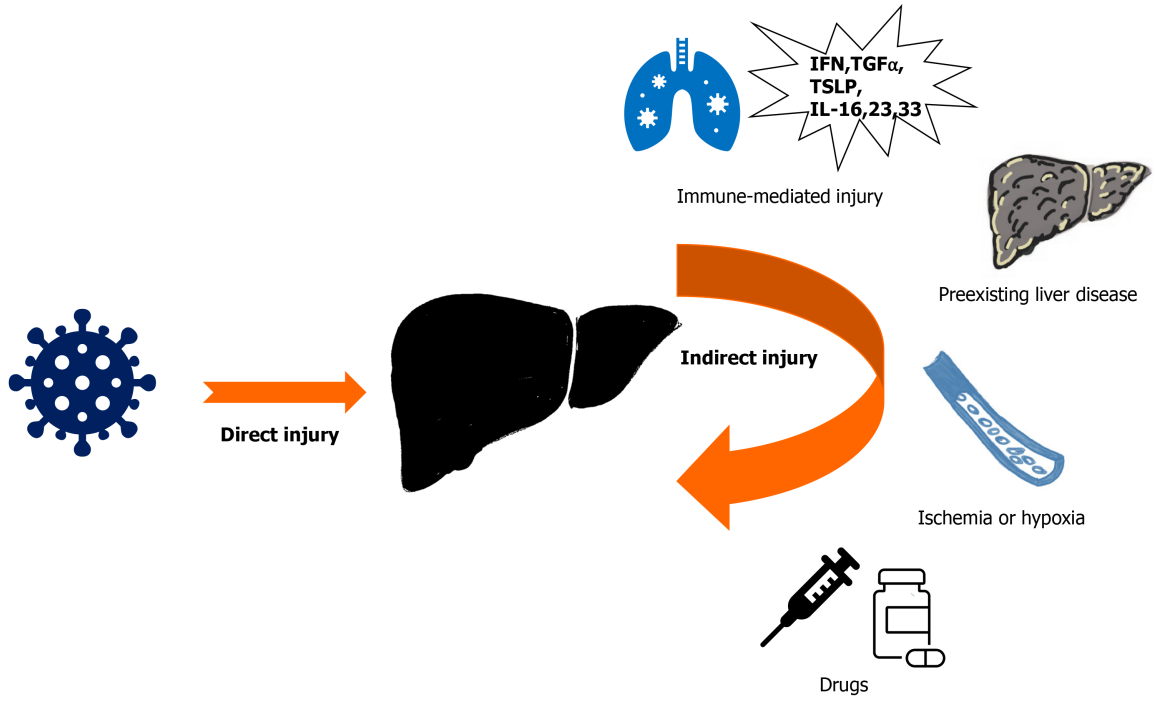

Figure 1 Mechanism of liver injury in coronavirus disease 2019.

the multifaceted impact of NAFLD on the pathophysiology and clinical course of COVID-19. However, effective treatment for metabolic disease can mitigate the increased risk from NAFLD[29,36].

COVID-19 and cirrhosis

Patients with cirrhosis are also at increased risk of decompensation with SARS-CoV-2 infection[38]. The presence of cirrhosis was also found to be an independent predictor of mortality in COVID-19[29,31]. In a study from the United States, the risk factors related to higher mortality in COVID-19 and CLD were alcoholic liver disease, decompensated cirrhosis, and HCC[39]. The worse outcomes in patients with cirrhosis can be multifactorial and likely due to cirrhosis-associated immune and inflammation modulation, limited physiological reserves, and increased risk of severe COVID-19[39]. Other large registries of patients with cirrhosis and COVID-19, like SECURE-cirrhosis and COVID-Hep.net, reported a case fatality rate of 38%, which may be as

high as 70% in the Child-Pugh C category[40].

Hepatocellular carcinoma

Patients with malignancy are vulnerable during the COVID-19 pandemic, with an increased risk of SARS-CoV-2 infection[41,42]. The overall prognosis of COVID-19 in cancer patients is poor, with high ICU admissions and mortality[41-43]. A small retrospective study of 28 cancer patients with COVID-19, including 2 HCC patients, had worse outcomes than the general population[43]. The increased risk may be attributed to age, multiple comorbidities, and the presence of cirrhosis. In patients with HCC, COVID-19 may exacerbate existing CLD and complicate the management of cancer.

PRESENTATION OF COVID-19 WITH PRE-EXISTING LIVER DISEASE

The SARS-CoV-2 infection in patients with pre-existing liver pathology may increase the risk of decompensation, acute liver injury, or a combination of both. Acute liver injury was the most observed presentation (43%) in CLD patients without cirrhosis, while acute-on-chronic liver failure (11.6%) and decompensation (9%) were more common in patients with cirrhosis[34]. The risk factors for decompensation include comorbid illnesses like diabetes or obesity. The AST/ALT ratio, total bilirubin, and R-value (ALT/ALP ratio) can predict survival in cirrhotic patients[34]. The residual hepatic synthetic function in CLD patients is inversely proportional to liver-related complications with COVID-19. Liver injury has been seen in the third week in CLD patients without cirrhosis and in the first week in cirrhotic patients[34].

COVID-19 AND LIVER TRANSPLANT RECIPIENT

Being an immunocompromised host, liver transplant recipients have an increased risk of acquiring SARS-CoV-2 infection and progression to severe disease. The outcome of COVID-19 in liver transplant patients was evaluated in a prospective study of 111 patients in Spain. Of 96 patients (86.5%) who were diagnosed with COVID-19 requiring hospital admission, 22 patients (19.8%) needed respiratory support, 12 (10.8%) required ICU admission, and the case fatality rate was 18% which was relatively lower than the matched general population despite higher severity of disease[44]. Similar results were found in another multicenter study of 112 patients from the United States. The hospital and ICU mortality rates were 22.3% and 26.8%, respectively, which was lower than the rates in matched patients of CLD without liver transplant[45]. The postulated hypothesis for better outcomes was ongoing immunomodulatory therapies that may ameliorate a harmful immune response (*i.e.* cytokine storm), reducing mortality[45,46]. However, immunosuppressants may delay viral clearance, explaining the severe disease[44]. The factors associated with mortality in liver transplant recipients were new liver injury, younger age, hispanic ethnicity, metabolic syndrome, vasopressor requirement, and antibiotic usage. Moderate liver injury [ALT 2-5 times the upper limit of normal (ULN)] and severe liver injury (ALT more than five times the ULN) was significantly associated (P = 0.007) with mortality and ICU admission[45].

MANAGEMENT OF CHRONIC LIVER DISEASE DURING COVID-19 PANDEMIC

The COVID-19 pandemic had a considerable impact on the management of CLD. Various factors must be considered and monitored while managing this group of patients. There is a potential threat of cross transmission of SARS-CoV-2 among patients and health care workers (HCWs) during physical assessment and treatment. However, it is imperative to maintain the continuity of care of patients with CLD to reduce the risk of decompensation and hospital admission. The measures recommended for safe and effective management of CLD patients can be divided into general and specific (Figure 2).

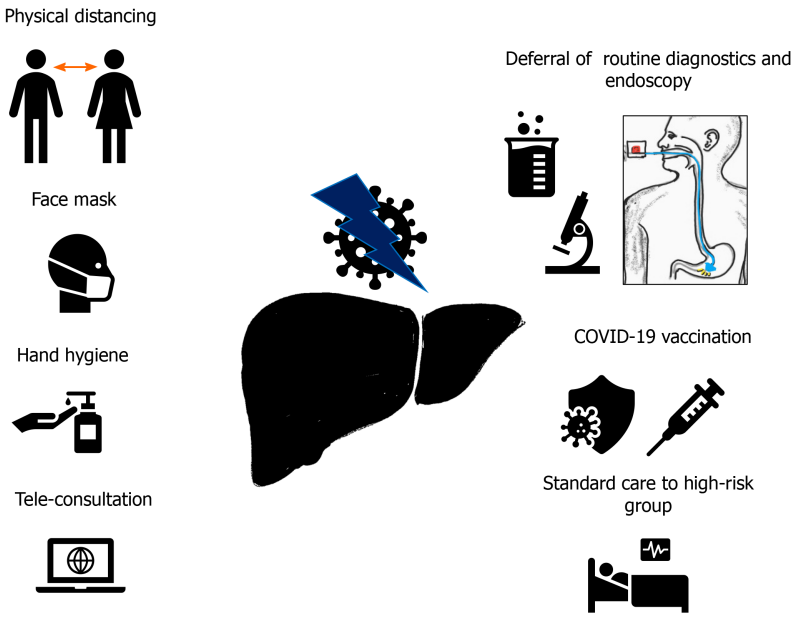

Figure 2 General measures for the safe management of patients with pre-existing liver disease during coronavirus disease 2019 pandemic. COVID-19: Coronavirus disease 2019.

General measures for all patients

Physical distancing, avoiding closed spaces without a face mask, and hand hygiene are vital pillars of SARS-CoV-2 infection prevention. Education on infection prevention measures should be included with other social measures like abstinence from alcohol and medication compliance. The screening of fever or respiratory symptoms should be performed on all patients and HCWs at the entrance of the hospital premises. Telemedicine, postponing routine outpatient visits, or periodic laboratory testing are other strategies that can be considered, depending on the available resources and patient condition[1]. The patient education must include prophylactic vaccination for streptococcus pneumonia or influenza.

Specific measures

Compensated liver disease: There is no evidence that initial clinical symptoms of SARS-CoV-2 are different in patients with CLD. Patients with NAFLD/MAFLD may suffer from other metabolic comorbidities like diabetes mellitus, hypertension, hyperlipidemia, and obesity, which need optimization and regular monitoring. Experts recommend against the alteration of immunosuppression in autoimmune hepatitis and liver transplant patients to reduce the risk of severe COVID-19[47]. The risk of aerosolization of SARS-CoV-2 during endoscopy must be considered during routine management of esophageal varices. Experts recommended non-endoscopic pathways to assess esophageal varices, especially during periods of high community transmission[47]. Any acute decompensation in patients with known CLD needs exclusion of SARS-CoV-2 coinfection. The potential reactivation of hepatitis B in patients with COVID-19 and chronic hepatitis B mandates monitoring of liver function tests and hepatitis B virus -DNA levels[28].

Decompensated liver disease: The care of the patients should follow standard guidelines while reducing direct visits to the healthcare facility (*e.g.*, using telemedicine or telephone consultation) wherever feasible. The standard management of these patients, like prophylaxis for variceal bleeding, spontaneous bacterial peritonitis, or hepatic encephalopathy, should be continued unaltered to prevent further worsening and reduce admissions[47].

Liver transplantation: The liver transplant recipients are at increased risk of contracting COVID-19, like patients with CLD. The general measures can include teleconsultation to shorten in-hospital stay and interactions with other HCWs. There were attempts to generate international consensus on treatment protocols of liver transplant recipients during this pandemic to reduce the risk of cross-transmission of SARS-CoV-2 and optimize healthcare resources[47]. The immunosuppression in liver transplant recipients may interfere with the immune response against the virus, while

any alteration in the treatment may cause acute graft rejection. Also, the use of various therapeutics to treat COVID-19 and drug-drug interactions with immunomodulators raises concerns of hepatotoxicity. In a prospective cohort study by Colmenero *et al*[44], mycophenolate at doses higher than 1000 mg/d was an independent predictor of severe COVID-19 in 111 liver transplant patients diagnosed with COVID-19. The synergistic effect of mycophenolate and SARS-CoV-2 may deplete peripheral lymphocytes responsible for an aberrant immune reconstitution to SARS-CoV-2[11,48]. In a multicenter study from the United States of COVID-19 in 112 liver transplant patients, new liver injury was associated increased mortality and ICU admission[45].

The close monitoring of liver enzymes in liver transplant patients and COVID-19 is suggested to watch for new liver injury or graft rejection. The immunosuppression regimen preferably should not be altered, except in the case of a mycophenolate-based regimen. Hypothermia is associated with worsening liver functions in severe COVID-19 and should be corrected with appropriate interventions[45].

Candidates for liver transplant: SARS-CoV-2 routine testing should be performed for both the recipient and donor before transplantation. However, a single negative RT-PCR test cannot exclude an asymptomatic infection[47]. During high community transmission or inundated healthcare resources, the transplantation should be offered only to select patients with poor short-term prognosis. It includes acute or acute-on-chronic liver failure, a high Model for End-stage Liver Disease score, or HCC with upper limits of the Milan criteria[45,49]. The diagnostic workup and procedure for the transplant program must be performed rapidly, with a short hospital stay[49].

Hepatocellular Carcinoma: Although the number of patients with HCC in the published COVID-19 studies is minimal, similar infection risk mitigation should be implemented in patients with CLD. The clinical services of cancer patients have been significantly affected by the current coronavirus pandemic, with decreased referral of the patients to the multidisciplinary tumor board (MTB), and treatment delays[50]. The evaluation, treatment and monitoring of HCC should be personalized based on the availability of medical resources and level of infection risk of SARS-CoV-2. Guidelines on the management of liver disease and HCC have been published by various academic societies[47,51]. The recommendations include virtual MTB meetings, prioritizing surgery on a case-to-case basis with preference to patients with low disease burdens and alternative therapies like radiofrequency and microwave ablation in selected patients. Treatment deferral or modification should be based on the best available evidence and availability of resources[52].

SARS-COV-2 VACCINES

Scientists developed vaccines against SARS-CoV-2 with unprecedented speed. The vaccines have been found effective in reducing the incidence of severe disease, hospitalization, and mortality. Vaccines based on various platforms, like mRNA, nonhuman viral vectors, and inactivated whole SARS-CoV-2 were developed. Despite more than 200000 participants in phase III trials, there is minimal data on efficacy in patients with pre-existing liver diseases. In the BNT162b2 (Pfizer/BioNTech) vaccination study, 217 participants (0.6%) had CLD and only 3 (< 0.1%) had moderate to severe liver disease[53]. Similarly, only 196 liver disease patients (0.6%) were included in the mRNA-1273 (Moderna) trial[54]. Data on patients with pre-existing liver disease is not available from the ChAdOx1-nCoV-19 (Oxford–AstraZeneca) vaccine trial[55]. Patients on systemic immunosuppression were excluded in all phase III trials, undermining the role of vaccines in the liver transplant recipients or patients with autoimmune liver disease on immunosuppressants. However, in the real world, millions are already vaccinated, including patients with liver disease; thus, data on safety and effectiveness are expected to be available soon. The deficiencies of innate or adaptive immune responses and an attenuated response to others vaccines are well recognized in CLD patients. A similar altered response to SARS-CoV-2 vaccines is also suspected. Nevertheless, based on an increased risk of severe disease, and in the absence of any data suggesting harm, the European Association for the Study of the Liver (EASL), the American Association for the Study of Liver Diseases (AASLD), and British Association for the Study of Liver currently recommend the available SARS-CoV-2 vaccines for patients with CLD, and liver transplant recipients[56-58]. Although the vaccines may be less effective in patients with CLD and liver transplant recipients, they still provide protection[58].

CONCLUSION

Emerging research suggests that liver injury is common in COVID-19 patients and associated with worse outcomes. Patients with CLD and post liver transplant patients are at risk of SARS-CoV-2 infection, with an increased risk of complications and mortality. The management of this vulnerable group of patients should be prioritized based on their clinical condition, strategies to reduce cross transmission, and optimizing limited resources. Liver transplant and HCC management programs should be modified depending on the prevalence of community transmission of SARS-CoV-2. Specific management issues should be considered during the treatment of COVID-19 in patients with pre-existing liver diseases.

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MINIREVIEWS

Direct, remote and combined ischemic conditioning in liver surgery

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Abstract

Liver ischemia-reperfusion injury is a major cause of postoperative liver dysfunction, morbidity and mortality following liver resection and transplantation. Ischemic conditioning has been shown to ameliorate ischemia-reperfusion injury in small animal models. It can be applied directly or remotely when cycles of ischemia and reperfusion are applied to a distant site or organ. Considering timing of the procedure, different protocols are available. Ischemic preconditioning refers to that performed before the duration of ischemia of the target organ. Ischemic perconditioning is performed over the duration of ischemia of the target organ. Ischemic postconditioning applies brief episodes of ischemia at the onset of reperfusion following a prolonged ischemia. Animal studies pointed towards suppressing cytokine release, enhancing the production of hepatoprotective adenosine and reducing liver apoptotic response as the potential mechanisms responsible for the protective effect of direct tissue conditioning. Interactions between neural, humoral and systemic pathways all lead to the protective effect of remote ischemic preconditioning. Despite promising animal studies, none of the aforementioned protocols proved to be clinically effective in liver surgery with the exception of morbidity reduction in cirrhotic patients undergoing liver resection. Further human clinical trials with application of novel conditioning protocols and combination of methods are warranted before implementation of ischemic conditioning in day-to-day clinical practice.

Key Words: Ischemic preconditioning; Ischemia-reperfusion injury; Hepatectomy; Liver transplant; Morbidity; Mortality

Core Tip: The concept of ischemic conditioning seems easy to apply and is an inexpensive method with the potential to protect the liver during hepatic surgery. It covers a wide spectrum of techniques and allows adjustment of the method to the

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particular patient. Unfortunately, despite promising animal studies in preventing ischemia-reperfusion injury by ischemic conditioning, currently there is a lack of sufficient data on its clinical efficacy in humans.

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INTRODUCTION

Ischemia-reperfusion injury (IRI) remains an important issue in hepatic surgery. IRI is a pathophysiological phenomenon where cellular damage is caused by reperfusion and reoxygenation following an ischemic period[1]. It is the most important pathogenetic factor occurring during the surgical procedure that impairs both functional reserve through loss of remaining hepatocytes and compromising liver capacity to regenerate. Thus, IRI is a major contributor to increased morbidity and mortality following liver resection and transplantation[2,3].

Ischemic preconditioning (IPC) is an adaptive pathophysiological mechanism based on a concept of preparation of the target organ for ischemic conditions in order to decrease the magnitude of IRI[4]. It was first described by Murry *et al*[5] in 1986. In a canine model, the authors demonstrated that short repetitive ischemic episodes protected the heart from subsequent sustained ischemic insult.

IPC can be either applied directly[5] or remotely[6]. Remote IPC (RIPC) is based on a concept of brief cycles of ischemia and reperfusion applied to a distant site or organ in order to exert a protective effect on another organ or site. Considering timing of the procedure, remote ischemic perconditioning (RIPer) refers to that performed over the duration of ischemia of the target organ[7].

Potential mechanisms responsible for the protective effect of tissue conditioning remain poorly understood. Regarding direct conditioning strategies, it is postulated that IPC suppresses cytokine release, enhances the production of hepatoprotective adenosine and nitric oxide and increases ATP availability by slowing the rate of ATP depletion, thus leading to upregulation of the process of cellular ATP production and liver regeneration and reduction of the liver apoptotic response[8,9]. The summary of IRI mechanism and pathways of IPC is illustrated in Figure 1[10]. In remote ischemic conditioning, reduction of hepatocellular injury in the early phase of IRI is achieved by improvement of parenchymal perfusion and oxygenation[11,12]. Interactions between neural, humoral and systemic pathways all lead to the protective effect of RIPC. In particular, these result in inhibition of the inflammatory response and activation of various hepatoprotective subcellular cascades[13].

In this review, we focus on clinical application of both, direct and remote, ischemic conditioning methods in hepatic surgery in humans. In the discussed papers we highlight clinical endpoints related to mortality, morbidity, intensive care unit (ICU) stay, hospital stay or intraoperative blood loss (in case of parenchymal resection). Postulated mechanisms of hepatocellular protection diminishing IRI are detailed in the referenced studies.

Hepatic steatosis has been associated with worse outcomes in liver surgery, and it is hypothesized that this is caused by a lower tolerance of steatotic livers to IRI[14,15]. Therefore special emphasis is put on outcomes achieved in patients undergoing liver resection and liver transplantation in humans with steatotic livers.

DIRECT IPC IN LIVER RESECTION

In 2000, Clavien *et al*[16] published the first non-randomized study on IPC in human liver[16]. Patients were subjected to IPC consisting of 10 min of clamping of the portal triad (Pringle maneuver) followed by 10 min of reperfusion before anatomical left or right hemihepatectomy. Liver cirrhosis, wedge or segmental resections were considered as exclusion criteria. The authors observed lower serum aminotransferase activities and reduced endothelial cell injury in the IPC group. No differences in

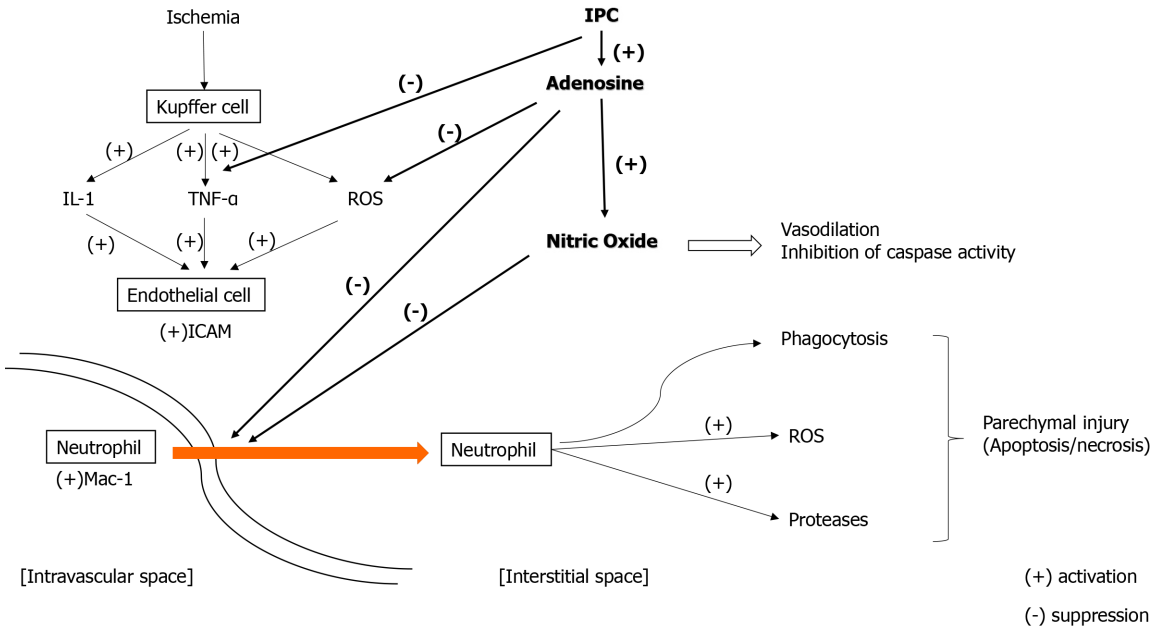

Figure 1 Summary of liver ischemia-reperfusion injury mechanisms and pathways of ischemic preconditioning interventions. Based on a paper by Montalvo-Jave *et al*[10]. ICAM: Intercellular adhesion molecule; IL-1: Interleukin-1; IPC: Ischemic preconditioning; TNF-α: Tumor necrosis factor-α; ROS: Reactive oxygen species.

mortality, hospital stay or blood loss were detected. These findings were followed by another study by Clavien *et al*[17]. In the randomized controlled trial (RCT), they confirmed previous results and highlighted younger patients and those with liver steatosis as subgroups who derived the most benefits from IPC. Nevertheless, no differences in mortality, hospital stay or blood loss were found. These promising results were followed by a number of studies exploring this field.

Cochrane meta-analysis included four RCTs published until 2008[18]. It assessed IPC followed by continuous clamping (CC) of the portal triad (135 patients) compared with CC alone (136 patients). All the included trials excluded liver resections performed in cirrhotic patients. IPC was achieved by 10 min of clamping followed by 10 min of unclamping, followed by CC in three trials[17,19-21]. In the fourth trial, the duration of initial clamping is likely to be 10 min, although it was not clearly stated. This was followed by 10 min of unclamping followed by CC[22]. The proportion of patients requiring blood transfusion was significantly lower in the IPC group, with no differences in mortality, posthepatectomy liver failure, morbidity, hospital stay or operative time.

Another meta-analysis, conducted by O'Neill *et al*[23], was published in 2013[23]. It comprised all the aforementioned studies and seven RCTs not included in the Cochrane Hepato-Biliary Group study, of which only one included patients with liver cirrhosis[24]. Ten minutes of the Pringle maneuver for IPC with 10 min of reperfusion was the most frequent strategy. In one study, IPC lasted 5 min with 5 min of reperfusion[24] and in another, IPC lasted 10 min with 15 min of reperfusion[25]. CC was used for parenchymal transection in seven studies[17,20-22,24-26], whereas intermittent clamping was used in the remaining four[27-30]. Eight studies that reported blood loss during liver resection found it to be nonsignificantly lower in the IPC group both in intermittent and CC. No differences in mortality, posthepatectomy liver failure, morbidity, operating time, hospital stay, prothrombin time, bilirubin concentration, aspartate aminotransferase (AST) or alanine aminotransferase (ALT) activities were detected (with and without patients with cirrhosis).

Another meta-analysis was published in 2017[31]. The authors focused only on RCTs investigating the role of IPC before CC. Pooled data were analyzed by combining the results of the 13 RCTs. Five trials enrolled both cirrhotic and noncirrhotic patients (91 in the IPC group and 90 in the control group)[21,32-35]. In three trials, IPC was performed through 5 min of inflow occlusion followed by 5 min of reperfusion[32,34,35]. In one study, IPC was done by inflow occlusion for 10 min followed by reperfusion for 15 min before CC[25]. Ten minutes of the Pringle maneuver for IPC with 10 min of reperfusion was used in nine studies[17,19,22,27-30]. In the case of underlying cirrhosis, IPC reduced postoperative morbidity. However, in

patients without cirrhosis, the analysis revealed no significant association between IPC and postoperative morbidity. There were also no differences in morbidity considering ischemia-reperfusion timing (10 + 10 *vs* 5 + 5). Mortality, operative time, total bilirubin concentration, AST or ALT concentration after postoperative day 1, and hospital and ICU stay were similar regardless of IPC.

Three studies focused on patients with steatotic livers in subgroup analyses. Two studies were RCTs[17,25], and one was a prospective nonrandomized study[16]. A total of 29 patients were analyzed as a subgroup (16 in IPC group and 13 in control group). Cutoff for liver steatosis was set as ≥ 30%, but the type of steatosis (micro- or macrovesicular) was not described. The protocol of IPC was 10 + 10 min in two studies[16,17] and 10 + 15 min in one study[25]. Only peak AST levels were measured as an endpoint in this subgroup comparison. IPC was associated with lower activity of AST after resections in steatotic livers[16,17,25], yet no results on clinical outcomes were provided.

In conclusion, there is currently no evidence supporting direct IPC as a protective strategy against mortality in patients undergoing liver resection, although it may be beneficial for patients with liver cirrhosis with respect to postoperative morbidity. Further investigation of applicability of direct IPC in cirrhotic and steatotic livers is warranted.

DIRECT IPC IN LIVER TRANSPLANTATION

In 2016, a meta-analysis on IPC in liver transplantation was published by Robertson *et al*[36]. Data from ten studies were analyzed (286 patients in IPC group and 307 patients in control group), four nonrandomized[37-40] and six RCTs[41-46]. Only transplantations of grafts procured from donors after brain death were included in these studies, and no grafts underwent machine perfusion. Grafts were preconditioned in the donor by portal triad clamping for 10 min in all but one study. In one study, IPC lasted for 5 min[46]. Time of reperfusion varied among studies from 10 to 39 min. Authors reported that IPC was associated with lower postoperative mortality, lower incidence of primary graft nonfunction and lower rate of retransplantation. None of these findings were statistically significant. Additionally, AST activity on the third postoperative day, length of ICU stay, length of hospital stay and incidence of acute rejection were all nonsignificantly lower in transplantations with IPC.

In living related liver transplantation, two prospective nonrandomized studies were published[47,48]. The protocol of IPC was 10 + 10 min in both studies. Only right lobes were procured from the donors (32 in IPC group and 32 in control group). There were no differences in graft survival, patient survival, morbidity, hospital stay, histological findings and liver function tests between recipients of IPC and non-IPC liver grafts.

Three studies focused on patients with steatotic donor livers in subgroup analyses. All donors were after brain death (25 in IPC group and 29 in control group). Two studies were RCTs[43,46], and one was a retrospective study[39]. The protocol of IPC was 10 + 10 min in one study[39], 10 + 30 min in second study[43], and in the remaining study IPC lasted for 5 min with ongoing reperfusion[46]. Definitions of significant steatosis varied among studies and comprised presence of any steatosis[39], > 15% of macrovesicular steatosis[43] and no specific definition[46]. None of the studies reported results on patient mortality. Clear conclusions cannot be drawn from these studies in terms of impact of IPC on steatotic liver grafts. Morbidity, graft survival, hospital stay, ICU stay and liver function tests seemed to be similar between IPC and non-IPC groups. However, there was a lack of uniform description of severity of hepatic steatosis, and the analyses were limited by small numbers.

In conclusion, there is currently no evidence that direct IPC decreases mortality after deceased and living donor liver transplantation. However, no trial provided data on recipient outcomes after more than 1 year postoperatively, and as such, the long-term effect of IPC on post-transplant outcomes remains to be elucidated. Also, there is insufficient data on IPC impact on steatotic grafts. Therefore, further analysis of this subgroup is warranted.

REMOTE IPC IN LIVER RESECTION

Only scarce data on remote IPC in liver resection in humans are available (Table 1). In five studies, the total number of 155 patients underwent RIPC with 160 patients serving as controls. Two studies had a third arm, direct IPC, including a total 52

Table 1 Randomized controlled trials on remote ischemic preconditioning in liver surgery

Ref.	Intervention (patients, *n*)	Ischemia-reperfusion	Place of ischemia	Cirrhosis, *n*	Pringle maneuver	Primary endpoint
Kanoria *et al*[49], 2017	RIPC (8)	2 × 10 + 10	Lower limb	-	No	Feasibility, safety
	Control (8)	-	-	-		
Rakić*et al*[50], 2018	RIPC (20)	3 × 5 + 5	Upper limb	-	Yes	Liver function tests
	IPC (20)	15 + 10	Portal triad	-		
	Control (20)	-	-	-		
Teo *et al*[51], 2020	RIPC (24)	4 × 5 + 5	Upper limb	13	Selectively	Serum ALT
	Control (26)	-	-	19		
Liu *et al*[52], 2019	RIPC (69)	3 × 5 + 5	Upper limb	56	Yes (in 20 min cycles)	Peak level of total bilirubin
	Control (67)	-	-	51		
Wu *et al*[53], 2020	RIPC (34)	3 × 5 + 5	Upper limb	23	Yes	Serum ALT and AST
	IPC (32)	10 + 10	Portal triad	26		
	Control (39)	-	-	25		

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; IPC: Ischemic preconditioning; RIPC: Remote ischemic preconditioning.

patients. In two studies, liver resection was performed due to colorectal metastases[49,50] and due to primary liver cancers in the others[51-53]. The most common protocol for ischemia-reperfusion was 5 min of upper limb ischemia followed by 5 min of reperfusion in 3 cycles in three studies[50,52,53] and 4 cycles in one study[51]. In the first published pilot randomized feasibility trial, authors applied 2 cycles of 10 min of the lower limb ischemia followed by 10 min of reperfusion[49]. Primary endpoints varied, with serum transaminase activities being the most common. Two studies found significant differences in the early postoperative ALT and AST activities in favor of RIPC[49] and IPC/RIPC over control[50]. In one study, significant differences in postoperative ALT and AST activities on days 1 and 3 in favor of ischemia group (either remote or direct) over control group were observed, but these were absent on postoperative day 7[53]. Analysis of the subgroup of patients with liver cirrhosis was performed in a single study pointing towards no effect of RIPC on ALT activity 24 h posthepatectomy[51]. Mortality, morbidity, blood loss and hospital stay were assessed in three trials, and no differences were found between groups[49,51,52].

Data on hepatic steatosis were provided in only two studies. In one trial, all specimens were evaluated for degree of steatosis[49], with minimal liver steatosis found in both groups. In the second study, etiology of liver cirrhosis was nonalcoholic fatty liver disease in 4 patients (2 in the study group and 2 in the control group)[51]. No further information was given.

In conclusion, there is still insufficient data supporting the use of RIPC in liver resection as protection against IRI in order to improve clinical outcomes.

REMOTE IPC IN LIVER TRANSPLANTATION

To the authors knowledge, only two studies addressed remote IPC in liver transplantation. In 2017, Robertson *et al*[54] published a pilot randomized controlled feasibility study on orthotopic liver transplantation from deceased donors (after either brain or cardiac death)[54]. Forty patients were randomized to a sham control group (20 patients) or an RIPC group (20 patients). The protocol for ischemia-reperfusion was 5 min of donor lower limb ischemia followed by 5 min of reperfusion in three cycles. Implantation of the liver graft was performed by standard piggy-back and caval replacement techniques. No differences in 90-d mortality, 90-d graft loss, complications, AST activity on the third postoperative day and hospital and ICU stay were detected.

In 2020, Jung *et al*[55] published an RCT on the application of RIPC in living donor liver transplantation[55]. In total, 148 donors were randomized to a sham control

group (73 donors) or an RIPC group (75 donors). The protocol for ischemia-reperfusion was 5 min of donor upper limb ischemia followed by 5 min of reperfusion in 3 cycles. For the recipients, the medical records were retrospectively analyzed. In the donors, no differences in complications, AST, ALT, total bilirubin and international normalized ratio within 7 postoperative days, incidence of delayed recovery of hepatic function and liver regeneration index depending on the use of RIPC were found. However, recipients who received preconditioned grafts had lower AST activity on postoperative day 7 and the maximal AST activity during the first postoperative week. No differences in other laboratory variables, early graft dysfunction, acute kidney injury, graft failure after 12 mo post-transplantation or hospital and ICU stay were detected.

In conclusion, there is no evidence supporting the use of RIPC in deceased and living donor liver transplantations as protection against IRI in order to improve clinical outcomes.

REMOTE ISCHEMIC PERCONDITIONING, ISCHEMIC POSTCONDITIONING AND COMBINED METHODS OF ISCHEMIC CONDITIONING IN LIVER SURGERY

In search of effective protection against liver IRI, novel concepts are being adapted from experience with other organs. Ischemic postconditioning (IPOS) applies brief episodes of ischemia at the onset of reperfusion following a prolonged ischemia and was first introduced in a rodent heart model[56]. Advantage of IPOS over IPC is that it can be easily applied with precisely controlled timing. Modification of the RIPC technique is RIPer, first applied by Schmidt *et al*[7] in the context of myocardial ischemia[7]. In a porcine model, alternating periods of occlusion and perfusion of the limb while the myocardium was under ischemia was examined. Little data exists on the efficacy of these methods alone or in combination in hepatoprotection against IRI.

In 2012, a mice liver resection study by Song *et al*[57] compared IPC, RIPC (hind limb), IPOS and the combination of IPC with IPOS[57]. The authors found that the combination of direct IPC with IPOS offered additional protection over the solo treatment. In contrast, no additive protection of IPOS was found when applied with RIPer in rat liver resection model[58]. In this study, the authors identified RIPer as the most promising technique to avoid hepatic IRI, in comparison with IPOS and combination of RIPer with IPOS. This was in accordance with other studies on rodent liver resection or transplantation, which confirmed a protective effect of RIPer against IRI[59-61]. Combination of different ischemic conditioning techniques in a mouse liver transplantation model was reported by Li *et al*[62]. By comparing IPC and RIPC with a combination of both methods, they found both techniques effective in hepatic IRI protection but no synergistic and additive effect of IPC and RIPC. Another study designed by this group assigned mice to direct IPC (donor), RIPer (recipients) and IPC + RIPer (donors and recipients were subjected to IPC and RIPer, respectively)[63]. By double protection of the graft, first by IPC in donor then by RIPer before reperfusion in recipient, they showed that combined treatment brought enhanced attenuation in IRI through additive effects on antioxidation, antiapoptosis, modulation of microcirculation disturbance and inhibition of innate immune response.

The aforementioned protocols have only been tested in animal models. No studies on humans have been published researching the possible application of IPOS, RIPer or combined ischemic conditioning. There are currently no ongoing clinical trials on that subject[64].

CONCLUSION

Direct IPC was not found effective in terms of decreasing mortality after liver resection or transplantation. Its role in specific subgroups of patients remains to be elucidated. Studies on remote IPC in liver resection pointed toward either no beneficial effects or effects limited to moderate reduction of IRI as indicated by serum transaminases and bilirubin concentration. Most studies used protocols with 5 min ischemic periods, which may indicate that this is an insufficient period.

In terms of liver transplantation, RIPC was found to be beneficial only in early graft function from living donors. Those were young, nonsteatotic grafts with relatively short periods of cold and warm ischemia. Other techniques of ischemic conditioning

are yet to be assessed in human clinical trials.

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ORIGINAL ARTICLE

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Bile acid indices as biomarkers for liver diseases II: The bile acid score survival prognostic model

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Abstract

BACKGROUND

Cholestatic liver diseases are characterized by an accumulation of toxic bile acids (BA) in the liver, blood and other tissues which lead to progressive liver injury and poor prognosis in patients.

AIM

To discover and validate prognostic biomarkers of cholestatic liver diseases based on the urinary BA profile.

METHODS

We analyzed urine samples by liquid chromatography-tandem mass spectrometry and investigated the use of the urinary BA profile to develop survival models that can predict the prognosis of hepatobiliary diseases. The urinary BA profile, a set of non-BA parameters, and the adverse events of liver transplant and/or death were monitored in 257 patients with cholestatic liver diseases for up to 7 years. The BA profile was characterized by calculating BA indices, which quantify the

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composition, metabolism, hydrophilicity, formation of secondary BA, and toxicity of the BA profile. We have developed and validated the bile-acid score (BAS) model (a survival model based on BA indices) to predict the prognosis of cholestatic liver diseases.

RESULTS

We have developed and validated a survival model based on BA (the BAS model) indices to predict the prognosis of cholestatic liver diseases. Our results demonstrate that the BAS model is more accurate and results in higher true-positive and true-negative prediction of death compared to both non-BAS and model for end-stage liver disease (MELD) models. Both 5- and 3-year survival probabilities markedly decreased as a function of BAS. Moreover, patients with high BAS had a 4-fold higher rate of death and lived for an average of 11 mo shorter than subjects with low BAS. The increased risk of death with high *vs* low BAS was also 2-4-fold higher and the shortening of lifespan was 6-7-mo lower compared to MELD or non-BAS. Similarly, we have shown the use of BAS to predict the survival of patients with and without liver transplant (LT). Therefore, BAS could be used to define the most seriously ill patients, who need earlier intervention such as LT. This will help provide guidance for timely care for liver patients.

CONCLUSION

The BAS model is more accurate than MELD and non-BAS models in predicting the prognosis of cholestatic liver diseases.

Key Words: Hepatobiliary diseases; Bile acid indices; Death; Liver transplant; Survival model; Prognosis

Core Tip: We have developed survival models based on bile acid (BA) indices to predict the prognosis of hepatobiliary diseases. Our BA models outperformed the model for end-stage liver disease and non-BA models in predicting the occurrence of the adverse events of death and/or liver transplant.

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INTRODUCTION

Cholestatic liver diseases are hepatobiliary diseases associated with a reducing in bile flow due to impairment in bile production or failure of bile flow into bile duct[1]. Chronic liver diseases account for greater than 41000 deaths in the United States in 2017, making it the 11th leading cause of mortality[2]. Most cholestatic diseases progress toward end stage liver failure, which likely requires liver transplantation. Even after liver transplantation, post-surgery complications are common, which may require liver re-transplantation[3].

Biomarkers currently used in the clinic for the diagnosis and prognosis of liver diseases are primarily serum liver enzymes such as aspartate transaminase (AST), alanine transaminase (ALT), and bilirubin. However, these markers have numerous shortfalls including the lack of specificity for liver or bile duct injuries as they can be elevated in hyperthyroidism, adrenal, heart, or muscle disorders. Also, severe cell injury has to occur before their levels increase[4,5]. Multifactorial models with multiple parameters based on these biomarkers are also frequently used and offer advantages compared to the use of their individual biomarker components such as the Child-Turcotte-Pugh score[2].

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More recently, the model for end-stage liver disease (MELD) was developed to predict three-month mortality of patients with end-stage liver disease[5,6]. MELD is calculated based on serum creatinine, bilirubin, international normalized ratio (INR), and Na^+, which are related to both liver and renal functions. MELD is currently used in many countries to classify patients awaiting transplantation to identify patients with the highest priority for liver transplant (LT)[6]. Since its implementation, MELD led to an intense reduction in the number of people waiting for liver transplant and decreased mortality on the waiting list without affecting post-transplant survival[7]. MELD is also an effective predictor of outcome in other conditions, such as patients have cirrhosis going for surgery and patients with alcoholic hepatitis or fulminant hepatic failure[7]. However, MELD is based on three objective laboratory variables, that are not necessarily liver specific. For example, serum bilirubin can be elevated in cases of hemolysis or sepsis. Serum creatinine can also be elevated from an underlying kidney disease that unrelated to hepatorenal syndrome and is a poor surrogate of renal function in cirrhotic patients[8]. In addition, patients may have an elevated INR which can be secondary to warfarin use. Any of these conditions can increase the MELD score and overestimate the liver disease severity[9]. Furthermore, several studies have shown that patients with cholestatic liver diseases may still have high mortality rates despite having low MELD scores[10,11].

Numerous clinical and preclinical studies have shown up to a 100-fold increase in BA concentrations in urine with various hepatobiliary diseases[12-16]. The impediment in bile flow associated with cholestatic liver diseases cause accumulation of toxic BA in the liver and blood, which can worsen the liver condition that lead to their accumulation and contribute to the unfavorable liver disease prognosis[17]. However, the potential use of BA as a marker for liver diseases have never translated into a widespread use in the clinic[18,19], due to major limitations including the major differences of the physiologic and pathologic effects of the various individual BA and the extremely high inter- and intra-individual variability of BA concentrations.

To this regard, we have developed the concept of "BA Indices", which are ratios calculated from the absolute concentration of individual BA and their metabolites. BA indices offered numerous advantages over absolute BA concentrations including low intra- and inter-individual variability and resistance to the influence of food consumption, age, gender, body mass index (BMI), and moderate alcohol consumption[19-21]. In the 1st part of this study, we have demonstrated that BA indices outperformed serum liver enzymes as biomarkers for the diagnosis of cholestatic liver diseases. In this second part of the study, we have developed survival models based on BA indices to predict the prognosis of hepatobiliary diseases. Our BA models outperformed the non-BA and MELD models in predicting the occurrence of the adverse events of death and/or LT.

MATERIALS AND METHODS

Study participants

New and existing patients of the University of Nebraska Medical Center (UNMC) hepatology clinic, who were diagnosed with one or multi-hepatobiliary conditions due to chronic hepatitis C (n = 63) , hepatitis B (n = 14), alcoholic liver disease/alcoholic cirrhosis (n = 103), primary biliary cholangitis (n = 11), primary sclerosing cholangitis (n = 13), autoimmune hepatitis (n = 24), alpha-1-antitrypsin deficiency (n = 5), nonalcoholic fatty liver disease/nonalcoholic steatohepatitis (n = 51), carcinoma (n = 24), cryptogenic cirrhosis (n = 10), polycystic liver disease (n = 5), elevated liver function test (n = 18), and unknown etiology (n = 5), were enrolled in this study. Table 1 shows a summary of our patient population characteristics. A total of 257 patients (121 female and 136 male) between the ages of 19 and 83 years, who were treated for cholestatic liver diseases in UNMC, over the period from November of 2011 to December of 2018, were recruited into the study. All participants were followed up for up to 7 years by collecting urine samples for BA analysis and monitoring non-BA parameters and adverse events including liver transplant, and death from their medical records.

The study was approved by the Institutional Review Board at UNMC and written informed consents were provided for all participating subjects. The registry URL was (https://www.clinicaltrials.gov/ct2/show/NCT01200082?term=alnouti&draw=2&rank=1). The clinical trial number was NCT01200082. Thirty milliliters of urine samples were collected from patients on their first visit to the hepatology clinic. All urine samples were stored in -80 °C until analyzed by liquid chromatography-tandem mass

Table 1 Patient population characteristics

	Patients	Death	Liver transplant
n	257	27	25
Gender			
Male	136	21	17
Female	121	6	8
Age (yr)			
mean ± SE	52.2 ± 0.71	55.9 ± 1.88	52.9 ± 2.1
Body mass index			
mean ± SE	30.7 ± 0.45	29.65 ± 1.19	29.11 ± 0.45
Race			
White	217	26	24
Black	11	0	0
Asian	7	0	0
Hispanic	4	0	1
Others	18	1	0
Non-BA parameters (mean ± SE)			
Creatinine (mg/dL)	1.02 ± 0.09		
Albumin (g/dL)	3.53 ± 0.04		
INR	1.19 ± 0.02		
Protime (s)	12.01 ± 0.42		
AST (U/L)	59.9 ± 4.07		
ALT (U/L)	54.9 ± 4.26		
Bilirubin (mg/dL)	1.75 ± 0.15		
AST/ALT	1.28 ± 0.04		
MELD	10.6 ± 0.34		
APRI	1.15 ± 0.11		

BA: Bile acids; INR: International normalized ratio; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; MELD: Model for end-stage liver disease; APRI: Aspartate aminotransferase/platelet ratio index.

spectrometry (LC-MS/MS).

Non-BA parameters

The performance of potential biomarkers from the urinary BA profile was also compared with and existing markers of liver function including ALT, AST, serum creatinine, albumin, protime, INR, bilirubin, AST/ALT ratio, and AST/platelet ratio index (APRI). These markers were monitored using the patients' medical records. Bile acid quantification by liquid chromatography–tandem mass spectrometry

Urine samples were extracted using solid phase extraction as described previously[8,22,23]. BA concentrations were quantified by LC-MS/MS, as we described previously.

Calculation of BA indices

BA profile in urine was characterized using BA "indices", which describe the composition, hydrophobicity, toxicity, and metabolism of total and individual BA as we have described previously[8,22,23].

Statistical analysis

All statistical analysis was performed using the Statistical Product and Service

Solutions software, version 25 (IBM corporation, Armonk, NY, United States) and R software, version 3.6.3 (R Foundation for statistical Computing). A *P* value of 0.05 was considered significant for all the statistical tests described below.

Survival model development

Cox proportional hazards (PH) regression was used to develop survival models to predict the prognosis of hepatobiliary diseases in terms of progressing specifically into the end points/adverse events of death.

For the "death" models, the only endpoint/adverse event recorded was death at 3 and 5 years. We only had 7 and 17 deaths occurring within earlier time points including 1 and 2 years, respectively, which was not enough to develop survival models. Patients who underwent liver transplant (LT) were censored with the date of transplantation. Patients still alive at the end of each period (3 and 5 years) were considered as censored at that time. The term ''censored'' indicates that the patient was alive at that date and that was the end of the follow-up[22]. Patients dropped off, not due to the occurrence of adverse event, i.e. death, before the end of the follow-up period, were censored at the last day they were seen in the clinic.

In addition to the "death only" models above, we also constructed models to predict death and/or LT. We followed the same approach as the "death" models, with the exception that the endpoint was the occurrence of the adverse events of either death or LT. Patients whom did not have either of the adverse events at the end of each period (3 and 5 years) were censored at that time.

Individual BA and non-BA variables were analyzed as possible predictors of survival in a univariate Cox regression analysis. Values of these variables included in the statistical analysis were obtained at the time of patients' first visits. Significant variables ($P < 0.05$), which were identified from the univariate analysis were included in the multivariate analysis. To build the multivariate model a backward elimination regression method was used to retain the most significant variables with retention criteria of $P < 0.05$.

Model performance, goodness of fit and validation

Goodness of fit was performed by testing PH assumption for each covariate included in the final Cox model and for the global model as a whole. We used the bootstrapping for model validation.

Receiver operating characteristic (ROC) curve analyses was performed on the scores from the various multivariate Cox models to determine their cut-off values in differentiating patients with *vs* without the adverse event. The cut-off values with optimum specificity and sensitivity were selected and the areas under the ROC curve (AUC) values were calculated.

Survival prediction

The average survival probability [$S_0(t)$] for a patient with an average score were calculated for different time points. To obtain the probability of survival for t years [S (*t*)], first the score *e.g.* bile-acid score (BAS) is calculated, and finally S (*t*) is calculated using this equation: Survival probability for *t* years: $S(t) = S_0(t)^{\exp(BAS - BAS0)}$.

Where, BAS_0 is the average score from all patients in this study.

Kaplan-Meier plots were used to display survival curves. We have divided patients into two categories of high *vs* low risk and compared their survival with the Log-rank test and Breslow test[22]. We have tried the median cut-off values of the model scores to define high *vs* low risk.

Models comparison

We have used multivariate cox regression analyses to build various models for the prediction of death. The performance of the different models in predicting the occurrence of death within 3- and 5-year periods were compared between the different models using the statistic outcomes from the Bootstrapping, Schoenfeld residuals, AUC, and Kaplan-Meier analyses.

RESULTS

Patient population characteristics

Table 1 shows a summary of the characteristics of the patient population in our study. The demographic variables were (age, BMI, gender, and race). Subjects were divided

into five race groups (White, Black, Asian, Hispanic, and others). During the 7-year follow-up period of 257 patients with cholestatic liver diseases, 27 patients (10.5%) died and 25 patients (9.7%) underwent liver transplantation.

We were interested in predicting the occurrence of adverse events of death within 3- and 5-year periods. During a 3-year follow-up period, 21 patients (8.2%) died and 19 patients (7.4%) underwent liver transplantation. While during a 5-year follow-up period, 25 patients (9.7%) died and 21 patients (8.2%) underwent liver transplantation.

Univariate Cox regression analysis for death prediction

Supplementary Table 1 shows the results of univariate Cox regression analyses for death prediction by BA Indices. Cox regression detects the risk of death associated with changes in BA indices. Positive regression coefficients imply that the risk of death increases with increasing the values of BA indices, while negative coefficients imply the risk of death increases with a decrease in the values of BA indices. We found correlation between the risk of death and many BA indices ($P < 0.05$).

The hazard ratio (HR) from Cox regressions analysis quantifies the magnitude of the risk of death per unit change in BA indices. Because BA concentrations and indices have different scales and units, we performed the same calculation per 10% and 20% of the mean value of each variable instead of per absolute unit. For example, for a 20% increase in the %CDCA, the risk of death increases 1.26-fold (HR: 1.26; $P < 0.05$).

We performed the same univariate cox regression analysis for demographics and non-BA parameters as well (Supplementary Table 2). Notably, the risk of death was significantly higher in males than females from this univariate analysis. Increasing levels of INR, protime, bilirubin, AST/ALT, APRI, and MELD also significantly increased the risk of death, whereas decreasing levels of albumin significantly increased the risk of death.

Multivariate Cox regression analysis for death prediction

In multivariate analysis, a backward elimination regression was used to retain the most significant BA variables. The only BA variables retained in the multivariate model were %CDCA and %Tri-OH, which were independently predictive of survival (Table 2). For example, a 20% increase in the %CDCA and %Tri-OH increases the risk of death by 1.34-fold (HR: 1.34; $P < 0.05$) and 1.14-fold (HR: 1.14; $P < 0.05$), respectively. The BAS for individual patients can be calculated from this equation: BAS for death = 0.039 × %CDCA + 0.052 × %Tri-OH.

For example, for a patient with %CDCA of 20%, and a %Tri-OH of 50%, the BAS would be 3.38.

We performed the same multivariate Cox regression analysis for demographics and non-BA parameters as well. For demographic variables, gender was significant in univariate analysis, but did not retain in multivariate analysis when included in the BA model building. In contrast, gender retained in the multivariate analysis for the non-BA model, but with minimal improvement of model goodness of fit and validation (the Bootstrapping, Schoenfeld residuals, AUC, and Kaplan-Meier analyses). Therefore, we did not include gender in the multivariate Cox models and AST/ALT ratio was the only significant predictive variable of death (Table 2). For example, a 20% increase in the AST/ALT, increases the risk of death by 1.36-fold (HR: 1.36; $P < 0.05$). The non-BAS for individual patients can be calculated from this equation: non-BAS for death = 1.236 × AST/ALT.

In addition, we used the same methodology to develop other models including: (1) mixed BA and non-BA variables including demographics to test how the performance of a global BA- and non-BA mixed model compares to the BA-only and non-BA-only models; (2) MELD variables with coefficients from our data set to create a model with the original MELD variables, but with model coefficients derived from our data set; and (3) original MELD modified with BA and/or non-BA variables including demographics, to test if the performance of the original MELD can be improved by adding significant BA and non-BA parameters from the univariate analysis and vice versa (Supplementary Table 3). Overall, none of these strategies produced any statistically significant models neither they did improve the BA or non-BA-only model; therefore, were not further evaluated or validated.

Model performance, goodness of fit and validation

Goodness of fit was performed by testing PH assumption for all the covariates of the final Cox model as well as for the global model as a whole, using a statistical test and a graphical diagnostic based on Schoenfeld residuals. A graphical diagnostic that shows a non-random pattern against time is evidence of violation of the PH assumption. The

Table 2 Multivariate Cox regression analysis for death prediction

BA indices (%) and non-BA parameters	B-value (regression coefficient)	Standard error	*P* value	Hazard ratio: Exp (B)		
				1 unit change	10% change	20% change
The BAS model						
%CDCA	0.039	0.010	0.000	1.040	1.159	1.344
%Tri-OH	0.052	0.016	0.001	1.053	1.069	1.142
The non-BAS model						
AST/ALT	1.236	0.303	0.000	3.442	1.165	1.357

Using the regression coefficients from this table: The bile-acids score (BAS) equation is: the non-BAS equation is: BAS: Bile acids score; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

PH assumption is supported by a non-significant relationship between residuals and time. The Schoenfeld residual plots and *P* values supported the validity of the BA and non-BA models (Supplementary Figure 1).

We also used the bootstrapping validation. Bootstrapping validation results for the BA and non-BA models indicate that our regression coefficients were in the range of the 95%CI, *P* values were statistically significant for each covariate, bias values and standard error values were very small (Supplementary Table 4). We can conclude that the Bootstrapping validation results supported the validity of the BA and non-BA models.

Figure 1 shows the ROC curves of the models for death prediction. For 5-year death prediction, the AUC for BAS, non-BAS, and MELD were 0.740, 0.653, and 0.683, respectively. For 3-year death prediction, the AUC for BAS, non-BAS, and MELD were 0.761, 0.664, and 0.715, respectively. Potential cut-off values selected based on the optimum sensitivity and specificity for different models. The ROC-optimum scores for BA, non-BA, and MELD models for death prediction were 2.71, 1.72, and 10, respectively (Table 3).

Survival prediction

Table 4 presents the estimated survival probability [$S_0(t)$] for a patient with an average BAS_0 of 2.24 (the average BAS from all 257 patients in this study) for different time points. To obtain the survival probability for *t* years [S (*t*)], first BAS is calculated, $S_0(t)$ is identified from Table 4, and finally S (*t*) is calculated using this equation: Survival probability for *t* years: $S(t) = S_0(t)^{\exp(BAS - BAS0)}$.

Where, BAS_0 is the average BAS from all patients in this study; namely 2.24, while BAS is the BAS for that particular patient. For the same example patient discussed above, the probability of surviving for at least 3 years is: Survival probability for (3) years = $0.934^{\exp(3.38 - 2.24)} = 0.81 = 81\%$

The relationship between estimated 5- and 3- year survival probability [S (t)] and the BAS in patients with liver disease are shown in Figure 2A. Survival probability decreases as a function of BAS. For example, the 5-year survival probability for patients with BAS of 1.2 (25th percentile of the population), 2.1 (50th percentile of the population *i.e.* median), and 3.1 (75th percentile of the population) are 97%, 93%, and 82%, respectively. Similarly, the 3-year survival probability for patients with the same BAS above, are 98%, 94%, and 85%, respectively.

Table 4 presents the estimated survival probability [$S_0(t)$] for a patient with an average non-BAS_0 of 1.58 for different time points. The survival probability for (*t*) years is calculated using this equation: Survival probability for *t* years: $S(t) = S_0(t)^{\exp(non-BAS - non-BAS0)}$.

The relationship between estimated 5- and 3- year survival probability [S (*t*)] and the non-BAS in patients with liver disease are shown in Figure 2B. For example, the 5-year survival probability for patients with non-BAS of 1.1 (25th percentile of the population), 1.4 (50th percentile of the population), and 1.9 (75th percentile of the population) are 92%, 90%, and 83%, respectively. Similarly, the 3-year survival probability for patients with the same non-BAS above, are 95%, 91%, and 86%, respectively.

By the end of the study, up to 7 years monitoring of 257 patients with cholestatic liver diseases, 27 patients (10.5%) have died. The Kaplan-Meier estimator was used to estimate subjects' survival free of adverse events over time. We have tried the median

Table 3 Receiver operating characteristics analysis of bile-acids score, non- bile-acids score, and models for end stage liver diseases for death prediction

Models	AUC (5-yr)	AUC (3-yr)	(Cutoff value; sensitivity, specificity)
BAS	0.740	0.761	(2.71; 74, 68)
non-BAS	0.653	0.664	(1.72; 67, 66)
MELD	0.683	0.715	(10; 62, 64)

AUC: Areas under the ROC curve; BAS: Bile acids score; MELD: Model for end-stage liver disease.

Table 4 Estimated survival probability [$S_0(t)$] for death prediction

t (mo)	5	7	14	24	36	60	76
The BAS							
S_0 (*t*)	0.993	0.985	0.971	0.948	0.934	0.916	0.901
The non-BAS							
S_0 (*t*)	0.989	0.978	0.958	0.924	0.902	0.876	0.855

BAS: Bile acids score.

of the BAS of the population (2.19) cut-off value to define high *vs* low risk of death (Figure 3A). The estimated mean survival time was 71 mo (5.9 years) for the high-risk group and 82 mo (6.8 years) for the lower risk group based on the median BAS of 2.19 (Table 5). The *P* value of the log rank test and Breslow test were statistically significant (*P* value < 0.05), indicating the median cut-off of BAS, can differentiate low *vs* high risk of death.

Figure 3B shows the Kaplan Meier survival for the high *vs* low risk of death groups based on the median (1.44) for the non-BAS. The estimated mean survival time was 74 mo (6.2 years) for the high-risk group and 79 mo (6.6 years) for the lower risk group based on the median non-BAS of 1.44. The *P* value from the log rank test and Breslow test was insignificant (*P* value > 0.05), indicating the median of non-BAS (1.44) cannot differentiate low *vs* high risk of death (Table 5).

Figure 3C shows the Kaplan Meier survival for the high *vs* low risk of death groups based on the median (11) for the MELD model. The estimated mean survival time was 74 mo (6.2 years) for the high-risk group and 78 mo (6.5 years) for the lower risk group based on the median MELD of 11. The *P* value from the log rank test and Breslow test was insignificant (*P* value > 0.05), indicating the median of MELD (11) cannot differentiate low *vs* high risk of death (Table 5).

Death and/or LT model

We have developed similar BAS and non-BAS multivariate cox models for the prediction of the adverse events of death and/or LT instead of death only (Supplementary Table 5). Both models were also validated using the same criteria (data not shown). For both 3 and 5-years prediction, AUC was > 0.74 for both models (Supplementary Figure 2 and Supplementary Table 6). Similar to the "death only" models, there were direct relationship between BAS and non-BAS and liver transplant-free survival (Supplementary Figure 3). The estimated mean liver transplant-survival time was 60 mo (4.9 years) for the high-risk group and 79 mo (6.6 years) for the lower risk group based on the median BAS (0.45), which were statistically different (Supplementary Figure 4 and Supplementary Table 7).

DISCUSSION

We developed a survival model based on BA indices to predict the prognosis of hepatobiliary diseases in terms of progressing into the end point/adverse event of death over a 3- and 5-year period of time. Using the multivariate Cox regression analysis, we have constructed these final models for death prediction: (1) The BAS

Table 5 Kaplan-Meier analysis for survival

Cutoff	Total *n*	*n* of events	Estimated mean (mo)	Standard error	95%CI
BAS					
Median cutoff of 2.19					
Low risk < 2.19	128	4	81.68	1.14	79.44-83.93
High risk > 2.19	129	23	70.72	2.5	65.81-75.62
Non-BAS					
Median cutoff of 1.44					
Low risk < 1.44	118	9	78.68	1.70	75.34-82.02
High risk > 1.44	139	18	73.97	2.21	69.64-78.29
MELD					
Median cutoff of 11					
Low risk < 11	133	11	78.06	1.71	74.71-81.42
High risk > 11	124	16	73.91	2.35	69.29-78.52

BAS: Bile acids score; MELD: Model for end-stage liver disease.

model for death prediction**:** BAS for death = 0.039 × %CDCA + 0.052 × %Tri-OH; (2) The non-BAS model model for death prediction**:** non-BAS (non-BAS) for death = 1.236 × AST/ALT. BAS in this population ranged from 0-4, while the non-BAS ranged from 0.44-4.98.

Cholestatic diseases are associated with impaired bile flow to the intestine, which is expected to translate into reduced transformation of primary BA including CDCA and CA into secondary BA by intestinal bacteria. Therefore, accumulation of primary BA in the blood may indicate further impairment in bile flow and worsening of the liver diseases[8,22,23]. This is in agreement with the BAS model, where increased %CDCA and %Tri-OH BA (primarily consists of CA) were the most significant predictors of liver disease prognosis into death. Another interpretation for the accumulation of CDCA could be related to the fact that CDCA is the best substrate for bile salt export pump (BSEP), which is responsible for the efflux transport of BA across the canalicular membrane from hepatocytes into bile. Therefore, loss of BSEP function could be associated with the progression of the liver disease[8,22], which leads to CDCA accumulation in the liver and eventually into the systemic circulation.

Goodness of fit was performed by testing PH assumption using a statistical test and a graphical diagnostic based on Schoenfeld residuals. For death prediction, the PH assumption was met in both BA and non-BA models supporting their validity (Supplementary Figure 1). In addition, we used the bootstrapping method for model validation. Bootstrapping validation results supported the validity of both the BA and non-BA models for death prediction (Supplementary Table 4). Further validation efforts are also ongoing to build internal and eventually external data sets for more rigorous model validation.

We used ROC analysis to compare the accuracy of our prognostic models. The higher the AUC under the ROC curve, the greater the overall accuracy of the marker in distinguishing between groups. For prognostic models, AUC of 0.9 or greater is rarely seen, AUC between 0.8 and 0.9 indicates excellent diagnostic accuracy, and any AUC over 0.7 may be considered clinically useful[23,24]. ROC curves are also used to determine cut-off values which quantify the normal ranges of biomarkers. The selection of optimum cut-off values is a tradeoff between sensitivity and specificity. Accordingly, scores for the BA, non-BA, and MELD models for death prediction of 2.71, 1.72, and 10, respectively, were identified as cut-off values with optimum sensitivity *vs* specificity (Table 3).

For 5-year death prediction, the AUC for BAS was 0.74 compared to 0.65 for non-BAS and 0.68 for MELD models (Figure 1A). Similarly, for 3-year death prediction, the AUC for BAS was 0.76 compared to 0.66 for non-BAS and 0.71 for MELD models (Figure 1B). In addition, BAS sensitivity in death prediction (74% *vs* 67% and 62%) was 7% and 12% higher than non-BAS and MELD, respectively. BAS specificity was also higher than non-BAS and MELD (68% *vs* 66% and 64%). Therefore, ROC analysis show

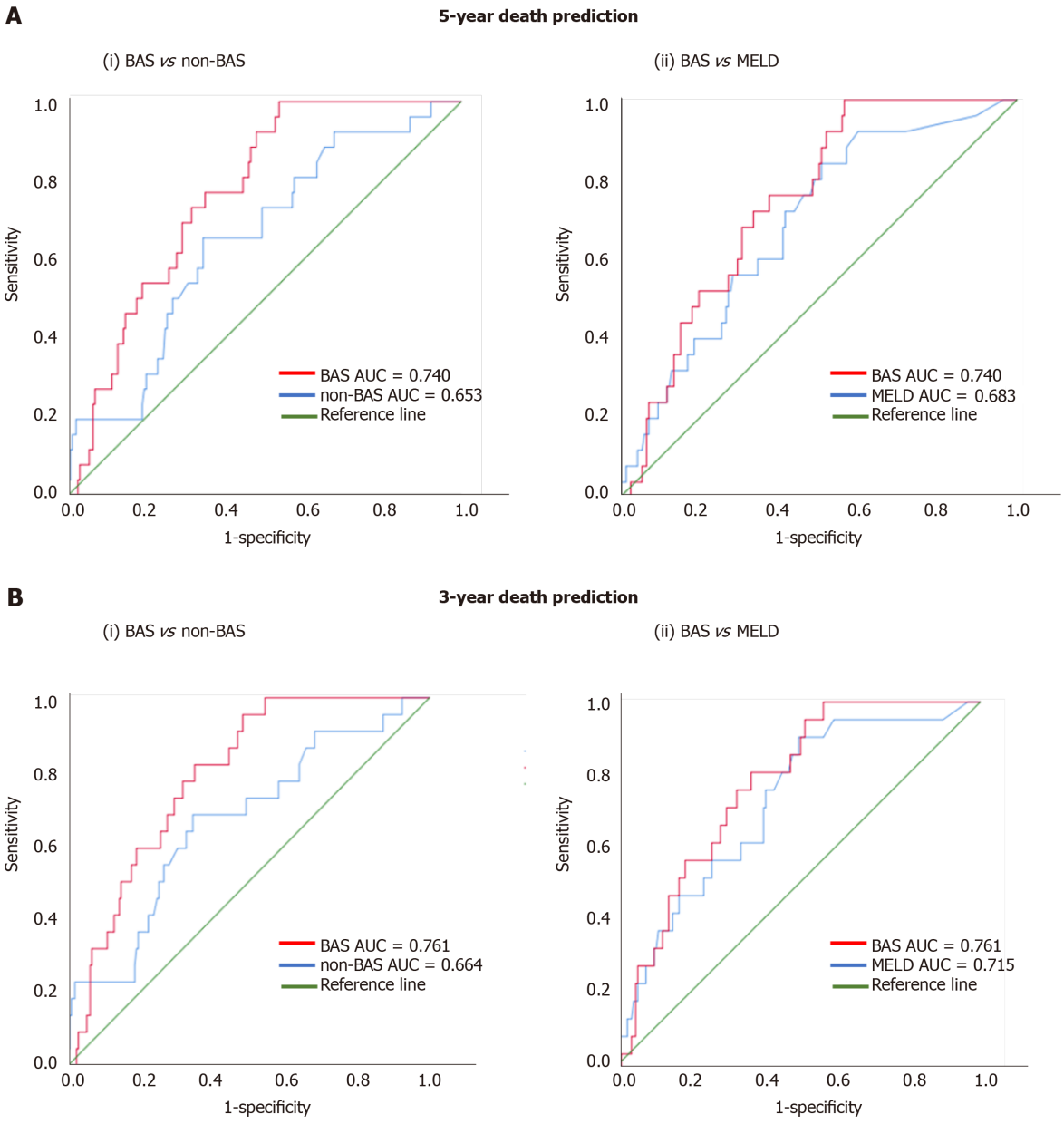

Figure 1 Receiver operating characteristics curves of bile-acids score, non- bile-acids score, and model for end stage liver diseases for death prediction. A: The area under the receiver operating characteristic curves (AUC) for bile-acids score (BAS), non-BAS, and model for end stage liver diseases (MELD) for 5-year death prediction; B: The AUC for BAS, non-BAS, and MELD for 3-year death prediction. AUC: Area under the receiver operating characteristic curves; BAS: Bile-acids score; MELD: Model for end stage liver diseases.

that BAS is more accurate and results in higher true-positive and true-negative prediction of death compared to both non-BAS and MELD.

The Cox survival model can be used to predict the survival probability at any time point. The survival probability for t years [S (t)] was calculated for every subject using both BAS and non-BAS models, as: Survival probability for (t) years: $S(t) = S_0(t)^{\exp(BAS - 2.24)}$, survival probability for (t) years: $S(t) = S_0(t)^{\exp(non\text{-}BAS - 1.58)}$.

Where $S_0(t)$ presents the estimated survival probability for a patient with an average BAS of 2.24 or non-BAS of 1.58 for different time points (Table 4).

As shown in Figure 2, both 5- and 3-year survival probabilities decrease as a function of both BA and non-BAS. For example, the 3-year survival probability for patients with BAS of 1.2 (25th percentile of the population), 2.1 (50th percentile of the population *i.e.* median), and 3.1 (75th percentile of the population) are 98%, 94%, and 85%, respectively. While, the 3-year survival probability for patients with equivalent non-BAS (25th, 50th, and 75th population percentiles) are 95%, 91%, and 86%, respectively.

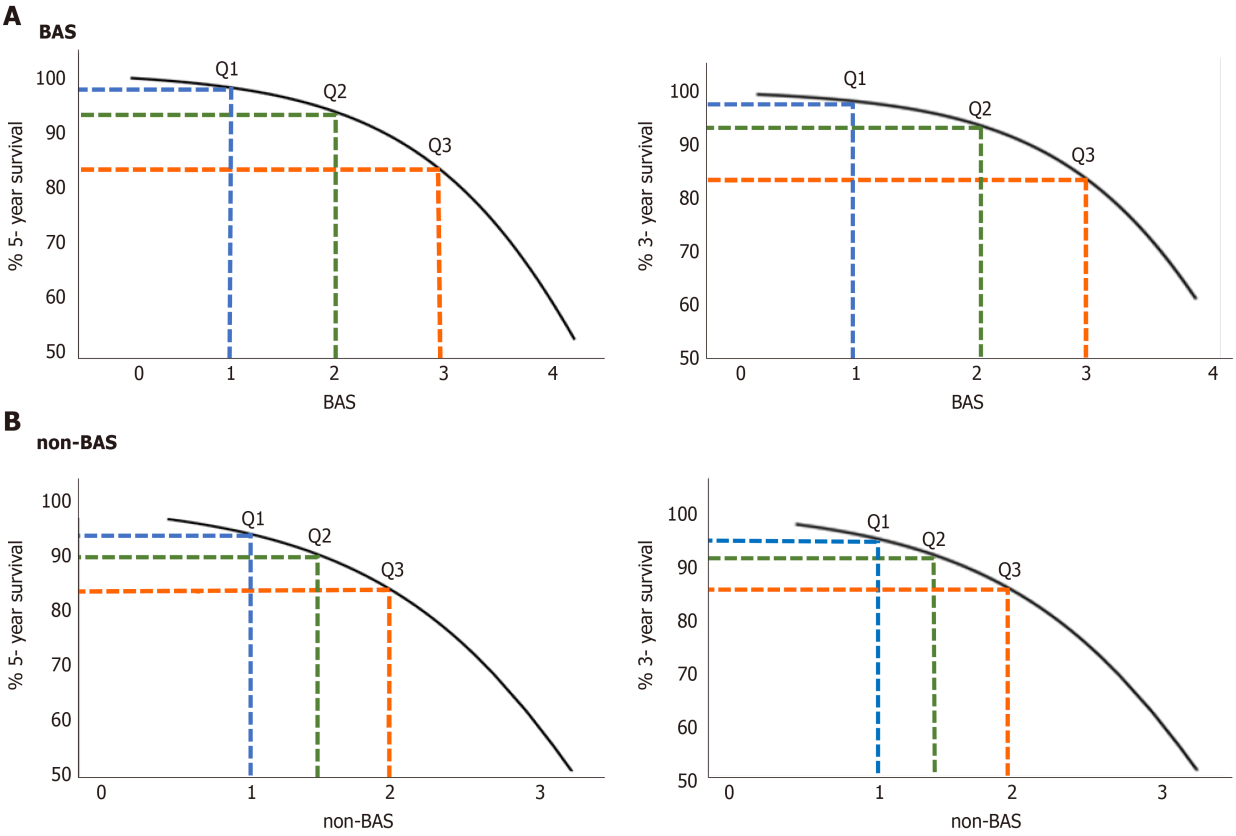

Figure 2 Estimated 5- and 3-year survival [S (*t*)] from the bile-acids score and non- bile-acids score models. A: The relationship between estimated 5- and 3- year survival probability [S (*t*)] as a function of bile-acids score (BAS); B: The relationship between estimated 5- and 3- year survival probability [S (*t*)] as a function of non-BAS. Q1, Q2, and Q3 are 25th, 50th, and 75th percentiles of the population, respectively. BAS: Bile-acids score.

The Kaplan-Meier estimator was used to estimate subjects' survival free of adverse event over time. Median cut-off for BAS (2.19) was able to differentiate low *vs* high risk of death. While the median cut-offs for non-BAS and MELD were not able to differentiate low *vs* high risk of death (Figure 3 and Table 5).

Twenty-three patients with high BAS (> the median BAS of 2.19) died *vs* four patients with low BAS (< the median BAS of 2.19) for the entire study. Therefore, 19 more patients died with high compared to low BAS. In contrast, nine and five more subjects with high non-BAS and high MELD have died compared to low non-BAS and low MELD, respectively. Also, patients with low BAS lived for an average of 82 mo, while patients with high BAS lived for an average of 71 mo since their diagnosis with the liver diseases. Therefore, patients with low BAS lived 11 mo longer than patients with high BAS. On the other hand, patients with low non-BAS or low MELD (< median score), lived, in average, for only five or four months longer, compared to the high non-BAS or high MELD (high score), respectively (Table 5). Consequently, the shortening of lifespan between patients with high *vs* low BAS was 6-7 mo more compared to high non-BAS or high MELD. Also, the number of deaths with high BAS is 2-4-fold higher than that with high non-BAS or high MELD. Therefore, it can be concluded that in this patient population, patients with high BAS are at a much higher risk of death compared to patients with high MELD or high non-BAS.

Similar conclusions can be made regarding the death and/or LT prediction models. Patients with high BAS lived without need for LT 2-5 mo less than patients with high non-BAS or high MELD. Therefore, patients with high BAS are at a higher risk of death and/or LT compared to patients with high MELD or high non-BAS (Supplementary Figures 2-4) and (Supplementary Tables 5-7).

CONCLUSION

In summary, we have developed and validated a survival model based on BA (the BAS model) indices to predict the prognosis of cholestatic liver diseases. Our results demonstrate that the BAS model is more accurate and results in higher true-positive

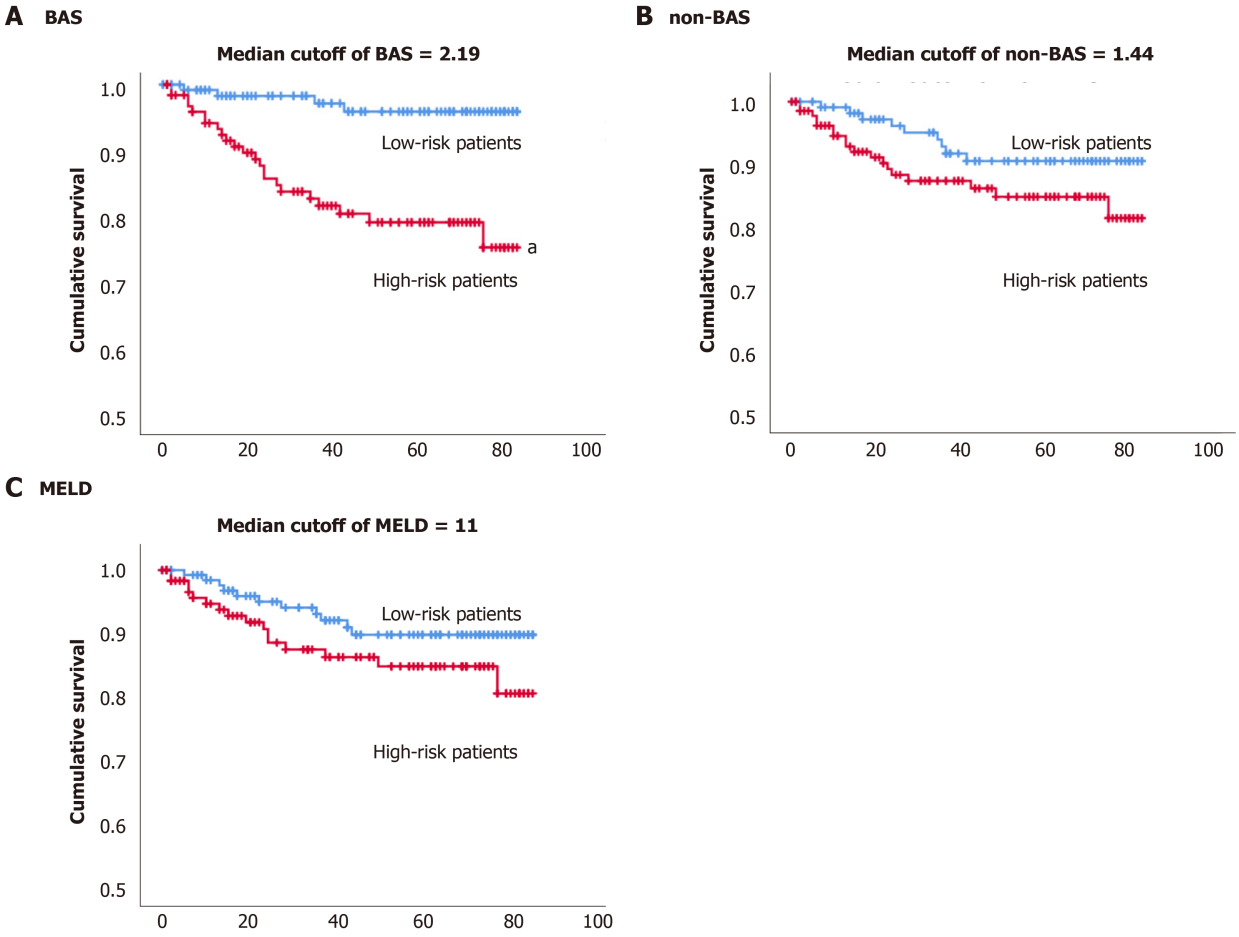

Figure 3 Kaplan-Meier survival plots for high *vs* low bile-acids score, non- bile-acids score, and models for end stage liver diseases. [a]*P* value < 0.05 from the Log rank and Breslow tests. A: The median cutoff value of the bile-acids score (BAS) was used to define high *vs* low risk of death; B: The median cutoff value of the non-BAS was used to define high *vs* low risk of death; C: The median cutoff value of the model for end stage liver diseases was used to define high *vs* low risk of death. BAS: Bile-acids score; MELD: Model for end stage liver diseases.

and true-negative prediction of death compared to both non-BAS and MELD models. Both 5- and 3-year survival probabilities markedly decreased as a function of BAS. Moreover, patients with high BAS had a 4-fold higher rate of death and lived for an average of 11 mo shorter than subjects with low BAS. The increased risk of death with high *vs* low BAS was also 2-4-fold higher and the shortening of lifespan was 6-7-mo lower compared to MELD or non-BAS. Similarly, we have shown the use of BAS to predict the survival of patients with and without LT. Therefore, BAS could be used to define the most seriously ill patients, who need earlier intervention such as LT. This will help provide guidance for timely care for liver patients.

ARTICLE HIGHLIGHTS

Research background

Most cholestatic diseases progress toward end stage liver failure, which likely requires liver transplantation. Numerous clinical and preclinical studies have shown up to a 100-fold increase in bile acids (BA) concentrations in urine with various hepatobiliary diseases. However, due to their high inter-and intra-individual variability, BA has not been used in clinic as markers for the diagnosis and prognosis of liver diseases. To this end, we have developed the concept of BA indices and utilized it to build a survival model to predict the prognosis of liver diseases.

Research motivation

Biomarkers currently used in the clinic for the diagnosis and prognosis of liver diseases are primarily serum liver enzymes. Model for end-stage liver disease (MELD)

was developed to predict three-month mortality of patients with end-stage liver disease. MELD is based on three objective laboratory variables that are not necessarily liver specific. The potential use of BA as a marker for liver diseases has never translated into a widespread use in the clinic. To this end, we have developed the concept of BA indices and utilized it to build a survival model to predict the prognosis of liver diseases.

Research objectives

The objective of this project was to discover and validate prognostic biomarkers of cholestatic liver diseases based on the urinary BA profile. We investigated the use of the urinary BA profile to develop survival models to predict the prognosis of hepatobiliary diseases. One application for BAS could be to define the most seriously ill liver patients, who may need earlier intervention such as liver transplantation.

Research methods

Sample analysis: Liquid chromatography-tandem mass spectrometry. Statistical analysis: univariate and multivariate Cox proportional hazards regression, testing proportional hazards assumption, receiver operating characteristic curve, survival probability, and Kaplan-Meier plots.

Research results

The bile-acid score (BAS) model (a survival model based on BA indices) was more accurate and results in higher true-positive and true-negative prediction of death compared to both non-BAS and MELD models. Both 3- and 5-year survival probabilities markedly decreased as a function of BAS. Patients with high BAS had a 4-fold higher rate of death and lived for an average of 11 mo shorter than subjects with low BAS. The increased risk of death with high *vs* low BAS was also 2-4-fold greater and the shortening of lifespan was 6-7-mo lower compared to MELD or non-BAS.

Research conclusions

We have developed and validated a survival model (the BAS model) based on BA indices to predict the prognosis of cholestatic liver diseases.

Research perspectives

BAS could be used to define the most seriously ill patients, who need earlier intervention such as liver transplant. This will help provide guidance for timely care for liver patients.

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ORIGINAL ARTICLE

Case Control Study

Gut dysbiosis is associated with poorer long-term prognosis in cirrhosis

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Abstract

BACKGROUND

Gut dysbiosis is common in cirrhosis.

AIM

To study the influence of gut dysbiosis on prognosis in cirrhosis.

METHODS

The case-control study included 48 in-patients with cirrhosis and 21 healthy controls. Stool microbiome was assessed using 16S ribosomal ribonucleic acid gene sequencing. We used modified dysbiosis ratio (MDR): [*Bacilli* (%) + *Proteobacteria* (%)]/[*Clostridia* (%) + *Bacteroidetes* (%)]. Patients with MDR more the median made up the group with severe dysbiosis, others did the group with non-severe dysbiosis. The follow-up period was 4 years.

RESULTS

The mortality rate of patients with severe dysbiosis was significantly higher than that of patients with non-severe dysbiosis (54.2% *vs* 12.5%; $P = 0.001$). The presence of severe dysbiosis was independent risk factors for death [hazard ratio = 8.6 × (1.9-38.0); $P = 0.005$]. The abundance of *Enterobacteriaceae* ($P = 0.002$), *Proteobacteria* ($P = 0.002$), and *Lactobacillaceae* ($P = 0.025$) was increased and the abundance of *Firmicutes* ($P = 0.025$) and *Clostridia* ($P = 0.045$) was decreased in the deceased patients compared with the survivors. The deceased patients had a higher MDR value than the survivors [0.131 × (0.069-0.234) *vs* 0.034 × (0.009-0.096); $P = 0.004$]. If we applied an MDR value of 0.14 as the cutoff point, then it predicted patient death within the next year with a sensitivity of 71.4% and a

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specificity of 82.9% [area under the curve = 0.767 × (0.559-0.974)]. MDR was higher in patients with cirrhosis than in health controls [0.064 × (0.017-0.131) *vs* 0.005 × (0.002-0.007); $P < 0.001$], and in patients with decompensated cirrhosis than in patients with compensated cirrhosis [0.106 × (0.023-0.211) *vs* 0.033 × (0.012-0.074); $P = 0.031$]. MDR correlated negatively with prothrombin ($r = -0.295$; $P = 0.042$), cholinesterase ($r = -0.466$; $P = 0.014$) and serum albumin ($r = -0.449$; $P = 0.001$) level and positively with Child–Turcotte–Pugh scale value ($r = 0.360$; $P = 0.012$).

CONCLUSION
Gut dysbiosis is associated with a poorer long-term prognosis in cirrhosis.

Key Words: Cirrhosis; Dysbiosis; Gut; ROC-analysis; Microbiota; Microbiome; Gut-liver axis

Core Tip: The mortality rate of patients with severe dysbiosis was significantly higher than that of patients with non-severe dysbiosis. The abundance of *Enterobacteriaceae*, *Proteobacteria*, and *Lactobacillaceae* was increased and the abundance of *Firmicutes* and *Clostridia* was decreased in the deceased patients compared with survivors. The abundance of *Bacilli*, *Enterococcaceae* and *Lactobacillaceae* was higher and the abundance of *Clostridia* was lower in those who died during the first year of follow-up compared with those who survived this year. The abundance of *Enterobacteriaceae* and *Proteobacteria* was higher in those who died in 2nd-4th years of follow-up compared with survivors.

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INTRODUCTION

Microbiota are stable ecological communities of microorganisms in certain habitats[1]. Recently, the human microbiota has attracted the attention of researchers. Previous studies have shown that its composition varies in different diseases, and it has been hypothesized that the pathology of the human microbiota (dysbiosis) can participate in the pathogenesis of these diseases[2].

As the gut microbiota is the richest human microbiota, most research has been devoted to it. The gut microbiota plays an important role in human life; it digests non-digestible carbohydrates as well as generates vitamins and short-chain fatty acids (butyrate is particularly prominent), which are used as a source of energy by colonocytes. This function is performed by strict anaerobes of the main taxa of normal microbiota, which belong to the *Clostridia* class and *Bacteroidetes* phylum. Nevertheless, the gut microbiota can also play a pathogenic role because it has potential pathogenic bacteria, which belong to the *Bacilli* class (*Streptococcaceae*, *Enterococcaceae*) and *Proteobacteria* phylum (*Enterobacteriaceae*). In addition, facultative anaerobes of the *Bacilli* class and *Proteobacteria* phylum can enter the gut wall, mesenteric lymph nodes, portal, and systemic blood flow. This phenomenon is called bacterial translocation. The gut microbiota is also the main source of endotoxin (lipopolysaccharide), a toxic substance of gram-negative bacteria, primarily *Proteobacteria*[3].

To date, several articles[4-6] have been published that describe alterations of the gut microbiome in cirrhosis. Researchers have shown that the abundance of harmful *Proteobacteria* increases, whereas the abundances of useful *Ruminococcaceae* and *Lachnospiraceae* belonging to the *Clostridia* class decrease in the gut microbiome in cirrhosis.

Analysis of the relationship between gut dysbiosis and the course of cirrhosis is complicated by several problems. The first is the fact that the only reliable method for

analyzing the gut microbiota is sequencing, which is very expensive and requires a rare bioinformatics specialist. Therefore, the study of dysbiosis has not yet transcended the walls of scientific laboratories and entered clinical medicine.

The second problem is the interpretation of obtained data. The researcher acquires a huge amount of redundant data after sequencing. A generalizing indicator should be used to simplify the analysis. Several such indicators have been proposed, including the richness and diversity of microbiota, the *Firmicutes/Bacteroidetes* ratio[7], and the cirrhosis dysbiosis ratio (CDR)[5]. However, these indicators have various disadvantages; first of all, many of them have a weak theoretical basis. Thus, the proliferation of harmful bacteria can lead to an increase in the richness and diversity of microbiota. However, the proliferation of beneficial bacteria can lead to similar changes; therefore, an increase or decrease in these indicators cannot be correctly interpreted. *Firmicutes* is too heterogeneous and represented by useful members of the *Clostridia* class as well as potentially pathogenic members of the *Bacilli* class. In addition, the *Firmicutes/Bacteroidetes* ratio does not take into account *Proteobacteria* that are the main potentially pathogenic bacteria. *Bacteroidetes* has a multifaceted effect on the macroorganism and cannot be considered as only harmful bacteria. Therefore, the *Firmicutes/Bacteroidetes* ratio may be useful for comparing the gut microbiota between countries, between individuals on different diets, or for assessing changes in the microbiota with age, but it cannot show how much better or worse the composition of the microbiota has become.

The CDR proposed by Bajaj *et al*[5] is based on the ratio of "good" to "bad" bacteria. However, it also has some disadvantages. Its values decrease with an increase in the severity of dysbiosis, which can lead to misinterpretation. *Bacteroidaceae* were among the "bad" bacteria, but they play a rather neutral role in the gut microbiome and are widely represented in the microbiomes of healthy individuals, especially in studies from Asian countries[4]. In addition, *Bacteroidaceae*, being strict anaerobes, cannot be subjects of bacterial translocation[8]. At the same time, the list of "bad" bacteria did not include *Bacilli*, which together with *Enterobacteriaceae* are responsible for bacterial translocation[8] and secondary infections[3,9,10] in cirrhosis.

Thus, the development and testing of a pathogenetically-based dysbiosis ratio remains an important task. With this ratio, it will be possible to replace expensive and inaccessible sequencing with polymerase chain reaction (PCR) for selected taxa *via* automatic ratio calculation, which will allow for the introduction of gut dysbiosis tests into clinical practice.

The second important task of studying gut dysbiosis in cirrhosis is to clarify whether its presence affects the prognosis of patients.

Identifying a solution to these two problems is the aim of the present research.

MATERIALS AND METHODS

Theoretical substantiation of the modified dysbiosis ratio

We used the CDR as a basis but flipped the equation such that the "bad" bacteria were in the numerator and the "good" bacteria were in the denominator. Therefore, our modified dysbiosis ratio (MDR) increased with aggravation of dysbiosis, which was less confusing in its interpretation. We considered *Proteobacteria* and *Bacilli* as "bad" bacteria since they are responsible for bacterial translocation as well as the development of secondary infections[3,8-10] and their contents increase in cirrhosis[4,5]. We used the dominant taxa in healthy individuals, *Clostridia* and *Bacteroidetes*, as "good" bacteria. These taxa are strict anaerobes; therefore, they do not undergo bacterial translocation and do not cause extraintestinal secondary infections in cirrhosis[3,8-10]. *Clostridia* predominate in the American population, where the Western diet is widespread, whereas *Bacteroidetes* are more common in the Asian population, where the Eastern diet is widespread[4,5]. Thus, the total accounting of these taxa is also intended to reduce the effect of diet on the value of the MDR. The abundance of *Clostridia* has been found to decrease with the development of cirrhosis[4,5]. The changes in Bacteroidetes abundance in cirrhosis appear to vary across different studies[4-6].

Thus, the pathogenesis- and evidence-based MDR was calculated as follows: [*Bacilli* (%) + *Proteobacteria* (%)]/[*Clostridia* (%) + *Bacteroidetes* (%)].

Patients

In this case-control prospective study, 113 consecutive patients with cirrhosis were admitted to the Department of Hepatology of Clinic for Internal Diseases, Gastroen-

terology and Hepatology at Sechenov University (Moscow, Russia) and screened for inclusion. The study procedures were explained to potential participants, and written informed consent was obtained before enrollment. The present study was approved by the Ethics Committee of Sechenov University in accordance with the Declaration of Helsinki (№03-16).

The inclusion criteria were as follows: diagnosis of cirrhosis verified by histology or clinical, biochemical, and ultrasound findings; and age between 18 and 70 years. The exclusion criteria were as follows: use of lactulose, lactitol, or other prebiotics, probiotics, antibiotics, or metformin in the past 6 wk; alcohol consumption in the past 6 wk; or inflammatory bowel disease, cancer, or any other serious disease. Of the original 113 patients screened for inclusion, 48 met the criteria and were enrolled in the study while 65 were excluded (Figure 1).

A study control group consisted of 21 healthy individuals who visited the clinic for routine health examinations during the same period.

The severity of liver disease was determined using the Child–Turcotte–Pugh (CTP) scoring system[11], in which class A was defined as compensated cirrhosis and classes B and C were defined as decompensated cirrhosis.

Gut microbiome analysis

The morning after admission, a stool sample was taken into a sterile disposable container and immediately frozen at -80 °C[12].

Deoxyribonucleic acid from the stool was isolated using the MagNa Pure Compact Nucleic Acid Isolation Kit I (Roche, Basel, Switzerland) according to the manufacturer's instructions. Libraries for sequencing were prepared by two rounds of PCR amplification. In the first round, specific primers for the v3-v4 region of the 16S ribosomal ribonucleic acid (RNA) gene were used: 16S-F: TCGTCGGCA-GCGTCAG-ATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and 16S-R: GTCTCGTGG-GCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC.

After amplification, the PCR product was purified using AMPure XP magnetic beads (Beckman Coulter, Brea, CA, United States). Then, a second round of PCR was performed to attach specific adapters and enable multiplexing of the samples. To begin, 5 μL of the first PCR product was added to the reaction after ball cleaning with primers containing Illumina indices (Nextera XT Index v2 Primers; San Diego, CA, United States) and adapter sequences as well as 2 × KAPA HiFi HotStart ReadyMix. The amplification products were also purified using AMPure XP beads (Beckman Coulter). The concentrations of the prepared libraries were then measured using a Qubit 2.0 fluorimeter (London, United Kingdom) and quantitative PCR. The quality of the libraries was assessed using the Agilent 2100 Bioanalyzer (Santa Clara, CA, United States). The libraries were mixed in equal proportions and diluted to the required concentration to be run on a MiSeq (Illumina) device. Pair-end readings of 300 + 300 nucleotides were obtained. Reads were trimmed from the 3'-tail with Trimmomatic (Illumina) and then merged into a single amplicon with the MeFiT tool[13,14]. We did not perform operational taxonomic unit picking; instead, we classified amplicon sequences with the Ribosomal Database Project (RDP) classifier and RDP database[15].

Follow-up

The patients were contacted by phone every 3 mo to confirm that they were alive. If there was no answer, we contacted the patient's relatives by phone to find out if the patient was alive or dead. If it was not possible to contact them, we studied patient electronic medical records in the United Medical Information and Analytical System of Moscow, in which death registration data are entered. The follow-up period was 4 years.

Statistical analysis

Statistical analysis was performed with STATISTICA 10 (StatSoft Inc., Tulsa, OK, United States) and SPSS Statistics (IBM SPSS, Armonk, NY, United States) software. The data are presented as medians (interquartile ranges). Differences between continuous variables were assessed with the Mann-Whitney test because many variables were not distributed normally. Fisher's exact test was used to assess the differences between categorical variables. Survival was assessed using the Kaplan-Meier estimator and Cox regression test. A Cox regression model was constructed to assess the influence of various factors on patient survival and hazard ratios (HRs). Correlations between variables were computed using Spearman's rank correlation. P values ≤ 0.05 were considered as statistically significant.

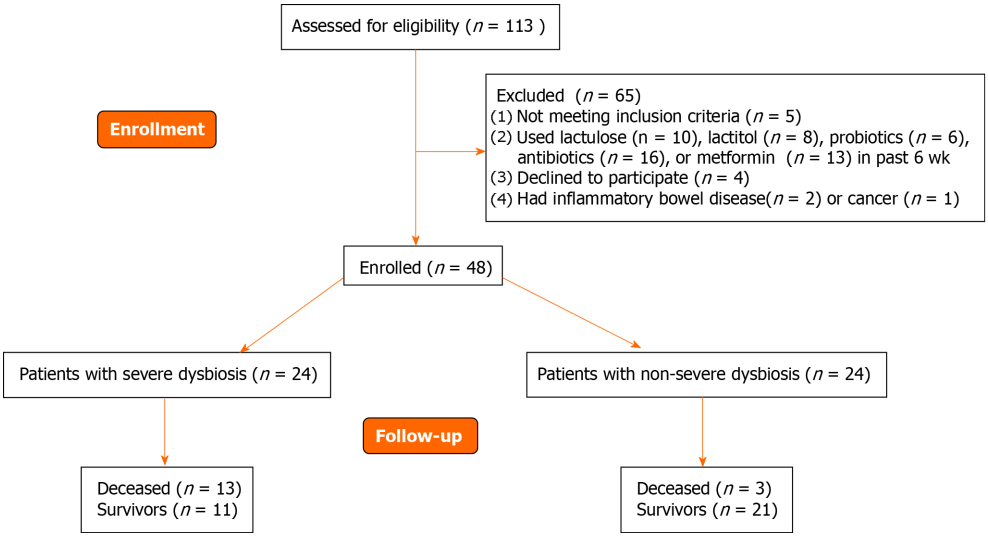

Figure 1 CONSORT 2010 flow diagram.

RESULTS

Participants with cirrhosis and healthy controls were comparable in age [51 (40-59) *vs* 46 (42-54) years; P = 0.489], body mass index [24.6 × (22.7-27.7) *vs* 26.3 × (25.1-29.0) kg/m^2; P = 0.110], and sex distribution (male/female: 23/25 *vs* 8/13; P = 0.313).

Seventeen participants with cirrhosis had compensated cirrhosis (CTP class A), and the remaining 31 had decompensated cirrhosis (19 class B and 12 class C). Participants with compensated cirrhosis and decompensated cirrhosis were also comparable in age [49 (38-55) years *vs* 52 (40-59) years, P = 0.316], body mass index [24.8 × (21.8-27.8) kg/m^2 *vs* 24.4 × (22.8-27.7) kg/m^2; P = 0.771], and sex distribution (6/11 *vs* 17/14; P = 0.160).

The MDR was higher in patients with cirrhosis than in healthy controls [0.064 × (0.017-0.131) *vs* 0.005 × (0.002-0.007); P < 0.001] and in patients with decompensated cirrhosis than in those with compensated cirrhosis [0.106 × (0.023-0.211) *vs* 0.033 × (0.012-0.074); P = 0.031]. When taken as the cutoff point, an MDR value of 0.01 made it possible to distinguish patients with cirrhosis from healthy individuals with a sensitivity of 81.3% and a specificity of 90.5% [AUC = 0.884 × (0.806-0.962)] (Figure 2). The specificity approached nearly 100% with a cutoff value of 0.02.

If we used the median MDR (0.064) as a cutoff point, then the group of patients with cirrhosis could be divided into patients with severe (n = 24) and non-severe (n = 24) dysbiosis (Figure 1).

The abundance of useful *Clostridia* was reduced and that of harmful *Bacilli* was increased, whereas the abundance of harmful *Enterobacteriaceae* was not significantly changed in patients with non-severe dysbiosis compared to healthy controls. The abundance of *Clostridia* further decreased, the abundance of *Bacilli* further increased, and the abundance of *Enterobacteriaceae* also increased in patients with severe dysbiosis. Interestingly, an increase in the abundance of *Bifidobacteriaceae* considered beneficial to the gut microbiome was also observed in cirrhosis without significant differences between groups with different degrees of dysbiosis. The abundance of *Bacteroidetes* did not differ significantly between patients with cirrhosis and healthy individuals (Table 1).

There were no significant differences in age, body mass index, sex distribution, and etiology of cirrhosis between patients with severe and non-severe dysbiosis. Patients with severe dysbiosis had lower serum albumin and cholinesterase levels, higher CTP scale values, and higher C-reactive protein levels. Although the incidences of ascites, esophageal varices, and hepatic encephalopathy were higher in patients with severe dysbiosis than in those with non-severe dysbiosis, these differences did not reach the significance level. There were no differences between the groups of patients in red blood cell, white blood cell, and platelet counts; creatinine, sodium, potassium, and glucose levels; and aminotransferase, alkaline phosphatase, and gamma-glutamate transferase activities (Table 2).

The MDR correlated negatively with prothrombin (r = -0.295; P = 0.042), cholinesterase (r = -0.466; P = 0.014) and serum albumin (r = -0.449; P = 0.001) levels and positively with CTP scale values (r = 0.360; P = 0.012).

Table 1 Comparison of the gut microbiome at different taxonomic levels between the groups

Taxa	Heath controls (*n* = 21)	Cirrhosis with non-severe dysbiosis (*n* = 24)	Cirrhosis with severe dysbiosis (*n* = 24)	*P* value, Non-severe dysbiosis *vs* controls	*P* value, Severe dysbiosis *vs* controls	*P* value, Severe dysbiosis *vs* non-severe one
Firmicutes	91.8 (89.3-96.4)	89.7 (73.0-93.6)	80.1 (62.7-88.1)	0.074	< 0.001	0.028
Clostridia	88.0 (86.6-91.7)	83.5 (69.8-88.7)	69.8 (57.4-77.2)	0.008	< 0.001	0.001
Ruminococcaceae	33.9 (28.1-41.6)	27.6 (19.2-36.5)	18.8 (7.9-31.7)	0.086	0.002	0.081
Lachnospiraceae	43.8 (37.2-54.6)	37.6 (27.2-60.5)	31.0 (22.1-46.0)	0.488	0.030	0.190
Bacilli	0.1 (0.0-0.2)	0.5 (0.2-1.9)	7.1 (1.3-14.8)	< 0.001	< 0.001	< 0.001
Streptococcaceae	0.03 (0.02-0.10)	0.29 (0.12-0.52)	3.20 (0.38-10.4)	< 0.001	< 0.001	0.002
Lactobacillaceae	0.00 (0.00-0.01)	0.02 (0.01-0.22)	0.47 (0.12-1.50)	0.002	< 0.001	< 0.001
Enterococcaceae	0.00 (0.00-0.00)	0.00 (0.00-0.03)	0.03 (0.01-0.08)	0.067	0.001	< 0.001
Bacteroidetes	5.6 (2.8-8.1)	5.7 (1.8-12.9)	6.1 (3.2-8.2)	0.954	0.829	0.959
Bacteroidaceae	2.5 (0.8-3.4)	1.3 (0.6-4.3)	1.4 (0.2-3.8)	0.991	0.432	0.261
Actinobacteria	0.2 (0.1-0.3)	0.8 (0.3-2.8)	0.7 (0.4-2.9)	< 0.001	< 0.001	0.687
Bifidobacteriaceae	0.1 (0.0-0.2)	0.6 (0.1-2.6)	0.5 (0.2-2.3)	0.002	0.001	0.687
Proteobacteria	0.39 (0.14-0.51)	0.15 (0.10-0.81)	3.57 (1.77-6.65)	0.869	< 0.001	< 0.001
Enterobacteriaceae	0.03 (0.01-0.05)	0.04 (0.02-0.61)	2.70 (1.58-6.24)	0.104	< 0.001	< 0.001

The mortality rate of patients with severe dysbiosis was significantly higher than that of patients with non-severe dysbiosis (54.2% *vs* 12.5%; $P = 0.001$). Moreover, the difference in mortality was insignificant in the first year of follow-up (20.8% *vs* 8.3%; $P = 0.092$) and significant in subsequent years of follow-up (33.4% *vs* 4.2%; $P = 0.002$) (Figure 3).

Deceased patients had a higher MDR value than the survivors [0.131 × (0.069-0.234) *vs* 0.034 × (0.009-0.096); $P = 0.004$]. Moreover, this was observed in the deceased in the first year of follow-up [0.191 × (0.035-1.126) *vs* 0.046 × (0.012-0.115); $P = 0.022$] as well as in subsequent years [0.115 × (0.074-0.144) *vs* 0.034 × (0.009-0.096); $P = 0.044$].

If we took an MDR value of 0.05 as the cutoff point, it predicted patient death within the next 4 years with a sensitivity of 65.2% and a specificity of 81.3%. If we used 0.11 for this, then the sensitivity was 81.3% and the specificity was 62.5% [AUC = 0.755 × (0.611-0.899); Figure 4A].

If we applied an MDR value of 0.14 as the cutoff point, then it predicted patient death within the next year with a sensitivity of 71.4% and a specificity of 82.9% [AUC = 0.767 × (0.559-0.974); Figure 4B].

The presence of severe dysbiosis [HR = 8.6 × (1.9-38.0); $P = 0.005$] and total serum bilirubin level [HR = 1.005 × (1.001-1.010); $P = 0.015$] were independent risk factors for death, unlike serum albumin ($P = 0.870$) and prothrombin ($P = 0.167$) levels, degrees of ascites ($P = 0.752$), and esophageal varices ($P = 0.230$).

In addition, death in the first year of follow-up was significantly determined by serum albumin level [HR = 0.83 × (0.71-0.97); $P = 0.020$], unlike degrees of ascites ($P = 0.619$), dysbiosis ($P = 0.241$), total serum bilirubin ($P = 0.742$) and prothrombin levels ($P = 0.386$), and esophageal varices ($P = 0.125$). However, mortality in subsequent years of follow-up was determined significantly by the degree of dysbiosis only [HR = 24.8 × (2.3-269.6); $P = 0.008$].

The abundances of *Enterobacteriaceae* [2.4 × (1.6-7.6) *vs* 0.4 × (0.0-1.7)%; $P = 0.002$], *Proteobacteria* [3.4 × (1.9-8.2) *vs* 0.6 × (0.1-2.0)%; $P = 0.002$], and *Lactobacillaceae* [0.35 × (0.12-0.81) *vs* 0.06 × (0.01-0.31)%; $P = 0.025$] were increased, and the abundances of *Firmicutes* [78.8 × (62.7-85.6) *vs* 87.1 × (71.7-93.6)%; $P = 0.025$] and *Clostridia* [73.0 ×

Table 2 Main indicators of patients with cirrhosis with severe and non-severe dysbiosis

	Severe dysbiosis (*n* = 24)	Non-severe dysbiosis (*n* = 24)	*P* value
Age, yr	51.5 (42.0-59.0)	50.0 (35.0-57.5)	0.392
Body mass index, kg/m^2	24.6 (22.8-27.7)	24.2 (22.7-27.7)	0.837
Male/female	12/12	11/13	0.500
Etiology of cirrhosis: Alcohol	9	9	0.617
Autoimmune	2	7	0.068
HBV	7	2	0.068
HCV	5	3	0.350
Cryptogenic	1	3	0.304
Child–Turcotte–Pugh score	9 (8-10)	7 (6-9)	0.047
Death	13	3	0.001
Death within the first year of follow-up	5	2	0.092
Death during the following years of follow-up	8	1	0.002
Esophageal varices (present/absent)	20/4	18/6	0.477
Hepatic encephalopathy (overt/minimal/absent)	11/9/4	6/11/7	0.288
Number connection test, seconds	87 (65-118)	79 (59-92)	0.248
Ascites (present/absent)	16/8	11/13	0.122
Spontaneous bacterial peritonitis (present/absent)	0/24	0/24	1.000
Red blood cells, 10^{12} cell/L	3.8 (3.4-4.0)	3.9 (3.6-4.5)	0.370
White blood cells, 10^9 cell/L	3.8 (2.7-5.3)	3.8 (3.1-5.2)	0.628
Platelets, 10^9 cell/L	87 (55-120)	76 (60-108)	0.860
Serum total protein, g/L	70 (61-76)	73 (64-78)	0.599
Serum albumin, g/L	31 (28-37)	38 (34-41)	0.009
Serum total bilirubin, μmol/L	47 (31-62)	31 (24-63)	0.375
Prothrombin index (Quick test), %	58 (48-67)	64 (54-71)	0.239
Creatinine, mg/dL	0.69 (0.53-0.87)	0.73 (0.66-0.90)	0.187
Serum sodium, mmol/L	141 (139-144)	141 (138-143)	0.795
Serum potassium, mmol/L	4.3 (4.0-4.7)	4.3 (4.1-4.7)	0.844
Serum glucose, mmol/L	5.1 (4.7-5.6)	5.3 (4.7-6.0)	0.260
Alanine aminotransferase, U/L	36 (25-72)	37 (23-60)	0.804
Aspartate aminotransferase, U/L	54 (41-98)	40 (26-67)	0.219
Gamma glutamyl transferase, U/L	77 (40-148)	76 (36-131)	0.621
Alkaline phosphatase, U/L	221 (188-340)	222 (166-298)	0.542
Cholinesterase, kU/L	2.7 (1.9-3.7)	4.0 (3.6-4.5)	0.031
C-reactive protein, mg/L	10.1 (2.1-16.1)	2.1 (0.3-8.9)	0.032
Splenic length, cm	15.4 (14.0-17.6)	16.1 (13.3-19.2)	0.841

HBV: Hepatitis B virus; HCV: Hepatitis C virus.

(51.9-78.2) *vs* 80.1 × (68.5-87.2)%; $P = 0.045$] were decreased in the gut microbiome of deceased patients compared to the survivors.

The abundances of *Bacilli* [14.0 × (1.4-18.4) *vs* 1.1 × (0.3-4.6)%; $P = 0.017$], *Enterococcaceae* [0.09 × (0.04-0.38) *vs* 0.01 × (0.00-0.04)%; $P = 0.005$], and *Lactobacillaceae* [0.45 × (0.24-1.52) *vs* 0.09 × (0.01-0.38)%; $P = 0.021$] were higher, and the abundance of *Clostridia* [67.1 × (31.2-78.2) *vs* 77.5 × (68.5-86.8)%; $P = 0.047$] was lower in those who

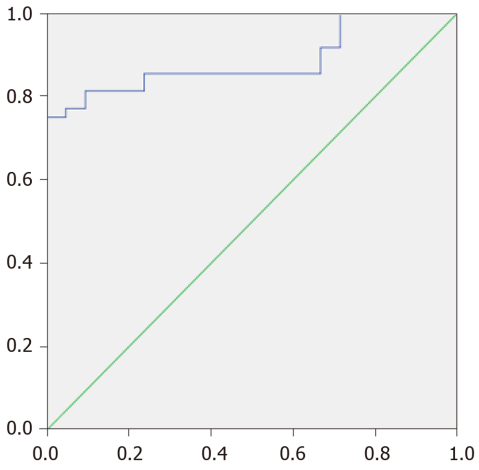

Figure 2 Receiver operating characteristic analysis of modified dysbiosis ratio for distinguish patients with cirrhosis from healthy individuals.

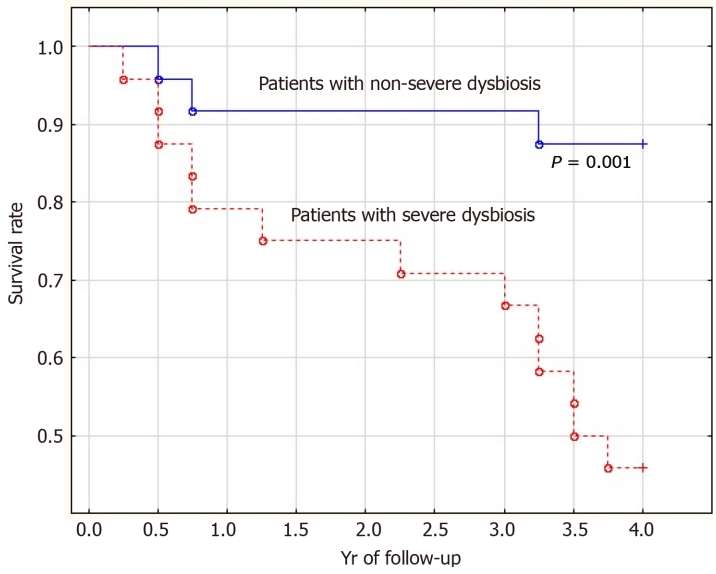

Figure 3 Survival curve (years) of patients with cirrhosis with severe (dotted line) and non-severe (solid line) dysbiosis.

died during the first year of follow-up compared to those who survived the first year. The abundances of *Enterobacteriaceae* [2.2 × (1.8-6.5) *vs* 0.4 (0.0-1.7)%; $P = 0.009$] and *Proteobacteria* [3.8 × (2.5-7.0) *vs* 0.6 × (0.1-2.0)%; $P = 0.010$] were higher in those who died in the second through fourth years of follow-up compared to the survivors. The deceased during the first year of follow-up had higher abundances of *Bacilli* [14.0 × (1.4-18.4) *vs* 0.5 × (0.4-4.2)%; $P = 0.026$] and *Enterococcaceae* [0.09 × (0.04-0.38) *vs* 0.00 × (0.00-0.05)%; $P = 0.002$] than those who died in the next 3 years of follow-up (Figures 5 and 6).

There was no significant difference in the *Firmicutes/Bacteroidetes* ratio between patients with cirrhosis and healthy individuals [13.3 × (7.8-40.9) *vs* 15.8 × (11.2-33.1); $P = 0.469$], the survivors and deceased patients [14.0 × (6.1-51.7) *vs* 12.7 × (8.0-26.4); $P = 0.938$], and patients with compensated and decompensated cirrhosis [16.0 × (7.8-68.7) *vs* 13.1 × (7.9-35.6); $P = 0.846$].

The CDR was significantly lower in patients with cirrhosis than in healthy individuals [16.4 × (7.2-39.0) *vs* 34.9 × (23.0-101.1); $P = 0.002$], in deceased patients than in the survivors [10.5 × (4.5-18.9) *vs* 19.7 × (10.7-57.6); $P = 0.041$], and in decompensated cirrhosis than in compensated cirrhosis [13.1 × (5.0-27.4) *vs* 22.5 × (14.1-65.4); $P = 0.039$]. Using the cutoff value of this ratio equal to 22, we could distinguish between patients with cirrhosis and healthy individuals with a sensitivity of 64.6% and a specificity of 85.7% [AUC = 0.735 × (0.620-0.850)]. The CDR was lower in patients who died in the first year of follow-up compared to those who survived the

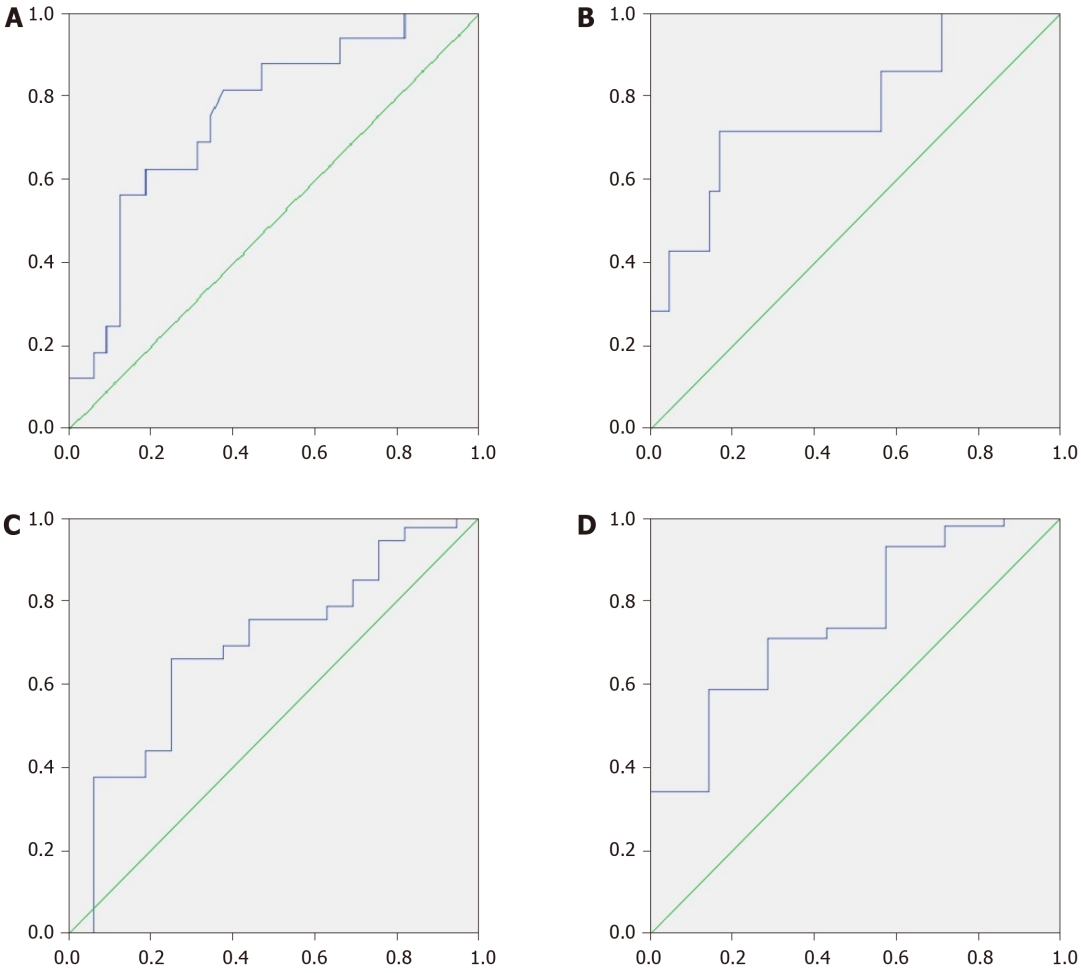

Figure 4 Receiver operating characteristic-analysis of modified dysbiosis ratio in predicting death. A: During 4 years; B: During 1 year; and of cirrhosis dysbiosis ratio in predicting death; C: During 4 years; and D: During 1 year.

first year [9.4 × (1.7-15.4) *vs* 17.7 × (9.0-54.8); $P = 0.035$] but did not differ significantly between those who died in the following years and those who survived [13.6 × (7.3-22.5) *vs* 19.7 × (10.7-57.6); $P = 0.321$].

If we used a CDR value of 15 as the cutoff point, then it predicted patient death within the next 4 years with a sensitivity of 68.8% and a specificity of 62.5% [AUC = 0.684 × (0.522-0.845); Figure 4C] as well as within the first year with a sensitivity of 85.7% and a specificity of 58.5% [AUC = 0.753 × (0.569-0.936); Figure 4D].

DISCUSSION

Translating scientific developments into clinical practice is a rather difficult task. The study of the gut microbiome in various diseases is becoming mainstream in modern science, but thus far, it has no applications in clinical practice. It is hindered by the high cost of sequencing the fecal microbiome and the shortage of bioinformatics specialists.

Therefore, an important step in introducing the study of gut dysbiosis into clinical practice is to replace this expensive method with a simpler and more affordable one. PCR is an ideal candidate to determine the content of selected taxa in feces, followed by a comprehensive assessment of the state of the gut microbiome.

The idea to conduct a comprehensive assessment of the state of the gut microbiome in cirrhosis originated with Bajaj and colleagues[5]. However, their CDR can be improved, which was one of the aims of our study.

Here, we modified the CDR to improve its analytical performance and show that it can be used to predict the death of patients.

First, we inverted the CDR equation, placing the abundance of "bad" bacteria in the numerator and the abundance of "good" bacteria in the denominator. Thus, the value

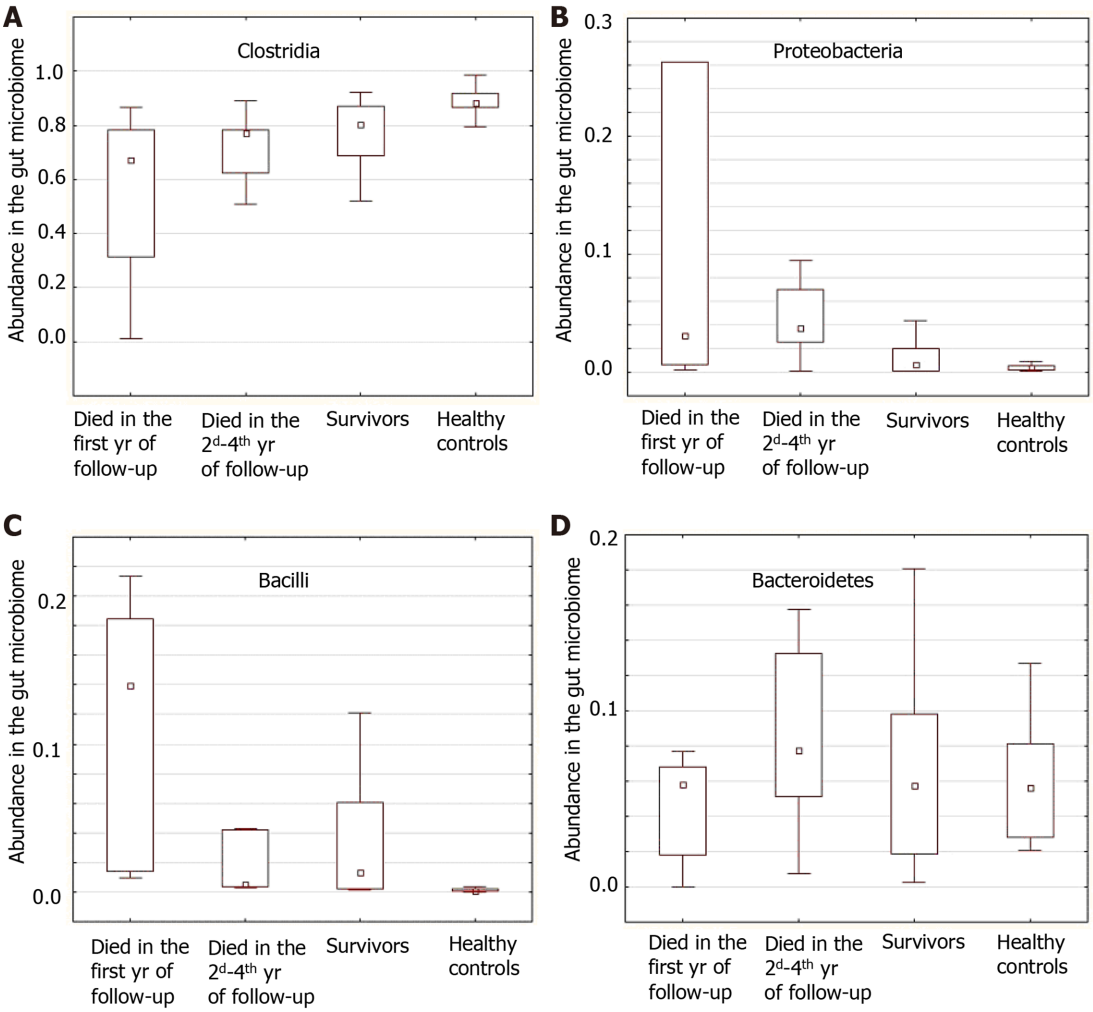

Figure 5 An abundance of the main taxa in patients who died during the first and the subsequent years of follow-up, survivors and healthy controls. The middle point is the median, the box is the interquartile range, the whiskers are non-outlier range. A: *Clostridia*; B: *Proteobacteria*; C: *Bacilli*; and D: *Bacteroidetes*.

of our MDR increases with the aggravation of dysbiosis, which is more logical. The original CDR decreases with the aggravation of dysbiosis, which can be confusing to interpret.

Our MDR is based on the data regarding the role of various taxa in the pathogenesis of cirrhosis complications and changes in their abundance in cirrhosis. We excluded *Bacteroidaceae* from the list of "bad" bacteria since their role in the pathogenesis of cirrhosis is not clear, and the change in their abundance in the gut microbiome in cirrhosis varies according to different researchers. According to our data, it does not change significantly, according to Chen *et al*[4], it decreases, and according to Bajaj *et al*[5], it increases in compensated cirrhosis and decreases in decompensated cirrhosis, becoming almost the same as that in healthy individuals. On the contrary, in a study by Kakiyama *et al*[6], the abundance of *Bacteroidaceae* decreased with compensated cirrhosis and increased with decompensated cirrhosis. Instead, we added *Bacilli* to the list of "bad" bacteria, which, like *Proteobacteria/Enterobacteriaceae*, are responsible for bacterial translocation and the development of extraintestinal infections in cirrhosis[8-10]. The abundances of both of these taxa increased with cirrhosis according to all studies[4-6], including ours.

As "good" bacteria, we used the higher-level taxon *Clostridia*, which includes all taxa accounted as "good" bacteria in the CDR. The main problem is that the abundance of these taxa is highly dependent on diet[16,17]. Among healthy individuals, it was 90% in the Russian population (our data), approximately 45% in the American population[5], and approximately 30% in the Chinese population[4]. However, if you add to them to the abundance of *Bacteroidetes*, which changes in the opposite direction relative to *Clostridia* and *Firmicutes*[16,17], then the differences were not so large: 95%, 80%, and 90%, respectively. This dependence of the *Bacteroidetes* and

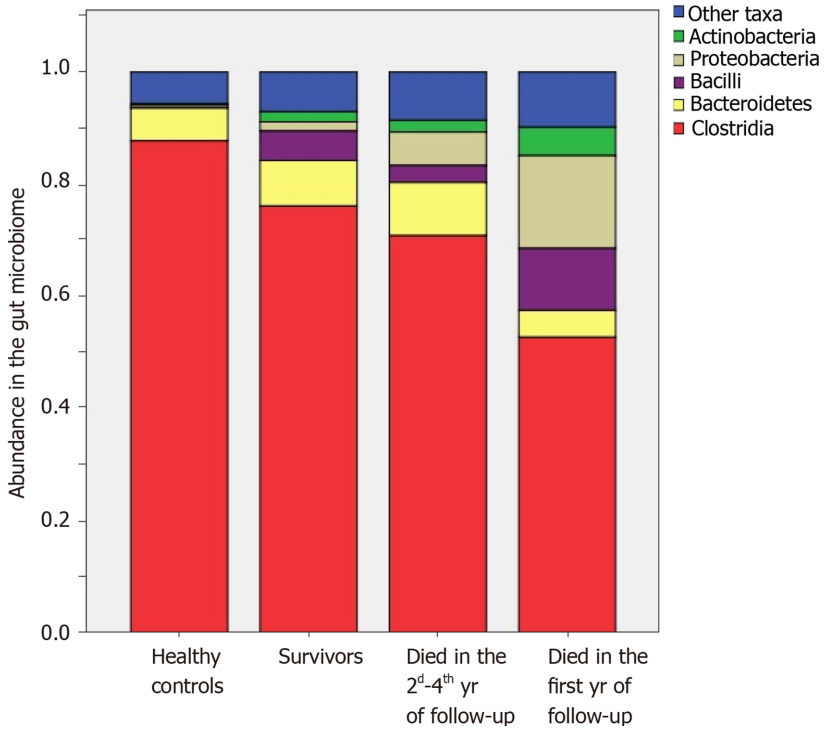

Figure 6 The composition of the gut microbiome in healthy individuals, survivors, and deceased in the first and subsequent 3 years.

Clostridia abundances on diet led to the fact that the value of the CDR in our population was more than an order of magnitude higher than in the original study. Thus, the addition of *Bacteroidetes* to the group of "good" bacteria can neutralize the effect of diet on MDR and allow it to be used in different populations.

In our study, we were able to show that despite the change in the order of values, the CDR retained its main characteristics: it was higher in healthy individuals, lower in patients with compensated cirrhosis, and minimal in patients with decompensated cirrhosis.

Both the CDR and MDR were useful in assessing the prognosis of patients with cirrhosis, but the analytical characteristics of our modification were higher. In particular, the MDR, unlike the CDR, made it possible to assess the long-term (more 1 year) prognosis of patients.

Interestingly, the different taxa included in the MDR had different effects on prognosis. *Clostridia* and *Bacilli* mainly determined the medium-term prognosis (death within a year), and *Proteobacteria* and *Enterobacteriaceae* determined the long-term prognosis (death over the subsequent 3 years). This finding may be due to the fact that *Bacilli* provide a more powerful translocation of living bacteria, which leads to faster death, whereas *Enterobacteriaceae* act mainly by translocating their endotoxin, which leads to a more delayed death.

Thus, we were able to show that the gut microbiome in cirrhosis can be comprehensively and reliably evaluated using targeted analysis of the most significant taxa, which will allow for replacing expensive and poorly available sequencing with cheaper and more affordable PCR for four indicators (*Proteobacteria*, *Bacilli*, *Clostridia*, and *Bacteroidetes*) that does not require interpretation by rare bioinformatics specialists.

This will be a big step forward in introducing the achievements of fundamental hepatology into clinical practice, as it will give doctors an instrument for assessing the state of the gut microbiome in their patients as well as determining how it is affected by drugs that are prescribed for the correction of dysbiosis. This reality reinforces the strength of our study.

In addition, our study is the first to describe the effect of gut dysbiosis on the prognosis of patients with cirrhosis, thereby confirming existing hypotheses about the important role of the gut-liver axis in the course of cirrhosis[3,18-21]. This is its second strong point.

The limitation of our study is its small sample size, which did not prevent us from obtaining significant results. It should also be noted that patients with severe hepatic encephalopathy (grades 2-4) are typically not admitted to our clinic, so these patients were not included in our study. The question of whether our results can be transferred

to this cohort of patients remains open. Since patients with infections received antibiotics before admission, which could change the composition of the gut microbiota, we excluded them from the study. None of the included patients developed infectious complications of cirrhosis during hospitalization. Thus, patients with infectious complications of cirrhosis were not included in our study, and it is not clear whether the results can be generalized to them. A larger study involving non-included patient populations should be provided to confirm the findings.

New studies are needed to evaluate how various methods (*e.g.*, probiotics, prebiotics, antibiotics, and fecal transplantation) can correct dysbiosis by analyzing the MDR and how this correction can improve the prognosis of patients with cirrhosis.

CONCLUSION

In conclusion, we were able to improve the CDR as well as show that gut dysbiosis is associated with poor prognosis in cirrhosis. Thus, we have developed a methodological apparatus and scientific basis for the correction of gut dysbiosis in such patients.

ARTICLE HIGHLIGHTS

Research background

Gut dysbiosis is common in cirrhosis.

Research motivation

The aim is to study the influence of gut dysbiosis on prognosis in cirrhosis.

Research objectives

The objectives include the development and test of a modified dysbiosis ratio (MDR) to distinguish between patients with cirrhosis and healthy controls, patients with compensated and decompensated cirrhosis, deceased and surviving patients.

Research methods

The case-control study included 48 in-patients with cirrhosis and 21 healthy controls. Stool microbiome was assessed using 16S ribosomal ribonucleic acid gene sequencing. We used MDR: [*Bacilli* (%) + *Proteobacteria* (%)]/[*Clostridia* (%) + *bacteroidetes* (%)]. Patients with MDR more its median made up the group with severe dysbiosis, others did the group with non-severe dysbiosis. The follow-up period was 4 years.

Research results

The mortality rate of patients with severe dysbiosis was significantly higher than that of patients with non-severe dysbiosis. The presence of severe dysbiosis was independent risk factors for death. The deceased patients had a higher MDR value than the survivors. MDR was higher in patients with cirrhosis than in health controls and in patients with decompensated cirrhosis than in patients with compensated cirrhosis.

Research conclusions

Gut dysbiosis is associated with a poorer long-term prognosis in cirrhosis.

Research perspectives

A larger study involving non-included patient populations should be provided to confirm the findings. New studies are needed to evaluate how various methods (*e.g.*, probiotics, prebiotics, antibiotics, and fecal transplantation) can correct dysbiosis by analyzing the MDR and how this correction can improve the prognosis of patients with cirrhosis.

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ORIGINAL ARTICLE

Retrospective Study

Combination of type IV collagen 7S, albumin concentrations, and platelet count predicts prognosis of non-alcoholic fatty liver disease

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Institutional review board statement: The study protocol complied with guidelines of the 1975 Helsinki Declaration and was approved by the Institutional Research Ethics Committee (approval No. 3027).

Informed consent statement: Written informed consent was obtained from all the patients.

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Abstract

BACKGROUND

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease and affects approximately 25% of the general global adult population. The prognosis of NAFLD patients with advanced liver fibrosis is known to be poor. It is difficult to assess disease progression in all patients with NAFLD; thus, it is necessary to identify patients who will show poor prognosis.

AIM

To investigate the efficacy of non-invasive biomarkers for predicting disease progression in patients with NAFLD.

METHODS

We investigated biomarkers associated with mortality in patients with NAFLD who visited the Kawasaki Medical School General Medical Center from 1996 to 2018 and underwent liver biopsy and had been followed-up for > 1 year. Cumulative overall mortality and liver-related events during follow-up were calculated using the Kaplan-Meier analysis and compared using log-rank testing. We calculated the odds ratio and performed receiver operating characteristic curve analysis with logistic regression analysis to determine the optimal cut-off value with the highest prognostic ability.

RESULTS

We enrolled 489 patients who were followed-up for a period of 1-22.2 years. In total, 13 patients died (2.7% of total patients enrolled); 7 patients died due to liver-related causes. Poor prognosis was associated with liver fibrosis on histological examination but not with inflammation or steatosis. Blood biomarkers associated with mortality were platelet counts, albumin levels, and type IV collagen 7S

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levels. The optimal cutoff index for predicting total mortality was a platelet count of 15 × 10^4/μL, albumin level of 3.5 g/dL, and type IV collagen 7S level of 5 mg/dL. In particular, only one-factor patients with NAFLD presenting with platelet counts ≤ 15 × 10^4/μL, albumin levels ≤ 3.5 g/dL, or type IV collagen 7S ≥ 5 mg/dL showed 5-year, 10-year, and 15-year survival rates of 99.7%, 98.3%, and 94%, respectively. However, patients with two factors had lower 5-year and 10-year survival rates of 98% and 43%, respectively. Similarly, patients with all three factors showed the lowest 5-year and 10-year survival rates of 53% and 26%, respectively.

CONCLUSION

A combination of the three non-invasive biomarkers is a useful predictor of NAFLD prognosis and can help identify patients with NAFLD who are at a high risk of all-cause mortality.

Key Words: Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Platelet count; Albumin; Type IV collagen 7S; All-cause mortality

Core Tip: We investigated biomarkers associated with mortality in non-alcoholic fatty liver disease (NAFLD) patients who underwent liver biopsy. Blood biomarkers associated with mortality were platelet count, albumin levels, and type IV collagen 7S levels. In particular, 5-year and 10-year survival rates were reduced for patients with all three factors: platelet counts below 15 × 10^4/μL, albumin levels below 3.5 g/dL, and type IV collagen 7S levels more 5 ng/dL. In summary, the combination of the three non-invasive biomarkers is a useful predictor of NAFLD prognosis and helps identify patients with NAFLD who are at high risk of death from all causes.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease and affects approximately 25% of the general global adult population[1]. The development of NAFLD is associated with lifestyle-related diseases, such as obesity, type 2 diabetes, hypertension, and dyslipidemia. Cardiovascular disease is the leading cause of death among NAFLD patients[2,3]. However, liver-related diseases are also a major cause of death among patients with NAFLD, and liver-specific and all-cause mortality rates are higher for these patients than for the general population NAFLD, and liver-specific and all-cause mortality rates are higher for these patients than for the general population[1]. The incidence of liver-specific and all-cause mortality among patients with NAFLD is generally 0.77 and 11.77 per 1000 years, respectively, while it is 15.44 and 25.56 per 1000 years, respectively, for patients with non-alcoholic steatohepatitis (NASH)[1].

The prognosis of NAFLD patients with advanced liver fibrosis is known to be poor[1,4-8]. Progression of liver fibrosis in patients with NAFLD is associated with mortality from various non-liver-related causes[6].

Liver biopsy is typically performed for diagnosing advanced fibrosis in patients with other liver diseases, such as NASH; however, it is not a practical tool for the diagnosis of NAFLD. In addition, the limitations of liver biopsies, such as invasiveness, poor patient tolerance, sampling variability, and high costs, are well known. Thus, there is increasing interest in developing and validating non-invasive methods for measuring liver stiffness, such as imaging and elastography techniques

based on ultrasonography or magnetic resonance imaging[3,4,9-14]. However, a limitation of these methods is that the images are visualized using an instrument that is not available in many institutions. Therefore, serum biomarkers that can assess the progression of liver fibrosis in patients with NAFLD may serve as important tools for identifying patients with advanced fibrosis. Some biomarkers of interest, such as procollagen type III N-terminal propeptide, type IV collagen 7S, hyaluronic acid, and Mac-2 binding protein [WFA(+)-M2BP] levels, and cytokeratin-18 have been used for identifying patients with NAFLD with advanced fibrosis. Other studies have used different biomarker scores, such as the BARD score, NAFLD fibrosis score, FIB-4 (fibrosis-4) index, aspartate aminotransaminase (AST) to alanine aminotransaminase (ALT) ratio, AST to platelet ratio index, FibroTest, and Enhanced Liver Fibrosis score, for the assessment of liver fibrosis[3,4,11,13,14-24]. However, none of these scores predict the prognosis of NAFLD patients. Hence, we aimed to investigate the efficacy of non-invasive biomarkers for predicting disease progression in patients with NAFLD.

MATERIALS AND METHODS

Patients

We retrospectively identified patients with NAFLD who underwent liver biopsy at the Kawasaki Medical School General Medical Center from 1996 to 2018 (Table 1). The exclusion criteria were as follows: history of other liver diseases including hepatitis B virus or hepatitis C virus infections, autoimmune liver diseases, drug-induced liver injury, metabolic liver diseases, or history of alcohol intake (men, ≥ 30 g/d and women, ≥ 20 g/d). Blood tests were performed before the liver biopsy, and we examined the prognostic factors based on the blood test results. The study protocol complied with the guidelines of the 1975 Helsinki Declaration and was approved by the Institutional Research Ethics Committee. Written informed consent was obtained from all the patients.

Clinical, biochemical, and histological parameters

We investigated the mortality rate and causes of death among the enrolled patients. We also investigated the development of any complications during the follow-up period. The start date of the follow-up period was defined as the date of liver biopsy and the end date of the follow-up period was defined as the date of last follow-up for surviving patients or the date of death for patients who died during the follow-up period. All NAFLD patients visited our hospital once every 3-6 mo. The following clinical parameters were included in the analysis: age at diagnosis of NAFLD; sex; body mass index calculated as weight (in kg) divided by height (in meters squared); and the presence of diabetes mellitus, hyperlipidemia, and dyslipidemia. We also included the following biochemical parameters in the analysis: platelet count, levels of albumin, total bilirubin, AST, ALT, gamma glutamyl transpeptidase, total cholesterol, cholinesterase, serum iron, ferritin, leptin, adiponectin, and high-sensitivity C-reactive protein, and homeostasis model assessment insulin resistance. The FIB-4 index was calculated as follows: age (years) × AST (U/L)/platelet count (× 10^4/μL) × √AST (U/L)[13,16,17]. Type IV collagen 7S and procollagen III peptide (P-III-P) were used as indicators of liver fibrosis.

Liver biopsy and histological analysis

All liver biopsies were performed using 16G or 17G biopsy needles with ultrasound guidance or using 14G needles with laparoscopic guidance. The histological examinations were performed by two experienced liver pathologists who were blinded to the patient details. The histological parameters included fibrosis, inflammation, steatosis, hepatocyte ballooning, and the NAFLD activity score (NAS) system[25]. The individual histological features of NAFLD were assessed using the following NAS system proposed by the NASH Clinical Research Network (NASH CRN): lobular inflammation (0-3), steatosis (0-3), and hepatocellular ballooning (0-2)[26,27]. The liver fibrosis stages were assessed according to Brunt's criteria.

Statistical analysis

The cumulative all-cause mortality and liver-related events during follow-up were assessed using the Kaplan-Meier method and compared using the log-rank test. The Kaplan-Meier analysis included the following variables: steatosis grade, ballooning

Table 1 Clinical and histological characteristics of the patient population (*n* = 489)

Characteristics	Values
Age	50.1 (14-82)
Male sex, %	54.6
Body mass index, kg/m^2	26.9 (20.8-49.5)
Fibrosis stage, 0/1/2/3/4	65/173/111/122/18
Grade, 0/1/2/3	45/204/178/62
Steatosis, 0/1/2/3	13/158/228/90
NAFLD activity score, < 4/≥ 5	265/224
ALT, IU/L	69 (2-563)
AST, IU/L	43 (13-312)
γ-GTP, IU/L	60 (12-736)
Total bilirubin, mg/dL	0.8 (0.04-2.7)
Total cholesterol, ng/dL	198 (102-317)
Cholinesterase, IU/L	205 (90-337)
Platelet count, × 10^4/μL	20.8 (6.6-44.7)
Albumin, g/dL	4.5 (2.5-5.4)
HOMA-IR	2.9 (0.7-22.4)
Iron, μg/dL	119 (13-295)
Ferritin, ng/dL	149 (3.9-983)
Leptin, ng/dL	9.3 (1.1-59.3)
Adiponectin, μg/mL	5.5 (2.0-27.5)
High-sensitivity CRP, mg/dL	0.117 (0.01-1.92)
P-III-P, U/mL	0.7 (0.28-3.8)
Type IV collagen 7S, ng/mL	4.1 (1.9-15)
Hyaluronic acid, ng/mL	28 (9-619)
Fibrosis-4 index	1.29 (0.17-1.29)

NAFLD: Non-alcoholic fatty liver disease; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; γ-GTP: Gamma-glutamyl transpeptidase; HOMA-IR: Homeostatic model assessment of insulin resistance; CRP: C-reactive protein; P-III-P: Procollagen-III peptide.

grade, NAS category, fibrosis stage, albumin, platelet counts, type IV collagen 7S levels, and FIB-4 index. We also calculated the odds ratio and performed receiver operating characteristic (ROC) curve analysis with logistic regression analysis to determine the cutoff values with the highest predictive ability. The optimal cut-off value was determined based on the Youden index. The prognostic performance of the optimal cutoff value was expressed as the diagnostic specificity, sensitivity, positive predictive value, and negative predictive value, using area under the ROC (AUROC) curve analysis. In univariate (unadjusted) and multivariate (adjusted) analyses, the hazard rate ratio estimates (relative risk) for outcomes were calculated using Cox proportional hazard regression analysis to control for the effect of potential risk factors (confounders) while considering the different follow-up durations. A P value < 0.05 was considered significant. All statistical analyses were performed using JMP (version 14.2, SAS system, United States). The statistical methods of this study were reviewed by Akiyoshi Izumi from Asahigawaso Rehabilitation and Medical Center, Okayama.

RESULTS

Survival rate

In total, 489 patients were enrolled in the present study; the 5-year survival rate was 98.5%, and the 10-year, 15-year, and 20-year survival rates were 95.4%, 91.9%, and 91.9%, respectively. The follow-up period varied between 1 and 22.2 years (Figure 1). In total, 13 (2.7%) patients died; of these, 7 patients died of liver-related causes [hepatocellular carcinoma (HCC) was observed in 1 patient; Table 2]. The complications that developed during the follow-up period were HCC (n = 12), other organ cancers (n = 13), and cerebrovascular disorders (n = 9).

Liver histological findings

Patients presenting with progression of advanced liver fibrosis after liver biopsy had increased mortality. The 5-year and 10-year survival rates of patients with NASH CRN Stage 4 disease were 81% and 41%, respectively. However, the degree of inflammation or steatosis was not associated with poor prognosis. The optimal area under the curve for albumin was 3.8 and 3.5 with specificities of 47% and 39%, sensitivities of 95% and 99%, positive predictive values of 98% and 98%, and negative predictive values of 21% and 56% (Figure 2).

Blood test factors

A univariate Cox hazard model was used for analyzing factors associated with mortality at the time of diagnosis of the NASH Clinical Research Network. We found that the ALT levels, platelet counts, albumin levels, and levels of liver fibrosis markers (P-III-P, type IV collagen 7S and FIB-4 index) were significantly associated with mortality (Table 3).

Survival curves were created using the following biomarkers: type IV collagen 7S, platelet count, albumin, and FIB-4 index. ALT was not included as a biomarker because the levels frequently varied. To investigate the predictive performance of these biomarkers with respect to NAFLD mortality, an optimal COI for type IV collagen 7S level, platelet count, albumin level, and FIB-4 index was determined based on the ROC curve analysis of all 489 patients with NAFLD. As shown in Figure 3A-D, the cutoff values for the platelet count, albumin level, type IV collagen 7S concentration and the FIB-4 index were set at 15×10^4, 3.8 g/dL, and 3.5 mg/dL, 5.0 ng/mL, and 1.3 and 2.61, respectively.

At the time of NASH diagnosis, patients with albumin levels < 3.5 mg/dL, platelet counts < 15×10^4, type IV collagen 7S levels ≥ 5 ng/dL, and FIB-4 indexes ≥ 2.67 clearly showed reduced survival (Figure 4A-D). Furthermore, we investigated the prognosis by combining type IV collagen 7S, which had a high AUROC among liver fibrosis markers (type IV collagen 7S, P-III-P, and FIB-4 index), the albumin level, and platelet count. Albumin level < 3.5 mg/dL, platelet count < 15×10^4/μL, and type IV collagen 7S levels ≥ 5 ng/dL were examined individually and in combination. The 5-year, 10-year, and 15-year survival rates for patients with only one factor were 99.7%, 98.3%, and 94%, respectively. However, survival rates were low for patients who presented with more than one factor. For these individuals, the 5-year and 10-year survival rates were 98% and 43%, respectively. For those who presented with two factors, the 5-year and 10-year survival were 53% and 26%, respectively, and for those presenting with three factors (Figure 5).

DISCUSSION

To the best of our knowledge, this study is the first study to evaluate the predictors of the prognosis of NAFLD based on the results of a blood test. We found that a combination of three non-invasive biomarkers, namely, platelet count, albumin level, and type IV collagen 7S level, is a useful predictor of NAFLD prognosis. The major causes of death in patients with NAFLD are cardiovascular events, organ cancers other than liver cancer, and liver-related disease. Among Japanese patients with NAFLD, the reported mortality rates associated with NAFLD are low during the follow-up period. The causes of death are more likely to be cancers of other organs and cerebral cardiovascular events than liver-related pathologies[28].

The most important predictor of outcomes among patients with NAFLD is the progression of liver fibrosis[1,5-7]. Angulo *et al*[6] retrospectively analyzed the long-term outcomes of 619 patients diagnosed with NAFLD in the United States, Europe, and Thailand during 1975-2005[6] and reported that only liver fibrosis, among various

Table 2 Summary of the causes of death

	n (%)
All deaths	13 (2.7)
Liver-related events	7 (1.4)
HCC + liver failure	3
HCC only	1
Liver failure	3
Cerebrovascular disease	1 (0.2)
Non-liver cancers	4 (0.8)
Pancreatic cancer	2
Bile duct cancer	2
Infection	1 (0.2)

HCC: Hepatocellular carcinoma.

Table 3 Factors associated with mortality among the patients with non-alcoholic fatty liver disease (*n* = 489)

	AUROC	Odds ratio	95%CI	*P* value
AST	0.57	1.00	0.99-1.02	0.9841
ALT	0.71	0.97	0.95-0.99	0.0026
γ-GTP	0.521	1.00	0.99-1.01	0.4259
Platelet count	0.748	0.78	0.69-0.88	< 0.0001
Total bilirubin	0.588	1.10	0.55-1.39	0.3208
Total cholesterol	0.580	0.99	0.98-1.01	0.2
Iron	0.553	1.01	1.02	0.1801
Albumin	0.815	0.093	0.04-0.20	< 0.0001
Ferritin	0.527	1.00	1.00-1.00	0.7651
Leptin	0.565	1.00	0.94-1.06	0.7441
HOMA-IR	0.731	1.04	1.009-1.06	0.0182
P-III-P	0.786	5.58	2.27-11.6	0.0014
Type IV collagen 7S	0.863	1.48	1.28-1.67	< 0.0001
Fibrosis-4 index	0.914	1.799	1.44-2.23	< 0.0001

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CI: Confidence interval; γ-GTP: Gamma-glutamyl transpeptidase; HOMA-IR: Homeostatic model assessment of insulin resistance; P-III-P: Procollagen-III peptide; AUROC: Area under the receiver operating characteristic curve.

longitudinal histological features, was associated with disease prognosis. Only liver fibrosis was independently associated with long-term all-cause mortality, liver transplantation, and liver-related events. Meta-analyses have also reported that liver fibrosis is an important risk factor for liver-related mortality[1,7]. Compared with NAFLD patients without fibrosis, NAFLD patients with fibrosis were at an increased risk of all-cause mortality, and the risk increased as fibrosis progressed[7]. In our study, patients with advanced liver fibrosis, especially cirrhosis, also showed poor prognosis; however, an association with inflammation, steatosis, or ballooning was not noted. Our findings further confirm that the progression of fibrosis markedly affects the prognosis of patients with NAFLD.

Several biomarkers can be used to evaluate liver fibrosis in patients with NAFLD[3,4,11,13,14-25,29]; however, previous studies have not examined disease prognosis using blood biomarker levels recorded at the time of NAFLD diagnosis

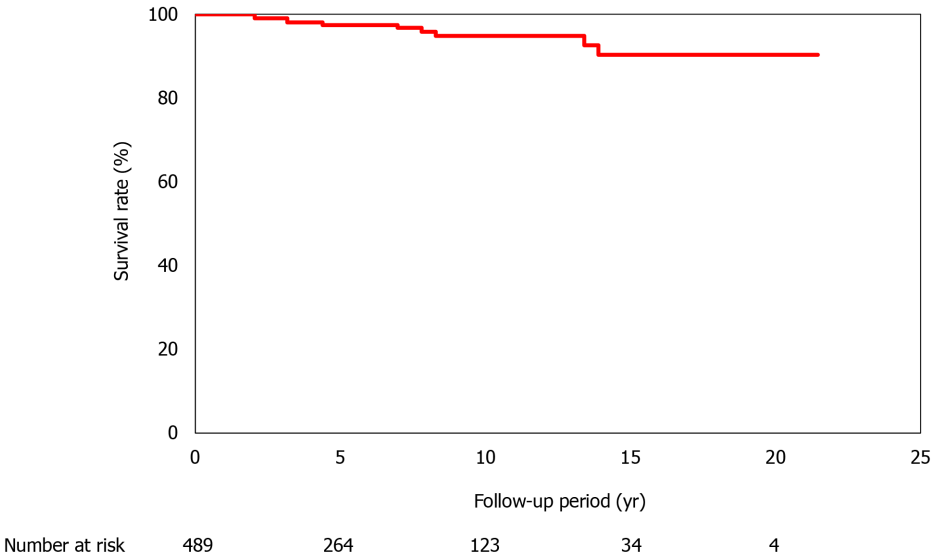


Figure 1 Survival of the 489 patients with non-alcoholic fatty liver disease. The follow-up period varied between 1 yr and 21.2 yr, and all-cause mortality was considered. The survival rates are 98.5% at 5 yr, 95.4% at 10 yr, 91.9% at 15 yr, and 91.9% at 20 yr.

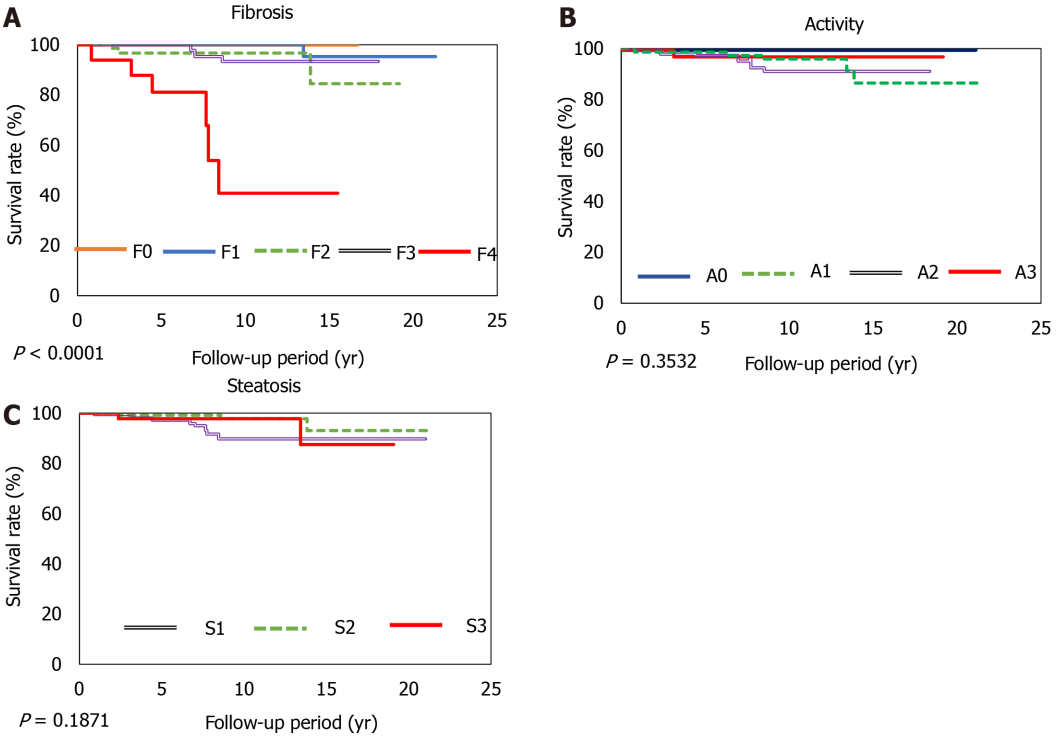


Figure 2 Survival rates according to the grading of fibrosis, inflammation, and steatosis. The overall survival rates for stage 4 liver fibrosis are 81% at 5 yr and 41% at 10 yr. A: Fibrosis (F0-4); B: Inflammation (A0-3); C: Steatosis (S1-3).

using liver biopsy.

NAFLD may progress rapidly in some patients and slowly in other patients. Singh *et al*[5] performed a systematic review and meta-analysis of 11 paired biopsy cohort studies that included 411 patients with > 2145 person-years of follow-up data and reported that approximately 30% of the patients developed advanced fibrosis and 70% of the patients remained stable or the stage of fibrosis in these patients improved. Furthermore, the annual fibrosis progression rates were 0.07 stages for patients with NAFLD and 0.14 stages for patients with NASH. Nasr *et al*[30] conducted a biochemical, clinical, and histological analysis of 129 patients with NAFLD who were enrolled between 1988 and 1993 in a prospective cohort study and followed them for 19.8 years. They reported that end-stage liver disease developed in 12 (9.3%) patients and advanced fibrosis developed in 34% of the patients. Furthermore, among the 113

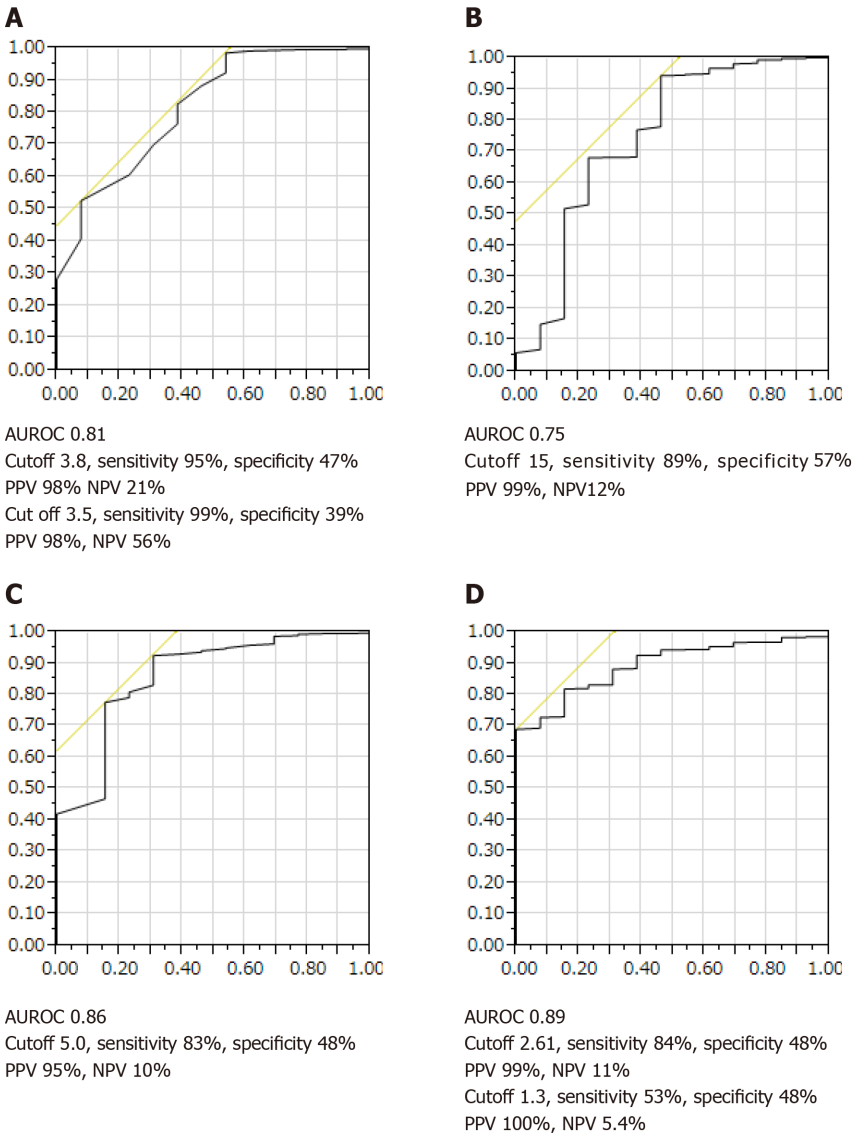


Figure 3 Receiver-operating characteristic curves for survival among patients with non-alcoholic fatty liver disease. A: Albumin concentration; B: Platelet count; C: Type IV collagen 7S concentration; D: Fibrosis-4 index. AUROC: Area under the receiver operating characteristic curve, PPV: Positive predictive value, NPV: Negative predictive value.

patients with low baseline fibrosis (stage 3), 16% of the patients developed advanced fibrosis. No differences in clinical, histological, or biochemical variables were observed between patients who developed liver fibrosis and those who did not. These studies did not examine the association of *PNPLA3* polymorphisms with menopause. Although the difference in the progression of NASH and NAFLD is not clear, racial differences and genetic factors, including *PNPLA3* expression[31], weight gain, onset and deterioration of diabetes[32], sex differences, and menopausal factors, affect prognosis[33].

It is necessary to consider the various factors that affect disease progress in each case of NAFLD. Although several studies have reported on the evaluation of biomarkers and elastography methods that can predict the progression of liver fibrosis[3,4,11,13,14-25], non-invasive biomarkers that can easily predict the prognosis of NAFLD have not been identified to date.

Our results indicate that patients with NAFLD who present with a combination of albumin level < 3.5 g/dL, platelet count < 15 × 10^4/μL, and type IV collagen 7S level ≥ 5 ng/mL show poor prognosis. In particular, the 10-year survival rate was only 43% for patients who presented with all three factors. We observed that type IV collagen 7S was a more useful indicator of advanced liver fibrosis than other biomarkers (Table 3). Yoneda *et al*[24] reported that the type IV collagen 7S level is a more useful marker of prognosis for patients with advanced fibrosis associated with NASH than for patients with mild fibrosis. Furthermore, a scoring system that uses type IV collagen 7S and AST levels, named the CA index, has been reported to predict NASH and fibrosis

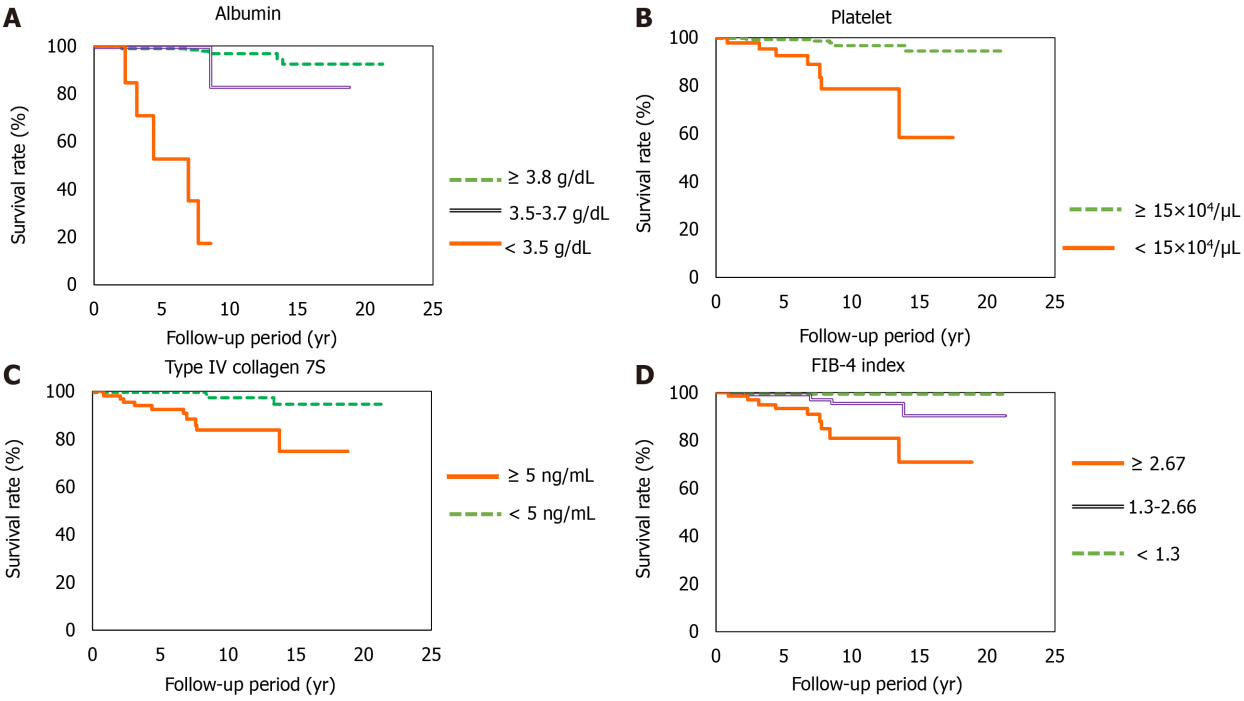
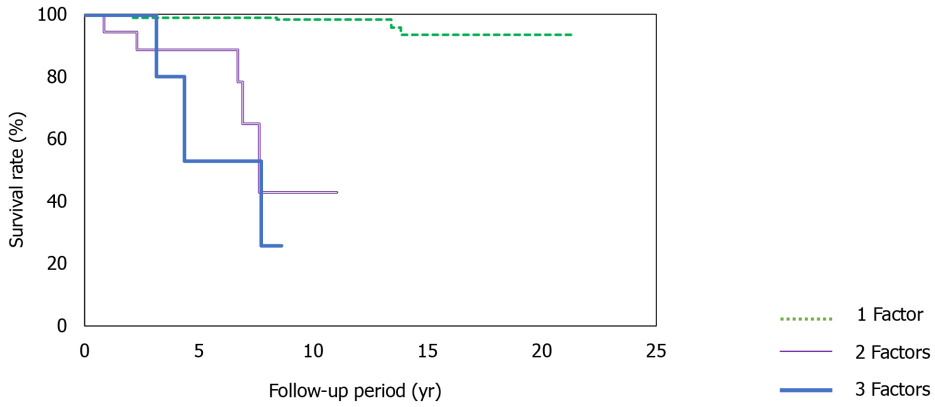


Figure 4 Survival rates. A: Albumin concentration (albumin ≥ 3.8 g/dL *vs* 3.5-3.7 g/dL; $P < 0.001$, albumin ≥ 3.8 g/dL *vs* < 3.5 g/dL; $P < 0.0001$, albumin 3.5-3.7 g/dL *vs* < 3.5; $P < 0.0001$); B: Platelet count (platelet ≥ 15 × 10^4/μL *vs* < 15 × 10^4/μL; $P < 0.0001$); C: Type IV collagen 7S concentration (type IV collagen 7S ≥ 5 ng/mL *vs* < 5 ng/mL; $P < 0.0001$); D: Fibrosis-4 index (Fibrosis-4 index ≥ 2.67 *vs* 1.3-2.67; $P < 0.001$, Fibrosis-4 index C1.3-2.67 *vs* < 1.3; $P < 0.0001$, Fibrosis-4 index ≥ 2.67 *vs* < 2.67; $P < 0.0001$). FIB: Fibrosis.



Number at risk

0 Factor	353	189	90	24	4
1 Factor	107	58	29	10	0
2 Factors	22	13	4	0	0
3 Factors	7	4	0	0	0

Figure 5 Survival rates according to positivity for the different biomarkers. Patients with only one risk factor have relatively good survival rates at 5 yr (99.7%), 10 yr (98.3%), and 15 yr (94%). However, patients with two risk factors have lower survival rates at 5 yr (98%) and 10 yr (43%), and patients with all three risk factors have even lower survival rates at 5 yr (53%) and 10 yr (26%) (1 factor *vs* 2 factors, $P < 0.0001$; 1 factor *vs* 3 factors, $P < 0.0001$; 2 factors *vs* 3 factors; $P < 0.05$).

associated with NAFLD with sufficient accuracy, thus allowing for convenient diagnosis and screening of NASH and associated fibrosis[21]. The same index was found to be useful in 400 Japanese patients from 18 institutes with biopsy-proven NAFLD and advanced liver fibrosis due to CA or FA fibrosis. The CA index is a combination of AST and type IV collagen 7S levels, and the FM fiber index includes type IV collagen 7S and hyaluronic acid levels and vascular cell adhesion[25]. The type

IV collagen 7S level is useful for determining advanced fibrosis in patients with NASH and was found to be more sensitive and specific than other fibrosis markers assessed in our study.

Albumin is also an important biomarker for predicting the prognosis of HCC in patients with NAFLD. Kawaguchi *et al*[34] analyzed the factors affecting survival by performing a random forest analysis for 247 NAFLD-HCC patients diagnosed between 2000 and 2014 and recruited from 17 medical institutions in Japan. The results showed that the best prognostic profile for patients with NAFLD-HC comprised treatment for HCC and serum albumin levels > 3.7 g/dL.

There are some limitations of this study. We did not classify prognosis according to all-cause mortality; moreover, the study population comprised patients from at a single center. Nevertheless, it is significant that the study followed a long-term course of up to 20 years.

In our study, the platelet count, albumin level, type IV collagen 7S level, and the FIB-4 index were important prognostic factors at the time of diagnosis of NAFLD. Our findings suggest that these factors should be recorded in patients with NAFLD at the time of diagnosis to determine future treatment strategies.

Studies conducted in the future should focus on assessing these biomarkers further and examining long-term prognosis using Fibroscan and magnetic resonance elastography. Further research is also needed to confirm these findings in other populations.

CONCLUSION

This study may prove useful in clinical practice because simple predictors of NAFLD progression, namely, albumin level, platelet count, and type IV collagen 7S level, were identified; all these parameters are can be easily assessed in daily practice.

ARTICLE HIGHLIGHTS

Research background

Non-alcoholic steatohepatitis has few symptoms until it progresses; thus, it is necessary to identify non-alcoholic fatty liver disease (NAFLD) patients who will show poor prognosis.

Research motivation

The limitations of liver biopsies, such as invasiveness, poor patient tolerance, sampling variability, and high costs, are well known. Thus, there is increasing interest in developing and validating non-invasive methods for measuring liver stiffness. However, many current methods involve instruments that are not available in many institutions.

Research objectives

Serum biomarkers that can assess the progression of liver fibrosis in patients with NAFLD may serve as important tools for identifying patients with advanced fibrosis. We aimed to investigate the efficacy of non-invasive biomarkers for predicting disease progression in patients with NAFLD.

Research methods

We investigated biomarkers with predictable prognosis for NAFLD patients who underwent liver biopsy. All patients were followed-up for > 1 year.

Research results

The combination of three non-invasive biomarkers involved in NAFLD prognosis comprised platelet counts, albumin levels, and type IV collagen 7S. Our results indicate that patients with NAFLD who present with a combination of albumin levels < 3.5 g/dL, platelet counts < $15 \times 10^4/\mu L$, and type IV collagen 7S levels ≥ 5 ng/mL show poor prognosis. In particular, the 10-year survival rate was only 43% for patients who presented with all three factors.

Research conclusions

The combination of platelet count, albumin level, and type IV collagen 7S was useful in further predicting the prognosis of NAFLD.

Research perspectives

Studies conducted in the future should focus on assessing these biomarkers further and examining long-term prognosis.

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ORIGINAL ARTICLE

Retrospective Study

Surgical treatment outcomes of primary hepatic sarcomas: A single-center experience

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Informed consent statement: Acquiring participant's consent seems to be realistically impossible and does not influence integrity of research. And there would be no reasons that participant would deny providing his or her consent; research involves no more than minimal risk to the patients. Therefore, the IRB of Samsung Medical Center approved that the participant's consent can be

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Abstract

BACKGROUND

Primary hepatic sarcoma is a rare tumor originated from mesenchymal tissue. There are various pathologic types of primary hepatic sarcoma and the treatment outcome of this tumor was usually disappointing. Unlike hepatocellular carcinoma, outcome of primary hepatic sarcoma is not well-known due to it's rarity. However, with development of medical technology, surgical treatment may lead to better survival.

AIM

To investigate the surgical outcomes of primary hepatic sarcoma, we gathered and analyzed the cases of a single institute.

METHODS

From August 2001 to September 2016, a total of nine patients were surgically treated for primary hepatic sarcoma after exclusion of cases with open and closure, early loss to follow-up and sarcomatoid hepatocellular carcinoma and sarcomatoid cholangiocellular carcinoma. Baseline characteristics, tumor characteristics such as tumor pathology, size and number, surgical and adjuvant treatments were reviewed. Tumor recurrence, and patient survival were analyzed with retrospective approach.

RESULTS

The enrolled participants included five patients with angiosarcoma and four patients with undifferentiated sarcoma. All patients experienced tumor recurrence at a median of 52 post-operative days. Only two patients survived and the 5-year survival rate was 29.6%. One patient with angiosarcoma who received central

waivered.

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hepatectomy for primary tumor and received radiofrequency ablation for recurrent tumor still lives for 11 years. One patient with undifferentiated sarcoma received Rt. lobectomy for primary tumor followed by chemotherapy and radiation therapy still lives around 30 mo even though she got additional operation for recurrent tumor. Two patients who received living donor liver transplantation due to angiosarcoma died. Only adjuvant therapy was associated with survival gain (P = 0.002).

CONCLUSION
Patients with primary hepatic sarcoma may gain survival benefit with surgical resection followed by adjuvant therapy, even though the outcome remains relatively poor.

Key Words: Liver; Angiosarcoma; Undifferentiated sarcoma; Operation; Survival; Recurrence

Core Tip: This is a retrospective study to analyze the outcomes of primary hepatic sarcoma. A total of nine patients were included, five of them are with angiosarcoma and four are with undifferentiated sarcoma. While all patients experienced tumor recurrence, one patient with angiosarcoma and another patient with undifferentiated sarcoma still survive for 11 years and 30 mo respectively, after receiving effective local treatment for recurrent tumors. Although the outcome of primary hepatic sarcoma is known to be poor, surgical treatment with appropriate adjuvant therapy may support the long-term survival.

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INTRODUCTION

Sarcoma is a malignant tumor that usually arises from mesenchymal tissue. Most sarcomas originate in the extremities, and the prognosis of sarcoma in these sites is well known due to its prevalence[1]. Primary hepatic sarcoma is a rare and aggressive tumor with poor outcomes. Most malignant tumors in the liver are hepatocellular carcinomas (HCC); hepatic sarcomas represent less than 1%[2]. Hepatic sarcoma has various pathologic types, including angiosarcoma, undifferentiated (embryonal) sarcoma, leiomyosarcoma, and epithelioid hemangioendothelioma, among others. For most of these tumors, the cause is still unknown, and there are usually no specific symptoms until abdominal pain presents due to the effect of the mass increasing in size[3,4]. The various types, the rarity, and the difficulty in diagnosis of primary hepatic sarcoma results in various prognoses, thereby making it difficult to set a treatment plan. The aim of our study is to analyze the outcomes of primary hepatic sarcoma following surgical resection in a single institute.

MATERIALS AND METHODS

Patient selection

From August 2001 to September 2016, a total of 43 patients underwent surgical treatment for primary hepatic sarcoma at Samsung Medical Center, South Korea. These patients were selected by searching the word "sarcoma" in liver pathological report through all time of our institute. Open and closure, inadequate medical chart, and sarcomatoid HCC or cholangiocellular carcinoma cases were excluded. Six early-follow up loss patients who were treated with resection in our center and then

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transferred to other hospitals were also excluded. Four patients who did not want additional therapy and did not come to outpatient clinic were also excluded. Data was collected by retrospective approach.

Patient age, sex, and pre-operative blood tests such as total bilirubin, aspartate transaminase, alanine aminotransferase, alpha fetoprotein, and CA 19-9 were evaluated. Operation types were reviewed, and pathological diagnosis, tumor size and number were evaluated. Disease recurrence was evaluated using computed tomography or positron emission tomography scan. Patient death was the primary end point.

Operation and adjuvant therapies

Operation type was decided by tumor size, location and liver function and the target of operation was R0 (microscopically margin-negative) resection. However, patients with R1 (macroscopically no remnant tumor, but margin-positive microscopically) and R2 (seen remnant tumor) resection were also included in the study population. Patients usually received chemotherapy when the pathologic report was diagnosed as primary hepatic sarcoma, unless the patient was in poor general condition and could not endure chemotherapy. For cases of recurrence or metastasis of cancer at the vertebrae, radiation therapy (RT) was performed. Resectable recurrent tumors were excised surgically. For small recurrences in the liver, radiofrequency ablation (RFA) could also be performed.

Statistical analysis

Baseline characteristics and tumor markers were compared using the Mann-Whitney test. Cox proportional hazards regression analysis and the Kaplan-Meier method were used to analyze disease-free survival, overall survival and corresponding risk factor. The independent variables were age, sex, tumor size and number, tumor markers and adjuvant therapy. Disease free-survival and overall survival was compared according to pathologic type of sarcoma and adjuvant therapy due to known different prognosis of each sarcoma type. Statistical analysis was executed using IBM SPSS-24 statistical program (IBM Institute, NY, United States).

RESULTS

Baseline characteristics

After exclusion of open and closure, early loss to follow-up, inadequate medical chart, and sarcomatoid HCC or cholangiocellular carcinoma among 43 patients, total nine patients with pure hepatic sarcomas were involved in this study (Figure 1). According to pathological diagnoses, we divided the patients into an angiosarcoma group (n = 5) and an undifferentiated sarcoma group (n = 4, Table 1). Baseline characteristics of each group showed no statistical differences in any variables including tumor size, number of tumors, and tumor markers (Table 2). Median size of the largest tumor was 13 cm. Median age of the patients was 57, and only one patient was pediatric (a 2-year-old female).

Angiosarcoma group

Among the five patients with angiosarcoma, only one patient survived. Median survival duration was 13 mo, and median disease-free survival was 53 d. Two patients (a 57-year-old male and a 52-year-old male, case 1 and 3, respectively) could not receive adjuvant chemotherapy due to poor general condition and expired relatively early (66 d and 127 d, respectively). The pediatric patient (case 2) underwent living donor liver transplantation (LDLT) for angiosarcoma and was diagnosed with multiple bone metastasis at the extremities on post-operative day 53. She received six cycles of ifosfamide/carboplatin/etoposide chemotherapy and expired at post-operative 31 mo. Case 5, a 60-year-old male, received LDLT and experienced recurrence at the liver and metastasis at the vertebra and rib at post-operative 11 mo. He received palliative RT on the bone metastases and expired at post-operative 13 mo.

Case 7, a 62-year-old male who received central hepatectomy for a 2 cm angiosarcoma on segment 8, is still alive after 11 years (Figure 2A). The pathologic resection margin had no cancer cells, and the tumor was the infiltrative type without invasion to any other organs. After operation, the patient experienced recurrence on segment 2 with a 0.9 cm tumor. Successful RFA was done, and he has been cancer-free for six years.

Table 1 Characteristics of the study population

Case	Sex	Age	Pathology	Size (cm)	Operation, R Status	Adjuvant therapy	Recurrence (mo)	Follow-up (mo)	Outcomes
1	M	57	HAS	13.5	Lt. lobectomy, R0		1.7	2.2	Dead
2	F	2	HAS	21	Living donor LT, R0	CTx[1]	1.8	31.2	Dead
3	M	52	HAS	7.3	Rt. Trisectionectomy, R0		1.2	4.2	Dead
4	F	48	UDS	13	Rt. lobectomy, R0	CTx[2]	1.6	68.2	Dead
5	M	60	HAS	2.4	Living donor LT, R0	RT	11.0	13.4	Dead
6	F	53	UDS	25	Rt. Trisectionectomy, R2		0	1.3	Dead
7	M	62	HAS	2	Central hepatectomy, R0	RFA	59.9	135.1	Alive
8	F	60	UDS	11.5	Rt. lobectomy, R0	CTx, RT, Exc[3]	14.6	29.9	Alive
9	M	60	UDS	24	Rt. lobectomy and Rt. Npx, R0	CTx[4]	1.2	9.9	Dead

[1]ICE (ifosfamide/carboplatin/etoposide) 6 cycles.
[2]VIP (etoposide/ifosfamide/cisplatin) 5 cylcles, AI (doxorubicin/ifosfamide) 5 cycles, docetaxel/gemcitabine 2 cycles.
[3]VIP (etoposide/ifosfamide/cisplatin) 6 cycles, RT on vertebral metastasis, abdominal wall metastatic tumor excision.
[4]AI (doxorubicin/ifosfamide) 4 cycles, docetaxel/gemcitabine 1 cycle. HAS: Hepatic angiosarcoma; UDS: Undifferentiated sarcoma; Npx: Nephrectomy; CTx: Chemotherapy; RT: Radiation therapy; RFA: Radiofrequency ablation; Exc: Excision.

Table 2 Characteristics of the groups

	Total (*n* = 9)	Angiosarcoma (*n* = 5)	Undifferentiated sarcoma (*n* = 4)	*P* value
Age (range)[1]	57 (2-62)	57 (2-62)	56 (48-60)	0.999
Sex, male (%)	5 (55.6)	4 (80)	1 (25)	0.120
Largest tumor size (cm)[1]	13.0	7.3	18.5	0.142
Tumor number[1]	1.0	2	1	0.371
AFP[1]	2.8	2.6	2.8	0.999
CA19-9[1]	13.2	3.2	1970	0.180
Recurrence (%)	9 (100)	5 (100)	4 (100)	0.999
Disease-free survival days[1]	52 (0-1798)	53 (36-1798)	35 (0-439)	0.221
Death (%)	7 (77.8)	4 (80)	3 (75)	0.866
Survival days[1]	402	402	596	0.806

[1]The numbers are median value. AFP: Alpha fetoprotein.

Undifferentiated sarcoma group

Among the four patients with undifferentiated sarcoma, only one patient survived. Median survival duration was 20 mo, and median disease-free survival was 53 d. Case 6, a 53-year-old female, received Rt. trisectionectomy for a 25 cm undifferentiated sarcoma associated with partial cholangiocellular carcinoma. The tumor was partially ruptured, and a cytology test of ascites was positive for malignant cells with two regional lymph node metastases. The patient expired at post-operative 40 d before receiving chemotherapy. Case 9, a 60-year-old male, received Rt. lobectomy, Rt. nephrectomy, and diaphragm resection due to a 24 cm undifferentiated sarcoma, followed by a diagnosis of lung metastasis at post-operative 35 d. He received four cycles of AI regimen (doxorubicin/ifosfamide) and one cycle of docetaxel/gemcitabine chemotherapy until he expired at post-operative 10 mo.

Case 4, a 48-year-old female, received Rt. lobectomy due to a 13 cm undifferentiated sarcoma. The tumor had already penetrated to the liver capsule and had a high mitotic count (10/10 HPFs), and tumor emboli were in the Rt. portal vein. Multiple tumors recurred on the liver resection margin and the remnant liver at post-operative 49 d.

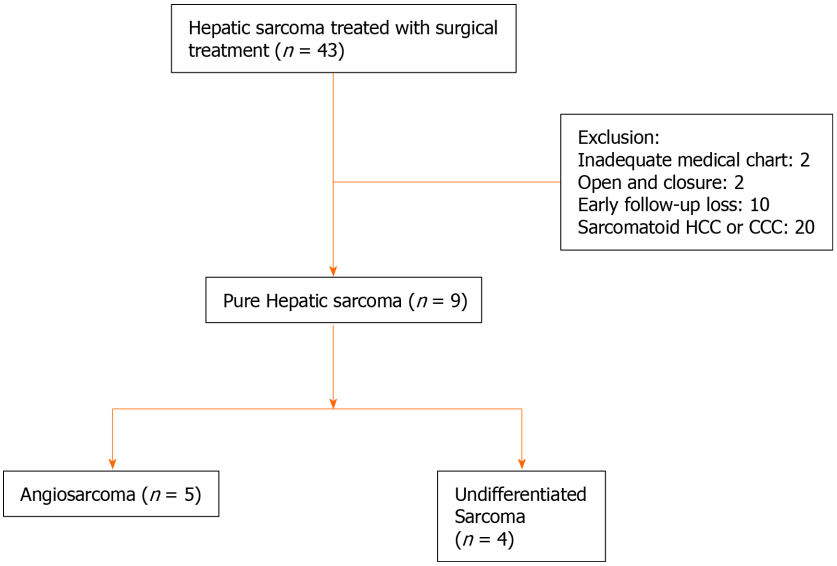


Figure 1 Flow chart of selecting pure hepatic sarcoma. 5 cases of angiosarcoma and 4 cases of undifferentiated sarcoma were included. HCC: Hepatocellular carcinoma; CCC: Cholangiocellular carcinoma.

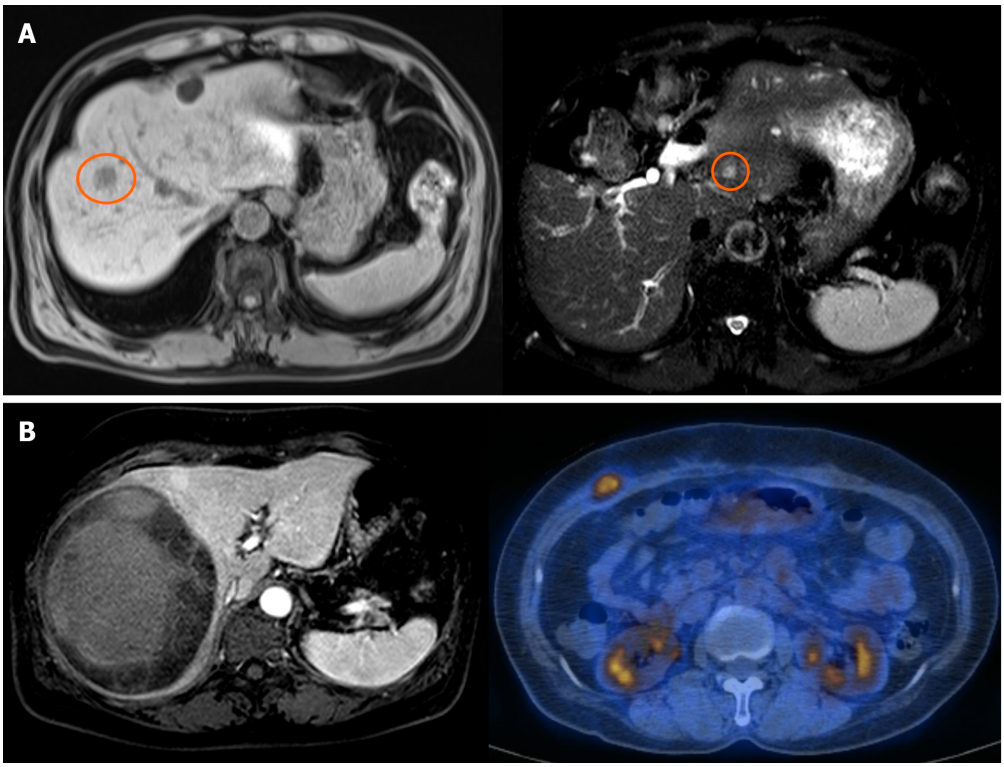


Figure 2 Images of a surviving patient (cases 7 and 8). A: Case 7. Left: T1 magnetic resonance imaging (MRI) of pre-operative angiosarcoma on S8 (orange circle). Right: T2 MRI of recurrence on S2 five years after central hepatectomy; B: Case 8. Left: MRI of pre-operative sarcoma. An 11.5 cm circumscribed mass with hemorrhage on the Rt. lobe. Right: Positron emission tomography-computed tomography of the recurrent mass. Focal fluoro-deoxyglucose uptake at the Rt. anterior abdominal wall.

The patient received multiple series of chemotherapy, including five cycles of VIP regimen (etoposide/ifosfamide/cisplatin) followed by five cycles of AI regimen (doxorubicin/ifosfamide) and two cycles of docetaxel/gemcitabine. The patient survived 68 mo until she died due to tumor progression, lung metastasis, and liver abscess.

Case 8, a 60-year-old female who received Rt. lobectomy for an 11.5 cm undifferentiated sarcoma, is still alive after 30 mo (Figure 2B). The tumor had high cellularity, moderate pleomorphism, and tumor necrosis of more than 50% with a negative resection margin. After operation, the patient received six cycles of chemotherapy with

the VIP regimen (etoposide/ifosfamide/cisplatin), followed by RT to the pre-operatively diagnosed vertebral metastasis (1.5 cm tumor at T9). She had no cancer recurrence until abdominal metastasis was detected at post-operative 14 mo. The metastatic tumor was 2 cm, located between the abdominal investing fascia and the external oblique muscle. After a wide excision, the patient has been cancer-free for 30 mo.

Cancer recurrence

All patients experienced recurrence of the primary cancer. Median disease-free survival was 52 d. Because one patient with angiosarcoma had tumor recurrence in the Left lobe at post-operative 4 years and 11 mo, the disease-free survival of angiosarcoma looks higher than that of undifferentiated sarcoma; however, this was not statistically significant (Figure 3). The age, sex, pathology type, and tumor markers showed no statistical influence on cancer recurrence (Table 3). Only the largest tumor size was associated with higher cancer recurrence, but only in univariate analysis (HR = 1.13, P = 0.49) and not in multivariate analysis.

Patient death

Among the total of nine patients, only two patients survived (one with angiosarcoma, one with undifferentiated sarcoma). The 5-year survival rate was 29.6% (Figure 4). There was no significant difference between survival of the angiosarcoma and undifferentiated sarcoma groups. Pathologic type, largest tumor size, number of tumors, and tumor markers did not influence patient death with univariate analysis (Table 4). Only adjuvant therapy had an effect on patient survival. The 5-year survival of patients who received adjuvant therapy was 44.4%, while all patients without adjuvant therapy expired within 1 year (Figure 5).

DISCUSSION

In our study, all patients who received surgical treatment for primary hepatic sarcoma had tumor recurrence, and the 5-year survival rate was relatively low (29.6%). However, one patient with angiosarcoma is still alive after 11 years, and one with undifferentiated sarcoma is still alive after 30 mo.

With recent medical advances, survival outcomes after surgical resection of primary hepatic sarcoma are slightly increasing. One study with 22 patients who received primary surgical treatment showed a 5-year survival rate of 65.2%[5]. However, that study population included various sarcoma types, including rhabdomyosarcoma, leiomyosarcoma, and hemangiopericytoma, while the five cases of angiosarcoma had much poorer outcomes with only one patient surviving for six months. Another review article including 64 cases of hepatic angiosarcoma showed a median survival time of five months[6]. In this article, median survival of patients who received local excision alone or local excision combined with chemotherapy was 17 mo. In our study, the angiosarcoma group had a median survival of 13 mo, with a 5-year survival rate of 20%. One patient who received central hepatectomy and RFA for recurrence at the Lt. lateral section still lives after 11 years.

Hepatic undifferentiated sarcoma is also known to have poor outcomes[7]. Sometimes, it is misdiagnosed as other cystic tumor on pre-operative images and revealed as undifferentiated sarcoma on pathologic review after surgical resection[8,9]. However, it may have better outcomes compared to angiosarcoma when surgically resected. A recent review article including 271 patients who received partial hepatectomy showed a 5-year survival rate of 70%[10]. The 5-year survival rate for the undifferentiated sarcoma group in our study was 50%. One patient who still lives, received Right lobectomy for an 11.5 cm tumor, followed by chemotherapy and RT for vertebral metastasis. About 14 mo later, she underwent a local excision at the abdominal wall metastasis and has maintained a disease-free state for 15 mo. A 48-year-old female patient who received Rt. lobectomy followed by chemotherapy lived more than five years but expired at 5 years and 7 mo due to tumor cachexia.

Even though the outcome of surgical resection for primary hepatic sarcoma is not ideal, surgical resection is still considered to be a better treatment than chemotherapy alone. In a study with 30 primary hepatic sarcoma patients, those who received R0-surgical resection experienced much better outcomes than those who did not, except for patients with the specific pathologic type of epithelioid hemangioendothelioma[2]. Another study in which 6 patients received transcatheter arterial chemoembolization or transcatheter arterial embolization alone for hepatic angiosarcoma resulted in all

Table 3 Risk factors for cancer recurrence

	Univariate HR (95%CI)	*P* value	Multivariate HR (95%CI)	*P* value
Sex (male)	0.82 (0.20-3.31)	0.779		
Age	0.99 (0.96-1.03)	0.694		
Pathology (HAS/UDS)	0.50 (0.12-2.07)	0.339	0.63 (0.11-3.55)	0.602
Largest tumor size	1.13 (1.00-1.27)	0.049	1.12 (0.97-1.28)	0.115
Tumor number	1.28 (0.79-2.07)	0.311		
AFP	1.02 (0.77-1.33)	0.917		
CA 19-9	1.00 (1.00-1.00)	0.527		
Adjuvant therapy	0.24 (0.04-1.47)	0.121	0.25 (0.04-1.71)	0.157

HAS: Hepatic angiosarcoma; UDS: Undifferentiated sarcoma; AFP: Alpha fetoprotein; CI: Confidence interval; HR: Hazard ratio.

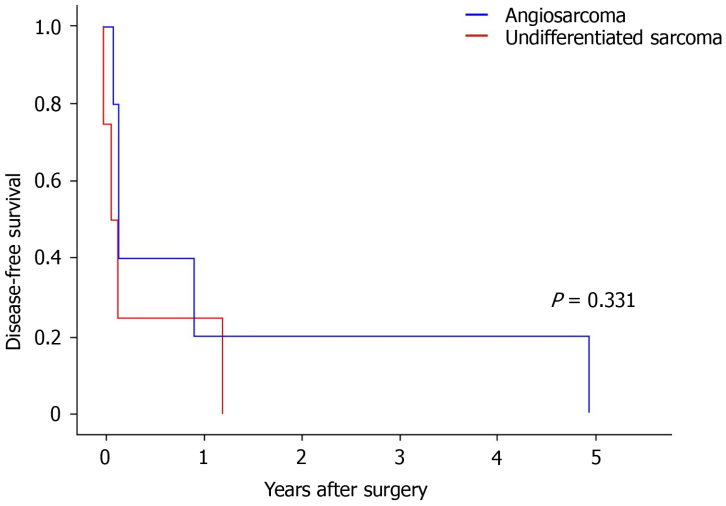
Table 4 Risk factors for patient death

	Univariate HR (95%CI)	*P* value
Sex (male)	1.36 (0.30-6.24)	0.693
Age	1.00 (0.96-1.04)	0.922
Pathology (HAS/UDS)	0.99 (0.22-4.47)	0.989
Largest tumor size	1.08 (0.97-1.20)	0.179
Tumor number	1.28 (0.79-2.07)	0.311
AFP	0.99 (0.74-1.31)	0.922
CA 19-9	1.00 (0.99-1.01)	0.677
Adjuvant therapy	0.00 (0.03-2779)	0.002

HAS: Hepatic angiosarcoma; UDS: Undifferentiated sarcoma; AFP: Alpha fetoprotein; CI: Confidence interval; HR: Hazard ratio.

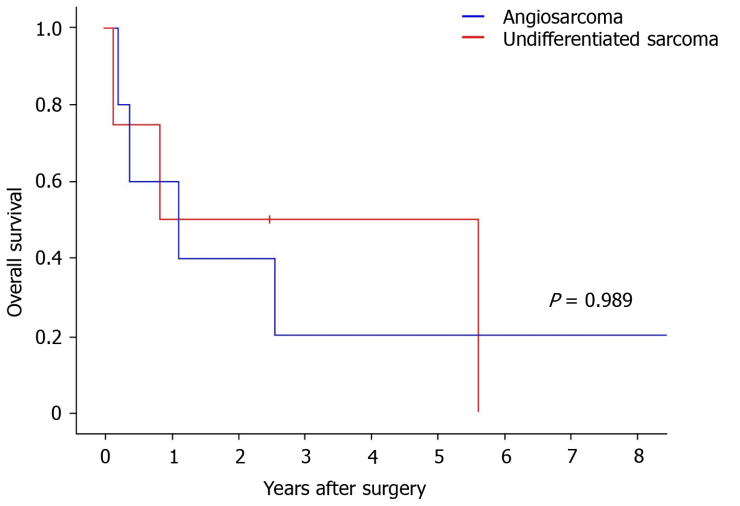
patients expiring within 1 year[11]. A recent study of 8 patients with R0-resected hepatic angiosarcoma showed median survival and disease-free survival of 59 and 11 mo which emphasizes the radical surgical resection is best approach for long-term survival[12].

Still, adjuvant chemotherapy after resection is recommended for hepatic sarcoma. In cases of angiosarcoma, one study showed that a combination of surgical treatment and adjuvant chemotherapy may be beneficial[13]. A review article with 64 cases of angiosarcoma suggested that surgery with chemotherapy is the optimal choice for survival[6]. May *et al*[14] studied five pediatric patients with hepatic undifferentiated sarcoma who underwent a uniform approach of local resection and vincristine, actinomycin-D, cyclophosphamide. All patients survived with median survival of 53 mo. Lenze *et al*[15] described 14 patients with undifferentiated sarcoma who remained alive for a median of 28.5 mo after receiving both surgical resection and adjuvant chemotherapy, which was a significantly better outcome compared to patients without adjuvant chemotherapy. However, the optimal regimen of chemotherapy for hepatic sarcoma has not yet been established. Kim *et al*[16] showed that palliative chemotherapy may be beneficial to survival even if the hepatic angiosarcoma is unresectable. Transarterial chemoembolization showed some effectiveness in acute intra-abdominal hemorrhage of hepatic angiosarcoma cases[17]. In our study, three patients who did not receive adjuvant therapy had poorer survival than patients who received adjuvant therapy. However, two of them could not receive chemotherapy due to poor general condition (angiosarcoma patients), and one patient expired before scheduled chemotherapy (undifferentiated sarcoma). There is a case report of immunotherapy about a patient with primary hepatic angiosarcoma with multiple liver metastasis treated by pazopanib plus procedural death factors-1 inhibitor and RAK cells showing stable disease after treatment[18]. Although this is only one case report, this study showed a hope of new era of treatment which may aid surgical



	Time (yr)	1	2	3	4	5
Angiosarcoma (n = 5)	survival rate	0.2	0.2	0.2	0.2	0
	# at risk	1	1	1	1	0
Undifferentiated Sarcoma (n = 4)	survival rate	0.25	0			
	# at risk	1	0			

Figure 3 Disease-free survival. Median disease-free survival was 52 d. There was no statistical difference between angiosarcoma and undifferentiated sarcoma groups.

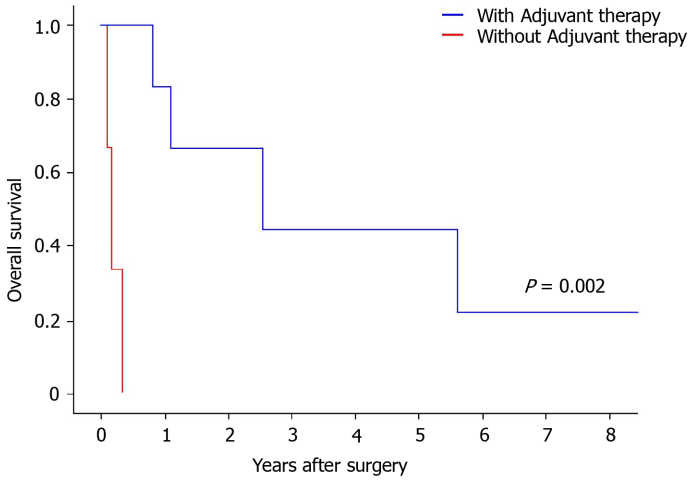


	Time (yr)	1	2	3	4	5
Angiosarcoma (n = 5)	survival rate	0.6	0.4	0.2	0.2	0.2
	# at risk	3	2	1	1	1
Undifferentiated Sarcoma (n = 4)	survival rate	0.5	0.5	0.5	0.5	0.5
	# at risk	2	2	1	1	1

Figure 4 Overall survival. The 5-year survival rate was 29.6%. There was no significant difference between survival of the angiosarcoma and undifferentiated sarcoma groups.

resection of hepatic angiosarcoma.

In our study, two patients with hepatic angiosarcoma received living donor liver transplantation (LT). The 2-year-old girl had recurrence at 53 d and expired at 31 mo after LT, while the 60-year-old male had recurrence at 11 mo and expired at 14 mo. Selection of treatment between surgical resection and LT is an issue of concern. A study of 237 patients registered in the National Cancer Database of North America concluded that both hepatic resection and LT may lead to similar long-term survival with selected pathologic cases such as epithelioid hemangioendotheliomas[19]. However, this study also found that the prognosis of angiosarcoma was worse with



	Time (yr)	1	2	3	4	5
Received adjuvant therapy (n = 6)	survival rate	0.83	0.67	0.44	0.44	0.44
	# at risk	5	4	2	2	2
No adjuvant therapy (n = 4)	survival rate	0.25	0.25	0		
	# at risk	1	1	0		

Figure 5 Overall survival depending on adjuvant therapies. Patients who received adjuvant therapy showed higher overall survival rate.

both resection and transplantation. A review article of 64 angiosarcoma cases did not recommend LT for angiosarcoma due to the higher recurrence rate[6]. This result accords with the poor outcomes of LT seen in the hepatic angiosarcoma patients in our study. For hepatic undifferentiated sarcoma, liver transplantation cases are rare. Walther *et al*[20] studied 3 patients who received LT and remained in clinical remission for a mean of 35 mo. Wu *et al*[10] showed 14 patients who received LT for hepatic undifferentiated sarcoma with a 5-year survival rate of 78.9%. When weighing the benefits of LT against the risks for the liver donor or the shortage of deceased donor, LT in hepatic undifferentiated sarcoma is still controversial and needs further research.

Our study has limitations in that it is a retrospective study and has a small number of patients due to the rarity of the tumor. Furthermore, three patients did not receive adjuvant therapy, not due to clinical decision, but due to either poor patient condition or the patient expiring prior to receiving the adjuvant therapy.

CONCLUSION

Primary hepatic sarcoma has poor outcomes even after surgical resection. However, surgical resection may have some benefit for extending long term life expectancy in some cases. Adjuvant therapy may support the outcomes. Liver transplantation for primary hepatic angiosarcoma also continues to have poor survival outcomes.

ARTICLE HIGHLIGHTS

Research background

Primary hepatic sarcoma is a malignant tumor which arises from hepatic mesenchymal tissue. It consists of angiosarcoma, undifferentiated (embryonal) sarcoma, leiomyosarcoma, and epithelioid hemangioendothelioma.

Research motivation

Due to it's rarity and various prognosis, the treatment plan of primary hepatic sarcoma is not established yet.

Research objectives

We aim to analyze the tumor characteristics, treatment and prognosis of the primary

hepatic sarcoma cases which was surgically resected in a single center.

Research methods

After exclusion of cases with open and closure, early loss to follow-up and sarcomatoid tumors, total nine cases of primary hepatic sarcoma were surgically resected from August 2001 to September 2016. The research data collection and analysis were achieved with retrospective approach. Baseline patient's characteristics, tumor characteristics and treatment modality with tumor recurrence and patient's survival were analyzed. The analysis was done separately according to tumor pathologic type.

Research results

Among five angiosarcoma and four undifferentiated sarcoma patients, only two patients survived and all patients experienced tumor recurrences (5-year survival rate: 29.6%). Follow-up post-operative durations of survived angiosarcoma patient and undifferentiated sarcoma patient were 11 years and 30 mo, respectively. Adjuvant therapy had a positive role on survival gain (P = 0.002). However, this study has a limitation of a retrospective approach and a small case number.

Research conclusions

In spite of known poor prognosis, surgical resection of primary hepatic sarcoma may help extending the life expectancy of patient. Aggressive adjuvant treatment after resection may aid the better outcome.

Research perspectives

Accumulation of primary hepatic sarcoma data followed by finding of specific prognostic factor should be researched. New era of adjuvant therapies, such as immunotherapy for primary hepatic sarcoma is also needed to be developed.

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META-ANALYSIS

Endoscopic retrograde cholangiopancreatography drainage for palliation of malignant hilar biliary obstruction — stent-in-stent or side-by-side? A systematic review and meta-analysis

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Abstract

BACKGROUND

Biliary drainage, either by the stent-in-stent (SIS) or side-by-side (SBS) technique, is often required when treating a malignant hilar biliary obstruction (MHBO). Both methods differ from each other and have distinct advantages.

AIM

To compare both techniques regarding their efficacy and safety in achieving drainage of MHBO.

METHODS

A comprehensive search of multiple electronic databases (MEDLINE, Embase, LILACS, BIREME, Cochrane) was conducted and grey literature from their inception until December 2020 with no restrictions regarding the year of

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Conflict-of-interest statement: The authors deny any conflict of interest.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

Manuscript source: Invited manuscript

Specialty type: Gastroenterology and hepatology

Country/Territory of origin: Brazil

Peer-review report's scientific quality classification

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

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publication or language, since there was at least an abstract in English. The included studies compared SIS and SBS techniques through endoscopic retrograde cholangiopancreatography. Outcomes analyzed included technical and clinical success, early and late adverse events (AEs), stent patency, reintervention, and procedure-related mortality.

RESULTS

Four cohort studies and one randomized controlled trial evaluating a total of 250 patients (127 in the SIS group and 123 in the SBS group) were included in this study. There were no statistically significant differences between the two groups concerning the evaluated outcomes, except for stent patency, which was higher in the SIS compared with the SBS technique [mean difference (d) = 33.31; 95% confidence interval: 9.73 to 56.90, I^2 = 45%, P = 0.006].

CONCLUSION

The SIS method showed superior stent patency when compared to SBS for achieving bilateral drainage in MHBO. Both techniques are equivalent in terms of technical success, clinical success, rates of both early and late AEs, reintervention, and procedure-related mortality.

Key Words: Endoscopic retrograde cholangiopancreatography; Biliary tract neoplasms; Biliary; Hilar; Stenting; Drainage

Core Tip: Biliary drainage is often required when treating a malignant hilar biliary obstruction. There are two types of drainage: Stent-in-stent (SIS) and side-by-side (SBS) techniques. Both of them differ from each other and have distinct advantages. This study aimed to compare both techniques regarding their efficacy and safety. Our systematic review and meta-analysis demonstrated no statistically significant differences between the SIS and SBS techniques; except for stent patency which was superior in the SIS technique. The choice of palliation for drainage must be guided by both local expertise and resource availability.

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INTRODUCTION

Malignant hilar biliary obstruction (MHBO) is a late manifestation of certain types of cancer. This is diagnosed as unresectable in up to 80% of cases, and capable of causing potentially fatal complications, such as cholangitis and sepsis[1-6]. Thus, aimed at improving the quality of life and survival rate of patients, a discussion on the optimal method for palliation of drainage is very valuable[7-10].

The endoscopic biliary stent, introduced at the beginning of the 1980s, was a significant advance in the treatment of extrahepatic obstruction[11-13]. In biliary obstruction, self-expandable metal stents (SEMS) seem to provide prolonged patency of drainage when compared to plastic stents[3,4,14-17]. The endoscopic approach is preferred for drainage over the percutaneous and surgical approaches due to its more physiological nature, minimal invasiveness[3,4,6,18-20], low rate of adverse events (AEs), and shorter hospital stays[21]. One predictor of the effectiveness of biliary drainage is when the drained hepatic volume is above 50%. This often requires a bilateral decompression[15,22], which is associated with a lower chance of reintervention when compared to unilateral drainage in the palliation of drainage of

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MHBOs[23].

Bilateral drainage of the bile ducts can be performed *via* two methods: Stent-in-stent (SIS) or side-by-side (SBS)[15] placement of metal stents (Figure 1). In the SIS technique, one of the stents is positioned through the wire mesh of the other, configuring into a Y-shaped aspect. On the other hand, in the SBS method, both stents are placed side by side[22]. The SIS technique, in contrast to the SBS technique, does not require a dilated common bile duct, and thus allows the placement of higher caliber biliary stents[17], and presents a more physiological nature of drainage[3]. The SBS technique provides an easier procedural execution[3,15], and in the case of stent occlusion, reintervention is often more feasible[17].

In theory, there are advantages to both techniques, which casts doubt whether there is enough evidence to favor one method to the detriment of the other. Furthermore, few comparative studies have addressed the subject, making it still unclear which of the two methods is the optimal approach. To gather the best available data in the literature, we have designed this systematic review and meta-analysis on the subject. We aimed to compare the feasibility, safety, and efficacy of both the SIS and SBS techniques for palliative drainage in MHBO.

MATERIALS AND METHODS

Protocol and registration

This study was performed in conformity with the PRISMA[24] and it was registered in the International Prospective Register of Systematic Reviews under the file number CRD42020191262. The study was approved by the Ethics Committee of Hospital das Cl ínicas, Faculty of Medicine at The University of Sa□o Paulo.

Eligibility criteria

The data search was made without limitations of publication date or language, since there was at least an abstract in English. We considered clinical trials or observational studies published either as full text or as an abstract with the necessary data, comparing SIS and SBS metal stent placement in patients with malignant hilar biliary strictures. The following outcomes were observed: Technical and clinical success, early AEs (occurring within the first month after the procedure), late AEs (occurring after 30 d), stent patency, reintervention, and procedural-related mortality.

The exclusion criteria were studies using non-human subjects and trials that evaluated percutaneous biliary access drainage.

Information sources

We identified the studies by searching electronic databases and scanning reference lists of the selected articles. This search strategy was applied in electronic databases [MEDLINE, Embase, Central Cochrane, LILACS (*via* BVS), BIREME, and Google Scholar] and grey literature from their inception until December 2020 (Figure 2).

Search strategy and study selection

The following search strategy was used in all databases: [(Neoplasia OR Neoplasias OR Neoplasm OR Neoplasms OR Tumors OR Tumor OR Cancer OR Cancers OR Malignancy OR Malignancies) AND (Biliary Tract OR Biliary Tree OR Biliary System OR Bile Duct OR Bile Ducts)] OR [(Bile Duct Neoplasms OR Bile Duct Neoplasm OR Bile Duct Cancer OR Bile Duct Cancers OR Biliary Tract Neoplasm OR Biliary Tract Neoplasms OR Biliary Tract Cancer OR Biliary Tract Cancers) AND (Prostheses and Implants)] OR Prosthetic OR Implants OR Implant OR Prostheses OR Prosthesis OR Endoprosthesis OR Endoprostheses OR Stent OR Stents OR Stent-in-stent OR Side-by-Side.

Data collection process and data items

Two researchers reviewed the title and abstract of each article after the removal of duplicated articles. Articles that were found to be relevant were selected for full-text review. The final decision on the selection of the studies was based on predetermined inclusion and exclusion criteria. Any disagreement on the selection of studies was resolved by consensus with a third experienced researcher. The target data of the selected studies were entered and organized in a Microsoft Excel spreadsheet by the same two reviewers who conducted the selection. The reviewers extracted from the articles the outcomes of interest and information concerning the population and study

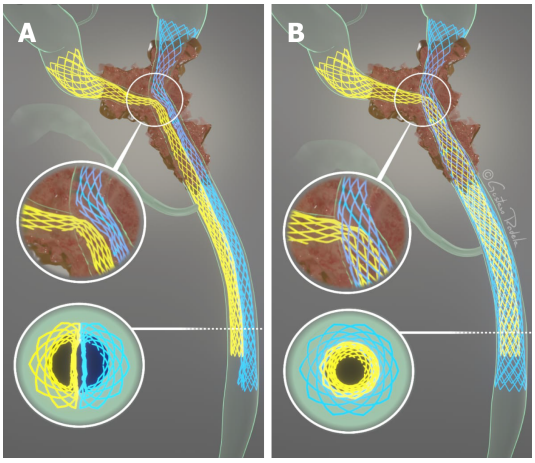

Figure 1 Two methods of bilateral drainage of the bile ducts. A: Side-by-side; B: Stent-in-stent.

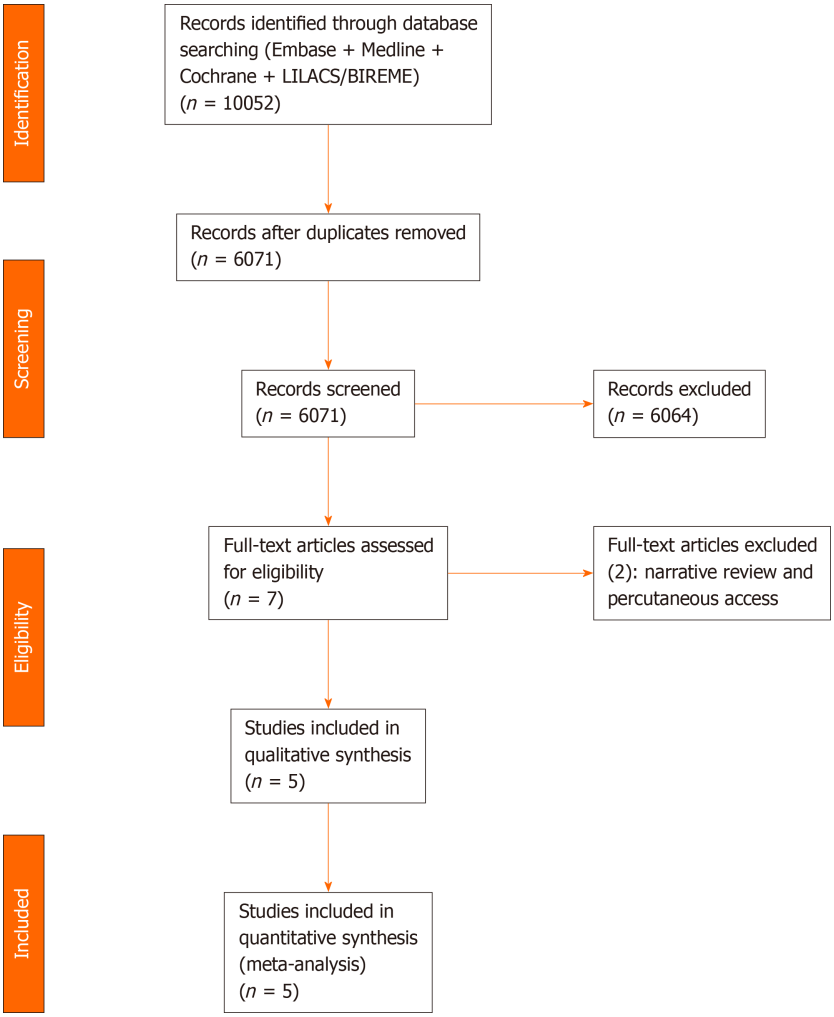

Figure 2 Flow diagram showing the article selection process.

characteristics. When the data of the published articles were insufficient, the corresponding authors were consulted by e-mail for further elucidation.

Risk of bias in individual studies and quality of evidence

The risk of bias in the cohort studies was assessed by the Risk of Bias in Non-randomized Studies-of Interventions (ROBINS I) Cochrane tool[25]. For randomized clinical trials, the risk of bias was defined by version 2 of the Cochrane Risk-of-Bias

tool for Randomized Trials (RoB2)[26].

The quality of evidence, expressed as high, moderate, low, and very low, was assessed utilizing the objective criteria from GRADE (Grading Recommendations Assessment, Development, and Evaluation) for each of the pre-specified results and outcomes using GRADEpro-Guideline Development Tool software (McMaster University, 2015; Evidence Prime, Inc., Ontario, Canada)[27].

Synthesis of results and data analysis

For continuous variables, we used mean or median values[28] along with the standard deviation and the total number of patients. Regarding the outcomes expressed by categorical variables, the absolute number of events and the total number of patients was employed, with calculation of the regular and absolute risk differences for each group utilizing the Mantel-Haenszel test. The mean values of each continuous outcome were calculated, as well as the 95% confidence interval (CI). P values < 0.05 were considered statistically significant and the results were exposed through forest plots.

Heterogeneity was calculated using the Higgins method (I^2). When heterogeneity < 50% was found, the fixed-effect model was used. In outcomes with high heterogeneity among studies (I^2 > 50%), sensitivity analysis employing funnel plots were conducted to identify publication bias (outliers). If the heterogeneity levels were still high even after outlier exclusion, we maintained the outlier and applied the random-effects model to express the results (true heterogeneity). If the heterogeneity levels were low after outlier exclusion, we applied the fixed-effects model.

The data of interest extracted from the selected studies were meta-analyzed using RevMan software (Review Manager Software version 5.4—Cochrane Collaboration Copyright© 2020).

RESULTS

Study selection and study characteristics

A total of 10052 articles were identified through our searches in the MEDLINE, Embase, LILACS, BIREME, and Central Cochrane databases. After the removal of duplicates, evaluation of the titles and abstracts, and text analysis, four retrospective cohort studies[29-32] and one randomized controlled trial (RCT)[33] were included in the meta-analysis (Figure 2). The characteristics of the included studies are summarized in Table 1.

Three[29,30,32] of the four retrospective studies presented a moderate overall risk of bias, assessed by the ROBINS-I tool, mainly due to confounding, the bias in the selection of participants, and bias in the selection of the reported results. The other included study[31] presented a serious risk of bias. The RCT study[33] presented a low risk of bias in our analysis (RoB2) (Tables 2 and 3). Detailed information concerning the risk of bias for each outcome is described in Table 4.

Technical success

All four cohorts[29-32] (181 patients) and the RCT study[33] (69 patients) assessed technical success. The overall analysis showed no difference between both SIS and SBS [risk difference (RD) = 0.06; 95%CI: -0.00 to 0.13, I^2 = 0%, P = 0.06] (Figure 3).

The overall certainty of the evidence was moderate for the cohorts and high for the RCT study, according to GRADE.

Clinical success

Three studies evaluated clinical success, namely two cohorts[30,32] (116 patients) and the RCT study[33] (69 patients). This outcome was similar for both SIS and SBS techniques in the overall analysis (RD = 0.07; 95%CI: -0.05 to 0.18, I^2 = 56%, P =0.26) (Figure 4).

The overall certainty of the evidence was low for the cohort and high for the RCT study, according to GRADE.

Early AEs

Three cohorts[30-32] (157 patients) and the RCT study[33] (69 patients) evaluated early complications. In the overall analysis, both SIS and SBS techniques performed similarly regarding this outcome (RD = -0.09; 95%CI: -0.19 to 0.01, I^2 = 2%, P =0.07) (Figure 5).

Table 1 Type of intervention and outcome of study

			Technical success		Clinical success		Rate of early adverse events		Rate of late adverse events		Stent patency		Reintervention		Procedure-related mortality	
Ref.	Design	Year	SIS	SBS	SIS	SBS	SIS	SBS	SIS	SBS	SIS	SBS	SIS	SBS	SIS	SBS
Lee *et al*[33]	RCT	2019	34/34	32/35	32/34	29/35	4/34	4/35	6/34	8/35	Median 253 d (28-420); SD 98; mean 253	Median 262 d (9-455); SD 111.5; mean 262	15/34	12/35	0/34	0/35
Naitoh *et al*[30]	Cohort	2012	24/24	25/28	24/24	24/28	1/24	3/28	2/24	8/28	Median 104 d (20-600); SD 145; mean 207	Median 155 d (15–881); SD 216.5; mean 155	NA	NA	0/24	0/28
Kim *et al*[31]	Cohort	2012	18/22	15/19	NA	NA	5/22	6/19	11/22	7/19	NA	NA	NA	NA	NA	NA
Law *et al*[29]	Cohort	2013	7/7	17/17	NA	NA	NA	NA	0/7	0/17	NA	NA	3/7	9/17	0/7	0/17
Ishigaki *et al*[32]	Cohort	2020	40/40	23/24	37/40	23/24	9/40	11/24	4/40	3/24	Median 169 d (108-445); SD 84.25; mean 169	Median 205 d (85-NA); SD 24.39; mean 123.75	NA	NA	NA	NA

SIS: Stent-in-stent; SBS: Side-by-side; RCT: Randomized controlled trial; NA: Not available.

The overall certainty of the evidence was moderate for both cohorts and the RCT study, according to GRADE.

Late AEs

Five studies[29-33] compared late complication rates, evaluating a total of 181 patients in the cohorts and 69 patients in the RCT. In the overall analysis, there was no significant difference between the two groups (RD = -0.04; 95%CI: -0.14 to 0.05, I^2 = 0%, P = 0.39) (Figure 6).

The overall certainty of the evidence was moderate for both cohorts and the RCT study, according to GRADE.

Stent patency

Three studies assessed stent patency: two cohorts[30,32] (116 patients) and the RCT[33] (69 patients). The overall analysis revealed increased stent patency when SIS was performed [mean deviation (MD) = 33.31; 95%CI: 9.73 to 56.90, I^2 = 45%, P = 0.006] (Figure 7).

The overall certainty of the evidence was moderate for the cohort and high for the RCT study, according to GRADE.

Reintervention

One cohort[29] compared reintervention rates, evaluating a total of 24 procedures—7 in the SIS group and 17 in the SBS group. We found no difference between the two groups in the overall analysis (RD = 0.05; 95%CI: -0.15 to 0.26, I^2 = 0%, P = 0.60)

Table 2 Risk of bias for ROBINS-I

Ref.	D1	D2	D3	D4	D5	D6	D7	Overall
Naitoh *et al*[30] 2012	Moderate	Moderate	Low	Low	Low	Moderate	Moderate	Moderate
Kim *et al*[31] 2012	Serious	Serious	Low	Serious	Serious	Serious	Serious	Serious
Law *et al*[29] 2013	Moderate	Moderate	Low	Low	Moderate	Moderate	Serious	Moderate
Ishigaki *et al*[32] 2020	Moderate	Moderate	Low	Low	Low	Moderate	Moderate	Moderate

D: Domains; D1: Bias due to confounding; D2: Bias due to selection of participants; D3: Bias in classification of interventions; D4: Bias due to deviations from intended interventions; D5: Bias due to missing data; D6: Bias in measurement of outcomes; D7: Bias in selection of the reported result.

Table 3 Risk of bias for RoB2

Ref.	D1	D2	D3	D4	D5	Overall
Lee *et al*[33], 2019	Low	Low	Low	Low	Low	Low

D: Domains; D1: Bias due to randomization process; D2: Bias due to deviations from intended interventions; D3: Bias due to missing outcome data; D4: Bias due to measurement of the outcome; D5: Bias due to selection of the reported result.

(Figure 8).

The overall certainty of the evidence was low for the cohort and high for the RCT study, according to GRADE.

Procedure-related mortality

Two cohorts[29,30] compared procedure-related mortality, evaluating a total of 76 procedures—31 in the SIS group and 45 in the SBS group. We found no difference between the two groups (RD = 0.00; 95%CI: -0.05 to 0.05, I^2 = 0%, P = 1.00) (Figure 9).

The overall certainty of the evidence was moderate for the cohorts and high for the RCT study, according to GRADE.

DISCUSSION

Despite being targeted by promising therapies in several clinical trials[34,35], bile duct tumors are often diagnosed as unresectable when they present with biliary obstruction. Therefore, internal drainage *via* the endoscopic deployment of stents has a pivotal role in this condition.

To the best of our knowledge, this is the first systematic review and meta-analysis comparing both the SIS and SBS techniques for the palliation of biliary drainage in MHBOs. This is a relevant topic for clinical practice, and many studies have non-comparatively evaluated these biliary drainage methods in the past. Despite presenting higher stent patency with the SIS method, we have found through our meta-analysis that there were no statistically significant differences concerning technical success, clinical success, early AEs, late AEs, reintervention, and procedure-related mortality.

For both groups, technical success was achieved in most cases, and we consider that the included studies were conducted at high-volume centers. The main challenge in the SBS method consists of the deployment of the second stent along with the first one. This is especially important since the distal end of both stents should ideally remain at the same level to facilitate an eventual reintervention. New devices have been developed, including systems with a thinner delivery system, which allows the simultaneous deployment of both prostheses. This system prevents the risk of a failed second placement and is associated with a shorter procedural time, as reported by Inoue *et al*[36]. Traditionally, the dilation on the wire mesh of the first stent before inserting the second one is necessary for the SIS technique. This prerequisite increases the difficulty and cost of the procedure. However, stents with larger cells have been developed, specifically for this usage, with high rates of technical success for the SIS method[37]. We consider that despite the fact that achieving bilateral biliary drainage

Table 4 Description of bias for each outcome (GRADE)

Certainty assessment							Summary of findings				
Participants (studies) follow up	Risk of bias	Inconsistency	Indirectness	Imprecision	Publication bias	Overall certainty of evidence	Study event rates (%)		Relative effect (95%CI)	Anticipated absolute effects	
							With SBS	With SIS		Risk with SBS	Risk difference with SIS
Early adverse events: Cohorts											
157 (3 observational studies)	Serious[1]	Not serious	Not serious	Not serious	None	Moderate	20/71 (28.2)	15/86 (17.4)	RR 0.54 (0.31 to 0.96)	282 per 1.000	130 fewer per 1.000 (from 194 fewer to 11 fewer)
Early adverse events: RCT											
69 (1 RCT)	Not serious	Not serious	Not serious	Serious[2]	None	Moderate	4/35 (11.4)	4/34 (11.8)	RR 1.03 (0.28 to 3.79)	114 per 1.000	3 more per 1.000 (from 82 fewer to 319 more)
Late adverse events: Cohorts											
181 (4 observational studies)	Serious[1]	Not serious	Not serious	Not serious	None	Moderate	18/88 (20.5)	17/93 (18.3)	RR 0.82 (0.46 to 1.47)	205 per 1.000	37 fewer per 1.000 (from 110 fewer to 96 more)
Late adverse events: RCT											
69 (1 RCT)	Not serious	Not serious	Not serious	Serious[2]	None	Moderate	8/35 (22.9)	6/34 (17.6)	RR 0.77 (0.30 to 1.99)	229 per 1.000	53 fewer per 1.000 (from 160 fewer to 226 more)
Procedural-related mortality: Cohorts											
76 (2 observational studies)	Serious[1]	Not serious	Not serious	Not serious	None	Moderate	0/45 (0.0)	0/31 (0.0)	Not pooled	Not pooled	Not pooled
Procedural-related mortality: RCT											
69 (1 RCT)	Not serious	Not serious	Not serious	Not serious	None	High	0/35 (0.0)	0/34 (0.0)	RR 0.00 (-0.05 to 0.05)	0 per 1.000	- per 1.000 (from 0 fewer to 0 fewer)
Technical success: Cohorts											
181 (4 observational studies)	Serious[1]	Not serious	Not serious	Not serious	None	Moderate	80/88 (90.9)	89/93 (95.7)	RR 1.06 (0.97 to 1.16)	909 per 1.000	55 more per 1.000 (from 27 fewer to 145 more)
Technical success: RCT											
69 (1 RCT)	Not serious	Not serious	Not serious	Not serious	None	High	32/35 (91.4)	34/34 (100.0)	RR 1.09 (0.97 to 1.22)	914 per 1.000	82 more per 1.000 (from 27 fewer to 201 more)
Clinical success: Cohort											
116 (2 observational studies)	Serious[1]	Serious[3]	Not serious	Not serious	None	Low	47/52 (90.4)	61/64 (95.3)	RR 1.05 (0.87 to 1.26)	904 per 1.000	45 more per 1.000 (from 118 fewer to 235 more)

Clinical success: RCT											
69 (1 RCT)	Not serious	Not serious	Not serious	Not serious	None	High	29/35 (82.9)	32/34 (94.1)	RR 1.14 (0.96 to 1.35)	829 per 1.000	116 more per 1.000 (from 33 fewer to 290 more)
Reintervention: Cohort											
24 (1 observational study)	Serious[1]	Not serious	Not serious	Serious[2]	None	Low	9/17 (52.9)	3/7 (42.9)	RR 0.81 (0.31 to 2.13)	529 per 1.000	101 fewer per 1.000 (from 365 fewer to 598 more)
Reintervention: RCT											
69 (1 RCT)	Not serious	Not serious	Not serious	Not serious	None	High	12/35 (34.3)	15/34 (44.1)	RR 1.29 (0.71 to 2.33)	343 per 1.000	99 more per 1.000 (from 99 fewer to 456 more)
Stent patency: Cohort											
116 (2 observational studies)	Serious[1]	Not serious	Not serious	Not serious	None	Moderate	52	64	-	The mean stent patency: Cohort was 0	MD 45.75 higher (18.92 higher to 72.58 higher)
Stent patency: RCT											
69 (1 RCT)	Not serious	Not serious	Not serious	Not serious	None	High	35	34	-	The mean stent patency: RCT was 0	MD 9 lower (58.49 lower to 40.49 higher)

[1]There are risk of bias in selection of the reported result, according to ROBINS-I tool.
[2]Wide confidence interval range.
[3]High heterogeneity, calculated using the Higgins method (*I2*).
CI: Confidence interval; RR: Risk ratio; MD: Mean difference; RCT: Randomized controlled trial.

in the MHBOs is technically challenging, technical success rates were increased and equivalent between both SIS and SBS, probably due to the endoscopist's vast expertise and the availability of suitable material.

Clinical success was defined in the studies as a total bilirubin decrease in the first month to at least 50% or 75% of the pre-treatment value. Although there was no statistical difference between the groups, we have reservations regarding this outcome definition and we think this outcome should be evaluated very carefully. One reason for this could be that the studies that evaluated this outcome opted for a conservative definition, based on a little significant drop in bilirubin levels, and not on laboratory level standards. Also, they failed to assess other laboratory or clinical parameters.

The use of uncovered SEMS is preferred over fully covered SEMS (FCSEMS) for palliative drainage of malignant biliary obstructions[21], just as it was done in the assessed studies. This is due to the risk of obstruction in intrahepatic lateral branches and cystic and pancreatic ducts, abscess-related factors, cholecystitis, and acute pancreatitis (AP). Inoue *et al*[38] and Yoshida *et al*[39] reported the occurrence of hepatic abscesses (11.8% and 6.3% of cases, respectively) when using 6 mm FCSEMS. Although these results cannot be attributed to the stents, they allow us to consider such a hypothesis. In our study, the SIS and SBS techniques presented similar results

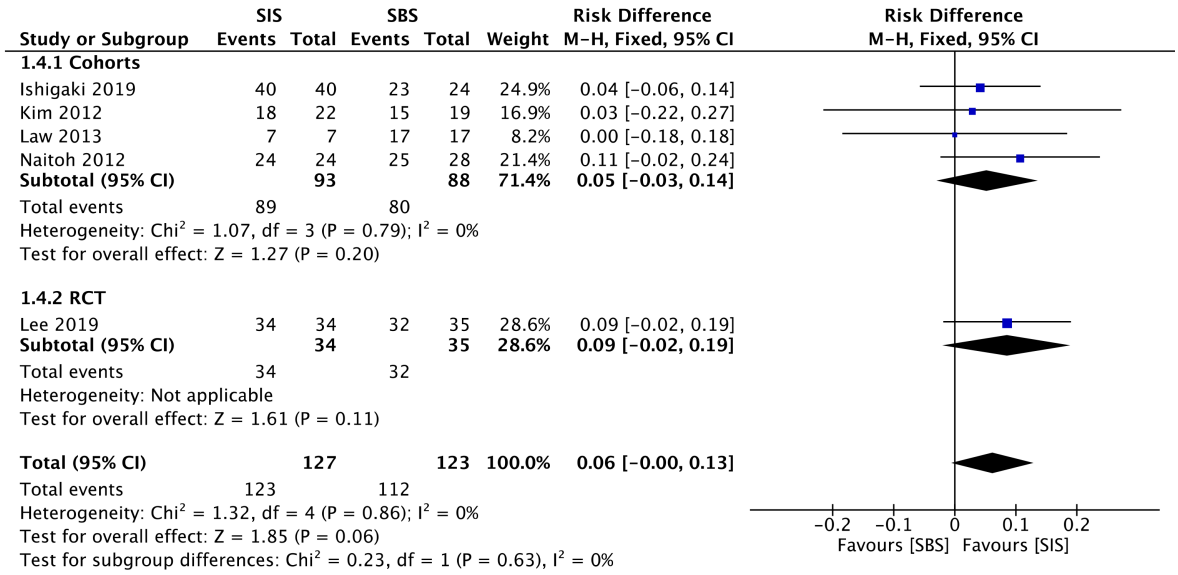

Figure 3 Forest plot — studies reporting rate of technical success using a fixed-effects model. CI: Confidence interval; SIS: Stent-in-stent; SBS: Side-by-side; RCT: Randomized controlled trial; M-H: Mantel-Haenszel test.

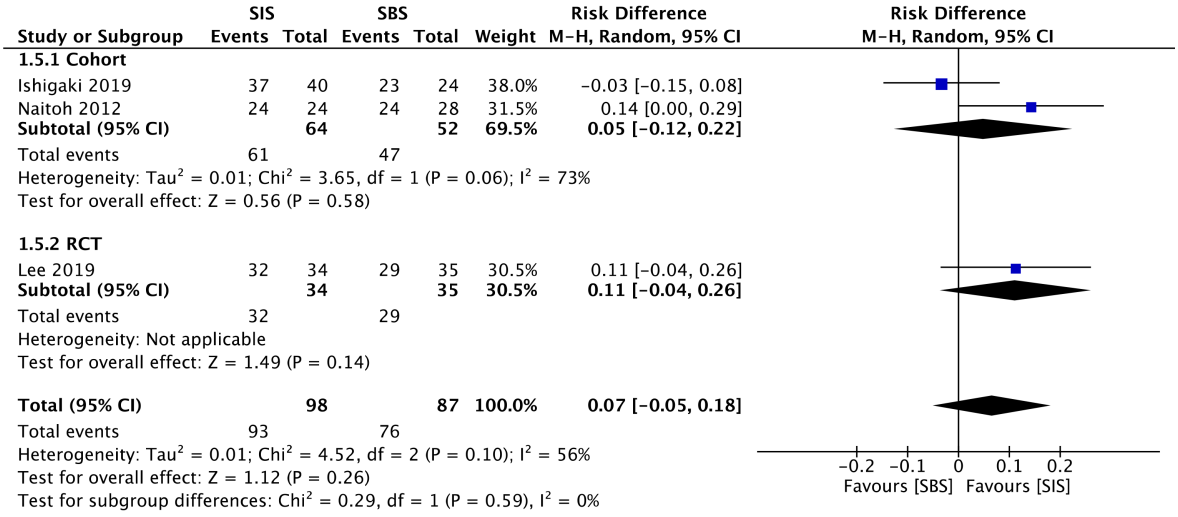

Figure 4 Forest plot — studies reporting rate of clinical success using a random-effects model. CI: Confidence interval; SIS: Stent-in-stent; SBS: Side-by-side; RCT: Randomized controlled trial; M-H: Mantel-Haenszel test.

regarding late complications, such as cholangitis, cholecystitis, and biloma formation. In the cohort meta-analysis, SBS resulted in higher early complication rates (RD = -0.14; 95%CI: -0.27 to -0.01, I^2 = 0%, P = 0.03), such as AP. Tarnasky *et al*[40] had already reported a higher risk of AP in patients referred to biliary stenting for hilar biliary stricture. Furthermore, stent deployment in SBS with the distal end of the stent across the papilla, instead of above the papilla, seems to raise the risk of AP[41]. Nevertheless, in the cohorts meta-analyzed in the present study both techniques were utilized, thus impeding the attribution of the aforementioned complication exclusively to that reason. However, a RCT and general analysis showed no statistically significant differences. These data suggest that both techniques are safe as part of a minimally invasive treatment, with no differences regarding the occlusion of intrahepatic, cystic, or pancreatic ducts. Even if it is not possible to arrive at this conclusion from only this meta-analysis, it seems to us that the stent type has more influence on the complication rates than the drainage technique itself. The safety of endoscopic treatment and each specific technique, is reinforced by the absence of procedural-related deaths in all the casuistry of this study.

The outcome of stent patency, evaluated as moderate and high levels of evidence for the cohort and the RCT, respectively, showed a MD = 33.31, favoring SIS, with a

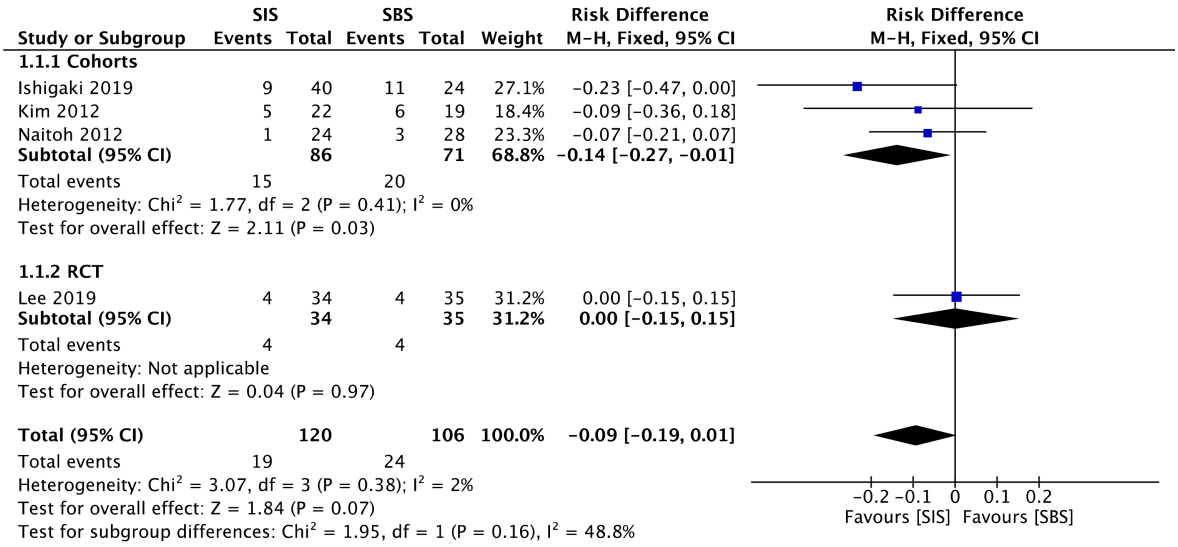

Figure 5 Forest plot — studies reporting rate of early adverse events using a fixed-effects model. CI: Confidence interval; SIS: Stent-in-stent; SBS: Side-by-side; RCT: Randomized controlled trial; M-H: Mantel-Haenszel test.

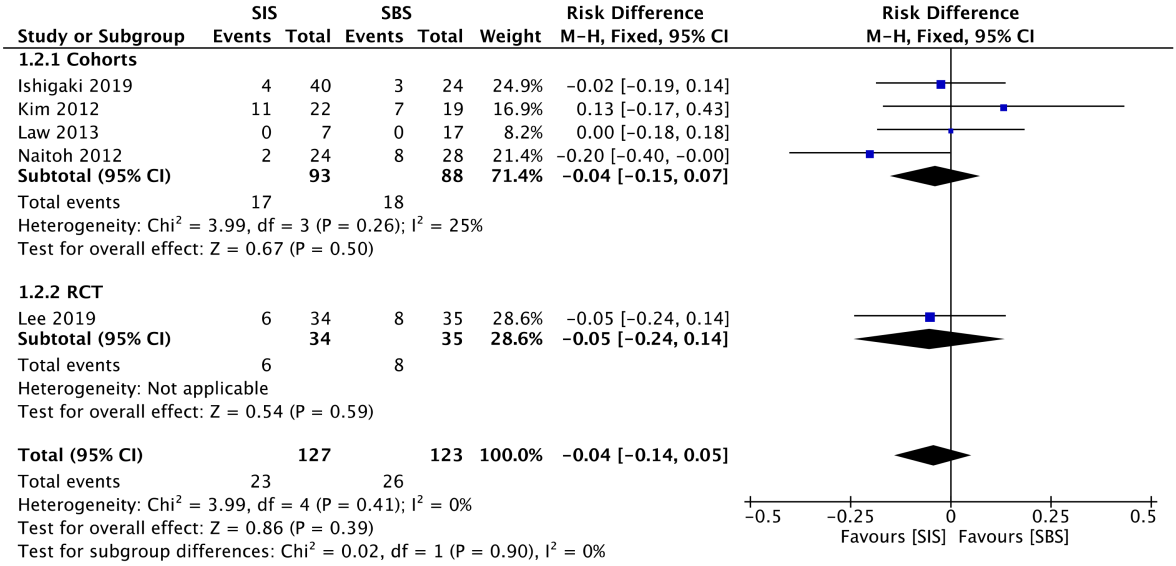

Figure 6 Forest plot — studies reporting rate of late adverse events using a fixed-effects model. CI: Confidence interval; SIS: Stent-in-stent; SBS: Side-by-side; RCT: Randomized controlled trial; M-H: Mantel-Haenszel test.

95%CI: 9.73 to 56.90. Although the reason behind such a difference is unclear, we believe that the SIS technique may allow greater stent expandability, and consequently larger internal caliber, in comparison with the SBS technique. Nevertheless, this result should be analyzed very carefully since some studies do not specify the exact caliber of the employed stent, and one of them disclosed the use of calibers slightly larger in the SIS technique. The use of SEMS in the studies is a positive factor regarding stent patency, corroborating the findings of the specific study that showed higher patency with these types of stents when compared with the plastic stents (131 d *vs* 47 d)[42]. Our study found no difference regarding the reintervention rate. The main cause of post-procedural obstruction was tumor progression (ingrowth or overgrowth) provoking cholestasis and cholangitis, and thus requiring reintervention. The reintervention approach usually adopted in these cases is the placement of an inner metallic stent, after the cleansing of ductal debris with a balloon extraction and/or cholangioscopy. Radiofrequency ablation can also be considered, but related studies are still scarce[21].

Our study has some limitations. There is only one RCT in the literature comparing both analyzed techniques. Besides the RCT, only 4 comparative retrospective observa-

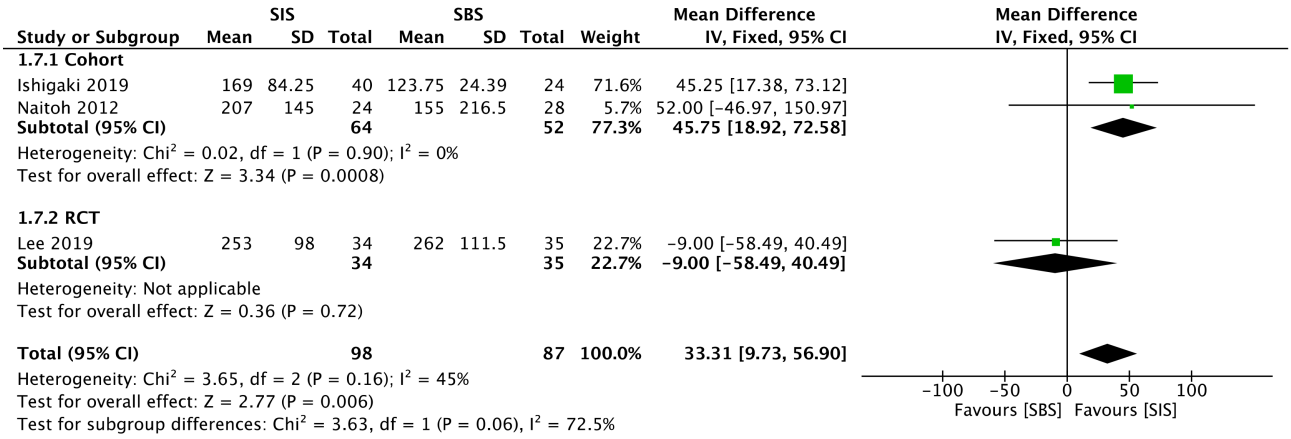

Figure 7 Forest plot — studies reporting the number of days of stent patency using a fixed-effects model. CI: Confidence interval; SIS: Stent-in-stent; SBS: Side-by-side; RCT: Randomized controlled trial.

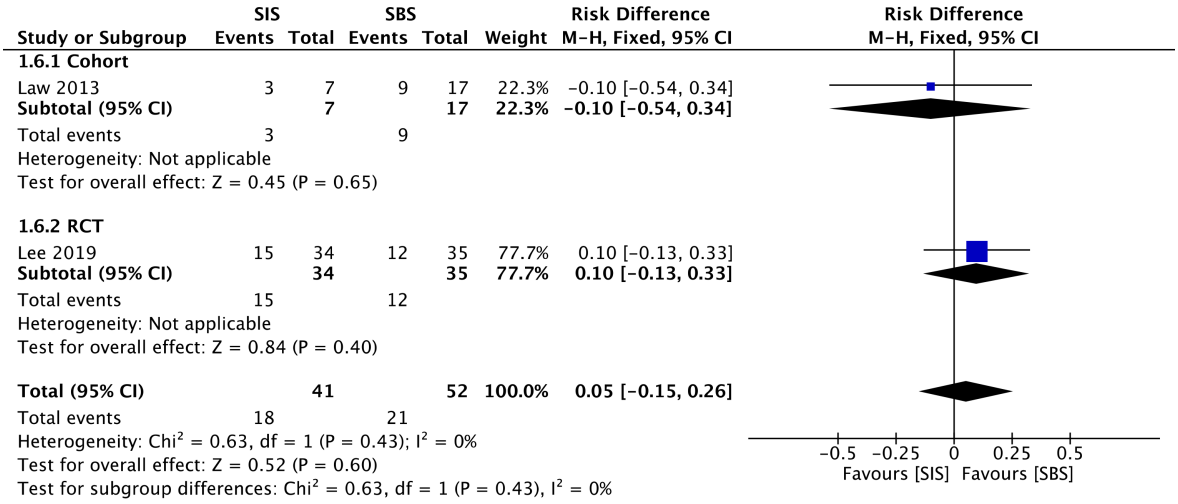

Figure 8 Forest plot — studies reporting the rates of reintervention using a fixed-effects model. CI: Confidence interval; SIS: Stent-in-stent; SBS: Side-by-side; RCT: Randomized controlled trial; M-H: Mantel-Haenszel test.

tional studies are available in the literature. Furthermore, the number of patients in the included studies is small, perhaps because this disease does not have a high prevalence. Although the study by Kim *et al*[31] is available only as an abstract, it possessed all the necessary data for this analysis. Moreover, the prostheses used in different studies were from different manufacturers, with no information on diameter measurements for comparison. Given such limitations, new RCTs may have a valuable role in new systematic reviews, thus improving the quality of evidence.

Despite the aforementioned limitations and to the best of our knowledge, our study is the first systematic review with a meta-analysis on this topic. We firmly believe this has significant clinical applicability given the increasing demand for bile duct drainage in the palliation of malignant hilar tumors.

CONCLUSION

There is no significant difference between the SIS or SBS techniques in terms of early and late complication rates, technical success, clinical success, reintervention, and procedural-related mortality. The SIS technique was superior in terms of stent patency when compared to the SBS technique, which may guide decision-making regarding the best therapeutic modality for each patient.

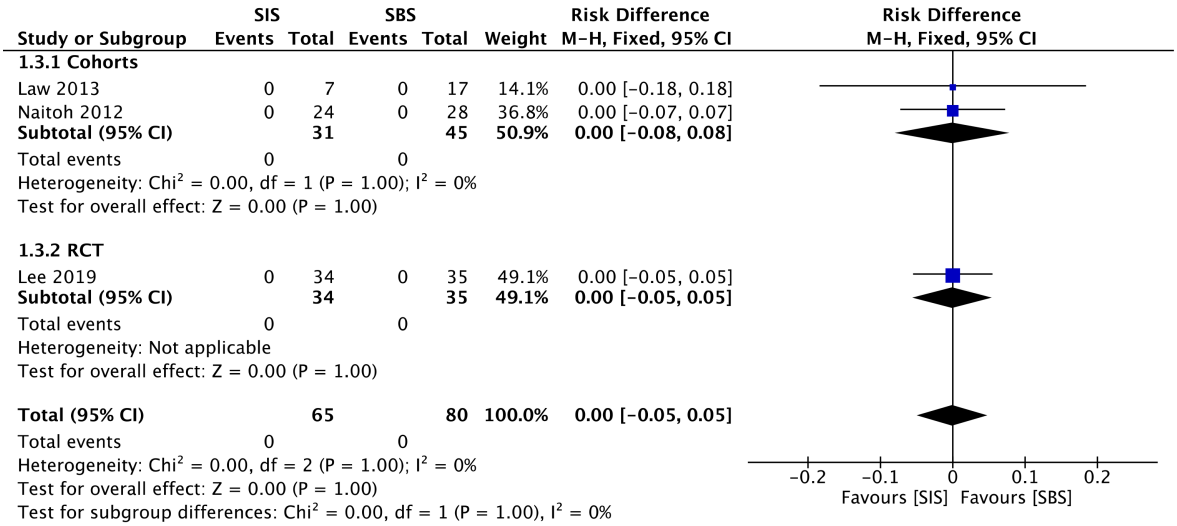

Figure 9 Forest plot — studies reporting the rates of procedural-related mortality using a fixed-effects model. CI: Confidence interval; SIS: Stent-in-stent; SBS: Side-by-side; RCT: Randomized controlled trial; M-H: Mantel-Haenszel test.

ARTICLE HIGHLIGHTS

Research background

Patients with malignant hilar biliary obstruction (MHBO) benefit from bilateral palliative endoscopic drainage. However, there is no consensus on which is the optimal technique for placing a metal stent: Stent-in-stent (SIS) or side-by-side (SBS).

Research motivation

Many patients undergo palliative endoscopic retrograde cholangiopancreatography (ERCP) drainage, due to the advanced stage of the disease at the time of diagnosis, unresectable in most cases. However, choosing the best management for drainage can be a real technical challenge. Therefore, we aimed to compare both drainage techniques in an attempt to identify the optimal approach.

Research objectives

To perform a systematic review and meta-analysis of available studies that compare SIS and SBS deployment in patients with MHBO undergoing ERCP drainage.

Research methods

The systematic review and meta-analysis followed the PRISMA Guidelines. Electronic searches were performed in MEDLINE, Embase, Cochrane, LILACS, and BIREME databases, and the grey literature. Comparative cohorts and randomized controlled trials (RCTs) were included. Studied outcomes were technical and clinical success, early and late adverse events (AEs), stent patency, reintervention, and procedure-related mortality.

Research results

Four comparative cohorts and one RCT were included in the final analysis with a total of 250 patients, of whom 127 belonged to the SIS group and 123 to the SBS group. Stent patency was significantly higher in the SIS group. Procedure-related mortality was similar in both groups, and no significant differences were found in the rates of technical success, clinical success, early AEs, late AEs, and reintervention.

Research conclusions

There was no difference between the groups concerning technical and clinical success, early and late AEs, reintervention, and procedure-related mortality. However, there was longer stent patency in patients undergoing the SIS technique. This result suggests that SIS may be the preferred technique for bilateral palliative metal stent deployment in patients with inoperable MHBO.

Research perspectives

Palliative biliary drainage is an increasingly performed procedure, but without consensus on the optimal technique, SIS or SBS. There is a small number of comparative studies in the literature. Future RCTs will have an important role in elucidating the most optimal drainage technique.

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CASE REPORT

Acquired hepatocerebral degeneration in a metastatic neuroendocrine tumor long-term survivor — an update on neuroendocrine neoplasm's treatment: A case report

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Abstract

BACKGROUND

Metastatic small bowel low-grade neuroendocrine tumors (NETs) have a good prognosis. Surgery is the only curative treatment; however, this may induce advanced liver disease, particularly in long-term survivor patients. Acquired hepatocerebral degeneration or Parkinsonism in cirrhosis is characterized by rapidly progressive extrapyramidal symptoms in patients with advanced liver disease.

CASE SUMMARY

A 70-year-old man presented to the emergency department with diminished

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consciousness and disorientation, and was diagnosed with hepatic encephalopathy. The patient was diagnosed in 1993 with a metastatic small bowel NET, for which he twice underwent hepatic surgery, with metastatic resection in 1993 and a right hepatectomy in 2002 to remove two hepatic metastases. In 2003, the patient started first-line chemotherapy and in 2004 started the first of three consecutive biological treatments, followed by radio-molecular therapy, achieving stable disease for 14 years. Disease progression was identified and he underwent an endoscopic retrograde cholangiopancreatography. However, in 2019 advanced liver disease was identified. We diagnosed the development of acquired hepatocerebral degeneration, an unusual long-term side effect after multiple hepatic procedures.

CONCLUSION

The importance of regular and ongoing surveillance in long-term NET survivors who undergo hepatic procedures should be integrated into the therapeutic management plan, as some of these negative outcomes could be prevented.

Key Words: Neuroendocrine tumors; Hepatocerebral degeneration; Parkinsonism; Somatostatin analogues; Everolimus; Hepatic metastases; Peptide radionuclide receptor therapy; Encephalopathy; Paramagnetic deposits; Case report

Core Tip: To the best of our knowledge, this is the first case report of acquired hepatocerebral degeneration in a metastatic small bowel neuroendocrine tumor long-term survivor, an uncommon irreversible extrapyramidal neurodegenerative condition encountered in patients with cirrhotic chronic liver disease, and resulting in widespread cerebral, basal ganglia, and cerebellar damage.

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INTRODUCTION

Neuroendocrine neoplasms (NENs) are a rare group of cancers accounting for about 0.05% of all newly diagnosed malignancies and 0.5% of all gastrointestinal and lung malignancies[1-3]. Nonetheless, the incidence rate increased 6.4-fold from 1973 to 2012[2,4]. NENs are a heterogeneous group of malignancies with a slightly higher female preponderance, and are most commonly found in the gastrointestinal tract and lungs[5].

The neuroendocrine system encompasses not only the endocrine glands but is also scattered throughout the exocrine parenchyma, the so-called diffuse endocrine system[6,7]. Histologically, NENs are clustered into two main groups. On one hand, neuroendocrine tumors (NETs) are typically well-differentiated tumors characterized by uniform nuclei with dense granules, histologically described as "salt and pepper." By contrast, neuroendocrine carcinomas have a poorly defined phenotype with a high mitotic index, and up to 40% do not express neuroendocrine markers[6,7]. Diagnosis confirmation must always be accompanied by a biopsy of the primary tumor or metastases. The 2017 World Health Organization classification takes into account the grade of differentiation and the Ki-67 mitotic proliferation index, distinguishing four groups; G1, G2 and G3 NETs and neuroendocrine carcinomas. Ki-67 grading is an important prognostic factor, and is therefore a mandatory biomarker in pathological reporting[8-10].

Liver metastases represent another crucial prognostic factor. Surgery of metastases

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is the only treatment that offers a cure[11]. For unresectable lesions, there are multiple treatment options such as somatostatin analogues (SSA), interferon α, local liver therapies, chemotherapy, peptide-receptor radionuclide therapy, angiogenesis inhibitors, and mammalian rapamycin inhibitors. SSA have both anti-secretory and antiproliferative effects, improving progression-free survival in both the PROMID trial (octreotide LAR *vs* placebo) and the CLARINET (lanreotide *vs* placebo) trial[12,13]. The NETTER-1 trial reported prolongation of progression-free survival (PFS) after treatment with ^{177}Lu-Dotatate compared to treatment with octreotide in patients with a well-differentiated midgut-NET[14]. Notably in the case of gastrointestinal NETs, the certainty of evidence is highest for the combination of SSA plus ^{177}Lu-dotatate[15].

Nonetheless, the downside of these options is that many of these treatments can result in injury of healthy liver parenchyma and development of sinusoid liver fibrosis, and consequently induce portal hypertension with progression to advanced liver disease. The main complications of chronic liver disease are hepatocellular carcinoma and portal hypertension[16,17]. According to a study published in 2013 by Tryc *et al*[17], about 4% of cirrhotic patients develop progressive hypokinetic-rigid syndrome, which is not present in hepatic encephalopathy, recently referred to in the literature as "cirrhosis-related-Parkinsonism" or "acquired hepatocerebral degeneration (AHD)." The most commonly reported symptoms of patients with AHD are bradykinesia, cerebellar symptoms, tremor, and rigidity[16,18,19].

It has been hypothesized that AHD originates from increased manganese deposits in the basal ganglia, particularly in the globus pallidum, damaging the presynaptic dopamine transporters and post-synaptic dopamine receptors in cirrhotic patients[17,20,21]. Treatment with levodopa can be effective when D2 receptors are available[17,22]. The study by Rose *et al*[20] analyzing postmortem human brain tissue, demonstrated an increase in manganese deposits in several brain structures of cirrhotic patients. The two main causes of increased manganese deposits that the authors found to be statistically significant resulted both from portocaval-shunt and liver dysfunction[20]. This manuscript is the first case in the literature to report AHD in a metastatic gastrointestinal NET long-term survivor.

CASE PRESENTATION

Chief complaints

A 70-year-old male patient presented in January 2019 to the emergency department with diminished consciousness and disorientation, without any other relevant symptoms.

History of present illness

Neurological symptoms were first reported in May 2017 and asymmetric Parkinson's disease diagnosed in June 2018 for which he received levodopa.

History of past illness

The patient had a medical history of high blood pressure, which was treated with diuretics. The patient denied use of potentially hepatotoxic drugs. He also had diabetes mellitus type 2 treated with metformin, without any other cardiovascular risk factors. He underwent a gastrectomy and Billroth II reconstruction in March 1993 for a gastric ulcer.

His oncological history started in 1993 when he was diagnosed with a metastatic midgut NET confirmed by a hepatic biopsy, in which the anatomic pathology reported a well-differentiated tumor with a Ki-67 expression of 1.26%, graded as a G1 tumor (Figure 1), and without hepatic enzyme alterations. He subsequently underwent two hepatic procedures: a single metastasis resection from the right hepatic lobe in July 1993 and a right hepatectomy was performed in 2002 to remove two hepatic metastases. There were no changes to laboratory data or computed tomography (CT) scans after both procedures.

In November 2003, the patient started first-line chemotherapy with streptozotocin and doxorubicin after a new hepatic lesion appeared. In 2004, the patient showed hepatic progression and began treatment with the biological agent octreotide. He achieved stable disease lasting until 2010, when a CT scan showed a new hepatic lesion in the surgical bed, three sub-centimeter hepatic lesions, and a new adenopathy in the hepatic hilum. The treatment was discontinued. Then the patient participated in the RAMSETE clinical trial evaluating the efficacy of everolimus at 10 mg daily in non-functioning extrapancreatic NETs, and was randomized to the active treatment arm.

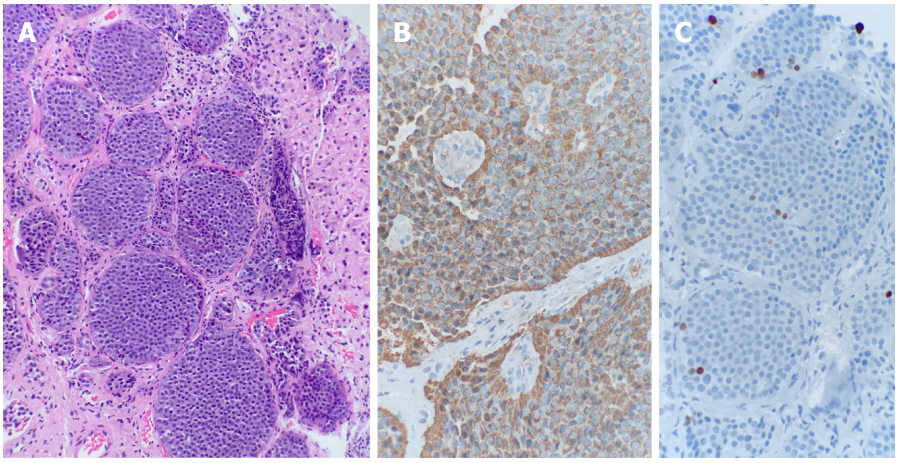

Figure 1 Anatomic pathology report of the tumor biopsy. A: Hematoxylin and eosin staining showing neoplastic neuroendocrine cells with a typical insular pattern infiltrating hepatic parenchyma; B: Chromogranin A positivity with granular/dot-like cytoplasmic staining; C: Ki-67 staining shows a proliferation rate of 1. 26% in the hot-spot, and the neoplasia was graded as G1.

The lesions showed tumor shrinkage with a total reduction of 23%, corresponding to stable disease per RECIST v1.1. The only side effects were low platelet counts and grade 1 pneumonitis (CTCAE v5.0). After 5 years with stable disease, a CT scan showed progressive disease in July 2015, and he changed to a third biological treatment, lanreotide at 120 mg monthly, again achieving stable disease as the best response.

In June 2017, he presented to the emergency department with cholangitis due to extrinsic compression of the bile duct from hepatic lesions, and a choledochal stent was inserted by endoscopic retrograde cholangiopancreatography. The CT scan showed progressive disease in the liver. A somatostatin receptor scintigraphy revealed liver, hepatic hilum and peritoneal uptake, and he started peptide receptor radionuclide therapy (PRRT) with ^{177}Lu-Dotatate for three sessions and 30 mg octreotide LAR monthly, achieving partial response as the best response. One year later in July 2018, the patient returned to the emergency room with a new episode of cholangitis. Hepatic magnetic resonance imaging (MRI) showed extensive progression in the surgical bed, invading the biliary stent and causing partial obstruction. A new endoscopic retrograde cholangiopancreatography was performed to unblock the bile duct and antibiotic treatment was administered. At discharge, the patient continued on octreotide LAR, and had an ongoing best response of stable disease at the time of presentation to the emergency department in January 2019.

It should be noted that in May 2017, the patient began to complain of memory loss and distal tremor, but it was not until June 2018 that he was diagnosed with asymmetric Parkinson's disease by a neurologist. At this time, the CT scan showed heterogeneous liver with signs of portal hypertension. The patient began treatment with levodopa for Parkinson's disease, without a significant clinical response.

Personal and family history

There was no relevant family history.

Physical examination

On physical examination, he presented with flapping, jaundice, facial amimia, and cogwheel rigidity in both arms. There were no signs of Kayser-Fleischer rings. All other neurological examinations were normal.

Laboratory examinations

Blood tests showed low platelet count, low albumin and evidence of cholestasis, with no other relevant alterations. Urine sediment and blood culture were negative, ruling out an infectious cause. The patient presented with Child-Pugh B and MELD 8 at admission. Ceruloplasmin was within normal ranges. There were no findings of hypovitaminosis, dyselectrolytemia, hypothyroidism, hepatitis virus serologies, or autoimmunity tests.

Imaging examinations

A head CT scan performed with intravenous contrast material revealed no evidence of intracranial hemorrhage, mass, or acute territorial infarct. However, an abdominal CT scan and brain MRI shed light on our case. The CT scan showed signs of portal hypertension, describing splenomegaly and splenic dilatation with collateral circulation, as well as an intrahepatic portosystemic shunt without biliary tract obstruction (Figure 2A and B). Surprisingly, brain MRI showed symmetric basal ganglia hyperintensity in a T1 alteration and asymmetric extension to cerebral peduncles, compatible with deposits of paramagnetic substances (Figure 3A and B) related to the intrahepatic shunt described in the previous CT scan. A video-electroencephalography was performed and showed neuronal dysfunction of metabolic-toxic origin. To complete our analysis, the patient underwent a gastroduodenoscopy, which showed grade 3 esophageal varices and the liver MRI revealed multiple irregular metastases (Figure 4).

FINAL DIAGNOSIS

The patient was diagnosed with hepatic encephalopathy and AHD secondary to advanced liver disease, most likely induced by a combination of previous hepatic resections, targeted therapies, and radionuclide treatment.

TREATMENT

We started treatment with both oral and rectal laxatives, banding, and beta blockers at increasing dosage, improving the hepatic encephalopathy symptoms without developing side effects, thus restoring the patient to his basal state.

OUTCOME AND FOLLOW-UP

The patient was discharged after 2 wk of treatment with only a remaining rigidity of the superior left extremity, a sign of non-reversible neuronal damage due to AHD. We did not administer any further oncological treatment, and best supportive care was maintained. Levodopa treatment was stopped. The patient was alive 6 mo after discharge.

DISCUSSION

NENs are rare and heterogeneous tumors with the particularity of secreting hormones, adding a further layer of complexity to their clinical management. On the other hand, this also gave the treating physician the opportunity to target these tumor cells with multiple approaches. We must consider carefully the treatment modalities available, since our choices will impact our patients' future. In this particular case, the patient developed an AHD after receiving multiple treatments for his metastatic midgut NET.

In particular, the patient underwent two hepatic surgeries and multiple hepatotoxic treatments, notably receiving four targeted therapies, three doses of PRRT, and lastly a somatostatin agonist. According to the NETTER-1 study, PRRT is superior to octreotide, but an important part of our treatment approach is to individualize the therapy according to the type of tumor, patient and treatments received previously. While PRRT treatment is a local radiotherapy that is highly selective for tumor tissue, it also affects the healthy surrounding hepatic parenchyma. Our patient had little healthy hepatic parenchyma left, and therefore had a greater susceptibility to local "ablative" therapies.

The patient experienced advanced liver disease after PRRT treatment, which likely acted as a trigger for AHD in a patient with unhealthy liver tissue, as we could identify in the CT scans and from consecutive liver laboratory tests before, during and after PRRT (Figure 5). The patient was not a candidate for closing the portosystemic shunt due to technical difficulties, and the patient's severe portal hypertension (Child B cirrhosis) and a high bleeding risk, so his only options were preventive medical treatment. Thus, the AHD symptoms of our patient persisted, since the cause was not

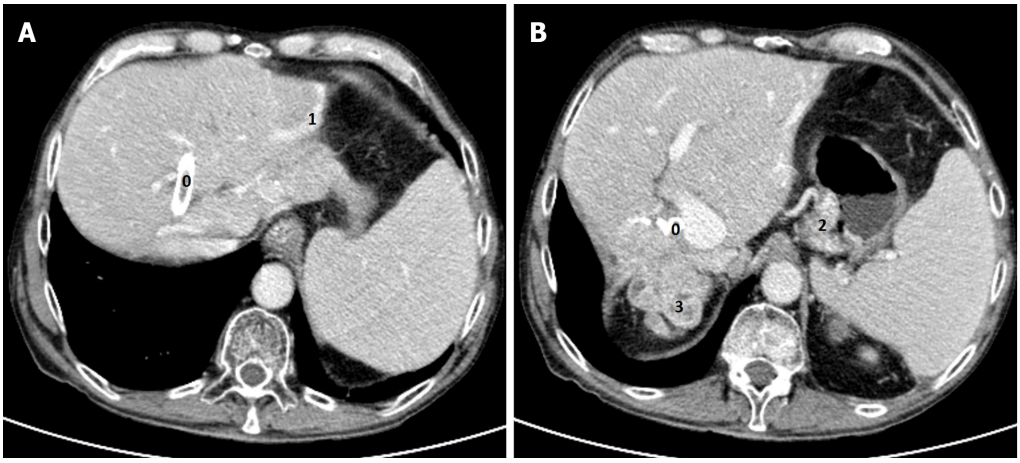

Figure 2 Contrast-enhanced computed tomography (portal phase). A: Biliary prosthesis (0), and abnormal vascular hepatic vein in the most marginal aspect of the left liver lobe related to a portosystemic shunt (1); B: Enlarged vein in the gastrohepatic ligament associated with small gastric mural varicose veins (2). Metastatic lesions at the hepatic hilum (3).

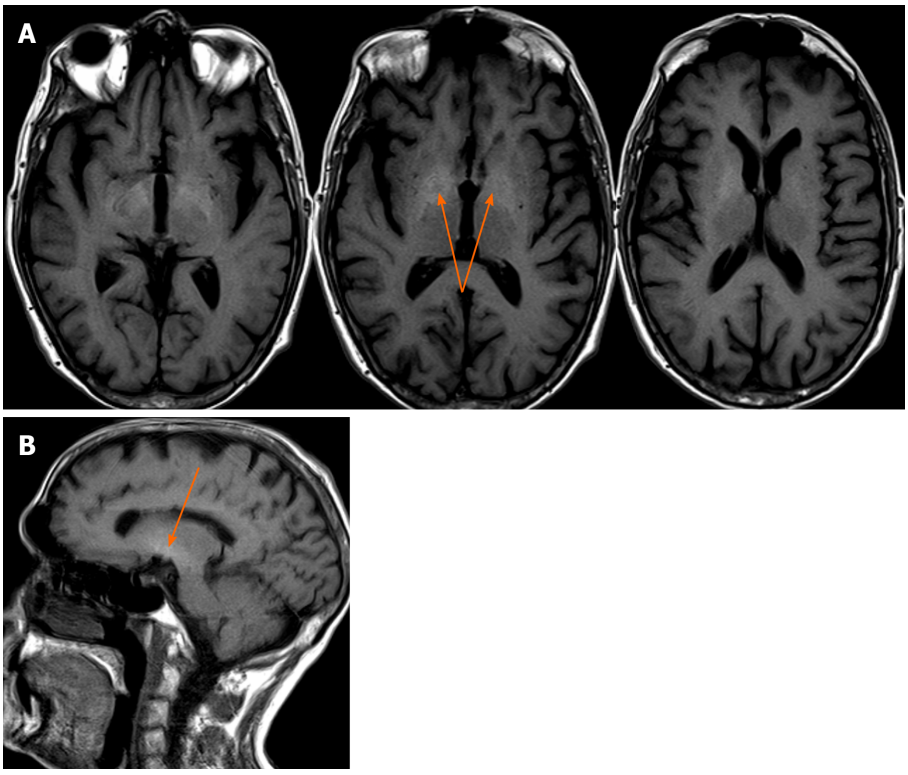

Figure 3 Brain magnetic resonance imaging. A: Axial; B: Sagittal projection showing T1-weighted imaging, hyperintense signal (arrow) within a lentiform nucleus extending into the midbrain.

treated. On the other hand, the hepatic encephalopathy responded excellently, within days, to ammonia-lowering agents. The Parkinson's disease was initially thought to be primary, but with the extensive paramagnetic deposits in the basal ganglia and the poor response to levodopa, it would be more reasonable to consider it a Parkinsonism secondary to the portosystemic shunts and hepatic cirrhosis. As stated previously, the presence of manganese in the basal ganglia of cirrhotic patients is diagnostic of AHD and can result in irreversible neuronal damage.

CONCLUSION

Herein, we report the first case described in the literature of AHD in a metastatic NET.

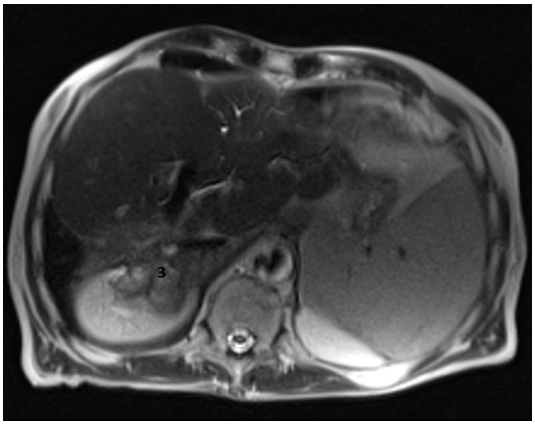

Figure 4 Liver magnetic resonance imaging. Axial T2-weighted imaging HASTE magnetic resonance imaging. Multiple irregular right liver metastatic lesions (3).

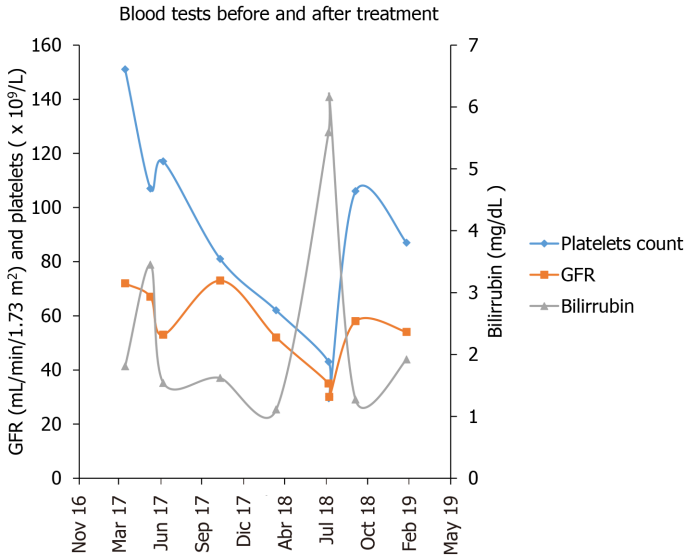

Figure 5 Blood tests before and after treatment. Laboratory test parameters showing platelet count (× 10^9/L), glomerular filtration rate (GFR) (in mL/min/173 m^2) and total bilirubin (mg/dL) before and after peptide-receptor radionuclide therapy treatment.

Patients with NETs are typically long-time survivors, and we currently have multiple treatment modalities to choose from. Selection should be based on maximizing survival and reducing both the potential immediate and long-term side effects. The negative outcomes relating to hepatic injury in long-term NET survivors resemble those of patients with advanced liver disease. As such, regular monitoring and surveillance for potential complications in long-term cancer survivors should be recommended to rule out negative outcomes that may appear following treatment.

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The primary aim of *World Journal of Hepatology* (*WJH*, *World J Hepatol*) is to provide scholars and readers from various fields of hepatology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

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REVIEW

Glutathione-S-transferases genes-promising predictors of hepatic dysfunction

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Abstract

One of the most commonly known genes involved in chronic diffuse liver diseases pathogenesis are genes that encodes the synthesis of glutathione-S-transferase (GST), known as the second phase enzyme detoxification system that protects against endogenous oxidative stress and exogenous toxins, through catalisation of glutathione sulfuric groups conjugation and decontamination of lipid and deoxyribonucleic acid oxidation products. The group of GST enzymes consists of cytosolic, mitochondrial and microsomal fractions. Recently, eight classes of soluble cytoplasmic isoforms of GST enzymes are widely known: α-, ζ-, θ-, κ-, μ-, π-, σ-, and ω-. The *GSTs* gene family in the Human Gene Nomenclature Committee, online database recorded over 20 functional genes. The level of GSTs expression is considered to be a crucial factor in determining the sensitivity of cells to a broad spectrum of toxins. Nevertheless, human *GSTs* genes have multiple and frequent polymorphisms that include the complete absence of the *GSTM1* or the *GSTT1* gene. Current review supports the position that genetic polymorphism of *GST* genes is involved in the pathogenesis of various liver diseases, particularly non-alcoholic fatty liver disease, hepatitis and liver cirrhosis of different etiology and hepatocellular carcinoma. Certain GST allelic variants were proven to be associated with susceptibility to hepatological pathology, and

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correlations with the natural course of the diseases were subsequently postulated.

Key Words: Glutathione-S-transferase; Non-alcoholic fatty liver disease; Drug induced liver disease; Liver cirrhosis

Core Tip: Current review provide data regarding impact of genetic polymorphism of glutathione-S-transferase (*GST*) genes in the pathogenesis of various liver diseases, particularly non-alcoholic fatty liver disease, hepatitis and liver cirrhosis of different etiology and hepatocellular carcinoma. Certain GST allelic variants were proven to be associated with susceptibility to hepatological pathology and correlations with the natural course of the diseases were subsequently postulated.

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INTRODUCTION

Glutathione-S-transferases (GSTs) are group of phase II detoxification enzymes that catalyses the conjugation of glutathione (GSH) to a variety of endogenous and exogenous electrophilic compounds. It is without doubts that phase I enzyme reaction catalyses the incorporation of a functional group to a foreign compound, resulting in the formation of an intermediate metabolite. However, many of intermediates contain high potent chemical groups that can react with different cellular components including DNA, proteins and lipids[1,2]. This presence of intermediate metabolites can lead to multiple adverse health effects. Intermediate substances undergo phase II metabolism to form highly hydrophilic and less chemically active compounds, facilitating their excretion through bile or urine. Moreover, before being eliminated from the body, an extraneous compound can directly take part in phase II bypassing phase I detoxification. Phase II enzymes deactivate and detoxify foreign compounds unlike phase I enzymes which serves as activation metabolism, and therefore referred to as detoxification enzymes[3-5]. The aim of the current review was to overview up-to-date data and sum up results of own investigations regarding the distribution of *GST* genes polymorphisms, possible mechanisms of their involvement in the processes of desintoxication, drugs metabolism and cancerogenesis, and their role in the natural course of various liver diseases.

GSTs are presented by the cytosolic and membrane-bound microsomal super-family members. The groups of microsomal GSTs are structurally distinct from the cytosolic enzymes as they are rather homo-and heterotrimerise than dimerise in order to form a solitary active site. Microsomal GSTs are known to be the primary players in the endogenous metabolism of certain important substances like prostaglandins and leukotrienes. In contradistinction to microsomal GSTs, cytosolic GSTs are highly polymorphic and can easily be divided into eight sub-classes: α, μ, ω, π, θ, ζ, σ-, and ω-. The π and μ classes of GSTs play a regulatory role in the mitogen-activated protein kinase pathway participating in cellular survival and death signaling *via* protein-protein interactions with c-Jun N-terminal kinase 1 (JNK1) and apoptosis signal-regulating kinase (ASK1). JNK and ASK1 are in turn activated in response to cellular stress[6-8].

GSTs are broadly distributed in the living world, from single cell organisms like bacteria to various plants, animals, and humans. Plant GSTs include the φ, τ, θ, ξ and λ classes; the θ and ξ have analogues in animals, too. Moreover, the ξ and θ classes are numerous in non-vertebrate animals. Advocating that the ancestral progenitor for mammalian GSTs, probably arose from the θ class GSTs based on significant homology between the θ class GST and a dichloromethane dehalogenase enzyme from the prokaryote methylobacteriaceae, belonging to the genus of rhizobiales which is

known to be able to undergo genetic transformation and become competent for DNA uptake close to the end of the exponential growth phase[9-12].

The review of the GSTs gene family in the Human Gene Nomenclature Committee (HGNC), online database, shows 23 (as for beginning of 2021) functional genes contained within the group[13], which is a minor upgrade from the last decade, when there were only 21 of such genes reported. However, the number of subfamilies varies from 16 to 26 in different sources, and some genes of the group were determined as encoding membrane-bound enzymes having GST-like activity, but these genes are not related to the *GSTs* gene family evolutionarily. These genes include *GST-κ1* [glutathione S-transferase kappa 1 (*GSTK1*), *GST13*, HGNC: 16906, Chromosome 7q34], and microsomal glutathione S-transferase 1 (*MGST1*, Chromosome12p12.3) microsomal glutathione S-transferase 1-like 1 (prostaglandin E synthase-PTGES, *MGST-IV, PIG12, MGST1-L1, TP53I12,* HGNC: 9599, Chromosome 9q34.11), microsomal glutathione S-transferase 2 (*MGST2*, *MGST-II*, HGNC: 7063, Chromosome 4q31.1), and microsomal glutathione S-transferase 3 (*MGST3, GST-III*, HGNC: 7064, Chromosome 1q24.1). The known human *GSTs* gene family consists of six subfamilies- α (GSTA-alpha), μ (GSTM-miu), ω (GSTO-omega), π (GSTP-pi), θ (GSTT-theta) and ξ (GSTZ-zeta)[14].

Probably, naming of GSTs genes can cause confusion, because both GSTW and GSTO names are similarly used for GST omega (ω) subfamily marking, and GSTT or GSTQ are concurrently used for GST theta (τ) subfamily listing in different sources. The reason for this lack of certainty originates from the HGNC's rules. Moreover, quite similar nomenclature problems were reported with the mouse *GST* genes[14,15].

Nonetheless, while only human GSTs are of valid clinical significance, other *GSTs* genes are of notable interest as this may explain both the connections and developments of human GSTs. The soluble GSTs can be subdivided into the cytosolic and mitochondrial forms, only GSTκ is exclusively mitochondrial, while *GTSA1*, *4*, *GSTM1* and *GSTP1* encode both cytosolic and mitochondrial forms. The rest of the *GSTs* genes encode cytosolic proteins only. Note worthily, a vast number of GSTs were first identified in non-mammalian organisms, and were later recognised in humans and mammals[16-18], however most of the mammalian GSTs have been extensively studied and classified according to commonly assented criteria.

NON-HUMAN GSTS

Reports concerning plant GST enzyme revealed its involvement in catalysing the detoxification of the herbicide atrazine by conjugation to the endogenous γ-L-glutamyl-L-cysteinyl-glycine in sorghum and maize plants, which initiated a research that focuses on the detoxification of various herbicides and other toxic xenobiotic compounds in plants[19]. GSTs exhibit catalysis of the conjugation between various xenobiotics with electrophilic centres and the nucleophilic GSH, tagging the xenobiotic for vacuolar sequestration. The resulting γ-L-glutamyl-L-cysteinyl-β-alanine conjugates were much less toxic and more water-soluble than the original xenobiotics. It was shown that multiple plant GSTs participate in antioxidative protection due to their glutathione peroxidase activity[20].

The floral GSTs are mostly cytosolic and can represent up to 2% of soluble proteins. They have the ability to manifest auxin-inducibility and have ligandin function as well to participate in auxin transport. GSTs play a significant role during the normal metabolism of plant secondary products like anthocyanins[21]. The understanding of GSTs' role in endogenous floral processes and metabolic substrates had been still far from complete in contrast to the vast knowledge collected about their detoxification function[20,22].

Likewise, in human genome, floral GSTs enzymes are encoded by large gene families. The genome of the model plant Arabidopsis thaliana harbors 54 *GST* genes, which are grouped into seven distinct classes in plants. The well-studied large GSTF and GSTU classes are specific to plants, whilst the smaller GSTZ and GSTT classes exist in animal and human tissues. Lesser data is obtainable about the three outlying minor classes including GSTL, dehydroascorbate reductases, and tetrachloro-hydroquinone dehalogenase[21,23].

HUMAN GSTS

Human GSTs genes have multiple and frequent polymorphisms, including the complete absence (up to 20%-50% in some groups and populations) of the *GSTM1* or the *GSTT1* gene. The prevalence of the null genotype of *GSTT1* and *GSTM1* genes are heterogeneous amongst different ethnic populations. The *GSTT1* deletion is found in 20% of Caucasians and 80% of Asians[24]. While *GSTM1* zero genotype is detected in 38%-67% of Caucasian individuals, 33%-63% in East Asians and 22% to 35% in Africans and African Americans[25]. The substitution of adenine for guanine in nucleotide position 313 in the *GSTP1* gene leads to a reduction in the GST enzymatic activity which plays a significant role in the development of various diseases[26].

Following deficit in evident GSTs activities may lead to impaired detoxication of environmental substances, like toxins, carcinogens or drugs that may consequently generate clinically worth problems in patients lacking these genes[14,27-29].

GSTA, GSTM, and GSTP are over expressed in rat model of hepatic neoplasms (preneoplastic nodules) and the increased levels of these isoenzymes are assumed to provide the multidrug-resistant phenotype observed in these lesions. The majority of human tumors and human tumor cell lines express significant amounts of GSTP. The mechanisms responsible for over expression of GSTs, implicate transcriptional activation, stabilization of either messenger ribonucleic acid or protein, and gene amplification. In humans, remarkable interindividual differences are present in the expression of GSTA, GSTM, and GSTT. However, the exact molecular basis for the variation in GSTA is not known; missing of certain GSTM and GSTT classes can be attributed to deletion of the *GSTMl* gene in 50% of the population and deletion of the *GSTTl* gene in 16% of the population. The biological consequences of failure to express hGSTMI or hGSTTI protein can include higher susceptibility to some types of malignancies including skin, colon, bladder, and possibly lung cancer[10,30].

The level of GSTs expression is considered to be a crucial factor in determining the sensitivity of cells to a broad spectrum of toxins. The most abundant mammalian GSTs are the GSTA, GSTM and GSTP, however the biological control of these families is complex as they exhibit species-, age-, sex-, tissue-, and tumor-specific patterns of expression. Moreover, GSTs as shown above are regulated up and down by a broad spectrum of xenobiotics and drugs, with a significant number of these substances occurring naturally as non-nutritional components in modern food. It is obvious that humans are exposed regularly to such compounds[10].

Majority of chemical compounds, acting as GSTs inducers or inhibitors, have effect on transcriptional activation of *GSTs* genes through either antioxidant-responsive element, xenobiotic-responsive element, GSTP enhancer l, or glucocorticoid-responsive element[31,32].

The probability of GSTs is regulated *in vivo* by reactive oxygen species which is based on evidence that is not only but some of the most potent. GSTs inducers are capable of generating free radicals by redox-cycling, but hydrogen peroxide has been shown to strongly induce GSTs in plant and mammalian cells. An induction of GST by reactive oxygen species would appear to represent an adaptive response as GSTs detoxify some of the toxic peroxide-, carbonyl-, and epoxide-containing metabolites produced within the cell during oxidative stress[33-35].

Several functional studies of individual GSTs showed that they can positively contribute to host resistance against various microorganisms, whereas some physiologic mechanisms undergo further studying. Notwithstanding, the elevated total GST enzyme activities and notable accumulation of multiple GST transcripts and proteins was often observed in numerous host-pathogen interactions[23,36]. GSH is the most important non-protein thiol compound in several organisms and plays an important role in signaling and host defense reactions in infection. GSTs' participation in antioxidative react together with the crucial cellular antioxidant GSH in order to eliminate lipid hydroperoxides that accumulate in infected tissues, is clearly their distinguishable function[37-39].

Substantiation of *GSTs* genes from some commensals and parasites that may have immunomodulatory effect towards the immune system is growing, based on the involvement of separate profiles of cytokine gene transcription and different patterns of cell growth. Both antioxidants and oxidative stress manifest prompt transcription effect on many of the *GSTs* genes, which leads to increased protection of the cell against insult caused by environmental chemicals and drugs[40-42].

Possible interactions between host and microorganisms may result in three different ways: resistance gene (R-gene) mediated resistance, basal resistance and virulence. The first one (R-gene mediated), hypersensitive-type resistance is based on a specific interaction of a bacterial effect or gene product with the R-gene of the host organism.

R-gene mediated type of resistance is commonly corresponded with the localised cell death in infected host. It is unspecific, in case of basal resistance recognition; opposite to the R-gene mediated cell death, as genetically alien organisms are recognised based on their common molecular patterns. Induction of basal resistance is not associated with perceptible symptoms, in contrast to the hypersensitive-type R-gene mediated cell death. Poor host defense results in virulence[32,43].

Several members of the cytosolic GSTA, GSTM, GSTP, GSTT, microsomal transferases MGST2 and MGST3, are up-regulated by a wide spectrum of foreign compounds including but not limited to fumaric acid, thiazolidinediones, dexamethasone, phenobarbital, β-naphthoflavone, oltipraz, sulforaphane, coumarin, *etc.*[42]. The mechanism explaining this gene expression induction includes the aryl hydrocarbon receptor, and rostane receptor, the Pregnane X receptor, nuclear factor erythroid 2-related factor 2, CAATT/enhancer binding protein-β, and peroxisome proliferator-activated receptor-γ, which connects GSTs with other pathogenetic mechanisms, genes, and clinical conditions that include insulin resistance, diabetes mellitus type 2, arterial hypertension and abdominal obesity[44].

Due to the fact that GSTs play a determinative role in the detoxification of xenobiotics, their down- or up-regulation may obviously affect biological effects and metabolism of many biologically active compounds, industrials and environmental pollutants. Several studies have demonstrated the potency of some flavonoids to modify the expression of GSTs and their activities. Furthermore, real effect of flavonoid compounds on GSTs strongly hinge on concentration, remedy administration duration, chemical structure of particular flavonoid, as well as on GST origin and isoform. To add confusion, *in vitro* and *in vivo* studies results are often inconsistent, incongruous or conflicting. Notwithstanding, prudential use of a flavonoid enriched diets, which may potentially induce GSTs are commonly beneficial, however the uncontrolled intake of certain flavonoids like catechins and quercetin in high doses as a dietary supplement may threaten health in consequence of GST inhibition. Moreover, combined use of certain flavonoids with drugs (acetaminophen, cisplatine, cyclophosphamide, and simvastatin) or xenobiotics (acrylamide, isocyanates polycyclic aromatic hydrocarbons, and chlorpyrifos), which are GSTs substrates, might have significant pharmacological and toxicological consequences[45].

GSTs genes often, demonstrate high inductivity through various stimuli of both abiotic and biotic origin. For example, salicylic acid (SA) showed prompt inducible effect on multiple GSTs. Some of the *GSTs* genes (*GSTF2, GSTF8, GSTF10, GSTF11*) are recognised determining SA-binding receptor proteins, though the biological relevance of SA binding to these GSTs needs further study[36,46-48].

Similar behavior may be observed in other genes involving in hepato-pancreatic conditions like angiotensin-converting enzyme gene and peroxisome proliferator-activated receptors-γ gene[49]. We can presume, that there is little evidence of specific precise cellular hepatic alteration mechanisms resulted from GST enzymes dysfunction or corresponding genetics' dysregulations.

NONALCOHOLIC FATTY LIVER DISEASE

Due to the studies of possible difference in the distribution frequency of allelic variations in the *GSTP1* A313G polymorphism, it has been established that G allele is spread significantly and more frequent in patients with nonalcoholic fatty liver disease (NAFLD) than in healthy individuals ($\chi^2 = 5.69$, $P = 0.017$) in Ukrainian population (Table 1)[50]. This data is consonant with the results of Hashemi *et al*[51], who have demonstrated that G allele of *GSTP1* gene is a risk factor for NAFLD formation. It was investigated, that total bilirubin level in blood of NAFLD patients with GG genotype of A313G polymorphism of *GSTP1* gene was higher as compared to AA genotype and AG genotype carriers. Presence of G allele was also associated with increased alanin-aminotransferase activity, which was noticed to be significantly higher in NAFLD patients AG, and GG genotypes carriers as compared to patients with AA genotype [52].

Pro-and anti-inflammatory cytokines and adipokines profile varies in NAFLD patients with different polymorphic variants of the *GSTP1* gene (A313G) in particular. Homozygous patients with G allele are characterised by higher level of interleukin-10 (I1-10) in the blood as compared to patients with the AA and AG genotypes, that may occur potentially in response to the increase in the tumor necrosis factor-α (TNF-α) concentration, which proved the increased activity of inflammation processes[53,54]. NAFLD patients were investigated with low adiponectin levels in the blood in

Table 1 Distribution of polymorphic variants of the A313G polymorphism of the *GSTP1* gene in patients with nonalcoholic fatty liver disease and healthy individuals

Genotypes of the gene *GSTP1*	Patients with NAFLD, *n* = 104		Healthy individuals, *n* = 45	
	Absolute number, *n*	%	Absolute number, *n*	%
AA	47	45, 2%	28	62, 2%
AG	42	40, 4%	16	35, 6%
GG	15	14, 4%	1	2, 2%
A-allele	136	65, 4%	72	80, 0%
G-allele	72	34, 6%	18	20, 0%

NAFLD: Nonalcoholic fatty liver disease.

comparison with healthy people[55]. Moreover, according to Li *et al*[56] low adiponectin level is associated with the progression of steatohepatitis. The adiponectin concentration was lower in patients with NAFLD and AG and GG genotypes than in those with the AA genotype, indicating a worse adipokine profile for the NAFLD natural course[50]. A reverse tendency has been determined for leptin, however its blood level was higher in NAFLD patients with AG and GG genotypes as compared to those with the AA genotype[50]. This elevation of the leptin content in the *GSTP1* G allele carriers was, probably, associated with a high TNF-α concentration stimulating leptin production[57]. The aforementioned can prove the development of the leptin-resistance syndrome more severe in this cohort of patients[58]. In general, these observations indicate the formation of adipokine imbalance in the examined patients with AA genotype, which is typical for patients with NAFLD[59] which causes elevated leptin concentration against decrease adiponectin level in the blood[60].

Deletion polymorphic variants of *GSTT1* and *GSTM1* genes prevalence amongst NAFLD patients was approximately the same as their distribution between healthy individuals in Ukrainian population. These data are partially different from those suggested by Hori *et al*[61] who reported higher frequency of *GSTM1* null genotype in NAFLD patients as compared to control in the Japanese. There were not any notable differences in the parameters of the synthetic, detoxification, excretory liver functions together with activity of cytolytic and cholestatic syndromes and lipid profile in NAFLD patients with deletion of *GSTT1* and *GSTM1* genes and patients with functional allele of these genes[62]. It agrees with Rafiee *et al*[63] who also did not define importance contrasts in cholesterol and triglycerides plasma levels in individuals with different polymorphic variants of the studied genes. Interestingly, earlier studies of Maciel *et al*[64] suggested that double deletion genotypes of *GSTM1* and *GSTT1* genes were associated with hypertriglyceridemia.

Elevated TNF-α level in the blood is typical for NAFLD patients as compared to healthy individuals[65]. Jamali *et al*[66] proposed an algorithm involving TNF-α for predicting NAFLD/non-alcoholic steatohepatitis. Importantly, that null-genotype of *GSTT1* gene goes with higher TNF-α concentration as compared with patients having allele variant of *GSTT1*, and thereby indicate the activation of proinflammatory segment of cytokine profile and inflammatory processes[62]. Note worthily, TNF-α is one of the key factors involved in the insulin resistance, inflammation and apoptosis in case of NAFLD[67], thus its elevated level could be a predictor of aggravated liver injury in NAFLD patients with null-genotype of *GSTT1* gene.

Certain peculiarities in adipokine profile were detected regarding *GSTM1* genotype. Leptin plasma level was significantly higher in patients with null-genotype of *GSTM1* gene as compared to NAFLD patients with functional allele. This elevation of leptin content in null-genotype *GSTM1* carriers was probably associated with a high TNF-α concentration that stimulates leptin production[57]. Deletion polymorphism of *GSTT1* and *GSTM1* genes in patients with NAFLD was associated with lower content of restored glutathione, catalase activity. And in the case of carrier of zero genotype of *GSTM1* gene; it was also with higher level of reaction products of thiobarbituric acid in blood as compared to patients with functional allele of the gene[68].

DRUG INDUCED LIVER INJURY AND HEPATITIS

Prevalence of G allele of *GSTP1* (A313G) gene did not differ notably in chronic hepatitis patients in comparison with healthy individuals in Ukrainian population, however, presence of G allele was associated with higher activity of cytolytic syndrome lower restored glutathione blood content in comparison with patients AA genotype carriers[69]. *GSTP1* IIe/Val genotype was significantly more frequent in the patients with chronic hepatitis B infection and in patients with cirrhosis than in healthy individuals in Turkey; *GSTP1* Val/Val genotype was even more frequent in these patients[70]. In addition, these authors denoted relation between *GSTP1* gene polymorphism and hepatitis stage. In fact, as IIe/Val and Val/Val genotype frequencies increased so did the stages of the disease and tendency grow towards cirrhosis[70].

In our previous study, it was found that deletion genotype of *GSTM1* and *GSTT1* in patients with chronic hepatitis were representative to those in healthy individuals. Qi *et al*[71], have discovered that the genes *GSTM3* and *GSTP1* promoter methylation, which causes dysfunction of intracellular antioxidant defense system, more frequently occurs in patients with acute and chronic liver failure in case of hepatitis B virus, compared to patients with compensated viral hepatitis. Determination of methylated promoters of *GSTP1* and *GSTM3* genes can serve as a prognostic factor in the development of acute and chronic liver failure in these patients. It was found that *GSTO2* mutant genotypes were increased with progression, and the degree of hepatitis B virus (HBV) infection and the patients had mutant *GSTO2* genotypes such as (A/G, and G/G) were more susceptible for more severe HBV disease progression. The authors of the aforementioned study concluded that people with A/G and G/G genotype for *GSTO2* are more prone to develop hepatic failure[72]. Certain investigations have driven to the relation of *GST* gene polymorphism and drug induced liver injury. It was discovered almost twenty years ago, that homozygous null mutation at the *GSTM1* gene might predispose to hepatotoxicity for drugs used for the treatment of tuberculosis[73]. This statement was supported in the following studies reviling *GSTT1* homozygous null polymorphism may be a risk factor of antituberculosis drug-induced hepatotoxicity in Caucasians[74]. Meanwhile, presence of at least one functional allele of *GSTM1* was significantly more frequent amongst the groups with higher grades of liver toxicity for antituberculosis drugs in Brazilians[75]. Contrarily, G*STT1* and *GSTM1* were not related to increased antituberculosis drug induced liver injury in Indian citizens[76]. By now, certain researchers[77] have linked troglitazone intoxication in the development of chronic diffuse liver diseases with the double-zero genotype *GSTT1* and *GSTM1* genes, considering its consequence of insufficient activity of detoxification defense systems, low activity of conjugation of sulfuryl groups. It has been shown that the zero genotype of *GSTT1* gene increases the risk of drug-induced liver damage in particular, due to the use of isoniazid[78]. Finally, in meta-analysis, it was found that null *GSTM1* genotype was responsible for higher susceptibility to drug induced liver disease related to antituberculosis medications in East Asian population, but not the Indians or Caucasians[79]. There were no confirmed relationships between null genotype of *GSTT1* gene and this kind of drug induced liver disease[79]. On the other hand, Wu *et al*[80] investigated that patients with tuberculosis A allele carriers of *GSTP1* gene (A313G) have a higher risk of anti-tuberculosis drug-induced hepatotoxicity development.

LIVER CIRRHOSIS

With regards to the report of Burim *et al*[81] study of susceptibility to cirrhosis and pancreatitis in alcoholic, concerning the GST and cytochromes 450 genes polymorphism, revealed that *GSTP1* Val allele carriers were at higher risk of both diseases. Ghobadloo *et al*[82] discovered the association of cryptogenic cirrhosis with Val/Val *GSTP1* genotype which might be explained by low detoxification activity of protein that implicate this polymorphism as a risk factor for occurrence of the disease. Goncharova *et al*[83] showed that patients with liver cirrhosis AA genotype carriers have 2.5 times higher survival rate compared with the patients with the GG and AG genotypes of *GSTP1* gene.

Khan *et al*[84] showed an increase in risk to alcoholic cirrhosis in patients with *GSTM1* null genotype when compared with non-alcoholic or alcoholic controls. A much higher risk to alcoholic liver cirrhosis was observed in patients carrying combination of null genotypes of *GSTM1* and *GSTT1*[84]. The authors of the

mentioned study found interaction of GSTs with variant genotype of manganese superoxide dismutase, which detoxifies free radicals, or cytochrome P450 2E1 that generates free radicals, and resulted in several fold increase in risk to alcoholic liver cirrhosis. Thus, conclude the possible gene-gene interaction in modulating the risk of the alcoholic liver cirrhosis development[84]. However, in another study from Brazil, no differences were found in the prevalence of the *GSTM1* and *GSTT1* null genotypes between control non-alcoholics and alcoholics with liver cirrhosis, as well as alcoholics without disease and alcoholics with liver cirrhosis[81]. Several older studies also have got different conclusions regarding the impact of *GSTM1* null genotype on the appearance of liver cirrhosis in patients with alcohol abuse. Specifically, Harada *et al*[85] in Japanese and Savolainen *et al*[86] in Finland found an increased risk of liver cirrhosis associated with the GSTM1 null genotype in chronic alcoholics. Whilst, Frenzer *et al*[87] in Caucasian population and Rodrigo *et al*[88] in Spanish adults have not reported any. Brind *et al*[89] have found higher prevalence of zero *GSTT1* genotype in patients with alcoholic liver disease compared to patients who do not consume alcohol. Meanwhile *GSTT1* null genotype was not found to vary importantly between liver cirrhosis related to hepatitis B infection and healthy individuals[90]. At the same time, patients with *GSTM1* null genotype are at risk of progression of liver disease as the frequency of *GSTM1* null genotype was found to be significantly higher in chronic hepatitis B, hepatitis B cirrhosis and cryptogenic cirrhosis as compared with controls [90]. Moreover, the link between *GSTM1*, but not *GSTT1* null genotype and cryptogenic cirrhosis was found in Iranian population[82]. Komuro *et al*[91] in their investigations of primary biliary cirrhosis concluded that genotypic difference of *GSTM1* and *GSTT1* did not relate to susceptibility of this disease, nevertheless serum titer of anti-mitochondrial antibody of *GSTM1* null and *GSTT1* null patients were significantly higher than those of *GSTM1* positive and/or *GSTT1* positive patients. Baclig *et al*[92] also postulated that polymorphism in *GSTM1* null genotype seems to be associated with an increased risk of chronic liver disease amongst Filipinos.

HEPATOCELLULAR CARCINOMA

The GST null genotype has been examined to have an association with various malignancies including cancers of the bladder[93], gastric[94], colon[95], and lung[96]. K. Wu *et al*[97] investigated that *GSTP1* 313 G/G polymorphism is a strong predisposing risk factor for bladder cancer. Meanwhile, data regarding the role of *GST* gene polymorphism on the hepatocellular carcinoma (HCC) is sporicidal. Qu *et al*[98] have found single nucleotide polymorphism (SNPs) *GSTO2* rs7085725 and *GSTP1* rs4147581 were significantly associated with the overall survival of HCC patients and suggested to use them alone or in combination as potential prognostic markers for HCC patients. Particularly, according to the author's suggestion, SNP of *GSTP1* (rs4147581) could have a predictive biomarker in HCC patients aged ≤ 55 years[98]. *GSTM1* and *GSTT1* polymorphisms appear to be associated with a modest increase in the risk of HCC in Egyptian patients[99]. *GSTT1* null genotype was associated with more than 2-fold increased risk for HCC development in patients with hepatitis associated with hepatitis C virus (HCV) as compared to the control group. However, *GSTM1* null genotype was found to have a protective effect when hepatitis patients were considered in Indian population[100]. Meanwhile, in older study it was found that the *GSTT1*-null genotype alone did not affect risk of HCC development in HBV, but the *GSTM1*-null genotype was associated with a decreased risk for early-onset HCC[101]. The meta-analysis by Li *et al*[102], involving results of 46 related studies with more than 15 thousands of patients showed that both *GSTM1* null genotypes and *GSTT1* null genotypes increased the risk of HCC, while *GSTM1-GSTT1* dual-null genotypes increased the risk of HCC to a higher extend. Interestingly, during ethnicity consideration, this connection was significant only for Asians, and not for Caucasians and Africans. In older meta-analysis by Shen *et al*[103] *GSTM1* and *GSTT1* null genotype was found to be associated with higher risk of HCC with a similar ethnic pattern. GST-P1 rs1138272 (341C>T) polymorphism was found to have a protective effect on liver cancer development in a high-risk HCV/HBV-positive population in Caucasian ethnicity[104]. GST-P1 genetic polymorphisms (*i.e.*, Ile105Val, rs1695) were not associated with HCC risk in Asian population, European and African[105,106]. Higher *GSTP1* levels in tumor tissues indicated a better overall survival and disease-free survival for HCC patients[107]. The mentioned authors have found that *GSTP1* could decrease p-Akt in liver cancer cell lines and may inhibit alfa-fetoprotein expression. *GSTP1's* inhibition on cancer progression may be accomplished by arresting the cell

cycle at the G1/S transition in HCC cells[108]. *GSTA1* TT genotype was more frequent in HCC than in non-HCC patients, suggesting that individuals carrying this genotype could be associated with 2-fold higher risk of developing HCCs[109]. *GSTM1* and *GSTT1* null genotypes are associated with an increased HCC risk in Chinese population with higher risk typical for double null genotype. Furthermore, in another meta-analysis, it was investigated that null genotype of *GSTT1* was associated with HCC susceptibility in Asians, and both *GSTT1* and *GSTM1* genes deletion were associated with higher susceptibility. *GSTP1* Ile105 Val gene polymorphism was not correlated with this disease, however, polymorphisms in *GSTM1* and *GSTT1* genes are not related to the incidence of HCC in a high-risk Spanish population[110]. Marahatta *et al*[111] provided the support for the difference in genotypic distribution for GSTO1* A140D between hepatocellular carcinoma and cholangiocarcinoma.

CONCLUSION

Current review supports the position that genetic polymorphism of *GST* genes is involved in the pathogenesis of various liver diseases, specifically in non-alcoholic fatty liver disease, hepatitis and liver cirrhosis of different etiology and hepatocellular carcinoma. Certain *GST* gene allelic variants were proven to be associated with susceptibility to hepatological pathology and correlations with the natural course of the diseases were postulated. Still the data obtained in different studies sometimes is controversial and even conflicting. Thus, more investigations involving larger numbers of patients are needed.

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MINIREVIEWS

Wilson's disease: Revisiting an old friend

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Abstract

Wilson's disease (WD) is a rare condition caused by copper accumulation primarily in the liver and secondly in other organs, such as the central nervous system. It is a hereditary autosomal recessive disease caused by a deficiency in the ATP7B transporter. This protein facilitates the incorporation of copper into ceruloplasmin. More than 800 mutations associated with WD have been described. The onset of the disease frequently includes manifestations related to the liver (as chronic liver disease or acute liver failure) and neurological symptoms, although it can sometimes be asymptomatic. Despite it being more frequent in young people, WD has been described in all life stages. Due to its fatal prognosis, WD should be suspected in all patients with unexplained biochemical liver abnormalities or neurological or psychiatric symptoms. The diagnosis is established with a combination of clinical signs and tests, including the measurement of ceruloplasmin, urinary copper excretion, copper quantification in liver biopsy, or genetic assessment. The pharmacological therapies include chelating drugs, such as D-penicillamine or trientine, and zinc salts, which are able to change the natural history of the disease, increasing the survival of these patients. In some cases of end-stage liver disease or acute liver failure, liver transplantation must be an option to increase survival. In this narrative review, we offer an overview of WD, focusing on the importance of clinical suspicion, the correct diagnosis, and treatment.

Key Words: Wilson´s disease; Copper; ATP7B; Ceruloplasmin; Chelator; Liver disease

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Core Tip: Wilson's disease (WD) is a rare metabolic disorder caused by the deposition of copper in organs, particularly in the liver and the brain. As the symptoms and clinical presentation can be highly variable, WD is not always suspected. A detailed but practical review is presented to assist clinicians in the diagnosis and management of WD.

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INTRODUCTION

Wilson's disease (WD) is an autosomal-recessive monogenic disorder characterized by an excessive accumulation of copper, firstly described in 1912 by Kinnear Wilson. The World Health Organization estimates the global prevalence of WD to be between 1/10000 and 1/30000[1]. It is caused by mutations in the *ATP7B* gene, which encodes a transporter protein with ATPase activity. This transporter is involved in incorporating copper into apoceruloplasmin, which is finally eliminated in bile. When a mutation affects the ATP7B transporter, free copper is released into the bloodstream and is removed by urine instead of feces[2]. Therefore, ATP7B is essential for copper biliary excretion[3].

In this review, we aimed to revise the clinical aspects of WD, including diagnosis, clinical manifestations, and the therapeutic approach, and discuss the future treatment of the disease.

GENETICS

The *ATP7B* gene is located on chromosome 13q14.3 and comprises 20 introns and 21 exons, encoding a protein of 165 amino acids[4,5], whose function is the incorporation of copper into ceruloplasmin. Currently, more than 800 mutations have been discovered in the gene[6], of which 380 have confirmed involvement in the pathogenesis of the disease[7,8]. Although mutations have been reported in almost all exons[5], they mainly affect the central regions of the gene (both 8 and 14 exons are the most frequently affected). The most common mutations are H1069Q and R778L in European and Asian populations, respectively[2,4]. Approximately 90%-98% of WD subjects are heterozygous, showing different mutations in each of the alleles encoding the ATP7B. On the other hand, the phenotype and the penetrance of WD can be extremely variable. Even patients carrying two disease-causing mutations do not necessarily have a demonstrable alteration of copper metabolism[9]. Some of the proposed reasons are differences in copper intake, individual antioxidant capacity or susceptibility to liver fibrosis, and hormonal influences[10].

The potential role that epigenetics could have in the gene expression of the disease should be highlighted. Some experimental models have shown changes in DNA methylation through breast milk enriched with methyl groups that could be related to the clinical manifestation of WD[11].

Considering the probability of late-onset, the fact of having asymptomatic cases, and the phenotypic variability, it seems vital to evaluate the previous and next generation of the index case[12]. Both the European Association for the Study of the Liver (EASL) and the American Association for the Study of Liver Diseases (AASLD) recommend an appropriate study of the index case taking into account the family history of liver- and brain-related disease[7,13]. These guidelines propose to assess the patient's siblings since the risk of WD is 25% (by presenting two mutations in both alleles). Subsequently, other first-degree family members should be evaluated, although the risk decreases to 0.5%[12].

CLINICAL MANIFESTATIONS

There is a wide variety of symptoms involved in WD, which predominantly affect the liver and brain (Table 1). Although WD may be present at any age, it is more common between the ages of 5 and 35. However, it should be investigated in patients with liver failure due to an unknown cause and those with liver disease and neuropsychiatric symptomatology[13]. Asymptomatic patients are commonly diagnosed during the family screening process[7].

Liver symptoms

Liver symptoms of WD occur mainly during childhood and adolescence[10]. In these cases, liver involvement appears up to 10 years before neurological manifestations[7]. The clinical spectrum ranges from asymptomatic patients, with mild analytical alterations, to subjects with fulminant liver failure. In this scenario, there are forms of acute (from acute hepatitis to fulminant liver failure) and chronic presentation (from steatosis to compensated and decompensated cirrhosis)[14].

Asymptomatic forms usually have only hepatomegaly, discretely elevated transaminases, or are identified during the screening of an index case[15].

Acute presentation: WD should be suspected in a patient with acute hepatitis, in which viral hepatitis is ruled out. Symptoms are similar to acute viral hepatitis, with jaundice and abdominal pain[14]. This situation, including acute liver injury (manifested by coagulopathy) or acute liver failure (with hepatic encephalopathy), occurs predominantly in women[16]. Beyond these signs and symptoms, the elevation of hemoglobin, cholinesterase, and low alkaline phosphatase are characteristic of acute WD. Sometimes, hemolytic anemia with a negative Coombs test is presented, one of the diagnostic criteria of WD[7]. WD causes 2%-5% of acute liver failure events, showing a fatal prognosis in the absence of liver transplantation (LT) [14].

Chronic hepatitis and cirrhosis: Typically, it starts as a slight transaminase elevation that progresses slowly to fibrosis and, finally, cirrhosis. When it manifests itself as cirrhosis, there is an increased risk of mortality[8]. Sometimes, patients may show splenomegaly uniquely as a sign of portal hypertension. In particular, young patients over three years of age showing cirrhosis should be evaluated for WD[15]. On the other hand, WD can initially be confused with autoimmune hepatitis, as they occur at a similar age and are manifested by jaundice and increased transaminases and gammaglobulins[14]. Also, WD has been described as causing hepatic steatosis, which is identified in up to 15% of biopsies[17].

Neurological symptoms

Neurological involvement typically appears after liver manifestations. WD affects the central nervous system mainly through extrapyramidal system dysfunction and bulbar involvement. The most common symptom is dysarthria, particularly in the early stages of the disease[8]. The neurological presentation can also be manifested by tremors, parkinsonism, or involuntary movements, even by cerebellar dysfunction, chorea, or hyperreflexia[14]. Furthermore, dystonia affecting the face and jaw is characteristic, producing a typical sign (Wilson's face)[15]. Also, a postural tremor is common in WD patients[7].

Psychiatric symptoms

Psychiatric symptoms must be considered in WD. In fact, patients showing these symptoms often suffer a delayed diagnosis[18]. In fact, approximately one third of patients develop psychiatric symptoms as the initial manifestation[7]. Typical symptoms are depression and anxiety[14], although changes in behavior or personality or impulsivity can occur[19]. In addition, affective disorders are more common than psychotic spectrum disorders.

Ocular manifestations

Kayser-Fleischer´s (KF) ring represents a frequent manifestation of WD, which affects the Descemet membrane of the cornea. The slit-lamp examination shows a brown-gold colored ring on the periphery of the cornea[20]. It is present in more than 90% of patients with WD showing neurological involvement but only in half of cases with liver disease. Notably, the KF ring does not affect vision, and its disappearance has been seen in patients undergoing effective treatment and LT[15]. Although it is one of the most typical features of WD, this ring has been described in cholestatic syndromes and other diseases[21].

Table 1 Clinical manifestations

Wilson's Disease Clinical Manifestations	
Liver	Hepatomegaly, jaundice, pain in right hypochondria, asthenia, elevation of transaminases, acute liver injury, acute liver failure, cirrhosis (compensated and decompensated), ACLF, steatosis
Neurological	Dystonia, tremor, dysarthria, dysphagia, Parkinson, chorea
Psychiatric	Behavioral changes, depression, anxiety, psychosis, school performance deficit, sexual disinhibition
Eye	Kayser-Fleischer Ring, Cataract
Hematologic	Hemolytic anemia, coagulopathy, thrombopenia
Renal	Acute renal failure, nephrolithiasis, urolithiasis, renal tubular acidosis
Musculoskeletal	Arthropathy, muscle weakness
Other	Heart disease, pancreatitis, hypoparathyroidism

ACLF: Acute-on-Chronic Liver Failure.

Other symptomatology

As copper can be accumulated in different organs and systems, WD has been associated with arthropathy[22], recurrent muscle weakness due to hypokalaemia[23], cardiomyopathy[24], symptomatic urolithiasis[25], pancreatitis[26], cases of hypoparathyroidism[27], and infertility[28,29].

DIAGNOSIS

There are no specific diagnostic tests for WD (Table 2). Instead, a combination of clinical signs and symptoms and some tests are required to achieve the final diagnosis.

Ceruloplasmin

Ceruloplasmin is the leading copper transporter protein, carrying 90% of serum circulating copper. It is synthesized in the liver and excreted into the circulation from hepatocytes, mostly as holoceruloplasmin (containing six copper atoms) and the remainder as apoceruloplasmin (not joined to copper)[30]. Ceruloplasmin levels may be determined enzymatically by its copper-dependent oxidase activity or by immunological assays. The immunological assay measures the total ceruloplasmin level but not the ceruloplasmin oxidase activity. Therefore, normal levels of ceruloplasmin do not rule out low oxidase activity and WD. For this reason, the use of enzymatic assays is more appropriate[31]. Blood ceruloplasmin levels are typically low (< 0.2 g/L) in patients with WD and neurological involvement. However, they may be higher in up to half of patients with WD[32]. On the other hand, ceruloplasmin levels are not decreased only in WD, but can be reduced in other conditions such as renal or enteric protein loss, malabsorption, end-stage liver disease, or aceruloplasminemia[33]. In addition, up to 20% of healthy heterozygous carriers have low non-pathological levels of ceruloplasmin. Ceruloplasmin is also an acute-phase reactant and may be elevated in inflammation or infections, resulting in false negatives in WD patients with both characteristics[7].

Serum copper

Serum copper decreases proportionally with ceruloplasmin levels. WD should be considered when normal or elevated serum copper levels along with decreased ceruloplasmin are identified, as this indicates an increase in the concentration of non-ceruloplasmin-bound copper[34]. However, in patients with deficient ceruloplasmin levels, low total serum copper levels can be found even though free copper (albumin-bound copper or non-ceruloplasmin bound copper) may be increased. For this reason, only the determination of free copper is important as total serum copper mostly reflects ceruloplasmin-bound copper. To calculate free copper, serum copper must be subtracted from the value of ceruloplasmin and multiplied by 3 (each ceruloplasmin molecule provides 3 mg of copper). Patients with WD have free copper levels between 10-20 mg/dL and symptomatic individuals have levels > 20 mg/dL[35]. Free copper levels may also be increased in cholestatic syndromes and copper intoxication[36] and

Table 2 Diagnosis tests for Wilson's disease

Test	Normal values	Wilson disease	False negative	False positive
Ceruloplasmin	0.2-0.4 g/L	< 0.2 g/L	Increased levels:	Low levels:
			Hepatic inflammation	Malabsorption
				Malnutrition
			Estrogen	Aceruloplasminemia
			Pregnancy	Menkes' disease
			Infection	Terminal liver disease
			Children	Nephropathy with renal protein loss
			Overestimation by immunological assay	Excess zinc ingestion
				Healthy heterozygotes WD
Non ceruloplasmin bound copper	< 0.3 μg/dL	> 10 μg/dL	Overestimation of ceruloplasmin by immunological assay	Increased levels:
				Cholestatic syndromes
				Acute liver failure
				Copper intoxication
Urinary copper excretion	< 0.6 μmol/24 h; < 40 μg/24 h	> 1.6 μmol/24 h; > 100 μg/24 h	Incomplete collection; Children	Increased levels:
				Cholestatic syndromes
				Autoimmune hepatitis
				Chronic active liver disease or hepatocellular necrosis
				Healthy heterozygotes WD
Liver biopsy	< 50 μg/g; < 0.8 μmol/g	> 250 μg/g; > 4 μmol/g	Uneven copper distribution	Increased levels:
				Cholestatic syndromes
				Idiopathic copper toxicosis disorders
Kayser Fleischer rings	Absence	Present: Neurological WD		Primary biliary cholangitis
		Absence:		
		50% hepatic WD		
		Asymptomatic WD		

WD: Wilson's disease.

strikingly elevated in acute WD liver failure due to the sudden release of copper from the liver.

Determining free copper is challenging due to the inadequacy of ceruloplasmin determination methods. It is preferable to use enzymatically determined ceruloplasmin levels when calculating free copper, but they do not detect apoceruloplasmin and overestimate ceruloplasmin. For this reason, the determination of ceruloplasmin non-bound copper is not commonly used as a diagnostic method[37]. In 2009, a new method called exchangeable copper (CuEXC) was proposed for the direct determination of labile copper. It can be performed routinely and allows a direct and accurate measurement of copper overload, representing an extrahepatic biomarker[38]. For instance, values greater than 2.08 mmol/L suggest a high risk of severe neurological disease[39]. Additionally, CuEXC facilitates calculation of the relative exchangeable copper. When its threshold is higher than 18.5%, this biomarker reaches a sensitivity and specificity close to 100% in WD diagnosis, without the presence of false negatives [40,41]. Therefore, it could differentiate WD from other liver diseases and healthy heterozygous subjects, representing a promising family screening marker[2,42].

Urinary copper excretion

Urinary copper excretion in 24 h reflects the amount of circulating non-ceruloplasmin

copper and, therefore, represents the excess copper excreted in the urine. In children, a value greater than 0.64 mmol/24 h or 40 g/24 h is suggestive of WD, while the cut-off for adults is 1.6 mmol/24 h (100 g/24 h)[16]. However, in up to 16%-23%, especially in asymptomatic children and siblings, urinary copper excretion may be lower than the values set[34,43]. After D penicillamine (DPA) administration (1.000 mg administered in two doses), urinary copper excretion consists of measuring urinary copper excretion within 24 h on the same day. It has been proven that urinary copper excretion values > 160 μg/24 h is compatible with WD in children[44]. However, this test is not standardized in adults, so it is not currently recommended in that population.

The determination of urinary copper excretion is challenging in some scenarios, such as the presence of renal failure and an incomplete or inadequate collection of urine. In addition, patients with autoimmune hepatitis, cholestatic diseases, acute liver failure, or asymptomatic heterozygous patients can show elevated urinary copper excretion[45].

Liver biopsy

Liver biopsy is a non-risk-free invasive technique; thus, it is not easy to perform in asymptomatic patients. Its use is limited to patients with compatible clinical or biochemical findings but without a definite diagnosis.

WD has no specific histological changes, although there are suggestive changes. Mild steatosis may be observed in patients without risk factors (alcohol, overweight, diabetes mellitus, or dyslipidemia) who are often mistaken to have non-alcoholic fatty liver disease. Furthermore, staining of metallothionein (protein-bound to intrahepatocyte copper) by orcein or lysosomal copper complexes, using rodain or rubenic acid, show liver copper deposits[35]. The sensitivity of these stains increases when the sample is deposited in xylol for 24 h[46]. Despite this, the hepatic accumulation of copper cannot be ruled out with histochemistry as staining only reveals copper deposits in less than 10% of patients. Thus, intrahepatic copper quantification is essential for the diagnosis of WD after a hepatic biopsy. For the determination of copper in dry weight, it is necessary to obtain a significant sample (at least 1 cm) and its placement in a copper-free and dry container. Values greater than 250 μg/g (4 mmol/g) are diagnostic, while values less than 50 μg/g (0.8 μmol/g) make the diagnosis highly unlikely. The major problem of the intrahepatic quantification of copper is the heterogeneity of distribution of liver copper deposits (which could be unrepresentative), as well as the elevation of intrahepatic copper deposits in cholestatic diseases[47].

Neurological and psychiatric assessment

Patients with WD, even if they have predominantly hepatic involvement, should be evaluated neurologically. The neurological symptoms in WD are varied, and include Parkinsonian motor alterations and psychiatric symptoms[18]. Magnetic resonance imaging (MRI) shows structural abnormalities with a hyperintensity in the T2 sequence in the basal ganglia, tectum, spinal bulb, thalamus, and brainstem. Also, there is a decreased intensity in the T1 sequence in the basal ganglia[48]. During MRI, the "giant panda face" sign, found in 14% of patients, is characterized by hyperintensity of the tegmentum of the midbrain, especially around the red nucleus, which maintains its normal hypointensity on T2-weighted imaging axial sections of the brain. This sign, along with the tectal and center-protuberance plaque's hyperintensity and the simultaneous involvement of the basal ganglia, thalamus, and brainstem, are practically pathognomonic of WD[49].

Genetic testing

Direct sequencing of the *ATP7B* gene provides the greatest efficiency in clinical molecular diagnosis. The most common mutation (H1069Q) is present in 40%-50% of patients in Western countries; however, 17% of patients with a diagnosis established by the Leipzig criteria do not have any identifiable *ATP7B* gene mutation[50]. This may be explained by the inability of genetic testing to distinguish disease-specific mutations from polymorphisms of the gene and the absence of analyzing the non-coding regions of the gene, which can also affect gene expression. However, next-generation sequencing is becoming a very useful, reliable, time-saving, and cost-effective tool for diagnostic testing in the future.

How is the diagnosis established?

As previously described, a single test does not allow a definite diagnosis of WD. For this reason, a scoring system that combines clinical parameters with biochemical and

imaging tests, known as the Leipzig criteria[7,13], is needed for patients (Table 3)[51]. More than 4 points are required to establish the diagnosis of WD according to these criteria, while an alternative diagnosis should be considered in individuals showing less than 4 points. Therefore, liver biopsy and the genetic assessment may not be needed if other test results add up to at least 4 points. However, the Leipzig criteria show some weaknesses that have to be taken into account, such as the lack of definition of the upper limit of normality of urinary copper excretion or the importance attributed to urinary copper excretion in 24 h after stimulation with DPA [52,53].

TREATMENT

Lifelong treatment is necessary even in asymptomatic patients. There are several treatments for WD, including DPA, trientine, and zinc salts. Figure 1 summarizes the therapeutic approach for patients with WD. Once treatment is indicated for WD, it should be monitored in terms of efficacy (including adherence to treatment) and side effects. Briefly, urinary copper excretion should be assessed every two weeks within the first 4-6 wk and every 2-3 mo during the next 6-12 mo[10,54]. The objectives of copper excretion, according to the drug, are described in Table 4. Similarly, side effects of treatment should also be monitored using blood tests and the liver profile, as well as copper and serum ceruloplasmin[13].

DPA

DPA is the first-line drug for WD, and its mechanism involves chelation of circulating copper which will subsequently be excreted in the urine. DPA reduces copper's affinity for proteins by facilitating the removal of copper from tissues, and it induces the synthesis of metallothionein in the liver, a cysteine-rich protein with a high affinity for metal ions. It is metabolized in the liver and is mostly excreted in the urine.

DPA is administered orally, and its absorption is 40%-70% of the administered dose. The dose in adults is 750-1500 mg, and in children is 20 mg/kg/d, given in 2 or 3 divided doses in both cases. DPA should not be taken with food, antacids, or iron supplements because they decrease its absorption. Notably, pyridoxine supplementation should be recommended during treatment with DPA[7].

Up to 90% of patients under DPA therapy have hepatic improvements. However, the efficacy of DPA in neurologic WD is less satisfactory, with an improvement rate of 55%[55]. On the other hand, DPA has numerous adverse reactions; many of them can be severe (Table 5). In those situations, DPA should be discontinued and replaced with another drug. One of the most concerning scenarios is the severe and irreversible neurological worsening at the start of treatment, which can occur in 10%-50% of patients with previous neurological symptoms[56].

Although neurological worsening typically occurs with DPA treatment, it has also been demonstrated with trientine and to a lesser extent with zinc salts[16,57]. Free copper induces oxidative stress which damages brain tissue. Consequently, the chelating agent should be started at a low dose (125 mg/d) and should be increased every 3-4 d.

Trientine

Trientine or triethylenetetramine dihydrochloride is a chelating agent with a similar mechanism of action to DPA. The efficacy of trientine is similar to DPA. It forms a complex with four nitrogen atoms and copper to be excreted in the urine. It is administered orally, and is poorly absorbed from the gastrointestinal tract. The usual dose is 900 to 2700 mg/d for the initial chelation phase and 750 to 1500 mg/d for the maintenance phase in adults, while 20 mg/kg/d is recommended in children (always divided into two or three doses a day). Similar to DPA, trientine should also be administered separately from food and other drugs. Recent studies propose administering a single daily dose of 15 mg/kg, which would significantly improve adherence to treatment[58]. A particular challenge in trientine treatment is its instability as it must be kept cold (2°C-8°C). On the other hand, trientine is a well-tolerated chelating agent that decreases the discontinuation rate up to 4 times compared to DPA, but higher rates of neurological deterioration have been observed than with PDA therapy [55] (Table 5).

Zinc salts

Zinc induces metallothionein synthesis in enterocytes, binding to copper and

Table 3 Leipzig scoring for Wilson's disease

Typical clinical signs and symptoms	
Kayser-Fleischer ring	
Present	2
Absent	0
Neurologic symptoms or typical abnormalities on MRI	
Severe	2
Mild	1
Absent	0
Serum ceruloplasmin	
Normal (> 0.2 g/L)	0
0.1-0-2 g/L	1
< 0.1 g/L	2
Coombs negative hemolytic anemia	
Present	1
Absent	0
Other tests	
Liver copper[1]	
> 4 μmol/g	2
0.8-4 μmol/g	1
< 0.8 μmol/g	-1
Rhodamine positive granules[2]	1
Urinary copper excretion[3]	
Normal	0
1-2 times ULN	1
> 2 times ULN	2
5 times ULN after penicillamine	2
Mutation analysis detected	
Both chromosomes	4
One chromosome	1
No mutations	0
Total Leipzig score	
Score	Evaluation
≥ 4	Diagnosis established
3	Diagnosis possible
≤ 2	Diagnosis very unlikely

[1]In the absence of cholestasis.
[2]If no quantitative liver copper available.
[3]In the absence of acute hepatitis. MRI: Magnetic resonance imaging; ULN: Upper limit of normal.

preventing its absorption into the portal circulation. It is then excreted in feces due to the natural flaking of enterocytes. Zinc also induces metallothionein synthesis in hepatocytes by neutralizing copper in the liver[59,60]. The recommended dose is 150 mg/d, divided into three doses, while 75 mg is adequate for children lower than 50 kg, at least 30 min before meals. In combination with some chelating agents, zinc should

Table 4 Monitoring urinary copper excretion in the treatment of Wilson's disease

Treatment	Initial treatment	Maintenance treatment	Undertreatment or non-compliance	Overtreatment or non-compliance
D penicillamine	> 500 μg/24 h	200-500 μg/24 h	> 500 μg/24 h	< 200 μg/24 h
Trientine	> 500 μg/24 h	200-500 μg/24 h	> 500 μg/24 h	< 100 μg/24 h
Zinc	> 100-500 μg/24 h	< 75 μg/24 h	> 15 μg/24 h	< 5 μg/24 h

Table 5 Adverse effects of medical therapy used in the treatment of Wilson's disease

Medication	Side effects
D penicillamine	Early (1-3 wk):
	Fever, cutaneous eruptions, myelosuppression, lymphadenopathy, proteinuria
	Late: (> 3 wk-yr)
	Renal: Nephrotoxicity, nephrotic syndrome
	Lungs: Goodpasture syndrome
	Bone marrow: Aplasia
	Eye: Optic neuritis, retinitis
	Skin: Pemphigus, pemphigoid lesions, aphthous stomatitis, hair loss
	Autoimmunity: Lupus erythematosus, myasthenia gravis, polymyositis, immunoglobulin A depression
	Dose-dependent:
	Pyridoxine deficiency
	Mammary hypertrophy
	Skin: Elastosis serpiginosa, lichen planus, progeria-like skin changes
	Neurological deterioration (10%-50%)
Trientine	Few side effects:
	Bone marrow depression
	Sideroblastic anemia
	Hemorrhagic gastritis, loss of taste, and skin rash
	Neurological deterioration is less common
Zinc	Very few side effects:
	Gastric irritation
	Elevation of serum amylase and lipase
	Bone marrow depression
	Neurological deterioration is very uncommon
Tetrathiomolybdate	Few side effects:
	Bone marrow suppression
	Increased serum aminotransferase levels
	Anemia
	No neurological deterioration

be administered separately to avoid neutralization of salts. Evidence shows that zinc salts have few side effects, with gastric irritation being the most common side effect. Zinc salts are not recommended as the initial treatment, particularly in acute liver failure. Therefore, it should be used as first-line therapy only in asymptomatic patients or as maintenance treatment after initiation with chelating agents[61,62].

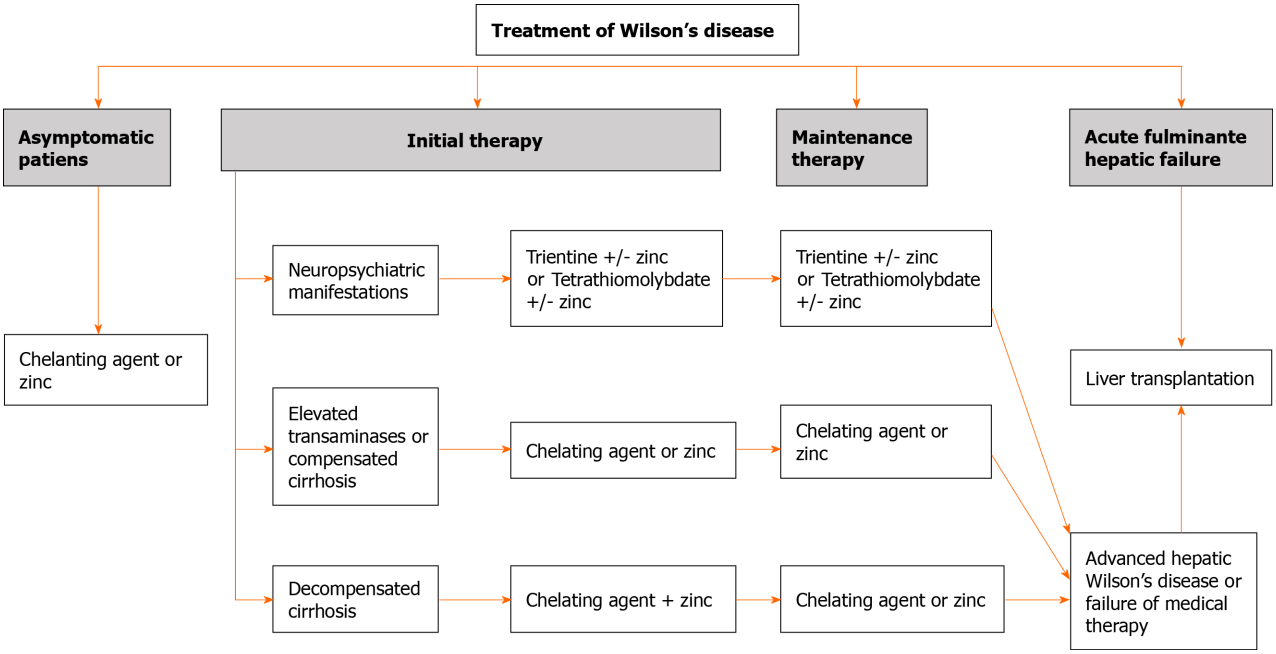

Figure 1 Therapeutic approach for Wilson's disease.

New treatment options

Trientine tetrahydrochloride is a new drug that is being studied in clinical trials. Compared with conventional trientine, it is stable at normal temperature. On the other hand, tetrathiomolybdate ammonium (TTM), a potent decoppering drug, reduces intestinal absorption of copper and forms a tripartite complex with proteins and copper that is subsequently excreted in bile. In contrast to chelating agents, TTM is not associated with neurological deterioration; thus, it can be used in the neurological phenotype of WD[63]. However, it has been associated with other side effects such as myelosuppression, anemia, and elevation of transaminases. Notably, the ammonium salt of TTM is unstable, although a new complex (Bis-choline TTM) is being developed to solve this issue[64]. Finally, methanobactins are a novel approach that is being investigated with positive results in WD treatment. They can remove copper from the mitochondria, avoiding cell toxicity and acute liver failure[65].

Is dietary copper restriction necessary?

As excessive accumulation of copper causes WD, it has been proposed that copper should be restricted in the diet. Significantly, foods to avoid are chocolate, fruits, nuts, mushrooms, liver, and seafood. Both AASLD and EASL guidelines recommend avoiding the intake of high-concentration copper foods or water, particularly within the first year of diagnosis[7,13]. Nevertheless, copper absorption depends on the content of copper in the diet, showing a self-regulatory mechanism. In fact, diets with a high copper concentration result in lower absorption by enterocytes and a higher copper excretion[66]. Thus, copper-rich foods should be consumed to generate excessive copper intake.

LT

LT has a particularly good survival rate in the WD setting[67]. It is indicated mainly in two situations: acute liver failure and end-stage liver disease. WD has a particular score (King's score) that should be used to decide on LT in the setting of acute liver failure, as an index greater than 11 is associated with a high risk of death without LT [68,69]. LT provides functionality for hepatic ATP7B, resulting in normalization of copper metabolism and removal; consequently, chelation therapy may be discontinued after LT. Although LT is controversial as a treatment for the neurological phenotype of WD, an improvement in neurological involvement has been documented[70,71].

Treatment in special situations: Pregnancy

Treatment should not be discontinued in pregnant patients as the risk is higher than with maintenance therapy, with acute liver failure cases described in patients after withdrawal of treatment[72]. Although DPA has teratogenic potential, a clear increase

in risk has not been observed in patients with this treatment, similar to trientine and zinc salts[73,74]. On the other hand, copper deficiency could have a teratogenic effect, so it is advised to reduce chelating therapy by 25%-50% during pregnancy.

PROGNOSIS

In the absence of adequate treatment, the prognosis of WD is fatal[7], but with treatment, this entity has an excellent prognosis. However, we should consider that severe neurological alterations may not be improved, although most patients show significantly improved neurological involvement. Similarly, psychiatric manifestations also improve and can even disappear. On the other hand, patients with cirrhosis often remain compensated and do not have cirrhosis complications, although patients with WD and liver cirrosis should be screened for HCC[54].

FUTURE TREATMENT APPROACHES FOR WD

To date, treatments for WD are based on removing excess copper from the body or LT. Currently, many clinical trials are investigating new treatments with higher efficacy and tolerance, but only a few studies have focused on copper metabolism restoration.

Liver-targeted gene therapy represents an attractive treatment option for many liver conditions[75,76]. Recently, Murillo *et al*[77] demonstrated that the use of recombinant adeno-associated viral vector (rAAV8), containing complementary DNA encoding copper transporting ATPase2, normalized soluble haloceruloplasmin, and hepatic parenchymal copper levels for more than six months after a single administration, in an animal model[77]. Related to these results, a phase I/II study in sixteen adult WD patients will start in 2021 (clinicalgov. Identifier: NCT04537377), where a single intravenous dose of a rAAV liver tropic capsid containing a single-stranded DNA genome carrying a shortened version of the *ATP7B* gene will be used.

The regenerative medicine field has progressed in the past two decades. The role of hepatocytes in liver repair is well known. In fact, hepatocyte transplantation has been proposed as an alternative approach to LT, but has some disadvantages such as weak viability in cell culture, the complexity of hepatocyte source, and the vulnerability to cryopreservation[78]. In this sense, stem-cell therapy has been shown to be a potential therapeutic approach in several liver diseases[79,80]. The differentiation potential of mesenchymal cells into hepatocytes has been demonstrated in several studies[81,82]. Indeed, mesenchymal cells can be easily isolated from visceral fat or bone marrow, expanded without losing their differentiation potential, and can migrate to injured areas[83]. The potential to ameliorate liver injury in preclinical and clinical studies has been previously described[84,85]. Recently, induced Pluripotent Stem Cells (iPSCs) have dominated the field of regenerative medicine. These cells have been isolated from patients with different liver diseases showing specific genotypes[86,87]. IPSCs can be isolated by non-invasive methods[88], providing a hepatocyte source for genetic disorders, protein dysfunction, and subsequent cellular defects responsible for specific diseases. A previous study described the generation of iPSCs from WD donor fibroblasts (skin samples) that bear the R778L mutation in the *ATP7B* gene and their differentiation into hepatocyte-like cells with defective copper transport[89]. They reported gene correction using a lentiviral vector. In the future, hepatocyte-like cells from similarly genetically corrected iPSCs could be an option for autologous transplantation in WD patients. In summary, the expanding tools of gene editing and cell therapy with promising results in other monogenic liver diseases provide a new approach in WD, which could improve the quality of life of these patients by restoring copper metabolism.

CONCLUSION

The knowledge on WD is increasing. The diagnosis of this entity is based on clinical features, biochemical parameters and genetic testing, although new biomarkers are on the horizon. The development of new and effective treatments, including gene therapy, is promising for the future treatment of this disease.

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MINIREVIEWS

Balloon-occluded retrograde transvenous obliteration for treatment of gastric varices

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Abstract

Rupture of gastric varices (GVs) can be fatal. Balloon-occluded retrograde transvenous obliteration (BRTO), as known as retrograde sclerotherapy, has been widely adopted for treatment of GVs because of its effectiveness, ability to cure, and utility in emergency and prophylactic treatment. Simplifying the route of blood flow from GVs to the gastrorenal shunt is important for the successful BRTO. This review outlines BRTO indications and contraindications, describes basic BRTO procedures and modifications, compares BRTO with other GVs treatments, and discusses various combination therapies. Combined BRTO and partial splenic embolization may prevent exacerbation of esophageal varices and shows promise as a treatment option.

Key Words: Gastric varices; Balloon-occluded retrograde transvenous obliteration; Balloon-occluded antegrade transvenous obliteration; Partial splenic embolization; Transjugular intrahepatic portosystemic shunt; Plug- and coil-assisted retrograde transvenous obliteration

Core Tip: Gastric varices (GVs) are a common complication of liver cirrhosis and their rupture is often fatal. Balloon-occluded retrograde transvenous obliteration (BRTO) has been widely adopted for treatment of GVs because of its effectiveness, ability to cure, and utility in emergency and prophylactic treatment. Various modifications of BRTO and combinations with other therapies are also beneficial. Combined BRTO and partial splenic embolization shows promise as a treatment option.

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INTRODUCTION

Gastric fundal varices (GVs) and esophageal varices (EVs) are two of the main presentations of cirrhosis-induced portal hypertension. Although the bleeding risk of GVs is relatively low, their rupture is associated with high mortality (14%–45%)[1-4], because of their larger shunt diameter and higher flow. Hemodynamically, the two types of varices are completely different. The left and right gastric veins comprise the inflow of EVs, with the azygos vein system serving as the outflow. In contrast, the short and posterior gastric veins comprise the main inflow of GVs, although the left gastric vein may also be involved; the gastrorenal shunt (GRS), which drains blood to the left renal vein *via* the descending branch of the left inferior phrenic veins (80%–85%), and the gastrocaval shunt (GCS), which runs below the diaphragm and drains into the inferior vena cava (10%–15%) serve as outflow[5]. Eradication of GVs is difficult endoscopically because of the large diameter and high flow velocity of the shunts. Balloon-occluded retrograde transvenous obliteration (BRTO), developed by Kanagawa in 1996, is a sclerotherapy technique that approaches the varices from the outflow side of the GRS[6]. Since then, BRTO has been widely accepted in Japan[7-9], Asia, and the United States[10,11] as an effective treatment for GVs. In Europe, however, BRTO is not well recognized and not a treatment option for GVs[12,13]. In this review, we outline the indications and contraindications for BRTO, describe basic BRTO procedures and modifications, compare BRTO with other GVs treatments, and discuss various combination therapies.

INDICATIONS AND CONTRAINDICATIONS FOR BRTO

According to Saad *et al*[14], the two clinical indications for BRTO are bleeding GVs (active, current, prior, and impending) and refractory hepatic encephalopathy involving the portosystemic shunt that forms GVs. Contraindications include: (1) Severe uncontrollable coagulopathy associated with liver failure; (2) Splenic vein thrombosis; (3) Portal vein thrombosis; and (4) Uncontrolled bleeding from EVs. In the case of uncontrolled bleeding from EVs, BRTO is contraindicated as a sole procedure; combined transjugular intrahepatic portosystemic shunt (TIPS) and BRTO or balloon-occluded antegrade transvenous obliteration (BATO) *via* the TIPS route are recommended instead.

We use BRTO for both emergency and elective treatment of ruptured GVs as well as prophylactic treatment according to the criteria described below[15,16]. Indications for prophylactic treatment of GVs include nodular form and red color spot lesions[17], increasing size over time, and hepatic encephalopathy. However, we do not treat patients with severe hepatic dysfunction (total bilirubin ≥ 4.0 mg/dL, Child-Pugh score ≥ 13), renal dysfunction (eGFR < 30 mL/min/1.73 m^2), or other serious diseases with poor prognosis as well as those without a portosystemic shunt amenable to a retrograde approach[15]. We consider the presence of contrast agent flowing freely from the GRS into the portal vein on balloon-occluded retrograde venography (BRTV) a relative contraindication[16].

ADVANTAGES OF BRTO OVER OTHER TREATMENTS

Although beta blockers are widely used to prevent bleeding in esophagogastric varices, based on a great deal of evidence[13,18], this review omits a description of them as its focus is interventional procedures.

TIPS is widely used in Western countries to treat portal hypertension in patients with esophagogastric varices and refractory ascites[19-24]. TIPS significantly reduces GVs rebleeding compared with pharmacotherapy and endoscopic treatments such as endoscopic variceal band ligation[19-21]. Although TIPS reduces portal venous pressure (PVP), GVs rebleeding and stent dysfunction are common[19-21,25]. Additionally, post-TIPS mortality is relatively high due to serious complications such as intraperitoneal hemorrhage, hemobilia, sepsis, hepatic failure, congestive heart

failure, and others[25,26]. However, the use of polytetrafluoroethylene-covered stents has improved the TIPS patency rate[27] and the complication rate has decreased in conjunction with more widespread use. Preemptive TIPS is also recommended to prevent esophagogastric varices rebleeding[13,28].

Endoscopic injection of n-butyl-2-cyanoacrylate (CA) has also been widely used to treat GVs[29]. In patients with acute bleeding, CA injection is reportedly more effective than pharmacotherapy alone[30,31] and is the therapy of choice[32,33]; however, CA injection for elective treatment is not recommended and only used when no other treatment is available[32,33].

BRTO is highly effective to eradicate GVs[6-8,15,34] and can be effective for prophylactic[7-9,34] as well as emergency bleeding treatment[15,35,36]. Several studies have shown that BRTO is superior to endoscopic interventions in terms of bleeding control and prognosis in patients with GVs[35,37,38]. Furthermore, several comparative studies have reported that BRTO has a slight advantage over TIPS in terms of rebleeding, hepatic encephalopathy, hepatic functional reserve, and survival[39-44]. These studies are summarized in Tables 1-4. Table 1 shows the study design and sample size. Table 2 summarizes the sclerosant used for BRTO, types of stents used for TIPS, and the technical success rate of each procedure. Table 3 shows the rebleeding rates of GVs and EVs. Table 4 shows the notable complications after each procedure. Recent meta-analyses[45-47] have concluded that BRTO in patients with GVs bleeding is associated with lower rates of rebleeding and postprocedural hepatic encephalopathy, as well as better survival than TIPS. Although BRTO is effective in eradicating GVs, it is associated with complications such as postprocedural EVs, ectopic varices, and intractable ascites. Further debate over the relative superiority of BRTO or TIPS is not constructive. Rather, clinicians should fully understand the characteristics, risks, and benefits of each and use them suitably according to individual patient therapeutic needs. Clinicians should also consider using them in various therapeutic combinations.

CONVENTIONAL BRTO PROCEDURE

BRTO drug preparation and procedures have been described in detail by Hirota *et al* [16]. In Japan, BRTO using ethanolamine oleate with iopamidol (EOI) became covered by insurance in 2018 after publication of a prospective multicenter clinical trial[48].

Our conventional BRTO method is described as follows[15,49]: GRS is diagnosed by computed tomography (CT). An 8 Fr long shepherd hook-shaped (Asato; Medikit, Tokyo, Japan) or cobra-shaped (S-one sheath; Terumo Clinical Supply Co., Gifu, Japan) sheath introducer is advanced into the left renal vein *via* the right femoral or internal jugular vein, respectively. A 6 Fr catheter with a 20 mm diameter balloon or 5.2 Fr catheter with a 9 mm diameter balloon (Selecon MP Catheter; Terumo Clinical Supply Co.) is then advanced into the GRS through the introducer in a retrograde fashion. BRTV (Figure 1) is then performed to identify shunts and their inflow and outflow. Before sclerosing the GRS, the route from the GVs to the GRS needs to be simplified. We use the down-grading method [50], selective coil embolization of the minor accessory draining veins[51], and/or the stepwise injection method [51] to downgrade the target shunt vessels to a relatively simple Hirota grade 1 or 2[52] (Figure 2A-D). If the coexisting GCS has a large diameter and selective coil embolization of the left inferior phrenic vein is impossible, the GCS is occluded with another balloon catheter [53] (Figure 2E). Under temporary balloon occlusion, contrast medium is injected *via* the balloon catheter to confirm stagnation of variceal flow for ≥ 10 min and evaluate the required volume of sclerosing solution. When stagnation of the contrast medium is confirmed, the same volume (10-40 mL) of 5% EOI is injected and remains stagnant in the vessels with overnight balloon occlusion. Human haptoglobin (4000 units) is administered prior to EOI injection to prevent acute kidney injury secondary to hemolysis caused by EOI[54]. The catheter is removed after overnight occlusion. Thrombosis of the GVs-GRS outflow (therapeutic effect) and thrombus formation elsewhere in the portal system (adverse effect) are confirmed by CT 3 to 7 d after BRTO. Eradication of GVs is confirmed by endoscopy after 2 to 3 mo.

BRTO MODIFICATIONS

BRTO is commonly performed overnight to prevent the outflow of sclerosant into the systemic circulation[15,16,48]. Alternatively, a vascular plug[55] or microcoils[56] can

Table 1 The studies comparing balloon-occluded retrograde transvenous obliteration and transjugular intrahepatic portosystemic shunt

Ref.	Journal	Country	Study design	Number of cases	
				BRTO	TIPS
Choi *et al*[39]	KJR 2003	South Korea	RCT, Single institution	8	13
Ninoi *et al*[40]	AJR 2004	Japan	Retrospective, Single institution	77 (BRTO: 49 / PTS: 28)	27
Sabri *et al*[41]	JVIR 2014	United States	Retrospective, Single institution	23	27
Kim *et al*[42]	KJR 2017	United States	Retrospective, Single institution	25	27
Lee *et al*[43]	JGH 2017	South Korea	Retrospective, Two institutions	95	47
Gimm *et al*[44]	Gut and Liver 2018	South Korea	Retrospective, Single institution	157	19

BRTO: Balloon-occluded retrograde transvenous obliteration; TIPS: Transjugular intrahepatic portosystemic shunt; RCT: Randomized controlled trial; PTS: Percutaneous transhepatic sclerotherapy.

Table 2 Materials used and technical success rate

Ref.	BRTO	Tips	Technical success rate	
	sclerosant	Stent type	BRTO	TIPS
Choi *et al*[39]	EO	Bare	8/8	13/13
Ninoi *et al*[40]	EO	Bare	49/58	27/27
Sabri *et al*[41]	STS	Covered	21/23	27/27
Kim *et al*[42]	EO, STS	Covered	22/25	27/27
Lee *et al*[43]	EO, STS, polidocanol	Covered	106/123	49/60
Gimm *et al*[44]	EO, STS	Bare, covered	159/166	19/22
Total			365/403	162/176
			90.6%[1]	92.0%[1]

[1]Not significant. BRTO: Balloon-occluded retrograde transvenous obliteration; TIPS: Transjugular intrahepatic portosystemic shunt; EO: Ethanolamine oleate, STS: Sodium tetradecyl sulfate.

be placed to occlude the GRS instead, allowing catheter system removal as soon as the treatment is complete (a single day procedure). The original methods of plug-associated retrograde transvenous obliteration (PARTO)[55] and coil-associated retrograde transvenous obliteration (CARTO)[56] (Figure 3A and B) emphasized their advantage of not requiring balloon catheters, sclerosants, or a long period of postprocedural bed rest and monitoring. However, these techniques have the disadvantage of high cost. By embolizing the small drainage vessels with gelatin particles, the selective coil embolization procedure can be omitted, and the procedure becomes easy and effective[55,57]. However, recurrence of GVs is lower when a surfactant such as sodium tetradecyl sulfate is used as a sclerosant in PARTO compared with use of gelatin alone[57]. Recurrence might be due to recanalization through the gelatin sponge which does not provide the permanent endothelial injury and thrombosis caused by sclerosants[58]. Injected gelatin has no direct effect on blood clot formation. Once the injected gelatin particles flow into the systemic circulation, they become emboli to the micro-vessels elsewhere. In contrast, sclerosant has a thrombus-forming effect on small drainage vessels, even in small amounts. However, if a small amount of sclerosant flows into the systemic circulation, it is often diluted with a large amount of blood and the effect of vascular endothelial damage can be ignored. Therefore, we believe that sclerosant should be used in BRTO rather than gelatin sponge alone.

Instead of downgrading by advancing the balloon catheter, a modified CARTO[59] in which embolization is performed using microcoils and sclerosant is injected upstream to the GVs has also been described (Figure 3C). Yamamoto *et al*[60] described CARTO-II, in which sclerosant is injected from a balloon catheter in the

Table 3 Rebleeding rate from gastric varices and esophageal varices

Ref.	Rebleeding rate from GVs		Rebleeding rate from EVs	
	BRTO	TIPS	BRTO	TIPS
Choi *et al*[39]	0/8	1/13	0/8	0/13
Ninoi *et al*[40]	1/77	6/27	3/77	2/27
Sabri *et al*[41]	0/23	3/27	0/23	0/27
Kim *et al*[42]	2/25	2/27	1/25	0/27
Lee *et al*[43]	7/95	6/47	4/95	7/47
Gimm *et al*[44]	8/157	3/19		
Total	18/385	21/160	8/228	9/141
	4.7%[1]	13.1%[1]	3.5%[2]	6.4%[2]

[1]P = 0.0005.
[2]Not significant. GVs: Gastric varices; EVs: Esophageal varices; BRTO: Balloon-occluded retrograde transvenous obliteration; TIPS: Transjugular intrahepatic portosystemic shunt.

Table 4 Complications after balloon-occluded retrograde transvenous obliteration or transjugular intrahepatic portosystemic shunt

Ref.	LF		HE		Ascites		EVs aggravation	
	BRTO	TIPS	BRTO	TIPS	BRTO	TIPS	BRTO	TIPS
Choi *et al*[39]	0/8	1/13	0/8	1/13	0/8	0/13	1/8	0/13
Ninoi *et al*[40]	3/77[1]	10/27[1]	0/77	5/27	6/77		14/77	
Sabri *et al*[41]	0/23	0/27	0/23	6/27				
Kim *et al*[42]	0/25	0/27	0/25	6/27	1/25	1/27	1/25	0/27
Lee *et al*[43]	0/95	1/47	0/95	14/47	13/95	2/47		
Gimm *et al*[44]	0/157	0/19	4/157	0/19	48/157	1/19	22/157	1/19

[1]Including long-term events. BRTO: Balloon-occluded retrograde transvenous obliteration; TIPS: Transjugular intrahepatic portosystemic shunt; LF: Liver failure; HE: Hepatic encephalopathy; EVs: Esophageal varices.

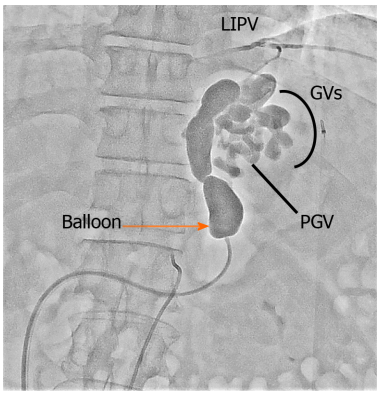

Figure 1 Balloon-occluded retrograde transvenous venography. When the gastrorenal shunt is balloon-occluded (arrow) and retrogradely imaged, the posterior gastric vein, which is the inflow vessel, is visualized *via* the gastric varices. A part of the left inferior phrenic vein as an outflow vessel is also demonstrated. PGV: Posterior gastric vein; GV: Gastric varices; LIPV: Left inferior phrenic vein.

same manner as conventional BRTO, coil-embolization is performed just above the balloon (Figure 3D), and the balloon catheter is finally removed. In CARTO-II, thrombosis has already occurred due to vascular endothelial damage caused by the sclerosant, and coil-embolization is performed to prevent the thrombus from flowing

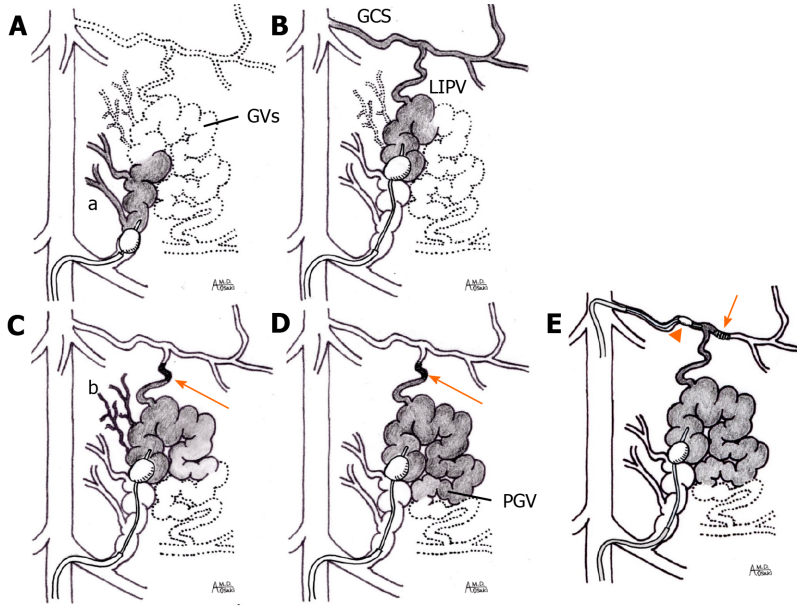

Figure 2 Illustration of the balloon-occluded retrograde transvenous obliteration procedure. A: Balloon-occluded retrograde transvenous venography (BRTV). The initial BRTV does not visualize the main body of the gastric varices (GVs) because multiple draining vessels are present (a); B: When the balloon catheter is advanced beyond the small drainage vessels (downgrading method), the relatively large diameter left inferior phrenic vein (LIPV) becomes visualized as another drainage route to the gastrocaval shunt (GCS); C: GVs become visualized when selective coil embolization (arrow) of the LIPV is performed. As small amounts of sclerosant are injected sequentially over time, the smaller drainage vessels (b) are gradually embolized (stepwise injection method); D: After stepwise injection, BRTV demonstrated the GVs in their entirety as well as the inflowing posterior gastric vein; E: If selective coil embolization of the LIPV is impossible, the GCS should be occluded with another balloon catheter for balloon-occluded retrograde transvenous obliteration (BRTO) (dual-BRTO). Selective coil embolization of the LIPV branch (arrow) is performed through the catheter *via* the GCS. PGV: Posterior gastric vein; GVs: Gastric varices; LIPV: Left inferior phrenic vein; GCS: Gastrocaval shunt.

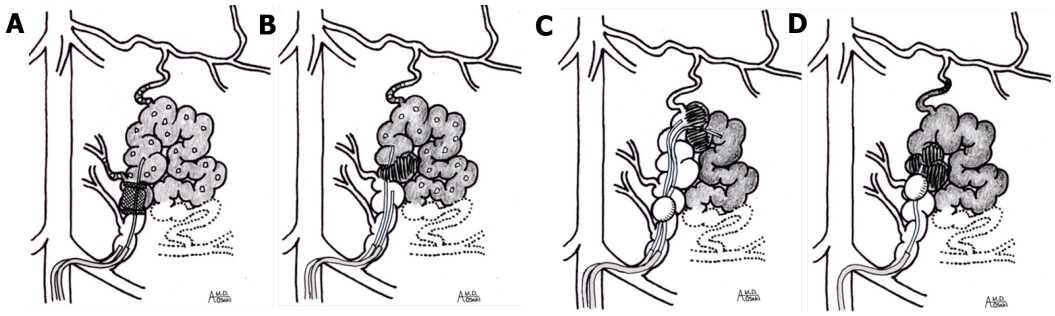

Figure 3 Schema of balloon-occluded retrograde transvenous obliteration modified variants. A: In plug-assisted retrograde transvenous obliteration, a vascular plug is placed instead of a balloon catheter to block shunt blood flow. In the original method, gelatin sponge suspension is injected instead of sclerosant; B: In coil-assisted retrograde transvenous obliteration (CARTO), shunt blood flow is blocked using microcoils and gelatin sponge suspension is injected to embolize the gastric varices; C: In modified CARTO, instead of downgrading by advancing the balloon catheter, embolization is performed using microcoils and sclerosant is injected upstream into the gastric varices; D: In CARTO-II, sclerosant is injected from a balloon catheter in the same manner as conventional balloon-occluded retrograde transvenous obliteration, coil embolization is performed just above the balloon, and the balloon catheter is finally removed.

to the systemic circulation after removing the balloon catheter. The same group also reported the utility of a mixture of low-dose gelatin sponge particles and 5% EOI in retrograde transvenous obliteration (GERTO)[61]. GERTO combines the advantages of gelatin particles and sclerosant, blocking small drainage vessels and causing reliable thrombosis *via* vascular endothelial damage.

Although these various BRTO modifications have appeared, their advantages and disadvantages have not yet been thoroughly evaluated. However, an advantage of both PARTO and CARTO is short indwelling balloon time; their disadvantage is high cost.

COMBINED TREATMENT

Various additional treatments have been performed in combination with BRTO. If BRTO alone is difficult, additional embolization of gastric vein inflow may be used to completely obliterate the GVs. Percutaneous transhepatic obliteration (PTO) may be used when conditions are unsuitable for BRTO, such as GVs without GRS[40,50,62]. Combined BRTO and PTO can obstruct both the feeding and draining veins of GVs to completely retain the sclerosant with in GVs, which may provide better control of variceal blood flow than either procedure alone[63]. However, the drawback of shunt embolization, including BRTO and PTO, is an increase in PVP. Although BRTO is associated with a lower rate of GVs rebleeding than TIPS[39-44] or endoscopic intervention[37,38], the increased PVP may cause enlargement of EVs[64-66]. Saad *et al* [67] therefore proposed use of BATO *via* the TIPS route, combined TIPS and BRTO, or combined BATO and BRTO, depending on the clinical situation. A recent study[68] has proposed a modified method, balloon-assisted antegrade transvenous obliteration (BAATO), in which balloon occlusion of the GRS is performed in retrograde fashion followed by antegrade trans-TIPS catheter injection of CA rather than sclerosant. The distribution of CA in GVs can be controlled by modifying blood flow velocity *via* balloon size adjustment. Thus, BAATO might be valuable alternative option as well. Although, TIPS certainly offsets the increase in PVP caused by BATO and/or BRTO, it can cause hepatic encephalopathy. Partial splenic embolization (PSE) also has a PVP-reducing effect, although weaker than TIPS, and combination with BRTO can be effective[69]. We previously reported that PSE can diminish the increase in PVP after BRTO[49] and that combined BRTO and PSE is a safe and effective treatment for GVs [15]. PSE is technically easier than TIPS and can be performed rapidly. Furthermore, the incidence of EVs exacerbation is lower and improvement in hepatic functional reserve is greater after combined BRTO and PSE than BRTO alone[15]. Increased portal venous flow after BRTO leads to improvement in the hepatic functional reserve [65,70] and is mainly due to increased splenic venous blood flow (Figure 4A and B) without a substantial increase in hepatopetal mesenteric venous blood flow. We speculate that hepatopetal mesenteric venous blood flow increases after PSE decreases the splenic venous blood flow (Figure 4C), which results in improved hepatic functional reserve. PSE has a PVP-reducing effect and can prevent exacerbation of EVs after BRTO. However, PSE-related complications may occur. According to a systematic review of 30 articles[71], the incidence of post-embolic syndrome, pleural effusion, ascites, thrombosis (mainly portal thrombosis), splenic abscess/bacterial peritonitis, and death after PSE is 73.4%, 9.4%, 8.1%, 2.4%, 1.3%, and 1.0%, respectively. Underlying liver dysfunction and splenic infarction rate (infarcted splenic volume/total splenic volume) greater than 70% may be risk factors for major complications[71,72].

CONCLUSION

GVs rupture is potentially fatal. Although various GVs treatments have been reported, BRTO is widely used because of its effectiveness, ability to cure, and utility for both emergency and prophylactic treatment. Recent BRTO modifications and combinations with other therapies are also beneficial. Although BRTO combined with TIPS and BRTO combined with PSE seem promising, randomized trials have not been performed and serious complications may occur. Their use should be approached with caution.

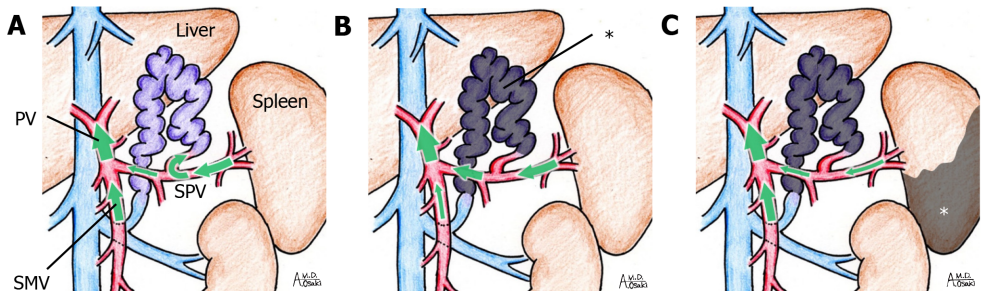

Figure 4 Schema of changes in portal hemodynamics due to combined balloon-occluded retrograde transvenous obliteration and partial splenic embolization. A: Before treatment, most of the splenic blood flow is short-circuited to the systemic circulation *via* the gastrorenal shunt (GRS); B: The GRS is embolized by balloon-occluded retrograde transvenous obliteration (BRTO) (black asterisk). The increase in portal venous flow after BRTO is mainly caused by increased splenic venous blood flow without a substantial increase in hepatopetal mesenteric venous blood flow; C: The lower half of the spleen is infarcted by partial splenic embolization (PSE) (white asterisk). Hepatopetal mesenteric venous blood flow increases after splenic venous blood flow is decreased by PSE. PV: portal vein, SPV: splenic vein, SMV: superior mesenteric vein.

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MINIREVIEWS

Role of chromosome 1q copy number variation in hepatocellular carcinoma

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Abstract

Chromosome 1q often has been observed to be amplified in hepatocellular carcinoma. This review summarizes literature reports of multiple genes that have been proposed as possible 1q amplification drivers. These largely fall within 1q21-1q23. In addition, publicly available copy number alteration data from The Cancer Genome Atlas project were used to identify additional candidate genes involved in carcinogenesis. The most frequent location for gene amplification was 1q22, consistent with the results of the literature search. The genes *TPM3* and *NUF2* were found to be candidates whose amplification and/or mRNA up-regulation was most highly associated with poorer hepatocellular carcinoma outcomes.

Key Words: Liver cancer; Chromosomal amplification; Hepatocellular carcinoma; The Cancer Genome Atlas

Core Tip: A list of candidate chromosome 1q amplification driver genes was compiled from the existing literature by PubMed search. Bioinformatics tools were used to identify additional candidates using publicly available genomics and transcriptomics data. Genes identified this way were largely distinct from those identified from the literature. Thus, these two strategies can be used in a complementary manner.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer related deaths worldwide. Liver cancers are the fourth most common cause of cancer related deaths (the sixth most commonly diagnosed type of cancer), and HCC accounts for between 75% and 85% of primary liver cancer cases[1]. About 54% of HCC cases worldwide are attributed to the hepatitis B virus (HBV) while 31% of cases are attributed to hepatitis C virus (HCV) infections[2]. Given the fact that chronic HBV infection presents as a significant risk factor for HCC, vaccination against HBV is recommended as a way to prevent HCC[3].

COMMON GENOMIC ALTERATIONS IN HCC

More recently, technological advances have permitted the sequencing of the genomes and transcriptomes of numerous cancers. Mutations in several genes have been detected repeatedly in HCC[4]. Common somatic changes include mutations to beta-catenin and p53, resulting in activation of the Wnt signaling pathway and dysregulation of the cell cycle, respectively. Mutations activating *TERT* gene expression are also common. Patterns of genetic alterations in individual tumors have been examined with the goal of classifying them, to predict outcome and potentially guide therapeutic decisions[5].

Over the past few decades, a significant amount of research has shown an association between HCC and specific chromosomal abnormalities. In particular, chromosomal gains have been noted for 1q, 6p, 8q, 17q, and 20q. Similarly, chromosomal losses have been detected for 1p, 4q, 6q, 8p, 13q 16p, and 17q[6-8]. Amplification of chromosome 1q21-23 has been identified as the most frequent chromosomal alteration associated with HCC[9]. Thus, we were interested in considering the evidence for which gene or genes is critical for driving this chromosomal abnormality.

AMPLIFICATION OF CHROMOSOME 1Q GENES

During the past two decades, several genes within or near the 1q21-23 range have been highlighted as potentially significant to HCC[10]. Many of these are highlighted in Table 1. In 2003, Wong *et al*[11] studied the 1q21-1q22 region using positional mapping by interphase cytogenetics. They identified significantly increased levels of gene expression of the *JTB*, *SHC1*, *CCT3*, and *COPA* genes in five cases of HCC compared to paired adjacent non-malignant liver tissues, and they concluded that these genes may represent targets in HCC progression[11]. More recently, *JTB* (Jumping Translocation Breakpoint) has been identified as a protein that negatively regulates the apoptotic process by affecting the activation of caspase 9[12]. *SHC1* is involved in signal transduction from receptor tyrosine kinases to various downstream proteins and has been identified in mitogenic signaling[13-15]. *CCT3* is involved in cell cycle regulation [16]. *COPA* is the α-subunit of the coatomer protein complex I which plays a role in retrograde protein trafficking from the Golgi to the endoplasmic reticulum[17].

In 2004, Midorikawa *et al*[8] used an expression imbalance map analysis [which they confirmed using genomic quantitative real-time polymerase chain reaction (qPCR)] to demonstrate amplification of the 1q21-12 region in HCC tumor samples. Moreover, they identified two new genes (*HAX-1* and *CKS1B*) as being as being highly expressed in HCC tissue compared with noncancerous tissues. They also described the amplification of *SHC1* and *CCT3* (previously identified by Wong *et al*[11]). *HAX-1* (*HCLS1* associated protein X-1, gene name *HAX1*) has been associated with activation of tyrosine kinases[18]. Like *CCT3*, *CKS1B* (CDC28 protein kinase regulatory subunit) plays an essential role in mediating a cell's progression through the cell cycle[19]. To further support the conclusions of Midorikawa *et al*[8], Shen *et al*[20] demonstrated that HCC cells had increased levels of *CKS1B* mRNA and protein compared to adjacent non-tumor liver tissue. Elevated *CKS1B* expression was also positively associated with poor differentiation features[20].

In 2008, Inagaki *et al*[21] analyzed a 700-kb DNA region located at 1q21 in 19 HCC-derived cell lines. Using high-density SNP microarray analysis, fluorescence in situ hybridization (FISH), and real-time quantitative PCR, they identified a significant increase in copy number at the 1q21 region. Using reverse transcriptase PCR, they identified three genes (*CREB3L4*, *INTS3*, and *SNAPAP*) that were significantly overex-

Table 1 Amplified genes within/near 1q21-23 that have been associated with hepatocellular carcinoma

Gene[1]	Location[2]	Description of protein product	Ref.
JTB	1q21.3	Promotes cell resistance to apoptosis	Wong *et al*[11] and Kanome *et al*[12]
SHC1	1q21.3	Downstream signaling from receptor tyrosine kinases	Wong *et al*[11], Midorikawa *et al*[8], Pelicci *et al*[13], Kavanaugh and Williams[14], and van der Geer *et al*[15]
CCT3	1q22	Associated with cell cycle regulation	Wong *et al*[11], Midorikawa *et al*[8], Won *et al*[16]
COPA	1q23.2	Assists in retrograde vesicular transport from Golgi to endoplasmic reticulum	Wong *et al*[11] and Vece *et al*[17]
CKS1B	1q21.2	Associated with cell cycle regulation	Midorikawa *et al*[8] and Ganoth *et al*[19]
HAX-1 (*HAX1*)	1q21.3	Plays a role in the activation of receptor tyrosine kinases	Midorikawa *et al*[8] and Suzuki *et al*[18]
CREB3L4	1q21.3	Associated with androgen receptor signaling	Inagaki *et al*[21] and Qi *et al*[22]
INTS3	1q21.3	Associated with RNA polymerase II	Inagaki *et al*[21] and Baillat *et al*[24]
SNAPAP (*SNAPIN*)	1q21.3	Part of SNARE complex (docking and fusion of synaptic vesicles)	Inagaki *et al*[21] and Ilardi *et al*[25]
ALC1 (*CHD1L*)	1q21.1	Facilitates DNA synthesis and cell cycle when over expressed	Ma *et al*[26]
ASH1L	1q22	Histone methyltransferase involved in gene expression	Elsemman *et al*[27] and An *et al*[29]
METTL13 (*EEF1AKNMT*)	1q24.3	Regulates protein synthesis in cancer cells; promotes tumor growth and metastasis	Elsemman *et al*[27]; Liu *et al*[30], and Li *et al*[31]
TARBP1	1q42.2	Double-stranded RNA binding protein; promotes HIV-1 and -2 and HCV replication	Elsemman *et al*[27], Zhang *et al*[50], and Christensen *et al*[32]
SMYD2	1q32.2	Part of the protein lysine methyltransferase family of enzymes	Elsemman *et al*[27] and Leinhart and Brown[34]
SMYD3	1q44	Part of the protein lysine methyltransferase family of enzymes	Elsemman *et al*[27] and Leinhart and Brown[34]

[1]Alternative gene designation provided in parentheses (see text).
[2]Chromosome locations are as found on the Genome Browser at http://genome.ucsc.edu[65]. HIV: Human immunodeficiency virus; HCV: Hepatitis C virus.

pressed in samples taken from HCC tumors[21]. Based on these findings, they concluded that these three genes are likely targets for the amplification mechanism, and they may be involved in HCC progression. *CREB3L4* (cyclic amplification responsive element binding protein 3-like 4) is part of the CREB/ATF family of transcriptional factors, and it is primarily expressed in the prostate gland in humans as well as prostate and breast cancer cell lines[22]. *CREB3L4* has been shown (by immunostaining) to have a higher expression level in cancerous prostate cells than in adjacent noncancerous cells[22] and it has also been shown to contribute to the progression of breast cancer[23]. INTS3 (integrator complex subunit 3) is part of the Integrator complex which is associated with the C-terminal domain of RNA polymerase II[24]. SNAPAP (snare-associated protein, gene name *SNAPIN*) is part of the SNARE complex of proteins that is involved in the docking and fusion of synaptic vessel[25]. At this point, little is known about the relationship of either INTS3 or SNAPAP with tumorigenesis.

Later in 2008, Ma *et al*[26] used microdissected DNA from 1q21 and hybrid selection to isolate ALC1 (also known as CHD1L) as a candidate oncogene. After confirming the amplification of *ALC1* using FISH, they transfected it into human liver cell lines resulting in the cells being able to form more colonies than vector-transfected cells when grown in soft agar[26]. They also demonstrated that *ALC1* overexpression plays a role in facilitating DNA synthesis, down-regulating p53 expression, promoting G1/S phase transition, and inhibiting apoptosis.

More recently, in 2016 Elsemman *et al*[27] were interested in S-adenosylmethionine (SAMe) which has been described by Lu *et al*[28] as playing a significant role in hepatic diseases including HCC. SAMe is synthesized from ATP and methionine by methionine adenosyl transferase genes including MAT1A which is significantly

downregulated in HCC. Elsemman *et al*[27] analyzed reactions containing SAMe, and using copy number variation analysis they identified five methyltransferase genes (*ASH1L*, *METTL13*, *TARBP1*, *SMYD2*, and *SMYD3*) located on chromosome 1q, all of which were amplified in samples of HCC relative the healthy tissue samples. ASH1L is a histone methyltransferase protein which is involved in the regulation of gene expression[29]. *METTL13* (gene name EEF1AKNMT) has been shown repeatedly to promote tumor growth and metastasis and is negatively associated with survival among lung and pancreatic cancer patients[30,31]. *TARBP1* is a double-stranded RNA binding protein that promotes the replication of human immunodeficiency virus-1 and -2 as well as HCV[32]. It has also been directly correlated with decreased survival rates in patients with HCC[33]. SMYD2 and SMYD3 are both members of the protein lysine methyltransferase family of proteins[34], and each has been associated with a variety of cancer types. SMYD2 has been shown to be overexpressed in esophageal squamous carcinoma, gastric cancer, and pediatric acute lymphoblastic leukemia[35-37]. SMYD3 is overexpressed in cancers including breast, liver, and colorectal cancer[38,39].

ANALYSIS OF GENOMIC AND TRANSCRIPTOMIC DATA

We were interested in what more recent genomic and transcriptomic studies have revealed about chromosome 1q amplification and HCC. The Cancer Genome Atlas (TCGA) Project has accumulated an important, publicly available genomic and mRNA expression data set which includes multiple cancers types including HCC (data set Liver Hepatocellular Carcinoma, LIHC)[40]. There is also a more recent version of this data, which is part of TCGA Pan-Cancer Clinical Data Resource[41], a subset of the LIHC data set that has been curated to include four major clinical outcome endpoints. We chose to use this data set to try to identify additional candidate amplification driver genes. This version of the LIHC patient cohort (PanCan-LIHC) has the following patient characteristics: 251 males/121 female with 241 living, and 131 deceased. Most individuals had a total of 10-140 mutations genome wide; 23 had 140-190, 18 had greater than 190, and 2 had fewer than 10 (14 did not have data available). Most PanCan-LIHC individuals exhibited genome alterations, with gains in 1q being the most common alteration: 225 individuals (60.5%) exhibited 1q gains, with 23.7% called as diploid and 15.9% with data not available).

The original publication reporting the LIHC cohort analyses identified copy number alterations (CNAs) in several likely driver genes spread across several chromosomes [40]. However, the only driver gene listed for 1q is MCL1 at 1q21.3. They also reported a short stretch of four genes that were significantly amplified at 1q22, but no candidate genes were indicated. In a report on the analysis of aneuploidy across TCGA cancer types, strong 1q amplification was noted in the PanCan-LIHC cohort (as well as in other epithelial breast and lung tumors)[42]. Using the Oncoprint tool at the cBioPortal for Cancer Genomics (https://www.cbioportal.org/), we could see that all of the genes listed in Table 1 were amplified in 7%-13% of tumors, with mRNAs overexpressed in 9%-41% of tumors (data not shown), consistent with the earlier reports described above.

STRATEGY TO IDENTIFY ADDITIONAL CANDIDATE DRIVER GENES

To further explore possible 1q amplification driver candidates, the frequency of CNAs in the Pan-Cancer version of LIHC sample set was explored using the cBioPortal suite of tools[43,44]. First, the CNA data set for all genes in the PanCan-LIHC was downloaded and imported into in an Excel spreadsheet. Second, all genes that had been scored as having an amplification or homozygous deletion with a frequency of at least 5% of tumor samples were sorted from those with lower frequency. This resulted in a list of 1871 genes meeting these criteria. Finally, this set of 1871 altered genes was sorted by chromosome and further restricted to those that were annotated as Cancer genes according OncoKB[45].

These steps produced a list of 49 candidate genes localized to chromosome 1q (not shown). These fell into two groups, a centromere proximal group spanning intervals 1q21.2-1q25.2 (28 genes), and a second group covering the distal interval of 1q31.1-1q44 (21 genes). Across the 1q region, the gene amplified in the highest percentage of tumors was *MUC1* located at 1q22 (11.7% amplification). This might correspond to the short stretch identified at 1q22 by the TCGA-LIHC paper referred to above. The overall frequency of amplification was greater in the proximal group of genes (mean of

10.29%, range of 8.2%-11.7%) *vs* the distal set (mean of 6.41%, range of 5.4%-7.4%). Of the 15 genes listed in Table 1, only two were present in the list of 49, *CKS1B* and *SMYD3*.

ANALYSIS OF NEW CANDIDATES

Using the Oncoprint visualization tool at cBioPortal, all 49 genes were examined to determine the putative CNAs from GISTIC2.0 calls[46], as well as the presence of non-synonymous mutations and altered mRNA expression (*z*-score threshold of +/- 2.0 relative to diploid samples). The total alteration percentages ranged from < 10% to 50% for the individual genes, with few non-synonymous mutations (not shown). The total number of genes under consideration was narrowed down to 12 by focusing on those with at least 25% of samples with one or more of the various alterations (Figure 1). All but one of these genes was derived from the centromere proximal half of the 1q arm (the exception was PARP1 at 1q42.12). All 12 genes exhibited numerous instances of mRNA upregulation, both with and without DNA amplification. Note that COP1 in Figure 1 at 1q25.1 is not the same as COPA at 1q23.3 (Table 1).

Each of the 12 genes was examined individually using the cBioPortal Comparison and Survival tools to determine whether the presence of alterations was associated with survival outcomes. There were only two genes where amplification, or mRNA increase, or both were associated with reduced survival compared with the samples without either type of alteration. These two were *TPM3* at 1q21.3 and NUF2 at 1q23.3 (Table 2, scores designated "all"). However, when the CNAs were examined separately from increased mRNA levels, amplification alone was not associated with any survival or outcome measure (not shown). Instead, the mRNA elevations clearly had a more significant correlation with patient outcome, as can be seen from the Logrank test q-values (Table 2, "mRNA"). Patients with *TPM3* mRNA elevation had an overall median survival of 25.15 mo *vs* 80.74 mo for those without the elevation. Patients with *NUF2* mRNA elevation had an overall median survival of 23.38 mo *vs* 70.06 mo for the unaltered group. Thus, altered expression of these two genes may contribute to clinical outcome.

COMPARING THE FREQUENCY OF TPM3 AND NUF2 ALTERATIONS IN HCC WITH OTHER CANCERS

We were interested whether *TPM3* and *NUF2* alterations were common in other types of cancer besides HCC. To explore the alteration frequencies in other cancer types, the entire Pan-Cancer patient cohort was analyzed using the cBioPortal suite of tools[41]. All 32 cancer types included in the Pan-Cancer sample set were selected, and the *TPM3* and *NUF2* genes were searched individually. The Cancer Types Summary produced a display showing the frequency of gene alterations (amplifications, deep deletions, non-synonymous mutations, structural variants) in all 32 types of cancer as well as the types of alterations identified (Figure 2). The PanCan-LIHC HCC dataset had the second highest percentage of TPM3 alterations and the third highest percentage of *NUF2* alterations. In the case of both genes, amplification of *TPM3* and *NUF2* was the most common type of alteration seen in the HCC patient sample. Interestingly, *NUF2* had a relatively higher frequency of mutations than amplifications in some cancer types.

PREVIOUSLY REPORTED ASSOCIATION BETWEEN *TPM2* OR *NUF2* AND HCC

Despite the low q-values, it remains possible that the association between *TPM3* and *NUF2* gene expression and patient survival is random. Therefore, we searched the literature to find whether either *TPM3* or *NUF2* genes had been associated previously with HCC. Kim *et al*[47] examined chromosomal alterations in 76 HCC, finding frequent gain of 1q. They found *TPM3* mRNA was elevated in tumors compared to normal tissue, and proposed that it might represent an oncogene in HCC, consistent with our analysis. A follow up study found that knock down of *TPM3* in HCC cells reduced migration and invasion capabilities[48].

Table 2 Correlation between *TPM3* and *NUF2* alterations and prognosis

Gene	*n* (affected)	Overall[1]	Disease-specific[1]	Progression free[1]	Disease free[1]
TPM3 (all)	92	1.283e-3	1.263e-3	0.108	0.0242
NUF2 (all)	87	4.931e-3	2.357e-4	6.231e-4	4.931e-3
TPM3 (mRNA)	78	4.046e-5	4.046e-5	1.231e-3	2.238e-3
NUF2 (mRNA)	65	3.898e-4	1.441e-5	3.374e-4	1.520e-3

[1]All Logrank test survival *P* and *q* values were generated using the Comparison/Survival tool at cBioPortal to compare survival rates between groups of patients. Various types of survival values were calculated for individuals with any type of alteration (all) or only those with altered mRNA levels (mRNA). The q-values shown were derived from the initially *P* values using the Benjamini-Hochberg False Discovery Rate correction procedure.

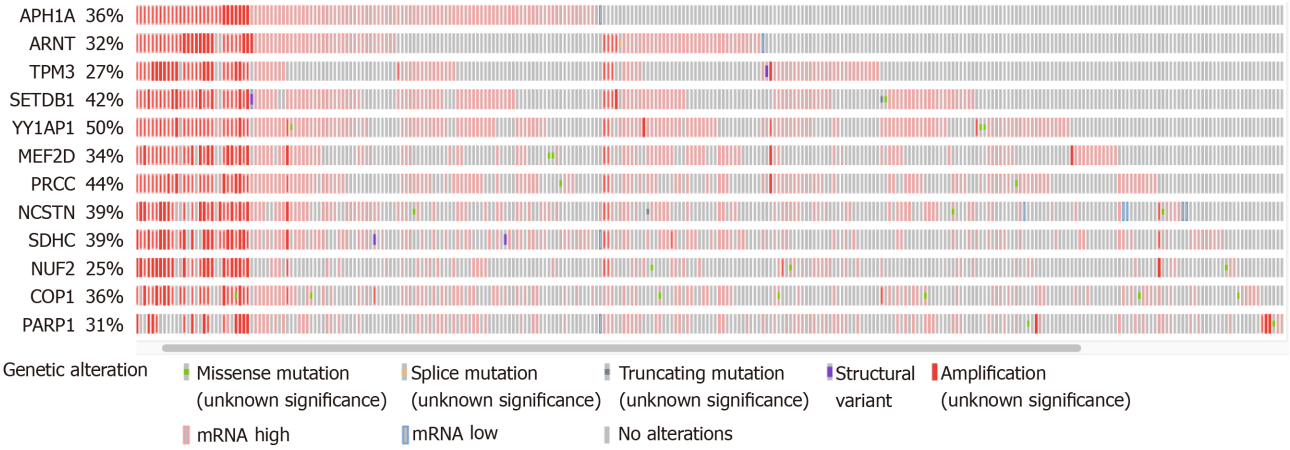

Figure 1 Oncoprint of genetic alterations and mRNA elevations. The alterations to the 12 genes identified by the amplification driver gene identification strategy were visualized using the cBioPortal Oncoprint tool. The nature of the alterations is indicated by the key below the Oncoprint. Note that some individuals display both amplification (solid red) plus elevated mRNA (red outline). Each vertical set of lines reflects the alterations occurring in a single hepatocellular carcinoma patient. Individuals with no alterations detected in any of the 12 genes are not shown.

NUF2 elevation was reported in micro-dissected malignant hepatocytes derived from HBV-associated tumors[49]. Analysis of the Gene Expression Omnibus HCC data also revealed upregulation of NUF2 in HCC compared with healthy colon epithelial cells[50]. An analysis of the original TCGA-LIHC data set, which has substantial overlap with the PanCan-LIHC samples that we explored, also found that *NUF2* was overexpressed compared with normal liver samples[51], and that overexpression was significantly associated with overall median survival. Other independent analyses of the same data set also reported *NUF2* upregulation and association with poorer prognosis[52-54]. It has been suggested that *NUF2* may represent a biomarker for early recurrence after HCC resection[55], and that it might represent a potential therapeutic target[56].

IMPLICATIONS OF *TPM3* AND/OR *NUF2* OVEREXPRESSION

The product of the TPM3 gene is tropomyosin3, an actin binding protein. The four TPM genes TPM genes produce 40 distinct protein isoforms by use of alternative promoters and extensive alternative mRNA splicing[57]. Changes in isoform production have been associated with cellular transformation[48,58]. The specific role of increased *TPM3* in cancer cells is unclear, as the protein is involved in numerous activities related to the actin cytoskeleton. Despite this, it is worth noting that small molecules that block the binding of isoform TPM3.1 to actin showed promise in perturbing the growth of cancer cells[59,60].

The protein encoded by the *NUF2* gene, along with those encoded NDC80, SPC24 and SPC25 form the Nuclear Division Cycle 80 complex. This complex plays an important role in mitotic spindle formation and chromosome segregation[61]. Over expression of other complex members, especially NDC80, has also been observed

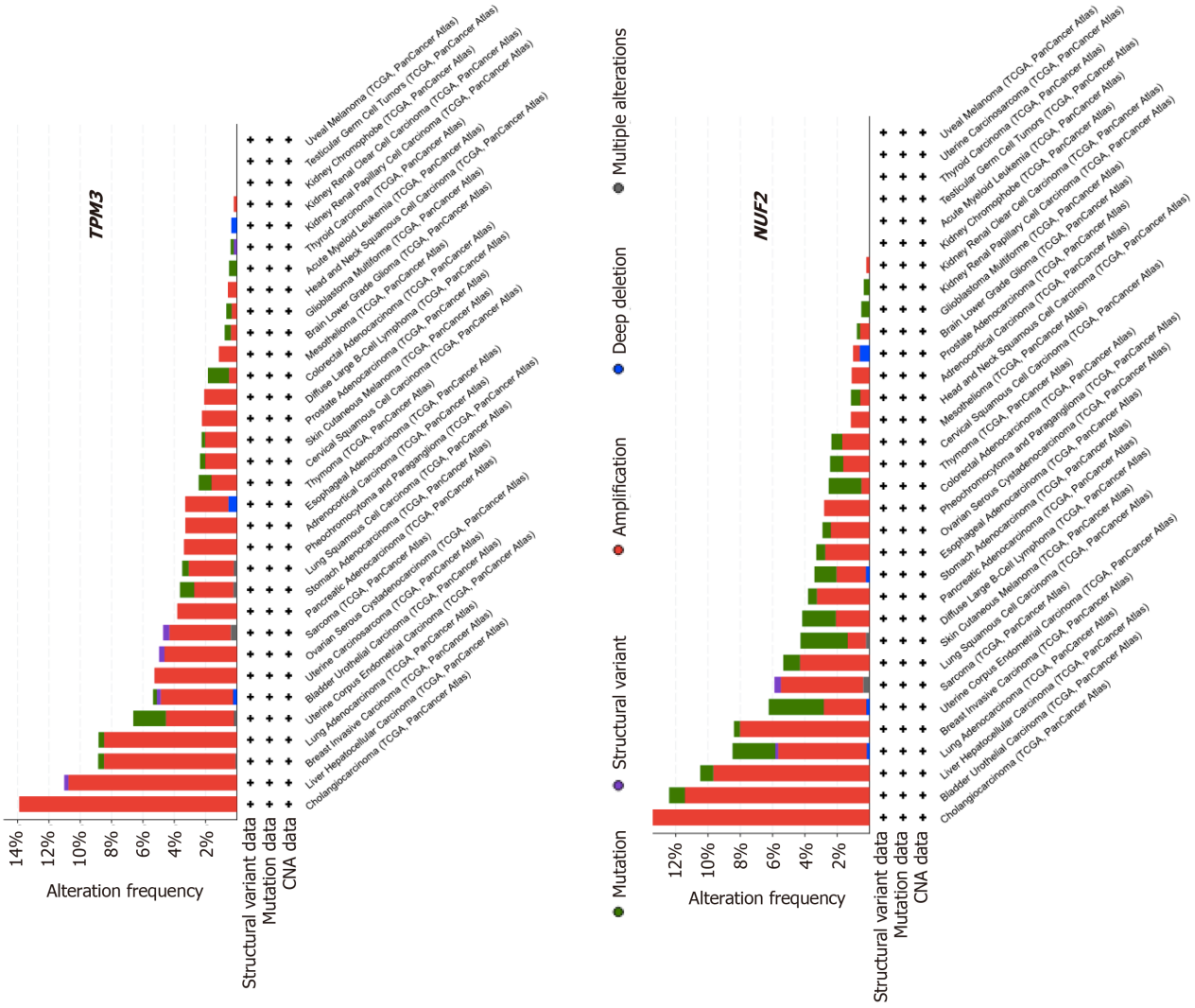

Figure 2 Frequency of *TPM3* or *NUF2* alterations in other cancers. Analyses from cBioPortal show the percent of cancer cases that include a *TPM3* gene alteration (upper plot) or a *NUF2* gene alteration. The results were generated by first selecting all 32 of The Cancer Genome Atlas PanCancer Atlas studies[41] from the cBioPortal Query page, and then searching for DNA alterations in the *TPM3* gene and *NUF2* gene individually. Graphs shown in this figure were taken from the Cancer Types Summary results page that is produced by cBioPortal following this search. The colors above represent the following: green, mutation; purple, fusion; red, amplification; blue, deep deletion; grey, multiple alterations.

frequently in multiple cancers, and it has been proposed that overexpression of NDC80 complex proteins leads to defective mitosis and may promote aneuploidy[62]. Screening in epithelial ovarian carcinoma cells of an siRNA library has identified *NUF2* as one of four genes that reduced cell viability and increased apoptosis when knocked down[63]. This study also found a correlation between *NUF2* mRNA elevation and poorer prognosis in ovarian carcinoma patients. *NDC80* (also known as Hec1) interacts directly with *NUF2* and may represent a therapeutic target. A screen of a small molecule library for inhibitors of the interaction between *NDC80* and mitotic kinase Nek2 identified a compound named INH1 as being able to disrupt the protein-protein interaction[64]. This study also showed that INH1 decreased proliferation of breast cancer cells in culture and in a mouse xenograft assay.

CONCLUSION

In conclusion, our review of the literature and independent analysis of the TCGA-LIHC PanCancer data set identified two non-overlapping sets of genes that reside on chromosome 1q and frequently undergo amplification in HCC (compare Figure 1 and Table 1). We found what appears to be a significant correlation between amplification and/or increased expression of *TPM3* and *NUF2* and poorer prognosis, which is consistent with previous reports in the literature. Amplification of 1q also is observed

frequently in other cancers. One limitation to our strategy to identify additional driver genes is that only genes previously identified as involved in cancer by OncoKB were considered. The absence of many genes in Table 1 suggests more candidate genes may still be identified. In the case of large chromosomal CNAs such as seen with 1q, it is truly challenging to identify the critical driver mutations involved. Further studies will be needed to understand the contributions of numerous genes amplified on chromosome 1q so as to effectively target therapeutics.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Impact of donor-specific antibodies on long-term graft survival with pediatric liver transplantation

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Abstract

BACKGROUND

In a previous paper, we reported a high prevalence of donor-specific antibody (DSA) in pediatric patients with chronic rejection and expressed the need for confirmation of these findings in a larger cohort.

AIM

To clarify the importance of DSAs on long-term graft survival in a larger cohort of pediatric patients.

METHODS

We performed a retrospective analysis of 123 pediatric liver transplantation (LT) recipients who participated in yearly follow-ups including Luminex testing for DSA at our center. The cohort was split into two groups according to the DSA status (DSA-positive n = 54, DSA-negative n = 69). Groups were compared with regard to liver function, biopsy findings, graft survival, need for re-LT and immunosuppressive medication.

RESULTS

DSA-positive pediatric patients showed a higher prevalence of chronic rejection (P = 0.01), fibrosis (P < 0.001) and re-transplantation (P = 0.018) than DSA-negative patients. Class II DSAs particularly influenced graft survival. Alleles DQ2, DQ7, DQ8 and DQ9 might serve as indicators for the risk of chronic rejection and/or

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allograft fibrosis. Mean fluorescence intensity levels and DSA number did not impact graft survival. Previous episodes of chronic rejection might lead to DSA development.

CONCLUSION

DSA prevalence significantly affected long-term liver allograft performance and liver allograft survival in our cohort of pediatric LT. Screening for class II DSAs in combination with assessment of protocol liver biopsies for chronic antibody-mediated rejection improved early identification of patients at risk of graft loss.

Key Words: Donor-specific antibodies; Graft rejection; Liver transplantation; Fluoroimmunoassay; Pediatrics; Graft dysfunction; Fibrosis

Core Tip: This was a retrospective study to evaluate the impact of donor-specific antibodies (DSAs) on graft survival with pediatric liver transplantation. Graft fibrosis and graft loss was significantly higher in patients with DSAs. Screening for DSAs should be included in follow-ups to avoid delayed identification of patients at risk of graft loss (rejection), and may be even more relevant for patients with early histological signs of possible allograft dysfunction (fibrosis). Moreover, patients with DSAs may be poor candidates for reduction of initial immunosuppression or even weaning.

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INTRODUCTION

The impact of anti-human leucocyte antigen (HLA) donor-specific antibodies (DSAs) on graft survival and graft function in kidney and heart transplantation is crucial[1-3]. However, their impact on liver transplantation (LT) is still controversial: Some research suggests that the overall impact on graft and patient outcome is limited[4], but other studies found that it may be an independent risk factor for patient death and graft loss[5-7].

For a long time, DSAs were suspected to have a minor influence on liver allografts [8,9], based on low vascular expression of HLA class II antigens, weak HLA class I expression on hepatocytes and a large endothelial surface diluting soluble antibodies and antigens[10]. However, HLA class II expression is present, especially on perivenular dendritic cells and endothelial cells of the septal venule, sinusoidal and central vein[11,12].

There is growing (but limited) data for pediatric LT suggesting that DSAs might have an impact on long-term graft survival by influencing the development of portal inflammation, portal fibrosis[13-15], perivenular or perisinusoidal fibrosis[15-18], or obliterative portal venopathy[19] and might lead to chronic antibody-mediated rejection (cAMR)[10,19,20].

According to Demetris *et al*[10,21-23] chronic rejection (CR) is characterized by a slow process based on alloreactivity. Histopathological findings of T-cell-mediated rejection (TCMR) include lesions or loss of small bile ducts, portal inflammation, venous endothelial inflammation, obliterative arteriopathy and low-grade necroinflammation. Since obliterative arteriopathy is rarely found in a percutaneous needle biopsy, ductopenia and signs of necroinflammation tend to be used for the diagnosis of chronic rejection in biopsy specimens[23]. According to O'Leary *et al*[24], ductopenia, biliary strictures and fibrosis are associated with DSAs in adult LT.

Initially, AMR was only suspected after ABO-incompatible transplantation, but it has since been reported in ABO-compatible LT as well[19,25,26]. It has also been

suggested that TCMR and cAMR are linked, and that any form of TCMR might channel cAMR by increasing the presentation of intra- and extracellular donor antigens on dendritic cells, which would then stimulate the production of DSAs (second-hit hypothesis)[10,23,24,27,28].

This paper reports the results of a retrospective cross-sectional study of the influence of DSAs developed after LT on long-term graft survival in pediatric LT recipients.

MATERIALS AND METHODS

Study design and study population

From 1993 to 2015, 765 pediatric LTs were performed at our tertiary center. Testing of DSAs started in 2013, mostly with the ELISA technique. We performed a cross-sectional retrospective chart analysis of all patients coming for check-ups at our pediatric department between 2013 and 2017. All charts were checked for DSA measurement with the Luminex technique (single antigen bead assay) as the most sensitive test, as well as donor HLA typing and complete laboratory values. We included 123 patients in the present study after exclusions due to change of residency, follow-up in other transplant centers, missing donor typing and missing Luminex testing as well as deaths within the first year after LT.

DSA testing usually took place as part of the yearly follow-up routine at 1 to 19 years (mean 8.9 years) after first LT. There were 55 female (44.7%) and 68 male (55.3%) participants. The main diagnosis leading to LT was biliary atresia (n = 40, 32.5%), followed by metabolic disorders (n = 40, 32.5%), acute liver failure (n = 10, 8.1%), Alagille syndrome (n = 9, 7.3%) and others (n = 64, 52%). LT was performed either as full-graft (n = 22) or technically modified as a split graft (n = 74), reduced-size graft (n = 10) or living donor LT (n = 14). The majority of patients are still living with the first transplant (n = 87, 70.7%); 26 patients were retransplanted once (21.1%), 8 patients twice (6.5%) and two patients three times (1.6%). The main cause for re-transplantation was chronic rejection with chronic graft dysfunction; see baseline characteristics in Table 1 for more details.

Yearly follow-ups included physical examination of the child, an ultrasound evaluation of the graft, extensive laboratory diagnostics (including detection of DSAs) and histopathological examination of liver biopsies if available.

Since we perform DSA detection with luminex testing as part of our routine clinical practice and because of the retrospective study design, our study was readily approved by the ethics committee.

Histological graft examination

In our center, we perform routine protocol liver biopsies every three to five years after LT. Liver biopsy is also indicated if there are laboratory signs of allograft dysfunction or if rejection is suspected. The histological features that we assessed are shown in Table 2. In case of fibrosis or rejection, grading and rating was performed using the Desmet score for fibrosis, the rejection activity index and Banff scores for chronic liver transplant rejection by experienced in-house pathologists.

HLA typing and luminex

We described the technique of HLA typing and HLA antibody testing in our previous paper[14]. In this study, we also used luminex single antigen class I and class II beads (One Lambda Inc., LABScreen®) for retrospective detection of anti-HLA-antibodies (A, -B, -Cw, -DR, -DQ, and -DP). Normalized mean fluorescence intensity (MFI) > 1500 was regarded as positive.

Statistical analysis

All data were statistically analyzed using IBM SPSS Statistics® software, Version 25. Due to their mainly Fisher's exact non-Gaussian character, variables were analyzed with Pearson's chi-square, Cramer and Phi, Mann-Whitney U and the Wilcoxon test. Freedom from events (graft loss and rejection) was estimated by the Kaplan-Meier method and was compared across groups with the log-rank test. Graft survival was computed from the date of LT to re-transplantation, or to biopsy-proven rejection. A P value < 0.05 was considered statistically significant. We performed binary correlation analysis and evaluation of odds ratios (95%CI, P < 0.05). Significant correlations (P < 0.02) were included to form predictive models for DSA development and chronic

Table 1 Baseline characteristics

	'DSA-positive' group 1 (*n* = 54)	'DSA-negative' group 2 (*n* = 69)
Age		
At LT (yr)	3.3 (1 mo-17 yr)	4.0 (1 mo-17.8 yr)
At follow-up (yr)	13.8 (2-23)	12.6 (1-24)
Gender		
Female	*n* = 21	*n* = 35
Male	*n* = 33	*n* = 35
Main diagnosis		
Biliary atresia	*n* = 20	*n* = 20
Alagille syndrome	*n* = 1	*n* = 8
Acute liver failure	*n* = 6	*n* = 4
Metabolic disorders[1]	*n* = 14	*n* = 26
Others[2]	*n* = 13	*n* = 11
Donor source		
Ldlt	*n* = 9	*n* = 11
Ddlt	*n* = 45	*n* = 58
Full-graft	*n* = 8	*n* = 12
Split size	*n* = 30	*n* = 43
Reduced size	*n* = 7	*n* = 3
Cold ischemic time (min)	543.3 (122-949)	572.5 (145-943)
Relt	*n* = 22	*n* = 14
Graft loss due to		
Cr	*n* = 23	*n* = 7
Alv	*n* = 7	*n* = 3
Thrombosis	*n* = 3	*n* = 3
Rond	*n* = 2	*n* = 0
Ssc	*n* = 2	*n* = 0
Time to DSA-testing		
Years after current LT	9.75 (1-19)	7.98 (1-19)
Anti-HLA antibodies	*n* = 54	*n* = 32
Previous episodes		
Of acute rejection	*n* = 12	*n* = 7
Of chronic rejection	*n* = 10	*n* = 3

[1]Such as carbamoyl phosphate synthetase defects, ornithine transcarbamylase deficiency, primary hyperoxaluria, alpha1-antitrypsin-deficiency, glycogen storage disease, maple syrup urine disease, citrullinemia, Wilson disease, others.
[2]Such as idiopathic cirrhosis, autosomal recessive polycystic kidney disease, Crijgler-Najar syndrome, progressive familiar intrahepatic cholestasis, malignancies, vascular dysfunction, neonatal hepatitis, primary sclerosing cholangitis, autoimmune hepatitis.
LDLT: Living donor liver transplantation; DDLT: Dead donor liver transplantation; relt: Retransplantation; HLA: Human leucocyte antigen; DSA: Donor-specific antibodies; CR: Chronic rejection; ALV: Acute liver failure; ROND: Recurrence of native disease; SSC: Secondary sclerosing cholangitis.

rejection with binary logistic regression and Cox regression analysis.

Group formation

The population was divided into two groups according to DSA status (group 1 = DSA-positive, *n* = 54; group 2 = DSA-negative, *n* = 69). If DSAs against a certain HLA locus

Table 2 Biopsy characteristics

	Group 1: 'DSA-positive' (*n* = 38)	Group 2: 'DSA-negative' (*n* = 34)	*P* value
Fibrosis[1]	*n* = 24	*n* = 6	
Low-grade	*n* = 23	*n* = 6	< 0.001
High-grade	*n* = 1	*n* = 0	0.5
Cirrhosis	*n* = 3	*n* = 0	0.5
Steatosis	*n* = 6	*n* = 6	0.8
Portal inflammation	*n* = 28	*n* = 18	0.005
Perivenular/perisinusoidal inflammation	*n* = 11	*n* = 2	0.011
Ductular inflammation	*n* = 13	*n* = 3	0.004
Endothelitis	*n* = 6	*n* = 2	0.1
Biliary lesions/ductulopenia	*n* = 6	*n* = 0	0.009
Ductular cholestasis	*n* = 12	*n* = 4	0.01
Biliary tract strictures	*n* = 9	*n* = 6	0.03
Single cell necrosis	*n* = 5	*n* = 0	0.02
Chronic rejection[2]	*n* = 7	*n* = 0	0.009
Possible camr[3]	*n* = 9	*n* = 0	0.002

[1]Fibrosis grading according to Desmet *et al*.
[2]Chronic rejection according to Banff criteria.
[3]Chronic antibody-mediated rejection (camr) according to Banff 2016 criteria, with absent C4d staining.
DSA: Donor-specific antibodies.

were found in more than four patients (n ≥ 5), they were analyzed separately to determine whether a single HLA locus was a common target for DSA formation or if it might be associated with histopathological changes, chronic rejection or retransplantation. To assess whether the number of DSAs influenced graft survival, we compared graft survival of patients with a single DSA (*n* = 26) with those who had multiple DSAs (*n* = 28). To determine whether high MFI levels influenced graft performance, we compared patients with very high MFI levels [MFI > 10000 (*n* = 24)] to patients with lower MFI levels [MFI < 10000 but > 1500 (*n* = 30)].

Not every patient with luminex testing received a liver biopsy, so we could not include every participant for histopathological analysis.

Immunosuppressive medication

Initial immunosuppression (IS) within the first year and also maintenance therapy 1 year post-LT has already been described by our group[14]. In the present study population, patients mainly received immunosuppressive therapy with CNI (group 1: CSA *n* = 27; Tac *n* = 24; group 2: CSA *n* = 29; TAC *n* = 36) which was either monotherapy (group 1: 53.7%; group 2: 56.5%) or in combination with other medications. Detailed information is provided in Table 3. IS was modified if there were side effects or rejection episodes.

RESULTS

HLA analysis and DSAs

There were 123 patients in the study. HLA antibodies were found in 74.1% of all patients (*n* = 106), and 43.9% (*n* = 54) presented with DSAs. The mean number of HLA antibodies per patient in group 1 was 10.9 (minimum of *n* = 1, maximum of *n* = 63, SD = 10.6) whereas group 2 had only 2.9 HLA antibodies per patient (minimum of *n* = 0, maximum of *n* = 33, SD = 6.2). The mean number of DSAs was 2.2 with a maximum of up to 6 DSAs per patient and graft (SD = 1.4).

Table 3 Immunosuppressive therapy

Group 1 'DSA-positive'		Group 2 'DSA-negative'
Monotherapy	*n* = 29	*n* = 39
CSA	*n* = 17	*n* = 20
Trough level	109.5 μg/L (23-674 μg/L) median 73 μg/L	58.0 μg/L (29-106 μg/L)
TAC	*n* = 10	*n* = 17
Trough level	4.4μg/L (1.0-7.3 μg/L)	5.5 μg/L (2.6-7.7 μg/L)
EVE		*n* = 1
Trough level		10.3 μg/L
SIR	*n* = 1	
Trough level	5.2 μg/L	
MMF	*n* = 1	*n* = 1
Combined therapy	*n* = 25	*n* = 30
CSA		
+ EVE	*n* = 3	*n* = 4
+ MMF	*n* = 3	*n* = 3
+ PRED	*n* = 2	*n* = 1
+ EVE + PRED		*n* = 1
+ MMF + PRED	*n* = 2	
Trough level	54.7μg/L (11-89 μg/L)	72.7 μg/L (23-137 μg/L)
TAC		
+ EVE	*n* = 3	*n* = 6
+ MMF	*n* = 5	*n* = 7
+ PRED	*n* = 5	*n* = 3
+ MMF + PRED	*n* = 1	*n* = 3
Trough level	6.1 μg/L (3.0-9.9 μg/L)	6.3 μg/L (2.1-15.1 μg/L)
EVE		
+ MMF		*n* = 1
+ MMF + PRED	*n* = 1	
Trough level	3.7 μg/L (1.0-5.0 μg/L)	4.3 μg/L (1.8-7.8 μg/L)
SIR		
+ MMF		*n* = 1 (trough levels, NA)
Adherence rates		
CSA	81.5%	72.4%
TAC	87.5%	88.9%
EVE	75%	84.9%
SIR	100%	100%

Trough levels given as mean (and range). MMF: Mycophenolate mofetil; CSA: Cyclosporin A; TAC: Tacrolimus; EVE: Everolimus; PRED: Prednisolone; SIR: Sirolimus; NA: Not available; DSA: Donor-specific antibodies.

All DSA-positive patients except one had DSAs of HLA class II (*n* = 53), while 14.8% (*n* = 8) had DSAs of both classes. Only one patient had DSAs exclusively in class I.

A detailed analysis of DSA HLA allele distribution showed that mainly HLA class II alleles, especially DR (*n* = 26 out of 54) and DQ (*n* = 39 out of 54) alleles were targeted. DP-HLA alleles could not be evaluated, because HLA donor typing was missing or

incomplete for the majority of DP alleles. We could count these as HLA antibodies, but could not determine donor specificity. Nevertheless, the DSA-positive group showed a 46.3% prevalence of anti-HLA DP antibodies (n = 25 of 54), while the prevalence of these antibodies was only 14.5% (n = 10 of 69) in the DSA-negative group (Figure 1).

Liver biopsies

Liver biopsies (group 1 n = 38; group 2 n = 34) were mostly performed as protocol biopsies (67.3% in group 1 *vs* 85.7% in group 2), followed by suspected rejection (16.3% *vs* 10.7%). Recurrence of native disease was suspected in four children and confirmed in three (PFIC2 n = 1; AIH n = 1; PSC n = 1).

Comparing both groups, we found that fibrosis, portal inflammation, perivenular or perisinusoidal inflammation, ductular inflammation, biliary lesions/ductulopenia, ductular cholestasis, biliary tract strictures, single cell necrosis and chronic rejection were significantly more common in the DSA-positive group. Fibrosis was significantly correlated to class II HLA-DSAs (P < 0.001), especially to alleles DQ2 (P = 0.03), DQ7 (P < 0.001), DQ8 (P = 0.02) and DQ9 (P = 0.007). Low-grade fibrosis (F1 and F2) in particular was significantly higher in DSA-positive patients (P < 0.001) and was found in 17 routine protocol biopsies in group 1 (F1 and F2), whereas only 3 protocol biopsies showed signs of low-grade fibrosis (F1) in group 2.

We also found a higher incidence of high-grade fibrosis (F3), cirrhosis and endothelitis in group 1, although the difference was not significant. Steatosis, hepatocyte ballooning and other signs of toxic damage to the graft were either comparable or more likely to be found in group 2 (Table 2).

Correlation analysis showed a significant connection between biopsy-proven rejection and DSAs of HLA class II (P = 0.005), in particular against DQ2 (P = 0.02), DQ8 (P = 0.02) and DR52 (P = 0.03).

Overall graft survival according to Kaplan-Meier estimates was significantly lower for patients with DSAs (Mantel-Cox test: P = 0.04, Figure 2).

Clinical evaluation of liver enzymes, liver synthesis parameters and ultrasound criteria in CR-positive patients was not consistent with the histopathological status. Elevated levels of ALT and AST were only found in 10%-20% of CR-positive patients; γGT- and GLDH-elevation occurred in 50%-66%. None of these enzyme elevations reached statistical significance, nor did aberration of liver function parameters (albumin, bilirubin, international normalized ratio).

Histopathological indicators of possible cAMR were found in 9 patients of Group 1 (5 male, 4 female). Of these, three patients received monotherapy (CSA n = 2, MMF n = 1), while the other patients were treated with CNI in combination with EVE or MMF. Combined therapy was introduced if renal function was impaired or if there were previous signs of rejection. Trough levels were in the therapeutic range in all but two patients. Liver enzymes were normal except for elevated γGT in four patients. Alleles most targeted by DSAs belonged to HLA class II. DSAs against DQ alleles were particularly prevalent (DQ2 n = 2; DQ7 n = 4; DQ8 n = 4, DQ9 n = 3).

There was a significantly higher incidence of previous episodes of chronic rejection in our DSA-positive patients (P = 0.015). The rate of previous episodes of acute rejection was comparable in both groups.

Influence of DSA number and MFI levels

There was no correlation between DSA number and MFI levels with chronic rejection or re-LT. The Mantel-Cox test showed no significant influence of the number of DSAs on graft survival (P = 0.7; Figure 3).

Single MFI levels ranged from 1668 to 31309 (cumulated MFI from 1678 to 120181; SD = 22333). Fibrosis, biopsy-proven chronic rejection, re-LT and numbers of re-LT were not significantly influenced by high MFI levels (MFI >10000 *vs* MFI > 1500 but < 10000). Graft survival was not significantly decreased in patients with high MFI levels (Mantel-Cox P = 0.7, Figure 4).

DSA-influence on re-LT

Comparing both groups, retransplantation was significantly more common in the DSA-positive group (n = 22 *vs* n = 14; P = 0.018). 79.9% of group 2 patients were able to maintain their first graft, but only 61.1% of group 1 maintained their initial grafts. The overall number of LTs was significantly higher in group 1 (1 to 4 LTs) than in Group 2 (1 to 3 LTs; P = 0.015). Also, the number of LTs was directly correlated with the presence of DSAs (P = 0.002). Retransplantation due to chronic rejection was significantly more common in group 1 (n = 9) than in group 2 (n = 2; P = 0.04).

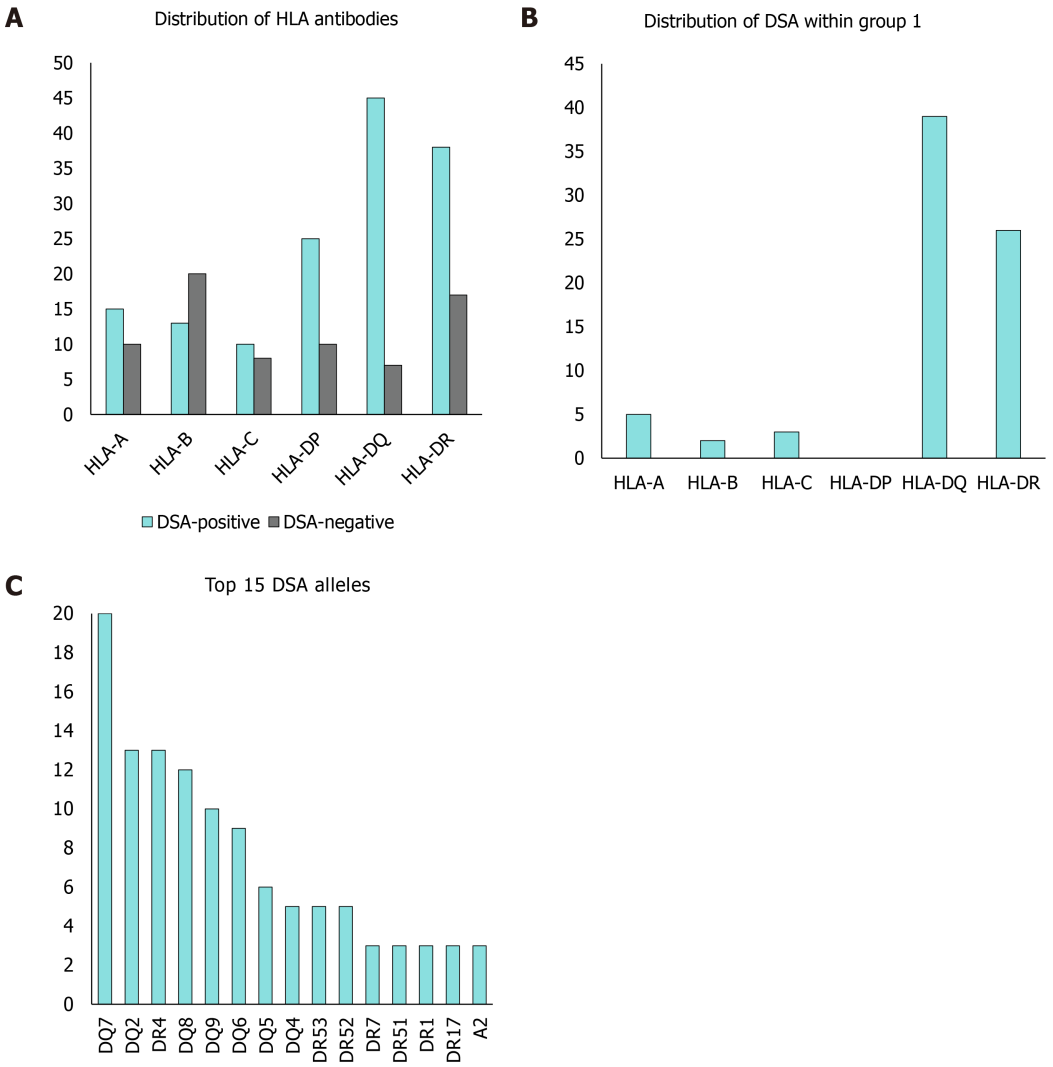

Figure 1 Allele distribution. A: Distribution of human leucocyte antigen (HLA) antibodies; B: Distribution of donor-specific antibodies (DSAs) within group 1; C: Top 15 DSA alleles. Vertical axis: numbers, quantitative; horizontal axis: categories of anti-HLA antibodies and subcategories of DSAs; columns: DSA-positive patients shown in green, DSA-negative patients shown in light grey. HLA: Human leucocyte antigen; DSA: Donor-specific antibody.

DISCUSSION

Rejection

The role of DSAs in the pathogenesis of chronic rejection in pediatric LT recipients has been subject to various studies but is still not fully understood. In a previous study, our group reported a higher prevalence of DSAs in patients with CR, although the statistical significance could not be determined due to the small cohort[14]. CR in DSA-positive patients was also described later by Wozniak *et al*[27] in a cross-sectional study of 50 pediatric patients.

The results of the present study show that histological indicators of CR have a significantly higher prevalence in DSA-positive patients, confirming O'Leary's findings in a pediatric population and reaffirming the results of our previous study in a larger pediatric cohort. Furthermore, in all cases of biopsy-proven CR, patient sera were positive for DSAs. We also identified nine DSA-positive patients who possibly suffered from chronic antibody-mediated allograft rejection. Even biopsies of DSA-positive patients who received routine protocol biopsies and had no laboratory signs of impaired allograft function exhibited histological signs of fibrosis or rejection. This shows that correlating the aberration of laboratory parameters and CR or fibrosis is not a reliable clinical procedure. Ohlsson *et al*[29] recently confirmed the value of protocol biopsies in detecting silent immune-mediated allograft injuries, regularly associated with the presence of DSAs.

As this study had a cross-sectional design, we could not examine the development of DSAs over the full study period, especially after rejection episodes. Nevertheless,

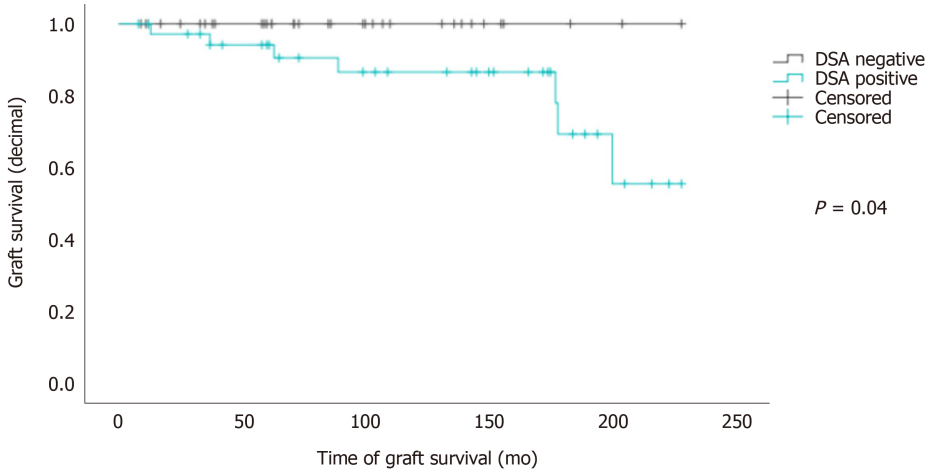

Figure 2 Donor-specific antibody presences on graft survival. Kaplan-Meyer survival plot; vertical axis: Graft survival (decimal); horizontal axis: Time of graft survival (months); graphs: Donor-specific antibody (DSA)-negative patients are shown in dark grey, numbers at risk *n* = 34; DSA-positive patients are shown in green, numbers at risk *n* = 38. DSA: Donor-specific antibody.

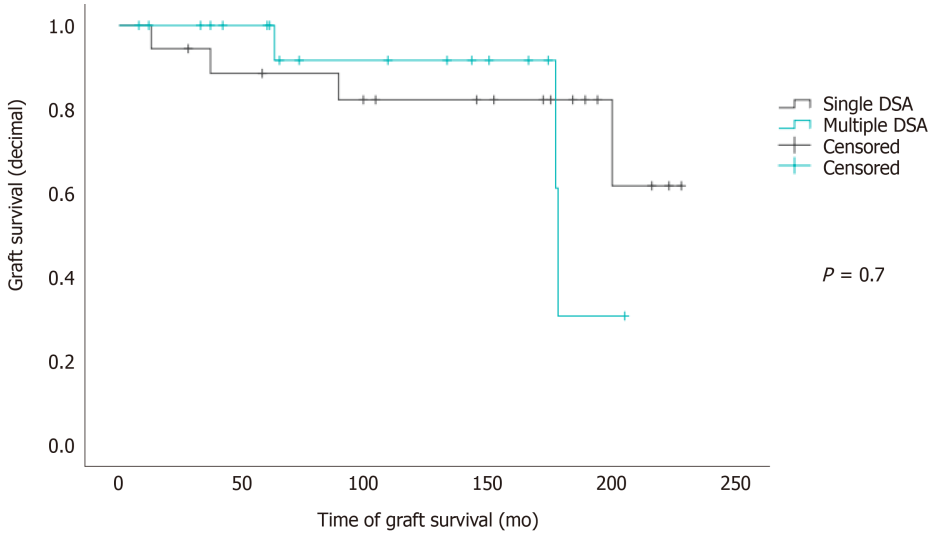

Figure 3 Number of donor-specific antibodies on graft survival. Kaplan-Meyer survival plot; vertical axis: Graft survival (decimal); horizontal axis: Time of graft survival (months); graphs: Patients with single donor-specific antibody (DSA) are shown in a dark grey, numbers at risk *n* = 19; patients with multiple DSAs are shown in green, numbers at risk *n* = 19. DSA: Donor-specific antibody.

previous episodes of CR with the same graft (prior to the current follow-up, which did not lead to graft loss or re-LT) were significantly more common in our DSA-positive patients ($P = 0.015$).

Although there is growing evidence that DSAs impact graft survival, it is still unclear whether DSA number or quality matter. While Couchonnal *et al*[30] reported poorer long-term graft survival in patients with high MFI (> 10000), Wozniak *et al*[27] described a significantly higher impact of the overall presence of DQ-DSAs on graft survival, as opposed to the presence of any DSAs with high MFI levels (threshold > 13000).

We also used MFI levels of > 10000 to identify strong DSA effects, but we found no statistically significant impact of MFI levels on biopsy-proven CR, graft survival or need for re-transplantation. However, anti-HLA class II antibodies and especially anti-HLA-DQ2 and -DQ8-antibodies were significantly correlated with graft survival. We therefore support Demetris' 'second-hit' hypothesis and regard it as probable that class II HLA-DSAs influence TCMR, cAMR and probably the need for reLT. This is supported by a significant influence of the presence of DSAs on graft survival in survival analysis. The use of anti-HLA-DQ2 and -DQ8-antibodies as screening markers needs to be assessed with further prospective studies.

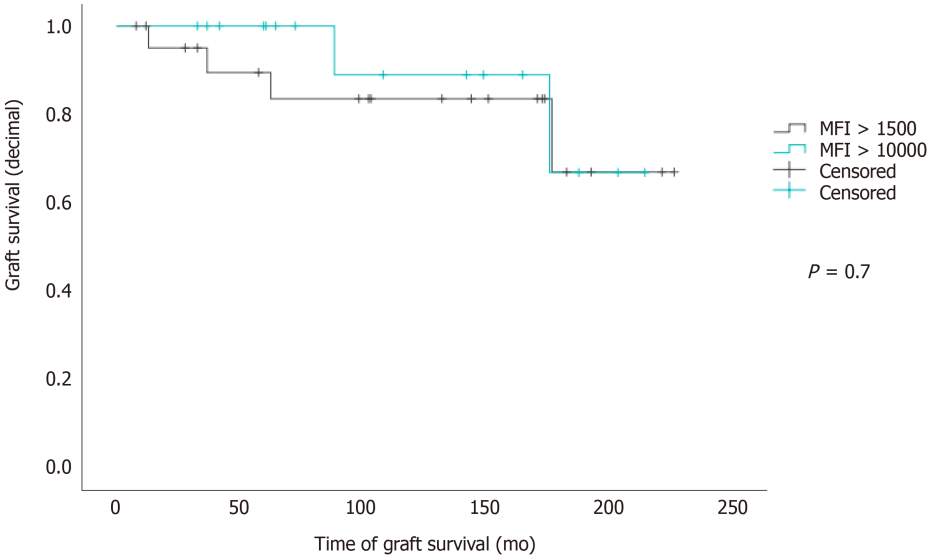

Figure 4 Mean fluorescence intensity levels on graft survival. Kaplan-Meyer survival plot; vertical axis: Graft survival (decimal); horizontal axis: Duration of graft survival (months); graphs: Patients who have donor-specific antibodies (DSAs) with mean fluorescence intensity (MFI) above 1500 are shown in dark grey; numbers at risk *n* = 22; patients who have DSAs with MFI above 10000 are shown in green; numbers at risk *n* = 16. MFI: Mean fluorescence intensity.

Fibrosis

Mild to moderate allograft fibrosis is a common finding in protocol biopsies obtained 5-10 years post-LT. So far, low IS trough levels or even weaning off are thought to promote the development of such fibrosis by enabling alloreactivity that leads to allograft inflammation[31] and fibrosis, with possible later development of cirrhosis and graft failure[17,20,32,33]. According to Briem-Richter *et al*[31], increasing IS dosage results in the resolution of histopathological signs of rejection and severity of fibrosis. In this present study, we found that portal, perivenular or perisinusoidal inflammation is very common in DSA-positive patients. Also, mild to moderate allograft fibrosis (grade 1-2) was significantly more common in DSA-positive patients. We therefore consider DSA presence as a symptom of such alloreactivity; this might help to identify children who are poor candidates for reducing IS levels.

Correlation between DSA and cirrhosis did not reach statistical significance, probably because of the small number of cirrhosis patients confirmed with histopathology (DSA-positive patients: *n* = 3 *vs* DSA-negative patients: *n* = 0).

The presence of HLA class II DSAs, especially anti-HLA-DQ antibodies, coincided significantly with allograft fibrosis. Furthermore, we were able to identify four specific HLA-DQ alleles that might serve as serological markers or have predictive value: DQ2, DQ7, DQ8 and DQ9.

Limitations

As a liver biopsy was not performed for every study participant, one might argue that there is selection of patients with poorer graft performance, leading to a biased correlation of DSA presence with CR. However, the general group of poor allograft performance is still relatively small, thus reversing the suspected bias with an overall representative selection of the study population. Other limitations of this study were the incomplete HLA typing of DP alleles, errors in sampling, and missing HLA donor typing in general, which led to exclusion of participants. Also, C4d staining was not performed on liver biopsies that were taken prior to the updated Banff criteria in 2016, so that these biopsies could not be fully included in the evaluation. As these are parts of the general restrictions of retrospective studies, we plan to conduct a prospective clinical trial to assess the new issues that this study has raised.

CONCLUSION

Long-term allograft survival is even more valuable in pediatric LT than in adult LT, and with the decreased graft survival and increased prevalence of allograft dysfunction and retransplantation in DSA-positive patients, this important subject should not be underestimated.

Screening for DSA must be included in follow-ups to ensure identification of patients at risk of potential graft loss (rejection), and may be even more relevant for patients with early signs of allograft dysfunction (fibrosis). Moreover, patients with DSA might not be good candidates for reduction of IS or even weaning. According to our results, in the presence of DSA, selected patients should be considered for additional graft biopsies including assessment with Banff chronic cAMR criteria after C4d-staining, since routine laboratory parameters are not sufficiently accurate for monitoring the allograft status and cannot identify patients with silent immune-mediated allograft injuries. Whether the latter could be detected by ultrasound elastography could be the subject of a future clinical trial.

Since HLA class I DSAs are less common and have less impact on allograft fibrosis or rejection, screening could be limited to HLA class II DSA (-DQ, -DR -DP).

ARTICLE HIGHLIGHTS

Research background

An impact of donor-specific antibodies (DSAs) on long-term liver allograft survival was found previously in a small cohort of pediatric patients, but the statistical significance was unclear.

Research motivation

The aim of this study was to clarify the importance of DSAs on long-term graft survival in a larger cohort of pediatric patients.

Research objectives

The objective of this study was to emphasize the importance of comprehensive follow-up examinations in clinical practice after pediatric liver transplantation (LT) and contribute to optimizing and standardizing LT follow-ups.

Research methods

This was a cross-sectional retrospective cohort study that compared the outcomes of two patient groups after pediatric LT.

Research results

Our study showed that DSAs significantly impact liver allograft survival. The presence of human leucocyte antigen class II DSAs is associated with chronic rejection, chronic antibody-mediated rejection, graft fibrosis, graft failure, graft loss and re-LT.

Research conclusions

Screening of DSAs and protocol liver biopsies including C4d immunostaining should be standard practice in follow-ups after pediatric LT.

Research perspectives

Further prospective studies should be conducted to explore whether certain DQ-DSAs could be used as a serological marker for the risk of graft loss.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Mortality and health care burden of Budd Chiari syndrome in the United States: A nationwide analysis (1998-2017)

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Abstract

BACKGROUND

The Budd Chiari syndrome (BCS) is a rare and potentially fatal disease, but there is a paucity of data on the in- hospital mortality as well its economic burden on the health care system.

AIM

To evaluate trends in mortality, length of hospital stays and resource utilization among inpatients with BCS.

METHODS

Data on all adult patients with a diagnosis of BCS were extracted from the National Inpatient Sample (NIS) from 1998 to 2017. To make inferences regarding the national estimates for the total number of BCS discharges across the study period, sample weights were applied to each admission per recommendations from the NIS.

RESULTS

During the study period, there were 3591 (8.73%) in-patient deaths. The overall in-hospital mortality rates among BCS patients decreased from 18% in 1998 to 8% in 2017; the mortality decreased by 4.41% ($P < 0.0001$) every year. On multivariate analysis, older age, higher comorbidity score, acute liver failure, acute kidney injury, acute respiratory failure, hepatic encephalopathy, hepatorenal syndrome, inferior vena cava thrombosis, intestinal infarct, sepsis/septic shock and cancer were associated increased risk of mortality. The average of length of stay was 8.8 d and it consistently decreased by 2.04% (95%CI: -2.67%, -1.41%, $P < 0.001$) from 12.7 d in 1998 to 7.6 d in 2017.The average total charges after adjusted for Medical Care Consumers Price Index to 2017 dollars during the time period was $94440

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and the annual percentage change increased by 1.15% (95%CI: 0.35%, 1.96%, P = 0.005) from $95515 in 1998 to $103850 in 2017.

CONCLUSION
The in-hospital mortality rate for patients admitted with BCS in the United States has reduced between 1998 and 2017 and this may a reflection of better management of these patients.

Key Words: National Inpatient Sample; Budd Chiari syndrome; Mortality; Length of stay; Total charges

Core Tip: Using a large administrative database, we were able to analyze the mortality and socioeconomic impact of Budd Chiari syndrome hospitalizations in the United States over a 19-year period with a high degree of granularity. We were able to show that while the mortality rate and length of stay has declined significantly, total charges continue to show an upward trend.

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INTRODUCTION

The Budd Chiari syndrome (BCS) is a rare but potentially fatal disorder that results from partial or complete obstruction of the hepatic venous outflow in the absence of right heart failure. Unlike Asian countries, the incidence and prevalence of BCS in Western countries is thought to be lower, but there are no large epidemiological studies[1]. BCS is a heterogeneous disease with a protean clinical presentation ranging from asymptomatic or chronic forms to fulminant liver failure[2,3]. Prognosis of BCS is highly variable and studies from large academic centers have reported mortality rates ranging anywhere between 13%-36%[4-9]. These wide ranges of mortality are more likely related to small sample size, variability in follow up period and publication selection bias. Risk factors such as ascites, hepatic encephalopathy, coagulopathy, elevated creatinine or bilirubin are considered to be independent risk factors for mortality[4-8]. A stepwise management approach consisting of anticoagulation, endovascular venoplasty, transjugular intrahepatic portosytemic shunts (TIPS) and liver transplantation (LT) has been proposed for the management of BCS[3,9-12]. However, this approach may not be applicable to all patients because of varying severity of presentation, the extent of venous occlusion and other serious comorbidities.

There are multiple studies that had investigated the mortality and economic burden of decompensated liver cirrhosis in the United States, but there is a paucity of data regarding the mortality burden and health care utilization for patients with BCS. The primary objective of our study was to assess the trends in in-hospital mortality, length of stay (LOS) and resource utilization among inpatients with BCS using the National Inpatient Sample (NIS) database.

MATERIALS AND METHODS

Study design and data source

This was a retrospective study where data were extracted from the NIS from 1998 to 2017. The NIS is the largest publicly available all-payer inpatient administrative database developed by the Agency for Healthcare Research and Quality (AHRQ) for

the Health Care cost and Utilization Project (HCUP). It represents approximately 20% stratified sample of discharges from community hospitals, but excludes long term acute care hospitals and rehabilitation facilities and contains information of more than 7 million hospital discharges annually. The number of states participating in the NIS grew from 8 in 1988 to 48 in 2017. The database captures information about primary and secondary diagnoses during each hospital stay as well as information about procedures. NIS also contains other valuable information such as severity and comorbidity measures, hospital characteristics (size, region, bed size, teaching/non-teaching), payment source (Medicare/Medicaid/private), total charges and length of hospital stay. In 2012, NIS revised the sample design so as to represent a sample of discharges rather than a sample of hospitals. This new strategy is expected to make the estimates more precise by reducing the sampling error. Starting October 1, 2015 all hospitals in the United States adopted International Classification of Diseases (ICD) 10 codes for disease classification as well as for procedures. The calendar year for 2016 and 2017 which is included in this study uses ICD 10 CM/PCS codes.

Population

Data were extracted from the NIS to identify patients ≥ 18 years of age using all listed diagnosis (primary or secondary diagnosis) of BCS from 1998 to 2017. The diagnosis of BCS was captured using the codes 453.0 (ICD-9) and I82.0 (ICD-10).

Variables

We obtained information on patient demographics (age, sex, race) and hospital characteristics (region of the country, bed-size, teaching status), patient disposition and insurance status (Medicare, Medicaid and private insurance). Study outcome included changes in inpatient mortality, LOS and total charges with time. We investigated if important complications such as acute liver failure, acute kidney injury, cirrhosis, ascites, hepatic encephalopathy, esophageal varices, portal vein thrombosis, inferior vena cava (IVC) thrombosis and spontaneous bacterial peritonitis had an impact on outcome. We also analyzed inpatient procedures such as liver biopsy, upper gastrointestinal endoscopy, paracentesis, TIPS and LT using appropriate ICD codes (Supplementary Table 1 shows the list of ICD-9 and ICD-10 codes). Severity of illness was measured using the Elixhauser comorbidity index after excluding liver diseases and this included 29 major Elixhauser comorbidity conditions[13].

Statistical analyses

Descriptive statistics are used to summarize patients' characteristics, hospital characteristics and utilization, comorbidities, complications, procedures and the outcome by using the weighted survey methods. Data are presented as mean and standard error for continuous variables, percentage and standard error for categorical variables. Standard errors of percentage or mean were estimated using Taylor series linearization method. To make inferences regarding the national estimates for the total number of BCS discharges across the study period, sample weights were applied to each admission per recommendations from the NIS. For the years from 1993-2011, AHRQ had developed discharge trend weights, specifically the NIS Trend Weight Files. Therefore, in our study for trend analyses spanning 2012 and earlier, NIS data trend weights were used to make estimates comparable to the new 2012 NIS design. We used the trend weight in place of the original discharge weights to create national estimates for trend analysis to make the data similar for the entire study period. For 2012 or later data, no trend weights were necessary and the discharge weight supplied on the NIS files were used directly[14]. We calculated BCS discharges rate per 1000000 US populations by dividing the estimated total BCS discharges by projected US population from the Census Bureau.

The annual percentage change (APC) was derived to compare the patients' characteristics, hospitals' characteristics and outcomes over time by using Poisson regression for categorical variables and linear regression with natural logarithm transformation for continuous variables. *P* value for APC was used to determine if the trends in the annual percentage change was significantly different from zero, the change was considered as statistically significant with *P* value of 0.05 or less.

The hierarchical generalized linear mixed model with hospitals as random effects was performed to evaluate the effects of potential associations between outcomes (mortality, length of stay and total charges) and patients' demographics (age, gender, and race), patient-level hospitalization variables (primary payer, disposition of patient), hospital-level variables (hospital region, bed size, location and teaching status), comorbidities, complications and procedures separately. Since race was not

available in some states, a dummy variable was created for missing data in the models to prevent the observation from being dropped. For mortality, binomial distribution and logit link was used. For length of stay, negative binomial distribution and log links were used. When analyzing the total charges, final total charges were adjusted to 2017 dollars based on medical care Consumer Price Index in US city average provided by the Bureau of Labor Statistics. We specified the models using gamma distributions and log links. A variable with *P* value of 0.05 or less was retained in the model and considered as statistically significantly associated with outcomes. All analyses were performed with SAS version 9.4 (SAS Institute, Cary, NC, United States)

RESULTS

Between 1998 and 2017, we identified a total of 8435 hospitalizations related to BCS. The mean age of the cohort was 50.5 years, 55% were women and 56 % were white. Nearly half (52%) the patients were covered by government funded health insurance (Medicare and Medicaid) (Table 1). A majority of the patients (59%) were discharged home, and an additional 13.5% were discharged with home health services. While the number of routine home discharges remained the same, there was a 3.31% increase in utilization of home health services ($P < 0.0001$) (Table 2).

Hospital mortality

Between 1998 and 2017, the in-hospital mortality was 8.74% ($n = 737$). Using the sample weights provided by HCUP, this corresponded to 3591 deaths (Table 2). Despite a significant increase in the comorbidity score during the time period, overall, in-hospital mortality rate among BCS patients decreased significantly by 4.41% per year ($P < 0.0001$) from 18% in 1998 to 8% in 2017, with the mortality rate being the lowest in 2015 (5%) (Figure 1A). There were no gender differences in mortality, but those who died were older than those were discharged from the hospital (mean age 58.7 years *vs* 49.7 years, $P < 0.001$). Of the patients who died, 53% were Caucasians, 13% were African Americans and 10% were Hispanics. Most deaths occurred in large hospitals (73%) or urban teaching hospitals (71%) (Supplementary Table 2). On multivariate analysis, older age, higher comorbidity score, acute liver failure, acute kidney injury (AKI), acute respiratory failure, hepatic encephalopathy, hepatorenal syndrome, intestinal infarct, IVC thrombosis, sepsis/septic shock and cancer were associated increased risk of mortality (Table 3).

Length of stay

The average of LOS was 8.8 days and it consistently decreased by 2% (95%CI: -2.67%, -1.41%, $P < 0.001$) per year from 12.7 d in 1998 to 7.6 d in 2017 (Figure 1B). The LOS in patients who died was longer compared to those who survived (13.54 d *vs* 8.38 d, $P < 0.0001$). On multivariate analysis primary payer, and hospital characteristics had impact on LOS. Important complications that had impact on LOS included AKI, acute liver failure, acute respiratory failure, ascites, spontaneous bacterial peritonitis, IVC thrombosis, comorbidity score and cancer (Table 4). Compared to the West, hospitals in the North East, Midwest and South had longer inpatient stays. LOS in urban teaching hospitals was significantly higher than urban non-teaching hospitals ($P < 0.0001$) (Supplementary Table 3).

Hospital costs

The average total charges after adjusted for Medical Care Consumers Price Index to 2017 dollars during the time period was $94440, and the APC increased by 1.15% (95%CI: 0.35%, 1.96%, $P = 0.005$) per year from $95515 in 1998 to $103850 in 2017 (Figure 1C). The hospital charge was higher in patients who died compared to those who survived ($190724 *vs* $85071, $P < 0.0001$). The charge was also higher in urban teaching hospitals than urban non-teaching hospitals ($P < 0.0001$). When stratified by different regions of the country, the charges were higher in the West compared to every other region in the country ($P < 0.001$, Supplementary Table 4). On multivariate analysis, race, hospital characteristics, number of procedures, length of stay, and comorbidity score were associated with total charges. Important complications that had an effect on total charges included AKI, acute respiratory failure, HRS, IVC thrombosis, cancer, and anemia due to acute blood loss (Table 5).

Table 1 Characteristics of patients and hospitals and individual effects on mortality, length of stay and total charges

Study time period	1998-2017	Individual effect (Type III test, *P* value)		
		Mortality	Length of stay	Total charges
BCS patients' characteristics				
Age	50.50 (0.19)	< 0.001	0.052	< 0.001
Female	55.19 (0.54)	0.003	0.001	< 0.001
Race		0.138	0.013	< 0.001
1: White	56.03 (0.54)			
2: Black	13.26 (0.37)			
3: Hispanic	9.56 (0.32)			
4: Asian/Pacific Islander	2.56 (0.17)			
6: Other	3.65 (0.2)			
9: Unknown	14.93 (0.39)			
Primary payer		< 0.001	0.002	< 0.001
1: Medicare	33.17 (0.52)			
2: Medicaid	18.42 (0.42)			
3: Private insurance	40.20 (0.54)			
6: Other	8.21 (0.3)			
Hospital characteristics				
Hospital size		0.014	< 0.001	< 0.001
1: Small	9.81 (0.32)			
2: Medium	18.92 (0.43)			
3: Large	71.27 (0.49)			
Hospital location and teaching status		0.195	< 0.001	< 0.001
1: Rural	6.89 (0.28)			
2: Urban nonteaching	24.24 (0.47)			
3: Urban teaching	68.87 (0.51)			
Hospital region		0.533	0.010	< 0.001
1: Northeast	21.84 (0.45)			
2: Midwest	22.15 (0.46)			
3: South	33.45 (0.52)			
4: West	22.57 (0.46)			
Clinical characteristics				
Ascites	29.93 (0.5)	< 0.001	< 0.001	< 0.001
Acute kidney injury	18.84 (0.43)	< 0.001	< 0.001	< 0.001
Hepatic cirrhosis with no mention of alcohol	18.65 (0.43)	0.901	0.031	0.838
Cancer	17.26 (0.41)	< 0.001	0.002	0.010
Portal hypertension	16.57 (0.41)	0.029	0.898	0.000
Hepatic encephalopathy	9.59 (0.32)	< 0.001	< 0.001	< 0.001
Portal vein thrombosis	7.92 (0.3)	0.006	0.372	0.073
Esophageal varices without bleeding	7.44 (0.29)	0.002	0.324	0.091
Acute respiratory Failure	7.03 (0.28)	< 0.001	< 0.001	< 0.001
HCC	6.93 (0.28)	< 0.001	< 0.001	0.543

Acute blood loss anemia/hemorrhagic	6.62 (0.27)	0.008	< 0.001	< 0.001
IVC thrombosis	6.39 (0.27)	< 0.001	< 0.001	< 0.001
Sepsis	6.10 (0.26)	< 0.001	< 0.001	< 0.001
Alcoholic cirrhosis	5.73 (0.25)	0.113	0.731	0.140
Acute liver failure	5.60 (0.25)	< 0.001	< 0.001	< 0.001
Hepatorenal syndrome	3.29 (0.2)	< 0.001	< 0.001	< 0.001
Variceal bleeding	3.20 (0.19)	0.107	0.160	0.001
Spontaneous bacterial peritonitis	2.83 (0.18)	< 0.001	< 0.001	< 0.001
Intestinal infarct/acute vascular insufficiency	2.11 (0.16)	< 0.001	< 0.001	< 0.001
Elixhauser Comoridity Score excluding liver disease	9.38 (0.12)	< 0.001	< 0.001	< 0.001

All data are presented as percentage (SE) for categorical variables and mean (SE) for continuous variables. BCS: Budd Chiari syndrome; HCC: Hepatocellular carcinoma IVC: Inferior vena cava.

Utilization of procedures

During their in-patient stay, patients underwent an average of 2.64 procedures per hospitalization. Paracentesis was the most frequent procedure (18.4%) followed by upper gastrointestinal endoscopy (10.9%), liver biopsy (6.2%), TIPS (3.6%) and LT (1.9%) (Table 2). Subgroup analysis showed that out of the 307 patients who underwent TIPS, 145 (47%) had LT.

While total number of procedures performed remained stable during the study period, there was a significant and notable reduction in the number of liver biopsies (APC: -4.01%, 95%CI: -5.42%, -2.58%, $P < 0.0001$), TIPS (APC: -4.95%, 95%CI: -6.78%, -3.09%, $P < 0.0001$) and LT (APC: -2.68%, 95%CI: -5.26%, -0.02%, $P = 0.05$). Hispanics underwent more procedures than Caucasians ($P < 0.001$) and Blacks ($P < 0.001$). Patients admitted to urban teaching hospitals underwent more procedures than urban non-teaching hospitals ($P < 0.0001$) and rural hospitals ($P < 0.0001$) (Supplementary Table 5).

DISCUSSION

In this large population-based study from the United States, we found that the overall in-patient mortality rate for an unselected group of patients with BCS was 8%. The mortality rates and LOS reduced significantly from 1998 to 2017, but total hospital charges, however, increased during the study period. The patients who survived hospitalization were younger than those who died (49.7 years *vs* 58.6 years), but race, hospital teaching status and hospital region did not impact survival. The reduction in mortality was multifactorial and possibly could be related to earlier detection of BCS, advances in therapeutic options and a better overall inpatient care.

To our knowledge, there are no prior studies that have exclusively analyzed inpatient mortality secondary to BCS, but multicenter studies in the recent era that investigated prognosis of BCS have reported improvement in survival rates with time [9,10]. A European study that consisted of 157 BCS patients, who were managed using a stepwise treatment algorithm over a median duration of 50 mo reported a mortality of 23%[9]. A majority of these patients succumbed to liver failure (33%) and the median time to death for the cohort was 10 mo. The study found that age, bilirubin and creatinine were independent risk factors for survival. Most patients (88.5%) in their study were on long term anticoagulation and those who did not respond to medical management were treated with percutaneous angioplasty/thrombolysis (n = 22), TIPS (n = 62) and LT (n = 20) in a step wise manner. Due to inherent limitations of the NIS dataset we were unable to determine how many patients in our study were on anticoagulation.

Overall, less than 5% of the patients underwent invasive procedures such as TIPS and LT. There were no significant differences in mortality between patients who underwent these procedures and those who did not. However, 89% of patients who underwent TIPS and 92% who had LT during their inpatient stay survived hospitalization. We also noticed a downward trend in the number of TIPS and LT in hospitalized BCS patients, perhaps because these procedures were done after patients were

Table 2 Trends in outcomes of interest

	1998-2017 (unweighted: 8435, weighted: 41119)	1998 (unweighted: 262, weighted: 1367)	2017 (unweighted: 680, weighted: 3400)	APC (95%CI)	*P* value for APC
Procedures					
Number of procedures	2.64 (0.03)	3.09 (0.20)	2.42 (0.13)	-0.51% (-1.09%, 0.06%)	0.082
Paracentesis	18.41 (0.42)	28.56 (2.82)	16.47 (1.42)	-1.67% (-2.53%, -0.81%)	0.000
Upper endoscopy	10.94 (0.34)	13.08 (2.06)	11.91 (1.24)	-0.17% (-1.31%, 0.97%)	0.766
Liver biopsy	6.24 (0.26)	10.35 (1.9)	5.15 (0.85)	-4.01% (-5.42%, -2.58%)	< 0.0001
Portosystemic shunt/TIPS	3.63 (0.2)	6.12 (1.54)	2.94 (0.65)	-4.95% (-6.78%, -3.09%)	< 0.0001
Liver transplantation	1.9 (0.15)	1.29 (0.75)	2.06 (0.54)	-2.68% (-5.26%, -0.02%)	0.048
Disposition of patient					
1: Discharged to home or selfcare	58.8(0.54)	50.71 (3.12)	55.96 (1.91)	-0.28% (-0.77%, 0.21%)	0.262
6: Home health care	13.49 (0.37)	12.04 (2.04)	17.23 (1.45)	3.31% (2.20%, 4.43%)	< 0.0001
5: Transfer: other type of facility	11.12 (0.34)	10.21 (1.89)	12.08 (1.25)	1.87% (0.70%, 3.06%)	0.002
20: Died in hospital	8.74 (0.31)	18.17 (2.44)	7.66 (1.02)	-4.31% (-5.50%, -3.10%)	< 0.0001
2: Transfer: short-term hospital	6.8 (0.28)	8.25 (1.71)	5.6 (0.88)	-1.26% (-2.66%, 0.17%)	0.084
7: Against medical advice	1 (0.11)	0.61 (0.43)	1.47 (0.46)	2.94% (-1.02%, 7.07%)	0.148
Outcomes					
Number of deaths	737 (Unweighted); 3591 (Weighted)	46 (Unweighted); 249 (Weighted)	52 (Unweighted); 260 (Weighted)		
Mortality rate per 1000000 United States populations		0.9	0.8	-0.29% (-0.86%, 0.27%)	0.309
Mortality rate per 1000000 inpatients		8.87	8.55	0.34% (-0.23%, 0.92%)	0.243
Mortality rate among BCS inpatients	0.09	0.18	0.08	-4.41% (-4.95%, -3.88%)	< 0.0001
Length of stay (d)	8.84 (0.13)	12.73 (1.01)	7.64 (0.36)	-2.04% (-2.67%, -1.41%)	< 0.0001
Average total charges in 2017 dollars	94440.04 (1996.06)	95515.01 (9483.24)	103850.98 (8183.79)	1.15% (0.35%, 1.96%)	0.005

All data are presented as percentage (SE) for categorical variables and mean (SE) for continuous variables. Annual percentage change (APC) > 0 means increasing, < 0 means decreasing. *P* value for APC measures if APC is significantly different from zero. *P* value ≤ 0.05 means the change is significant. BCS: Budd Chiari syndrome; APC: Annual percentage change.

discharged and hence was not captured by the NIS database. Nearly half (47%) the patients who had TIPS underwent LT, and it possible that TIPS was done in these patients as a bridge to LT, or perhaps they had more complications such as variceal bleeding or refractory ascites.

A management strategy that consists of a stepwise invasive treatment algorithm guided by response to prior treatment have resulted in better short- and long-term outcome in BCS patients[3,9-12,15]. This consists of early and prompt initiation of anticoagulation with low molecular weight heparin to prevent extension of thrombosis, referral to a hematologist for treatment of specific underlying clotting disorders and treatment of portal hypertension related complications. Patients who

Table 3 Multivariate model on mortality

	Response	Beta estimate	Standard error	*P* value for beta	Odds ratio (95%CI)
Age		0.024	0.003	< 0.0001	1.024 (1.019, 1.029)
Acute respiratory Failure	Yes (reference = No)	1.652	0.109	< 0.0001	5.219 (4.211, 6.468)
Intestinal infarct/acute vascular insufficiency	Yes (reference = No)	1.422	0.201	< 0.0001	4.143 (2.795, 6.142)
Acute liver failure	Yes (reference = No)	1.286	0.119	< 0.0001	3.617 (2.864, 4.567)
Hepatorenal syndrome	Yes (reference = No)	1.123	0.147	< 0.0001	3.072 (2.302, 4.101)
Cancer	Yes (reference = No)	0.882	0.098	< 0.0001	2.415 (1.993, 2.927)
Acute kidney injury	Yes (reference = No)	0.803	0.092	< 0.0001	2.232 (1.862, 2.675)
Sepsis/severe sepsis/septic shock	Yes (reference = No)	0.635	0.122	< 0.0001	1.886 (1.484, 2.398)
Hepatic encephalopathy	Yes (reference = No)	0.280	0.117	0.020	1.323 (1.052, 1.662)
Elixhauser Comorbidity Score excluding liver disease		0.026	0.004	< 0.0001	1.027 (1.019, 1.034)

deteriorate despite optimal medical management are considered for percutaneous or transhepatic angioplasty, TIPS and/or LT. The NIS data set did not include data on venoplasty or stenting perhaps because many of these procedures are done in the outpatient setting. Several studies have reported excellent outcome following LT in patients with BCS. In a previous study, using United Network of Organ Sharing (UNOS) datasets, we had reported 85% 3-year survival in patients with BCS who underwent LT in the United States[16]. Our group recently analyzed outcome of LT in 55 BCS patients who presented with fulminant hepatic failure using the UNOS database and found that expeditious LT in this subset of patients was associated with excellent long-term patient and graft survival. We also found that despite the presence of 3 or more organ failures, LT in these patients was associated with good outcome. They also achieved excellent post LT functional status as determined by the Karnofsky performance status scores[17]. A European series that investigated outcome of LT in 248 patients report actuarial survival of 76% at 1 year, 71% at 5 years and 68% at 10 years, with majority of the deaths occurring in the first 3 mo[18].

In our study we found that the average LOS was 9 d and this reduced consistently with an APC of 2% during the 19-year period. The reduction is consistent with nationwide efforts to reduce LOS for hospitalized patients. Multivariate analysis showed significant association between LOS and complications such as AKI, acute liver failure, acute respiratory failure, SBP and IVC thrombosis. The LOS was longer in medium and large sized hospitals compared to smaller hospitals probably because these hospitals were tertiary care centers and BCS patients admitted in those hospitals were perhaps more sicker requiring prolonged inpatient stay. This would also explain why urban hospitals had a longer LOS compared to hospitals in rural areas. Longer LOS in such hospitals was associated with higher total charges as expected. We also noticed a geographical variation in the LOS, as hospitals in the North East, Midwest and South had longer inpatient stays compared to the West. Although it is difficult to explain this particular finding, a similar observation was made by the HCUP report on US hospital LOS variation by region in 2016 and could be related to physician practice patterns, access to health care services, treatment preferences and cost of living that varies by geographic location in a diverse country like United States[19].

The average total costs for BCS hospitalizations between 1998 and 2017 was $94440 and this continued to show a significant upward trend. We found that compared to the West, hospitals in the Northeast, Midwest and South of United States had lower total charges. We do not have a good explanation for this finding. The increasing financial burden of BCS hospitalizations to the US health care system in our study, despite a reduction in the average LOS, is consistent with other studies that have analyzed the economic impact of hospitalizations related to decompensated cirrhosis and can be attributed to the increasing hospitalization rate as well as increasing severity of disease burden as indicated by comorbidity score[20,21].

Our study has a few limitations most of which are inherent to the use of a large administrative database. The use of ICD codes to capture the diagnosis of BCS could result in coding errors potentially resulting in misclassification. We could not perform a sensitivity analysis because of the absence of patient identifiers in the datasets. Another major shortcoming is that the NIS reports every hospitalization as a separate

Table 4 Multivariate model on length of stay

	Response	Beta estimate	Standard error	*P* value for Beta	*P* value for type 3 test
Primary payer	1: Medicare (reference)	0.000	-	-	0.022
	2: Medicaid	0.053	0.037	0.144	
	3: Private insurance	0.084	0.030	0.005	
Hospital bed size	1: Small (reference)	0.000	-	-	< 0.0001
	2: Medium	0.113	0.049	0.021	
	3: Large	0.293	0.042	<.0001	
Hospital location and teaching status	1: Rural (reference)	0.000	-	-	< 0.0001
	2: Urban nonteaching	0.206	0.054	<.0001	
	3: Urban teaching	0.433	0.050	<.0001	
Hospital region	1: Northeast	0.171	0.038	<.0001	< 0.0001
	2: Midwest	0.017	0.038	<.0001	
	3: South	0.055	0.034	<.0001	
	4: West (reference)	0.000			
Complications					
Acute liver failure	Yes (reference = No)	0.223	0.057	< 0.0001	< 0.0001
Acute respiratory Failure	Yes (reference = No)	0.380	0.052	< 0.0001	< 0.0001
Acute kidney injury	Yes (reference = No)	0.255	0.035	< 0.0001	< 0.0001
Ascites	Yes (reference = No)	0.118	0.028	< 0.0001	< 0.0001
Spontaneous bacterial peritonitis	Yes (reference = No)	0.480	0.076	< 0.0001	< 0.0001
IVC thrombosis	Yes (reference = No)	0.138	0.052	0.008	0.008
Intestinal infarct/acute vascular insufficiency	Yes (reference = No)	0.383	0.088	< 0.0001	< 0.0001
cancer	Yes (reference = No)	-0.278	0.036	< 0.0001	< 0.0001
Elixhauser Comorbidity Score excluding liver disease		0.019	0.001	< 0.0001	< 0.0001

IVC: Inferior vena cava.

encounter and not as a unique patient. It is possible that many of these patients were readmitted and were counted more than once. We were also unable to obtain information regarding therapeutic data with respect to anticoagulation and specific pharmacological agents used to treat underlying thrombophilia. Nonetheless, the NIS database is considered to be a powerful research tool providing robust clinical data about real world scenarios and its reliability has been extensively validated[22].

CONCLUSION

In conclusion, this is the first study from the United States to illustrate reducing mortality related to BCS hospitalizations as well as a reduction in the average LOS. While these findings are reassuring, BCS continues to have a significant economic impact as indicated by the rising healthcare costs.

Table 5 Multivariate model on total charges

	Response	Estimate	Standard error	*P* value for beta	*P* value for type 3 test
Race	1: White (reference)	0.000	-	-	< 0.0001
	2: Black	-0.037	0.026	0.162	
	3: Hispanic	0.015	0.031	0.613	
	4: Asian/Pacific Islander	0.136	0.058	0.019	
	6: Other	0.082	0.046	0.077	
	9: Unknown	-0.185	0.025	< 0.0001	
Hospital bed size	1: Small (reference)	0.000	-	-	< 0.0001
	2: Medium	0.080	0.034	0.018	
	3: Large	0.169	0.029	< 0.0001	
Hospital location and teaching status	1: Rural (reference)	0.000	-	-	< 0.0001
	2: Urban nonteaching	0.428	0.037	< 0.0001	
	3: Urban teaching	0.552	0.034	< 0.0001	
Hospital region	1: Northeast	-0.195	0.027	< 0.0001	< 0.0001
	2: Midwest	-0.333	0.027	< 0.0001	
	3: South	-0.330	0.024	< 0.0001	
	4: West (reference)	0.000	-	-	
Complications					
Acute liver failure	Yes (reference = No)	0.078	0.039	0.044	0.044
Acute respiratory Failure	Yes (reference = No)	0.204	0.036	< 0.0001	< 0.0001
Acute kidney injury	Yes (reference = No)	0.146	0.025	< 0.0001	< 0.0001
Hepatorenal syndrome	Yes (reference = No)	-0.132	0.050	0.008	0.009
IVC thrombosis	Yes (reference = No)	0.075	0.035	0.035	0.035
Acute blood loss anemia/ hemorrhagic	Yes (reference = No)	0.155	0.035	< 0.0001	< 0.0001
Cancer	Yes (reference = No)	-0.052	0.025	0.037	0.037
Elixhauser Comoridity Score excluding liver disease		0.005	0.001	< 0.0001	< 0.0001
Other variables					
Number of procedures		0.118	0.004	< 0.0001	< 0.0001
Length of stay		0.054	0.001	< 0.0001	< 0.0001

IVC: Inferior vena cava.

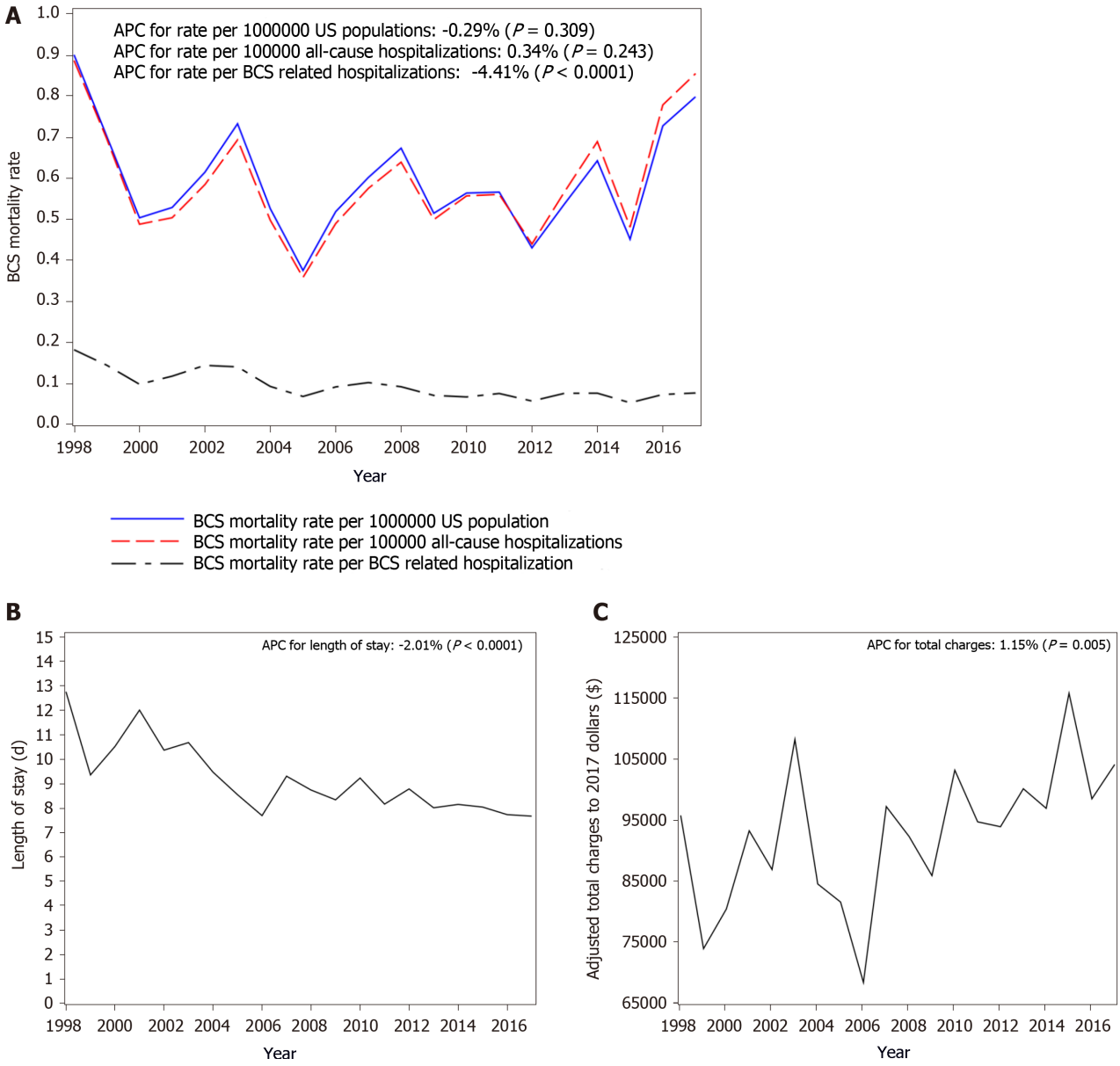

Figure 1 Annual percentage changes. A: Annual percentage change for mortality in patients with Budd Chiari syndrome (BCS) (per 1000000 United States population, per 100000 all cause hospitalizations and BCS related hospitalizations; B: Length of hospital stay for patients with BCS from 1998 to 2017; C: Adjusted (charges adjusted to 2017 dollars) hospital charges from 1998 to 2017 in patients with BCS. APC: Annual percentage change; BCS: Budd Chiari syndrome; US: United States.

ARTICLE HIGHLIGHTS

Research background

The Budd Chiari syndrome (BCS) is a rare disorder that results from partial or complete obstruction of the hepatic venous outflow in the absence of right heart failure.

Research motivation

There is a paucity of data on the in-hospital mortality of BCS as well its economic impact on the United States health care system.

Research objectives

This study aimed to evaluate trends in mortality, length of hospital stays and resource utilization among inpatients with BCS.

Research methods

Retrospective study where data were extracted from the National Inpatient Sample

(NIS) from 1998 to 2017. To make inferences regarding the national estimates for the total number of BCS discharges across the study period, sample weights were applied to each admission per recommendations from the NIS.

Research results

During the study period, there were 3591 (8.73%) in-patient deaths. The overall in-hospital mortality rate among BCS patients decreased from 18% in 1998 to 8% in 2017; the mortality decreased by 4.41% every year. The average of length of stay was 8.8 d and it consistently decreased by 2.04% from 12.7 d in 1998 to 7.6 d in 2017.The average total charges during the time period was $94440 and the annual percentage change increased by 1.15%

Research conclusions

The in-hospital mortality rate for patients admitted with BCS in the United States has reduced between 1998 and 2017 while total charges continued to increase.

Research perspectives

Using a large national database, we analyzed the mortality and socioeconomic impact of BCS hospitalizations in the United States with a high degree of granularity.

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ORIGINAL ARTICLE

Retrospective Study

Comparison of unenhanced magnetic resonance imaging and ultrasound in detecting very small hepatocellular carcinoma

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Author contributions: Tarao K summarized the data and wrote the paper; Nozaki A, Komatsu H, Komatsu T, Tanaka K, Chuma M, Numata K, and Maeda S followed up the patients; Taguri M conducted statistical analysis; Yoshida T and Koyasu H made suggestions regarding the MRI technique.

Institutional review board statement: The study was reviewed and approved by the Ethics Committee of Yokohama Municipal Citizen's Hospital Institutional Review Board (Approval No. 21-02-01).

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Abstract

BACKGROUND

In hepatocellular carcinoma (HCC), detection and treatment prior to growth beyond 2 cm are important as a larger tumor size is more frequently associated with microvascular invasion and/or satellites. In the surveillance of very small HCC nodules (≤ 2 cm in maximum diameter, Barcelona clinical stage 0), we demonstrated that the tumor markers alpha-fetoprotein and PIVKA-II are not so useful. Therefore, we must survey with imaging modalities. The superiority of magnetic resonance imaging (MRI) over ultrasound (US) to detect HCC was confirmed in many studies. Although enhanced MRI is now performed to

study used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

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accurately diagnose HCC, in conventional clinical practice for HCC surveillance in liver diseases, unenhanced MRI is widely performed throughout the world. While, MRI has made marked improvements in recent years.

AIM

To make a comparison of unenhanced MRI and US in detecting very small HCC that was examined in the last ten years in patients in whom MRI and US examinations were performed nearly simultaneously.

METHODS

In 394 patients with very small HCC nodules, those who underwent MRI and US at nearly the same time (on the same day whenever possible or at least within 14 days of one another) at the first diagnosis of HCC were selected. The detection rate of HCC with unenhanced MRI was investigated and compared with that of unenhanced US.

RESULTS

The sensitivity of unenhanced MRI for detecting very small HCC was 95.1% (97/102, 95% confidence interval: 90.9-99.3) and that of unenhanced US was 69.6% (71/102, 95% confidence interval: 60.7-78.5). The sensitivity of unenhanced MRI for detecting very small HCC was significantly higher than that of unenhanced US ($P < 0.001$). Regarding the location of HCC in the liver in patients in whom detection by US was unsuccessful, $S_{7\text{-}8}$ was identified in 51.7%.

CONCLUSION

Currently, unenhanced MRI is a very useful tool for the surveillance of very small HCC in conventional clinical follow-up practice.

Key Words: Comparison of magnetic resonance imaging and ultrasound; Surveillance of very small hepatocellular carcinoma; Magnetic resonance imaging; Ultrasound; Unenhanced magnetic resonance imaging

Core Tip: Recent technological development of magnetic resonance imaging (MRI) scanners has been excellent. The 3.0-tesla (T) MR scanner with a higher field strength has been increasingly used because improved lesion detection can be expected as a result of the increased signal-to-noise ratio, which is theoretically twice with 3.0-T compared with 1.5-T. Another important improvement in MRI is the practical use of diffusion-weighted imaging. In this study, a comparison of unenhanced MRI and ultrasound in detecting very small hepatocellular carcinoma (2 cm in maximum diameter) was made. The sensitivity of unenhanced MRI for detecting very small hepatocellular carcinoma was as high as 95.1% as compared with 69.6% of unenhanced ultrasound ($P < 0.001$).

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INTRODUCTION

If hepatocellular carcinoma (HCC) tumors are growing up to more than 2 cm in diameter, they are often associated with microvascular invasion and/or satellites, which are major predictors of recurrence after initial effective treatments[1]. The same tendency was observed by Stravitz *et al*[2], who reported that the early detection of HCC improves the prognosis. Therefore, we must identify very small HCC nodules (≤

2 cm in maximum diameter) in the surveillance of HCC.

Recently, we demonstrated that more than one third of patients with very small HCC nodules were dropped from surveillance using the tumor markers alpha-fetoprotein (AFP) and PIVKA-II[3]. Therefore, we must survey patients with liver diseases using imaging modalities.

Surveillance of HCC in liver diseases, especially in liver cirrhosis, has been conducted by ultrasound (US) or magnetic resonance imaging (MRI) throughout the world.

Although US was performed more popularly than MRI in the surveillance of HCC, the superiority of MRI over US has been demonstrated in many studies since 2001-2003[4,5]. Although enhanced MRI is now performed for the accurate diagnosis of HCC[5-9], in conventional clinical practice for HCC surveillance in liver diseases, unenhanced MRI is widely performed throughout the world. On the other hand, MRI has made much progress in recent years.

In this study, a comparison of unenhanced MRI and US in surveying very small HCC was made. In order to conduct precise evaluation, we selected patients in whom MRI and US were performed at about the same time.

MATERIALS AND METHODS

Study population

This was a retrospective observational study that included 403 patients with small single HCC nodules (≤ 2 cm in maximum diameter, Barcelona clinical stage 0) who visited the following three hospitals and one clinic in Yokohama City for the first time between January 2008 and September 2020: Gastroenterological Center, Medical Center, Yokohama City University; Department of Gastroenterology, Yokohama Municipal Citizen's Hospital; Department of Gastroenterology, National Hospital Organization, Yokohama Medical Center; and Tarao's Gastroenterological Clinic. Of the 403 patients with very small HCC, 102 were selected in whom MRI and US were conducted simultaneously (on the same day or at least within 14 days of one another) (Figure 1). In this series of the study, MRI and US were performed in unenhanced states because we wanted to study the usefulness to survey HCC in routine follow-up study. In the unenhanced MRI, a very small HCC usually appears as a dark spot in T_1 image and light white spot in T_2 image (see Figures 2-5). It is important that characteristics of both T_1 and T_2 images were present at the same time. In the US images, it usually appears as a dark round spot.

HCCs were diagnosed chiefly by dynamic computed tomography (CT) and abdominal angiography, which showed early enhancement and early washout. This work was performed in accordance with the Declaration of Helsinki.

Previously diagnosed HCC was excluded from the protocol. This study was performed after approval by the respective institutional review boards.

The patients were classified according to the etiologies of liver diseases (Table 1).

HCC detection

The diagnosis of HCC was confirmed by US, MRI, CT, enhanced dynamic CT, and abdominal angiography. All patients underwent abdominal angiography to confirm the single nodules. The maximum diameter of the HCC nodules was scaled by US or MRI.

Helical dynamic CT and abdominal angiography were performed in almost all patients except those with hypersensitivity to iodine and advanced kidney disease. In the helical dynamic CT, an intravenous bolus injection of contrast material and sequential scanning were performed, and an intense homogenous arterial phase (early enhancement) and early washout in the venous phase were considered to be characteristic of HCC[10-12]. Abdominal angiography was also performed to exclude the benign nodular lesions and exclude HCC patients with macrovascular invasion. Of course, the characteristic features of very small HCC in unenhanced MRI as mentioned above were taken into account.

Patients with macrovascular invasion or extrahepatic metastasis were excluded. In patients undergoing hepatectomy, the final decision on HCC was made by pathological diagnosis, and cases of benign nodules were excluded.

Statistical analysis

We calculated the detection rate and its 95% confidence interval (CI) for each method. We then compared the detection rates between MRI and US using McNemar's test.

Table 1 Background of hepatocellular carcinoma patients (≤ 2 cm in diameter) who underwent unenhanced magnetic resonance imaging and unenhanced ultrasound simultaneously

Background of patients	
Number of patients	102
Age (yr)	72.4 ± 9.6
Sex (%)	
Male	52 (51.0)
Female	50 (49.0)
Etiology (%)	
HBV	13 (12.9)
HCV	61 (60.3)
Alcohol	14 (13.9)
NBNC	7 (6.9)
Autoimmune	2 (2.0)
NASH	2 (2.0)
PBC	1 (1.0)
Others	2 (2.0)

HBV: Hepatitis B virus; HCV: Hepatitis C virus; NBNC: Non-B non-C; NASH: Nonalcoholic steatohepatitis; PBC: Primary biliary cirrhosis.

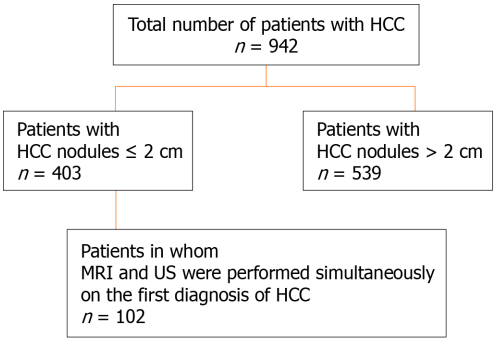

Figure 1 Patient selection. HCC: Hepatocellular carcinoma; MRI: Magnetic resonance imaging; US: Ultrasound.

RESULTS

The sensitivity of unenhanced MRI for detecting very small HCC (≤ 2 cm in diameter) was 95.1% [97/102, 95%CI: 90.9-99.3] and that of unenhanced US was 69.6% (71/102, 95%CI: 60.7-78.5) ($P < 0.001$).

Table 2 shows the location of the HCC in the liver of patients in whom detection by US was unsuccessful. $S_{7\text{-}8}$ was the site in 51.7% of these patients. Thus, HCC lesions in $S_{7\text{-}8}$ may be difficult to identify by US. Representative images of four cases of very small HCC (A, B, C, and D) by unenhanced MRI are shown in Figures 2-5. In all the four cases, HCC was confirmed using hepatectomized specimens.

Moreover, the treatment methods for 102 HCC patients are shown in Table 3.

DISCUSSION

For the surveillance of very small HCC, US was hitherto performed worldwide. However, in recent years, the superiority of MRI over US to detect very small HCC has been reported in many articles.

Table 2 Location of hepatocellular carcinoma in the liver in patients for whom detection by ultrasound was unsuccessful

Location in the liver	Number of patients (%)
S_{1-4}	6 (20.7)
S_{5-6}	8 (27.6)
S_{7-8}	15 (51.7)

Table 3 Treatment methods for hepatocellular carcinoma in 102 very small hepatocellular carcinoma patients

Therapy	Number of treated patients
Hepatectomy	19
RFA	58
TACE	14
TACE + RFA	2
TAI	1
Chemotherapy	2
BSC	3
Others	3

BSC: Best supportive care; RFA: Radiofrequency ablation; TACE: Transcatheter arterial chemoembolization; TAI: Transcatheter arterial infusion.

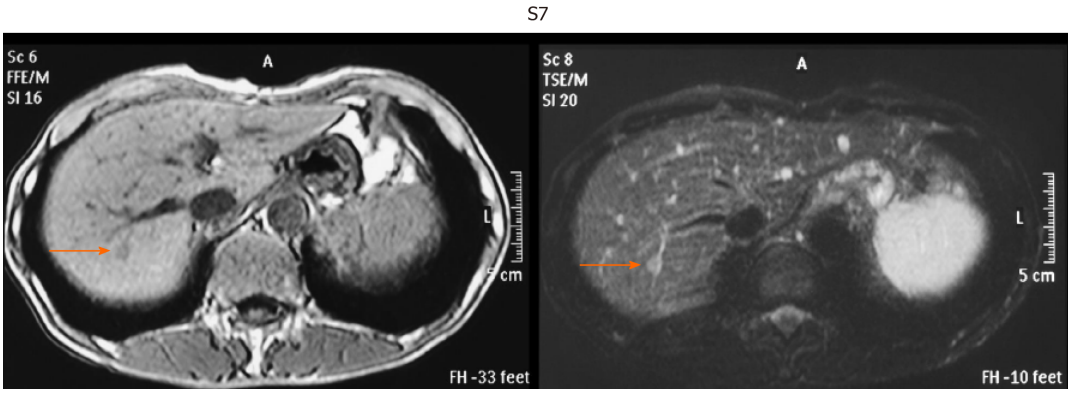

Figure 2 Representative image of very small hepatocellular carcinoma by unenhanced magnetic resonance imaging. Hepatocellular carcinoma in S7 segment. T1-weighted image (left, light dark spot). T2-weighted image (right, light white spot).

Colli *et al*[4] conducted a systemic review on this issue, and found that the pooled estimate of 14 US studies was 60.5% (95%CI: 44-76) for sensitivity[13-25], and that of 9 MRI studies was 80.6% (95%CI: 70-91) for sensitivity[9,23,24,26-31]. The difference in sensitivity between US and MRI may be due to the fact that MRI is less influenced by the operator's technique, patient's body type, and location of HCC lesions.

More recently, in 2017, Kim *et al*[5] compared MRI and US in a cohort of 407 patients with cirrhosis who underwent 1100 surveillance examinations, and found that MRI had a sensitivity of 83.7% (95%CI: 69.7-92.2) for early HCC detection, which was significantly higher than that of US (25.6%, 95%CI: 14.8-49.4).

We demonstrated in this study that 95% of cases with very small HCC can be detected by unenhanced MRI. This figure is very high compared with previous reports published between 2001 and 2003 concerning the sensitivity of unenhanced MRI for detecting very small HCC. Table 4 shows the reported sensitivity of unenhanced MRI for detecting very small HCC between 2001 and 2003 when MRI used 1.5-tesla (T) imaging. The average sensitivity in that period was 60.3% (95%CI: 52.2-68.4)[25,27,28,30,31].

Table 4 Reported sensitivity of unenhanced magnetic resonance imaging to detect very small hepatocellular carcinomas (≤ 2 cm in diameter) between 2001 and 2003

Ref.	Sensitivity (%)
Krinsky *et al*[27], 2001	7/15 (46.7)
de Lédinghen *et al*[28], 2002	33/54 (61.1)
Libbrecht *et al*[25], 2002	7/10 (70.0)
Bhartia *et al*[30], 2003	15/21 (71.4)
Burrel *et al*[31], 2003	23/41 (56.1)
Pooled estimates	85/141 (60.3)
	95%CI: 52.2-68.4

CI: Confidence interval.

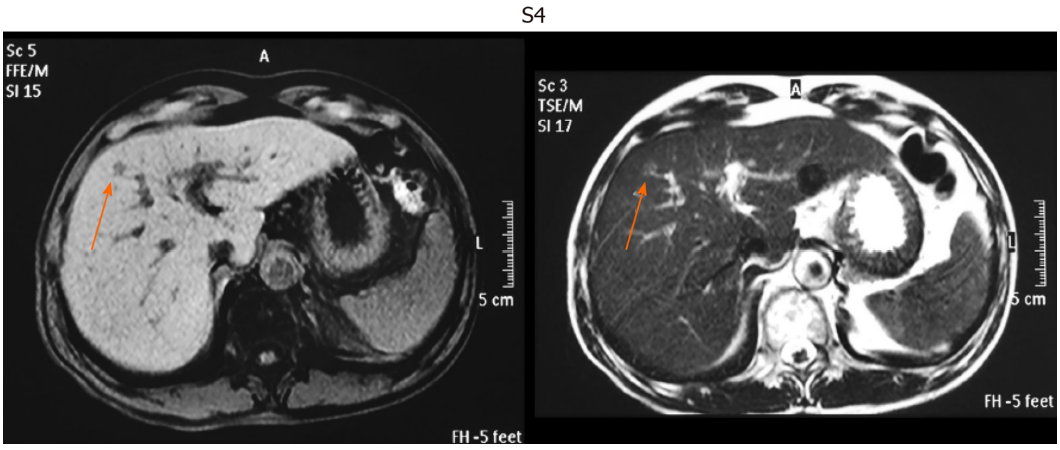

Figure 3 Representative image of very small hepatocellular carcinoma by unenhanced magnetic resonance imaging. Hepatocellular carcinoma in S4 segment. T1-weighted image (left, light dark spot). T2-weighted image (right, light white spot).

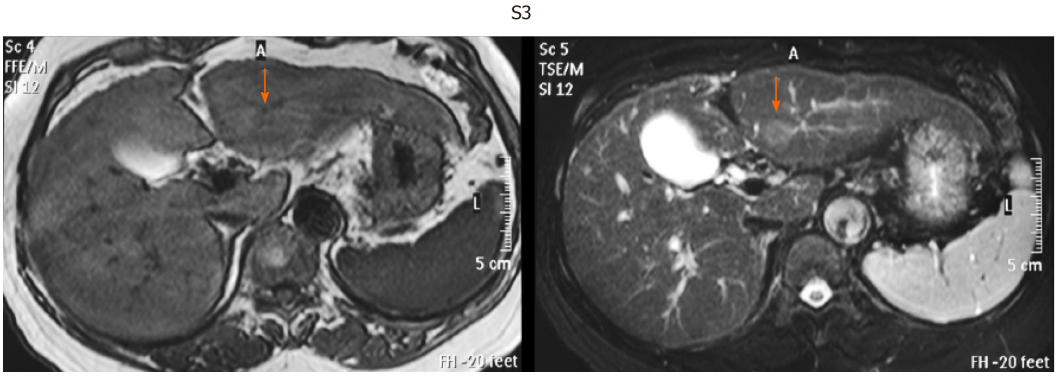

Figure 4 Representative image of very small hepatocellular carcinoma by unenhanced magnetic resonance imaging. Hepatocellular carcinoma in S3 segment. T1-weighted image (left, light dark spot). T2-weighted image (right, light white spot).

The reasons why this marked improvement appeared in the sensitivity of unenhanced MRI with regard to detecting very small HCC must be considered.

First of all, MRI has made marked progress in its ability in recent years. Recent technological development of MRI scanners has allowed high-quality multiphasic imaging of the entire liver. Since 2003-2005, the 3.0-T magnetic resonance (MR) scanner with a higher field strength has been increasingly used because improved lesion detection can be expected as a result of the increased signal-to-noise ratio (SNR), which is theoretically twice the SNR at 1.5-T[32,33]. Indeed, it was demonstrated that

S8

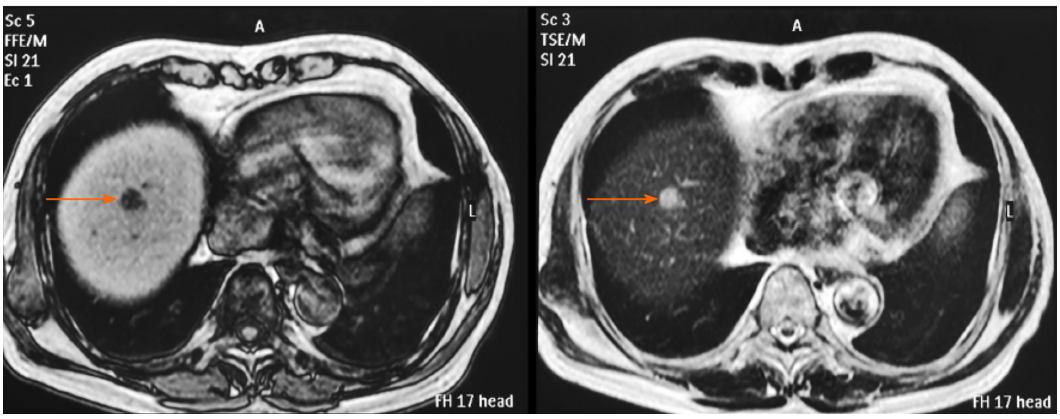

Figure 5 Representative image of very small hepatocellular carcinoma by unenhanced magnetic resonance imaging. Hepatocellular carcinoma in S8 segment. T1-weighted image (left, light dark spot). T2-weighted image (right, light white spot).

3.0-T images were superior to 1.5 T images for detecting hepatic metastases[34]. Previous misdiagnoses of HCC on MRI maybe have been due to poor patient compliance, especially the inability to suspend respiration. These problems can be resolved by the new advancements mentioned above to develop faster and motion-robust sequences.

Another important improvement of MRI is the practical use of diffusion-weighted imaging. Indeed, it was demonstrated that the sensitivity of detecting pancreatic cancer rose with the use of diffusion-weighted imaging[35].

On the other hand, the sensitivity of unenhanced US in our study for detecting very small HCC was 69.6%, which was nearly the same as those in previous reports[9,22,24,26-29]. One of the reasons for the inferiority of US may be the location of HCC in the liver. A lesion located at $S_{7\text{-}8}$ (the most frequent HCC lesion in the liver) may be difficult to identify by US.

Our present study indicates the importance of unenhanced MRI in detecting very small HCC, because more than one third of these patients were dropped from surveillance by tumor markers AFP and PVKA-II. However, there are two limitations of unenhanced MRI. First, it is more expensive than US. Second, in case of very tiny HCC (3-5 mm), it is difficult to find HCC by unenhanced MRI.

CONCLUSION

Considering the above-mentioned facts, unenhanced MRI is a very useful tool for detecting very small HCC in the conventional follow-up of patients with liver diseases, especially liver cirrhosis.

ARTICLE HIGHLIGHTS

Research background

Nowadays advancement of magnetic resonance imaging (MRI) has markedly improved the quality of liver imaging. We believe that a high-speed scan and diffusion-weighted imaging are two major factors that have contributed to the improved detection of hepatocellular carcinomas (HCCs). In early MRI, a respiration artifact was the most troublesome factor deteriorating the quality of images of the liver. A high-speed scan brought by the conversion from 1.5-tesla (T) to 3.0-T facilitates whole-liver MRI while patients hold their breath. Breath-holding scans reduce motion and misregistration artifacts, and create high-quality liver images. In addition, the practical use of diffusion-weighted imaging has contributed to the detection of cell-rich lesions. Tumors are proper objects of these sequences. There is a report (or several reports) that the sensitivity of detecting pancreatic cancer rose with the use of diffusion-weighted imaging. We believe that the same can be applied to detect HCC. Currently, dynamic MRI with contrast media is considered the standard procedure to diagnose HCC. However, with improved images, non-contrasted liver MRI is still a

useful modality to detect HCCs.

Research motivation

Previous reports in 2001-2003 stated that the sensitivity of unenhanced MRI to detect very small HCC (≤ 2 cm in diameter) was about 60%. Since then, there have been few reports on the sensitivity to detect very small HCC, especially in recent years.

Research objectives

Surveillance of HCC in liver diseases, especially in liver cirrhosis, has been conducted by ultrasound (US) or MRI throughout the world. Although US was performed more popularly than MRI in the surveillance of HCC, the superiority of MRI over US has been demonstrated in many studies since 2001-2003. Although enhanced MRI is now performed for the accurate diagnosis of HCC, in conventional clinical practice for HCC surveillance in liver diseases, unenhanced MRI is widely performed throughout the world. On the other hand, MRI has made marked improvements in recent years. In this study, a comparison of unenhanced MRI and US in detecting very small HCC was made. In order to conduct precise evaluation, we selected patients in whom MRI and US were performed at about the same time (on the same day whenever possible or at least within 14 d of one another).

Research methods

Out of the 403 patients with very small HCC nodules (≤ 2 cm in maximal diameter), 102 who underwent unenhanced MRI and US at nearly the same time (on the same day whenever possible or at least within 14 d of one another) at the first diagnosis of HCC were selected. The detection rate of HCC by unenhanced MRI was studied in comparison with unenhanced US.

Research results

We found that the sensitivity of unenhanced MRI for detecting very small HCC was as high as 95.1%, as compared with 69.6% by unenhanced US ($P < 0.001$).

Research conclusions

Currently, unenhanced MRI is a very important imaging modality for picking up very small HCC in usual clinical practice.

Research perspectives

As in this study, the marked superiority of unenhanced MRI to detect very small HCC as compared with unenhanced US was confirmed, and it may be desirable to perform routine surveillance of HCC in liver diseases by unenhanced MRI.

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CASE REPORT

Distant metastasis of hepatocellular carcinoma to Meckel's cave and cranial nerves: A case report and review of literature

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Abstract

BACKGROUND

Metastasis occurs as a late event in the natural history of hepatocellular carcinoma (HCC), and most patients die of liver failure attributed to the tumor supplanting the liver. Conversely, the brain is a less common metastatic site.

CASE SUMMARY

We describe a rare case of hepatitis C virus-related multiple HCC metastasizing to the cavernous sinus, Meckel's cave, and the petrous bone involving multiple cranial nerves in an 82-year-old woman. At admission imaging studies including Gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid-enhanced magnetic resonance imaging (MRI) revealed multiple HCC nodules in both right and left lobes. Ultrasound guided biopsy of the left lobe revealed moderately differentiated HCC. Molecular targeted therapy with Lenvatinib (8 mg/d for 94 d, *per os*) and Ramucirumab (340 mg/d and 320 mg/d, two times by intravenous injection) were administered for 4 mo, resulting in progression of the disease. Three months after the start of molecular target therapy, the patient presented with symptoms of hyperalgesia of the right face and limited abduction of the right

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eye, indicating disturbances in the right trigeminal and abducens nerves. Brain MRI disclosed a mass involving the cavernous sinus, Meckel's cave and the petrous bone. Contrast-enhanced MRI with gadolinium-chelated contrast medium revealed a well-defined mass with abnormal enhancement around the right cavernous sinus and the right Meckel's cave.

CONCLUSION
The diagnosis of metastatic HCC to the cavernous sinus, Meckel's cave, and the petrous bone was made based on neurological findings and imaging studies including MRI, but not on histological examinations. Further studies may provide insights into various methods for diagnosing HCC metastasizing to the craniospinal area.

Key Words: Meckel's cave; Abducens nerve; Trigeminal nerve; Hepatocellular carcinoma; Magnetic resonance imaging; Case report

Core Tip: We describe a case of hyperalgesia of the right side of the face and limited abduction of the right eye caused by hepatocellular carcinoma (HCC) metastasizing to the right cavernous sinus, the right Meckel's cave, and the right petrous bone diagnosed through neurological findings and imaging studies. Although HCC metastasizing to the cavernous sinus, Meckel's cave and the petrous bone is rare, clinicians need to be vigilant when the patients show neurological dysfunction, especially cranial nerve involvement.

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INTRODUCTION

Hepatocellular carcinoma (HCC), the most common liver cancer, is considered to bring more than 25 hundred thousand deaths worldwide every year. Metastasis is one of the most major points influencing prognosis. HCC often involves metastasis in the liver, but metastasis out of the liver to the lung, bone, and adrenal glands is less frequent, whereas the brain is commonly not connected. The authors report a case of hyperalgesia of the right side of the face and limited abduction of the right eye caused by HCC metastasizing to the right cavernous sinus, the right Meckel's cave, and the right petrous bone diagnosed through neurological findings and radiological studies.

CASE PRESENTATION

Chief complaints

An 82-year-old woman was in November 2019 admitted to Kobe Asahi Hospital for the treatment of HCC with molecular targeted therapy such as Lenvatinib (LEN) (8 mg/d).

History of present illness

She had overcome hepatitis C virus infection (HCV) 10 years earlier with interferon treatment, but still retained Child A liver cirrhosis.

History of past illness
She has suffered from chronic obstructive pulmonary disease for 20 years.

Personal and family history
Nothing particular.

Physical examination
She had no hepatomegaly and no splenomegaly.

Laboratory examinations
Laboratory examinations at admission revealed the following: Total protein 7.3 g/dL (normal 6.5-8.3), albumin 3.6 g/dL (3.8-5.3), aspartate aminotransferase 92 IU/L (10-40), alanine aminotransferase 172 IU/L (5-40), gamma-glutamyl transpeptidase 90 IU/L (< 35), alkaline phosphatase 422 IU/L (115-359), T-bil 1.3 mg/dL (0.2-1.2), NH3 163 μg/dL (< 130), pertussis toxin 88.3% (70-130), white blood cell 67 × 10^3/μL (36-90), Hb 13.6 g/dL (11.5-15.0), platelets 32.0 × 10^4/μL (13.4-34.9), hepatitis B surface antigen (-), HCVAb (+), HCV RNA (-), tumor markers were as follows: Alpha-fetoprotein (AFP) 30332.7 ng/mL (< 10.0), PIVKA-II 1395 mAU/mL (< 40) (Table 1).

Imaging examinations
Imaging examination 1: At admission imaging studies including Gadolinium-ethoxy-benzyl-diethylenetriamine pentaacetic acid-enhanced magnetic resonance imaging (MRI) showed multiple HCC nodules in both right and left lobes (Figure 1A). Gastrointestinal fiberscope revealed atrophic gastritis.

Imaging examination 2: Brain MRI revealed high intensity in the bilateral globus pallidus on T2-weighted images (T2WI), ascribed to elevated serum ammonia (163 μg/dL), but no findings in the cavernous sinus or Meckel's cave (Figure 1B), and marrow in the petrous bone was intact (Figure 1C).

Imaging examination 3: Brain MRI revealed a low intensity mass around the right Meckel's cave on T2WI (Figure 1D) and loss of normal fatty bone marrow signal intensity in the right petrous bone on T1-weighted images (T1WI) (Figure 1E).

Imaging examination 4: MRI revealed a low intensity mass around the right cavernous node, the right Meckel's cave, and the right petrous bone on T2WI (Figure 1F). Based on MRI findings, the rapid increase in the size of the lesions over 1 mo and the onset of neurologic dysfunction, such as impairment of right trigeminal and abducens nerves, were most likely due to the metastasizing HCC.

Histopathological examinations
Ultrasound guided biopsy of the left lobe revealed moderately differentiated HCC (Figure 1G).

FINAL DIAGNOSIS
Contrast-enhanced MRI with gadolinium-chelated contrast medium revealed a well-defined mass with abnormal enhancement around the right cavernous sinus and the right Meckel's cave (Figure 1H).

TREATMENT
Molecular targeted therapy with LEN (8 mg/d for 94 d, *per os*) and Ramucirumab (340 mg/d and 320 mg/d, two times by intravenous injection) were administered for 4 mo, resulting in progression of the disease. Two months after the start of molecular targeted therapy, tumor markers were as follows: AFP 3830 ng/mL, PIVKA-II 3782 mAU/mL.

Three months after the start of molecular targeted therapy, tumor markers were as follows: AFP 25761 ng/mL, PIVKA-II 13045 mAU/mL. The patient demonstrated hyperalgesia of the right side of the face and limited abduction of the right eye.

Four months after the start of molecular targeted therapy, tumor markers were as follows: AFP 226112 ng/mL, PIVKA-II 268638 mAU/mL, carcinoma embryonic

Table 1 Laboratory data on admission

Parameters	Results	Parameters	Results
WBC	67 × 10^3/μL	ALP	422 IU/L
Hb	13.6 g/dL	γ-GTP	90 IU/L
Platelets	32.0 × 10^4/μL	NH3	163 μg/dL
PT	88.3%	HBsAg	(-)
TP	7.3 g/dL	HCVAb	(+)
ALB	3.6 g/dL	HCV RNA	(-)
T-bil	1.3 mg/dL	AFP	30332.7 ng/mL
AST	92 IU/L	PIVKA-II	1395 mAU/mL
ALT	172 IU/L		

WBC: White blood cell; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALB: Albumin; TP: Total protein; PT: Pertussis toxin; AFP: Alpha-fetoprotein; HCV: Hepatitis C virus; HBsAg: Hepatitis B surface antigen; ALP: Alkaline phosphatase; γ-GTP: Gamma-glutamyl transpeptidase.

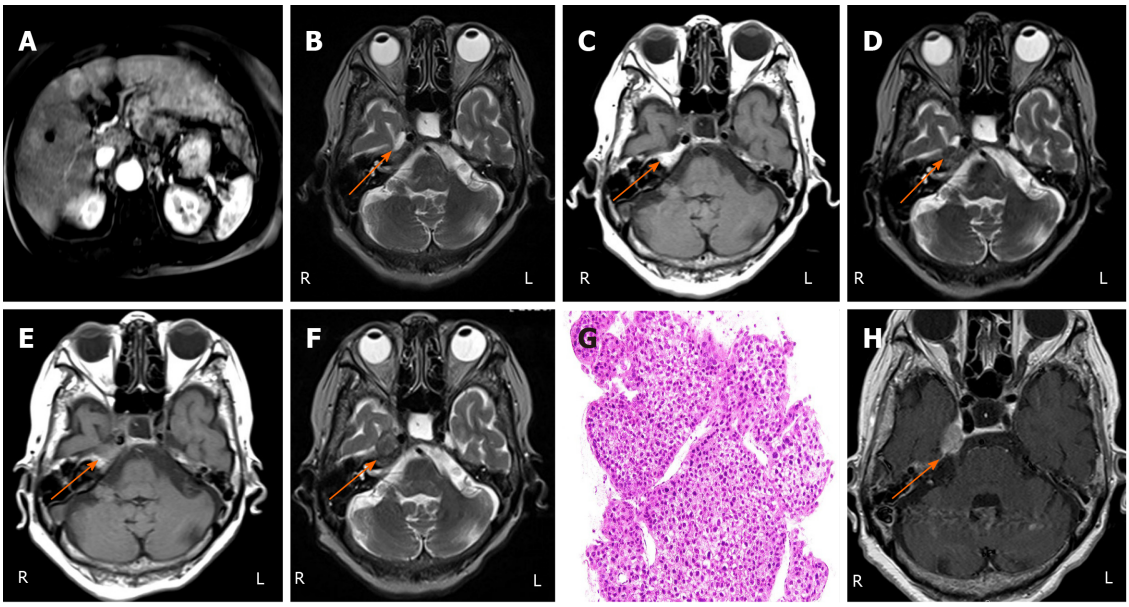

Figure 1 Imaging findings and histopathological findings. A: Ethoxybenzyl magnetic resonance imaging (MRI), hypervascular hepatocellular carcinoma (HCC) in the right and left lobes; B: Brain MRI [T2-weighted image (T2WI)], no findings in the cavernous sinus or Meckel's cave; C: Brain MRI [T1-weighted image (T1WI)], intact findings of bone marrow in the petrous bone; D: Brain MRI (T2WI), low intensity mass in the right Meckel's cave (arrow); E: Brain MRI (T1WI), loss of normal fatty bone marrow signal intensity in the right petrous bone (or apex); F: Brain MRI (T2WI), low intensity mass around the right cavernous node, the right Meckel's cave, and the right petrous bone on T2WI; G: Histopathological finding (hematoxylin and eosin staining), moderately differentiated HCC; H: Contrast enhanced MRI, well-defined mass with abnormal enhancement in the right cavernous sinus, and the right Meckel's cave (arrow). L: left; R: Right.

antigen 3.7 ng/mL (< 5.0), CA19-9 126.8 U/mL (< 37.0), interleukin-2R 824 U/mL (122-496).

Five months after the start of molecular targeted therapy, tumor markers were as follows: AFP 26795 mg/dL, PIVKA-II 258061 mAU/mL.

OUTCOME AND FOLLOW-UP

Based on the diagnosis, γ knife treatment was performed resulting in relief of the right side of the hyperalgesia. Fourteen days after γ knife treatment, the patient died due to the worsening of general condition.

DISCUSSION

Metastasis occurs as an advanced incident in the clinical course of liver cancer, and most patients expire because of hepatic insufficiency due to the cancer supplanting the liver. Distant metastases are routinely discovered at autopsy in over 50% of the cases [1-3]. On the contrary, the brain is an uncommon metastatic location. Accidental distant lesions at such more unusual locations are less a considered as possible metastases when metastatic HCC is not discovered at the more usual locations (the lungs, lymph nodes, and bone)[1-3].

The central nervous system is an uncommon location of metastatic HCC[4-8]. Before 1990, the diagnosis of HCC metastasizing to the craniospinal place was evidenced by histopathological findings of biopsy, operative and post-mortem tissues. Lately diagnosis is confirmed by neurologic tests and radiological findings, including computed tomography (CT) and MRI due to advances in such examinations[9-13]. In the 20th century, seven cases of HCC presenting as brain metastasis with no overt liver connection have been reported: Distant metastasis of liver cancer to the cerebrum in one case, and to the cranium in 6 cases[8]. Each showing slightly unusual hepatic examination early assessed, led to the diagnosis that in brain metastasis of obscure origin in a place where it is a usual illness, liver cancer should be viewed in differential diagnoses[8]. In Japan as in Taiwan, the place where liver cancer is a usual illness, HCC metastasizing to the cranium base relating to plural cranial nerves has not been described until now, but one case of cranium metastasis related to emergent epidural HCC[9].

After the 20th century, several cases of metastatic HCC to the cranial nerves have been reported: A 50-year-old female with HCV-associated recurrent multiple HCC metastasizing to the skull base involving multiple cranial nerves shows with conditions drop of eyelid, settlement of the right eyeball, and left abducens paralysis, suggesting disabilities of the right oculomotor and trochlear nerves, and both side abducens nerves. Contrast-enhanced CT of the brain shows an indistinct tumor with unusual increase surrounding the sella turcica. Brain MRI reveals that the tumor involves the clivus, the cavernous sinus, and the petrous apex. On contrast-enhanced MRI with gadolinium-chelated contrast medium, the tumor shows imbalanced middle increase. The diagnosis of metastatic liver cancer to the skull base is done based on of neurologic studies and radiological findings such as CT and MRI, but not on histopathological findings[13].

Two patients with HCC metastasizing to the skull base, the pituitary gland, the sphenoid sinus, and the cavernous sinus present with diplopia, retro-orbital headache, and multiple cranial nerve palsies. One is diagnosed with HCC prior to transsphenoidal operation of the pituitary metastasis. The second patient is, with histopathological examination, diagnosed to have HCC signs and symptoms associating with the primary tumor[14].

Two cases of HCC metastasizing to the cavernous cavity and the sphenoid cavity presenting with double vision and back eye socket headache, are performed operation for primary pituitary gland tumors. After operation, both cases are diagnosed as metastases from HCC[15].

A 73-year-old woman with HCV-related HCC shows a slightly limited abduction, more focused on the left eye with horizontal double vision. MRI of the face and paranasal cavity reveals a tumor in the left sphenoid cavity (22 mm × 16 mm × 16 mm) that invades the cavernous cavity and the forward slope of Meckel's cave[16,17]. HCC cases of metastasis to the brain from literature were summarized in Table 2 [Age: 56 (25-82), male: 16, female: 7]. Meckel's cave, a natural mouth-shaped aperture measuring 4 mm × 9mm wide at its opening and 15 mm in length within petrous apex's meningeal dura propria and periosteal layers, is the central part of the mid cranial fossa; it plays as a main route for the biggest cranial nerve (the fifth)[18,19]. The cavernous sinus is an important element of the cranial vascular organization, having immediate or indirect relations with the cerebrum, cerebellum, brainstem, face, eye, eye socket, nasopharynx, mastoid, and middle ear[20,21].

The neural components inside the cavernous sinus contain the sympathetic carotid plexus and 4 cranial nerves. The sites of these nerves, in superior to inferior turn, are the oculomotor (the third), trochlear (the fourth), abducens (the sixth), and ophthalmic divisions of the trigeminal (the fifth)[20].

Differential diagnosis of Meckel's cave lesions includes neoplastic and non-neoplastic ones.

Meckel's cave tumors account for only 0.5% of all intracranial tumors. Neoplastic lesions are trigeminal schwannoma (the most common with -33% of cases)[22], meningioma[22,23], pituitary macroadenoma, metastases: Including retrograde spread

Table 2 Hepatocellular carcinoma cases of metastasis to the brain from literature

No	Age	Sex	Presenting symptoms	Site of metastasis	Survival (from the onset of symptoms)	Ref.
1	25	M	Headache and left weakness	Right temporoparietal brain	1 d	Chang and Chen[5], 1979
2	50	M	Weakness of right leg, focal seizure of right leg	Calvarium of the skull, dura, brain	3 mo	Chang and Chen[5], 1979
3	51	F	Epistaxis, ptosis, diplopia, facial weakness in the left side	Skull base	6 mo	Chang and Chen[5], 1979
4	64	M	Loss of vision in the left eye, anorexia, weight loss	Lateral aspect of the temporal fossa and in the anterior portion of the middle cranial cavity	3 mo	Zubler *et al* [7], 1981
5	59	M	Left arm weakness and numbness, headache with left weakness, disturbed consciousness	Brain parenchyma (right frontotemporal parietal) with intracranial haemorrhage	2 mo	Lee[8], 1992
6	58	F	Progressive enlarging scalp mass over vertex for 4 mo	Calvarium, dura, brain parenchyma	10 mo	Lee[8], 1992
7	48	F	Progressive enlarging scalp mass over the left parietal and right frontal region for 6 mo	Calvarium	8 mo	Lee[8], 1992
8	36	M	Progressive enlarging scalp mass in right occipital region for 2 mo	Calvarium	3 mo	Lee[8], 1992
9	60	M	Diplopia and proptosis for 2 mo. Ophthalmoplegia for 1 mo	Skull base (retrobulbar)	7 mo	Lee[8], 1992
10	54	M	Progressive dysarthria and atrophy of left tongue for 2 mo	Skull base (jugular fossa hypoglossal canal)	4 mo	Lee[8], 1992
11	47	M	Right hemicrania for 3 mo blurred vision with ptosis and limitation of eye movement (OD) numbness on the right forehead for one month	Skull base (parasellar)	6 mo	Lee[8], 1992
12	70	M	Left-sided weakness	Acute epidural hematoma adjacent to the right parietal bone	2 mo	Hayashi *et al* [9], 2000
13	58	F	Progressive weakness of her right leg, right hemianesthesia and weakness	Left parietal region, left high parietal area	6 mo	Lee and Lee [11], 1988
14	50	M	Hemiparesis and numbness of left upper arm, explosive headache and vomiting, disturbance of consciousness	Right frontotemporoparietal area	2 mo	Lee and Lee [11], 1988
15	65	M	Progressive painful right sided proptosis and ptosis, intermittent right temporal and facial pain, loss of sensation on the right side of the face	Right orbital apex	9 d	Phadke and Hughes[12], 1981
16	55	M	Mild right weakness	Left fronto-parietal cerebral hemisphere	11 d	Phadke and Hughes[12], 1981
17	50	F	Ptosis, diplopia, left abducens palsy	Clivus, cavernous sinus, petrous apex	Not described	Kim *et al*[13], 2006
18	40	M	Diplopia, retro-orbital headache, and occasional vomiting	Pituitary fossa, clivus, sphenoid sinus, and right petrous apex	3 mo	Aung *et al* [14], 2002
19	71	M	Headache, diplopia, ptosis of the right eye	Pituitary gland, optic chiasma, cavernous sinus	1 yr	Aung *et al* [14], 2002
20	67	M	Diplopia, left retro-orbital headache	Sphenoid sinus, pituitary gland, clivus	15 mo	Tamura *et al* [15], 2013
21	58	M	Headache, visual disturbance, general fatigue, diplopia, oculomotor nerve palsy	Pituitary fossa, cavernous sinus	3 wk	Tamura *et al* [15], 2013
22	73	F	Frontotemporal and left periorbital headache with associated photophobia	Left sphenoid sinus, cavernous sinus	Not described	Morais *et al* [16], 2018
23	82	F	Hyperalgesia of the right face and limited abduction of the right eye	Cavernous sinus, Meckel's cave, petrous bone	5.5 mo	Our case

of head and neck tumors[24-27], epidermoid cysts[28], lipoma, base of skull tumors. All these tumors should be differentiated from Meckel's cave tumors.

Non-neoplastic lesions include internal carotid artery aneurysms/vascular malformation[29,30], and petrous apex cephalocele.

In our case, benign neoplasms such as schwannoma, meningioma, pituitary macroadenoma, epidermoid cyst, lipoma, base of skull tumors, as well as internal carotid artery aneurysms, vascular malformation and petrous apex cephalocele were ruled out in differential diagnosis.

In our case, brain MRI (T1WI and T2WI) disclosed a mass involving the right cavernous sinus, the right Meckel's cave and the right petrous bone; MRI with contrast medium revealed abnormal enhancement around the right cavernous sinus, and the right Meckel's cave.

Moreover, no other malignancies, or lymphoma, have been observed clinically; metastasis from HCC is most likely, irrespective of the absence of histological findings.

CONCLUSION

Taken together with neurological and imaging findings, our case was diagnosed as metastatic HCC to the right cavernous sinus, the right Meckel's cave and the right petrous bone involving multiple cranial nerves including the right fifth, and sixth.

The diagnosis of HCC metastasizing to this area is difficult to confirm by histopathological examination because of the deep-seated location and the neurovascular structures; nevertheless, histopathological diagnosis of HCC metastases to the pituitary gland bone has been reported[13,14].

In a previous study, the reason for HCC metastasis to the skull base was explained by the long survival of 15 years with various treatment regimens of chemotherapy and chemoembolization[13]. In our case, HCC metastasis may be due to the biological behavior of HCC such as being moderately differentiated and the failure of molecular targeted therapy, resulting in disease progression.

To our knowledge, our case is the second case of HCC metastasizing to the cavernous sinus, and Meckel's cave.

Although HCC metastasizing to the cavernous sinus, Meckel's cave and the petrous bone complicating multiple cranial nerves is very exceptional, medical professionals should be careful and good at managing radiological examinations including CT and MRI, when the patients show neurologic dysfunction, especially cranial nerve connection.

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