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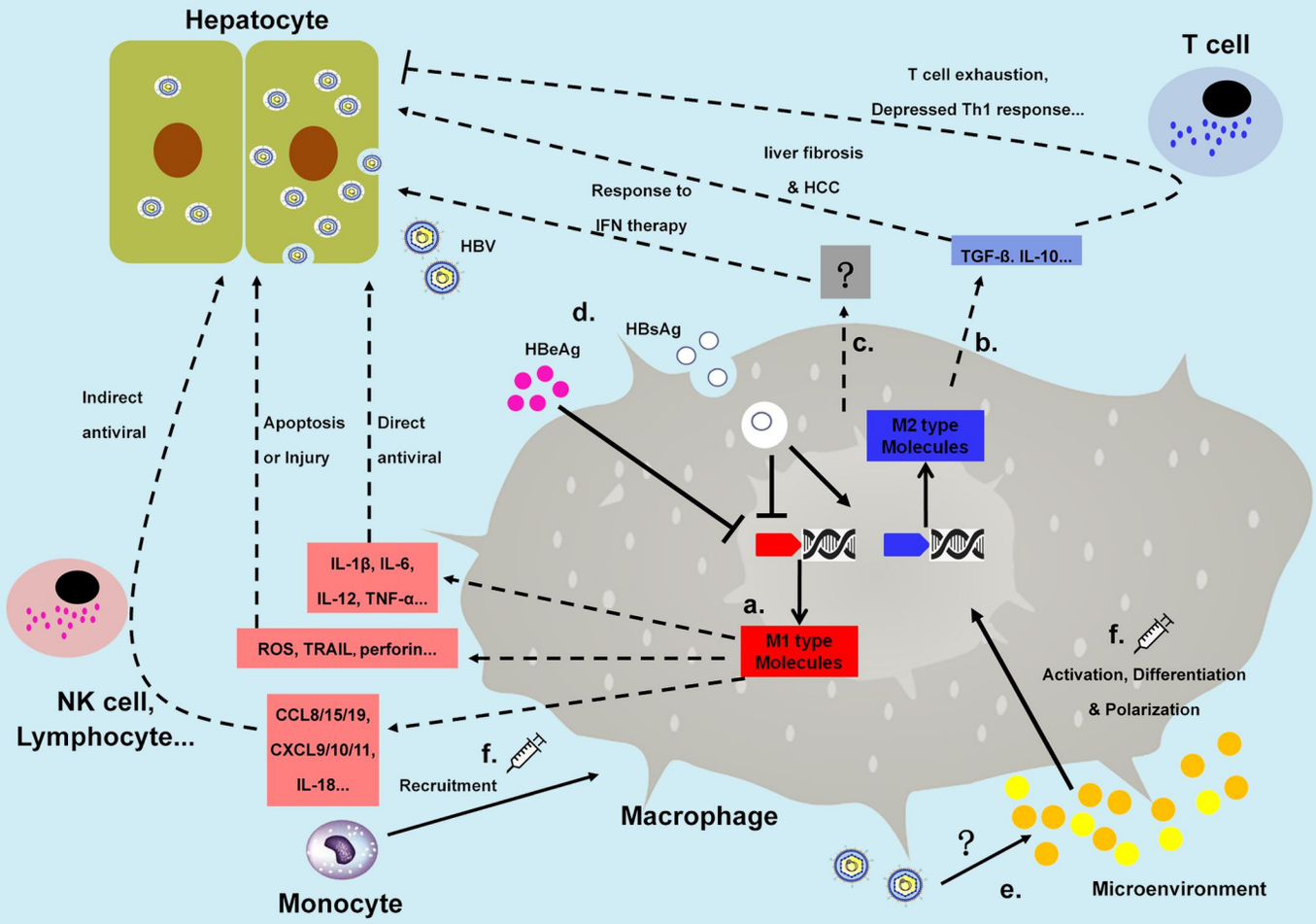

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Aims and Scope

Journal of Clinical and Translational Hepatology (JCTH, J Clin Transl Hepatol) publishes high quality, peer-reviewed studies in the clinical and basic human health sciences of liver diseases. *JCTH* welcomes submissions of articles within its topical scope including: novel discoveries in clinical and basic hepatology; liver disease mechanisms; novel techniques in research and management of liver diseases; epidemiological/environmental factors of liver diseases; role of immune system function in liver diseases; acute and chronic hepatitis; cirrhosis; genetic and metabolic liver diseases and their complications; hepatobiliary diseases; liver cancer; drug metabolism; biliary disease; peritoneal tuberculosis. *JCTH* publishes various types of articles, including original article, review, short communication, systematic review, meta-analysis, case report, methodology article, letter to the editor, and editorial.

Indexing & Abstracting

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JOURNAL OF CLINICAL AND TRANSLATIONAL HEPATOLOGY

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Journal of Clinical and Translational Hepatology Has Been Indexed in SCIE: A Milestone towards a Greater Academic Goal

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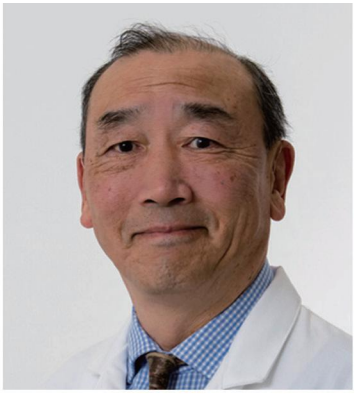

Prof. George Y. Wu

Dr. Harry Hua-Xiang Xia

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On November 10, 2020, the Editorial Office received a notification letter from Clarivate Analytics stating that *Journal of Clinical and Translational Hepatology* (*JCTH*) had been selected to be included in citation indexes in the Web of Science, and that articles published in *JCTH* after December 30, 2017 will be listed in some of the most influential and widely used databases in the world, including Current Contents/Clinical Medicine, Science Citation Index Expanded (SCIE), Essential Science Indicators, and Journal Citation Reports Science.

While indexing in the major databases, including PubMed/PubMed Central in 2015, Emerging Science Citation Resources in 2018 and Scopus in early 2020, was testimony to the excellence of *JCTH*, there was no assurance that the Journal would be accepted by the most exclusive of indices. We — Prof. Hong Ren, the General Editor-in-Chief; Prof. George Y. Wu, Comprehensive Editor-in-Chief; and Dr. Harry Hua-Xiang Xia, Co-Editor-in-Chief — have attempted to provide strategic vision, effective leadership, and stringent ethical standards for the Journal. But, all of this would have been of little consequence had it not been for the tremendous contributions of the associate editors, editorial board members, reviewers, authors, and staff of the editorial office. It is primarily because of their collective efforts that *JCTH* has achieved worldwide recognition in the competitive environment of academic publishing. Therefore, we, on behalf of the *Journal*, wish to take this opportunity to thank all of those individuals who have worked so hard and done so well to date.

On this momentous occasion, it is fitting to review the history of *JCTH* and the journey that led to its current status. The initiation and preparation for the creation of the Journal began in the Spring of 2010, when Dr. Xia was told by Dr. Qingfeng Sun from The Third Affiliated Hospital to Wenzhou Medical University that Prof. Ren, the Editor-in-Chief of a prestigious Chinese journal named *Chinese Journal of Hepatology*, intended to launch an English language journal in hepatology for The Second Affiliated Hospital of Chongqing Medical University, China. He approached Prof. Dazhi Zhang, Director of the Editorial Office of *Chinese Journal of Hepatology*, and the two had a very productive conversation. After several rounds of teleconferences and personal meetings in

Abbreviations: JCTH, Journal of Clinical and Translational Hepatology; SCIE, Science Citation Index Expanded.

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Xiamen, Chongqing, and Guangzhou, a general consensus was achieved that the project was worthwhile and feasible.

Xia & He Publishing Limited was engaged to undertake the task. Xia & He Publishing Limited had been originally registered since July 29, 2011 in Hong Kong, and was later established as Xia & He Publishing Inc. on January 8, 2015 in the USA under the very capable management of Dr. Hua He. The contract for publishing *JCTH* was signed by Prof. Ren and Dr. Xia on November 29 and December 8, 2011, respectively. In the meantime, Dr. Xia cordially invited Prof. Wu to be the Comprehensive Editor-in-Chief, owing to his remarkable academic record and international reputation. Dr. Xia and Prof. Wu, along with his wife, Prof. Catherine Wu, first met on November 27, 2010. In that and subsequent meetings and relaxed conversations — of course, over lunch at Chinese restaurants — numerous common views and perspectives were shared on the proposed journal.

After more than 2 years of preparation, *JCTH* was officially launched in September 2013, with an initial issue that featured an editorial entitled "Found in Translation" by Prof. Wu.[1] Since then, *JCTH* has published quarterly, yielding 29 issues with 361 peer-reviewed articles as of November 10, 2020. Of these articles, 92.0% are original and review articles, and 54.6% have been financially supported from various research funding sources, with The National Institutes of Health and National Natural Science Foundation of China listed as sponsors of the research in 24.9% of the articles. From the outset, *JCTH* sought to be diverse, international, and inclusive in both its articles and review panels; for the latter, the success of this approach is reflected by the composition of the Editorial Board, which now consists of 143 experts from 23 countries and regions, with 37.1% from China and 32.2% from the USA, followed by Egypt, India, Italy, *etc*. The corresponding authors of the articles published in *JCTH* to date are from 32 countries and regions, with 35.5% of them from the USA and 23.0% from China, followed by India, Egypt, Italy, *etc*. The reviewers are from 40 countries and regions, with 24.8% of them from the USA and 19.2% from China, followed by Japan, India, Italy, *etc*.

According to the Web of Science, as of November 10, 2020, the most cited *JCTH* article has been referenced 205 times,[2] and one of the articles on COVID-19 published in the early 2020 has already been cited 56 times.[3] The readership interest in and academic influence of *JCTH* have also been reflected by estimated unofficial impact factors for 2017, 2018 and 2019 of 3.615, 3.489 and 4.546, respectively, with a self-citation rate of only 1.4%. In June 2021, *JCTH* will receive its first official impact factor, which is expected to be around 3.5.

While indexing in SCIE is a major achievement and a remarkable milestone for *JCTH*, it is obviously not the final goal. As stated in our inaugural issue, "the objective of the *Journal of Clinical and Translational Hepatology* is to identify and publish articles that represent translations of fundamental research to contributions of direct practical value".[1] In all that *JCTH* does, this is and will remain the fundamental and paramount mission. That objective is also symbolized in our logo, in which translational research promotes the proper placement of pieces of a puzzle to form an ever more accurate picture of the liver. However, in the pursuit of this objective, we are planning to further increase the international influence of *JCTH* by inviting more high-profile experts to the editorial board and further increasing the standards for manuscripts to be published in the Journal. Within the next 10 years, we will strive for the Journal to become listed among the top 25% of journals in gastroenterology and hepatology, according to the Web of Science Journal Citation Reports.

In closing, we wish to share reflections that encapsulate the vision for the future of *JCTH* as composed by Dr. Xia in Chinese, with English translation in parentheses:

同谋肝胆志,
共表中华情.
十年磨一剑,
携手启新程.

(With a graceful ambition and careful preparation,
We determined to launch an internationally
influential hepatology journal in China.
With such a major milestone met after 10
years of persistence and endeavor,
We start a new journey toward a greater goal together.)

Funding

None to declare.

Conflict of interest

The authors have no conflict of interests related to this publication.

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Original Article

A 3D Human Liver Model of Nonalcoholic Steatohepatitis

Marion Duriez[1], Agnes Jacquet[1], Lucile Hoet[1], Sandrine Roche[1], Marie-Dominique Bock[1], Corinne Rocher[1], Gilles Haussy[1], Xavier Vigé[1], Zsolt Bocskei[1], Tamara Slavnic[2], Valérie Martin[3], Jean-Claude Guillemot[1], Michel Didier[1], Aimo Kannt[4,5], Cécile Orsini[1], Vincent Mikol[1] and Anne-Céline Le Fèvre*[1]

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Abstract

Background and Aims: To better understand nonalcoholic steatohepatitis (NASH) disease progression and to evaluate drug targets and compound activity, we undertook the development of an *in vitro* 3D model to mimic liver architecture and the NASH environment. ***Methods:*** We have developed an *in vitro* preclinical 3D NASH model by coculturing primary human hepatocytes, human stellate cells, liver endothelial cells and Kupffer cells embedded in a hydrogel of rat collagen on a 96-well plate. A NASH-like environment was induced by addition of medium containing free fatty acids and tumor necrosis factor-α. This model was then characterized by biochemical, imaging and transcriptomics analyses. ***Results:*** We succeeded in defining suitable culture conditions to maintain the 3D coculture for up to 10 days *in vitro*, with the lowest level of steatosis and reproducible low level of inflammation and fibrosis. NASH disease was induced with a custom medium mimicking NASH features. The cell model exhibited the key NASH disease phenotypes of hepatocyte injury, steatosis, inflammation, and fibrosis. Hepatocyte injury was highlighted by a decrease of CYP3A4 expression and activity, without loss of viability up to day 10. Moreover, the model was able to stimulate a stable inflammatory and early fibrotic environment, with expression and secretion of several cytokines. A global gene expression analysis confirmed the NASH induction. ***Conclusions:*** This is a new *in vitro* model of NASH disease consisting of four human primary cell-types that exhibits most features of the disease. The 10-day cell viability and cost effectiveness of the model make it suitable for medium throughput drug screening and provide attractive avenues to better understand disease physiology and to identify and characterize new drug targets.

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Keywords: 3D liver model; Human primary cells; Key features of NASH.

Abbreviations: ECM, extracellular matrix; HSC, hepatic stellate cell; KC, Kupffer cell; LEC, sinusoidal endothelial cell; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PHH, primary human hepatocytes.

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is an umbrella term that comprises a large spectrum of liver injuries, varying in severity but all leading to fibrosis. Among these, nonalcoholic fatty liver (NAFL) refers to hepatic steatosis alone, which is very common[1] and driven by the accumulation of intracellular lipid droplets. Furthermore, nonalcoholic steatohepatitis (NASH) is defined as a more serious pathogenesis, having inflammatory foci, hepatocyte damage, and fibrosis. Adverse hepatic outcomes related to NASH may include cirrhosis, liver failure, and hepatocellular carcinoma.[2] NAFLD is associated with obesity and features of metabolic syndrome, including hypertension, dyslipidemia, central adiposity, insulin resistance, or diabetes.[3,4] Moreover, NASH with advanced fibrosis has been linked to increased overall and liver-related mortality.[5]

Today, bariatric surgery is the most efficient procedure to reverse NASH and fibrosis in obese patients.[6] Weight loss induced by diet and exercise has also been shown to be effective in resolving NASH and improving hepatic fibrosis.[7] Despite a marked increase in prevalence, though NASH is still an orphan disease, with no approved drugs for its treatment. Thus, a better management approach for NAFLD and the development of new drugs and therapeutic options are urgently needed.[8] Although there has been steady progress in understanding NASH pathogenesis, the identification of therapeutic targets and the advancement of drug development have shown limited progress, mainly due to the lack of predictive preclinical models. Several animal models have been developed to study NAFL and NASH, but they do not accurately depict the human pathology, presumably because of NAFL/NASH heterogeneity.[9]

In drug discovery, hepatic *in vitro* models have been used to assess drug clearance and hepatotoxicity by investigating metabolism, enzyme induction, and transporter function. Monolayer cultures of isolated primary rat or human hepatocytes remain the main investigative tools for drug testing. These 2D models have shown several limitations, including a short lifetime and loss of function, likely resulting from dedifferentiation of primary hepatocytes (PHH).[10] Precision-cut liver slices, which contain PHH as well as liver nonparenchymal cells have also shown reduced lifetime; thus, impairing the development of liver chronic disease models and robust drug testing.

More recently, 3D cultures have been developed to improve cell survival and to provide a more natural tissue-like environment. In the liver, the extracellular matrix (ECM) behaves as a scaffold for surrounding cells, and *in vitro* artificial matrix aims to support ECM functions by promoting cell adhesion, cell differentiation, and cell-to-cell communication.[11–13] 3D cultures of hepatic cell lines or human PHH embedded in artificial scaffolds were shown to modify gene and cell surface receptor expression toward more mature-like phenotypes, resulting in the maintenance of hepatocyte polarization and functionality. Furthermore, it has been shown that collagen gels enhanced mechanical properties with good cell adhesion and a high survival rate for hepatocytes.[10] Thus, the 3D cell culture systems appear to be more representative of hepatocyte physiology in liver tissue and provide opportunities to develop extended *in vitro* models of NASH and NAFLD.

The development of *in vitro* human 3D models to mimic liver architecture has been undertaken by several groups, especially for drug safety assessment.[14] The process has included layered cocultures and cocultures on micropatterned surfaces, spheroids and bioprinted liver tissue.[14] Many of these models have been grown within specialized microfluidic devices, to provide nutrients and oxygen transport.[14] Few 3D coculture studies have been designed as NAFL or NASH disease models to display key pathogenic phenotypes. Thus, steatosis has been observed in NAFLD models supplemented with high concentrations of oleic and palmitic acids. Increased lipid accumulation was found to be associated with altered gene expression and activity of several CYP450 enzymes. Only limited cytokine release was reported, likely due to the absence of Kupffer cells (KCs) in these *in vitro* systems. Therefore, such 3D models highlighted the need to coculture additional cell types that could further incorporate features of inflammation and fibrosis and better reflect disease progression.

Feaver *et al.*[15] set up a 3D model with PHH, monocyte-derived macrophages and hepatic stellate cells (HSCs) in hemodynamic and transport conditions. Correlations between the *in vitro* model and human biopsies were evidenced by transcriptomics, lipidomics, and functional analysis. This model requires differentiating monocytes into macrophages, which is time consuming, and yields only M2 macrophages, a nonphysiological macrophage subpopulation. In addition, this hemodynamic system is unsuitable for high-throughput screening.

In this paper, we report on a new 3D coculture model combining PHH with HSCs, sinusoidal endothelial cells (LECs) and KC. Activated HSCs play a principal role in fibrosis initiation and development through the production of collagen, while KCs are involved in liver damage and inflammatory processes. LECs were more recently shown to play a pivotal role in NAFL/NASH progression.[16] This model was characterized biochemically and transcriptionally, and displayed some key features of hepatic injury, steatosis, inflammation, and early fibrosis. Its 10-day cell viability, as well as its reasonable cost-effectiveness, make it compatible for medium throughput screening in 96-well plates. This coculture model thus provides a valuable platform to better understand NASH disease progression, and to evaluate drug targets and compound activity.

Methods

The liver microenvironment under NAFLD and NASH disease conditions includes many circulating risk factors, which were incorporated into cell media to promote *in vitro* NAFLD or NASH-relevant phenotypes. Those risk factors include high glucose and insulin concentrations, excess of free fatty acids and endotoxins. For steatosis induction, free fatty acids (palmitic and oleic acids) were used, which lead to the accumulation of intracellular lipid droplets. This lipotoxic phenotype is the most commonly used stimuli for NAFLD *in vitro* models, as it can also lead to an increase of inflammatory cytokines levels.[17,18] Tumor necrosis factor-α was added to induce the inflammation process and activate the KCs. Cells were embedded in a hydrogel of rat collagen in 96-well plates and the NASH environment was induced by adding a media containing free fatty acids and tumor necrosis factor-α. Considering the main characteristics of NASH pathology, hepatocyte injury, steatosis, inflammation and fibrosis were assessed biochemically and via transcriptomics.

Cell 3D coculture in hydrogels/matrix of collagen

PHH were obtained from Lonza (Walkersville, MD, USA), and HSCs, KCs and LECs were provided by Samsara (San Diego, CA, USA). PHH, HSCs and LECs were seeded in red phenol-free William E medium (A12176; Gibco, Invitrogen Corp., Waltham, MA, USA) supplemented with 5% fetal bovine serum, primary hepatocyte thawing and plating supplements solution (CM3000; ThermoFisher Scientific, Waltham, MA, USA) and 1% of a non-essential amino acids solution (11140050; Gibco).

PHHs, LECs and hepatic stellate primary cells were embedded at 0.5×10^6, 0.1×10^6 and 0.1×10^6 cells/mL, respectively, in a half RAFT™ 3D collagen hydrogel (016-0R92; Lonza) in 96-well plates, as recommended by the provider. Cells were cocultured in DMEM (low-glucose, pyruvate, no glutamine, no red phenol; 11054; Gibco) supplemented with bovine serum/free fatty acid-free 0.125% solution (A7030; Sigma-Aldrich, St Louis, MO, USA), 50 U/mL penicillin/streptomycin (15140122; Gibco), dexamethasone 0.1 μM, ITS-G 1X (41400045; Gibco), GlutaMAX™ 1X (35050061; Gibco), HEPES 15 mM (15630080; Gibco), non-essential amino acids solution 1X (11140050; Gibco), acid L-ascorbic 2.5 mg/mL (A4403; Sigma-Aldrich) and glucagon 0.1 μg/mL (G2044; Sigma-Aldrich). This liver coculture medium is named "healthy media". After 3 days of culture, the cocultures were incubated either in healthy media or in a media mimicking the NASH environment and supplemented with glucose 25 mM (Sigma-Aldrich), oleate acid 40 μM (Sigma-Aldrich), palmitate acid at 60 μM (Cayman Chemical, Ann Arbor, MI, USA) and tumor necrosis factor-α 5 ng/mL (PeproTech, Cranbury, NJ, USA). Healthy and NASH medium were changed every 2-3 days. At day 6, KCs were added to the coculture at 0.2×10^6 cells/mL. Supernatants and embedded cells were sampled on days 3, 6, 8, 10, 13 and 15 for analysis.

Viability and hepatocyte metabolism

Viability was assessed by measurement of adenosine triphosphate using the CellTiter-Glo 3D Assay (G9681; Promega, Madison, WI, USA). PHH metabolism was measured through CYP3A4 activity with the Luciferin-IPA CYP3A4-P450 Glo Assay (V9002; Promega).

3D primary liver cell coculture immunostaining

3D hydrogels were fixed with 4% Paraformaldehyde/phosphate-buffered saline for 15 min at room temperature and

immunostainings were performed. PHH cell membrane staining was performed with a rabbit anti-cytokeratin 18 antibody (EPR17347, #Ab181597; Abcam, Cambridge, UK) and an Alexa Fluor 594 conjugated goat anti-rabbit IgG secondary antibody (#A11012; Molecular Probes, Eugene, OR, USA). HSCs were stained with a mouse anti-α-smooth muscle actin antibody (#A5228; Sigma-Aldrich) and an Alexa Fluor 680 conjugated goat anti-mouse IgG secondary antibody (#A21057; Molecular Probes). KCs were stained with an FITC conjugated anti-CD68 antibody was used (KP1, #FCMAB205F; Sigma-Aldrich), nuclei were labeled using NucBlue live (#R37610; ThermoFisher Scientific). Image acquisitions were performed with a Leica SP8X confocal microscope, using ×40 water objective, Z-stack of Z=30 μm zoom 0.8. 3D reconstitution was performed with IMARIS software 9.1.2.

Triglyceride content

Triglycerides content was measured on coculture supernatants with the PicoProbe Triglyceride Quantification Assay (K614-100; BioVision Inc., Milpitas, CA, USA). Intrahepatic lipid droplets were stained with the LipidTox Green probe (H34475; ThermoFisher Scientific) and PHH cell membrane with CK18 immunostaining. Images of 3D cocultures were acquired with a Leica SP8X confocal microscope, using ×40 water immersion objective and a Z-stack of Z=30 μm, zoom 0.8. 3D reconstitution was performed with IMARIS software 9.1.2.

Cytokine/metalloprotease release

The cytokine content in coculture supernatants were measured using the U-plex Biomarker Human Group 1 Kit (K15067L-2; MSD Technology, Rockville, MD, USA). Samples were diluted at 1/4 for IL6, CXCL8 and CCL2 measurement, and no dilution was performed for CXCL10 quantification. The concentration of secreted metalloproteinase MMP2 was quantified with the Human MMP2 Ultrasensitive Kit (K151FYC-2; MSD Technology) with a ½ dilution.

Statistical methodology of biochemical parameters

To compare NASH and healthy models, a two-way ANOVA was performed on each parameter, with group treatment (healthy or NASH), day and their interaction as fixed-effects factors, and with experiment and interaction of experiment, group and day as random effect factors. Comparisons of NASH versus healthy were provided for each day and no correction for multiplicity was done. To analyze the kinetics of the healthy model, a one-way ANOVA was performed on each parameter, with day as the fixed-effect factor and experiment and experiment by day as random effect factors. A Bonferroni-Holm's correction was applied on *p*-values to compare the following: day 6 vs. day 3, day 8 vs. day 6, day 10 vs. day 8 for comparison of both conditions; and day 10 vs. day 13 and day 13 vs. day 15 for healthy time course assessment. Either a log or rank transformation was applied on studied parameters, and fold-changes were calculated. For log-transformed variables, the differences estimated from the model and their confidence intervals were back-transformed by using an exponential function. For rank-transformed variables, fold-change and confidence intervals were estimated by Hodges-Lehmann's method.

RNA extractions

Hydrogels embedded cocultures containing 0.2×10^6 cells per well, were rinsed twice with cold phosphate-buffered saline and stored at -80°C until use. For RNA extraction, two wells were lysed in a final volume of 700 μL of Qiazol lysis reagent (Qiagen, Hilden, Germany). Lysates were homogenized using CK14-2 mL tubes in a Precellys tissue homogenizer instrument (Bertin Instruments, Montigny-le-Bretonneux, France). The lysates were collected and 140 μL of chloroform were added, vortexed and centrifuged at 12000 rpm for 15 minutes at 4°C. Recovered aqueous phase was processed with an on-column DNAse treatment and a RNeasy mini-kit (Qiagen) as recommended by the provider. RNAs were recovered in 30 μL of RNase-free water. RNA quality was determined by RNA LabChip with a 2100 Bioanalyzer (Agilent Scientific Instruments, Santa Clara, CA, USA). Samples with RNA integrity number >7.4 were further processed and their concentration was quantified by Xpose spectrophotometer (Trinean, Unchained labs, France).

RNA libraries and sequencing

RNA-Seq libraries were generated with 15 ng of total RNA. cDNAs were generated with the SuperScript VILO cDNA Synthesis Kit (ThermoFisher Scientific) and the following steps for library construction were performed using the "AmpliSeq for Illumina Transcriptome Human Gene Expression Panel" according the accompanying reference guide (Illumina, San Diego, CA, USA).

Libraries were quantified and qualified, respectively, by Qubit (Invitrogen) and Bioanalyzer (Agilent). Libraries were pooled (equimolar concentration at 4 nM), denatured and diluted to a final concentration of 1.4 pM. Sequencing was performed on the Illumina NextSeq500 with NextSeq 500/550 High Output v2 Kit and sequencing parameters of 2×151 base pair pair-end, dual index (2×8 base pairs).

Generated raw files were converted into FASTQ files and analyzed on Array Studio (V10.0.1.81; Omicsoft, Qiagen). Briefly, raw data QC was performed, then a filtering step was applied to remove reads corresponding to rRNAs as well as reads having low quality score. Mapping and quantification were performed using OSA4 [1C] (Omicsoft Sequence Aligner, version 4).[19] Reference Human.B38 genome was used for mapping and genes were quantified based on RefSeq gene annotations. Differentially-expressed transcripts were identified with DESeq2.[20] The variable multiplicity being taken into account and false discovery rate adjusted *p*-values calculated with the Benjamini-Hochberg correction.[21] Significant differentially-expressed genes were defined as $p<0.05$ after adjustment for false discovery and average fold-change between condition replicates of >1.8. The differentially-expressed genes were further analyzed using Ingenuity Pathway Analysis (Qiagen; https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis).[22]

Results

Human liver primary cells 3D coculture characteristics in healthy conditions

Setting up a human *in vitro* NASH disease model requires developing a coculture containing the different cells involved in the pathogenesis and maintaining it over an extended

period of time in culture to induce this chronic disease. We developed a 3D coculture with primary cells using the RAFT™ biological rat collagen hydrogel embedding PHH, HSCs, KCs and LECs. The addition of LECs as feeder cells in the 3D liver coculture allowed us to stabilize the model by improving cell viability from 1 week up to 2 weeks (data not shown). PHH, HSCs and LECs were seeded in the hydrogel of collagen at a 5:1:1 ratio, respectively, reproducing the human liver ratio, and cultured in a 96-well plate in a homemade cell culture media named 'healthy media' (Fig. 1A). At day 6, KCs were added to the coculture since they did not tolerate the embedding process (data not shown) but were able to enter the hydrogel once formed. Several media, where Obeticholic acid/Palmitic acid concentrations, glucose concentration and tumor necrosis factor were varied, were tested to maintain viability of the four cell types. Only the homemade liver coculture optimized media, described in the Materials and Methods section, allowed us to obtain a viable coculture of the four cell types up to day 10 (Fig. 1B).

To assess the suitability of the model, coculture viability, cytokine secretion and lipid droplet content were measured from day 3 to day 15 in four independent experiments. The human 3D liver coculture at a basal level displayed a good viability from day 3 to day 15, as reflected by adenosine triphosphate level (Fig. 2A). PHH CYP3A4 enzymatic function was evaluated and it remains stable from day 3 up to day 13 (Fig. 2B). Inflammatory cytokines and chemokines IL6, CXCL8 and CXCL10 were measured in coculture supernatants. Basal levels of secreted IL6 and CXCL8 remained stable from day 3 to day 15, with a nonsignificant increase at day 8 resulting from the addition of KCs at day 6 (Fig. 2C and 2D). CXCL10 was almost undetectable before day 8, after KCs addition, and its secretion in cell supernatants remained stable from day 8 to day 15 (Fig. 2E). The triglyceride content of the human liver 3D coculture was quantified, and PHH lipid droplets were observed by immunofluorescence via Green LipiTox and a cytokeratin-18 containing (Fig. 2F and 2G, respectively). A high basal level of triglycerides with no significant variation was detected from day 3 to day 15 (Fig. 2F). This result was correlated with the observation of an important intracellular lipid droplet staining in PHH from day 3 to day 10 (Fig. 2G). Of note is that the PHH triglyceride content in human liver 3D coculture was higher than expected. Several media were tested on PHH, including William's E media. All of them induced an elevated intracellular lipid droplet content (Supplementary Fig. 1) for unknown reasons and the cell coculture showed a lower viability than the homemade media.

Together, these results support a long-term viability and the stable expression of NASH-induced key features that can be directly quantified in the human 3D liver primary coculture under healthy conditions up to 15 days (Supplementary Table 1).

A transcriptomic analysis of human 3D liver primary coculture under the healthy condition was performed. Gene expression analysis was only performed on samples from days 3, 8 and 10 (n=3/condition) as the total RNA quantity extracted at day 13 and day 15 was too low for gene expression studies.

The expression of genes related to PHH activities, including the efflux transporters ABCC2, ABCB1 and ABCB11, was first investigated. These genes are consistently detected during the time course of the coculture until day 10 (Supplementary Table 2) as well as UGT1A1, a gene encoding the phase II UDP-glucuronosyltransferase enzyme, with a fold-change <2 and/or with a p-value >0.05. Furthermore, CYP7A1 cytochrome P450 gene was expressed from day 3 to day 10, with no significant difference (Supplementary Table 2), CYP3A4 expression, which was decreased at day 8 and day 10 compared to day 3, was stable from day 8 to day 10 (Supplementary Table 2) and was previously observed to be functional from day 3 to day 13 in 3D coculture (Fig. 2B). Together, these genes reflect the polarization, functionality and metabolic activity of mature PHH in human 3D liver primary coculture up to 10 days *in vitro*.

As expected, the expression of IL6, CXCL8 and CXCL10 inflammatory cytokine genes is observed. IL6 expression was stable between day 3 and day 10 (Supplementary Table 2), whereas CXCL8 and CXCL10 were significantly down- and up-regulated from day 3 to day 8, resulting from the addition of KCs but which remained stable from day 8 to day 10 (Supplementary Table 2). IL6, CXCL8 and CXCL10 gene expression results are in line with their secretion observed in 3D coculture supernatants (Fig2. C, D and E). The expression of

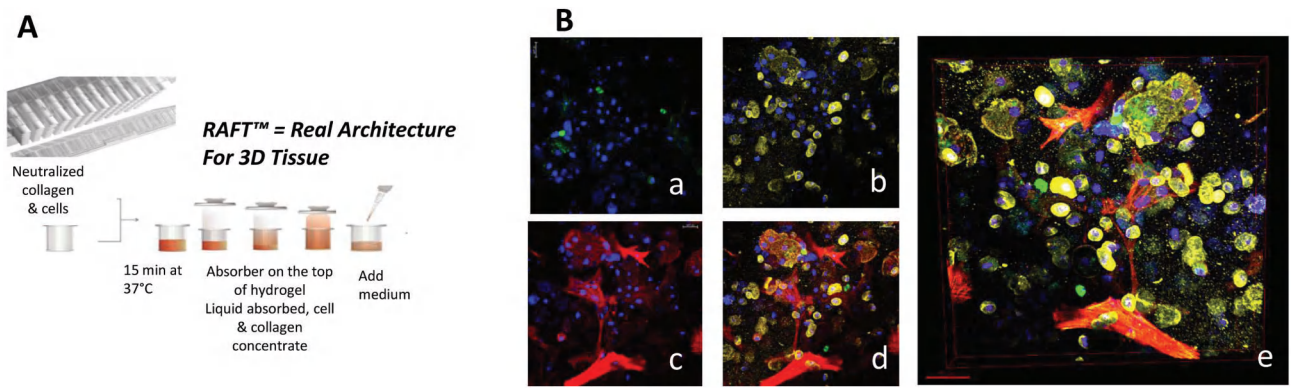

Fig. 1. Set-up of human liver primary coculture in a 3D environment.

(A) Real architecture for 3D model with RAFT™ system methodology illustration. (B) PHH-KC-HSC-LEC coculture at 10 days in 3D collagen matrix with healthy media. (a) KC staining with CD68 in green and nucleus-labeled in blue. (b) PHH staining with CK18 in green and nucleus labeled in blue. (c) HSC staining with α-smooth muscle actin in red and nucleus labeled in blue. (d) Merge. (e) 3D reconstitution was performed with IMARIS software 9.1.2.

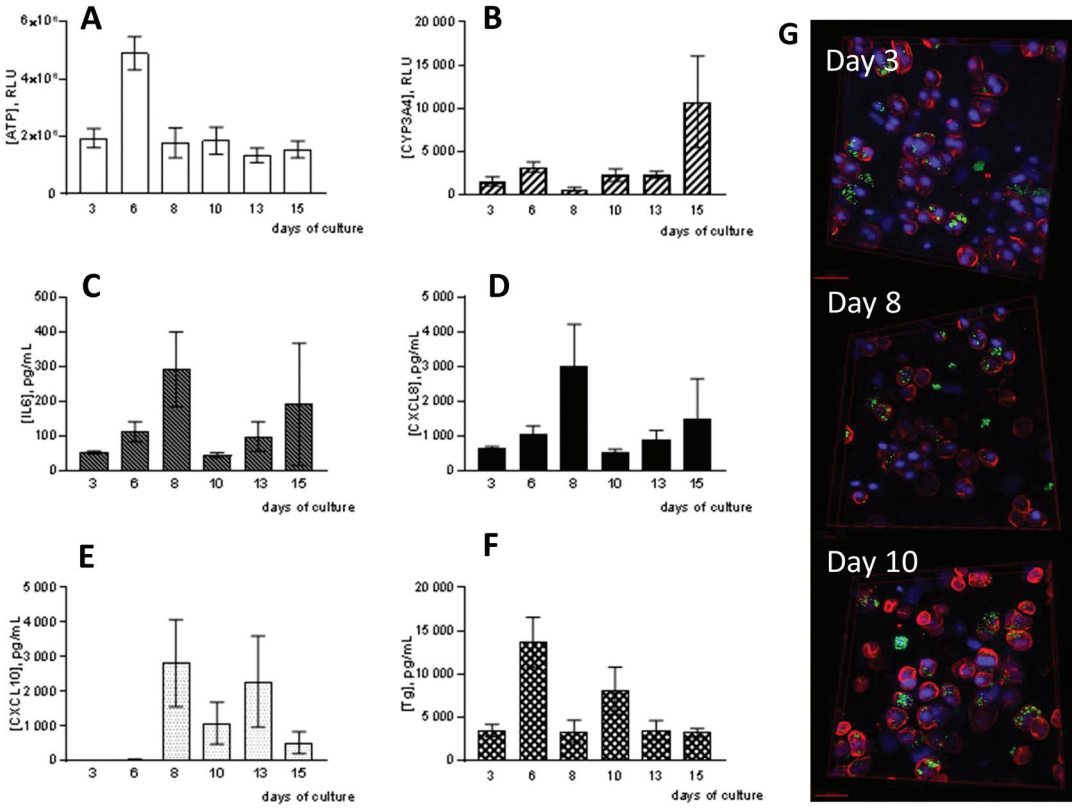

Fig. 2. Human 3D liver primary coculture characterization.

(A) Adenosine triphosphate intracellular in RLU. (B) CYP3A4 activity in RLU. (C) IL6 (D) CXCL8 and (E) CXCL10 in pg/mL. (F) Triglyceride content in pg/mL. (G) Kinetics of lipid droplet content in liver coculture in 3D collagen matrix, healthy condition. PHH staining of CK18 in red, nuclear in blue and lipid droplet in green. *p<0.050, **p<0.010, ***p<0.001, two-way ANOVA with random effect followed by Bonferroni-Holm correction

Col1A1 gene, encoding collagen compounds remained stable from day 3 to day 10 (Supplementary Table 2), whereas ACTA2 gene, encoding the α-smooth muscle actin protein, slightly increased with a 2.8-fold-change and 2.4-fold-change at day 8 and day 10, as compared to day 3, but which remained stable from day 8 to day 10 (Supplementary Table 2). The ACTA2 gene increased from day 3 to day 8, probably resulting from the addition of KCs since the latter have been shown previously to activate HSCs through soluble factors.[23] Together, these results showed that HSCs are not activated by a bystander effect of the coculture under healthy conditions.

We succeeded in defining suitable culture conditions, including medium composition and chronological cell addition, to maintain the four liver primary cell types in 3D coculture up to 10 days *in vitro*. Furthermore, these culture conditions gave the lowest level of steatosis, together with reliable low levels of inflammation and fibrosis.

Human 3D liver NASH model display PHH injury and steatosis

The human *in vitro* 3D NASH model was set up by culturing the human primary 3D liver coculture with a custom medium mimicking a NASH-like disease environment added from day 3. This medium contained free oleic and palmitic fatty acids at 100 μM with a 2:3 ratio, as well as tumor necrosis factor-α at 5 ng/mL.

The adenosine triphosphate levels measured in the human 3D liver coculture did not show a significant difference between the healthy and NASH culture conditions and indicated a good viability which was maintained until day 10 (Fig. 3A). ABCC2 and ABCB11 gene expressions were not modulated during the coculture, neither in the NASH nor in the healthy models, showing a stable PHH mature phenotype up to 10 days *in vitro* (Fig. 3B and Supplementary Table 3). Cytochrome P450 CYP3A4, the expression of which is known to be reduced in NASH, displayed a reduced activity and gene expression in the NASH model, as compared to healthy coculture but without reaching statistical significance (Fig. 3C). However, at day 10, three out the four experiments showed a decreased CYP3A4 activity (Fig. 3C), which correlated to a reduced gene expression at day 10 (Fig. 3D and Supplementary Table 3).

PHH steatosis assessed by triglyceride quantification in coculture did not show significant differences between healthy and NASH culture conditions (Fig. 4A). Intracellular lipid droplet staining is observed in both conditions but without noticeable differences at day 8 and day 10 (Fig. 4B). As mentioned before, PHH display a high basal level of triglycerides. This feature was observed for all the media tested, supporting the finding of PHH viability in the 3D collagen matrix and being consistent with biochemical quantification of steatosis.

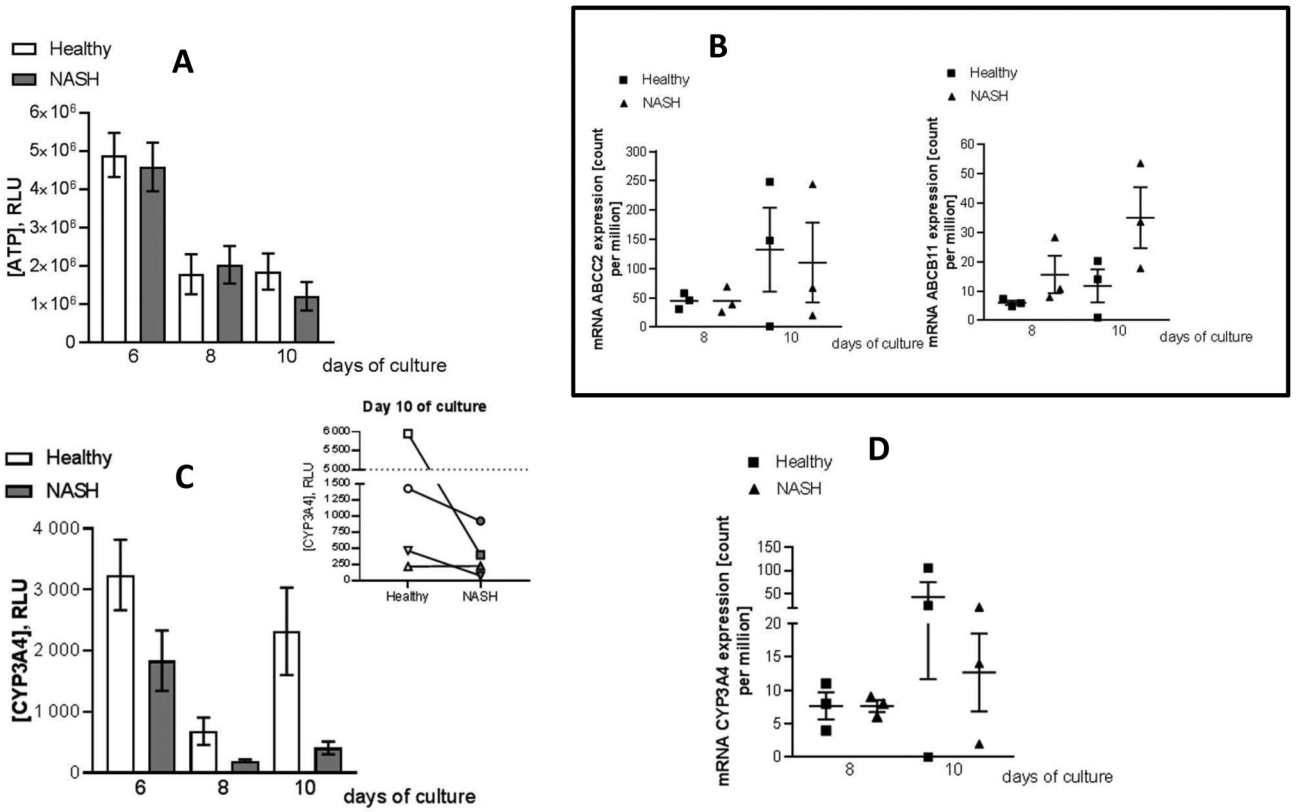

Fig. 3. PHH injury characterization in human 3D liver NASH model.

(A) Time course of adenosine triphosphate mean concentration (in RLU, +/-standard error of the mean) in 3D healthy and NASH models. (B) ABCC2 (left panel) and ABCB11 (right panel) mRNA expression in counts per million at days 8 and 10 in healthy and NASH culture. (C) Time course of CYP3A4 mean concentration (in RLU, +/-SEM) in 3D healthy and NASH models (left panel) with a focus on the fourth independent experiment trend on day 10 (right panel, each symbol represents an independent experiment). (D) CYP3A4 mRNA expression in counts per million at days 8 and 10 in healthy and NASH culture conditions. *p<0.050, **p<0.010, ***p<0.001, Student's test and DESeq2, respectively for panels A and C and panels B and D.

The human *in vitro* 3D NASH model displayed a similar stable viability as observed in the healthy 3D liver coculture model, whereas it showed a decreased CYP3A4 activity in three out of four experiments. Lipids droplets, characteristic of steatosis, were observed in both conditions; high basal level in healthy coculture does not allow detection of an increase of triglyceride content in NASH conditions.

Human 3D liver NASH model expression of inflammatory and tissue remodeling factors

The ability of human 3D liver coculture to react to a proinflammatory environment was explored next. The secretion of CXCL8, IL6, CXCL10 and CCL2 in supernatants of healthy and NASH 3D coculture was examined by quantifying multiplex assay at days, 6, 8 and 10. Tumor necrosis factor-α (5 ng/mL) was added to the medium and changed every 2-3 days to induce an inflammatory process.

IL6 secretion showed a significant 5-fold increase at day 6 in the 3D NASH model, as compared to the healthy condition (Fig. 5A and Supplementary Table 4), and a significant increase in gene expression was observed at days 8 and 10 (Fig. 5B). The secretion of CXCL8 was significantly up-regulated in NASH coculture at day 6 and day 8, with a 10.3-fold and 4.7-fold increase, respectively (Fig. 5A and Supplementary Table 4). This observation correlated to an up-regulation in the expression of CXCL8 in 3D NASH coculture from day 8 to day 10, with a respective fold-increase of 13.4 and 14.6 (Fig. 5B). Finally, CXCL10 secretion was also significantly up-regulated in the 3D NASH model, being 32.5-fold and 22.6-fold at day 6 and day 10, respectively (Fig. 5A and Supplementary Table 4). A significant 8.7-fold increase of CCL2 at day 6 was also observed in coculture supernatant in the 3D NASH model (Fig. 5C and Supplementary Table 3).

The expression of the metalloproteinases MMP2 and MMP9 involved in tissue remodeling and markers of early fibrotic events was also explored. MMP2 secretion in the human 3D NASH coculture significantly increased by 1.2-fold, 1.7-fold and 1.4-fold at days 6, 8 and 10m respectively (Fig. 6A and Supplementary Table 4), and correlated with a significative up-regulation in MMP2 gene expression at day 10 (Fig. 6B and Supplementary Table 3). Finally, MMP9 transcripts significantly increased both at days 8 and 10, with a 3.2-fold and 6.6-fold change in the NASH environment (Fig. 6C and Supplementary Table 3).

Together, these results show that the human *in vitro* 3D NASH model is able to simulate a stable inflammatory and early fibrotic environment with the secretion of IL6, CXCL8, CXCL10, CCL2, MMP2 and MMP9 expression, respectively, as compared to the healthy model.

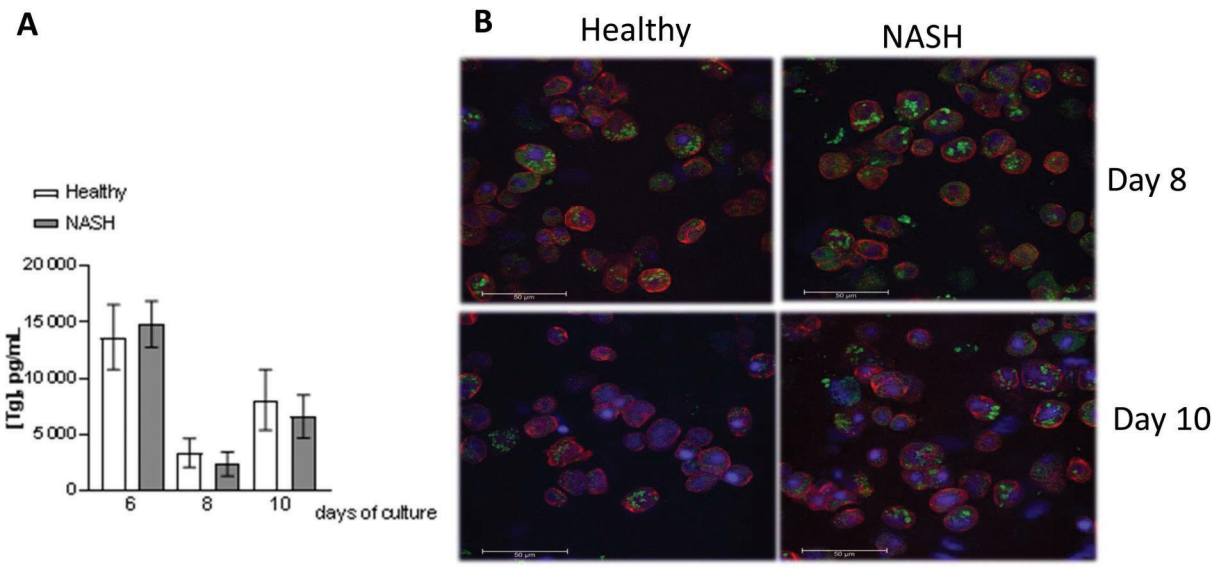

Fig. 4. Assessment of steatosis feature.

(A) Time course of triglyceride mean concentration content (in pg/mL, +/-standard error of the mean) in 3D healthy and NASH models. (B) Lipid droplet staining in healthy and NASH culture conditions (left and right panels respectively) at days 8 and 10 (upper and lower panels, respectively). Lipid droplets are stained in green, nucleus in blue, and CK18 staining at PHH cell surface in red. Images were acquired with a Leica SP8X confocal microscope. *p<0.050, **p<0.010, ***p<0.001, Student's test.

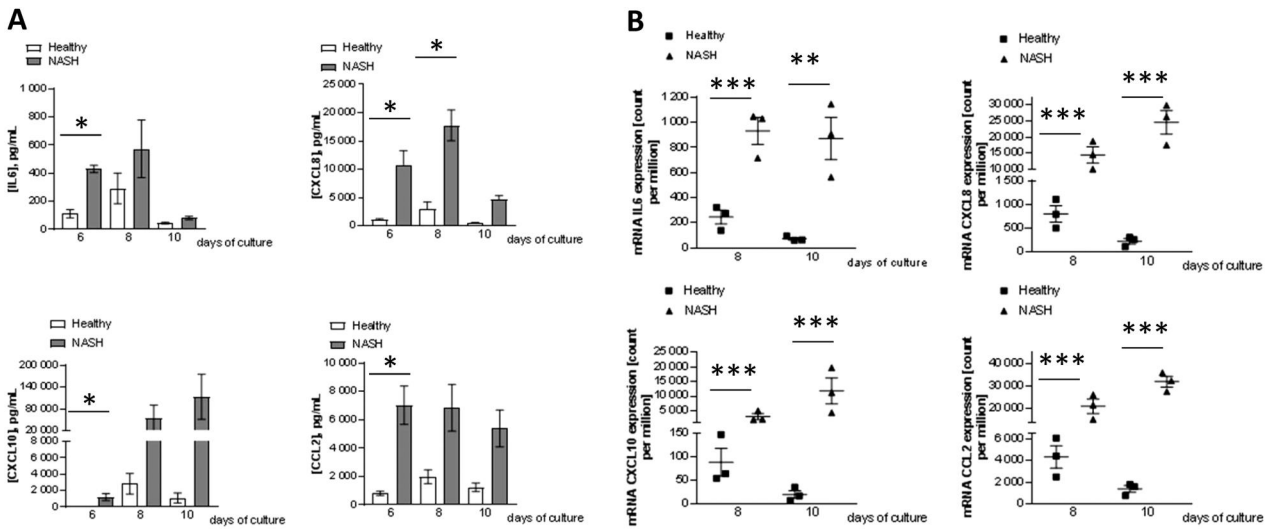

Fig. 5. Inflammatory environment induced in 3D liver NASH model.

(A) Time course of secreted IL6, CXCL8, CXCL10 and CCL2 mean concentration (in pg/mL, +/-standard error of the mean) at days 6, 8 and 10 in healthy and NASH models. (B) IL6, CXCL8, CXCL10 and CCL2 mRNA expression in count per millions at days 8 and 10 in healthy and NASH conditions. *p<0.050, **p<0.010, ***p<0.001, Student's test and DESeq2, respectively, for panel A and panel B.

Human 3D NASH model regulates a gene set related to pathways involved in NASH development

To better characterize the human *in vitro* 3D NASH model, a global gene expression analysis has been performed by RNA-Seq. For this purpose, three samples from days 3, 8 and 10 of the healthy 3D cocultures and from days 8 and 10 of the *in vitro* 3D NASH model were processed. Global RNA-Seq datasets from the 3D healthy and NASH models were first interrogated using a principal component analysis to cluster these two models during *in vitro* development (Fig. 7A). A distinct sample separation was observed between 3D healthy and NASH models (represented by circles and triangle, respectively, in Fig. 7A), highlighting that NASH culture condition alters the overall gene expression pattern of 3D liver cocultures. A time effect on both the healthy and NASH 3D liver model was also visualized, being more noticeable between days 8 and 10 in the 3D NASH coculture.

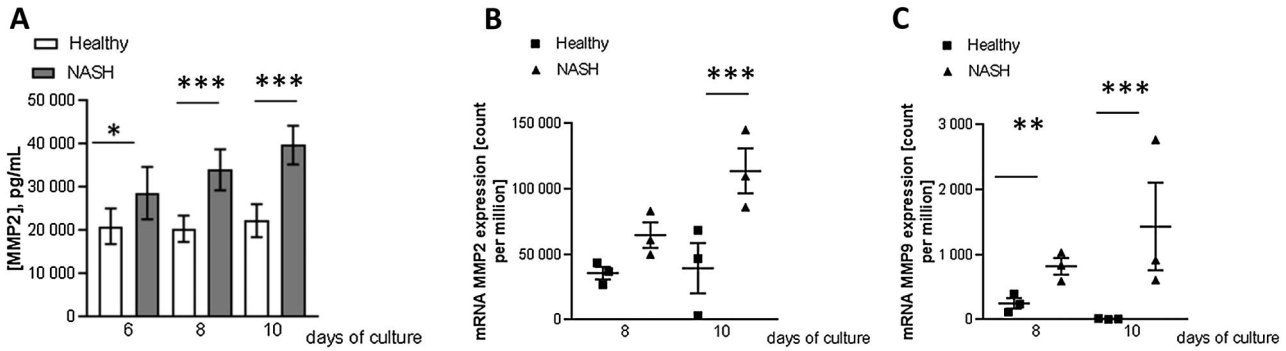

Fig. 6. Early fibrotic tissue remodeling factors induced in 3D liver NASH model.

(A) Time course of secreted MMP2 mean concentration (in pg/mL, +/-standard error of the mean) at days 6, 8 and 10 in healthy and NASH models. (B) MMP2 and (C) MMP9 mRNA expression level in count per millions at days 8 and 10 in healthy and NASH conditions. *p<0.050, **p<0.010, ***p<0.001, Student's test and DESeq2, respectively, for panel A and panels B and C.

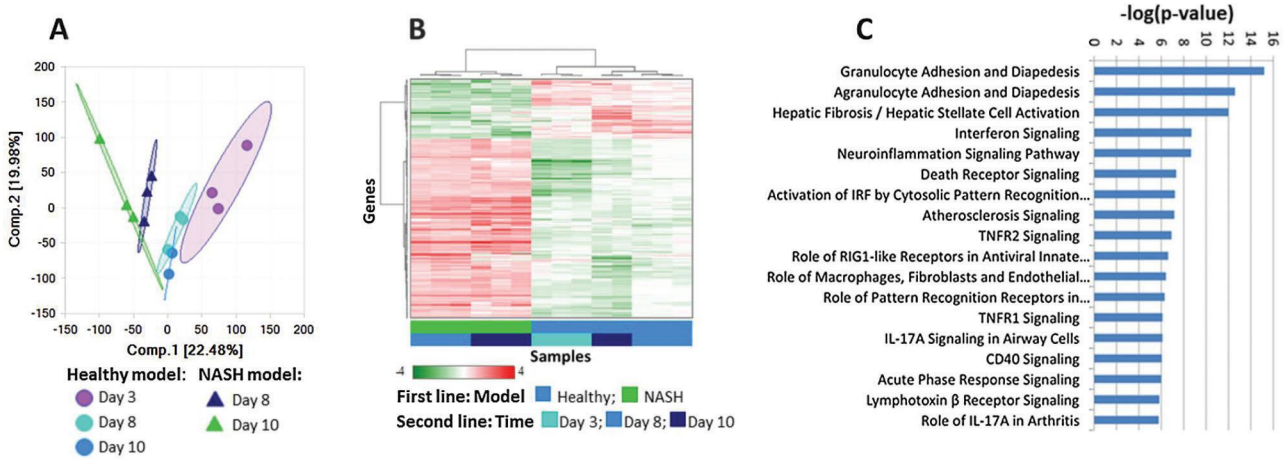

Fig. 7. RNA-Seq analysis of NASH and healthy 3D models.

(A) Principal component analysis (PCA) for gene expression in the 3D model of NASH versus healthy state at day 3, 8 and 10. The PCA was performed using DESeq2 normalized expression data. (B) Clustering analysis of differentially-expressed genes between the 3D model of the NASH disease and biological healthy models, at day 8. Heat map illustrating unsupervised hierarchical clustering of the 468 genes specifically regulated in NASH model versus the biologically healthy model at day 8 (log2 of DESeq normalized data). (C) Enrichment analysis of molecular pathways in NASH model at day 8. Visualization of top 18 enriched canonical pathways in human NASH patients as compared to normal controls. Values are expressed as –log (p-value).

To identify pathways modulated under NASH-inducing conditions, differentially-expressed genes between 3D healthy and NASH models were identified. A total of 659 significant differentially-expressed genes were detected between 3D healthy and NASH conditions at day 8 (Supplementary Fig. 2). A gene subset was already modulated between day 3 and day 8 in the healthy 3D model and, thus, could not be assigned to the NASH culture condition. In the end, we selected 468 differentially-expressed genes in the 3D NASH model that were not found to be regulated over time in the healthy model. This list included 351 up-regulated and 117 down-regulated genes (Supplementary Fig. 2). A hierarchical clustering analysis of healthy and NASH 3D liver models using these 468 differentially-expressed genes confirmed a good separation of the samples from healthy and NASH 3D coculture conditions (Fig. 7B). In addition, most of the differentially-expressed genes after 8 days *in vitro* are still differentially regulated at day 10.

We ran further pathway analysis of the 468-gene set differentially regulated in NASH conditions. Interestingly, among the top 18 enriched pathways, genes involved in hepatic fibrosis/hepatic stellate cell activation were found to be modulated, and this pathway was ranked in third position (Fig. 7C). The first two most strongly modulated gene clusters are associated with the activation of immune cell adhesion and the inflammatory process via the diapedesis pathways (Fig. 7C).

More precisely, genes encoding for claudin adhesion proteins, together with members of the immunoglobulin superfamily, such as ICAM1, ICAM2 and VCAM1, were up-regulated (Fig. 8). Other additional up-regulated inflammation/immune pathways included interferon signaling, IRF activation, tumor necrosis factor-receptor, and IL17 signaling.

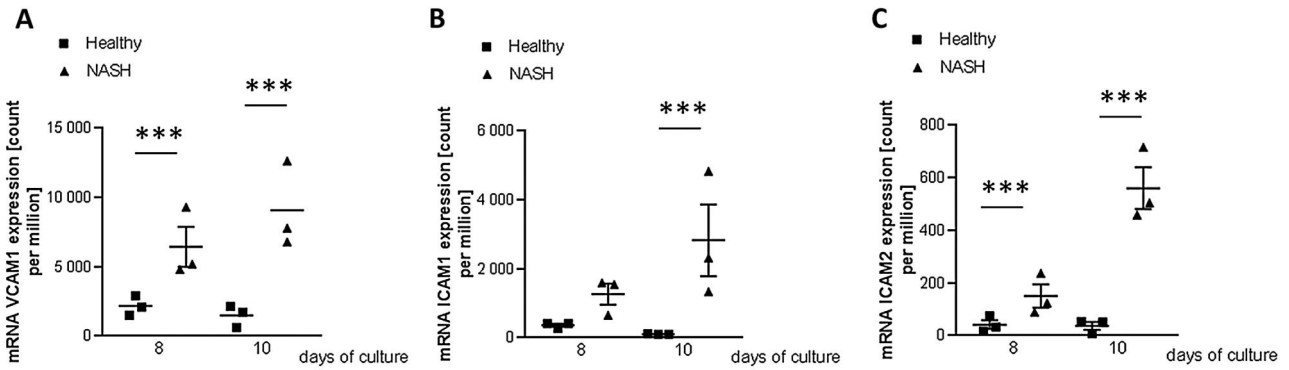

Fig. 8. mRNA expression level.

The values are expressed in counts per million at days 8 and 10 in the healthy and NASH conditions, with a scatter plot representation for VCAM1 (A), ICAM1 (B) and ICAM2 (C). *p<0.050, **p<0.010, ***p<0.001, DESeq2 test.

In conclusion, global gene expression analysis showed similar results to biochemical analysis. Together with the steatosis pathways observed under healthy conditions and maintained in the NASH 3D coculture, inflammation and early induction of fibrosis were shown to be induced in the human primary 3D liver NASH model.

Discussion

In our present study, we have set up an *in vitro* 3D NASH model by coculturing four human primary liver cell types, including PHH, HSCs, LECs, and KCs. The major challenge prior to this had been to develop a medium able to maintain these four primary cell types in culture, during an extended period of time, and to preserve the PHH mature phenotype.

Our custom Liver CoCulture media, optimized for 3D coculture, enabled us to successfully ensure cell viability up to 2 weeks *in vitro*. Transcriptomic analyses performed at day 10 indicated that PHH were still polarized and functional, with the detection of ABCC2, ABCB11, CYP3A4, and CYP7A1 gene expression.

In NASH, injured steatotic hepatocytes induce an inflammatory environment, leading to HSC activation and fibrosis. Since this mechanism results from hepatocyte ballooning, reflecting cell death, and the 3D NASH model requires a sustained viability to perform analysis, an inflammatory stimulus was provided by adding tumor necrosis factor-α cytokine to the NASH culture media. PHH injury was mainly characterized by a decrease of CYP3A4 activity and mRNA expression at day 10.[24]

Intriguingly, the 3D liver coculture exhibited high basal levels of triglycerides, as well as significant lipid droplet staining related to steatosis. An enrichment of pathways governing HSC activation and promoting early fibrosis was also observed. Thus, the human 3D liver coculture model displays steatosis and an activation of HSC leading to fibrosis, which are two main characteristics of NAFL/NASH. This model is able to respond to the inflammatory environment and to enhance the expression of proinflammatory cytokines, like IL6, as described in the literature.[25,26] Several previous studies have associated an increased CCL2 chemokine with steatohepatitis in chronic hepatic injury through an enhancement of proinflammatory monocyte/macrophage influx in the liver.[27–30] Thus, CCL2 is thought to link steatosis and inflammation, and accordingly, its expression is up-regulated in the human 3D NASH model.

An increase of CXCL8 and CXCL10 was also observed in the 3D liver NASH model, confirming the induction of an inflammatory environment, which is a key characteristic of NASH disease.[31] As previously described, CXCL8 could be a marker of NASH combined with diabetes.[32] Recently, Zimmermann *et al.*[33] established a positive correlation between CXCL8 mRNA expression and liver fibrosis stages, showing that a high level of transcript is found in a mouse model of severe F4 fibrosis; furthermore, an up-regulation of CXCL8-binding CXCR1 and CXCR2 receptors was positively correlated with chronic liver diseases. CXCL10 cytokine expression has also been correlated with fibrosis score.[34] The pivotal role of CXCL10 in NASH has also been shown *in vivo* using CXCL10-deficient mice, since a decrease in liver steatosis, injury, inflammation and fibrosis have been reported in this model compared to wild-type animals.[35]

The increase of CXCL10 mRNA and secreted protein in 3D coculture maintained in the NASH-like environment, together with the key role of CXCL10 in NASH disease reported in the literature, underscores the relevance of our human 3D NASH model.[35,36] Fibrogenesis is known to be associated with the synthesis and the activity of matrix metalloproteases that regulate ECM turnover during hepatic fibrosis. Among them, MMP2 and MMP9 are induced in fibrotic livers and are involved in the early disruption of the ECM in "pathologic" liver. Moreover, up-regulation of MMP2 has been identified in human fibrotic liver, whereas MMP9 induction has been highlighted in the rodent NASH model.[37–40] Phenotypes that manifest later in the disease lifetime, such as collagen formation, have not been observed in this model because of the limited duration of the coculture.

The translatability of the model is further strengthened by the differential regulation of the serum endothelial dysfunction markers, ICAM-1 and VCAM-1. These two markers are of major interest to validate the induced fibrosis in the NASH model. Indeed, ICAM-1 has been found to be significantly higher in serum from NASH patients compared to serum of NAFL and healthy patients,[41] supporting the role of ICAM-1 as a potential disease progression biomarker. Regarding VCAM-1, it has also been recently validated as an accurate biomarker of fibrosis in NASH patients.[42] Furthermore, ASK1/MAP3K5

Table 1. Comparison of characteristics of several models

Characteristics	Models				
	Liver-on-a-chip[49]	Spheroids[50,51]	Hemoshear model (Transwell)[15]	3D Insight™ Human Liver Microtissues (spheroids)[48]	Our 3D model
Cell types	HepG2 cell line	PHH and small amounts of KCs and HSCs	PHH, MΦ and HSCs	PHH, KCs, HSCs and LECs	PHH, KCs, HSCs and LECs
Stability time	8 days	35 days	10 days	9 days	10 days
Hepatocyte integrity	✓	✓	✓	✓	✓
Steatosis induction	✓	✓	✓	✓	✓
Inflammation induction	*x*	IL6 secretion only	✓	✓	✓
Fibrosis induction	*x*	*x*	✓	✓	✓
Disease induction	NAFL	NAFL	NASH	NASH	NASH
NASH markers	*x*	*x*	*x*	*x*	✓

has been largely described in the literature as a pharmacological target for NASH disease treatment[43–45] and, interestingly, its up-regulation is observed in our human 3D NASH model (Supplementary Table 2).

Finally, PD-L1 (CD274) gene expression was shown to be enhanced in the human 3D NASH model, and this finding is relevant for the induction of fibrosis. An up-regulation of the PD-L1 gene has been described previously in injured liver and associated with HSC immunomodulatory activity[46] and hepatocyte damage leading to inflammatory processes.[47] The induction of PD-L1 and PDCD1LG2 in the human 3D NASH model suggests that this pathway could qualify as an attractive avenue for NASH treatment.

Several 3D models for NASH have been developed and a recent comprehensive comparison was reported by Oseini *et al.*[14] Most of these models are used to measure drug toxicity and clearance but do not mimic disease progression. Few 3D culture systems have been set up for NAFLD disease modeling. The main features are summarized in Table 1. For example, liver-on-a-chip and spheroid technology have been used with cell lines or primary cell types to induce steatosis and recapitulate NAFLD. Two different commercial technologies, the Transwell™ from Hemoshear device and 3D Insight™ Human Liver Microtissues from InSphero (Table 1, rows 3 & 4), are comparable to our model in terms of model viability and NASH features. However, in the Transwell model, it is necessary to differentiate monocytes in macrophages. Furthermore, the use of a microfluidic system hampers its application for screening purposes. More recently, Mukherjee *et al.*[48] has developed a 3D model using 3D Insight Human liver microtissues that is the closest to the one reported in this paper. With the four human primary cell types, they described NASH features and use reference molecules to reverse the disease. However, transcriptional characterization was not reported. In our model, the translatability, as typified by NASH transcriptional markers, is more thoroughly defined. Our model is also distinguished by the fact that embedded endothelial cells were used. These cells have been shown to play a pivotal role in NAFLD/NASH progression from the simple steatosis to the early NASH stage probably by activating HSCs and KCs.[16,18] It has been suggested that coculture of primary human HSCs and LECs from cirrhotic livers promote fibrillar collagen production by HSCs. In addition, it is thought that liver sinusoidal endothelial cells injuries in the early stage of NAFLD are necessary for the activation of KCs and HSCs, and therefore for the NAFLD/NASH progression.

In conclusion, we have developed a new 3D NASH model with four human primary cell types, which include liver sinusoidal endothelial cells.

Optimization of the model, by using microfluidic devices, could be an option to increase cell survival and to mimic chronic disease, but it will likely not be suitable for screening purposes. Our 3D NASH model could be maintained for 10 days *in vitro* and showed triglyceride content leading to steatosis, an inflammatory response and activation of fibrosis-related pathways that are also associated with NASH. The relative extended life time of the 3D model culture makes it an attractive platform to evaluate preventive and curative treatments with drug candidates. These experiments will be reported in due course.

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Conflict of interest

All of the authors except TS were employees of Sanofi during the course of the study, and all are or have been shareholders of Sanofi.

Author contributions

Study design, data acquisition, and biochemical data analysis (AJ, LH), transcriptional data acquisition and analysis (SR, MDB, CR), imaging data (GH), critical revision of the manuscript (XV and ZB), statistical analysis (TS, VM), analyzing and interpreting data, critical revision of the manuscript, and funding acquisition (JCG, MD, AK, CO, VM), study supervision, study design, data acquisition analysis and interpretation, and drafting the manuscript (MD, ACLF).

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Original Article

Association of TCF7L2 rs7903146 Gene Polymorphism with the Risk of NAFLD and CAD in the Chinese Han Population

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Abstract

Background and Aims: Coronary artery disease (CAD) is a major cause of morbidity and mortality in patients with non-alcoholic fatty liver disease (NAFLD). Previous studies have suggested that *TCF7L2* rs7903146 was related to the risk of developing NAFLD but the conclusions are not consistent and no related study has been conducted in Chinese populations. The aim of this study was to investigate the association between *TCF7L2* rs7903146 and the risk of developing NAFLD and CAD in a Chinese Han population. ***Methods:*** *TCF7L2* rs7903146 genotypes were measured by the MALDI-TOF-MS from 143 NAFLD patients, 159 CAD patients, 131 NAFLD + CAD patients, and 212 healthy controls. The demographic data and serum lipid profiles of all subjects were collected. The distributions of genotype and allele frequency in each group were also tested. Logistic regression was used to investigate the risk of *TCF7L2* rs7903146 with NAFLD and CAD. All statistical analyses were conducted using SPSS 23.0. ***Results:*** There were no significant differences in the distributions of *TCF7L2* rs7903146 genotype and allele frequency in each of the two groups, and the *TCF7L2* rs7903146 CT + TT genotype did not increase the risk of developing NAFLD, CAD, and NAFLD + CAD. Except for body mass index in the control group, the differences of clinical parameters between the *TCF7L2* rs7903146 T allele carriers and non-carriers in each group were not significant. In the non-obese group, the *TCF7L2* rs7903146 CT + TT genotype was a protective factor for the development of NAFLD in the non-obese subjects (odds ratio=0.359, 95% confidence interval: 0.134-0.961, p = 0.041). ***Conclusions:*** *TCF7L2* rs7903146 was not associated with the risk of developing NAFLD, CAD, and NAFLD + CAD in the Chinese Han population. In the non-obese population, the *TCF7L2* rs7903146 CT + TT genotype was a protective factor against the development of NAFLD.

Keywords: Nonalcoholic fatty liver disease; Coronary artery disease; *TCF7L2*; Single nucleotide polymorphism.

Abbreviations: ALP, alkaline phosphatase; BMI, body mass index; CAD, coronary artery disease; FPG, fasting plasma glucose; GGT, gamma-glutamyltransferase; GIP, glucose-dependent insulinotropic polypeptide; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NAFLD, non-alcoholic fatty liver disease; SD, standard deviation; TBIL, total bilirubin; TC, total cholesterol; TCF-4, transcription factor-4; TG, triglyceride.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is the clinicopathological syndrome, characterized by excessive intracellular fat deposition that occurs with the exclusion of alcohol and other specific liver damaging factors, such as viruses, drugs, autoimmune factors, and genetic factors.[1] According to the clinical statistic results, coronary artery disease (CAD) causes the highest mortality in the patients with NAFLD, and the incidence of CAD in patients with NAFLD has increased significantly in recent years.[2,3] A previous study showed that NAFLD can act as one of the independent risk factors for CAD, after excluding hypertension, obesity, diabetes, and other factors.[4] Both NAFLD and CAD are influenced by the interaction of genetic and environmental factors, and they share some pathological mechanisms, such as obesity, insulin resistance, dyslipidemia, inflammation, and oxidative stress.[3,5,6] A large number of studies have investigates the role of NAFLD in the development of CAD. NAFLD was reported to be related to the formation of coronary artery plaques and impaired coronary blood flow reserve.[4,7,8] In addition, genetic pathogenesis in NAFLD and CAD is drawing more attention, and the gene polymorphisms of *ADIPOQ*, *LEPR*, *APOC3*, *PPAR*, *SREBP*, *TM6SF2*, and *MTTP* have been reported as risk factors for the development of NAFLD and CAD.[5]

TCF7L2 is located on chromosome 10q25 and encodes the transcription factor-4 (TCF-4) (also known as lymphocyte factor-4), which is an important member of the transcription factor family.[9–11] The TCF-4 family can participate in a variety of physiological pathways in different types of cells.[12,13] In 2009, Musso *et al.*[14] first reported that *TCF7L2* rs7903146 T allele frequency was higher in NAFLD patients than in healthy controls. In addition, they found that *TCF7L2* rs7903146 T allele carriers possessed higher levels of low-density lipoprotein (LDL), very low-density lipoprotein, triglyceride (TG), and cytokeratin 18 than non-carriers in the NASH subgroup. Subsequently, Giovanni *et al.*[15] reported that *TCF7L2* rs7903146 T allele carriers had the prolonged elevation of post-meal glucose-dependent insulinotropic polypeptide (GIP). The elevated GIP was demonstrated in other studies

to exacerbate liver steatosis and increase the levels of postprandial resistin and free fatty acids.[16,17] Besides, some evidence has suggested that *TCF7L2* rs7903146 is associated with serum lipid indexes, including TG, total cholesterol (TC), LDL, and apolipoprotein B, all of which are associated with risk of CAD development.[18,19] Corella *et al.*[20] demonstrated that the relationship between *TCF7L2* rs7903146 and serum lipid profiles was influenced by adherence to the MedDiet. When adherence was low, the levels of TC and LDL were higher in TT carriers than CT/CC carriers. But when adherence was high, no difference was observed between TT carriers and CT/CC carriers. Up to now, the association of *TCF7L2* rs7903146 with the risk of developing NAFLD and CAD was investigated only in a limited amount of studies, and no related report was available in Chinese populations. In addition, the effect of *TCF7L2* rs7903146 polymorphism on serum lipid profiles is still unclear.[21–23] In consideration of the increased incidence of NAFLD and CAD in China, illuminating the association of *TCF7L2* rs7903146 with the risk of NAFLD and CAD in Chinese may provide some new ideas for the treatment of NAFLD and CAD.

The aim of this study was to investigate the relationship of *TCF7L2* rs7903146 with the risk of developing NAFLD and CAD in a Chinese Han population, and explore the effect of *TCF7L2* rs7903146 on the serum lipid profiles.

Methods

Subjects

This study was conducted according to the principles of the declaration of Helsinki and its appendices.[24] Subjects were composed of 143 patients with NAFLD, 159 patients with CAD, 131 patients with NAFLD + CAD, and 212 healthy controls, who were enrolled at the Qingdao Municipal Hospital (China) from December 2017 to December 2018. The Guidelines for the Diagnosis and Treatment of Non-alcoholic Fatty Liver Disease (2010 Revision) and the Guidelines for the Diagnosis and Treatment of Acute ST-Segment Elevation Myocardial Infarction (2015 Version) were used as exclusion and inclusion criteria for NAFLD and CAD. Patients with NAFLD were included from the Department of Gastroenterology, in a random manner, and diagnosed by B-type ultrasonography. Combining the medical history and laboratory testing, subjects with alcoholic fatty liver, viral hepatitis, autoimmune liver disease, drug-induced liver disease and hereditary metabolic disease were excluded. Patients with CAD were included from the Department of Cardiology, in a random manner, and diagnosed by coronary angiography. We divided the CAD patients into with or without NAFLD groups, respectively. The healthy controls were included from the Physical Examination Center of the Qingdao Municipal Hospital, in a random manner. All the subjects signed informed consent forms after participating in this study.

Biochemical analyses

The demographic data (gender, age, height and weight) were obtained by questionnaire. The body mass index (BMI) was calculated by weight (kg)/height $(m)^2$. Fasting venous blood samples of each subject were collected after a 12-h overnight fasting and kept in ethylene diamine tetraacetic acid-containing tubes. The biochemical data, such as TG, TC, fasting plasma glucose (FPG), high-density lipoprotein (HDL), LDL, alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), and total bilirubin (TBIL) were measured through standard laboratory method.

Genomic DNA extraction and genotyping

Genomic DNA was extracted from each blood sample and stored at -80°C until use. The primers for PCR amplification were designed and synthesized by Bomiao Biotechnology (Beijing, China) as: 5′-ACGTTGGATGAACTAAGGGTGCCTCATACG-3′ and 5′-ACGTTGGATGGCCTCAAAACCTAGCACAGC-3′. The detailed process of PCR amplification consists of the following steps: an initial denaturation at 94°C for 5 m, followed by 45 cycles of denaturing at 94°C for 20 s, annealing at 56°C for 30 s, and extending at 72°C for 1 m. After that, a final extension at 72 °C was conducted for 5 m. The genotype *TCF7L2* rs7903146 was detected by DNA sequence using the MALDI-TOF-MS (MassARRAY System; Agena Bioscience, Shanghai, China) and raw data was acquired by TYPER4.0.

Statistical analysis

The measurement data were expressed as mean ± standard deviation (SD) for those conforming to the normal distribution, otherwise as median quartile. Homogeneity of variance was tested between each of two groups. The independent samples *t*-test was used for data conforming to normal distribution with homogeneity variances, while the rest were tested by rank sum test. Chi-square test was used to analyze the gender distribution differences. Hardy-Weinberg equilibrium was performed by chi-square test. The distribution of genotypes and allele frequency between two groups were also tested by chi-square. The correlation between genotype and risk of diseases were estimated by binary logistic regression modeling. The demographic and biochemical data of different genotypes were tested by independent sample *t*-test and rank sum test. A *p*-value of <0.05 was considered to indicate statistical significance. All the statistical analyses were conducted by SPSS 23.0 software (IBM Corp., Armonk, NY, USA).

Results

Demographic and biochemical characteristics of subjects

A total of 457 subjects were included in this study. As the results show in Table 1, differences of gender distribution were observed between the NAFLD group and control group, and between the NAFLD + CAD group and the control group. The NAFLD group had higher levels of BMI, TC, TG, LDL, alanine aminotransferase, GGT and FPG than the control group, while age and HDL level were lower in the NAFLD group compared to the control group (all $p < 0.05$). No significant differences were observed for aspartate aminotransferase, ALP and TBIL between the NAFLD group and the control group. Age, BMI and levels of TG, alanine aminotransferase, ALP, GGT, and FPG in the CAD group were high compared to those in the control group, and the levels of TC, HDL, and LDL were low in the CAD group compared to those in the control group (all $p < 0.05$). There were higher age, BMI, and levels of TG, alanine aminotransferase, ALP, GGT and FPG, and lower levels of TC, HDL and LDL in the NAFLD + CAD group compared to the control group (all $p < 0.05$). The NAFLD + CAD group had higher age and levels of ALP and FPG, and lower BMI and levels of HDL, LDL, alanine aminotransferase and TC than the NAFLD group (all $p < 0.05$).

Table 1. Demographic and biochemical characteristics of subjects

	Control group, n = 212	NAFLD group, n = 143	CAD group, n = 159	NAFLD + CAD group, n = 131	p_1	p_2	p_3	p_4
Male (%)	121 (57.06)	100 (69.93)	96 (60.38)	90 (68.70)	0.014	0.523	0.032	0.826
Age (yr)	47 (41-57.75)	42 (37-45)	66 (61-74)	62 (55-67)	<0.001	<0.001	<0.001	<0.001
BMI (kg/m^2)	23.90 (21.82-25.91)	26.61 ± 5.50	25.28 ± 3.25	25.19 ± 2.54	<0.001	<0.001	<0.001	<0.001
TC (mmol/L)	5.11 (4.59-5.74)	5.48 ± 0.82	4.64 ± 1.28	4.31 (3.76-5.54)	<0.001	<0.001	<0.001	<0.001
TG (mmol/L)	1.12 (0.87-1.69)	1.54 (1.16-2.20)	1.43 (1.03-1.85)	1.46 (1.01-2.18)	<0.001	0.002	<0.001	0.328
HDL (mmol/L)	1.30 ± 0.29	1.21 (1.08-1.35)	1.01 (0.86-1.16)	1.02 (0.85-1.17)	0.031	<0.001	<0.001	<0.001
LDL (mmol/L)	3.10 (2.67-3.58)	3.25 ± 0.61	2.64 (2.09-3.41)	2.64 (2.12-3.43)	0.047	<0.001	<0.001	<0.001
ALT (U/L)	18.28 (12.87-25.87)	23.80 (18.42-43.65)	20.73 (14.72-31.36)	22.67 (15.63-33.44)	<0.001	0.015	0.001	0.021
AST (U/L)	20.79 (18.15-24.68)	21.64 (18.33-26.78)	21.70 (16.99-31.08)	22.17 (16.80-32.72)	0.139	0.335	0.107	0.659
ALP (U/L)	71.17 ± 20.35	68.63 (58.99-83.39)	79.78 ± 22.87	82.50 (70.52-98.63)	0.785	<0.001	<0.001	<0.001
GGT (U/L)	22.12 (16.19-30.21)	30.63 (20.77-47.73)	26.35 (18.16-36.84)	27.70 (18.54-39.98)	<0.001	0.001	<0.001	0.084
TBIL (μmol/l)	13.20 (9.43-16.88)	11.9 (9.9-14.9)	13.50 (10.20-18.28)	13.10 (10.48-16.30)	0.398	0.169	0.448	0.111
Glu (mmol/L)	4.48 (4.04-4.96)	4.79 (4.50-5.18)	5.20 (4.55-6.43)	5.42 (4.75-6.40)	<0.001	<0.001	<0.001	<0.001

Data are expressed as mean ± standard deviation for those conforming to the normal distribution, otherwise as median quartile.

p_1: NAFLD group vs. control group; p_2: CAD group vs. control group; p_3: NAFLD + CAD group vs. control group; p_4: NAFLD + CAD group vs. NAFLD group. $p < 0.05$ was considered statistically significant.

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CAD, coronary artery disease; GGT, gamma-glutamyltransferase; Glu, glucose; HDL, high density lipoprotein; LDL, low density lipoprotein; NAFLD, nonalcoholic fatty liver disease; TBIL, total bilirubin; TC, total cholesterol; TG, triglyceride.

TCF7L2 rs7903146 genotype and allele frequency distribution

The genotype frequency distribution of *TCF7L2* rs7903146 was in accordance with Hardy-Weinberg equilibrium in each group (all $p > 0.05$) (Table 2). As the results show in Table 3, there was no significant difference in the genotype distribution of *TCF7L2* rs7903146 (CC vs. CT + TT) between the NAFLD group and the control group, the CAD group and the control group, the NAFLD + CAD group and the control group, and the NAFLD + CAD group and the NAFLD group (all $p > 0.05$). In addition, the difference of *TCF7L2* rs7903146 allele frequency distribution in each group was also not significant (all $p > 0.05$). When we analyzed the association of *TCF7L2* rs7903146 genotype and the risk of NAFLD, CAD, and NAFLD + CAD, the results suggested that the CT + TT genotype did not increase the risk of developing NAFLD, CAD, and NAFLD + CAD in the general population, and the CT + TT genotype also did not increase the risk of developing CAD in the patients with NAFLD (Table 4).

Association of TCF7L2 rs7903146 genotype with the risk of NAFLD in the non-obese subjects

The difference of clinical parameters between the T allele carriers and non-carriers in each group was analyzed, and no significant difference was observed in the NAFLD group, the CAD group, and the NAFLD + CAD group (data not shown). However, the BMI of the T allele carriers was lower than in non-carriers in the control group ($p = 0.027$). To investigate the potential relationship of *TCF7FL2* rs7903146 genotype with the BMI value, we divided the whole subject population into two groups: obese subjects (BMI ≥25) and non-obese subjects (BMI <25). In the obese group, no significant difference was observed for the genotype distribution of *TCF7L2* rs7903146 (CC vs. CT + TT) between the NAFLD patients and non-NAFLD patients. In the non-obese group, distribution of the CT + TT genotype and CC genotype was significantly different ($p = 0.035$) (Table 5). Logistic regression model analysis suggested that the CT + TT genotype was a protective factor against the development of NAFLD in the non-obese subjects (odds ratio=0.359, 95% confidence interval: 0.134-0.961, $p = 0.041$). After adjustment for gender and age, and serum lipids, the CT + TT genotype was still a protective factor against the development of NAFLD in the non-obese subjects (odds ratio = 0.245, 95% confidence interval: 0.072-0.837, $p = 0.025$) (Table 6).

Table 2. Result of Hardy-Weinberg Equilibrium analysis

Gene locus	Group	X^2	p
rs7903146	NAFLD group	0.39	0.53
	Control group	0.63	0.43
	CAD group	0.03	0.86
	NALFD + CAD group	0.07	0.81

*$p < 0.05$ was considered different.

Abbreviations: CAD, coronary artery disease; NAFLD, nonalcoholic fatty liver disease.

Table 3. Distribution of genotype and allele frequency of TCF7L2 rs7903146 in each group*

	Genotype frequency, *n* (%)						Allele frequency, *n* (%)					
	CC	CT + TT	p_1	p_2	p_3	p_4	C	T	p_1	p_2	p_3	p_4
NAFLD	125 (89.93)	14 (10.07)	0.93	0.28	0.06	0.08	263 (94.60)	15 (5.40)	0.90	0.22	0.06	0.06
Control	190 (89.62)	22 (10.38)					402 (94.81)	22 (5.19)				
CAD	134 (85.90)	22 (14.10)					289 (92.63)	23 (7.37)				
NAFLD + CAD	110 (95.65)	5 (4.35)					225 (97.83)	5 (2.17)				

p_1: NAFLD group vs. control group; p_2: CAD group vs. control group; p_3: NAFLD + CAD group vs. control group; p_4: NAFLD + CAD group vs. NAFLD group.
*SPSS 23.0 was used for testing and $p < 0.05$ was considered statistically significant.
Abbreviations: CAD, coronary artery disease; NAFLD, nonalcoholic fatty liver disease.

Table 4. Association between genotype and the risk of NAFLD, CAD, and CAD & NAFLD*

	OR (95% CI)	p_1	OR (95% CI)	p_2	OR (95% CI)	p_3	OR (95% CI)	p_4
CC	1	0.93	1	0.29	1	0.07	1	0.09
CT + TT	0.97 (0.48-1.96)		1.42 (0.75-2.67)		0.39 (0.15-1.07)		0.41 (0.14-1.16)	

p_1: NAFLD group vs. control group; p_2: CAD group vs. control group; p_3: NAFLD & CAD group vs. control group; p_4: NAFLD + CAD group vs. NAFLD group.
*Binary logistic analysis was used for testing.
Abbreviations: CAD, coronary artery disease; CI, confidence interval; NAFLD, nonalcoholic fatty liver disease; OR, odds ratio.

Table 5. Distribution of genotype frequency and allele frequency of TCF7L2 rs7903146 in obese and non-obese subjects*

	NAFLD group, *n* (%)	Non-NAFLD group, *n* (%)	X_2	*p*
Obese				
Genotype				
CC	151 (91.5)	149 (90.9)	0.045	0.832
CT + TT	14 (8.5)	15 (9.1)		
Allele				
C	316 (95.76)	313 (95.43)	0.043	0.836
T	14 (4.24)	15 (4.57)		
Non-obese				
Genotype				
CC	84 (94.38)	175 (85.78)	4.466	0.035
CT + TT	5 (5.63)	29 (14.22)		
Allele				
C	171 (96.07)	378 (92.65)	2.451	0.117
T	7 (3.93)	30 (7.35)		

*SPSS 23.0 was used for testing.
$p<0.05$ was considered a statistically significant difference.
Abbreviation: NAFLD, nonalcoholic fatty liver disease.

Discussion

Genetic factors significantly participate in the development and progression of NAFLD and CAD.[25,26] In this study, we investigated the relationship of *TCF7L2* rs7903146 with the risk of developing NAFLD and CAD in a Chinese Han population. As the results showed, there were no differences in the *TCF7L2* rs7903146 genotype and allele distribution between the NAFLD group and the control group, the CAD group and the control group, the NAFLD + CAD group and the control group, and the NAFLD + CAD group and the NAFLD group. In addition, the CT + TT genotype did not increase the risk of

Table 6. Association of TCF7L2 rs7903146 genotype and NAFLD in non-obese subjects*

	Unadjusted			Adjusted*	
	OR (95% CI)	*P*		OR (95% CI)	*P*
CC	1	0.041	CC	1	0.025
CT + TT	0.359 (0.134-0.961)		CT + TT	0.245 (0.072-0.837)	

*Binary logistic regression model was adjusted for age, gender, TC, TG and LDL.

Abbreviations: CI, confidence interval; LDL, low-density lipoprotein; NAFLD, nonalcoholic fatty liver disease; OR, odds ratio; TC, total cholesterol; TG, triglyceride.

developing NAFLD, CAD, and NALFD + CAD in the general population, and did not increase the risk of developing CAD in the patients with NAFLD. However, the *TCF7L2* rs7903146 CT + TT genotype was a protective factor for NAFLD in the non-obese subjects.

As a component of the Wnt/beta-catenin signaling pathway, TCF7L2 participates in a variety of physiological pathways, such as cell proliferation, differentiation, apoptosis, and oxidative stress, all the effect of TCF7L2 which can be exerted throughout growth, zoning, heterogenesis, and other intrinsic metabolism processes of liver.[27–29] The relationship of TCF7L2 with metabolism was first found by Grant *et al.*,[30] who found that the microsatellite of *TCF7L2* intron 3, DG10S478, was associated with type 2 diabetes in an Icelandic population. The gene's expression product, a high-mobility family box, contains transcription factors that influence blood glucose homeostasis. Subsequent studies revealed several potential functions of TCF7L2, including decreasing insulin secretion, impairing incretin effect, and increasing insulin resistance.[31–33] In addition, *TCF7L2* rs7903146 has been reported to be relevant to lipid disorders, NAFLD, obesity and hypertension.[14,18,34] Corella *et al.*[20] reported the correlation between *TCF7L2* rs7903146 and multiple lipid indexes, but the potential molecular mechanism by which *TCF7L2* rs7903146 induces abnormal hepatic metabolism remains unknown.

Some researchers reported that *TCF7L2* rs7930146 can regulate open chromatin and genetic transcription in pancreas, thus increasing the risk of diabetes.[31,35] In addition, Dorota *et al.*[36] found that the expression of TCF7L2 in liver may be regulated by weight loss. However, the evidence of how *TCF7L2* rs7903146 participates in hepatic lipid metabolism remains unclear. Musso *et al.*[14] found that the *TCF7L2* rs7903146 CT/TT genotype was a risk factor for NAFLD. In that study, the results indicated that the *TCF7L2* rs7903146 polymorphism did not associate with the risk of developing NAFLD, CAD, or NAFLD + CAD in the general population, and did not associate with the risk of developing NAFLD + CAD. Except for BMI in the control group, the differences of clinical parameters between the *TCF7L2* rs7903146 T allele carriers and non-carriers in each group were also not significant. Further analysis found that the distribution of the CT + TT genotype and the CC genotype was significantly different between the NAFLD patients and non-NAFLD patients in the non-obese group. After adjustment for gender and age, and serum lipids, the CT + TT genotype was still a protective factor for the development of NAFLD in the non-obese subjects. Previous study determined that the minor allele frequency of the rs7903146 T allele varied according to the difference of region (the minor allele frequencies in Vietnam, Estonia, Sweden, Caucasia, India, East Asia were 4.63, 19.82, 22.5, 29, 28.1, and 3.2, respectively).[37] In this study, the minor allele frequency of rs7903146 was 4.63, which was similar to the minor allele frequency of East Asia. Development of NAFLD was affected by ethnic, genetic, dietary, and environmental factors; therefore, the difference of the effect of *TCF7L2* rs7903146 polymorphism on the risk of developing NAFLD in Chinese and other countries may be affected by ethnic and genetic factors.

There were several limitations in this study. First, all the NAFLD patients were diagnosed by ultrasound rather than the liver biopsy; therefore, the diagnosis of NAFLD may not be very accurate. Second, the sample size was relatively small, which may affect the conclusion. Third, our conclusion cannot reflect the association between *TCF7L2* rs7903146 polymorphism and the risk of developing NAFLD in other countries, due to all the subjects in this study being of the Chinese Han population.

Conclusions

This study investigated the relationship of *TCF7L2* rs7903146 polymorphism with the risk of NAFLD, CAD, and NAFLD + CAD in a Chinese Han population, for the first time. The results suggest that *TCF7L2* rs7903146 was not associated with the risk of developing NAFLD, CAD, and NAFLD + CAD in our Chinese Han population, and *TCF7L2* rs7903146 did not affect the serum lipid metabolism. In the non-obese subpopulation, the *TCF7L2* rs7903146 CT + TT genotype was a protective factor for the development of NAFLD. More subjects of other ethnicity should be included to further investigate the association of *TCF7L2* rs7903146 with the risk of NAFLD, and the potential mechanism underlying the *TCF7L2* rs7903146 variant's affect the lipid metabolism should be illustrated.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study concept and design (YX and SL), acquisition of the data (XY, WJ, JZ, and MW), analysis and interpretation of the data (YX and WJ), drafting of the manuscript: (XY), critical revision of the manuscript for important intellectual content (YX and LS), supervision (YX). All the authors read and approved the final manuscript.

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Original Article

Clinical Outcome Event Adjudication in a 10-Year Prospective Study of Nucleos(t)ide Analogue Therapy for Chronic Hepatitis B

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Abstract

Background and Aims: In the REALM (Randomized, Observational Study of Entecavir to Assess Long-Term Outcomes Associated with Nucleoside/Nucleotide Monotherapy for Patients with Chronic HBV Infection) study, 12,378 patients with chronic hepatitis B virus (HBV) infection received up to 10 years of randomized therapy with entecavir or another HBV nucleos(t)ide analogue. Monitored clinical outcome events (COEs) included malignant neoplasms, HBV disease progression events, and deaths. An external event adjudication committee (EAC) was convened to provide real-time review of reported COEs to optimize data quality, and minimize potential adverse effects of the large cohort, interdisciplinary outcome assessments, geographic scope, and long duration. **Methods:** The EAC comprised an international group of hepatologists and oncologists with expertise in diagnosis of targeted COEs. The EAC reviewed and adjudicated COEs according to prospectively defined diagnostic criteria captured in the EAC charter. Operational processes, including data collection and query procedures, were implemented to optimize efficiency of data recovery to maximize capture of adjudicated COEs, the primary study outcome measure. **Results:** A total of 1724 COEs were reported and 1465 of these events were adjudicated by the EAC as reported by the investigators (85.0% overall concordance). Concordance by COE type varied: deaths, 99.6%; hepatocellular carcinoma (HCC), 83.3%; non-HCC malignancies, 88.0%; non-HCC HBV disease progression, 68.2%. Reasons for lack of concordance were most commonly lack of adequate supporting data to support an adjudicated diagnosis or evidence that the event pre-dated the study. **Conclusions:** The REALM EAC performed a critical role in ensuring data quality and consistency; EAC performance was facilitated by well-defined diagnostic criteria, effective data capture, and efficient operational processes. **Trial registration:** ClinicalTrials.gov NCT00388674.

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Introduction

Chronic hepatitis B virus (HBV) infection (CHB) remains a global health challenge, with an estimated 240 to 400 million infected individuals worldwide.[1–3] HBV nucleos(t)ide analogues (NUCs) introduced over the past two decades can help reduce this burden of disease; suppression of viral replication with HBV NUCs for 3-5 years can reverse HBV-associated liver fibrosis and reduce the incidence of hepatocellular carcinoma (HCC).[4–6]

Entecavir (ETV) is a third-generation NUC approved for treating hepatitis B e-antigen (HBeAg)-positive, HBeAg-negative, and lamivudine-experienced adults with CHB, based on phase 3 results demonstrating histologic, virologic, and biochemical benefits.[7–9] Virologic breakthrough was rare after 5 years of ETV treatment in NUC-naive patients; consistent with this durable antiviral effect, HBV disease progression was reduced.[10–12] ETV safety was favorable in randomized trials and long-term follow-up studies; no association between ETV and risk of specific adverse events was identified with therapy of up to 5 years.[13] However, in 2-year preclinical carcinogenicity studies, benign and malignant tumors involving lung, liver, and brain were observed in ETV-exposed mice and rats.[14] Excepting lung tumors, which were limited to male mice, rodent tumors occurred only at significantly higher

Keywords: Hepatitis B; Antiviral therapy; Clinical outcomes; Event adjudication; Liver cirrhosis.

Abbreviations: AASLD, American Association for the Study of Liver Diseases; CHB, chronic hepatitis B virus infection; CI, confidence interval; COE, clinical outcome event; CRF, case report form; CRO, clinical research organization; DRF, data request form; EAC, event adjudication committee; EASL, European Association for Study of the Liver; ETV, entecavir; HBV, hepatitis B virus; HBeAg, hepatitis B e-antigen; HCC, hepatocellular carcinoma; HR, hazard ratio; NUC, nucleos(t)ide analogue; REALM, Randomized, Observational Study of Entecavir to Assess Long-Term Outcomes Associated with Nucleoside/Nucleotide Monotherapy for Patients with Chronic HBV Infection; SAE, serious adverse event.

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ETV exposures than those occurring with approved doses in humans.

These findings prompted initiation of the REALM study ((Randomized, Observational Study of Entecavir to Assess Long-Term Outcomes Associated with Nucleoside/Nucleotide Monotherapy for Patients with Chronic HBV Infection; NCT00388674) of HBV-associated and non-HBV-associated clinical outcomes in adults with CHB who received ETV or non-ETV HBV NUCs for up to 10 years.[15,16] Planned enrollment was 12,500 patients in up to 500 research centers globally. To ensure data quality and consistency from this large and geographically diverse investigator group during the extended follow-up, an independent event adjudication committee (EAC) was established to review investigator-reported clinical outcome events (COEs). EACs have proven valuable in randomized trials in other fields, particularly those with substantial risk of misclassifying events that are crucial for study outcome assessment.[17–21] However, independent EACs have seldom been used in studies of liver disease; to our knowledge, REALM is the first HBV therapeutic study to utilize an EAC for evaluating COEs. Herein, we report the structure and processes utilized by the REALM EAC, the COEs submitted for EAC review, outcomes of their assessments, and key lessons learned.

Methods

Study design

Eligible patients were adults with HBeAg-positive or -negative CHB, who, in their physician's opinion, were eligible for monotherapy with an approved HBV NUC. Patients could be HBV treatment-naive or experienced, with or without compensated or decompensated cirrhosis, or coinfected with hepatitis C and/or hepatitis delta viruses. Patients were ineligible if coinfected with human immunodeficiency virus, had expected liver transplant-free survival of less than 1 year, a history of malignant neoplasm or dysplastic liver nodule, prior ETV use, or intention to receive interferon monotherapy.

Patients were randomly assigned 1:1 to receive ETV or other standard-of-care HBV NUC that was selected at each investigator's discretion and dosed per the product label. Concurrent interferon-alfa-2b or pegylated interferon-alfa-2a treatment was allowed. After receiving the first dose of study therapy, patients' HBV treatment regimens could be modified by switching to or adding alternate HBV NUC(s) or by terminating treatment altogether. Patient observation continued regardless of such modifications.

Data collection

After randomization, patients were followed for up to 10 years after enrollment of the first patient. With full enrollment anticipated to take 3 years, individual patients were expected to receive 7 to 10 years of follow-up. Participating sites monitored for and reported treatment-related serious adverse events (SAEs) and COEs; COEs included malignant neoplasms (non-HCC, HCC), deaths, and non-HCC liver-related manifestations of HBV disease progression, which included development or progression of compensated or decompensated cirrhosis. Patients were assessed for COEs through twice-yearly in-person visits and two interim telephone interviews. Although the specific means for evaluating patients was at the investigators' discretion, site guidance was provided regarding COEs to be monitored and diagnostic criteria.

EAC structure and review process

Due to the complexity of COE definitions and the unblinded study design, an EAC comprising two subcommittees (hepatology, oncology) was established to adjudicate all investigator-reported COEs per criteria outlined in the EAC charter (Supplemental Appendix). The EAC was co-chaired by a hepatologist and an oncologist and composed of experts in those fields; each member also served on the EAC subcommittee relevant to their clinical expertise. The hepatology subcommittee reviewed non-HCC liver-related events of HBV disease progression and liver-related deaths; the oncology subcommittee reviewed events of non-HCC malignant neoplasms and malignancy-related deaths. The subcommittees shared responsibility for reviewing new reports of HCC, dysplastic liver nodules, and non-liver/non-malignancy-related deaths. EAC members and the sponsor remained blinded to HBV therapies received throughout the study. Full committee meetings were initially held quarterly; however, due to increasing case volume, beginning in 2009, meetings were held every other month. Full committee meetings were teleconferences, except for one face-to-face meeting annually, which typically occurred before the American Association for the Study of Liver Diseases (commonly known as AASLD) or European Association for Study of the Liver (commonly known as EASL) meeting.

Each reported COE was reviewed independently by two subcommittee members, who recommended an adjudicated diagnosis (Fig. 1). With concordant reviews, the relevant Chair completed an adjudication case report form (CRF) which certified the adjudicated diagnosis and date of diagnosis, after which these data were entered into the clinical database. Discordant reviews prompted full committee review; the final decision was based on majority votes. The sponsor's Global Pharmacovigilance Group reviewed treatment-related SAE reports. If SAE details were consistent with a study-defined COE, the investigator was asked to re-report the event as a COE. If an event was reported as both an SAE and a COE, and subsequently confirmed to meet COE criteria, the SAE report was withdrawn.

Investigators reported events to the sponsor and/or a contracted clinical research organization (CRO) on standardized COE CRFs in the form of diagnostic questionnaires and COE workbooks. These documents formed the core contents of a case packet created for each reported COE and submitted for EAC review. The diagnostic questionnaire contained the reported COE and date of diagnosis; COE workbooks contained the investigator-reported event term, the primary evaluation method supporting the diagnosis (e.g. histology, imaging, laboratory assessments) and test date, and any secondary diagnostic information.

Following sponsor/CRO review of submitted CRFs, sites were contacted as necessary using standardized data request forms (DRFs) for further case information and source documents required for EAC review and adjudication. Specific DRFs were developed for each type of COE; DRFs requested case information, such as liver imaging or biopsy data for cirrhosis, ascitic fluid neutrophil counts for spontaneous bacterial peritonitis, or upper endoscopy results for variceal bleeding. The CRO provided an important link between

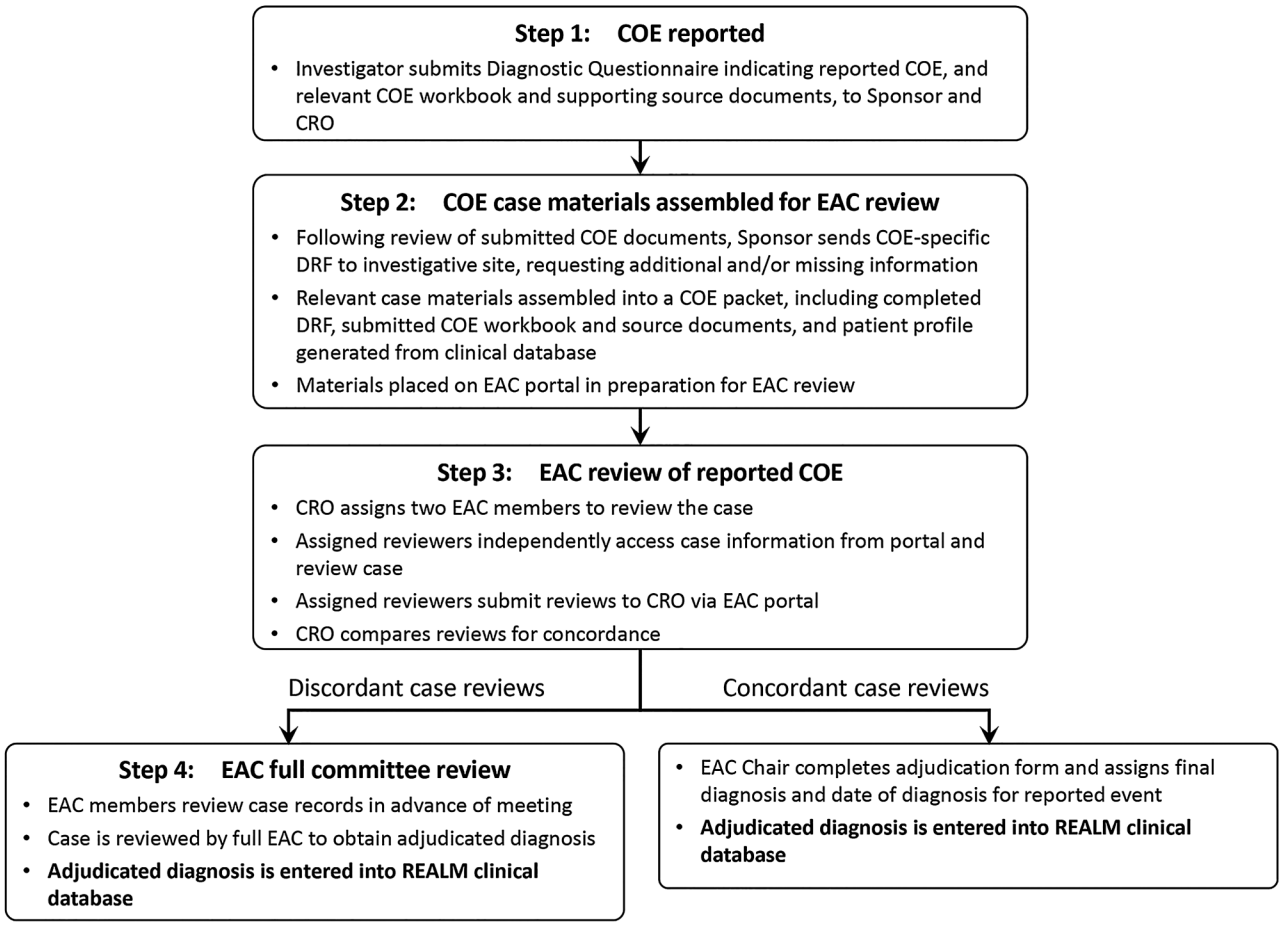

Fig. 1. Reporting and processing of COEs for adjudicated diagnosis. Flow chart indicates the standardized procedure developed for data collection and EAC adjudication. In addition, during steps 3 and/or 4, reporting sites were contacted as required to obtain further case information needed for adjudication, as described in step 2.

Abbreviations: COE, clinical outcome event; CRO, clinical research organization; DRF, data request form; EAC, event adjudication committee.

investigators and the EAC for acquisition of event documentation.

Additional COE capture methods for EAC adjudication

During the study, at least annually, the EAC reviewed evolving literature addressing HBV and HCC outcomes, including society practice guidelines, to assess the need to modify EAC charter diagnostic criteria. The charter was amended twice during the study, in October 2009 and May 2014. The first amendment added liver stiffness measurement by transient elastography (FibroScan®) as a supportive finding for diagnosis of cirrhosis. The second amendment allowed definitive diagnosis of cirrhosis based on FibroScan® findings alone, and HCC diagnosis based on detection of a characteristic focal lesion (arterial phase enhancement with venous phase washout) by a single contrast-enhanced, cross-sectional imaging procedure. Additional steps undertaken to maximize COE data capture are described below.

HCC events: Following the 2014 EAC charter amendment that resulted from updated AASLD practice guidelines for HCC,[22,23] the EAC reassessed investigator-reported HCC events that were assessed previously as "unable to adjudicate" using the original charter criteria. Reporting investigative sites were requested to provide any new relevant data, and the full EAC assessed whether the revised diagnostic criteria could now support an adjudicated diagnosis of HCC. Transarterial chemoembolism reports were also reviewed for relevant angiography findings, and dysplastic liver nodule reports were added to case packets of subsequently reported HCC events to support HCC diagnosis and timing.

Non-HCC HBV disease progression events: Initially, diagnosis of cirrhosis was based on liver histology and/or ultrasound. During the study, use of ultrasound-based elastography to assess liver fibrosis and cirrhosis became common practice. The EAC charter was modified twice, adding ultrasound-based elastography data as probable and definitive criteria for diagnosis of cirrhosis. However, the EAC did not retrospectively re-evaluate cirrhosis events that were previously assessed as "unable to adjudicate" because the technology was not used consistently before the charter amendment.

Deaths: The EAC assigned a primary cause of death to events with more than one investigator-reported cause and, to the extent permitted by supporting data, a primary cause

of death to events reported with an "unknown" cause. Most death reports were submitted with source documents, e.g. hospital discharge summaries and/or death certificates, allowing the EAC to affirm the death and assess the primary cause. However, some deaths were reported only with the death CRF and lacked source documents. Most such cases were reported by Asian sites, and the absence of source documents was often related to the death occurring at an outlying medical facility, where the investigator lacked attending privileges. In such cases, investigators were asked to verify the death in writing and, where possible, provide the most likely cause.

Non-HCC malignancy events: Some events of non-HCC malignancies were reported with general cancer diagnoses, even when supporting pathology reports allowed greater specificity regarding tumor type. To enhance the accuracy of data capture and analyses for these events, the EAC oncology chair and subcommittee members were trained on MedDRA coding of malignant and premalignant tumors. Heightened attention to investigator-reported diagnoses versus those on pathology reports and other source documents was reinforced. Secondarily, the sponsor independently reviewed investigator reports and adjudicated tumor diagnoses prior to addition to the study database. The EAC oncology chair was asked to reassess cases of concern, particularly histology data, and amend adjudicated diagnoses where appropriate.

Results

Patient disposition

Among the 12,485 randomized patients, 6216 and 6162 initiated treatment with ETV or a non-ETV nuc, respectively (Fig. 2).[15] Patients continued their randomly assigned treatment prior to switching to an alternate HBV treatment for a median 98.7 months (range: 0-114.4) with ETV or 94.0 months (range: 0-113.1) with a non-ETV NUC; median cumulative duration of either HBV therapy was 99.1 and 96.7 months, respectively. The results of the REALM study have been published elsewhere, and revealed no significant differences in clinical endpoints between ETV and non-ETV nucleos (t)ide analogue treatment, and virologic response independent of treatment group was associated with a reduced risk of liver-related HBV disease progression (hazard ratio (HR): 0.09, 95% confidence interval (CI): 0.038-0.221) and HCC (HR: 0.03, 95% CI: 0.009-0.113).[24]

COE outcomes

EAC-reviewed and -adjudicated COEs of liver-related HBV disease progression (HCC, non-HCC HBV disease progression, or liver-related death) were reported in 442 patients (7.1%) in the ETV group and 464 patients (7.5%) in the non-ETV group (Table 1).

Overall, 504 patients died, 502 of whom received an EAC-adjudicated diagnosis regarding cause of death; two events were assessed as unable to adjudicate due to lack of data verifying the death. Among these 502 patients, 328 (65%) were adjudicated with cause of death as reported by the investigator, and the remaining 174 (35%) were determined to have an alternate cause of death (Table 2). The most common causes of death in the ETV and non-ETV groups, respectively, were HCC (n=43 and 69), liver-related conditions (n=46 and 48), and non-HCC malignancies (n=17 and 15). Among the 179 adjudicated deaths with discrepant diagnoses between the reporting investigator and the EAC, most often the cause of death was reported as being due to a specific event such as HCC (n=66), a liver-related condition (n=30) or a malignancy (n=12) but was adjudicated as unknown due to inadequate evidence. The EAC adjudicated a specific cause of death for 6 patients with a reported unknown cause.

Among the 606 EAC-reviewed HCC events, 504 were adjudicated as HCC. One event was adjudicated as pre-existing; none were assessed with an alternate diagnosis. The EAC could not adjudicate 104 HCC events, predominantly due to inadequate case documentation. The EAC pursued various avenues to limit the number of HCC events assessed as unable to adjudicate. All HCC reports reviewed by the EAC after the 2014 charter amendment were assessed using the revised criteria for HCC diagnosis. In addition, using the revised diagnostic criteria, the EAC reassessed 31 previous HCC events that could not be adjudicated because only a single imaging study had been conducted; four (13%) were reassessed as meeting criteria for HCC.

The EAC reviewed 404 investigator-reported events of non-HCC HBV disease progression, 202 in each treatment group. Among these, 283/404 were adjudicated as a new event of disease progression, either as reported (n=175), as reported with a modified date of diagnosis (n=78), or with the disease progression manifestation changed (n=28). Of the remaining 124/404 events, 29 were adjudicated as pre-existing (most commonly events of cirrhosis or ascites), 3 received an alternate diagnosis, and 92 were assessed as unable to adjudicate due to inability to satisfy EAC diagnostic criteria.

The EAC reviewed 200 events of non-HCC malignancy. Among these, 145 were adjudicated as reported, or as reported but with an alternate diagnosis date. Thirty-one were adjudicated with an alternate diagnosis of non-HCC malignancy, providing greater specificity regarding tumor type. One was adjudicated as an alternate event (endometrial hyperplasia), and the remaining twenty-three were assessed as unable to adjudicate due to inability to satisfy EAC diagnostic criteria. All reported non-HCC malignancies assessed as unable to adjudicate lacked histologic data or other means for confirming the reported diagnosis.

Concordance between investigator and EAC assessments of HBV-associated COEs was high overall (85.0%; 1465/1724 COEs). However, concordance varied substantially by COE type. Concordance was highest for deaths (99.6% agreement; 502/504 COEs), followed by non-HCC malignancies (88.0% agreement; 176/200 COEs), HCC (83.3% agreement; 504/605 COEs), and non-HCC HBV disease progression (68.2% agreement; 283/415 COEs). In addition, EAC adjudication resulted in modification of the COE (e.g., hepatic decompensation), COE manifestation (e.g., ascites), and/or COE diagnosis date in 26.7% cases (461/1724 COEs).

Discussion

This is the first prospective observational cohort study of CHB therapy that has employed an EAC to affirm study endpoints. The standardized adjudication process was custom-tailored to meet the challenging aspects of study design. These include the diverse clinical outcomes assessed, the global scope that involved 24 countries and 299 sites in the Americas, Europe and Asia-Pacific, a large cohort of approximately 12,500 patients, and a long (10-year) study duration.

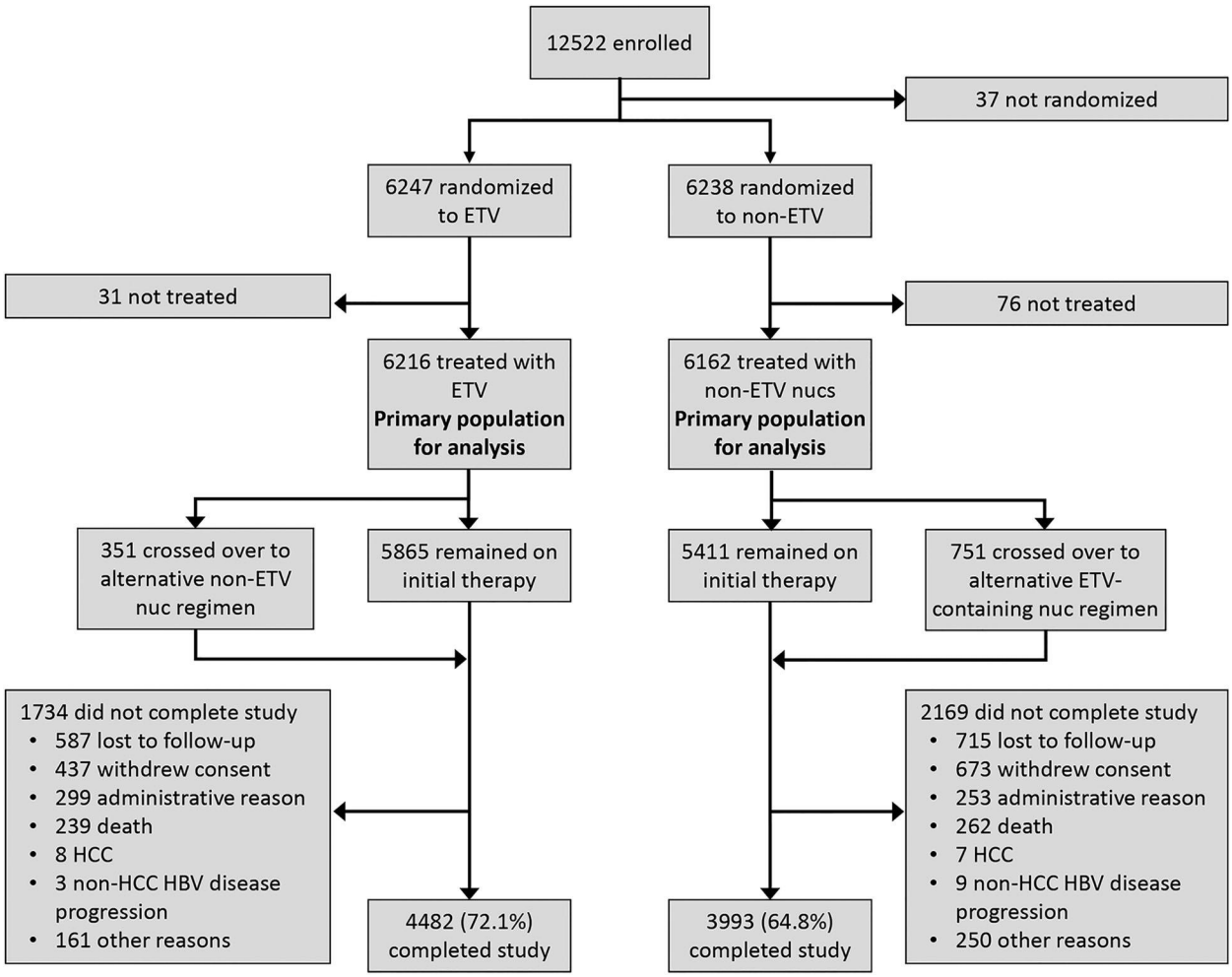

Fig. 2. Patient disposition. The flow of patients through the study is shown. Outcome analyses were based on all patients who were randomized and treated. Reasons for not completing the study are shown for each treatment group; discontinuations for administrative reasons were due primarily to early site closures associated with site conduct issues, dissolution of site ethics committees, or related issues.

Abbreviations: ETV, entecavir; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; NUC, nucleos(t)ide analogue.

The sponsor and CRO worked closely with the EAC to establish operational and administrative processes in accordance with the EAC charter, and to ensure that the EAC was provided with the means to perform its role efficiently. Despite the long study duration, attrition of EAC membership during the study was very limited.

The EAC reviewed and adjudicated cases independently of the sponsor and remained blinded to treatment-specific results throughout the study. The sponsor performed annual data assessments to share interim results of adjudicated COEs with the EAC, the data monitoring committee, and regulatory authorities; however, these interim assessments were based on pooled treatment results and study blinding was maintained.

EAC participation in clinical studies is well established in other fields, particularly cardiovascular medicine,[17–21] to promote consistency, objectivity, accuracy, and reliability of study outcomes. This is accomplished through standardized application of prespecified clinical event definitions that reflect best evidence from published literature and consensus among expert physicians, medical societies, and regulators. Event adjudication may also contribute to patient protection through collection of safety data used by oversight committees and regulatory agencies in real-time clinical event monitoring.

However, event adjudication is resource-intensive, time-consuming, and potentially subject to bias. To promote consistency and accuracy of event adjudication, the REALM EAC applied several strategies representing best practice that may support future observational cohort studies of CHB infection. The most pertinent challenges faced in the adjudication experience, the measures employed to address these challenges, and lessons learned are summarized below.

Optimizing efficiency

As the study progressed, substantial differences between sites were observed in the quality and availability of primary data needed for COE adjudication. To address this issue, standardized DRFs for each COE category and category

Table 1. Summary of EAC-reviewed COEs

EAC-reviewed COEs, number of patients	ETV, *N*=6216	Non-ETV, *N*=6162
Deaths	240	264
Liver-related	46	48
HCC	290	316
Non-HCC malignant neoplasms	109	91
Non-HCC HBV disease progression	202	202

Abbreviations: COE, clinical outcome event; EAC, event adjudication committee; ETV, entecavir; HBV, hepatitis B virus; HCC, hepatocellular carcinoma.

subtype were used to obtain additional data deemed critical for EAC adjudication. This element of the adjudication process enhanced the quality and efficiency of EAC case review and limited the need for additional site queries. Guidance in the application of COE criteria and event reporting were made available to investigators, and study coordinators made more frequent visits to sites where data collection was problematic.

The large volume of study data presented logistical challenges associated with acquisition and distribution of documents for EAC review, assessment, and documentation. These challenges were addressed through the rigorous, step-wise EAC process established for COE reporting, source document submissions, data querying and retrieval of responses, and use of adjudication CRFs specific to each COE type. A secure web-based portal provided efficient storage and recall of COE documentation. Through the portal, EAC members accessed event-related documents and COE case packets for review, and EAC chairs filed completed adjudication CRFs.

Adjudication efficiency was enhanced by periodic assessment to ensure adequate EAC membership and appropriate distribution of case assignments across the EAC, including hepatology and oncology experts. Although oncologists were initially responsible primarily for reviewing reported non-HCC malignancies and malignancy-related deaths, as the case load increased, the oncologists also assumed shared responsibility with hepatologists for reviewing HCC events.

Assignment of adjudicated diagnoses

Event adjudication entailed determining the diagnosis and the date of diagnosis of investigator-reported events, and assessment of whether reported cases met EAC charter-defined COE criteria. Investigator-reported dates of diagnosis were verified by primary review of radiographic, laboratory, and other source documents from the REALM database.

Due to international enrollment (299 site investigators, 24 countries, 4 continents), the committee faced challenges in resolving significant geographic variability in language, clinical practice standards, and definitions of liver-related events. The rigorous assembly of COE-related information, using standardized DRFs for COE category and type, and adherence to EAC charter-defined criteria for COEs facilitated report consistency. Furthermore, international representation in the composition of the EAC helped promote consistency in event adjudication across global regions.

Table 2. Summary of COE reviews by the EAC in the REALM study

Patients with events, *n* (%)	ETV, *N*=6216	Non-ETV, *N*=6162
Reported deaths	**240 (4)**	**264 (4)**
EAC reviewed	240 (4)	264 (4)
Adjudicated as death	238 (4)	264 (4)
Adjudicated as reported	156 (3)	169 (3)
Adjudicated as reported with alternative diagnosis date	0	3 (<0.1)
Adjudicated with alternate diagnosis	82 (1)	92 (1)
Unable to adjudicate	2 (<1)	0
Reported HCC events	**289 (5)**	**316 (5)**
EAC reviewed	290 (5)	316 (5)
Adjudicated as HCC	241 (4)	263 (4)
Adjudicated as reported	122 (2)	146 (2)
Adjudicated as reported with alternative diagnosis date	119 (2)	117 (2)
Adjudicated with alternate diagnosis	1 (<1)	
Unable to adjudicate	51 (1)	53 (1)
Pre-existing events	0	1 (<1)
Reported non-HCC malignant neoplasm events	**109 (2)**	**91 (1)**
EAC reviewed	109 (2)	91 (1)
Adjudicated as non-HCC malignant neoplasm	95 (2)	81 (1)
Adjudicated as reported	35 (1)	24 (<1)
Adjudicated as reported with alternative diagnosis date	48 (1)	38 (1)
Adjudicated with alternative diagnosis	12 (<1)	19 (<1)
Adjudicated as alternate event	1 (<1)	0
Unable to adjudicate	13 (<1)	10 (<1)
Reported non-HCC events of HBV disease progression	**208 (3)**	**207 (3)**
EAC reviewed	202 (3)	202 (3)
Adjudicated as non-HCC events of HBV disease progression	137 (2)	146 (2)
Adjudicated as reported	87 (1)	88 (1)
Adjudicated as reported with alternative diagnosis date	34 (1)	44 (1)
Adjudicated as reported with alternative disease progression manifestation	16 (<1)	12 (<1)
Adjudicated with alternate diagnosis	0	3 (<1)
Pre-existing events	21 (<1)	8 (<1)
Unable to adjudicate	44 (1)	48 (1)

EAC reviewed events were reviewed by two EAC members, as described in the Methods. Adjudicated events were those that had adequate supporting information to permit an adjudicated decision by the committee.

Abbreviations: COE, clinical outcome event; EAC, event adjudication committee; ETV, entecavir; HBV, hepatitis B virus; HCC, hepatocellular carcinoma.

Event capture and prespecified data analyses

Data analyses rely heavily on the quality and consistency of source data. In this study, primary and key secondary endpoint analyses were based on adjudicated COE data; hence, minimizing the number of unadjudicated events was critical. After initial establishment of diagnostic criteria, adjustments were implemented to address subsequent changes that emerged in clinical practice guidelines, diagnostic testing, and treatment over the 10-year study period. Ultimately, the EAC charter was amended only twice, to incorporate new guideline criteria for HCC diagnosis and add ultrasound-based elastography to diagnostic criteria for cirrhosis. EAC reassessment of cases following charter updates ensured uniform application of charter criteria across time.

Adjudicating cause of death was the situation that most frequently required full-committee discussion to resolve discordant reviews. Inadequate hospital documentation was a frequent source of uncertainty, particularly in regions where death certificates were not regularly available. In addition, in patients with cirrhosis and HCC, distinguishing liver-related death from HCC-related death required careful evaluation of primary documents to assess primary contributing factors to fatality. For example, a fatal variceal hemorrhage associated with portal hypertension, although generally liver-related, could be adjudicated as HCC-related if malignant invasion of the portal vein was documented. Furthermore, the EAC would request primary medical documents for subjects preceding study enrollment to distinguish clinical events as pre-existing, recurrent, or new.

As described in Methods, a secondary, independent review process was implemented to ensure that adjudicated diagnoses of non-HCC malignancies were as specific as possible, based on supporting pathology data. Of the 176 reported non-HCC malignancies that were adjudicated as a non-HCC malignancy (ETV: n=95; non-ETV: n=81), 31 of these events (ETV: n=12; non-ETV: n=19) were assigned an alternate tumor diagnosis by the EAC. In all cases, this was based on selection by the reviewing EAC oncologist of a more specific tumor diagnosis regarding the organ site and/or tissue diagnosis.

Maintaining consistency in COE adjudication over the duration of the study was essential. Monthly teleconferences and annual face-to-face meetings supported regular engagement of EAC members and minimized turnover. The teleconferences provided a live record of adjudication decisions and approach, and helped to promote homogeneity of the adjudication process over time. The EAC charter, adjudication process, and committee performance was reviewed annually.

The relatively frequent modifications of some categories of investigator-reported COEs demonstrate the impact of external adjudication on endpoint assessment in observational cohort studies. Most differences between investigator and EAC assessments of death were related to inadequate documentation to support investigator conclusions regarding cause of death. In contrast, discrepancies regarding HCC, non-HCC malignant neoplasms, and non-HCC events of HBV disease progression were more commonly related to pre-existing diagnoses, alternative dates of diagnosis, unacceptable diagnostic methods, or alternative disease progression manifestations.

In summary, if supported by an effective administrative infrastructure, EACs can enhance the consistency and validity of clinical outcome assessment and strengthen regulatory review of therapeutic interventions in liver disease. The REALM study represents possibly the first application of EAC adjudication in HBV therapeutic research, and is unique due to its large cohort, geographic scope, outcome assessments bridging hepatology and oncology, and long-term follow-up over 10 years. The challenges and accomplishments of this EAC may provide useful insights for future cohort studies in liver disease.

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Conflict of interest

JKL, CMV, and SM have received research support, consulting fees, and/or personal fees from Bristol-Myers Squibb and Gilead. PM has received research support and consulting fees from AbbVie, Gilead, and Merck. TN and EC are employees of the sponsor, Bristol-Myers Squibb. AYC, AZ, MK, JMF, and VN report no conflicts. The other authors have no conflict of interests related to this publication.

Author contributions

Study concept and design, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content (JKL). Study concept and design, analysis and interpretation of data, critical revision of the manuscript for important intellectual content (AYC, AZ, PM, CMF, MF, SR, JFM, TN, EC, VN). Study concept and design, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, study supervision (LC).

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Original Article

Long-term Outcome of Autologous Hematopoietic Stem Cell Infusion in Cirrhosis: Waning Effect over Time

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Abstract

Background and Aims: Long-term data on cell-based therapies, including hematopoietic stem cell infusion in cirrhosis, are sparse and lacking. ***Methods:*** Patients with cirrhosis of non-viral etiology received either standard-of-care (n = 23) or autologous CD34+ cell infusion through the hepatic artery (n = 22). Study patients received granulocyte colony-stimulating factor (commonly known as G-CSF) injections at 520 μgm per day for 3 days, followed by leukapheresis and CD34+ cell infusion into the hepatic artery. The Control group received standard-of-care treatment. ***Results:*** Mean CD34+ cell count on the third day of G-CSF injection was 27.00 ± 20.43 cells/μL 81.84 ± 11.99 viability and purity of 80-90%. Significant improvement in the model of end-stage liver disease (commonly known as MELD) score (15.75 ± 5.13 vs. 19.94 ± 6.68, p = 0.04) was noted at end of 3 months and 1 year (15.5 ± 5.3 vs. 19.8 ± 6.4, p = 0.04) but was not statistically different at end of the second (17.2 ± 5.5 vs. 20.3 ± 6.8, p = 0.17) and third-year (18.4 ± 6.1 vs. 21.3 ± 6.4, p = 0.25). No difference in mortality (6/23 vs. 5/23) was noted. ***Conclusions:*** Autologous CD34+ cell infusion effectively improved liver function and MELD score up to 1 year but the sustained benefit was not maintained at the end of 3 years, possibly due to ongoing progression of the underlying disease.

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Keywords: Stem cells; Cirrhosis; Hepatic artery; Bridge to liver transplantation.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CD, cluster of differentiation; CLD, chronic liver disease; DDLT, deceased donor liver transplantation; G-CSF, granulocyte colony stimulating factor; GMP, Good Medical Practice; HBA1C, glycosylated hemoglobin; INR, international normalized ratio; LDLT, living donor liver transplantation; MELD, model for end-stage liver disease.

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Introduction

The rising incidence of chronic liver disease (CLD) has increased the need for liver transplantation. The unmet need has resulted in numerous studies targeting the regenerative potential of the liver as an alternative or as a bridge to liver transplantation.[1,2] Stem cells are cells that reside in the human niche and have an intrinsic ability of self-renewal, but they maintain their undifferentiated state and have potency to transform into specialized cells, like hepatocytes.[3]

The capacity of the liver to regenerate can be traced back to the two Greek mythological characters, Tityus and Prometheus, who were both banished to the mountains to suffer and die. In mythology, a vulture used to come and feed on their livers, only to find that the liver regenerated the next day and thereby giving the first documentation of the regenerative potential of the liver.[4,5] The demonstration that hepatic oval cells can regenerate was shown biologically by hepatocyte proliferation in rats.[6]

Human studies using hematopoietic stem cells identified a subset of CD34+ cells, which have the potential to differentiate into liver cells. These CD34+ cells were identified based on their distinct appearance, immunophenotype, and gene expression. For proof of concept, safety study was first conducted with five patients, opening the doors for further research in this field.[7] After that, numerous studies have looked into the short-term outcome of infusion of hematopoietic or mesenchymal stem cells in patients with cirrhosis of the liver.[8–10] However, the route by which stem cells were infused, the method of calculation of the dose of cells, and the stage of liver cirrhosis when these cells were infused were heterogeneous. Studies using granulocyte colony-stimulating factor (G-CSF) alone to mobilize hematopoietic stem cells from bone marrow to the liver have shown an increased number of CD34+ hematopoietic stem cells in the liver tissue in a paired-biopsy study, thereby suggesting that there is a definite increase in the number of stem cells in the liver following mobilization.[11] Whether mere mobilization of the cells is enough or whether the separation of specific stem cells is required along with direct infusion into the target organ (liver) would yield better results is a matter of debate.

Our previous study with autologous hematopoietic stem cell infusion through the hepatic artery showed significant

short-term improvement in model for end-stage disease (MELD) score, serum albumin, and serum creatinine. These benefits were noticed at the beginning of the first month after autologous CD34+ cell infusion and were maintained at 3 and 6 months.[12]

Here, we present the long-term data of the same cohort of patients who underwent autologous hematopoietic stem cell infusion and were followed up for 3 years.

Methods

Patients with decompensated cirrhosis of the liver having a non-viral etiology, who attended the Liver Clinic of our institute, were screened for eligibility to be enrolled in the study. The screening was carried out between June 2012 and July 2013, and those patients with a MELD score of greater than 14 or a score of greater than 10 with evidence of ascites or hepatic encephalopathy were counseled to undergo liver transplantation. A significant proportion of patients were not willing to undergo transplantation, either due to financial constraints, lack of donor, or a combination of both. These patients were provided an explanation about stem cells and counselled that by enrolment in the study, there may be a beneficial effect on the natural history of their disease. The experimental nature of this modality of treatment was explained in detail, including the concept that they may not benefit at all from this study. Those patients who agreed to be a part of the study were enrolled in the study group. The remaining patients who were willing only to receive deceased donor liver transplantation (DDLT) were included in the liver transplantation waiting list for DDLT and registered with the centralized organ allocation authority of the state. These patients were considered as the control group in the study. A small number of patients who had a first degree-related liver donor underwent liver transplantation. This study was approved by the Institutional Review Board, Institutional Ethics Committee and the Institutional Committee of Stem Cell Research, and informed written consent was obtained from all patients.

Inclusion criteria

i. Adult patients between 18 to 70 years of age with a MELD score of >14 and with no donor or finance for immediate liver transplantation within in the next 3 months;
ii. A MELD score-based life expectancy of at least 3 months;
iii. Ability to give written informed consent.

Exclusion criteria

i. Any patient with hepatocellular carcinoma or any other malignancy within the last 5 years;
ii. Presence of ongoing infections, including retroviral, hepatitis B or hepatitis C;
iii. Co-existent cardiac and/or pulmonary co-morbidities related or unrelated to liver cirrhosis;
iv. Recent history of upper or lower gastrointestinal bleeding, acute kidney injury or hepatorenal syndrome with the last month;
v. Main portal vein thrombosis, either partial or complete, on cross-sectional imaging;
vi. Patients with a manual platelet count below 50,000/mm^3;
vii. Any patient with pregnancy or lactating mothers.

Control group

Patients between 18-70 years, who were enrolled in the waiting list for DDLT during the study period. Informed consent also was taken from all participants in the control group.

All the patients underwent liver protocol investigations, which included complete hemogram, kidney and liver function tests, serum alpha-fetoprotein, coagulation profile, Doppler-ultrasonography of the whole abdomen, and contrast-enhanced computed tomography scan along with quantitative estimation of hepatitis B DNA and hepatitis C RNA. Serology tests for syphilis, herpes simplex virus, and cytomegalovirus were conducted, as per protocol. The control group patients were investigated and treated with standard-of-care treatment for liver cirrhosis, which included diuretics, lactulose, beta-blockers, and low salt (<2 g of sodium chloride) and high-protein diet.

The study was approved by the Asian Institute of Gastroenterology Institutional Review Board, Asian Institute of Gastroenterology Ethics Committee, and the Institutional Committee of Stem cell Research, which was registered with the National Apex Committee for stem cell research in India. The study was registered with the clinical trial registry of India (CTRI/2017/11/010429).

Methodology

Study group patients received G-CSF at 520 μg/day (Neupogen, Filgrastim, Roche) for 3 consecutive days, in an effort to mobilize the hematopoietic stem cells' bone marrow niche to the peripheral circulation. Serial monitoring of complete blood counts was carried out, along with liver and kidney function tests. Doppler ultrasonography of the abdomen was done on the third day. Any adverse effects due to administration of G-CSF, like fever, symptomatic increase in spleen size and sepsis, were documented and reported. The estimation of serum CD34+ cells was measured daily and 1 h before leukapheresis. A predefined level of greater than 2 cells/μL was achieved. For leukapheresis, a central line was secured, and 1 h after the third dose of G-CSF, the patient was moved to the phlebotomy unit. Leukapheresis, under the supervision of a hematologist, was done using an MCS-3P magnetic cell separator (Hemaneics, USA). The amount of peripheral blood collected after leukapheresis varied between 30 mL to 60 mL. Side effects related to leukapheresis, like hypotension, tachycardia, shortness of breath, etc., were documented, and corrective measures were taken as per standard operating procedures. The leukapheresis product underwent mononuclear cell isolation in a cleanroom in a Good Medical Practice (commonly known as GMP)-certified laboratory of the institute.

Hi-Sep method (HiSep LSM1077, LS001; Himedia Laboratories, India) was used for mono-nuclear cell isolation, with washing with phosphate buffered saline and diluting with CliniMACS buffer (Miltenyi Biotech, GmbH, Germany). These cells underwent centrifugation and incubation with $CD34^+$ labeled monoclonal antibodies microbeads (MACS; Miltenyi Biotech) for 30 m. CliniMACS buffer was used to wash these cells, which were then processed in a CliniMACs cell separator. A high-gradient magnetic field was used to separate the CD34 + cells. The cell adequacy was calculated, and viability of the cells was assessed using the trypan blue dye exclusion method. Cell viability was higher than 80% in all cases.

Flow cytometry was used to ascertain cell purity. The CD34+ hematopoietic stem cells were diluted with 10 mL of phosphate buffered saline supplemented with 2% human serum albumin and dispatched for use on the same day, within 1 h. The hepatic artery was catheterized through the femoral route under fluoroscopic guidance by the interventional radiologist and infused into the hepatic artery. The patients were observed overnight and discharged the subsequent day, after a screening Doppler ultrasonogram to rule-out hepatic artery thrombosis. Any procedure-related adverse effects, like pain (recorded by visual analog scale) or bleeding from the femoral catheterization site, etc., were noted.

Follow-up visits of patients

Weekly follow-up of the patients was carried out through the first months, followed by quarterly evaluation for 1 year and then half-yearly assessment for the subsequent 3 years. During each follow-up visit, the patient underwent a complete hemogram, along with renal and liver function test, serum alpha-fetoprotein test, and Doppler ultrasonography of the abdomen. The presence or appearance of any new complaints was noted. As stem cells have a potential for carcinogenesis, any suspicious nodule or rise in alpha-fetoprotein was further evaluated by cross-sectional imaging to rule-out hepatocellular carcinoma. Worsening of ascites, need for large-volume paracentesis, evidence of spontaneous bacterial peritonitis late-onset or recurrent hepatic encephalopathy, an/or any gastrointestinal bleed was noted. Patients in the control arm underwent similar follow-up visits. For both groups, any emergency hospital visits, either to our center or any hospital locally, were noted.

Statistical analysis

A comparison of the clinical and laboratory findings was performed at the end of 1 month, 3 months and 3 years, to assess the long-term outcome. All nominal values were expressed as mean with standard deviation and as median with range. Fisher's exact test was used for categorical variables, and a two-tailed p-value of <0.05 was considered to be significant. GraphPad software 2019 was used for this per-protocol statistical analysis.

Results

One hundred patients were screened for eligibility to be enrolled in the study. The inclusion criteria were not met by 30 patients, while another 25 patients refused to participate in the study. Two patients in the study group underwent living donor liver transplantation (LDLT), as they were able to find a suitable donor. Another patient died due to hepatorenal syndrome, and one patient was lost to follow up in the initial 1-3 months period. Out of the 23 patients in the control arm (liver transplantation waiting list), there were no dropouts at the end of 1 month. However, between 1 and 3 months, two patients died due to sepsis and one due to hepatorenal syndrome. Another patient in the control group underwent DDLT, while two patients were lost to follow-up. In the initial data analysis that was published in 2015,[12] there were 17 and 18 patients in the control and study groups in the per-protocol analysis. These patients have now been followed up to the end of 3 years. During this extension period of the study, a total of 6/23 patients in the study group and 5/23 in the control group died due to complications of portal hypertension. Sepsis was the most common cause of death (four patients in both groups. Two patients in the study group and one patient in control died due to acute upper gastrointestinal bleed. Three patients in the control group underwent liver transplantation while on the waiting list, as shown in the consort diagram. During the follow-up of 3 years, two patients were lost to follow-up in the control group and one in the study group. So, in the final analysis at the end of 3 years, there were 13 patients in the study group and 12 in the control group.

In the control and study groups, at the beginning of the study, the cause of cirrhosis was cryptogenic in 18 (78.2%) and 16 (72.72%), and alcohol use disorder-related in 5 (21.7%) and 6 (27.27%) respectively. In the final analysis at the end of 3 years, there were 9/12 (75%) cryptogenic and 4/12 (25%) alcohol use disorder-related cirrhosis cases in the study group, and 8/12 (66.7%) and 4/12 (33.3%) respectively in the control arm.

There was no difference in the baseline characteristics at the initiation of the study, nor when the primary analysis was done at the end of 3 months nor at the end of the study at 2 years. The MELD score in both groups was similar. Median alcohol abstinence was 7 months (range, 5-11 months) in the control group. In comparison, it was 6 months (range, 4-12 months) in the study group, and all the patients maintained the alcohol abstinence during the study period. Seven patients in the control group and six in the study group had well-controlled type 2 diabetes mellitus [mean glycosylated hemoglobin (HBA1c): 6.45 (range, 5.3-8.3) and 6.63 (range, 5.1-9), respectively]. Ascites was present in 95.6% (n = 22) and 100% (n = 22) of patients in the control group and study group respectively. Patients with refractory ascites requiring therapeutic paracentesis for respiratory easement and impaired quality of life was 22.7% and 21.7% respectively in the study and control groups. Limitation of an increase in diuretic dose due to adverse effects was the cause of refractory ascites in all patients. Overt hepatic encephalopathy within the last 3 months before enrolment in the study was 26% (n = 6) and 7 (31.81%; n = 7) in the control and study groups respectively.

Baseline CD34+ cell count (cells/μL) before G-CSF administration was 2.3 ± 2.56 (mean ± standard deviation). Cell viability at baseline was 48.17 ± 23.95%. Peripheral CD34+ cell count measured on the third day after G-CSF infusion was 27.00 ± 20.43 (cells/μL), and the cell viability was improved to a value of 81.84 ± 11.99%. Cell purity varied between 80% and 90% among all patients. A minimum of 1×10^6 hematopoietic stem cells per kg dry body weight of the patient were infused through the hepatic artery.

Results after CD34+ cell infusion

Primary endpoint analysis of the original published data revealed an increase in the mean serum albumin in the study group (2.83 ± 0.36 vs. 2.43 ± 0.42, p = 0.001), which was not maintained at the end of the first year but showed statistically significant improvement at the end of second year. This improvement seen between the first and second year was not maintained at the end of the third year, as shown in Figure 2 (Table 2) when compared with controls. Further, at 3 months, significant improvement in serum creatinine was noted in the study group (0.96 ± 0.33 vs. 1.42 ± 0.70, p = 0.01), which was not maintained at any other point

during the study period, as shown in Table 2. Serum bilirubin, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) did not show any significant differences. Platelet count and international normalized ratio (commonly referred to as INR) showed some improvement but did not reach statistical significance at any point in time. However, when these patients were followed-up for 3 years, there was no difference in the MELD score and mortality (6/23 vs. 5/23) between the two groups.

There was a significant improvement in the MELD score (15.75 ± 5.13 vs. 19.94 ± 6.68, p = 0.04) at the end of 3 months. When these patients were followed-up for 3 years, the improvement in MELD score was maintained at the end of 1 year (15.5 ± 5.3 vs. 19.8 ± 6.4, p = 0.04) but was not statistically different at the end of 2 years (17.2 ± 5.5 vs. 20.3 ± 6.8, p = 0.17) and upon final analysis at the end of the third year (18.4 ± 6.1 vs. 21.3 ± 6.4, p = 0.25). Though there was no difference in the mortality (6/23 vs. 5/23) between the two groups, the maximum number of deaths was three in each group, occurring between the second and third years.

The most frequent cause of death in both groups was sepsis, followed by one case of upper gastrointestinal bleeding. Patients in both the groups were regularly followed-up with screening endoscopy, and the need for endoscopic variceal ligation was similar in the two groups. No case of new-onset development of gastric varices was noted in our series. The incidence of hepatic encephalopathy was also not different between the two groups (study 3/12 vs. control 4/13).

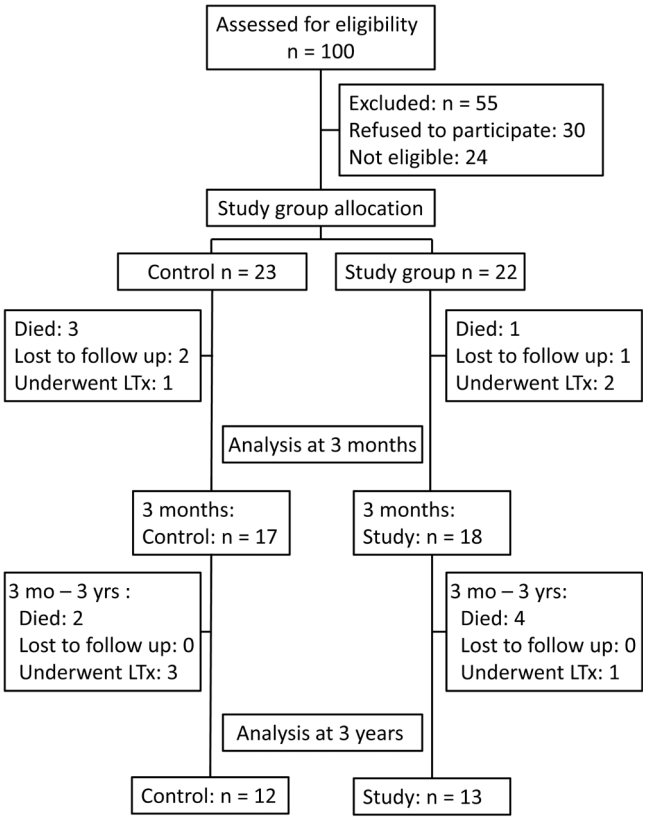

Fig. 1. Consort flow diagram showing patients at the time of recruitment and at the end of 3 years.

Abbreviation: LTx: Liver transplantation.

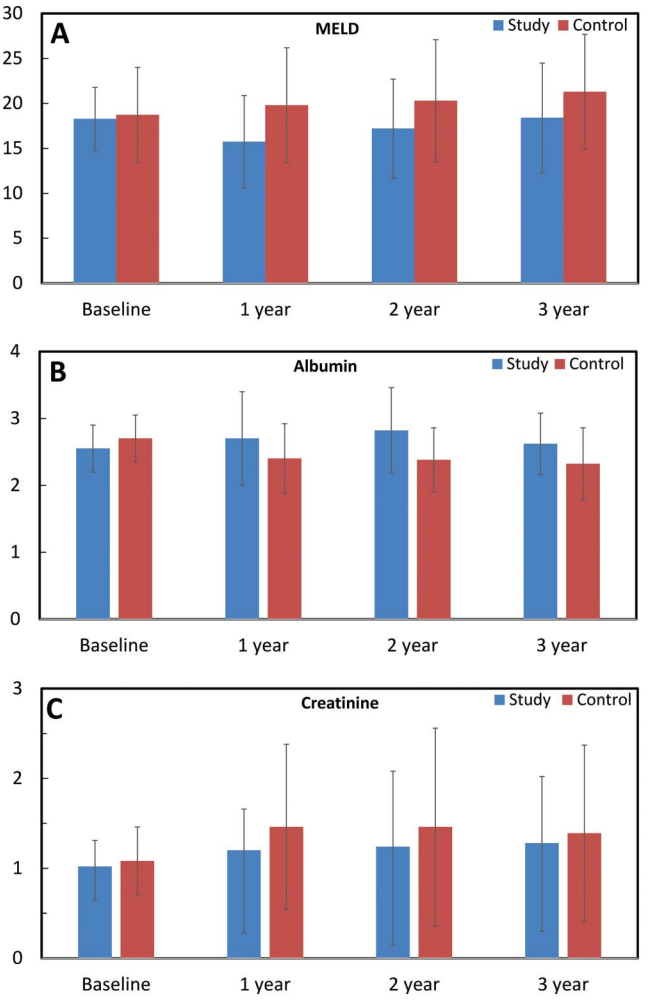

Fig. 2. Improvements. (A) The MELD improvement was maintained at 1 year. (B) Albumin improvement was noted at end of 2 years. (C) No difference in serum creatinine was noted in long-term outcome.

Abbreviation: MELD: Model for end-stage liver disease.

Discussion

Autologous CD34+ cell infusion is a minimally manipulated cell-based procedure that is safe and improves liver function in the short term, and has been suggested to serve as a bridge to liver transplantation.[12] But, all studies to date have published their outcomes at a maximum of up to the end of 3 to 6 months.[13–15] No studies have mentioned the long-term outcome of these patients after the end of the short study period.

A recent retrospective study that used peripheral mobilized stem cell infusion in patients with decompensated cirrhosis assessed a 5-year outcome. The study found that after propensity score matching, survival was significantly higher in patients receiving stem cell infusion (71.2% vs. 52.1%, p = 0.001) than in the control group.[16] The beneficial effect of this study, when compared with our research, could be due to the patient population having had a lower Child-Pugh score, and the majority of the patients being without ascites. So, it could be hypothesized that if stem cells are used early in the course of the natural history of cirrhosis, when the inflammatory milieu[17] of the liver is still less

Table 1. Baseline characteristics at recruitment of the control and study groups

Parameter	Control group, *n* = 23	Study group, *n* = 22	*p* value
Age in years	47.35 ± 12.54	48.91 ± 9.25	0.62
Sex, Male:Female	20:3	16:6	0.072
Hemoglobin, g/dL	9.29 ± 1.86	9.15 ± 1.60	0.79
Platelet count, lakh/mm^3	0.92 ± 0.27	1.1 ± 0.72	0.24
Total bilirubin, mg/dL	4.78 ± 4.06	3.55 ± 2.12	0.21
Albumin, mg/dL	2.7 ± 0.35	2.55 ± 0.35	0.16
INR	1.72 ± 0.53	1.80 ± 0.52	0.62
Serum creatinine, mg/dL	1.08 ± 0.38	1.02 ± 0.29	0.56
MELD score	18.73 ± 5.29	18.28 ± 3.50	0.74

Abbreviations: INR, international normalized ratio; MELD, model for end-stage liver disease.

Table 2. Results comparison at different time points after autologous hematopoietic stem cell infusion

Follow up parameter	Three months	One year	Two year	Three year
MELD score				
Study group	15.75 ± 5.13	15.75 ± 5.13	17.2 ± 5.5	18.4 ± 6.1
Control group	19.94 ± 6.68	19.8 ± 6.4	20.3 ± 6.8	21.3 ± 6.4
p-value	0.04	0.04	0.17	0.25
Albumin, mg/dL				
Study group	2.83 ± 0.36	2.7 ± 0.7	2.82 ± 0.64	2.62 ± 0.46
Control group	2.43 ± 0.42	2.4 ± 0.52	2.38 ± 0.48	2.32 ± 0.54
p-value	0.001	0.17	0.03	0.14
Creatinine, mg/dL				
Study group	0.96 ± 0.33	1.2 ± 0.46	1.24 ± 0.84	1.28 ± 0.74
Control group	1.42 ± 0.70	1.46 ± 0.92	1.46 ± 1.1	1.39 ± 0.98
p-value	0.01	0.42	0.461	0.78

hostile to the mobilized or infused stem cells, the regenerative power of the cells could be better. This theory needs to be validated in a prospective randomized trial with paired-biopsies taken pre- and post-stem cell infusion to document increase in the number of stem cells in the liver tissue. The study also did not record any adverse effect of stem cell infusion in the large cohort of patients.

Though initial isolated reports in stem cell studies have reported some adverse effects, like splenomegaly, splenic rupture, and hepatic artery thrombosis, multiple other studies, including randomized control trials, have not found any adverse effects on these patients. However, a multicenter study that did not see any improvement in MELD score and liver function when they compared peripheral CD133+ stem cell infusion mobilized by G-GSF compared to the group receiving G-CSF alone standard-of-care in compensated cirrhosis reported increased adverse effects.[18] The study also reported a significant worsening of ascites in the stem cell infusion group. As the majority of the patients were compensated, the development of ascites could have been a part of the natural history of cirrhosis, or a precipitating factor may be caused by acute-on-chronic liver failure, which was not addressed in the study. Besides, that study had used an infusion of stem cells through a peripheral vein and the percentage of the cells that reached the hepatic tissue remains unknown as there was no way the cells could be tracked. There was no paired biopsy to document homing-in of the stem cells.[19] Besides, as three doses of CD133+ hematopoietic stem cell infusion at a dose of 0.2×10^6 cells per kg on days 5, 30 and 60, was used, as multiple infusions, then comparing the results and extrapolating the same to studies with single infusion and different dosing is not correct.[20]

Long-term outcomes of autologous stem cell infusion have been studied in another retrospective study, which involved 23 patients undergoing stem cell infusion. Though the overall mortality was less in the stem cell group (55.1 % vs. 73.9%), there was no significant difference found in the 10-year long-term survival rate ($p > 0.05$). There was also a higher incidence of hepatocellular carcinoma in stem cell patients (47.8% vs. 21.7%, $p < 0.05$).[21] Though theoretically, there is a possibility of uncontrolled malignant proliferation of an aged stem cell niche,[22] the development of hepatocellular carcinoma in a 10-year period could develop as a part of the natural history. This needs further investigation, though a close watch on the development of hepatocellular carcinoma is always required following any cell-based therapy.[23]

Despite multiple studies, results with isolated hematopoietic stem cell or mesenchymal stem cell infusion in cirrhosis

have not shown very promising long-term results. The questions of whether it is the wrong timing of injections or whether the cells get destroyed in the hepatic tissue have paved the way for ongoing study on the combination of mesenchymal and hematopoietic stem cells, as the former has an immunomodulatory effect[24,25] which can possibly benefit the higher regenerative potentials of the hematopoietic stem cells.

One of the drawbacks of this study was the lack of paired-biopsies to demonstrate the homing-in of the stem cells or to demonstrate an increase in the number of CD34+ cells in the liver tissue. Unfortunately, there is no tracking system by which we can track these infused cells once they are injected. Since this was a long-term data study, multiple biopsies would have been needed at different times, which would have not been practical or feasible.

In conclusion, autologous hematopoietic stem cell infusion in patients with cirrhosis is still considered a safe procedure, despite isolated reports on safety. It is promising to serve as a bridge to liver transplantation. Since cirrhosis is an ongoing process, the answer to whether or not repeated infusions will help (the current evidence does not support it) requires further studies. Combination stem cell therapy or hepatic organoids and mini-liver development in laboratories contain a lot of promise.[26,27]

Conflict of interest

The authors have no conflict of interests related to this publication.

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Author contributions

Conceptualized and designed the study (MS, MiS, PNR, DNR), wrote the manuscript (MS, MiS), contributed to data collection, patient recruitment and follow-up (NJ, AK, PK, RG), involved in stem-cell harvesting and infusion (SJ, KP, SD, GJ), designed the figures (NJ, AK), analyzed the data (SF, PNR), critically reviewed and provided intellectual input to the paper (DNR, PNR, GVR, MS, MiS).

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Original Article

Hispanic Patients with Primary Biliary Cholangitis Have Decreased Access to Care Compared to Non-Hispanics

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Abstract

Background and Aims: Hispanic patients with primary biliary cholangitis (PBC) have reduced rates of biochemical response to ursodeoxycholic acid (UDCA) and increased risk of disease progression compared to non-Hispanic patients. In this study, we sought to identify differences in demographics, comorbidities, environmental risk factors and socioeconomic status between Hispanic and non-Hispanic patients with PBC. ***Methods:*** In a case control study, we analyzed data from Hispanic (n=37 females and 1 male) and non-Hispanic (n=54 females and 4 males) patients with PBC seen at the University of Miami/Jackson Memorial Hospital from January 1998 through January 2013. Data were obtained by filling out a questionnaire either via phone call, mail, or e-mail. Odds ratios were calculated to measure the association between exposure and outcomes.***Results:*** Baseline demographics, environmental risk factors and comorbidities were similar between Hispanic and non-Hispanic patients with PBC. Hispanic patients were less likely to be married and fewer Hispanics had education beyond high school level compared to non-Hispanics. Sixty four percent of Hispanic patients had a household income of less than $50000, compared to 19.5% of non-Hispanics. Fewer Hispanic patients with PBC had health insurance coverage compared to non-Hispanics (86.5% vs. 98.1%; odds ratio: 0.1, 95% confidence interval: 0-0.9). ***Conclusions:*** Differences in disease severity and response to therapy observed in prior studies could not be explained by environmental exposures. In addition to genetic variation, socioeconomic discrepancies (access to care) may further explain these differences.

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Keywords: Primary biliary cholangitis; Ethnicity; Health disparity.

Abbreviations: AASLD, American Association for the study of Liver Diseases; ALP, alkaline phosphatase; CI, confidence interval; NAFLD, nonalcoholic fatty liver disease; OR, odds ratio; PBC, primary biliary cholangitis; UDCA, ursodeoxycholic acid.

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Introduction

Primary biliary cholangitis (PBC) is a chronic immune-mediated liver disease characterized by inflammation and progressive loss of small intrahepatic bile ducts, eventually leading to biliary cirrhosis. PBC is a rare disease, affecting mainly women in their middle age, and the exact pathogenesis remains incompletely understood. Little information is available regarding the effect of race or ethnicity on susceptibility or progression of PBC.

A multicenter study by Peters *et al.*[1] described the differences in severity and disease progression by race and ethnicity in patients with PBC. The study concluded that progression to advanced liver disease, ascites, hepatic encephalopathy, and variceal bleeding was more frequent in the non-Caucasian group. Non-Caucasians also had lower levels of physical activity and more severe pruritus. Notably, among the 535 patients enrolled in the study, 86.4% were Caucasians. Non-Caucasians included 42 (7.9%) Hispanics, 21 (3.9%) African Americans, and 10 (1.8%) other. We have previously shown in a cross-sectional study that Hispanic patients with PBC have reduced response rates to UDCA.[2] In that study, Hispanic patients were more likely to have complications of advanced liver disease, such as portal hypertension, as compared to non-Hispanics. The reasons for such disparities were not addressed.

In the present study, we aimed to evaluate the demographics, comorbidities, environmental risk factors and socioeconomic status of Hispanic patients with PBC compared to non-Hispanic ones.

Methods

We identified all patients with diagnosis of PBC seen at the University of Miami/Jackson Memorial Hospital (Miami, FL, USA) between January 1998 and January 2013, through ICD-9 codes. Diagnosis of PBC was confirmed by chart review. As recommended by the American Association for the study of Liver Diseases (AASLD), diagnosis of PBC was confirmed when patients met two out of the following three criteria: 1) chronic cholestasis, evidenced by persistent increase in serum alkaline phosphatase (ALP); 2) positive serology for anti-mitochondrial antibodies; and 3) histologic confirmation of PBC, with the presence of nonsuppurative destructive cholangitis involving interlobular and septal bile ducts.

We developed a questionnaire-based survey, available in English and Spanish, to evaluate patients' demographics, comorbid conditions, environmental risk factors and

socioeconomic status, and contacted patients by mail, e-mail, or phone call. A pre-designed consent form was read over the phone to patients, and a verbal consent was obtained prior to collection of information. If the questionnaire was sent by e-mail or mail, this form was included for patients to review, and return of questionnaire implied that consent was provided. We collected data on patients' demographics, clinical presentation, comorbidities, risk factors for PBC, and socio-economic variables.

Once patients were identified and diagnosis confirmed by chart review, they were contacted via telephone call, e-mail communication, or postal mail. Multiple attempts were made to reach each patient. Of 265 subjects with confirmed diagnosis, 10 were deceased, 5 did not want to participate, and 26 had incorrect contact information in the medical records and could not be reached. Thus, the study population consisted of 224 patients with confirmed PBC. The study protocol and questionnaires were approved by the University of Miami Institutional Review Board.

Statistical analysis

Descriptive statistics were used to characterize the study population. Results were provided as *n* (%) or median (interquartile range). Odds ratios (ORs) and their 95% confidence intervals (Cis) were calculated to measure the association between exposure and outcome.

Evidence in favor of an association was determined by the exclusion of 1 from this interval. Male patients were excluded from the data analysis, since there were so few of them (*n*=5), to preserve homogeneity of the sample and generalizability of the result to the majority of PBC patients who are female. All analyses were performed using R (version 3.2.2) and the 'epitools' package.[3]

Results

Demographics

Two hundred and twenty-four patients were given the questionnaire and ninety-six (43%) completed it (Fig. 1). Of the responders, 39.6% were Hispanic (38 in total; 37 females and 1 male) and 60.4% were non-Hispanic patients (58 in total; 54 females and 4 males); forty-nine (91%) in the non-Hispanic group were White, 3.7% Black, 1.9% American Indian, and 3.7% other. Hispanic patients were all White. Median age of Hispanic patients was 51 years-old (age range: 30-75). Non-Hispanics had a median age of 53.5 (age range: 28-74). Among Hispanics, 58.3% were married, as compared to 75.4% of non-Hispanic patients. one patient had prior hepatitis B exposure and one had prior HCV infection; both were non-Hispanics. Twenty-six patients had radiographic evidence of cirrhosis; among them, 54% of these were Hispanic (*n*=14). Table 1 summarizes the demographics of our study population.

Among Hispanic patients, 45.9% were from the Caribbean, 32.4% from South America, and 10.8% from Central America; only four were born in the United States. The vast majority (83.8%) have lived in the USA for more than 15 years. Hispanic patients were diagnosed in a more recent era than the non-Hispanics. Ninety-two percent of Hispanic patients were diagnosed after year 2000, as compared to 42.6% of non-Hispanics.

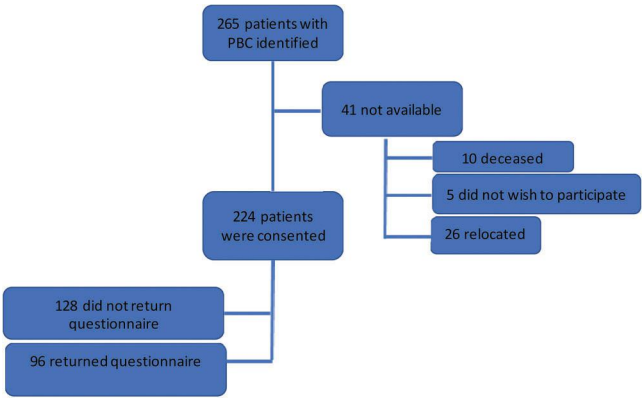

Fig. 1. Flow chart showing study enrollment.

Table 1. Baseline characteristics of Hispanic and non-Hispanic patients with PBC

Baseline characteristics	Hispanics, *n*=37	Non-Hispanics, *n*=54
Age, median (range)	51 (30-75)	53.5 (28-74)
Race, *n* (%)		
White	38 (100%)	49 (90.7%)
Black		2 (3.7%)
Asian		1 (1.9%)
Other		2 (3.7%)
Marital status, *n* (%)		
Single	10 (27.8%)	6 (11.3%)
Married	21 (58.3%)	40 (75.4%)
Other	5 (13.9%)	7 (13.2%)
Years lived in the USA, *n* (%)		
Less than 15 years	6 (16.2%)	0
15 years or more	31 (83.8%)	53 (100%)
Year of diagnosis, median (interquartile range)	2007 (2005-2008)	2002 (1997-2002)
Place of living at the time of diagnosis, *n* (%)		
USA	36 (97.3%)	86 (100%)
Outside of USA	1(2.7%)	0
Years lived in the location prior to diagnosis, median (range)	18.6 (2-44)	22.7 (1-55)

Hispanic patients were diagnosed with PBC mainly when living in the USA. Only 2.7% of Hispanic patients had been diagnosed outside of the USA. Average years lived in the location where patients were diagnosed with PBC was 18.7 years (range: 2-64) and 22.7 years (range: 1-62) for Hispanic and non-Hispanic patients, respectively.

Comorbidities and potential risk factors

There were no differences between groups with respect to hair dye and nail polish use, alcohol and tobacco use, age of menarche or menopause, number of miscarriages or pregnancies, or itching during pregnancy. Although non-Hispanics were more likely to use birth control pills or hormone replacement therapy, this difference did not reach statistical significance. There was no statistically different difference in history of urinary tract infections, vaginal infections, sexually transmitted diseases, concomitant medical conditions, or surgical procedures (Fig. 2). Symptoms at the time of presentation, diagnosis of PBC or autoimmune disease in a first degree relative were also not statistically significant between the two groups. Table 2 details the frequency of symptoms, comorbidities and risk factors in the two Ethnic groups.

Socioeconomic factors

In our study, 27% of Hispanic patients had a high school education or less as the highest level of education, as compared to only 17% of non-Hispanics. There was no statistically significant difference in the employment status between the two groups (83.3% of Hispanics were employed, as compared to 92.3% of non-Hispanics). Among Hispanics, 62.8% had a household income of less than $50,000, as compared to only 19.5% of non-Hispanics (OR: 6.7, 95% CI: 2.5-19.2). Most patients in both Hispanic and non-Hispanic groups were not the sole provider of income in the family. The median number of family members supported by the family income was the same in both the Hispanic and non-Hispanic groups. Only 1.9% of non-Hispanics did not have any health care insurance, as compared to 13.5% of Hispanics (OR: 0.1, 95% CI: 0-0.9) (Table 3).

Treatment with UDCA

Median dose of UDCA was the same for both Hispanic and non-Hispanic groups (1000 mg). Hispanic patients were numerically more likely to miss their medications, as compared to non-Hispanics, but this did not reach statistical significance (35.5% vs. 22.6%). The proportion of patients going without medications to afford food, housing and bills was similar in both groups (82.9% in Hispanics vs. 87% in non-Hispanics).

Discussion

In the current study, we investigated differences in demographics, comorbidities, environmental risk factors and socioeconomic status between Hispanic and non-Hispanic patients with PBC. We could not demonstrate any significant difference in comorbidities or environmental risk factors between the two groups. However, we found important socio-economic disparities, including lower level of education, lower income, and decreased availability of health care insurance among Hispanic patients. Furthermore, Hispanic patients were less likely to be married and frequently had a "recent" diagnosis of PBC compared to the Non-Hispanic patients. All of these characteristics could have a major role in disease progression and their medical management.

Carrion *et al.*,[4] studied chronic liver disease with different etiologies in Hispanic patients. The investigators found higher incidence and more aggressive course as well as worse outcomes despite treatment for many of these diseases. Specifically, Hispanic patients with nonalcoholic fatty liver disease (commonly known as NAFLD) had more advanced fibrosis and Hispanic patients with chronic hepatitis C infection had faster progression to cirrhosis. Furthermore, incidence and mortality from hepatocellular carcinoma were higher in Hispanics compared to non-Hispanic whites.

Peters *et al.*[1] studied patients with PBC from 11 of the states in the USA, including 501 females and 34 males, mean age of 52, from an ethnically diverse background. In that study, most patients were Caucasian, although their cohort also included 42 Hispanics. They showed more severe disease in Hispanics and African Americans, with greater limitation on activity level, worse pruritus, and

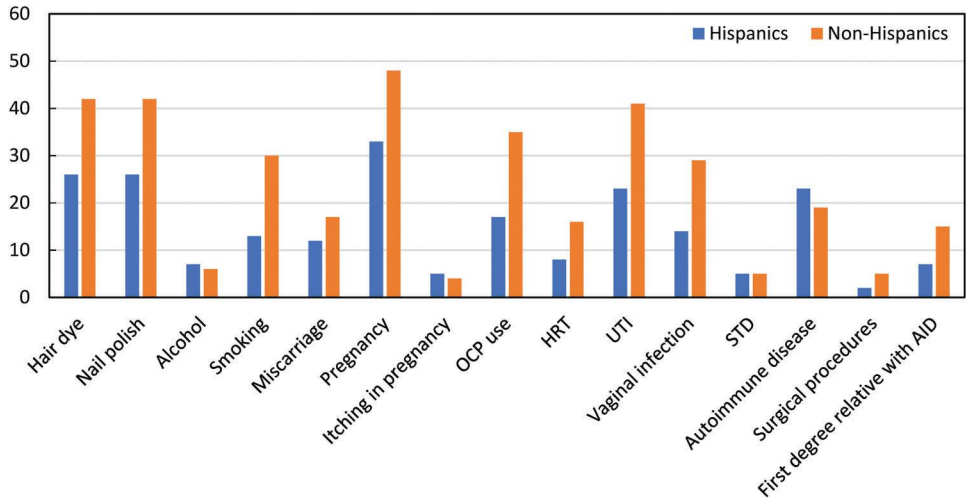

Fig. 2. Comorbidities and risk factors in Hispanic patients with PBC compared with non-Hispanics.

Abbreviation: AID, autoimmune disease; HRT, hormone replacement therapy; UTI, urinary tract infection; STD, sexually transmitted disease; PBC, primary biliary cholangitis.

Table 2. Comorbidities and risk factors in Hispanic and non-Hispanic patients with PBC

Comorbidities and risk factors	Hispanics	Non-Hispanics	OR (95%CI)
Hair dye, *n* (%)			
Never, rarely	11 (30%)	12 (23%)	1.48 (0.56-3.89)
Sometimes, often, always	26 (70%)	42 (77%)	
Nail polish, *n* (%)			
Never, rarely	11 (29.7%)	12 (22.2%)	1.47 (0.56-3.89)
Sometimes, often, always	26 (70.3%)	42 (77.8%)	
Tanning salon use, *n* (%)			
Never, rarely	37 (100%)	54 (100%)	
Sometimes, often, always	0	0	
Alcohol use, *n* (%)			
Never, rarely	30 (81.1%)	48 (88.9%)	0.54 (0.16-1.82)
Sometimes, often, always	7 (18.9%)	6 (11.1%)	
Smoking, *n* (%)			
Never, rarely	22 (62.9%)	23 (43.4%)	2.18 (0.91-5.37)
Sometimes, often, always	13 (37.1%)	30 (56.6%)	
Age of menarche, median (range)	12 (9-20)	12 (10-15)	
Age of menopause, median (range)	48 (37-60)	50 (35-60)	
Number of living children, median (range)	2 (0-5)	2 (0-4)	
History of miscarriage, *n* (%)	12 (33.3%)	17 (32.7%)	1.03 (0.41-2.56)
History of pregnancy, *n* (%)	33 (91.7%)	48 (92.3%)	0.91 (0.18-5.20)
History of itching during pregnancy, *n* (%)	5 (16.1%)	4 (9.5%)	1.79 (0.42-8.24)
History of birth control use, *n* (%)	17 (45.9%)	35 (66%)	0.44 (0.18-1.05)
HRT, *n* (%)	8 (21.6%)	16 (30.2%)	0.64 (0.23-1.70)
UTI, *n* (%)	23 (62.2%)	41 (75.9%)	0.52 (0.21-1.32)
Vaginal infection, *n* (%)	14 (38.9%)	29 (55.8%)	0.51 (0.21-1.21)
STD, *n* (%)	5 (13.9%)	5 (9.6%)	1.51 (0.38-6.05)
Comorbidity, *n* (%)*	8 (21.6%)	10 (18.5%)	1.21 (0.41-3.49)
Surgery, *n* (%)	2 (5.4%)	5 (9.3%)	0.59 (0.07-3.03)
Itching at diagnosis, *n* (%)	15 (40.5%)	26 (50.0%)	0.69 (0.29-1.61)
First degree relative with PBC, *n* (%)	7 (18.9%)	15 (32.6%)	0.49 (0.16-1.35)

Abbreviations: HRT, hormone replacement therapy; UTI, urinary tract infection; STD, sexually transmitted disease.
*Comorbidity includes Sjogren's syndrome, hypothyroidism, scleroderma, CREST, Raynaud's, rheumatoid arthritis, systemic lupus erythmatosos, polymyositis, diabetes, hypertension, coronary artery disease, hypercholesterolemia, history of cancer, asthma, herpes zoster, celiac disease.

severe disease as evidenced by laboratory data and exam findings (including splenomegaly, telangiectasia, peripheral edema, icterus, ascites and clubbing). Along these lines, Hispanics and African Americans were more likely to be excluded from treatment trials due to more severe liver disease. The investigators postulated that this increased disease severity noted among Hispanics and African Americans could have been due to delayed referral to tertiary care center, although this could not be evaluated. Another hypothesis was that Hispanics and African Americans had earlier onset PBC and were misdiagnosed. It was unclear from the study if these patients had more limited access to care, faster disease progression, initial misdiagnosis, or different comorbidities affecting their progress.

Although we have previously shown that Hispanic patients with PBC are more likely to have complications of advanced liver disease,[2] the potential factors contributing to this observation were not studied. Thus, we were interested in understanding how one's ethnicity and social environment could influence the clinical presentation and progression of PBC. Potential environmental risk factors affecting development of PBC have been extensively evaluated and include smoking,[5–9] tonsillectomy,[5] vaginal or urinary tract infections (especially if multiple),[5–9] having a first degree relative with PBC,[6,7,9] use of hormone replacement therapy,[7] history of psoriasis and other autoimmune diseases,[6,8,9] and shingles.[8] Prince *et al.*[8] found no association with obstetric risk factors. Interestingly, in one study, the longer use of oral contraceptive pills was actually protective against development of PBC.[6] In addition to being a risk factor for the development of PBC, smoking is also an independent risk factor for development of fibrosis in PBC patients.[10]

Table 3. Socioeconomic characteristics of Hispanic and non-Hispanic patients with PBC

Socioeconomic variables	Hispanics	Non-Hispanics	OR (95% CI)
High School education and below College education and above	27% 73%	17% 83%	1.7 (0.6-5.1)
Employed Unemployed	83.3% 16.7%	92.3% 7.7%	2.3 (0.6-10.2)
Household income less than 50,000	62.9%	19.6%	6.7 (2.5-19.2)*
Health care insurance	86.5%	98.1%	0.1 (0-0.9)*
Missing medications	35.5%	22.6%	0.5 (0.2-1.4)
Missing medications to afford essentials	17.1%	13%	1.3 (0.4-4.6)

Data from recent studies also indicates that Hispanic patients with PBC are less likely to undergo liver transplantation than Caucasian patients. Using the United Network for Organ Sharing registry, Cholankeril *et al.*[11] performed a retrospective review of the PBC liver transplant waitlist registrant's cohort, from 2000 to 2014. During this period, of a total of 156,624 adult patients listed for liver transplant, only 3.5% were waitlisted for a primary diagnosis of PBC. Overall, their mean age was 55.6 years, with women representing 86.2% of these patients, and 76.4% of all patients were Caucasian (vs. 14.5% Hispanics). Compared with white registrants, Hispanic registrants were significantly younger in age, had a higher proportion of females, were noted to have a higher mean model for end-stage liver disease score at listing, were more likely to develop complications related to portal hypertension at the time of listing, and were less likely to have private insurance. The authors found that the proportion of Hispanic registrants increased from 10.7% to 19.3% in this period of time, despite an overall decrease in the total number of PBC registrants; they also had the highest percentage of waitlist deaths, the lowest rate for undergoing LT, a significantly higher risk of death while on the waitlist, and the highest proportion of waitlist removals due to clinical deterioration.[11]

In the current study, Hispanics and non-Hispanics were not different with respect to the multiple risk factors and comorbidities thought to be associated with PBC and reported similar rates of pruritus and fatigue. Importantly, the main findings in the present study relate to differences in socioeconomic factors. Namely, Hispanics with PBC were less likely to be married (58% vs. 75%), indicating less help with responsibilities at home. These patients also had a lower level of education and lower income, and as expected were less likely to have medical insurance. All of these factors point towards more difficult access to health care among Hispanics, which could indeed explain the difference in disease severity noted by our group and by Peters *et al.*[1,2] Decreased access to health care can potentially lead to late presentation, more severe disease and lack of response to available therapies. In addition, the fact that the vast majority of Hispanics were diagnosed with PBC only when living in the USA also raises concern for missed diagnosis at their country of origin and longer disease duration prior to diagnosis. Finally, given that there was no difference in the mean age at diagnosis, a delayed diagnosis among Hispanics would imply initial presentation at age younger than 50, and it is well known that age is a determinant of PBC progression and response to UDCA.[12]

Our study was limited by a relatively low response rate, the potential for recall bias and the inability to provide strong evidence of cause and effect, as is characteristic of survey studies. To enhance our response rate, we attempted to contact patients through three different methods (mail, e-mail, and phone). It is possible that a lower income was also associated with decreased access to communication, in the form of e-mail or phone; if that is the case, lower response rates might be expected in this subgroup of patients. For others, legal status in this country could also play a role in their willingness to participate in a research study, and this in turn could have affected the response rates of the immigrant population. Trained personnel conducted all of the phone interviews in a standardized fashion. In addition, when appropriate, these interviews were conducted by a native Spanish speaking investigator to improve patients' level of comfort with the study.

We also acknowledge that there was not sufficient information on the prevalence of NAFLD or NASH and its effect on progression of PBC in this cohort of patients.

It has been reported that both Hispanics and African Americans have a more severe presentation of PBC compared to Caucasian patients. In the analysis by Peters *et al.*,[1] when comparing with Caucasian patients, the African American and Hispanic patients were significantly more likely to be limited in their physical activity level, to have severe or difficult-to-control pruritus, or to have a history of ascites, hepatic encephalopathy, or variceal bleeding.[1] Ethnic disparities are also evident in terms of in-hospital mortality for PBC patients; despite a reported increase in hospitalizations of non-Hispanic PBC patients from 57.8% to 71.2% in a 7-year period (from 2007 to 2014) compared to 4.1-6.3% for African-Americans and 8.6-10.9% for Hispanics, the highest in-hospital mortality was observed in African-American PBC patients.[14] In the present study, both Caucasian and African American non-Hispanics were analyzed together; although this could result in an overestimation of severity for the non-Hispanic group, it is unlikely to have a significant effect in our study, given that only 3.1% of the non-Hispanic patients self-identified as African American.

To our knowledge, this is the first study to compare risk factors and comorbidities between Hispanic and non-Hispanic patients with PBC. Our results should be validated in a larger cohort.

In summary, we have shown that access to health care was lower in the Hispanic population compared to the non-Hispanic population with PBC, which at least in part explains the differences in disease severity seen in previous studies. Environmental risk factors and comorbidities do not seem to significantly explain the variability in the disease course in different ethnic populations. Future studies should focus on evaluating different genetic backgrounds between Hispanic

and non-Hispanic patients with PBC and its role in the severity and progression of PBC.

Funding

None to declare.

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Designed the study and wrote the manuscript (AR), (CL) performed the study and analyzed the data (AR, NAPP, AFDLV, CL).

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Review Article

2019 Chinese Clinical Practice Guidelines for the Prevention of Mother-to-child Transmission of Hepatitis B Virus

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Abstract

To develop the evidence-based guidelines for managing mother-to-child transmission of hepatitis B virus in China, a multidisciplinary guideline development group was established. Clinical questions were identified from two rounds of surveys on the concerns of first-line clinicians. We conducted a comprehensive search and review of the literature. A grading of recommendations' assessment, development, and evaluation system was adopted to rate the quality of evidence and the strength of recommendations. Recommendations were formulated based on the evidence, overall balance of benefits and harms (at individual and population levels), patient/health worker values and preferences, resources available, cost-effectiveness, and feasibility. Eventually, recommendations related to 13 main clinical concerns were developed, covering diagnostic criteria, treatment indications, antiviral therapy choice, timing to initiate and discontinue treatment, immunoprophylaxis strategy at birth, and how to deal with special situations, such as unintended pregnancy, assisted reproduction, and breastfeeding. The guidelines are intended to serve as guidance for clinicians and patients, to optimize the management of majority of pregnant women who are positive for hepatitis B surface antigen. **Guideline registration:** International Practice Guide Registration Platform (IPGRP-2018CN040).

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Introduction

As a primary cause of liver cirrhosis and cancer, chronic hepatitis B (CHB), accounting for about 1 million deaths per year, remains a severe public health problem and presents a heavy disease burden and economic burden to society and families.[1] With an extensive hepatitis B vaccination program implemented, mother-to-child transmission (MTCT) has become the key obstacle to realizing the World Health Organization's goal of reducing the prevalence of hepatitis B surface antigen (HBsAg) among children aged 5, to 0.1%.[2] Furthermore, MTCT is responsible for familial clustering of hepatitis B virus (HBV) infection[3] in which the risk of cirrhosis and hepatocellular carcinoma increase significantly and the age of onset of end-stage liver diseases was advanced dramatically.[4] Elimination of MTCT is crucial to decreasing new HBV infections and to minimizing the burden of HBV-related diseases.

As the most principal strategy to prevent new HBV infections, the hepatitis vaccine has reduced the rate of MTCT by more than 80%[5] Whereas, among infants born to hepatitis B e antigen (HBeAg)-positive mothers, there are still 8% becoming CHB after vaccine immunoprophylaxis, and 4% after immmunoprophylaxis of vaccine combined with human hepatitis B immunoglobulin (HBIG).[5] Annually, there are almost 2 million new infections in children younger than 5 years. Antiviral intervention during pregnancy has been widely adopted to interrupt MTCT; nevertheless, there is still controversy about treatment indications, antiviral therapy choice, and the timing to initiate and discontinue the treatment, and so on.

Keywords: Hepatitis B virus; Mother-to-child transmission; Clinical practice guidelines; Prevention; GRADE.

Abbreviations: ADV, adefovir dipivoxil; AGREE II, Appraisal of Guidelines for Research and Evaluation; ALT, alanine aminotransferase; CHB, chronic hepatitis B; CK, creatine kinase; ETV, entecavir; GRADE, grading of recommendations assessment, development, and evaluation; HBeAg, hepatitis B e antigen; HBIG, human hepatitis B immunoglobulin; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; IFN, interferon; LAM, lamivudine; LdT, telbivudine; MTCT, mother-to-child transmission; PICOs, population, intervention, comparator, outcomes; RIGHT, Reporting Items for Practice Guidelines in Healthcare; TAF, tenofovir alafenamide; TDF, Tenofovir disoproxil fumarate; TFV, tenofovir; ULN, upper level of normal.

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To further standardize the clinical recommendations for top concerns of first-line clinicians, a multidisciplinary guideline development group was established to comprehensively evaluate the evidence and overall balance of benefits and harms, while the guidelines do not cover the whole spectrum of prevention and treatment of MTCT. As with clinical practice guidelines, they provide general guidance to optimize management of the majority of pregnant patients infected with HBV, while clinical judgement considering a unique patient and reliability of clinical care should be considered. In addition, despite accumulated knowledge, areas of uncertainty still exist and therefore health care workers, patients, and public health authorities must continue to make choices based on evolving evidence. The guidelines have two versions: the Chinese language version published in the *Chinese Journal of Infectious Diseases* and the English language version, which is the current version.

Methods

The guidelines were launched by the Society of Infectious Diseases, Chinese Medical Association, supported by the Chinese Grading of Recommendations Assessment, Development and Evaluation (GRADE) Center in methodology, and developed according to the World Health Organization's Handbook for Guideline Development (2014).[6] Appraisal of Guidelines for Research and Evaluation (known as AGREE II)[7] and Reporting Items for Practice Guidelines in Healthcare (known as RIGHT) tool[8] were also referred to. Three groups were established for developing the guidelines: steering committee, guidelines development panel, and guidelines secretary group. The steering committee consisted of 3 well-known experts in the field, with the following missions: 1) approve the use of population, intervention, comparator, outcomes (PICOs), 2) supervise the literature search and systematic reviews, 3) check the grade of the evidence, 4) finalize the recommendations using a modified Delphi approach, and 5) approve the publication of the guidelines. A multidisciplinary guidelines development panel, including experts from across the country in infectious diseases, hepatology, obstetrics, pediatrics, and methodology, was established, and tasked with the following missions: 1) generate the scope of the guideline and draft the PICOs, 2) grade the quality of the evidence, 3) draft the preliminary recommendations, and 4) write and publish the draft guideline. The guidelines secretary group conducted systematic reviews and investigated patients' views and preferences. All members involved in guidelines development were required to disclose any potential conflicts of interest, which were reviewed by the chairs (Yingren Zhao and Yaolong Chen). No relevant conflict of interest was reported.

Before initiating the guidelines, we wrote the protocol and registered it in the International Practice Guidelines Registry Platform (http://www.guidelines-registry.org, IPGRP-2018CN040). First, we collected questions reflecting clinicians' concerns through two rounds of questionnaire survey. Two hundred sixty-one copies of the questionnaire were collected from 98 facilities across mainland China, covering 26 provinces, municipalities, and autonomous regions. After deduplication and combination, 16 PICO questions were identified from among 68 clinical questions, based on importance grade. Published articles and conference abstracts were identified from PubMed, Embase, the Cochrane Library, and three Chinese literature databases (CNKI, WanFang, and CBM). The evidence synthesis group conducted systematic reviews and other literature searches for each question. We finally conducted 11 new systematic reviews. The GRADE was used to evaluate and rate the quality of evidence body (Table 1).[9] We then formulated recommendations and rated their strengths after comprehensive assessment of the quality of evidence, consideration of the overall balance of benefits and harms, patient/health worker values and preferences, cost-effectiveness, and feasibility. Finally, the guidelines development panel reached a consensus on each recommendation based on three rounds of Delphi survey and also reached a consensus on management algorithm for MTCT of HBV (Fig. 1). A flow chart describes the process of the guidelines development (Fig. 2).

Recommendations

The guidelines contain 24 recommendations on the top 13 concerns of clinicians, covering diagnostic criteria, monitoring and prevention during pregnancy, and breastfeeding, as well

Table 1. Grades of evidence and recommendations

Grade of evidence	Notes
High quality (A)	Further research is very unlikely to change our confidence in the estimate of effect.
Moderate quality (B)	Further research is likely to have an important impact on our confidence in the estimate of effect and there is a possibility that it may change the estimate.
Low quality (C)	Further research is very likely to have an important impact on our confidence in the estimate of effect and may be substantially different from the estimate of the effect.
Very low quality (D)	The estimate of effect is very uncertain, and the true effect is likely to be substantially different from the estimate of effect.
Grade of recommendation	
Strong (1)	The Guideline Panel is confident that the desirable effects of an intervention outweigh its undesirable effects (strong recommendation for an intervention), or that the undesirable effects of an intervention outweigh its desirable effects (strong recommendation against an intervention).
Weak (2)	The desirable effects probably outweigh the undesirable effects (weak recommendation for an intervention) or undesirable effects probably outweigh the desirable effects (weak recommendation against an intervention) but less uncertain higher cost or resource consumption exists.

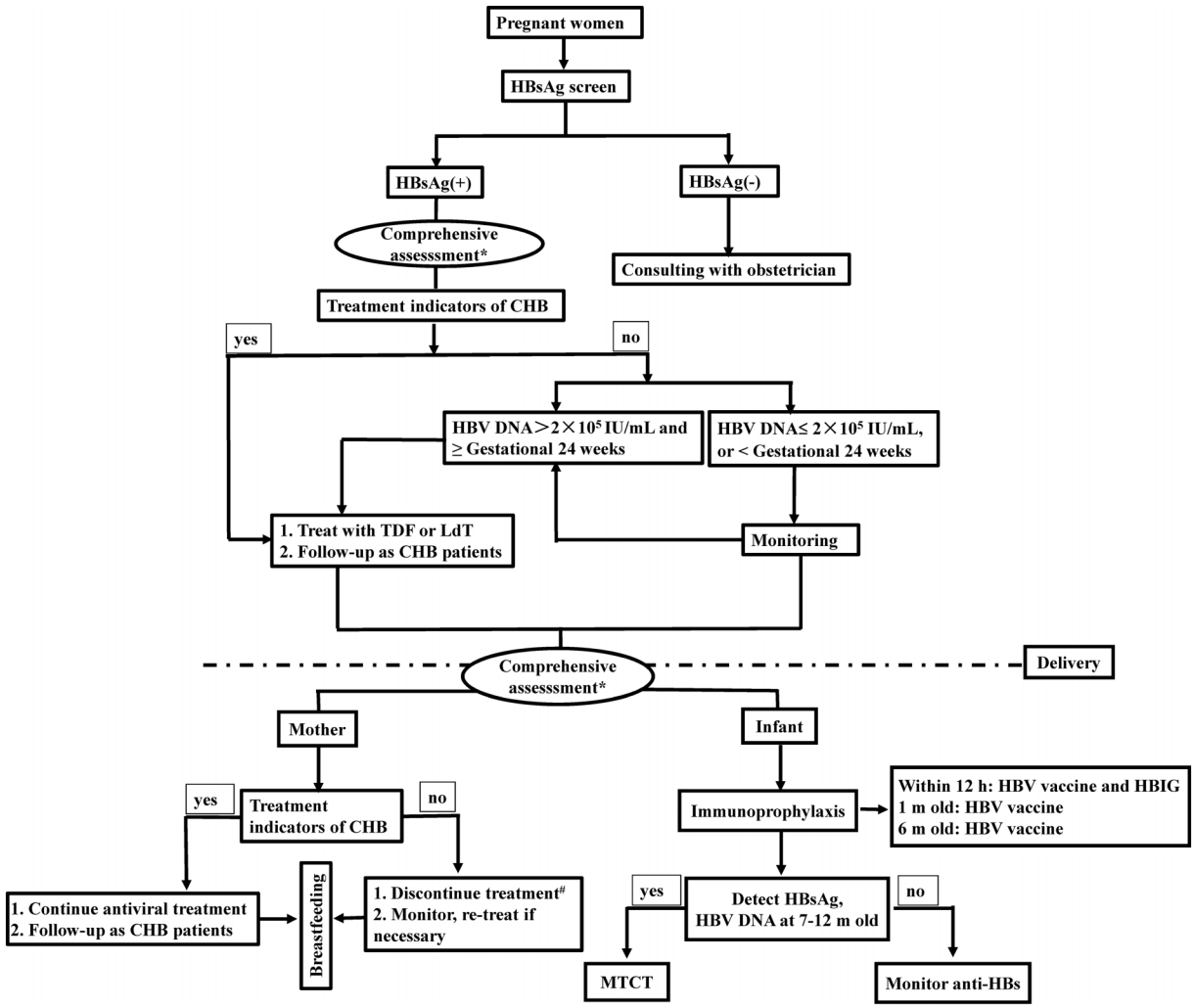

Fig. 1. Management algorithm for mother-to-child transmission of hepatitis B virus. *Comprehensive assessment: liver biochemical function, HBV DNA, imaging assessment; #Time to discontinue treatment: at delivery, postpartum 1 or 3 m old.

Abbreviations: CHB, chronic hepatitis B infection; HBIG, human hepatitis B immunoglobulin; HBsAg, hepatitis B surface antigen; HBV, hepatitis B Virus; m, months; MTCT, mother-to-child transmission; TDF, tenofovir disoproxil fumarate; LdT, telbivudine.

as immunoprophylaxis strategy at birth. All the recommendations are as follows.

Question 1: How to diagnose MTCT of HBV?
Recommendation: Infants with HBsAg and/or HBV DNA positive at 7-12 months-old are diagnosed as having CHB infection due to MTCT (1B).

Recommendation evidence

The previous reported rate of MTCT varied markedly, owing to inconsistence in specimens, timepoints of detection, and diagnostic criteria. Currently, HBsAg and/or HBV DNA positive at 7-12 months-old is deemed as having obtained CHB transmitted from mothers.[10,11] Whereas, there is no systematic review to assess the criterion. A systematic review and network meta-analysis showed the rate of HBsAg and/or HBV DNA positive at birth in cord blood or venous blood was significantly higher than that at 6, 7, or 12 months-old,[12] which indicated the excessive positive rate at birth may be attributed to false positivity caused by contamination of maternal blood or transient viremia due to placental abruption at birth.[13,14] In addition, there was no significant difference among the positive rates at age of 6, 7 and 12 months.[12] Therefore, HBV serological markers and HBV DNA should be tested at 7-12 months-old, namely 1-6 months after three dosages of vaccination, to determine the immune results and infection status. Moreover, the infants over 12 months-old with HBsAg and/or HBV DNA positive at first visit are also supposed to acquire HBV infection by MTCT.

Question 2: What is the vaccination schedule for infants of HBsAg(+) mothers?
Recommendations:
2.1: The infants of HBsAg(+) mothers should receive hepatitis B vaccine and 100 IU HBIG within 12 h after birth, and the following two doses of vaccine at 1 and 6 months-old, respectively (1A).
2.2 For premature or low-birth weight infants, the combined immunoprophylaxis should be administered within 12 h after birth when the vital signs are stable or after the

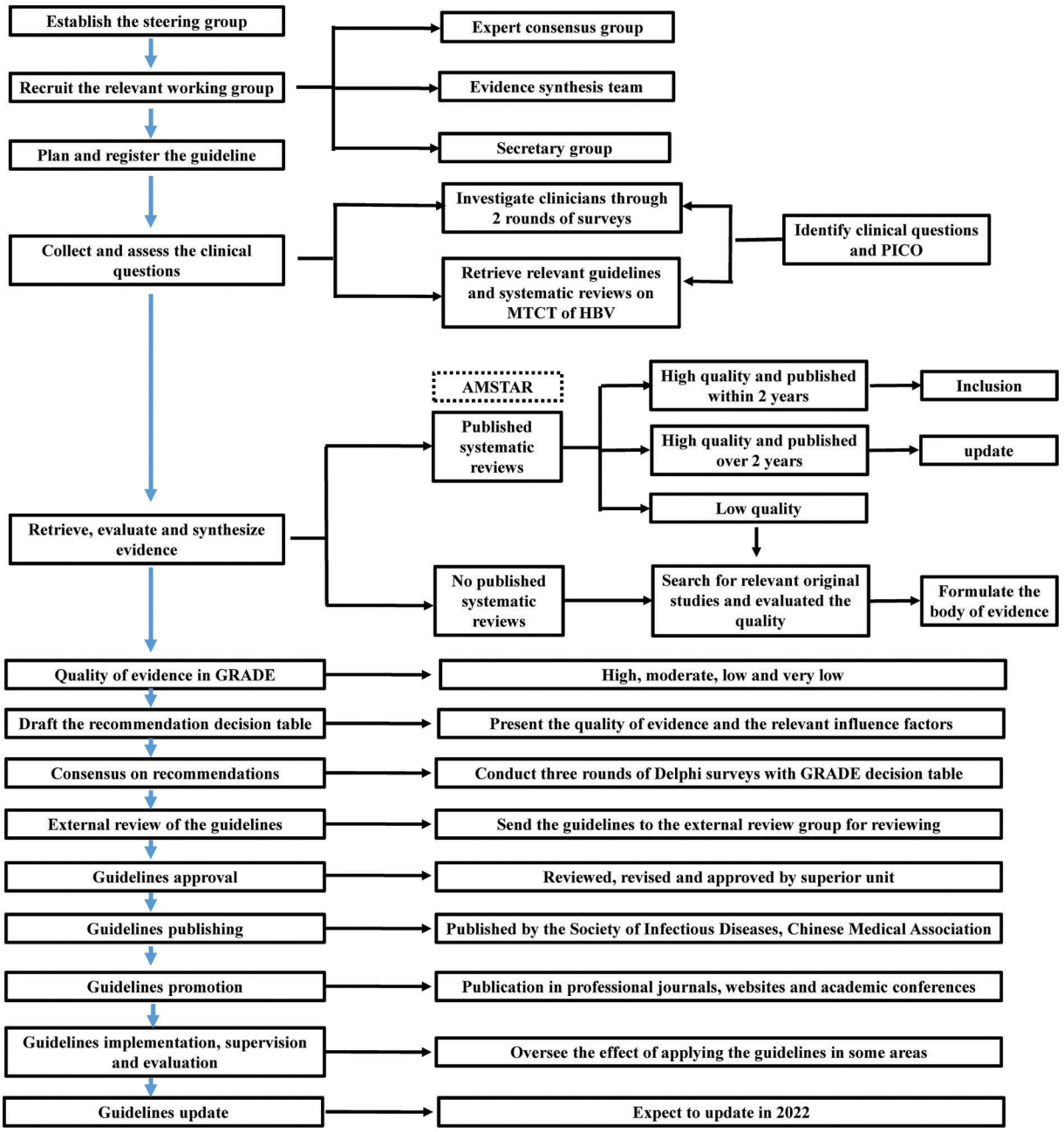

Fig. 2. Flowchart of the process of the guidelines development.

Abbreviations: GRADE, the Grading of Recommendations Assessment, Development and Evaluation; HBV, hepatitis B virus; MTCT, mother-to-child transmission; PICO, population, intervention, comparator, outcomes.

viral signs become stable. Three doses of full-course vaccine should be administered subsequently (1A).

Recommendation evidence

Combined immunoprophylaxis is the current standard immunoprophylaxis strategy for preventing MTCT in HBsAg(+) mothers. Several sets of guidelines[15–19] recommend newborns of HBsAg(+) mothers receive hepatitis B vaccine (10 μg recombinant yeast-derived hepatitis B vaccine or 20 μg recombinant Chinese hamster ovary cells hepatitis B vaccine) and HBIG, and vaccines are administered at 0, 1, and 6 months-old, respectively. For the premature or low-birth weight infants, one dose of vaccine is implemented as soon as possible within 12 h after birth (when the viral signs are stable) and another three doses of full-course vaccine are conducted after 1 month-old age is reached. For the very low-birth weight infants, those with severe birth defects, severe

asphyxia, or respiratory distress syndrome, should receive four doses of vaccine, administered after the vital signs become stable. By systematic review, we found 200 IU HBIG shows equivalent preventive effectiveness with 100 IU HBIG in infants born to CHB mothers (relative risk: 1.08, (0.64-1.82)) and HBeAg(+) mothers (relative risk: 0.84 (0.39-1.77)).[20] Considering cost-effectiveness, 100 IU HBIG is recommended to newborns of HBsAg (+) mothers.

Whether vaccine should be boosted in infants born to HBsAg(+) mothers is unclear. Systematic review also showed that the response to vaccine is similar between infants born to HBsAg(+) mothers and the general population.[21] While the titer of anti-hepatitis B surface antibody should be regularly assessed, boost could be considered when the titer is less than 10 IU/mL, regarding the high-risk circumstances of infection.

Question 3: What is the threshold of HBV DNA for antiviral intervention during pregnancy?
Recommendations:
3.1 Antivirals should be recommended to pregnant women with HBV DNA >2×10^5 IU/mL (1B).
3.2 Antiviral intervention could be decided after discussion with pregnant women with HBV DNA of 1×10^4- 2×10^5 IU/mL (2C).

Recommendation evidence

Maternal high viremia is an independent risk factor for MTCT.[22] The majority of guidelines from associations for the study of liver diseases, such as the American Association for the Study of Liver Diseases, the European Association for the Study of the Liver, the Asian Pacific Association for the Study of the Liver, and the National Institute for Health and Care Excellence, recommend antiviral intervention during pregnancy for preventing MTCT, whereas, the threshold of HBV DNA ranges from 2×10^5 IU/mL to 10^7 IU/mL.[17,23–25] In addition, the recommendations are mainly based on clinical trials aiming to evaluating efficacy of antivirals[11,26] or single retrospective cohort investigation.[27,28]

A systematic review including 6027 patients from 18 studies indicated the rates of MTCT were 0, 0.88%, 1.15%, 4.81%, 10.04% and 18.80% in pregnant women with antenatal HBV DNA <1×10^4 IU/mL, 1×10^4 IU/mL-1×10^5 IU/mL, 1×10^5 IU/mL-1×10^6 IU/mL, 1×10^6 IU/mL-1×10^7 IU/mL, 1×10^7 IU/mL-1×10^8 IU/mL, and >1×10^8 IU/mL, respectively.[5] The pooled rate of MTCT was as high as 10% in women with antenatal HBV DNA ≥1×10^6 IU/mL, which was remarkably higher than those with HBV DNA <1×10^6 IU/mL. Antivirals should be recommended for these pregnant women. Pregnant women with HBV DNA of 1×10^4 IU/mL-2×10^5 IU/mL still carry risk for transmitting the virus to their infants. Considering the high prevalence of HBsAg in China, antiviral intervention during pregnancy could be suggested in those with family history of HBV infection or history of MTCT. The benefit of antiviral treatment in terms of protecting newborns from HBV infection and controlling hepatitis activity in pregnant women with hepatitis should be explained before administration. At the same time, the patients should be notified of the side effects of antivirals, drug-resistant mutations, potential harms to the fetus, and hepatic flare after antivirals discontinuation, and so on.

Question 4: Which antivirals should be recommended for preventing MTCT?
Recommendations:
4.1. Tenofovir disoproxil fumarate (TDF) or telbivudine (LdT) is recommended for pregnant women with HBV DNA >2×10^5 IU/mL (1B).
4.2 TDF is preferred in pregnant women with drug-resistance to lamivudine (LAM) or LdT (2C).

Recommendation evidence

TDF and LdT do not show reproductive toxicity in animal experiments.[29,30] Plenty studies have manifested undifferentiated effectiveness of TDF and LdT in preventing MTCT.[10,11,31] In addition, the frequency of adverse events in pregnant women receiving TDF or LdT, such as abnormal creatinine, postpartum hemorrhage, rate of cesarean section, birth defects, and preterm birth were comparable with general population.[26] A prospective cohort has demonstrated undifferentiated growth and development in infants born to LdT-treated pregnant women during 5 years' follow-up.[32] For the pregnant women at high risk of MTCT, TDF or LdT should be administered to inhibit virus replication and reduce the transmission risk.

LAM and LdT present high potential of drug-resistance. Previous studies have confirmed the advantage of TDF over LAM and LdT as antiviral therapy.[33] Furthermore, the superiority of TDF has also been demonstrated in pregnant women with LAM or LdT-resistant mutants.[34] Hence, TDF could be suggested to pregnant women with drug-resistance to LAM or LdT.

As the first-line antiviral medicine for CHB patients, tenofovir alafenamide (TAF) has no influence on reproductive function in animal experiments. A study in human immunodeficiency virus-infected pregnant women assessed the safety of TAF but had small sample size.[35] The undergoing prospective, multicenter clinical trials will provide evidence for efficacy and safety of TAF in pregnant women with CHB.

Question 5: When should the antiviral be initiated to prevent MTCT during pregnancy?
Recommendation: The antiviral should be initiated at 24-28 weeks of gestation for preventing MTCT (2C).

Recommendation evidence

Head-to-head comparison of the efficacy and safety of different timepoints to initiate antiviral intervention is lacking. A Bayesian network meta-analysis and system review showed that the risk of MTCT decreased significantly in pregnant women accepting intervention before 28 weeks of gestation, as compared to those initiating after 28 weeks (relative risk: 0.019, (0.00034-0.19)).[36] Additionally, the efficacy and safety of antiviral therapy initiated from 24 weeks of gestation have been identified in cohort studies and case-control studies.[10,11,31,37,38] Therefore, pregnant women with high HBV DNA levels (>2×10^5 IU/mL) are recommended to initiate antiviral intervention at 24-28 weeks of gestation. For pregnant women with high viremia who are visiting the hospital after 28 weeks of gestation, antiviral intervention should be initiated immediately. For pregnant women with HBV DNA of 1×10^4 IU/mL -2×10^5 IU/mL who agree to take antivirals, the intervention could be initiated no later than 28 weeks of gestation.

Question 6: How to manage unintended pregnancy during antiviral therapy?
Recommendation: For patients who become unintentionally pregnant during antiviral therapy, TDF or LdT treatment should be continued (2B); adefovir dipivoxil (ADV) or entecavir (ETV) should be switched to TDF (2C); the potential risks of interferon (IFN) should be fully informed, and the patient should switch to TDF if the patients and/or family members decide to carry on the pregnancy (2C).

Recommendation evidence

Data from the Antiretroviral Pregnancy Registry and well-controlled studies have revealed superior safety of TDF and LdT in pregnant women.[10,11,31] Additionally, TDF shows great advantage in antiviral treatment because of superior resistance profile and more extensive safety data in pregnant women. A systematic review[39] showed the rate of birth defects as 0.66% in pregnant women exposed to nucleos(t)ide analogues, which are undifferentiated from the rate in Chinese population (5.6%)[40] and Metropolitan Atlanta Congenital Defects Program (2.8%).[41] Therefore, childbearing women with unintended pregnancy during antiviral therapy should continue TDF or LdT.

The safety of ADV and ETV in pregnancy has not be elucidated clearly. A systematic review[39] including safety data from the Antiretroviral Pregnancy Registry showed the rate of birth defects in pregnant women exposed to ADV or ETV is comparable with that among the general population. Hence, women undergoing treatment of ADV or ETV could continue a pregnancy under doctors' guidance. However, regarding the risks of birth defects associated with high dose of ADV or ETV in animal experiments, switching to TDF is recommended.

We found low-quality evidence about the safety of IFN during pregnancy. Randomized controlled study of IFN administration during pregnancy is unlikely to be conducted, given the ethical concerns of such a trail. Guidelines suggest that IFN is contraindicated during pregnancy and contraception is recommended during IFN treatment,[24,42,43] while how to deal with unintended pregnancy during IFN treatment causes substantial controversy between obstetricians and hepatologists. In a Rhesus monkey model, 90-180 times the recommended dosage of IFN led to increased rate of abortion. A series of cohort studies had displayed undifferentiated rates of adverse effects, including birth defects in pregnant women with essential thrombocythemia[44,45] or multiple sclerosis[46,47] following exposure to INF. In addition, in case reports, the infants born to HBV or hepatitis C virus/human immunodeficiency virus-infected mothers exposed to IFN during first trimester did not display abnormal rates of birth defects.[48–50] The data from Bayer HealthCare's global pharmacovigilance database have not revealed an obvious increase of adverse effects in pregnant women exposed to IFN during the early trimester.[47,51] With comprehensive assessment of toxicology, clinical reports, and views of obstetric experts, we suggest the risk of IFN should be fully informed to the pregnant women and their family members, and the TDF should be recommended instead of IFN if the family decides to continue the pregnancy.

Question 7: How to deal with HBsAg(+) pregnant women with hepatic flare?

Recommendations:
7.1: For pregnant women with HBV DNA $\leq 2\times10^5$ IU/mL and ALT $<2\times$ the upper limit of normal (ULN), close monitoring should be conducted (2C).
7.2: If a hepatic flare is confirmed to be associated with immune activation, antiviral treatment and monitoring should be initiated as CHB patients (1C).

Recommendation evidence

We found low-quality evidence on management of hepatic flare during pregnancy. About 10% of pregnant women presents hepatic flare and the majority of those cases involve mild ALT elevation.[52,53] For pregnant women with HBV DNA $\leq 2\times10^5$ IU/mL, mild ALT elevation ($<2\times$ULN) and no cirrhosis, we suggest close monitoring without antiviral treatment, according to existing evidence.[52–54] A proportion of CHB patients with mild ALT flare experience disease progression.[24] More evidence is required for treatment consideration in pregnant women with mild hepatitis flare.

For pregnant women with $2\times$ULN $\leq$ ALT $<5\times$ULN, close monitoring is recommended. If ALT continues fluctuating and hepatitis is attributed to immune activation, a treatment decision should be made.[24,55] Pregnant women with advanced fibrosis or cirrhosis should initiate antiviral treatment immediately, and close monitoring is required throughout the pregnancy.

Question 8: Which indicators should be monitored during antiviral therapy for pregnancies?
Recommendations:
8.1: For pregnant women taking antivirals to prevent MTCT, tests of liver biochemical function and HBV DNA should be conducted after 4 weeks (2C).
8.2: For those with hepatitis flares, more frequent monitoring and follow-up is recommended (2D). Renal function and serum phosphorus should be examined in patients receiving TDF, and creatine kinase (CK) should be measured in patients receiving LdT (2C).

Recommendation evidence

There is no consensus on monitoring timepoint and indicators examined in pregnant women undergoing antiviral treatment. A systematic review[56] showed a mean decrease of 3.16 $\log_{10}$ IU/mL (95% confidence interval: 2.97-3.35) in HBV DNA after 4 weeks of antiviral treatment; therefore, HBV DNA level after 4 weeks of treatment can be tested to forecast the risk of transmission. HBV DNA levels should be tested again before delivery to further assess the risk.

There is potential influence on renal function and bone turnover[57] during long-term TDF treatment and risk of CK increase during LdT treatment.[58] In terms of the potential adverse effects, renal function and serum phosphorus should be examined in pregnant women with TDF treatment and CK during LdT treatment.

Question 9: Does HBV infection influence assisted reproduction?
Recommendation: Considering the comparable cleavage rate, embryo implantation rate, pregnancy rate, and abortion rate in infertile women with CHB, assisted reproduction could be conducted following the same

intervention and monitoring algorithm as in other CHB pregnant women (2C).

Recommendation evidence

The impact of HBV on assisted reproduction and pregnancy outcomes is uncertain. Studies about MTCT rate in infertile women are scarce. One case-control study[59] showed lower rate of fertilization, cleavage, high-quality embryos, and pregnancy in infertile women with HBV infection. In women with HBV DNA $\geq 5\times10^2$ IU/mL, few investigations have shown that ovarian reserve was lower and the rate of fertilization and high-quality embryos was decreased.[60,61] Nevertheless, the systematic review we performed showed the rate of fertilization to be only a little lower, while there were not significant differences in the rate of cleavage, high-quality embryos, implantation, pregnancy, and abortion.[62] In these circumstances, the infertile women with HBV infection could accept assisted reproduction. The intervention strategy should follow the recommendations for CHB pregnant women.

Question 10: Could amniocentesis be conducted in CHB pregnant women?
Recommendations:
10.1: Amniocentesis can increase the risk of MTCT in pregnant women with HBV DNA $\geq 1\times10^6$ IU/mL and can be conducted only if the potential benefit is considered definite after assessment by an obstetrician (2B).
10.2: Amniocentesis is feasible after weighing the benefits and harms in pregnant women with HBV DNA $<1\times10^6$ IU/mL (2B).

Recommendation evidence

A previous systematic review[63] concluded that the risk of HBV transmission in amniocentesis was low in women with HBV DNA $<1\times10^6$ IU/mL, whereas the risk increased significantly in HBeAg(+) mothers with HBV DNA $\geq 1\times10^6$ IU/mL (relative risk: 3.41-9.54). The 2018 updated American Association for the Study of Liver Diseases Guidelines recommend the risk of MTCT be considered when assessing the potential benefit of amniocentesis in women with high viremia.[17] Therefore, for pregnant women with low viral load, amniocentesis could be conducted with signed written content; while for the women with high viremia ($\geq 1\times10^6$ IU/mL), the risk of MTCT should be assessed thoroughly and amniocentesis could be conducted for screening inherited and chromosomal diseases after consultation with an obstetrician.

Question 11: What is the influence of delivery mode on MTCT?
Recommendation: Cesarean section may reduce the risk of MTCT in pregnant women with antenatal HBV DNA $>2\times10^5$ IU/mL without intervention during pregnancy, and could be considered when there is fetal distress, macrosomia, or overdue pregnancy (2C).

Recommendation evidence

With appropriate intervention and close monitoring, the risk of MTCT has decreased by a great degree, and the delivery mode does not affect MTCT. However, a portion of pregnant women do not undertake regular follow-up and appreciate intervention during pregnancy, especially in underdeveloped regions, in this case, cesarean section could reduce the risk of MTCT (relative risk: 0.41, 95% confidence interval: 0.25~0.67, $p<0.001$) in the pregnant women with antental HBV DNA $> 2 \times 10^5$ IU/mL.[63] This population could benefit from cesarean section for reducing MTCT. After comprehensive assessment of the evidence and standpoints expressed by obstetricians, we suggest that cesarean section may be considered when there is fetal distress, macrosomia, or overdue pregnancy. To prevent excessive cesarean section, obstetric indications should also be followed.

Question 12: When should the antiviral be discontinued after delivery?
Recommendations:
12.1: Pregnant women taking antivirals for preventing MTCT can discontinue antiviral treatment immediately after delivery, 4 weeks postpartum, or 12 weeks postpartum (2C), and should be monitored closely for hepatitis flare and rebound of HBV DNA (2C).
12.2: Pregnant women accepting antivirals owing to hepatic flare should be monitored and treated according to guidelines for CHB patients after delivery (2D).

Recommendation evidence

There are a series of changes to the immune system and body function during pregnancy. No consensus has been reached about the timepoint to discontinue antiviral treatment because of the insufficient evidence on this issue.[17,24,64] Previous studies determined that about 20% of parturient women present ALT flare, regardless of antiviral treatment or not, and that there are two flare peaks, at 1 month postpartum and 3 months postpartum; the majority recover spontaneously.[52,53,65,66] The net-meta analysis showed no difference in rate of hepatitis flare among mothers discontinuing antiviral treatment immediately after delivery, 4 weeks postpartum, and 12 weeks postpartum, and those without antiviral intervention during pregnancy.[67] In view of this, the pregnant women receiving antivirals for interrupting MTCT can discontinue treatment immediately after delivery, 4 weeks postpartum, or 12 weeks postpartum. HBV DNA could rebound after antiviral discontinuation; close monitoring should be conducted and re-antiviral treatment can be considered when meeting treatment indications for CHB therapy.[24,55]

Previous studies of women who manifested ALT flare during pregnancy identified the occurrence as a risk factor for postpartum hepatic flare[66] and severe hepatitis has also been reported.[68] Therefore, it is suggested that pregnant women with active hepatitis should undergo monitoring and continue treatment after delivery, following guidelines for CHB patients.

Question 13: Could CHB mothers breastfeed?
Recommendations:
13.1: Breastfeeding is recommended after newborns accepting HBV vaccine and HBIG (2B).
13.2: The mother undergoing TDF treatment could give breastfeeding (2C).

Recommendation evidence

The research about breastfeeding in HBsAg(+) mothers is insufficient. One case-control study reported that the viral load in breast milk was related with maternal viral load[69] while the risk of transmission did not increase with high

maternal viremia.[70,71] Further systematic review found that the risk of MTCT did not increase in infants accepting breastfeeding (relative risk: 0.73, p=0.21).[72] Considering the significant benefit identified, breastfeeding is recommended to infants who undergo combined immunoprophylaxis.

The concentration of tenofovir (TFV) in breast milk and infants were 0.03% and 0.01% of recommended dose for infants from mothers with human immunodeficiency virus, respectively.[73–75] In an investigation with small size of HBsAg (+) mothers undergoing TDF treatment, the TFV was undetectable in infants accepting breastfeeding. In addition, TFV cannot be absorbed via gastrointestinal tract. Hence, the mothers undergoing TDF treatment after delivery could give breastfeeding. Studies on infants accepting breastfeeding from LdT-treated mothers are scarce.

Conclusions

Clinical guidelines are derived from clinical concerns and are intended to direct practice. Major guidelines of prevention and treatment of CHB provide recommendations for pregnant women as a special population.[17,23,24,55] The purpose of these guidelines is to provide scientific and specific guidance for the management of MTCT of HBV. Based on current clinical research outcomes, a preliminary exploration of the standardizations for managing MTCT has been established. Substantial relevant research evidence from China and other countries was fully retrieved and evaluated. Focusing on pregnant women with CHB, an expert panel from multiple disciplines proposed recommendations for the top 13 clinical concerns. Following accumulation of additional evidence and research findings, we plan to update the guidelines (at minimum) within 5 years of this publication (estimated 2022).

There are inevitably limitations to the process of guidelines' development that mainly reflect the low quality of the existing clinical studies and the small number of rigorously designed and implemented randomized controlled trials in this special population. For ethical issues, there is a lack of clinical trials on monitoring and treating CHB pregnant women with hepatitis flare; therefore, weak recommendations were proposed. In addition, scarce evidence exists on the rate of MTCT in infertile women with assisted reproduction, limiting the strength of recommendations. For infants born to CHB mothers, it is still essential to evaluate the necessity of booster vaccination. However, further scientific research will gradually address these shortcomings. Beyond that, as principal strategy, coverage of screening for HBsAg (+) in childbearing women and the vaccination program requires more efforts.

Development of these guidelines is a small step toward the goal of standardized diagnosis and optimized management of MTCT. It is hoped that these guidelines will facilitate clinical research, accumulate more high-quality evidence in the future, and ultimately promote the elimination of MTCT.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study concept and design (YRZ, GQW, WHZ, HR), drafting of the manuscript (JFL, TYC), critical revision of the manuscript for important intellectual content (YLC, WHZ, GQW, HR), administrative, study supervision (YRZ, GQW, WHZ, HR).

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Review Article

Clinical Considerations of Coagulopathy in Acute Liver Failure

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Abstract

Acute liver failure (ALF) is the rapid onset of severe liver dysfunction, defined by the presence of hepatic encephalopathy and impaired synthetic function (international normalized ratio of ≥1.5) in the absence of underlying liver disease. The elevated international normalized ratio value in ALF is often misinterpreted as an increased hemorrhagic tendency, which can lead to inappropriate, prophylactic transfusions of blood products. However, global assessments of coagulopathy via viscoelastic tests or thrombin generation assay suggest a reestablished hemostatic, or even hypercoagulable, status in patients with ALF. Although the current versions of global assays are not perfect, they can provide more nuanced insights into the hemostatic system in ALF than the conventional measures of coagulopathy.

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Introduction

Acute liver failure (ALF) is a rare, rapidly progressive syndrome that results from an acute onset of severe liver dysfunction. The most commonly accepted definition of ALF includes the development of hepatic encephalopathy and coagulopathy (international normalized ratio [INR] of ≥1.5).[1,2] The onset of acute symptoms occurs within 26 weeks, according to the American Association for the Study of Liver Diseases (AASLD).[1] But different societies have slightly different variations on the temporal classifications; for example, the European Association for the Study of the Liver (EASL) suggests three separate temporal subclassifications,[2] while the International Association for the Study of the Liver (IASL) and the Asian Pacific Association for the Study of the Liver (APASL) both employ a timeline of 4 weeks (Table 1).[3–5] Unless otherwise specified, the present review will focus on the AASLD definition of ALF. INR reflects the disruptions in hepatic synthetic function in ALF and is an essential and useful clinical prognosticating tool. Clinicians often rely on INR to assess bleeding risk in ALF.[6] However, recent studies have demonstrated that a careful approach is indispensable when interpreting raw INR values in the context of hemostasis and bleeding diathesis in ALF. In this review, we present the utility of INR as a reflection of coagulopathy, the arguments for reestablished hemostatic system in ALF, and the suggested tools for evaluating coagulopathy in ALF.

Keywords: Acute liver failure; Coagulopathy; Thrombin generation assay; Viscoelastic test.

Abbreviations: AASLD, American Association for the Study of Liver Diseases; ALF, acute liver failure; ALI, acute liver injury; APASL, Asian Pacific Association for the Study of the Liver; aPTT, activated partial thromboplastin time; EASL, European Association for the Study of the Liver; HE, hepatic encephalopathy; IASL, International Association for the Study of the Liver; ICP, intracranial pressure; INR, International Normalized Ratio; MELD, Model for End-Stage Liver Disease; PAI-1, plasminogen activator inhibitor-1; PT, prothrombin time; ROTEM, rotational thromboelastometry; TAFI, thrombin activatable fibrinolysis inhibitor; TEG, thromboelastography; TFPI, tissue factor pathway inhibitor; TGA, thrombin generation assay; tPA, tissue plasminogen activator; TPO, thrombopoietin; VET, viscoelastic test; vWF, von Willebrand factor.

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Interpretation of the INR value

Several reasons preclude the use of INR as the measure of coagulopathy in ALF. First, INR was designed for the specific indication of evaluating the interference of vitamin K-dependent clotting pathway, such as in warfarin-induced coagulopathy.[7] INR is less relevant in ALF because both vitamin K-dependent and -independent factors contribute to the coagulopathy. Second, INR reflects only the changes in procoagulant factors. INR arises from prothrombin time (PT) and is calculated as a ratio of patient's PT to standardized PT.[8] The laboratory measures of PT and activated partial thromboplastin time (aPTT) capture only the reduction in procoagulant factors.[9] These conventional studies of "coagulation" do not reflect any deficiencies in anticoagulant factors such as protein C, protein S, antithrombin, and tissue factor pathway inhibitor (commonly referred to as TFPI) that are also substantially reduced in ALF.[10] Dynamic interactions between all these cellular components do not fully enter the INR.[11] Lastly, INR is unreliable. There can be large interlaboratory discrepancies between INR measurements in patients with liver disease because this test was not developed to reflect coagulopathy in liver disease.[12] Robert and Chazoulleres[12] demonstrated that INR provided inadequate normalization of PT in patients with liver failure, whereas INR normalized PT in anticoagulated patients. Trotter *et al.*[13] additionally showed a significant inconsistency in INR results by sending a sample of blood to three reference laboratories. The laboratory variability resulted in different Model for End-Stage Liver Disease (commonly known as MELD) scores and an average change in organ allocation priority from 58th to 77th percentile (p=0.01). The irregularities in PT/INR were thought to be due to different sample storage time,

Table 1. Definitions of ALF

Society	ALF definition	Time course	Notes
AASLD	Presence of INR ≥1.5 and any degree of HE	Illness duration of <26 weeks	• Without preexisting cirrhosis except for patients with Wilson's disease, vertically-acquired hepatitis B virus, or autoimmune hepatitis • AASLD does not formally endorse ALF subgroups based on time course
EASL	Presence of acute abnormality of liver blood tests associated with coagulopathy (INR of >1.5) of liver etiology and HE/jaundice	• Hyperacute: development of HE within 7 days of jaundice • Acute: development of HE between 8 and 28 days of jaundice • Subacute: development of HE within 5-12 weeks of jaundice	• Without previous severe fibrotic or cirrhotic chronic liver disease, except for patients with acute *de novo* autoimmune hepatitis, Budd-Chiari syndrome and Wilson's disease • Jaundice is considered the first symptom
IASL	Presence of sudden and progressive liver dysfunction characterized by HE	Development of HE within 4 weeks of onset of symptoms • Hyperacute: <10 days • Fulminant: 10-30 days	• Without preexisting liver disease, except for patients with Wilson's disease and drug/toxic or viral hepatitis superimposed on preexisting liver disease
APASL	Presence of severe liver injury, coagulopathy INR of ≥1.5, and any degree of HE	Illness duration up to 4 weeks	• Without chronic liver disease or cirrhosis
Acute Liver Failure Study Group of Japan	Presence of fulminant hepatitis with HE and PT time less than 40% of standardized value	Development of grade II or more severe HE within 8 weeks of onset of disease symptoms • Acute: HE within 10 days • Subacute: HE later than 11 days	• Exclude acute liver failure caused by drug/chemical intoxication and microcirculatory disturbances, Wilson's disease, acute fatty liver of pregnancy and Reye's syndrome • Include asymptomatic hepatitis B virus carriers showing acute exacerbation of hepatitis

Abbreviations: ALF, acute liver failure; AASLD, American Association for the Study of Liver Diseases; INR, international normalized ratio; HE, hepatic encephalopathy; EASL, European Association for the Study of the Liver; IASL, International Association for the Study of the Liver; APASL, Asian Pacific Association for the Study of the Liver; PT, prothrombin time.

international sensitivity index of the thromboplastin, instrumentation, and the methodology used.[10]

The differences in PT/INR values within subclasses of ALF yield an interesting observation. In a study of 131 patients at the King's College in England, PT was more prolonged in fulminant hepatic failure (development of hepatic encephalopathy [HE] within 8 weeks) at median of 58 seconds when compared to PT in late-onset hepatic failure (development of HE between 8 and 24 weeks) at median of 32 seconds ($p<0.01$).[14] Similarly, the landmark Lancet study that first described the temporal subclassifications of ALF adopted by the EASL guidelines showed that the admission PT value was highest in the ALF group (development of HE within 8-28 days of jaundice), followed by the hyperacute liver failure group (development of HE within 0-7 days of jaundice) and the subacute liver failure group (development of HE within 29-72 days of jaundice).[15] Despite this interesting observation, the clinical significance in terms of coagulopathy and bleeding risk behind these differences in PT/INR values in ALF subgroups has not been explored.

Elevated INR is frequently observed in ALF but bleeding complications are uncommon. Munoz *et al.*[7] studied more than 1,000 patients with ALF from the Acute Liver Failure Study Group, a consortium of 24 tertiary care liver centers collecting data on patients with ALF. The mean INR of this cohort was 3.8 (ranging from 1.5 to >10.0). At admission, 81% of their cohort had an INR value between 1.5 and 5.0. Fourteen percent had an INR value ranging from 5.0 to 10.0, and 5% had an INR >10.0.[7] Another study on 2,095 ALF patients who presented to the Liver Intensive Therapy Unit at Kings College Hospital between 1973 and 2008 showed a similar INR profile. The mean INR in their cohort of 840 non-paracetamol ALF patients was 3.5 (range of 2.3 to 6), and the mean INR in their cohort of 1,255 paracetamol ALF patients was 6.2 (range of 3.9 to 9.3).[16] Despite the elevated INR values, spontaneous overt bleeding in ALF has been reported to be uncommon.[17–20] Bleeding in ALF is usually silent or manifested as mucosal membrane bleeding, often gastrointestinal in origin.[7,19,21] In the ALF Study Group, the INR values of ALF patients who experienced bleeding were not significantly different from those who did not experience bleeding.[7] Bleeding complications from invasive procedures such as the placement of an intracranial pressure (ICP) monitor is also comparable to those without invasive procedures. In a cohort of 58 ALF patients, bleeding from ICP monitor placement was 10.3%, and half of the complications

were incidental radiological findings.[22] More recently published in 2018, the overall incidence of bleeding was 10.6% during the first 7 days of admission, 89% spontaneous and 11% post-procedural, in a cohort of 1,770 adult patients with ALF in the ALF Study Group Registry. Bleeding complications were the cause of death in 2.1% of their patients. Importantly, INR was not statistically different between bleeders and non-bleeders.[6]

Rebalanced hemostasis in ALF

The exact mechanism of coagulopathy in ALF remains to be fully elucidated. However, current evidence suggests that the coagulopathy in ALF is derived from a complex and delicate interplay between decreased synthesis of procoagulant factors and anticoagulant factors, impaired fibrinolytic systems, defective platelets, and thrombocytopenia.[7,17,23] A significant alteration to the hemostatic system between procoagulant and anticoagulant pathways in ALF results in a delicate balance.[24] Any insult to this newly established system can tip the scale toward either thrombotic or bleeding complications.[19]

Acute hepatocellular injury leads to a considerable reduction in coagulation factor levels, as reflected by the prolonged PT/INR values. Hepatocytes synthesize most coagulation factors, including fibrinogen and factors II (prothrombin), V, VII, IX, X, XI, and XII.[25] In the 1970s, Boks *et al.*[26] reported that the levels of clotting factors were extremely depressed in their cohort of 7 ALF patients. In another study, 31 patients with acute paracetamol overdose showed reduced coagulation factors II, V, VII, and X but increased levels of factor VIII, an acute phase reactant synthesized in endothelial cells.[19,25,27,28] Coagulation factors also have a short half-life,[23] which augments the effect of reduced production of coagulation factors in ALF. These changes in coagulant factors are offset by decreased anticoagulant proteins in ALF.[19] Anticoagulant proteins, such as protein C, protein S, protein Z, protein Z-dependent protease inhibitor, antithrombin, heparin cofactor II, and α2-macroglobulin, are all synthesized by the liver;[10,29,30] an acute injury to hepatocytes leads to a diminished generation of these factors.

In addition to the decreased levels of coagulant and anticoagulant factors, fibrinogen is affected qualitatively and quantitatively in ALF. Fibrinogen is a glycoprotein that is cleaved by thrombin into fibrin to form a blood clot.[31] Green *et al.*[32] first reported the primary abnormality in fibrinogen in ALF by demonstrating varying degrees of disturbances in fibrin polymerization. A follow-up study modified the original calorimetry technique by Green *et al.* and reported dysfibrinogenemia in 86% of their 29 ALF patients.[33] These two studies confirmed the high incidence of acquired dysfibrinogenemia in ALF. Furthermore, fibrinogen produced in patients with ALF has increased amounts of sialic acid, which results in abnormal fibrinogen function and prolonged thrombin time.[25] Quantitatively, fibrinogen levels are typically normal or slightly reduced in ALF,[10,25] likely related to the fact that fibrinogen is an acute-phase protein.[19] Qualitatively, the disturbance in fibrinogen may contribute to coagulopathy in ALF.

Fibrinolysis, a process that prevents clotting, is also affected in ALF. All proteins involved in fibrinolysis, except for tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1), are synthesized by the liver.[24] The plasma levels of plasminogen, antiplasmin (α-2 plasmin inhibitor or α-2 PI), thrombin activatable fibrinolysis inhibitor (TAFI), and factor XIII are all significantly reduced in ALF.[10,24,26] The plasma levels of tPA and PAI-1 (inhibitor of tPA) are increased during ALF, due to their release by activated endothelium and reduced hepatic clearance.[10,19,25] However, PAI-1 levels are even more substantially increased than tPA levels in ALF, resulting in impaired fibrinolysis and hypofibrinolysis in ALF.[10,19,24]

Platelet dysfunction is routinely observed in ALF.[23] In addition, there may be mild to moderate reduction in platelet count,[17,19,24] though some patients may still retain normal platelet counts. According to data of more than 1,000 ALF patients from the Acute Liver Failure Study Group, the median platelet level was 132,000/mL (range of 1,000 to 533,000)[7]. Thrombocytopenia in ALF is thought to result from impaired platelet production and thrombin-mediated platelet consumption, though the exact mechanism is not yet known.[23] Initially, it was hypothesized that the decreased synthesis of thrombopoietin (TPO) was responsible for thrombocytopenia in ALF because TPO is produced by the liver. However, Schiødt *et al.*[34] measured the TPO level in 51 patients with ALF and reported that TPO level was above the upper limit of normal in 22 patients, normal in 24 patients, and below normal in only 5 patients. TPO levels did not correlate with platelet count in ALF. However, the level and function of platelet adhesive protein von Willebrand factor (vWF) and its cleaving protease ADAMTS13 in plasma have shown to affect platelet function in acute liver injury (ALI) and ALF. ALI is defined as INR of $\geq$1.5 in the absence of prior liver disease and illness duration of $\leq$26 weeks but without hepatic encephalopathy. vWF is a multimeric protein that is essential to platelet adhesion, and its reactivity towards platelets is proportional to its size, which is regulated by ADAMTS13. When compared to control subjects, patients with ALI and ALF had highly elevated vWF levels but reduced vWF function and reduced ADAMTS13 level and function. The overall platelet activity was normal or perhaps even increased; the rise in the concentration of vWF and decreased ADAMTS13 level and function more than compensated for the decrease in vWF function.[11,35]

There is also evidence suggesting that ALF may be a hypercoagulable state. Stravitz *et al.*[36] conducted a study on 50 ALI/ALF patients assessing the level of microparticles in their plasma. Microparticles are procoagulant membrane fragments (ranging in size from 0.1 to 1.0μm) derived from various cells. In their cohort, three dominant sizes of microparticles (0.27, 0.28 to 0.64, >0.64μm) were detected in ALI/ALF patients and healthy controls, and the ALI/ALF patients had a significantly higher concentration of all sizes of microparticles. When displaying tissue factor, a membrane protein vital in initiating coagulation[37,38] these highly procoagulant microparticles released from acutely injured liver potentially mediate the activation of coagulation and result in intravascular coagulation. The process further exacerbates liver damage in ALF.[36]

Overall, there is an overwhelming amount of evidence to suggest that the hemostasis in ALF is complex and rebalanced (Table 2, Fig. 1). Reduction in procoagulant factors counters diminished anticoagulant factors. Decreased levels of antiplasmin and TAFI offset increased levels of PAI-1 and reduced plasminogen. The increased amount of vWF compensates for the platelet dysfunction. Microparticles may even

Table 2. Changes of hemostasis in ALF

Factors	Factors contributing to anticoagulation	Factors contributing to coagulation
Coagulation factors	• Reduced procoagulant factors	• Reduced anticoagulant factors • Increased factor VIII
Fibrinolytic pathway	• Increased tPA • Reduced TAFI • Reduced antiplasmin	• Increased PAI-1 (more than tPA) • Reduced plasminogen
Fibrinogen	• Dysfibrinogenemia	N/A
Platelets	• Platelet dysfunction • Thrombocytopenia	• Increased vWF • Reduced ADAMTS13
Microparticles	N/A	• Increased microparticles

Abbreviations: ALF, acute liver failure; N/A, not applicable; PAI-1, plasminogen activator inhibitor-1; TAFI, thrombin activatable fibrinolysis inhibitor; tPA, tissue plasminogen activator; vWF, von Willebrand factor.

play a role in normalizing coagulopathy. Hence, an elevated INR does not fully represent the cellular processes in ALF.

Assessing hemostasis in ALF

Viscoelastic tests

Conventional coagulation tests, such as PT/INR, do not entirely represent the *in vivo* process in the *in vitro* setting. Global assays that consider all aspects of coagulopathy, including pro/anticoagulation mechanisms and fibrinolysis, offer many advantages in ALF. More recently, viscoelastic tests (VET) of coagulopathy, including thromboelastographic (TEG) and rotational thromboelastometry (ROTEM), have emerged for non-surgical applications in acute and chronic liver diseases. VET is a single point-of-care assay that allows for real-time functional evaluation of viscoelastic properties of coagulation, including dynamics of clot formation, ultimate clot strength, clot stability, and degradation.[11,39–42] TEG and ROTEM have long been utilized in liver transplantation as its use reduces blood and fluid infusion volume during surgery.[43–45]

In ALF, the parameters reflecting primary and secondary hemostasis are typically normal on TEG. Stravitz *et al.*[11] conducted a prospective ancillary project to The Acute Liver Failure Study Group and performed TEG on 50 patients with ALI/ALF on admission. The mean INR was elevated at 3.4 (range of 1.5 to 9.6) but the mean and median TEG parameters were normal for the entire population. Thirty-two patients (63%) had normal TEG studies, and four patients (8%) actually had hypercoagulable TEG parameters. Normal clot formation was observed without activation

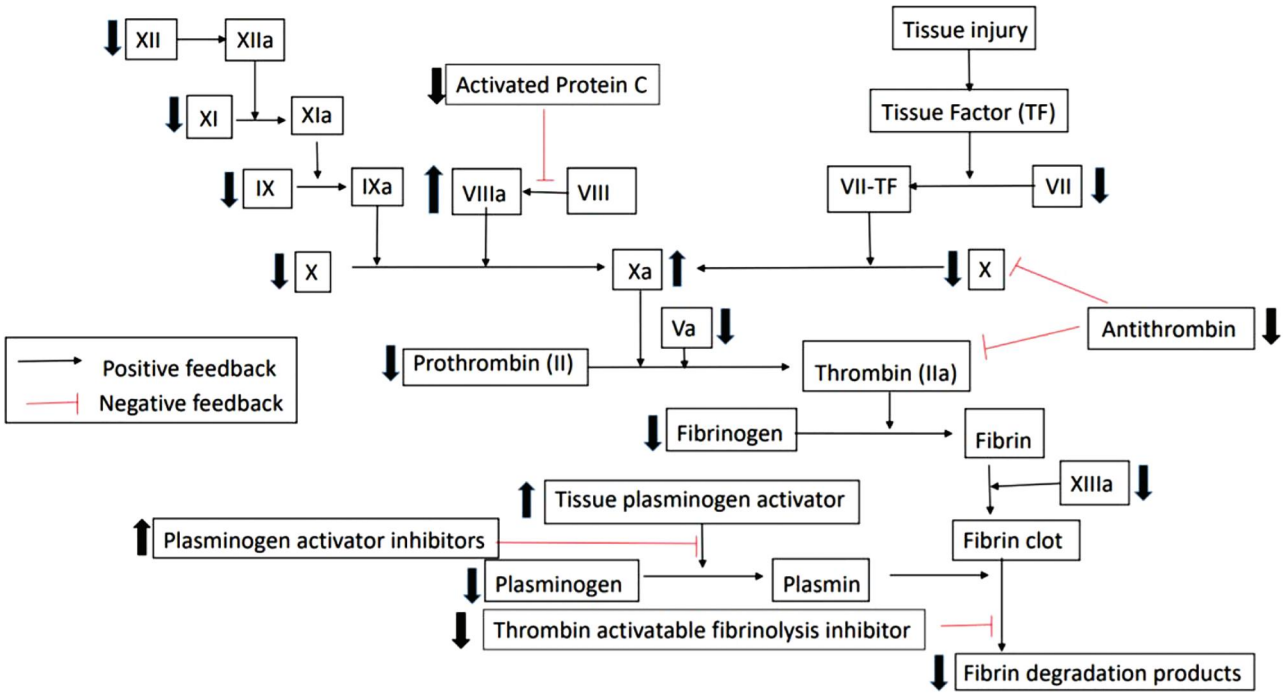

Fig. 1. Coagulation cascade in acute liver failure

Table 3. Different modalities of coagulopathy measurement in ALF

	Pros	Cons
Conventional tests (platelets/PT/INR)	• Quick • Widely available	• Inability to measure platelet dysfunction • Variability in PT/INR in liver disease rendering inaccurate measurements
VET	• Quick • Clinically available • Widely used in trauma, liver transplantation, cardiac surgeries to guide transfusions	• Insensitive to vWF • Lack of protein C activation • Absence of precise molecular mechanism behind tracings
TGA	• Ability to study precise molecular mechanisms • More global assessment of coagulopathy including protein C activation and vWF	• Time consuming • Only available in research setting

Abbreviations: INR, international normalized ratio; PT, prothrombin time; TGA, thrombin generation assay; VET, viscoelastic tests; vWF, von Willebrand factor; TGA, thrombin generation assay.

of the protein C system.[11] Furthermore, the authors reported that the number of thrombotic complications was higher than bleeding complications. Bleeding was reported in six patients, while thrombosis occurred in eleven patients.[11] In another study, plasma samples from 20 ALF patients showed similar results. The median INR in their cohort was 4.1 (interquartile range from 2.2 to 6.1) but it did not correlate with the TEG parameters. The authors demonstrated hypocoagulable TEG tracing in 20%, normal TEG tracing in 45%, and hypercoagulable tracing in 35%.[46] No significant bleeding complications or need for blood transfusions occurred in their study.[46] In summary, TEG tracings suggest perhaps a reestablished hemostasis system in ALF despite the elevated INR values.

Thrombin generation assay

VET is a useful bedside tool, where the results are typically available within minutes and the tracings available in real-time. However, one critique is that VET may not represent the true hemostatic balance in ALF because it lacks protein C activation and is insensitive to vWF in cirrhotic patients.[47] Thrombin generation assay (TGA) overcomes the inherent weaknesses of VET by providing a more accurate interplay between pro- and anticoagulant factors in ALF, thus evaluating the coagulopathy globally. However, unlike VET, TGA can be time-consuming and is currently only available in research settings.[48]

Lisman *et al.*[49] performed TGA using the Calibrated Automated Thrombogram on 50 patients with ALI/ALF and 40 healthy controls. Thrombin generation in patients with ALI/ALF was not significantly different from thrombin generation in control subjects when thrombomodulin was added to test mixture.[49] The presence of thrombomodulin allowed for full activation of protein C in ALF, a condition known to have protein C deficiency.[9] This finding of indistinguishable thrombin generation between ALI/ALF patients and control subjects supports the state of reestablished hemostasis in ALF. Fibrinolytic capacity was also significantly impaired in ALI/ALF patients, supporting hypofibrinolysis in these patients. No lysis was observed within 3 h in 73.5% of ALI/ALF patients but in only 2.5% of healthy controls. This phenomenon was associated with decreased levels of plasminogen and increased levels of PAI-1.[49] Moreover, the intact thrombin-generating capacity and hypofibrinolytic status persisted throughout the first week of admission in ALI/ALF patients.[49] Habib *et al.*[50] conducted a similar study on 32 patients with ALI/ALF and 40 control subjects utilizing TGA. Patients with ALI/ALF had a median INR of 3.36 (interquartile range 2.67 to 7.01) and decreased coagulation factors, except for factor VIII, as expected. The authors confirmed that thrombin generation in the presence of thrombomodulin in ALI/ALF patients was not significantly different from healthy controls. The ratio of thrombin generation with thrombomodulin to thrombin generation without thrombomodulin was significantly elevated in patients with ALI/ALF, suggesting hypercoagulable state in these patients.[50] Again, study data showed that TGA demonstrates a rebalanced hemostatic system in ALF that is not reflected in elevated INR values.

Conclusions

Based on the current evidence, global assessment of hemostasis in ALF indicates a "rebalanced" state. Therefore, prophylactic transfusion of blood products is unwarranted and may expose patients to harmful effects, such as volume overload and transfusion reaction, without a clear benefit.[17,23,47] Global tests of hemostasis have gained more recognition as potential tools in the evaluation of coagulopathy in patients with liver disease (Table 3). American Gastroenterological Association acknowledges the potential role of global assessment when evaluating clotting in patients with cirrhosis.[51] Both the European Association for the Study of the Liver and the Society of Critical Care Medicine also recommend the use of thromboviscous technology, such as VET and TGA, to assess bleeding/thrombotic risks in critically ill patients with ALF.[2,42] Neither the latest study data nor the most professional society guidelines support relying on INR as the sole measure of coagulopathy in ALF. Future iterations and standardization of VET and TGA are likely to provide a more comprehensive representation of coagulopathy in ALF.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Conceptualization and writing the original draft preparation (AK and PHC), data curation and visualization (AK), funding acquisition and supervision (PHC), writing, review and editing (AK, BN, TW, and PHC).

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Review Article

Skeletal Muscle Dysfunction in the Development and Progression of Nonalcoholic Fatty Liver Disease

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Abstract

The association between the pathogenesis and natural course of nonalcoholic fatty liver disease (NAFLD) and skeletal muscle dysfunction is increasingly recognized. These obesity-associated disorders originate primarily from sustained caloric excess, gradually disrupting cellular and molecular mechanisms of the adipose–muscle–liver axis resulting in end-stage tissue injury exemplified by cirrhosis and sarcopenia. These major clinical phenotypes develop through complex organ–tissue interactions from the earliest stages of NAFLD. While the role of adipose tissue expansion and remodeling is well established in the development of NAFLD, less is known about the specific interplay between skeletal muscle and the liver in this process. Here, the relationship between skeletal muscle and liver in various stages of NAFLD progression is reviewed. Current knowledge of the pathophysiology is summarized with the goal of better understanding the natural history, risk stratification, and management of NAFLD.

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Introduction

Nonalcoholic fatty liver disease (NAFLD) has become the most prevalent liver disorder of our time, affecting more than one billion people worldwide and with an estimated 30% prevalence in the adult US population, representing a significant global healthcare burden.[1,2] NAFLD is a manifestation of metabolic syndrome in the liver, having complex pathobiology. Its clinical outcomes are strongly linked to visceral obesity, type 2 diabetes mellitus, dyslipidemia, and endothelial dysfunction.[3] The original term of NAFLD includes a spectrum of disease, ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), which also features hepatocellular injury, inflammation, and a variable degree of fibrosis.[4] NAFLD may progress to cirrhosis, and it confers an increased risk of hepatocellular carcinoma.[4,5] However, prediction of clinical outcomes in NAFLD has proven challenging as environmental and genetic drivers of its progression are not fully identified.[6,7]

Inter-tissue crosstalk of the liver with adipose tissue and skeletal muscle plays a fundamental role in the pathobiology and natural course of NAFLD.[8–10] Cellular and molecular mechanisms governing the interplay of these organs in health and disease are therefore of significant interest.[11–13] Escalating dysfunction in the adipose–muscle–liver triangle results in increasingly severe pathophenotypes and clinical outcomes (Fig. 1). Skeletal muscle disorders in the form of myosteatosis, sarcopenia, and sarcopenic obesity are associated with this process.[10,14,15] Disruption of the complex physiological relationship between skeletal muscle and the liver is mutually detrimental and promotes the progression of NAFLD.[13,16–18]

This paper reviews the association between skeletal muscle dysfunction and the liver in various stages of NAFLD. We also summarize the pertinent aspects of NAFLD pathophysiology, which may help prognostication and identify new therapeutic targets.

Assessment of skeletal muscle mass and performance in metabolic dysfunction

Definition of myosteatosis, sarcopenia, and sarcopenic obesity

Myosteatosis is characteristically associated with liver steatosis in NAFLD, resulting from ectopic fat accumulation in skeletal muscle when available lipids exceed the disposal capacity of adipose tissue.[13] Depending on the type of fat deposition, myosteatosis may feature microscopic and macroscopic changes in muscle composition and architecture.[19] Intramyocellular fat is not necessarily abnormal as it serves as an energy source to fuel muscle contraction.[8,20] The

Keywords: Myosteatosis; Sarcopenia; Myokines; Nonalcoholic fatty liver disease; Cirrhosis; Adipose–muscle–liver axis.

Abbreviations: 12,13-diHOME, 12,13-dihydroxy-9Z-octadecenoic acid; AMPK, 5′-adenosine monophosphate activated protein kinase; ATP, adenosine triphosphate; ASM, appendicular skeletal muscle mass; BAT, brown adipose tissue; BCAA, branched chain amino acid; BIA, bioimpedance analysis; BMI, body mass index; CT, computer tomography; DXA, dual-energy X-ray absorptiometry; EGF, epidermal growth factor; EWGSOP, European Working Group on Sarcopenia in Older People; FFA, free fatty acid; FGF, fibroblast growth factor; GH, growth hormone; HR, hazard ratio; IGF, insulin-like growth factor; IL, interleukin; IRF, insulin regulatory factor; JNK, c-Jun N-terminal kinase; LXR, liver X receptor; MAFLD, metabolic dysfunction-associated fatty liver disease; MELD, Model for End-stage Liver Disease; MR, magnetic resonance; mTOR, mammalian target of rapamycin; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; Nrg, neuregulin; OR, odds ratio; PI3K, phosphatidylinositol-3-kinase; PKC, protein kinase C; PPAR, peroxisome proliferator activating receptor; SMI, skeletal mass index; SPPB, Short Physical Performance Battery; SREBP, sterol regulatory element-binding protein; TACE, transarterial chemoembolization; TCA, tricarboxylic acid; TGF, transforming growth factor; TNF, tumor necrosis factor.

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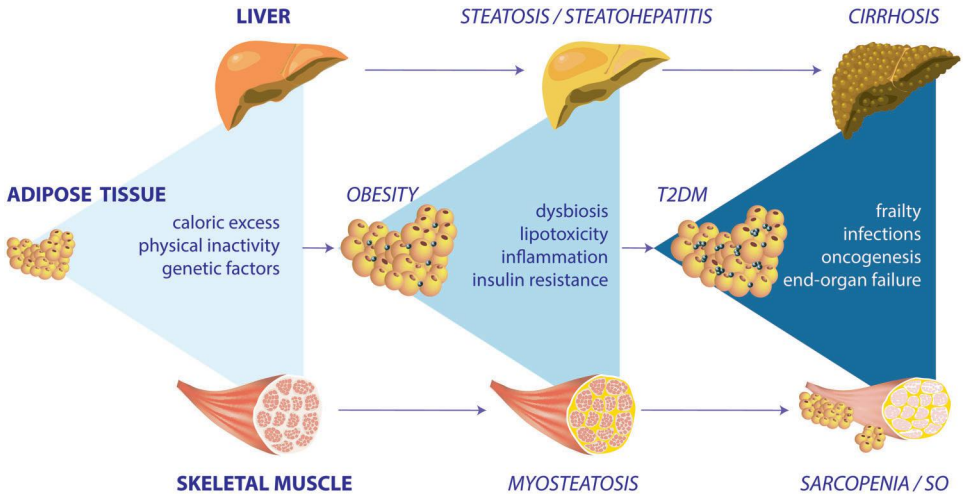

accumulation of excess fat in extramyocellular compartments is mostly pathologic. It can be defined as intramuscular (between muscle fibers) or intermuscular (between muscle fascicles) (Fig. 2). Myosteatosis may affect many individuals who do not meet the anthropometric criteria for sarcopenia or obesity. However, it is associated with lower muscle function and strength, muscle atrophy, and physical disabilities.[21] Myosteatosis has been described in many cirrhotic patients undergoing liver transplant evaluation, and studies have associated it with more complications and poor survival.[22]

As the name implies, sarcopenia is a condition with diminished skeletal muscle mass, first associated with older age.[23] Today, sarcopenia has a complex meaning as a progressive and generalized disorder seen in various chronic illnesses.[15] Sarcopenia may affect up to 70% of patients with cirrhosis and is an independent predictor of morbidity and mortality in this population.[13,24] In 2010, the European Working Group on Sarcopenia in Older People (EWGSOP) recommended using both low muscle mass and low muscle function (strength or performance) to diagnose sarcopenia.[25] In 2018, the EWGSOP updated the definition of sarcopenia so that the primary parameter is low muscle strength, the diagnosis is confirmed by the evidence of low muscle mass, and the disease state is characterized as severe if low physical performance is also present.[19]

Sarcopenic obesity is a term used to denote the simultaneous presence of skeletal muscle loss and excess body fat. Sarcopenia and obesity continue to be independently defined by their respective criteria, and there is no consensus on whether sarcopenic obesity impacts clinical outcomes more than the sum of its components.[10] Importantly, sarcopenia, obesity, and obesity-related disorders, including NAFLD, share several pathophenotypes, such as systemic low-grade inflammation and insulin resistance via overlapping cellular and molecular mechanisms.[10,26] Sarcopenic obesity reaches a prevalence of 20% to 35% among patients with cirrhosis and is associated with increased mortality.[21]

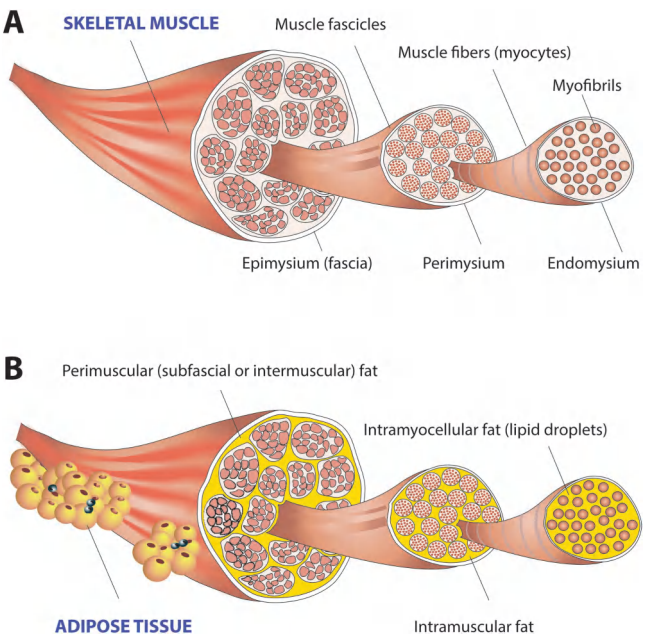

Fig. 2. Skeletal muscle and fat deposition. (A) Skeletal muscle is made up of intramyocellular myofibrils, muscle fibers and fascicles bound together by successively thicker connective tissue layers as endomysium, perimysium, and epimysium. (B) Skeletal muscle fat may be classified as intramyocellular (lipid droplets filling the cytoplasm between myofibrils of elongated myocytes) and extramyocellular components. Adipocytes may infiltrate muscle fibers (intramuscular fat), fascicles (intermuscular fat), or exist around the epimysium as extramuscular fat depots of adipose tissue.

Methods of analyzing skeletal muscle mass and function

There are many methods to measure skeletal muscle mass and many ways to report the findings, creating challenges in the literature.[27] A commonly used parameter is appendicular skeletal muscle mass (also referred to as ASM) adjusted for height to yield appendicular skeletal muscle index (also referred to as SMI).[28] Skeletal muscle cross-sectional imaging with magnetic resonance (MR) or computed tomography (CT) has become the gold standard, focusing on specific muscle groups or body locations, such as the psoas muscle or third lumbar (L3) region.[29] Multiple studies found that using CT imaging to assess skeletal muscle index at the L3 level is the most accurate method to evaluate sarcopenia in cirrhosis.[30] However, MR and CT imaging may not be widely available and/or expose patients to radiation.

Dual-energy X-ray absorptiometry (DXA) is another instrument available to measure skeletal muscle mass. It uses multiple low-dose X-rays to create a 3-D compartmental model and is highly accurate at differentiating fat, fat-free mass, and bone mineral mass.[12] The benefits of DXA are that it is inexpensive, widely available, and carries low radiation exposure. However, a disadvantage to using this method is that different instrument brands may not give consistent results, and it is less sensitive than CT in case-finding.[19,31] Further limitation of DXA is its inability to differentiate muscle from water; thus, ascites can distort muscle mass readings, a major concern in cirrhosis. However, this concern may be resolved by using appendicular lean mass to measure skeletal muscle indices.[32]

Bioelectrical impedance analysis (BIA) is a simple, affordable, non-invasive, and portable tool that has been used to estimate total body skeletal muscle mass or ASM. BIA measurements are indirect as they use whole-body electrical conductivity and a conversion equation that is calibrated with a reference of DXA-measured lean body mass in a specific population.[33] The disadvantages of BIA include that its accuracy is affected by hydration status and may both underestimate or overestimate fat-free mass. Additionally, prediction equations used to derive muscle mass from BIA may require adjustment for different ethnic populations.[34] Ultrasound is another tool available to evaluate muscle mass by measuring the thickness of muscles in the leg and/or the arm.[33] Assessment of the quadriceps femoris can detect muscle thickness and the cross-sectional area within a short period. Ultrasound is safe, inexpensive, portable, and has the advantage of assessing both muscle quality and quantity, while its disadvantages are that it is highly operator dependent and there is no standardized technique among examiners for measuring muscle thickness by ultrasound.[35]

In line with EWGSOP recommendations, functional assays of muscle strength and performance are now increasingly utilized to assess myosteatosis and sarcopenia. Muscle strength can be conveniently measured by grip strength using a calibrated handheld dynamometer.[36,37] In several studies, grip strength is defined as the maximum value of three serial attempts using the non-dominant hand.[38] Another method to measure muscle strength is the chair stand test (chair rise test), which uses the leg muscles instead and measures the time it takes for a patient to rise five times from a seated position without using their arms.[39] Tools available to assess physical performance related to skeletal muscle dysfunction include the Short Physical Performance Battery (SPPB), gait speed, get-up-and-go test, 6-m (i.e. minute) walk test, and stair climb power test. SPPB is a test that involves evaluating gait speed, balance, and a chair stand test. Gait speed is widely used in practice since it is easy to measure and highly reliable. A widely accepted gait speed test is the 4-m (i.e. meter) usual walking speed test. EWGSOP recommendation is a cut-off of 0.8 m/s (i.e. meter per second) for gait speed when defining severe sarcopenia.[19]

While myosteatosis is essentially a histological diagnosis, biopsy remains a rare option to assess excess fat deposition in skeletal muscle.[40] Similar to assessing hepatic fat content, non-invasive measurements of myosteatosis include CT, MR spectroscopy, and MR imaging.[41,42] Skeletal muscle attenuation measured by CT shows good correlation with intramyocellular fat content compared to MR spectroscopy findings or percutaneous muscle biopsy.[41,42] Further clinical studies may be necessary before assessing myosteatosis becomes part of the routine evaluation in NAFLD.

Clinical evidence of skeletal muscle dysfunction in NAFLD

Population-based and targeted studies on sarcopenia and NAFLD

There have been relatively few studies that have aimed at analyzing the relationship between myosteatosis and NAFLD. Instead, many studies have focused on the relationship between NAFLD and sarcopenia or sarcopenic obesity; this association has been extensively studied in several cohorts from Asian-Pacific countries. In a prospective observational cohort of 452 healthy adults from the Korean Sarcopenic Obesity Study, individuals with sarcopenia (defined by having a DXA-estimated skeletal muscle mass index 1 standard deviation below the reference) had significantly greater body mass index (BMI), waist circumference, total body fat mass, HOMA-IR score, and adverse cardiovascular indices compared to the non-sarcopenic group.[43] In a retrospective analysis of the Korean National Health and Nutrition Examination Surveys (2008-2011), sarcopenia was diagnosed by DXA in 337 (12.2%) of 2,761 participants with NAFLD.[44] That study found that sarcopenia carries an approximately 2-fold risk of significant fibrosis, independent of obesity and insulin resistance [odds ratio (OR)=1.76 to 2.68].[44] In a South Korean cohort of 309 subjects with biopsy-proven NAFLD, the prevalence of sarcopenia increased as liver disease progressed from the absence of NAFLD (8.7%) to steatosis (17.9%) and NASH (35.0%).[45] Moreover, significant fibrosis ($\geq$ F2) was more prevalent in subjects with sarcopenia than in those without (45.7% vs. 24.7%).[45]

In a large, longitudinal cohort study that followed 15,567 subjects from Seoul over 7 years, an inverse association was found between SMI (identified by BIA) and incident NAFLD (determined by the hepatic steatosis index) that developed in 1,864 of the 12,624 subjects (14.8%) without baseline NAFLD [adjusted hazard ratio (HR)=0.44].[46] In this study, a positive association between higher skeletal muscle mass index and the resolution of NAFLD was observed in 79 of the 2,943 subjects (2.7%) with baseline NAFLD (adjusted HR=2.09). These relationships persisted even after adjusting for baseline skeletal muscle mass index, suggesting that skeletal muscle mass impacts NAFLD's natural history.[46] In a prospective cross-sectional cohort study from China with 5,132 participants aged 18 to 80 years, sonographically-diagnosed

NAFLD was increasingly found among individuals with low SMI identified by DXA (OR=2.57) and low muscle strength based on weight-adjusted handgrip strength (OR=1.47). This association was even more robust with sarcopenia (OR=3.91) and sarcopenic obesity (OR=10.42 when defined by BMI and OR=11.64 when defined by waist circumference), indicating that the concurrence of sarcopenia and obesity represents an exceptionally high risk of NAFLD.[47]

Clinical evidence for the link between skeletal muscle dysfunction and NAFLD is not confined to the Asia-Pacific region. SMI defined sarcopenia based on BIA (≤ 37 in men and ≤ 28 in women) in a retrospective study of 225 Italian adults with biopsy-proven NASH, and its prevalence increased with the severity of liver fibrosis from 20.4% (≤ F2) to 48.3% (≥ F3). Multivariate regression analysis indicated that sarcopenia is associated with an OR of 2.88 for having at least F3 fibrosis in this cohort.[48] In the Rotterdam Study, a large ongoing population-based cohort of participants aged 45 years or older in the Netherlands, 161 lean, and 1,462 overweight or obese participants with NAFLD were analyzed for the prevalence of pre-sarcopenia and sarcopenia, which was relatively low (5.9% and 4.5%, respectively). SMI calculated from DXA measurements showed an inverse relationship with NAFLD in normal-weight women (OR=0.48).[38] In general, fat mass rather than lean mass was a predictor for NAFLD in this cohort, and the android-fat-to-gynoid-fat ratio was the best performing predictor for NAFLD prevalence (ORs ranging from 1.97 in lean men to 4.81 in lean women), suggesting that android fat is more likely to cause NAFLD.[38] Cross-sectional data analysis of 11,325 American participants of the third NHANES found that NAFLD was more prevalent with sarcopenia than without (46.7% vs. 27.5%). After adjustment for confounders, the association of sarcopenia with NAFLD remained significant (OR=1.24). Moreover, advanced liver fibrosis was more common in participants with sarcopenia than in those without (7.8 vs. 1.6%). The data indicate that sarcopenia in NAFLD represents a risk factor of advanced fibrosis independent of metabolic risk factors (OR=1.79).[49]

Sarcopenia in patients with established cirrhosis

Sarcopenia is a common feature of cirrhosis, and the transition from normal body composition to sarcopenia and from obesity to sarcopenic obesity has been repeatedly observed with the progression of liver disease. In contrast, healthier skeletal muscle indices are associated with long-term survival in patients with cirrhosis.[22,50,51] There have been few studies focusing on the specific relationship between skeletal muscle dysfunction and advanced liver disease associated with NAFLD. Recently, a study done at Mayo Clinic in Rochester, Minnesota (USA), evaluated the presence of sarcopenia and myosteatosis in patients awaiting liver transplant with a primary diagnosis of NASH (n=136) vs. alcohol-associated liver disease (n=129).[52] This study showed that while NASH patients had a higher Rockwood frailty score (49% vs. 34%, p=0.03), sarcopenia was less frequent in this group than among patients with alcohol-associated liver disease (22% vs. 47%, p<0.001). Moreover, myosteatosis (diagnosed by CT) was present to a similar degree in both groups and showed no association with adverse events such as increased length of stay or poor survival.[52]

The presence of myosteatosis, sarcopenia, and sarcopenic obesity was analyzed in a cohort study of 678 patients with cirrhosis in Edmonton (Canada). The etiology of cirrhosis was primarily chronic hepatitis C (40%), alcohol (23%), and NASH or cryptogenic (14%). Myosteatosis was found in 353 patients (52%), while 292 patients had sarcopenia (43%), and 135 had sarcopenic obesity (20%). The median survival of patients with myosteatosis, sarcopenia, and sarcopenic obesity was significantly worse than those without muscular abnormalities (28±5, 22±3 and 22±3 vs. 95±22 months, respectively). Multivariate analysis indicated an increased risk of mortality associated with myosteatosis and sarcopenia (HR=1.42 and 2.00, respectively).[22] Another retrospective analysis done in Kentucky (USA) focused on 207 adult patients who received liver transplantation (male, 68%; mean age, 54±8 years) due to cirrhosis from alcohol-related liver disease (38.6%), chronic hepatitis C (38.2%), and NASH (21.7%) or based on hepatocellular carcinoma (24.6%). In this cohort, 48% of patients were obese, 59% had sarcopenia, and 41.7% had sarcopenic obesity during transplant evaluation. Additionally, it was observed that sarcopenia was still present in 56 out of 59 (95%) patients who received CT scan at 6 months posttransplant.[53] Multivariate analysis found that obesity was an independent predictor of pretransplant sarcopenia and NASH was associated with a 6-fold increased risk of having sarcopenic obesity in cirrhotic patients.[53]

Pathogenesis of skeletal muscle dysfunction in NAFLD

Key mediators of adipose–muscle–liver crosstalk

Cellular and molecular mechanisms provide a complex interplay between adipose tissue, skeletal muscle, and the liver (Fig. 3). Adipose tissue as an endocrine organ secretes many bioactive substances termed adipokines that relay information to other metabolically active organs, including skeletal muscle and the liver.[54] Adiponectin, a major adipokine with many beneficial properties, activates 5′-adenosine monophosphate-activated protein kinase (AMPK), a master regulator of energy metabolism. Adiponectin receptor type 1 is highly expressed in skeletal muscle, while type 2 is mostly expressed in the liver, allowing for separate regulatory mechanisms.[55] Adiponectin promotes insulin sensitivity via cellular uptake and processing of glucose and fatty acids.[56] Adiponectin negatively regulates cell apoptosis via the mammalian target of rapamycin (mTOR) pathway and c-Jun N-terminal kinase (JNK)/caspase 3 pathways.[57] Moreover, adiponectin binding may activate ceramidases that degrade harmful ceramide and its derivatives.[9] Leptin is another major adipokine with beneficial effects, such as regulation of appetite, energy metabolism, and body weight.[58] Circulating leptin is believed to exert anabolic effects and decrease the impact of atrophy-related factors in skeletal muscle.[59] Obesity-associated leptin resistance results in hyperleptinemia, promoting insulin resistance by upregulating pro-inflammatory cytokines IL-6 and tumor necrosis factor alpha (TNF-α).[60] High leptin levels diminish the anabolic actions of insulin-like growth factor 1 (IGF1) in skeletal muscle and may increase frailty.[34] Moreover, high leptin levels promote inflammation and fibrogenesis in the liver and have been linked to the progression of NAFLD in experimental and clinical studies.[61,62]

Hormonally-active substances secreted by skeletal muscle cells are termed myokines, with remote actions on adipose tissue, liver, pancreatic beta cells, and the gut microbiota.[63,64] The growing list of myokines includes myostatin,

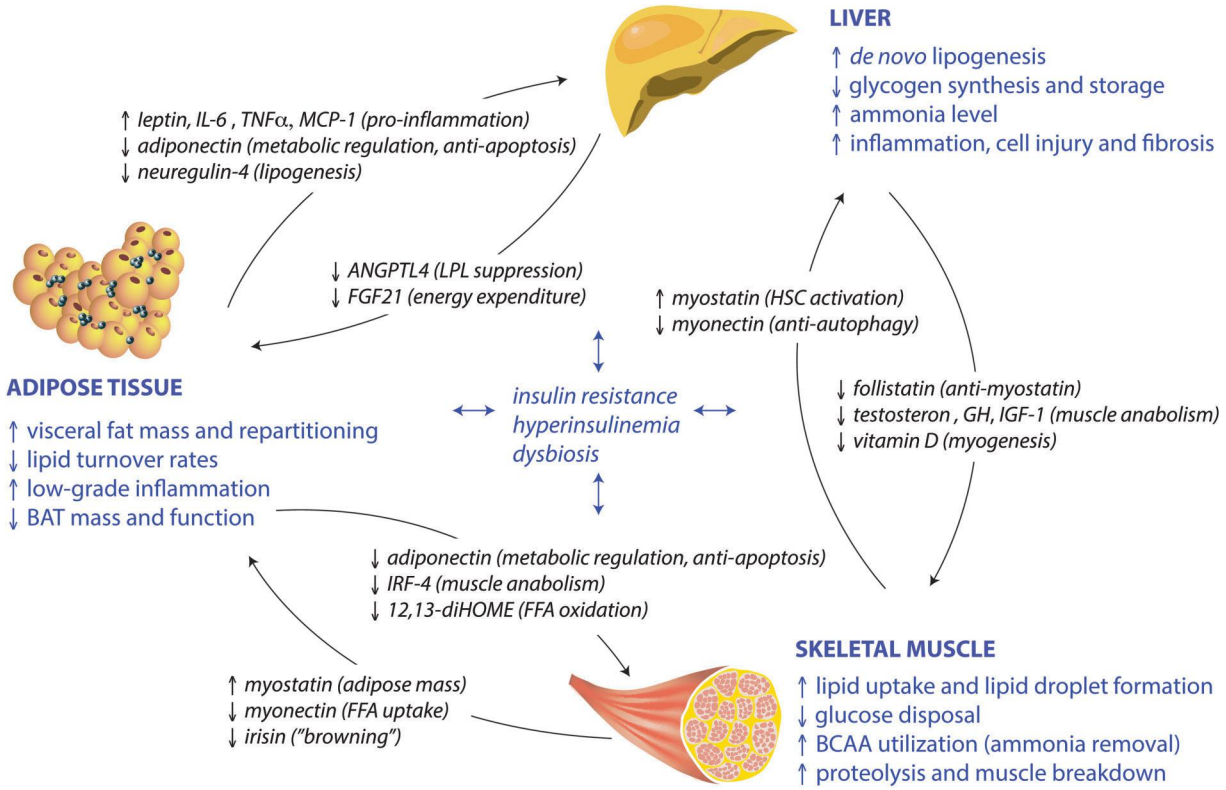

Fig. 3. Adipose–muscle–liver crosstalk in NAFLD: Mechanisms and mediators of pathogenesis. Schematic depiction of major structural and functional changes in adipose tissue, skeletal muscle, and the liver (blue) due to sustained energy surplus with key molecular mediators and mechanisms of interplay (black arrows). Input from other body components, such as pancreatic beta cells (hyperinsulinemia due to insulin resistance) and gut microbiota (dysbiosis), may affect all elements of the adipose–muscle–liver triangle (blue arrows). See details in the main text.

Abbreviation: 12,13-diHOME, 12,13-dihydroxy-9Z-octadecenoic acid; ANGPTL4, angiopoietin-like 4; BAT, brown adipose tissue; BCAA, branched-chain amino acids; FFA, free fatty acids; GH, growth hormone; HSC, hepatic stellate cell; IGF-1, insulin-like growth factor 1; IL-6, interleukin 6; IRF-4, interferon regulatory factor 4; LPL, lipoprotein lipase; MCP-1, monocyte chemoattractant protein 1; NAFLD, nonalcoholic fatty liver disease; TNFα, tumor necrosis factor-alpha.

irisin, myonectin, and various interleukins (IL-6, IL-7, IL-8, and IL-15). Myostatin is a TGF-β superfamily member and a negative regulator of skeletal muscle mass.[63] Myostatin interferes with mTOR signaling and activates skeletal muscle proteolysis through autophagy and the ubiquitin-proteasome pathway.[65] Myostatin promotes liver inflammation and fibrosis via activin IIbr receptors on hepatic stellate cells.[66] Irisin is an exercise-inducible myokine with the ability to increase total body energy expenditure by stimulating "browning" and uncoupling protein-1 expression in subcutaneous white adipose tissue and thus improving insulin sensitivity.[67] One of the molecular targets of irisin is the nuclear hormone receptor PPAR-α, which explains its stimulatory impact on fatty acid oxidation.[68] Myonectin, another exercise-inducible myokine, promotes the uptake of free fatty acids in adipose tissue and the liver.[63] Also, myonectin activates the PI3K/Akt/mTOR pathway and inhibits autophagy.[69] It has been suggested that myonectin is a nutrient-sensing myokine, coordinating nutrient uptake and storage among various tissues.[67]

Brown adipose tissue (BAT) is an increasingly recognized player in the adipose–muscle–liver triangle. BAT is a key regulator of energy homeostasis, due to its abundance of uncoupling protein-1 which enables the breakdown of lipids and other nutrient substrates at high rates by dissipating biochemical energy as heat rather than capturing it through ATP synthesis.[70] This profound thermogenic and energy-wasting ability of BAT may lower the risk of obesity-associated disorders, such as NAFLD.[71] Several molecular links between BAT, skeletal muscle, and the liver have been recently identified. 12,13-dihydroxy-9Z-octadecenoic acid (12,13-diHOME), an exercise-induced lipokine released by BAT, promotes fatty acid uptake and oxidation in skeletal muscle.[72] Moreover, interferon regulatory factor 4 (IRF4) in BAT prevents loss of exercise capacity by repressing the transcription of muscle function inhibitor myostatin.[73] IRF4 regulates exercise capacity, mitochondrial function, ribosomal protein synthesis, and mTOR signaling in skeletal muscle.[73] Finally, neuregulin 4 (Nrg4), a member of the epidermal growth factor (EGF) family, is highly expressed in BAT under physiological conditions and has reduced levels during obesity. Nrg4 has a beneficial impact on NAFLD by controlling the activity of liver X receptor (LXR) and sterol regulatory element-binding protein 1c (SREBP-1c), key regulators of de novo lipogenesis in the liver.[74]

Disruption of the adipose–liver–muscle axis in NAFLD

Expansion and remodeling of white adipose tissue in obesity leads to the development of prominent visceral fat depots,

which result in lipotoxicity, inflammation and altered neuroendocrine function.[9] Increased triglyceride storage and reduced lipid turnover leads to accumulation of potentially toxic lipid molecules, such as ceramides, diacylglycerol and long-chain acyl CoA.[75] Lipotoxicity promotes infiltration of adipose tissue with macrophages and other immune cells causing chronic, low-grade inflammation.[76] This injury is aggravated by oxidative stress on mitochondria, which process the breakdown of excess lipids, and by reduced tissue perfusion, leading to hypoxia in fat depots grown out of proportion.[75] Adipocytes in enlarged visceral fat depots have an altered adipokine secretion profile, characterized by diminished levels of adiponectin and increased secretion of leptin as well as other potentially harmful adipokines. like chemerin and resistin.[10] Adipocyte injury results in recruitment of macrophages and other immune cells associated with inflammation and the release of IL-6, TNF-α and plasminogen activator inhibitor-1.[77] Liver-derived fibroblast growth factor 21 (FGF21) has also been implicated in regulating energy metabolism in adipose tissue. Serum FGF21 levels increase in NAFLD but its effect may be lost due to peripheral resistance.[78]

Since adipose tissue has limited expandability, surplus lipids that are associated with obesity accumulate in non-adipose tissues, such as the liver and skeletal muscle.[9] Steatosis in the liver and skeletal muscle has complex pathophysiology, and its cause and consequence is challenging to distinguish.[26] Physiological amounts of intramyocellular lipids are stored as triglycerides in lipid droplets and used as a fuel source during exercise.[8] Lipid droplets are highly dynamic organelles involved in cell signaling and vesicle trafficking.[79] Intramyocellular lipid droplets are more abundant in type I compared to type II fibers, reflecting the greater oxidative capacity of type I fibers.[80] Perilipins are proteins associated with the surface of lipid droplets; they regulate lipid traffic and composition.[11] High levels of perilipin 2 have been detected in patients with sarcopenia and hepatic steatosis.[81] Physical exercise may increase intramuscular triglycerides' turnover and prevent the accumulation of lipotoxic intermediates, thus reducing the risk of insulin resistance.[11] Low-grade inflammation and excess lipid deposition in skeletal muscle results in mitochondrial dysfunction, myocellular apoptosis, and an adverse secretory pattern of myokines, which further disrupts endocrine interactions with adipose tissue and the liver.[10]

Insulin resistance is the most consequential biochemical pathophenotype associated with obesity, leading to progressive dysfunction of the adipose–muscle–liver axis.[82] Insulin resistance in obesity develops from the combined impact of toxic lipid molecules and pro-inflammatory mediators. Diacylglycerol, ceramides, and long-chain acetyl coenzyme A interfere with physiological insulin signaling by activating atypical isoforms of protein kinase C (PKC), setting off a series of deleterious molecular events in the liver and skeletal muscle.[83] PKC-mediated serine phosphorylation of insulin-receptor substrates in skeletal muscle and liver inhibits PI-3-kinase/Akt signaling. In contrast, PKC-mediated activation of protein phosphatase 2A dephosphorylates Akt at a key serine residue.[84] These changes prevent glucose uptake in skeletal muscle and lead to diminished glycogen synthesis and increased gluconeogenesis rates in the liver.[75] Peripheral insulin signaling is also weakened by additional serine/threonine kinases or 'stress kinases,' such as c-Jun N-terminal kinase (JNK), p38-mitogen-activated protein kinase (p38-MAPK), and IκB kinase (IKK), activated by TNF-α and other pro-inflammatory cytokines.[82] Compensatory hyperinsulinemia further promotes steatosis. Critical regulators of lipid metabolism, like SREBP-1c, remain responsive to insulin; they stimulate *de novo* lipogenesis and inhibit β-oxidation in the liver, creating a vicious cycle between lipotoxicity and insulin resistance.[75,82]

Disruption in the composition and function of gut microbiota (dysbiosis) may occur in response to various environmental factors.[85] Sustained nutrient excess has been linked to dysbiosis with an essential role in the pathophysiology of obesity-associated disorders.[86] Aberrant host-microbiome interactions may contribute to all NAFLD stages by altering intestinal bile acid metabolism, weakening of the intestinal epithelial barrier, and compromising innate immunity of the gut mucosa as fundamental underlying mechanisms.[87,88] Dysbiosis promotes chronic inflammation and insulin resistance, at least in part via modulation of the skeletal muscle composition and function.[89] Although there is no direct evidence of a link between human gut microbiota composition and sarcopenia, experimental studies indicate that administration of *Faecalibacterium prausnitzii,* a bacterial strain known for its beneficial effects through abundant production of short-chain fatty acids, increases gastrocnemius muscle mass and mitochondrial oxidative phosphorylation capacity in mice fed with a high-fat diet.[90]

Adipose–muscle–liver axis dysfunction in advanced stages of NAFLD

The adipose–muscle–liver axis becomes increasingly disrupted as NAFLD progresses into cirrhosis, and it is characterized by end-organ damage, frailty, infections, and oncogenesis. The relationship between cirrhosis and sarcopenia has a complex pathophysiology.[13,91] Liver dysfunction and reduced skeletal muscle mass intensify insulin resistance, which advances to type 2 diabetes and possibly leads to pancreatic β-cell failure.[12,92] Nutrient intake is reduced in cirrhosis due to anorexia, nausea, and malabsorption, which results from the congestion of gastrointestinal mucosa due to portal hypertension, impaired gut motility, and altered enterohepatic biliary physiology.[13] Cirrhotic patients have insufficient hepatic glycogen reserves due to the impaired synthetic capacity of hepatic cells.[91] Due to reduced hepatic ability to store, synthesize and mobilize carbohydrate stores, even after an overnight fast, patients with cirrhosis quickly shift their energy source to fat and protein catabolism, leading to rapid muscle breakdown.[21] Ongoing mitochondrial dysfunction, generation of reactive oxygen species, and impaired bioenergetics in skeletal muscle may all contribute to impaired protein synthesis and activate autophagy as a metabolic adaptive response.[13] Reduced cellular amino acid concentrations activate adaptive responses, including increased skeletal muscle autophagy, which has been reported in cirrhosis.[91]

Increasing hepatocellular dysfunction in cirrhosis may also result in decreased levels of potent anabolic factors, such as testosterone, and a relative lack of follistatin, a natural antagonist myostatin and activin(s) antagonist that contributes to skeletal muscle wasting by lowering protein synthesis and inducing myostatin expression.[93] High levels of FFA, in turn, inhibit the growth hormone (GH)/ IGF-1 axis, which generally plays a protective role in age-related muscle loss and muscle regeneration.[12] Decreased hepatic production of IGF-1 has been associated with sarcopenia in experimental

NAFLD.[94] Additionally, cirrhosis is associated with systemic inflammation that directly promotes muscle wasting.[13,91] Testosterone increases muscle protein synthesis by increasing amino acid utilization in skeletal muscle and increasing androgen receptor expression.[95] Gut barrier function in cirrhosis is compromised due to dysbiosis and portal hypertension, allowing the translocation of bacterial products recognized as pathogen-associated molecular patterns by immune cells in the gut and the liver leading to activation of pro-inflammatory state.[88,91]

Additionally, there are specific metabolic changes seen in cirrhosis that may worsen sarcopenia. Hepatocellular dysfunction and portosystemic shunting impair the rate of ureagenesis, which is a key metabolic pathway for ammonia disposal.[13] In order to compensate for this, skeletal muscle converts excess ammonia into glutamate by removing α-ketoglutarate from the tricarboxylic acid (TCA) cycle, which weakens myocellular ATP production capacity, leading to reduced myocellular protein synthesis and increased autophagy.[96] Due to branched-chain keto-dehydrogenase availability, skeletal muscle cells can channel branched-chain amino acids (BCAA) into the TCA cycle and sustain mitochondrial oxidative phosphorylation.[97] However, BCAA levels are already decreased in cirrhosis and may be further reduced due to increased utilization for ammonia disposal in the skeletal muscle, whereby reduced muscle mass contributes to hyperammonemia.[13] Sarcopenia is also worsened by a deficiency in vitamin D since it is a ligand of the nuclear vitamin D receptor, which regulates the expression of genes involved in cell proliferation and differentiation and affects myogenesis and muscle inflammation.[12,98] Lower levels of vitamin D are typically seen in the elderly but may get worse when absorption and metabolism of vitamin D become impaired in severe liver disease.[10,99]

Cirrhotic patients are increasingly prone to frailty and infections. Frailty, a condition distinct from disability, has been defined as a biological syndrome of decreased reserve and resistance to stressors. It is characterized by a cumulative decline across multiple physiologic systems and increased vulnerability to adverse outcomes.[100] Frailty that predates a patient's stated age significantly affects clinical performance in cirrhosis and is closely associated with sarcopenia. Physical exercise dramatically improves components of frailty, which is much more challenging with the coexistence of sarcopenia.[101] Susceptibility to infections is another major determinant of clinical outcomes in cirrhosis. Dysbiosis and breakdown of the intestinal barrier combined with portosystemic shunting promote bacterial infections in cirrhosis.[102] Immune functions in cirrhosis are further compromised by malnutrition and alcohol drinking.[103] Finally, myosteatosis and sarcopenia predict increased risk and worse prognosis of hepatocellular carcinoma in cirrhosis associated with NAFLD and other liver diseases, although the precise molecular mechanisms of this process are not entirely understood.[104,105]

Prevention and management of skeletal muscle dysfunction in NAFLD

It is now clearly established that progression and clinical outcomes in NAFLD are affected by skeletal muscle health, which in turn may benefit from effective treatment of liver disease.[13] Finding therapeutic strategies that address the pathobiology of both organs in metabolic dysfunction is therefore desirable. The main clinical objectives and specific treatment targets in this effort may differ depending on the stage of liver disease. Effective management of non-cirrhotic NAFLD is expected to diminish myosteatosis and prevent cellular and molecular mechanisms that would ultimately result in sarcopenia with or without concomitant obesity. In NAFLD patients with established cirrhosis, halting the progression of skeletal muscle dysfunction may be a more appropriate role than reversing the course of the disease. However, improved muscle function is likely to have a positive impact on liver disease progression and outcomes in all stages of NAFLD.[12,17,18,106]

Lifestyle modification approaches to skeletal muscle dysfunction in NAFLD

There are multiple lines of evidence that lifestyle interventions aimed at improving skeletal muscle mass, strength, and function positively impact the course of NAFLD.[34,106,107] These approaches, such as sustained physical activity, adequate nutrition, monitored weight loss, control of diabetes, and alcohol abstinence, usually promote whole health with combined benefits, and it may be challenging to decipher specific effects on skeletal muscle or the liver. Nevertheless, skeletal muscle is a major energy expenditure site and a key component in balancing excess nutrient intake to limit the buildup of adipose tissue and lipid spillover with ectopic deposition. Moreover, aerobic exercise has been associated with lower levels of circulating pro-inflammatory mediators, such as IL-6, TNF-α, and C-reactive protein, thus reducing systemic low-grade inflammation, restoring adipokine and myokine balance, and improving insulin resistance.[34,106,107] Regular exercise is known to modulate the composition and function of gut microbiota, improving bacterial richness and balance towards health-promoting taxa.[108]

Physical activity is also critical to mitigate the potential drawbacks of weight management strategies in metabolic dysfunction.[10,12] Even if it is controlled and incremental, weight loss induced by caloric restriction alone may lead to sarcopenia or make it worse since it usually results in the loss of both fat tissue (75%) and fat free mass (25%).[109] Fortunately, however, several studies indicate that these concerns are not necessarily substantiated. In a North American study, nondiabetic, severely obese individuals were enrolled in a 6-month lifestyle modification interventional trial to analyze the association between NAFLD and body composition before and after weight loss.[110] Subjects with NAFLD had greater visceral adiposity and experienced more significant improvements in visceral fat mass, waist circumference, hepatic insulin sensitivity, and serum transaminases than those without NAFLD. However, there was no baseline difference in muscle mass between the two groups. Perhaps more importantly, no disproportionate loss of lean mass and skeletal muscle mass occurred among participants with NAFLD resolution, suggesting that weight loss interventions may be accomplished with muscle preservation.[110] Initial reports about the impact of bariatric surgery on skeletal muscle mass are also promising. In a retrospective European study from a single center analyzing 69 patients who underwent bariatric surgery (gastric bypass or sleeve gastrectomy), the percentage of BMI loss and improvement in comorbidities were similar in the sarcopenic and non-sarcopenic subgroups, with no significant differences in skeletal muscle mass indices at 1-year postoperative follow-up.[111]

Pharmacotherapy in skeletal muscle dysfunction associated with advanced liver disease

Lifestyle interventions represent a logical approach to improve liver disease and metabolic comorbidities that result from increased caloric intake and physical inactivity. However, there are immense efforts to find efficient and safe pharmacotherapy for obesity-associated disorders, such as NAFLD, and restore homeostasis within the adipose–muscle–liver triangle.[112] Several phase III clinical trials for NAFLD aim to find drugs that target lipid accumulation, lipotoxicity, insulin resistance, liver inflammation, and fibrosis alone and in combination.[113,114] By contrast, less advanced are therapeutic efforts to improve myosteatosis and sarcopenia associated with metabolic dysfunction through molecular targets. Vitamin D supplementation to correct low levels to improve the liver–muscle interplay and reduce NAFLD's progression and severity has yielded controversial results.[12,99] Similarly, additional clinical evidence is needed to support the use of testosterone and GH to counteract the effects of myostatin in advanced liver disease.[12,91] In a proof-of-concept multicenter clinical trial, 24-week treatment of patients aged 75 years and older with a humanized monoclonal antibody against myostatin significantly increased lean mass and improved skeletal muscle performance measured by fast gait speed, chair stand tests, and other functional assays.[115] The impact of targeted molecular interventions on skeletal muscle dysfunction associated with NAFLD remains to be seen.

As discussed above, hyperammonemia in end-stage liver disease represents harm to skeletal muscle homeostasis as it interferes with myocellular energy metabolism and promotes protein breakdown and autophagy.[96] Ammonia-lowering measures, such as lactulose or rifaximin, and supplementation with protein-based calories or BCAA provide an opportunity to mitigate the loss of sarcopenia in cirrhosis by reducing the rate of protein catabolism and improving muscle mass.[91,116] However, nutritional parameters and quality of life do not necessarily follow the improvement in hepatic encephalopathy, and slow skeletal muscle turnover has been considered as an explanation for the apparent need of long-term management of hyperammonemia to affect sarcopenia in cirrhosis.[12] Liver transplantation may be the ultimate solution to eliminate the impact of diseased liver on skeletal muscle dysfunction; although, it is essential to realize that pre-existing sarcopenia may significantly hamper postoperative recovery after liver transplantation, and the adverse impact of immunosuppressive therapies on skeletal muscle is not negligible.[18,91]

Conclusions

We have come a long way in understanding NAFLD's pathophysiology since its original description 40 years ago. There is emerging interest in renaming NAFLD to metabolic dysfunction-associated fatty liver disease (also referred to as MAFLD) and recognizing this liver condition as part of a multisystem metabolic disorder that represents a continuum, beginning at early changes due to sustained caloric excess to severe outcomes from end-stage organ damage.[117] Since so many people are affected by this highly prevalent condition, risk assessment and prognostication is critically important and may be enhanced by better understanding the cellular and molecular mechanisms governing the relationship between skeletal muscle and the liver.

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Conflict of interest

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Author contributions

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Review Article

Macrophage Phenotypes and Hepatitis B Virus Infection

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Abstract

Globally, hepatitis B virus (HBV) infection and its related liver diseases account for 780,000 deaths every year. Outcomes of HBV infection depend on the interaction between the virus and host immune system. It is becoming increasingly apparent that Kupffer cells (KCs), the largest population of resident and monocyte-derived macrophages in the liver, contribute to HBV infection in various aspects. These cells play an important role not only in the anti-HBV immunity including virus recognition, cytokine production to directly inhibit viral replication and recruitment and activation of other immune cells involved in virus clearance but also in HBV outcome and progression, such as persistent infection and development of end-stage liver diseases. Since liver macrophages play multiple roles in HBV infection, they are directly targeted by HBV to benefit its life cycle. In the present review, we briefly outline the current advances of research of macrophages, especially the studies of their phenotypes, in chronic HBV infection.

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Introduction

As a major world health problem, hepatitis B virus (HBV) infects 257 million people, representing about 3% of the world's population (https://www.who.int/en/news-room/fact-sheets/detail/hepatitis-b). While approximately 95% of HBV infections acquired during adulthood are resolved, the virus cannot be cleared in most individuals infected in perinatal period or early childhood.[1] It is widely accepted that the virus-host interaction, which is affected by age, transmission route, immune status and other factors, determines the outcome of infection.[2,3]

Both adaptive and innate immunity are involved in anti-HBV immune response. On one hand, antigen-presenting cells (APCs), including macrophages and dendritic cells (DCs), initialize the virus-specific adaptive immunity characterized by activation of T helper lymphocytes and secretion of various cytokines, which then mobilize the cytotoxic T lymphocyte (CTL) to kill the HBV-infected cells. Additionally, HBV-specific antibodies are developed by the humoral immune system to neutralize the virus and facilitate its clearance.[4] On the other hand, the essential role of non-specific defense, especially the function of the liver macrophages (i.e. KCs), has gained growing attention (reviewed in Faure-Dupuy *et al.*[5]), albeit the precise mechanism remains incompletely elucidated because of the difficulty in identifying asymptomatic early infections in human studies.[6] Unlike hepatitis C virus (HCV), HBV was once considered as a "stealth virus", due to the fact that HBV could not induce significant innate immune response in an acute HBV-infected chimpanzee model.[7] Limited evidence from clinical study[8] also showed that no intense cytokine storm, such as type I interferon (IFN) and type III IFN production, occurs in patients with acute HBV infection. Nevertheless, one typical characteristic of HBV infection is macrophage hyperplasia in the liver,[9,10] suggesting an important role of macrophages in HBV pathogenesis. It has been demonstrated that some effecter molecules, such as interleukin (IL)-6,[11] were produced by KCs to replace IFNs to control HBV infection.[11] Another interesting study[12] showed that HBV DNA in the liver and blood were cleared before the adaptive immune response was elicited, indicating that innate immune response is much more than a simple branch to control virus invasion until onset of the adaptive response.

Herein, we will review the effects of liver macrophages on HBV infection, focusing on macrophage phenotypes in HBV persistent infections.

Macrophages: Functions and phenotypes

In the healthy liver, the compartment of liver macrophages is dichotomic, involving tissue-resident macrophages (i.e. KCs) and monocyte-derived macrophages (MDMs). KCs, as well as the liver DCs and sinusoidal endothelial cells (LSECs), are mainly localized in the sinusoids of the liver and they form the

Keywords: Hepatitis B virus; Chronic HBV infection; Macrophage; Phenotype.

Abbreviations: ALT, alanine aminotransferase; APC, antigen-presenting cell; ATP, adenosine triphosphate; CHB, chronic HBV infection; CHC, chronic hepatitis C virus; CTL, cytotoxic T lymphocyte; CXCL, chemokine (C-C motif) ligand, CCL, C-X-C motif chemokine ligand; DC, dendritic cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HSPG, heparan sulfate proteoglycan; IFN, interferon; IL, interleukin; IRF3, interferon regulatory factor 3; ISGs, IFN-stimulated genes; KC, Kupffer cell; LBP, LPS binding protein; LCMV, lymphocytic choriomeningitis virus; LPL, lipoprotein lipase; LSEC, liver sinusoidal endothelial cell; M-CSF, macrophage-colony stimulating factor; MDM, monocyte-derived macrophage; MHC, major histocompatibility complex; MHV, murine hepatitis virus; MIF, macrophage migration inhibitory factor; MR, mannose receptor; MxA, myxovirus resistance gene; NF-κB, nuclear factor kappa-B; NK, natural killer; NKT, natural killer T; NTCP, sodium taurocholate cotransporting polypeptide; OXPHOS, oxidative phosphorylation; PBMC, peripheral blood mononuclear cell; PEM, peritoneal exudative macrophage; PGE2, prostaglandin E2; PTH, primary tupaia hepatocytes; ROS, reactive oxygen species; STING, stimulator of IFN genes; TLR, toll-like receptor; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand.

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first line of defense to diverse antigens and toxic components contained in portal venous blood.[13] MDMs are mainly localized near the portal triad. When the KCs are depleted experimentally or pathologically, the MDMs can be infiltrated from the peritoneal cavity, replacing KCs by acquiring virtually the same phenotype.[14] In fact, the liver macrophages (i.e. KCs and MDMs) are very plastic and no specific marker is used to discriminate KCs from MDMs in human.[5]

As an important component of innate immunity, liver macrophages can function as: 1) phagocytes to remove dead cells, debris and pathogens;[15] 2) effective APCs, with their expression of major histocompatibility complex (MHC) and co-stimulatory molecules; 3) immune mediators involved in immune suppression and allograft tolerance of liver; and, 4) key players in rapid erythrocyte removal and iron recycling (mainly the MDMs).[16]

Polarization, which is equally vague as activation, is indispensable before the macrophages achieve their different functions. Generally speaking, macrophages can be polarized into two major subsets with different combinations of stimuli:[17] M1 macrophages of classical activation, which induce inflammation and cause tissue damage by facilitating Th1 response, and M2 macrophages of alternative activation, which maintain tissue integrity by promoting the Type 2 T helper cell response (Fig. 1). M1 and M2 type cytokines or surface markers are referred to, to differentiate different macrophage activation phenotypes. There are other subsets, such as M2a, M2b, M2c, etc. What has been overlooked, however, is that polarization is a process which changes continuously[18] and various mixtures of M1 and M2 type macrophages may result in confusion. As a matter of fact, much more effort is required to define the criteria for assessing phenotypes. However, for the rest of this review, we will discuss the association between macrophages and HBV infection on the basis of the current understanding of M1/M2 type macrophages.

Do liver macrophages sense HBV infection?

First of all, although the exact interaction between liver macrophages and HBV is still unclear, nonhepatic cell surface presentations of molecules interacting with PreS or hepatitis B core antigen have been documented. Peripheral blood mononuclear cells (PBMCs), the monocytic cell line THP-1 and U937[19–23] were reported to express the PreS-binding receptor of HBV. Lipoprotein lipase (LPL), which can be produced by THP-1 macrophages,[24,25] has an linear motif for PreS binding and may interact with HBV particles during infection.[23] Additional candidate Pre-S receptors, which can be expressed by KCs,[26] are lipopolysaccharide (LPS) binding protein (LBP), the LPS receptor CD14,[22] and mannose receptor (MR).[27,28] These receptors are involved in the binding of hepatitis B surface antigen to macrophages, monocytes or DCs. Hepatitis B core antigen was also reported to bind to PBMCs and trigger the release of IL-18.[29] Consistently, Cooper *et al.*[30] demonstrated that hepatitis B core antigen could bind to receptor(s), like the Toll-like receptor (TLR)2 and heparan sulfate proteoglycan (HSPG), on THP-1 macrophages by its arginine-rich domain at the C-terminal and effectively induce expression of pro-inflammatory molecules. Given the fact that hepatitis B core antigen mainly exists within the hepatocytes and viral particles, whether liver macrophages interact with hepatitis B core antigen during HBV infection in patients is still not clear. Accordingly, although there is a probable involvement of HBV antigen receptors in initializing viral infection, it is more likely that these receptors only mediate cellular recognition or internalization of HBV/HBV antigens. Little work has addressed the expression of the recently identified HBV functional receptor sodium taurocholate cotransporting polypeptide (NTCP)[31,32] in liver macrophages. In an interesting study, Neurath *et al.*[20] reported that HUT-78 and MOLT3 cells (both T cell lines) could covalently attach to PreS-cellulose or hepatitis B surface antigen-cellulose after treatment with concanavalin A linked with a peptide of HBV PreS1. This result suggests a similar possibility that HBV receptors could be induced by appropriate stimulations in liver macrophages or monocytes. Moreover, HBV antigens and nucleic acid have been detected in macrophages and monocytes,[21,33–35] raising the possibility that HBV might be "taken into" the macrophages or monocytes.

Secondly, the ability of macrophages to produce cytokines upon exposure to HBV potentially renders them as indispensable immune cells sensing and discriminating invading HBV. Hösel's group11 observed an early-time, nuclear factor kappa-B (NF-κB)-dependent induction of inflammatory mediators in primary human KCs stimulated with HBV inoculum generated from the HepG2.2.15 cell line. This cluster of soluble inflammatory cytokines, including IL-6, IL-8, IL-1β and tumor necrosis factor (TNF)-α but no type I IFN, inhibited HBV replication significantly. In a more recent study,[21] KCs isolated from patients with persistent HBV infection showed a higher activation status (characterized by elevated expressions of CD40, HLA-ABC and HLA-DR) than those of healthy control. And, in accordance with previous report, their experiments[21] also revealed obvious inductions of IL-6, IL-15, TNF, chemokine (C-C motif) ligand 4 (CCL4), C-X-C motif chemokine ligand 8 (CXCL8), as well as IL-10 in human primary KCs and PBMC-generated macrophages cultured with patient plasma-derived hepatitis B surface antigen. Most recently,

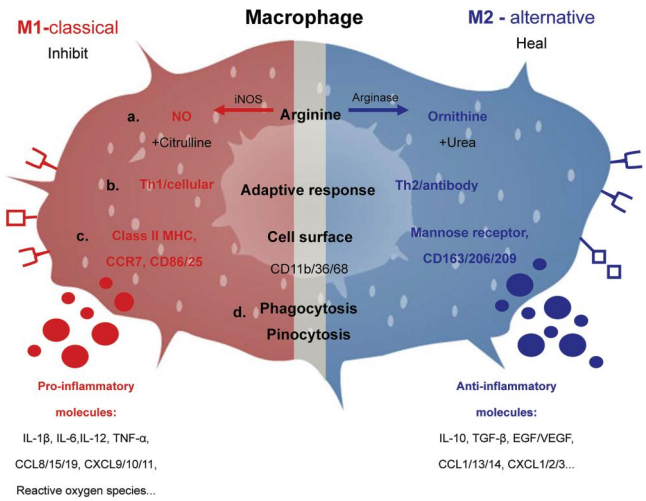

Fig. 1. Characteristic products and functions of M1 and M2 macrophages. Macrophages can metabolize arginine with the inducible nitric oxide synthase enzyme into nitric oxide and citrulline or with arginase into ornithine and urea which is the biochemical basis of the M1 or M2 macrophage responses, respectively (a). M1- or M2-dominant macrophages stimulate the Type 1 T helper cell or Type 2 T helper cell responses (**b**). Also shown are the major molecules involved, including cell surface molecules, cytokines, chemokines and so on, which are closely associated with the M1 or M2 phenotypes (**c**). Phagocytosis and pinocytosis are general properties of macrophages, which are not dependent on M1 or M2 type responses (**d**).

Cheng *et al.*[36] reported that human macrophages showed an inflammatory cytokine storm when stimulated with high level HBV, while the hepatocytes sensed HBV DNA poorly. Other *in vitro* studies demonstrated that HBV antigens (e.g., HBV envelop protein, PreS and HBV core antigen) were able to induce cytokine secretion in monocytes and MDMs after binding to the receptors (see below).

M1 and M2 macrophages involved in HBV infection

As a major source of cytokines and immune regulators, macrophages are involved in HBV infection in at least two aspects: (1) antiviral effects, mainly mediated by M1 type molecules (Fig. 2a); and, (2) immunotolerance, mediated by M2 type molecules (Fig. 2b).

Antiviral effects

Activation of M1-type macrophages and production of pro-inflammatory cytokines usually indicate a robust immune response to HBV infection. CD16+ is one of the M1-like phenotype markers. Zhang's group[37] investigated 110 hepatitis B e antigen-negative chronic hepatitis B (CHB) patients and found that the immune-activated group was characterized by lower HBV DNA and that high alanine aminotransferase (ALT) is associated with more CD16+ monocytes and/or macrophages in the peripheral blood and liver, when compared with the immune tolerant group. High level of M1-like CD16+ macrophages was an indicator for immune activation that helped patients to defend against the virus.

Direct antiviral effects: Agonists of TLRs[38–41] and HBV antigens (as discussed above) can induce macrophages to express soluble inflammatory mediators and other effective molecules, which are the major effectors to assume direct antiviral activity of macrophages. With diverse mechanisms, these effective molecules can either control HBV without obvious cytotoxicity or result in injury or apoptosis of the infected hepatocytes.

Type I IFN, for example, one of the key cytokines potently inhibiting HBV replication in hepatocytes, is routinely used in the clinic to treat HBV patients. Despite plenty of work having addressed the anti-HBV mechanisms of type I IFN, there is limited clinical or *in vivo* evidence for the idea that after the lag phase of HBV replication with negligible release of type I IFN, liver macrophage-synthesized IFN α/β may act as essential controller for HBV. Fortunately, circumstantial evidence is

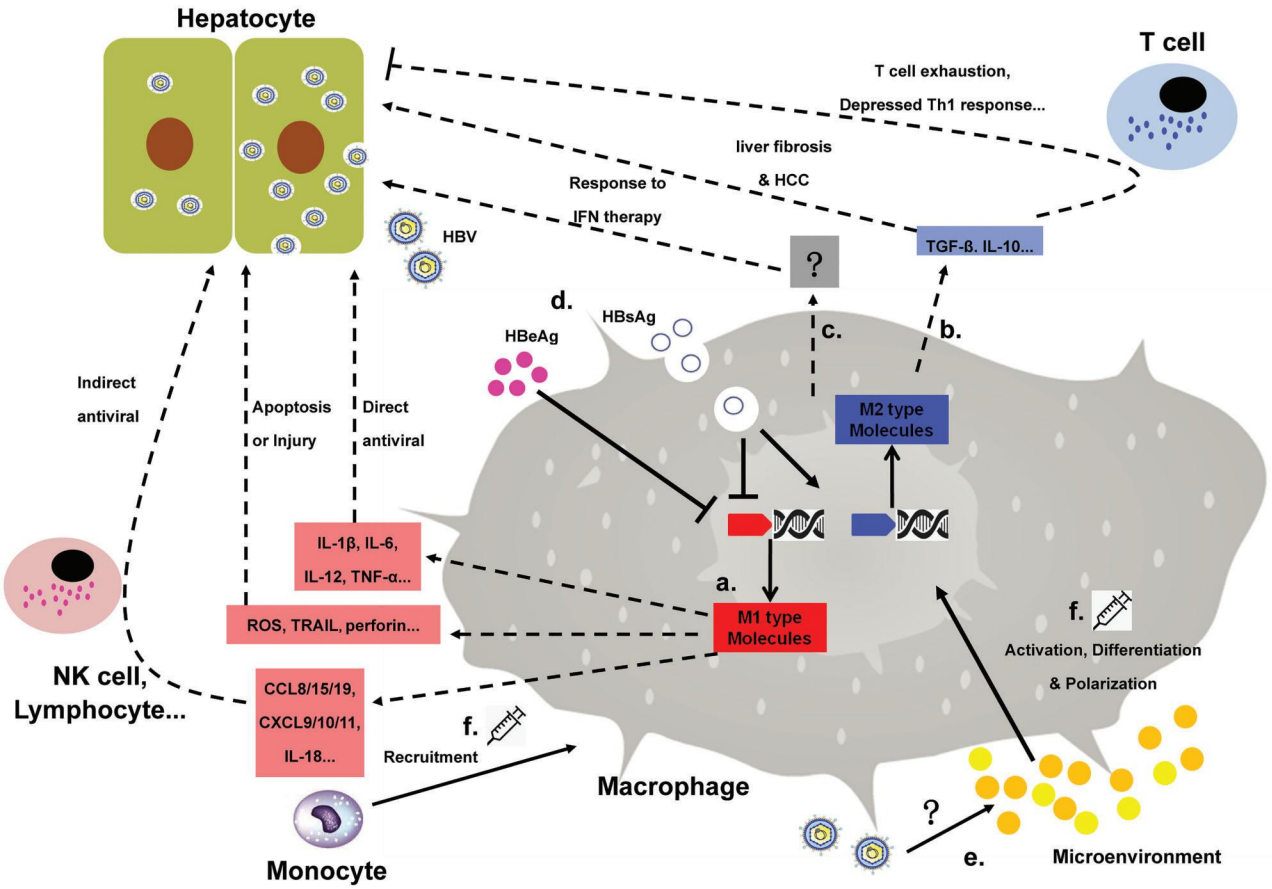

Fig. 2. Macrophage involvement in HBV infection. The anti-HBV effect of macrophages is mediated mainly by pro-inflammatory cytokines inducing a direct antiviral response or molecules recruiting or activating other immune cells. Meanwhile, another group of M1 KCs produces molecules that may result in injury or apoptosis of the hepatocytes (a). Immunomodulatory mediators, such as IL-10 and TGF-β, are closely associated with suppressed antiviral T cell responses and/or end-stage HBV liver disease (**b**).Macrophages may also contribute to the inflammatory or anti-inflammatory liver microenvironment and, consequently, alter hepatic response to IFN treatment (**c**). The phenotype and function of macrophages can be modified by either HBV itself (**d**) or the microenvironment (**e**). Thus, the therapeutic strategies targeting macrophages in an HBV infection may aim at modulating macrophage polarization/phenotype, monocyte recruitment/activation and so on (**f**).

available. First of all, type I IFNs induced by *in vitro* activated KCs effectively suppress HBV production. Injections of diverse TLR (TLR3/4/5/7/9) agonists could control HBV replication, which is IFN α/β-dependent in transgenic mice.[40] Wu's group[38] confirmed and extended these findings by collecting the supernatants of primary C57BL/6 mouse KCs after stimulation with ligands specific for TLR1 to TLR9 and evaluating their effects on HBV-Met cells. Their study showed a significant TLR3- or TLR4-mediated suppression of HBV replication, which can be abolished, or at least partially abolished, by IFN-β antibodies.

Another study[42] using KCs activated by the agonist of stimulator of IFN genes (STING) revealed a predominant type I IFN production and subsequent inhibition of HBV replication in the AML12HBV10 cell line. The anti-HBV effect of STING agonist was further confirmed in the HBV DNA hydrodynamic NOD/SCID mouse model.[42] Secondly, unrelated viral infection may activate KCs and noncytopathically inhibit HBV production via IFN α/β. Guidotti *et al.*[43] used lymphocytic choriomeningitis virus (LCMV) to infect HBV transgenic mice and assayed the production of HBV. They found that 3.5- and 2.1-kb HBV mRNAs were decreased or even absent, as well as HBV DNA replication forms. Similar results were found in HBV transgenic mouse model with malaria infection.[44] Recruitment of macrophages and subsequently elevated expressions of IFN α/β/γ suppressed HBV gene expression and replication *in vivo*. Thirdly, HBV has evolved some strategies specifically targeting IFN production in KCs, indicating the potential anti-HBV effects of macrophage-derived IFNs. Using the murine nonparenchymal liver cells, Wu's group[45] demonstrated that hepatitis b surface antigen, hepatitis B e antigen, as well as HBV virion, could suppress TLR3-mediated IFN-β, IFN-γ and IFN-stimulated gene (ISG) production by interfering with the activation of interferon regulatory factor 3 and NF-κB.

Activated macrophages are the major source of TNF-α,[46] which has been identified as a potent anti-HBV molecule. It has been well established that TNF-α production increases in the primary KCs[47] or PBMCs[48] isolated from CHB patients. *In vitro* studies also demonstrated that the expression levels of TNF-α in primary KCs,[11,21] MDMs[21] and THP-1[30] cells were up-regulated in response to HBV challenge. In addition, HBV replication in primary tupaia hepatocytes (PTHs) was partially inhibited by recombinant tupaia TNF-α.[49] In transgenic mice, TNF-α produced by macrophages during LCMV,[43] adenovirus or cytomegalovirus[50] infection inhibited HBV gene expression and DNA replication noncytopathically. Furthermore, substantial clinical data also raised the importance of TNF-α in HBV infection.

Elimination of hepatitis B e antigen and suppression of HBV replication in patients receiving IFNα treatment was accompanied by spontaneously induced TNF-α in PBMCs.[51] Anti-TNF-α therapy in patients with chronic inflammatory diseases was associated with higher risk of HBV activation, reactivation and hepatotoxicity,[52–55] which may be attributed to the setting of immune suppression. Accumulating evidence also suggests an important role of TNF-α gene polymorphisms in HBV infection.[56] These clinical studies, together with the data from basic research and animal models, mirror the fact that TNF-α, as well as the macrophages, is one of the prerequisites for virus clearance and permanent control of HBV.

Other macrophage-derived anti-HBV cytokines include IL-1β,[11,51] IL-6,[11,21,30] IL-12,[57] IL-15,[21] and macrophage migration inhibitory factor (MIF),[58] some of which may perform synergistic actions with each other.[59] Meanwhile, another group of KCs produced molecules, such as reactive oxygen species (ROS),[10] Fas-ligand,[60] TNF-related apoptosis-inducing ligand (TRAIL),[61] granzyme B and perforin,[62] may result in injury or apoptosis of the hepatocytes.

Indirect antiviral effects through recruiting or activating other immune cells: Liver macrophages synthesize several cytokines and chemokines to activate or recruit inflammatory cells involved in the anti-HBV roles. IL-18, an inflammatory cytokine belonging to the IL-1 family, is mainly expressed by liver macrophages.[63] Previous studies have demonstrated that IL-18 plays a powerful anti-HBV role by inducing cytokine production (e.g. IFN-γ, IFN α/β, TNF-α)[64] in some immune cells. Kakimi *et al.*[65] showed that IL-18 was a type I- and type II-IFN inducing factor, acting on both intrahepatic natural killer (NK) and natural killer T (NKT) cells in a transgenic mouse model, resulting in suppressed HBV replication. Interestingly, this inhibitory effect of IL-18 on HBV replication is dependent on IL-12, which is able to be released by activated macrophages. Boltjes *et al.*[21] analyzed the function of human primary KCs and *in vitro*-generated MDMs. They found both could be activated by exposure to patient-derived hepatitis B surface antigen, resulting in activation of NK cells characterized by up-regulation of CD69 and IFN-γ.

It has been well established that Type 1 T helper cells, B cells and DCs can also produce IFN-γ in response to IL-18 stimulation.[66–68] In addition, activated liver macrophages also produce CXCL, CXCL-9 and CXCL-10, which assist in trafficking of lymphocytes and monocyte/macrophages into the tissue.[69,70] Another study from Kakimi group[71] demonstrated that CXCL-9 and CXCL-10 derived from nonparenchymal cells (including KCs) chemoattracted lymphomononuclear inflammatory cells into the liver in a transgenic murine model.

Immunotolerance/immunosuppressive activity

Constantly exposed to diverse antigens derived from food or microbial products, the immune cells, in addition to other cells[72] in the liver, develop some mechanisms to prevent excessive activation and continuous pathology, known as inherent tolerogenicity of the liver. KCs are involved in the well-known tolerogenic milieu by secreting soluble immunoregulators (e.g., IL-10, TGF-β, and amphiregulin) or expressing inhibitory molecules on the membrane, both of which could be exploited by HBV for their favorable immunosuppressive microenvironment.

For instance, IL-10 could depress inflammation response by inhibiting Type 1 T helper cell cytokine expression. An HBV-carrier mouse model showed no significant immune response to hepatitis B surface antigen vaccination, which could be reversed by KC depletion or IL-10 deficiency.[73] Clinical data[74] also revealed an association between CHB and elevated plasma IL-10 level, though it was uncertain whether the increased IL-10 was derived mainly from macrophages or not. Consistently, Li's group[75] also reported that the increased production of IL-10 by KCs, which was stimulated by HBV core antigen, resulted in inhibition of the antiviral function of CD8+ T cells in mice.

Interestingly, IL-10 gene promoter polymorphisms were reported to be associated with HBV progression.[76] It was reported that murine KCs preferred to produce TGF-β, which is able to restrain immune response and to develop tolerance towards self-antigens,[77] rather than functioning as a

pro-inflammatory cytokine in response to HBV infection.[78] Although the precise mechanism remains unclear, the tolerogenic role of HBV by modulating liver macrophage polarization should not be ignored, since IL-10 and TGF-β are typical cytokines of M2 macrophages. Previous studies also suggested that KCs were primarily immunosuppressive, mediated by prostaglandin E2 (PGE2);[13] accordingly, HBV may maintain or even promote this immunosuppressive status by regulating macrophage polarization and/or PGE2 production to benefit its replication.

Bility and colleagues[79] found that CHB patients with fibrosis and/or hepatocellular carcinoma (HCC) and patients with acute HBV-associated liver failure experienced a M2 phenotype, including increased M2 macrophages in the liver infiltration and predominate M2-type gene expression profile in the liver. The authors developed a humanized mouse model, supporting HBV replication to investigate the HBV-associated immunopathogenesis. They found that impaired immune response in parallel with robust M2-type macrophage activation in the liver contributed to the development of persistent HBV infection, indicating the M2 macrophages might act as immune suppressors. Nowadays, accumulating evidence indicates that macrophages play an important role in HBV-induced immune suppression, not only in persistent infection establishment but also in the development of the end-stage liver diseases, such as liver fibrosis and HCC.[80]

Potential role of macrophages in response to IFNα treatment in CHB patients

Type I IFN is still one of the most important therapies for CHB infection or chronic hepatitis C virus (CHC). However, only a subset of the patients respond. Our previous studies showed that cell-type specific ISGs' expression in the liver predicts whether a given patient will respond to IFN treatment among CHC[81] or CHB[82] patients. We analyzed the pre-treatment gene expression in 38 CHB livers by immunohistochemical staining and found that in the treatment responders, increased ISG15 and myxovirus resistance gene (MxA) protein expression was more pronounced in macrophages than that in hepatocytes. In contrast, in the non-responders, elevated expression of ISG15 and MxA was more pronounced in hepatocytes compared with that in macrophages. A similar result was found in CHC patients before receiving pegylated-IFN/ribavirin treatment, indicating that the liver macrophages might be involved in mediating patients' response to IFN and other anti-viral therapy.

Many studies correlated IFN and a subset of typical ISGs (e.g., ISG15, USP18) with macrophage phenotypes and functions. Fleetwood *et al.*[83] reported that the type I IFN signaling pathway played an essential role in regulating phenotype and function of macrophage-colony stimulating factor (M-CSF)- or granulocyte-macrophage colony-stimulating factor (GM-CSF)-treated bone marrow-derived macrophages in mice. ISG15, a typical ISG, may play an important role in macrophage polarization and function. Macrophage polarization is characterized by mitochondrial functions regulated by different metabolic patterns, and the lack of ISG15 was responsible for mitochondrial dysfunction, including diminished oxidative phosphorylation (OXPHOS), depressed oxygen consumption rate, as well as reduced adenosine triphosphate (ATP) and ROS production in bone marrow-derived macrophages in mice.[84] Macrophages from the ISG15-deficient mice have been shown to have depressed phagocytic capacity, which is dependent on protein kinase AKT.[85] Moreover, we previously described USP18, another typical ISG, as a modulator of macrophage activity in mice. Compared with wild type control, both primary KCs or peritoneal exudative macrophages (PEMs) from USP18-/- preferred to polarize to the M2-like phenotype, producing more anti-inflammatory cytokines (e.g., IL-10 and IL-4) and less inflammatory cytokines (e.g., TNF-α and IL-12) in response to murine hepatitis virus (MHV)-3 infection (unpublished data).

The inflammation microenvironment may be changed by macrophages, influencing the response of hepatocytes to IFN treatment. Our previous study[86] found that pre-treatment with TNF-α or LPS led to an IFNα refractory state in human hepatoma cells and primary murine hepatocytes. We have also investigated the response of primary murine hepatocytes to IFNα after co-culturing with the primary murine hepatocytes and the primary murine USP18-/- (M2 like) or wide type (M1-like) PEMs, using the Transwell co-culture system. We found that hepatocytes co-cultured with USP18-/- PEMs experienced much higher expression of ISGs (including of ISG15, USP18 and MxA) with IFNα stimulation (unpublished data).

We therefore hypothesize that liver macrophages regulate inflammatory and anti- inflammatory responses, contributing to the liver microenvironment and, consequently, alter hepatic response to IFN treatment. However, more in-depth investigations are needed to uncover the underlying molecular mechanism (Fig. 2c).

Effect of HBV on macrophage phenotype

The distinct roles of M1 and M2 macrophages involved in HBV infection raise the possibility that HBV may promote M2 polarization of macrophages to impair the Type 1 T helper cell immune response, resulting in persistent infection and disease progression. A most recent study[87] supported the hypothesis that HBV suppresses M1 macrophage cytokine (IL-6 and IL-1β) expression and promotes M2 macrophage cytokine (IL-10) expression to favor HBV infection. Although the precise mechanism remains unclear, several *in vitro* studies have revealed that either hepatitis B surface antigen or hepatitis B e antigen may make a contribution. Expression of M1-type cytokines, such as TNF-α, IL-1b and IL-8, was inhibited by hepatitis B surface antigen in PBMCs,[19] while the expression of IL-10 was not affected or even promoted.[19,88] Similar results were observed in THP-1-derived macrophages: hepatitis B surface antigen acted as a potent suppressor of M1-type cytokines, including IL-12, TNF-α, IL-1β and IL-6.[88–90] Moreover, Yu *et al.*[91] reported a decreased IL-1β secretion in liver macrophages induced by hepatitis B e antigen. However, it has been reported that pro-inflammatory macrophages and monocytes expressing TNF-α and GM-CSF accumulated in chronic HBV or HCV-related liver disease.[92] Moreover, high level of IL-23 (as well as IL-1β, IL-6 and IL-17) expression in liver inflammatory macrophages was demonstrated to be associated with HCC development.[93] These contradictory lines of evidence have indicated the complicated association between different macrophage phenotypes and disease progression of hepatitis B virus infection (Fig. 2d). In addition, the microenvironment altered by HBV infection may also contribute to the activation, differentiation and polarization of macrophages (Fig. 2e).

Conclusions

Although our knowledge about the exact immunological pathogenesis during chronic HBV infection is limited, the remarkable heterogeneity of liver macrophages concerning not only the defense but also the homeostasis and metabolism make it a promising option for treating HBV infection[14] (Fig. 2f). Binding and/or up-taking virus/ viral antigens, as well as the signaling from infected hepatocytes, may result in the activation of liver macrophages. The activated liver macrophages, on one hand, enforce virus clearance by producing pro-inflammatory cytokines targeting hepatocytes to suppress HBV directly or recruiting, interacting or activating other immune cells to get rid of the virus, and on the other hand, modulating immunotolerance as a negative feedback to avoid unchecked inflammation. This functional diversity or contrary action makes it possible that liver macrophages may be exploited by HBV.

HBV is a stealth virus which "hides" itself in the early stage of life cycle to escape macrophage defenses, and may then manipulate the polarization/phenotype of macrophages to benefit its persistent infection. It is important to note that the response of liver macrophages to HBV infection is not only limited to innate defense but also links the innate immunity with acquired immunity. However, whether macrophages resolve virus, worsen liver immunopathogenesis, promote persistent infection or modulate the response to IFNα therapy depends on a combination of various factors and is finely tuned. This is indeed a complicated process and the interaction between macrophages and HBV deserves further study.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

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Review Article

Nontumoral Portal Vein Thrombosis: A Challenging Consequence of Liver Cirrhosis

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Abstract

Nontumoral portal vein thrombosis (PVT) is an increasingly recognized complication in patients with cirrhosis. Substantial evidence shows that portal flow stasis, complex thrombophilic disorders, and exogenous factors leading to endothelial dysfunction have emerged as key factors in the pathogenesis of PVT. The contribution of PVT to hepatic decompensation and mortality in cirrhosis is debatable; however, the presence of an advanced PVT increases operative complexity and decreases survival after transplantation. The therapeutic decision for PVT is often determined by the duration and extent of thrombosis, the presence of symptoms, and liver transplant eligibility. Evidence from several cohorts has demonstrated that anticoagulation treatment with vitamin K antagonist or low molecular weight heparin can achieve recanalization of the portal vein, which is associated with a reduction in portal hypertension-related events and improved survival in cirrhotic patients with PVT. Consequently, interest in direct oral anticoagulants for PVT is increasing, but clinical data in cirrhosis are limited. Although the most feared consequence of anticoagulation is bleeding, most studies indicate that anticoagulation therapy for PVT in cirrhosis appears relatively safe. Interestingly, the data showed that transjugular intrahepatic portosystemic shunt represents an effective adjunctive therapy for PVT in cirrhotic patients with symptomatic portal hypertension if anticoagulation is ineffective. Insufficient evidence regarding the optimal timing, modality, and duration of therapy makes nontumoral PVT a challenging consequence of cirrhosis. In this review, we summarize the current literature and provide a potential algorithm for the management of PVT in patients with cirrhosis.

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Keywords: Portal vein thrombosis; Liver cirrhosis; Clinical course; Anticoagulation; Transjugular intrahepatic portosystemic shunt.

Abbreviations: AASLD, American Association for the Study of Liver Diseases; CI, confidence interval; CT, computed tomography; CTP, Child-Turcotte-Pugh; DOAC, direct oral anticoagulant; EASL, European Association for the Study of the Liver; HCC, hepatocellular carcinoma; HR, hazard ratio; LMWH, low molecular weight heparin; MELD, model for end-stage liver disease; MRI, magnetic resonance imaging; MTHFR, methylene tetrahydrofolate reductase; OD, odds ratio; PVT, portal vein thrombosis; SMV, superior mesenteric vein; TEG, thromboelastography; TIPS, transjugular intrahepatic portosystemic shunt; VKA, vitamin K antagonist.

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Introduction

Portal vein thrombosis (PVT) is characterized by thrombus formation within the trunk of the portal vein or its main branches, which may extend to the splenic or superior mesenteric veins (SMVs).[1–3] It is further classified according to site, degree, extent, and functional relevance of the thrombosis, as well as the presence of underlying liver disease (Supplementary Table 1).[4–12] Recently, an "anatomico-functional classification system" that incorporates anatomic descriptors, timing of the thrombosis, and the relationship to clinical sequelae, was proposed (Supplementary Fig. 1).[12] PVT represents a well-known complication during the natural history of patients with liver cirrhosis. Evidence is accumulating that the rebalanced hemostasis system in cirrhosis is prone to hypercoagulability.[13] In patients with cirrhosis, the development of PVT is a milestone in the progression of advanced liver disease and increases the risk of death.[14] The complex hemostatic state in chronic liver disease makes it challenging to manage PVT in cirrhotic patients. The international guidelines provide brief recommendations on many aspects of treating PVT.[2,3,11,15] This review aims to address the essential knowledge for the management of PVT in patients with cirrhosis.

Epidemiology

The prevalence of nontumoral PVT increases with severity of the liver disease, being approximately 1% in patients with compensated cirrhosis and 8-25% in candidates for liver transplantation.[16–21] Different types of diagnostic approaches used in various studies may be responsible for heterogeneity in the reported prevalence, ranging from 0.6–16% using angiography or surgery to 10–25% using ultrasonography.[22]

The incidence of nontumoral PVT in liver cirrhosis has been reported in a limited number of studies. Among patients with virus-related cirrhosis, the cumulative incidence of *de novo* PVT was 12.8%, 20%, and 38.7% at 1, 5, and 8-10 years, respectively.[20] A longitudinal assessment of PVT in 1,243 cirrhotic patients with Doppler ultrasonography revealed that overall 1-, 3- and 5-year cumulative incidence rates of PVT were 4.6%, 8.2%, and 10.7%, respectively.[23] The incidence of nontumoral PVT in liver transplant candidates was reported as 2.1-23.3% per year.[5,24–30] Part of these differences may be due to different transplant policies. Nearly half of the nontumoral PVT was discovered at the time of liver transplantation.[31] Of these, 58.3% was partial, and 41.7% was complete PVT.[24] Recently, a multicenter prospective study PRO-LIVER (PVT Relevance On Liver cirrhosis: Italian Venous thrombotic Events Registry) involving 753 cirrhotic patients assessed

with Doppler ultrasound reported the incidence rate of PVT as 6.05 per 100 patient-years.[32] The incidence of PVT was higher in patients with a history of PVT, indicating that PVT per se carries a risk for recurrence.

Pathophysiology

In general, the predisposing factors of PVT are categorized into local and systemic factors.[33] The portal venous system in cirrhosis represents a local environmental factor particularly prone to thrombus formation by reduced blood flow from portal hypertension and the inflammatory milieu secondary to hepatic injury and gut translocation of bacteria or their by-products. A wide variety of systemic factors are described, including inherited and acquired thrombophilic disorder, extra-abdominal cancer, hormonal therapy, and autoimmune disorder.[34] The risk of a thrombotic event is substantial with the presence of any components of Virchow's triad, including venous stasis, hypercoagulability, and endothelial dysfunction. The role of the three components contributing to PVT development has been extensively investigated in cirrhosis (Fig. 1).

Portal venous stasis secondary to the liver architectural derangement and the splanchnic vasodilatation seems to be the most crucial local factor responsible for the development of PVT in the setting of cirrhosis.[35] Reduced portal flow velocity was identified as an independent factor associated with the development of PVT.[18] This finding was supported by the evidence that a portal flow velocity of less than 15 cm/s at Doppler ultrasonography is the most important risk factor for developing PVT in patients with cirrhosis.[36] The flow in the portal vein becomes further decreased by a "steal effect" due to a spontaneous portosystemic shunt. The presence of collateral vessels, with flow volume of more than 400 mL/min and a flow velocity of more than 10 cm/s, was found to be a significant predictive factor for the occurrence of PVT in cirrhosis.[20]

The decreased levels of most coagulation factors, except factor VIII and von Willebrand factor, are characteristic hallmarks of hemostasis in cirrhosis.[37,38] Also, a parallel reduction of natural anticoagulant factors, such as protein C and S, is observed. However, the contribution of hemostatic alterations to PVT development is challenging to evaluate because these may be due to co-existing liver dysfunction in advanced cirrhosis, rather than a primary disturbance.[15] The conventional coagulation assays reflect only the clot formation time in a plasma environment. The tests do not include thrombomodulin measurement; therefore, they are unsuitable for investigating acquired deficiency of both pro- and anticoagulants, as occurs in cirrhotic patients.[13] Thromboelastography (TEG), known as the viscoelastic test, can offer a global assessment of the hemostatic pathways.[39] This whole blood test allows a dynamic assessment of clot formation and dissolution that might help assess the relative contribution of the coagulation components to overall clot formation and dissolution in cirrhotic patients.[39] It has been solidly demonstrated to be useful in guiding transfusion for gastrointestinal bleeding and high-risk liver invasive procedures.[40,41] Few studies use TEG as the reference method for the function evaluation of multiple clotting components in patients with PVT.[42–44] A recent study evaluated thromboelastographic parameters among cirrhotic patients with variceal bleeding.[44] TEG showed a shortening of initial fibrin formation time in cirrhotic patients with PVT, indicating activation of plasma clotting

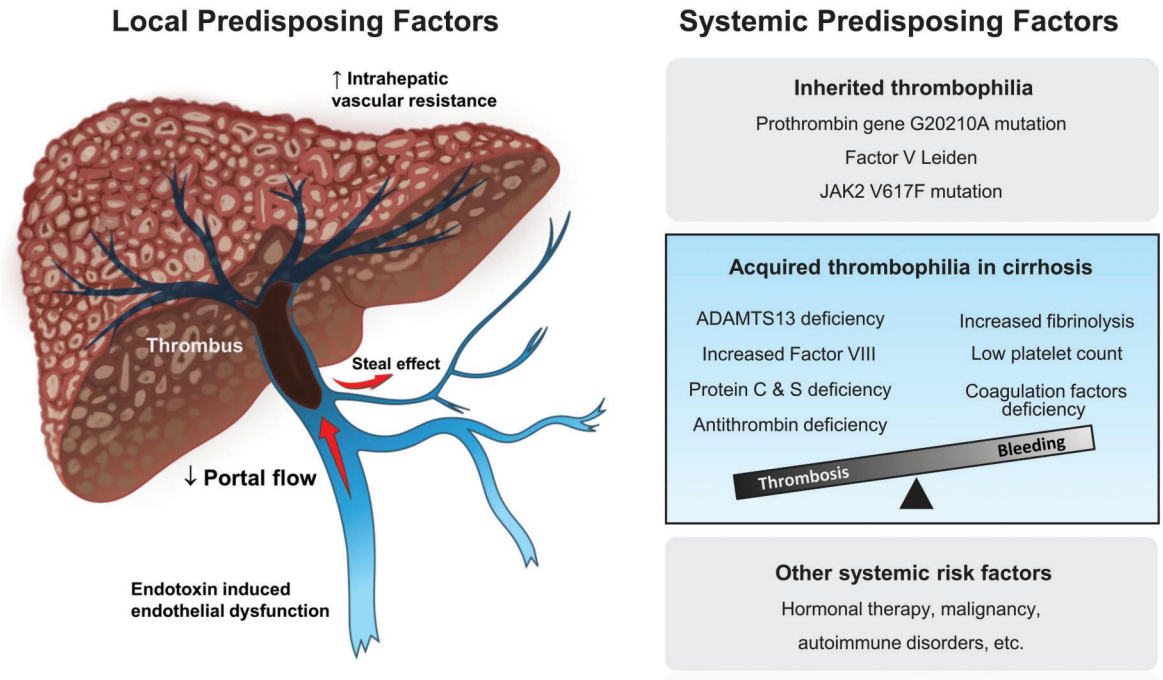

Fig. 1. Pathogenesis of nontumoral portal vein thrombosis (PVT) in liver cirrhosis. Both local and systemic factors have been involved in the development of PVT in patients with cirrhosis. The portal venous system in cirrhosis represents a local predisposing factor prone to thrombus formation by reduced portal blood flow from portal hypertension and increased intrahepatic vascular resistance with the inflammatory milieu secondary to gut-derived bacterial lipopolysaccharide. Cirrhotics have been traditionally considered prone to bleeding due to thrombocytopenia, defects of procoagulant factors, and fibrinolysis. However, there is growing evidence that hypercoagulability is an important part of the hematological spectrum in cirrhosis. The unstable coagulation balance can be tiled toward thrombosis if any acute insult ensues.

factors and inhibiting circulating inhibitors in this population. However, further studies are needed to define the appropriate TEG-guided approach to managing PVT in cirrhotic patients.

An early study revealed the high possibility of 69.5% to detect at least one thrombophilic genotype, including factor V Leiden, 20210A prothrombin gene mutation, and methylene tetrahydrofolate reductase (MTHFR) gene mutation associated with high plasma homocysteine, in cirrhotic patients with PVT.[45] This homeostatic profile was not consistent with a later study demonstrating that thrombophilic mutation was present in only 12% of cirrhotic patients with PVT.[46] Among various inherited thrombophilic disorders, the G20210A prothrombin gene variant is the most common underlying hypercoagulable disorder in cirrhotic patients and carrying an odds ratio (OR) of 5.94 for the development of PVT.[17] Myeloproliferative disorder secondary to the JAK2 V617F mutation was found in a significant proportion of cirrhotic patients with PVT.[47] Other thrombophilic conditions, such as low level of ADAMTS13 (known as von Willebrand factor-cleaving protease) and resistance to the anticoagulant action of thrombomodulin, were observed in cirrhotic patients with PVT.[48,49] The results of studies investigating the role of inherited thrombophilic disorder were summarized in Supplementary Table 2.[17,45–49]

The unstable coagulation balance can be tilted toward bleeding or thrombosis if any acute insult ensues. "Low-grade" endotoxemia may play a pivotal role in activating the clotting system in the portal and systemic circulation and could represent an underlying mechanism for PVT in advanced liver disease. Lipopolysaccharide derived from gut microbiota has been shown to increase the systemic levels of factor VIII via stimulating its release by endothelial cells.[50] Endotoxemia may be a determinant for splanchnic vasodilatation, which is a key factor for portal venous stasis.[51] Together these findings indicate that endotoxemia is a plausible mechanism accounting for the increased risk of thrombosis in the portal circulation of cirrhotic patients.

Risk factors of PVT other than thrombophilia

The unbalanced hemostasis and alteration in splanchnic hemodynamic are more apparent in patients with advanced liver disease. An experimental study showed that factor II, antithrombin, and protein C decreased progressively from Child-Turcotte-Pugh (CTP) class A to C.[38] Furthermore, the decreasing plasma level of protein C and antithrombin was well correlated with an increase in the model for end-stage liver disease (MELD) score.[18] Additionally, cirrhotic patients with higher CTP scores are possibly more likely to have reduced portal vein flow associated with steal syndrome.[20] Data from a recent large prospective study showed that the severity of liver disease at baseline was a significant predisposing factor associated with the development of PVT.[23] Moreover, CTP class C was a significant predictor of mortality (hazard ratio [HR] 11.5, 95% confidence interval [CI]: 6.95-18.9).[32]

The etiology of liver disease also influences the occurrence of PVT. According to a study of 885 cirrhotic patients who underwent liver transplantation, PVT was found in 3.6% of patients with primary sclerosing cholangitis, 8% with primary biliary cholangitis, 16% with alcoholic and hepatitis B virus-related cirrhosis, and mounting to 35% in patients with hepatocellular carcinoma (HCC).[5] Emerging information from large transplant registries suggests that nonalcoholic steatohepatitis may be an independent risk factor for the development of nontumoral PVT in patients with decompensated cirrhosis.[29,30] A recent cohort in the United States also showed that nonalcoholic steatohepatitis-related liver cirrhosis was significantly associated with the development of PVT (HR of 5.34, 95% CI: 1.53-18.7).[36]

Intraabdominal surgery (hepatectomy, shunt surgery) and local regional therapy for HCC have been reported as determinants of PVT, due to venous injury and disturbance of blood flow after intervention.[33]

Clinical manifestations of PVT in patients with cirrhosis

The clinical presentation of PVT is variable. PVT in patients with cirrhosis is frequently asymptomatic due to splanchnic decompression through an existing spontaneous portosystemic shunt. In the completely acute occlusion of the portal vein, PVT may develop acute abdominal pain, which raises a concern of the extension to the SMV and mesenteric arches, causing intestinal ischemia and, ultimately, bowel infarction. In a previously stable cirrhotic patient, new onset of symptoms related to worsening portal hypertension, such as the development of variceal bleeding and refractory ascites, may suggest the development of PVT and should be thoroughly evaluated.

After a few weeks, the obstructed part of the portal vein is bypassed through the formation of venous collaterals that bring blood — in a hepatopetal manner — around the area of obstruction, known as portal cavernoma. The network of collateral portal veins characterizes chronic PVT. In most cirrhotic patients, chronic PVT is asymptomatic and discovered incidentally during abdominal imaging for HCC surveillance. Patients with chronic PVT frequently have esophageal or gastric varices, and the most common clinical presentation is gastrointestinal bleeding.[17] Patients may have symptoms related to cirrhosis or other conditions, such as HCC, that predispose the development of PVT. Portal cholangiopathy, which compresses the large bile ducts by the paracholedochal collaterals, is also common in cirrhotic patients with long-standing chronic PVT.[52] Some patients with portal cholangiopathy develop biliary complications, including pruritus, obstructive jaundice, and cholangitis.[53,54]

Natural history of PVT in cirrhosis

Spontaneous resolution of PVT has been described from 45% to 70% of cases in different cohorts.[29,55,56] The spontaneous recanalization was reported to occur after a median follow-up of 5 months.[1] To date, data regarding predictors of spontaneous recanalization is limited. In cohort studies evaluating the natural course of PVT, spontaneous recanalization was not associated with thrombus age, degree of PVT, location of thrombosis, and portal cavernoma.[55,57] Only a cohort study by Maruyama *et al.*[20] demonstrated that the diameter and flow volume in the largest collateral vessel at diagnosis of PVT was inversely associated with spontaneous improvement of PVT; however, the data require confirmation.

Recurrence of PVT after spontaneous recanalization has been reported in some cohorts, ranging from 21.3% during the mean follow-up of 47 months in the prospective cohort[23] to 45% over an average follow-up of 63.3 months in the retrospective study.[20] Hence, continuous monitoring of portal vein patency after spontaneous recanalization should be maintained at regular intervals.

Clinical impact of PVT in cirrhosis

The impact of PVT on the natural course of cirrhosis is still debatable. PVT is generally thought to have a negative effect on prognosis because of a further increase in portal hypertension and worsening liver function caused by decreased liver perfusion and parenchymal atrophy. In particular, intrahepatic microvascular thrombosis secondary to liver necroinflammation may lead to liver ischemia, cell death, loss of functioning hepatic mass, and enhanced fibrogenesis through a process termed as "parenchymal extinction".[58] This hypothesis has been supported by evidence that has indicated that primary prophylaxis of PVT with low dose low molecular weight heparin (LMWH) was effective in reducing mortality and risk of hepatic decompensation in patients with advanced cirrhosis.[59] A recent meta-analysis involving 2436 cirrhotic patients demonstrated a significant association of PVT with both mortality and ascitic decompensation; it did not, however, evaluate the pooled effect of PVT on other features of hepatic decompensation, such as variceal bleeding.[14] A prospective study by D'Amico *et al.*[60] showed a more than 3-fold higher risk of failure to control active variceal bleeding in cirrhotic patients with PVT, irrespective of treatment modality. Subsequently, a retrospective analysis by Dell'Era *et al.*[61] highlighted that PVT was associated with a longer time to eradicate esophageal varies. Contrarily, a large prospective multicenter study following the incidence of PVT in cirrhosis overtime did not find a prognostic role of PVT, but mainly partial PVT on mortality and hepatic decompensation.[23] Furthermore, Luca *et al.*[55] found that spontaneous improvement of PVT did not provide any benefit in terms of the development of cirrhotic complications and survival. Based on these findings, it has been speculated that the progression or regression of partial PVT has no impact on the natural history of cirrhotic patients. However, evidence from a systematic review of the literature concluded that the presence of PVT might be associated with the long-term mortality in nontransplant patients with liver cirrhosis but not with the short-term mortality.[62] Considering heterogeneity in data reporting and lengths of follow-up among studies, the reproducibility of these findings remains to be confirmed.

Historically, PVT poses relevant challenges during liver transplantation due to an increase in operative technical complexity, transfusion requirements and re-interventions, and lowers it the survival rate.[63] According to the results of many transplant centers, the survival rates in the transplant setting mainly depend on PVT type and surgical technique.[64,65] In particular, the presence of PVT, especially complete occlusion, negatively affected the 1-year survival of liver transplant recipients with no impact on 5-year survival.[64] Furthermore, several alternative surgical techniques, other than conventional portal vein end-to-end anastomosis, were found to be associated with low survival rates.[65,66] In an analysis of the registry of transplant recipients in the USA during 2001-2007, PVT was found to be associated with significantly higher posttransplant mortality but to not affect waiting list mortality.[24,28,67] This finding was further extended by a recent analysis of the USA's transplant registry, which demonstrated that preexisting PVT significantly increased liver allograft failure and risk of death after liver transplant at 90 days, 1 year, 3 years, and 5 years.[27]

Diagnosis

The diagnosis of PVT includes abdominal imaging to demonstrate portal vein occlusion. As such, patients should undergo an evaluation to identify conditions that may predispose to PVT formation. In acute PVT, there will be evidence of portal vein occlusion without radiographic signs suggestive of chronic PVT, such as cavernous portal transformation. A Doppler ultrasound is a reasonable initial approach. The characteristic ultrasound findings are the presence of solid echo within the portal vein or branches combined with the absence of portal flow (Fig. 2A-B). The ultrasound has a reported overall sensitivity of 89-93% and specificity of 92-99% for the detection of PVT.[68,69] However, it is not sensitive for determining the extent of thrombus, especially in the SMV.[70]

If the ultrasound suggests PVT, an abdominal computed tomography (CT) scan can then be obtained. The classic feature of acute PVT is the presence of hyperattenuating material in the portal vein in a CT scan without contrast. Imaging after intravenous contrast injection may reveal a lack of luminal enhancement, increased hepatic enhancement in the arterial phase, and decreased hepatic enhancement in the portal phase.[71] However, it is observed when the imaging study is done within 30 days after the onset of symptoms.[72] Chronic thrombosis is characterized by the presence of portal cavernoma, reportedly seen as soon as 6 days after portal vein occlusion (Fig. 2C-D).[70] However, chronic PVT may be difficult to define accurately because enlarged collateral vessels may preexist as a consequence of cirrhosis.[73]

Contrast-enhanced CT and magnetic resonance imaging (MRI) are excellent modalities to evaluate the extension of thrombus and may detect predisposing conditions or intestinal ischemia. CT angiography has a reported 90% sensitivity and 99% specificity for the diagnosis of PVT, according to operative findings being used as a reference.[69] MRI has 100% sensitivity and 98% specificity for detecting PVT.[74,75] Overall, various imaging modalities have higher sensitivity in detecting complete PVT when compared to partial PVT (65% and 39%, respectively) with comparable specificity (99% and 97%, respectively).[76]

A new probability assessment tool for the development or presence of PVT in patients with cirrhosis was recently proposed.[12] Three major criteria include CTP class B or C cirrhosis, prior history of resolved PVT, and presence of thrombophilic disorder. In contrast, seven minor criteria are the evidence of portosystemic shunt, active hepatocellular malignancy, history of systemic venous thrombosis or abortion, recent abdominal intervention, reduced portal flow velocity <15 cm/s, and clinical presentation with acute abdomen or worsening of portal hypertension in cirrhotic patients. The presence of two major, or one major and two minor or four minor criteria indicates a high probability. However, further validation from a prospective study is needed.

Accurate differentiation between nontumoral and malignant PVT in cirrhotic patients is of paramount importance. Visualized thrombus in the portal vein is considered nontumoral PVT when all of the following characteristics are present: lack of enhancement of endoluminal material during the arterial phase of contrast administration, absence of mass forming features, and absence of wall disruption of portal vein or tumor encroaching on the portal vein.[77] The presence of neovascularization or main portal vein diameter >23 mm showed a sensitivity of 86% and specificity of 100%

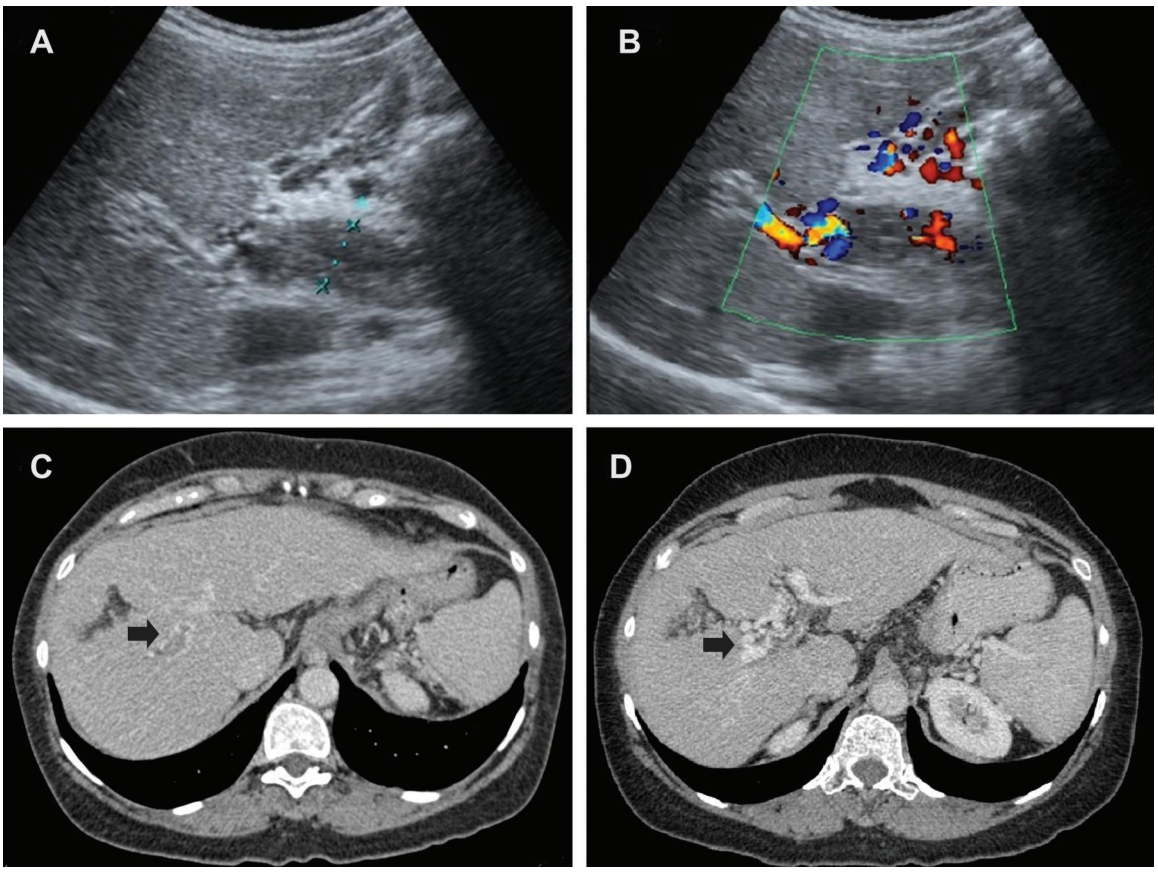

Fig. 2. Imaging findings of nontumoral portal vein thrombosis (PVT) in liver cirrhosis. (A) Ultrasound of the abdomen shows an echogenic material within the dilated portal vein, indicating PVT. (B) Doppler ultrasound of the abdomen shows decreased color flow within the main portal vein and demonstrates color-filled dilated collateral vessels around the porta hepatis consistent with cavernous transformation. (C) Computed tomography of the abdomen on portal venous phase shows a filling defect in the right branch of the portal vein (arrow), indicating thrombus. (D) Contrast-enhanced computed tomography depicts cavernous transformation (arrow) following portal venous thrombosis.

for the diagnosis of malignant PVT.[78] If uncertainty persists, a CT-guided biopsy for histological examination may be required.

Management

The optimal management of PVT in the setting of liver cirrhosis regarding the appropriate strategies, the magnitude of PVT (occlusive versus nonocclusive, acute versus chronic), type and timing of anticoagulation, and the role of a transjugular intrahepatic portal shunt (TIPS) are lacking. In 2009, the American Association for the Study of Liver Diseases (AASLD) published guidelines for the management of PVT in cirrhosis. They did not provide specific anticoagulation guidance for PVT but recommended clinical decisions be made on a case-by-case basis depending on the presence of thrombophilic conditions, symptoms, or extension to the SMV.[2] The European Association for the Study of the Liver (EASL) published guidelines on vascular disorders of the liver in 2016 and recommended evaluating for the presence of at-risk varices and initiating therapy with band ligation or nonselective β blocker before initiation of anticoagulation treatment for PVT in cirrhosis.[3] According to the EASL guideline, anticoagulation treatment is advised for at least 6 months in cirrhotic patients with PVT and should be continued for some months after portal vein repermeation or until transplant in candidates for liver transplantation.[3] Like AASLD and EASL guidelines, the Baveno VI consensus statement does not make recommendations on the choice of anticoagulation therapy for PVT due to limited data.[11] The indication, contraindication, and currently available therapeutic agents are summarized in Supplementary Table 3.[1–3,11,25,46,79–93]

Anticoagulation

Anticoagulation is the primary management of acute PVT, with supporting evidence of high efficacy and a favorable safety profile (Table 1). The objective is to achieve recanalization of the portal vein and prevent the extension of the thrombus to decrease the notorious consequences of portal hypertension and mesenteric ischemia and allow conventional end-to-end portal vein anastomosis to be technically possible in transplant candidates.[35] Currently, available guidelines recommend that anticoagulation should be considered in liver transplantation candidates with thrombosis of the main portal vein trunk or progressive PVT.[2,3,11] For noncandidates to liver transplantation, no recommendation regarding anticoagulation treatment has been made. However, anticoagulation could be considered in selected cases with symptomatic acute occlusive PVT, the extension to the SMV, or known strong prothrombophilic conditions.[11]

Clinical data suggest that anticoagulation and recanalization of the portal vein are associated with reduced portal hypertension-related events and improved survival.[80] Anticoagulation therapy in cirrhotic patients with PVT has shown the variability in the resolution of thrombosis. The degree of PVT at diagnosis does not predict the likelihood of response to anticoagulation,[81,94] but extensive PVT before treatment decreases the likelihood of recanalization.[46,57] The successful management of PVT in cirrhosis is strongly associated with early diagnosis and initiation of anticoagulation within the first 6 months.[46] The presence of portal cavernoma indicates a long-standing PVT that is unlikely to recanalize completely with anticoagulation. A relatively low recanalization rate of complete PVT after anticoagulation therapy suggests its limited usefulness in cirrhotic patients with complete PVT. Anticoagulants evaluated in these studies included vitamin K antagonist (VKA), LMWH, and direct oral anticoagulant (DOAC).[1,25,46,57,79–86,94–97]

In the acute setting of PVT, LMWH is the preferred agent, typically followed by VKA. LMWH has the advantage of a fixed-dose regimen without laboratory monitoring; however, daily subcutaneous administration may reduce compliance and require dose adjustment according to renal function that is relatively fragile in patients with advanced liver cirrhosis. VKA is generally considered for long-term anticoagulation therapy, but maintaining the international normalized ratio in the therapeutic range throughout treatment and interference with the MELD score makes its use challenging. The risk and benefits of treatment with anticoagulants for PVT in cirrhosis have been debated. Compelling evidence from two meta-analyses showed that traditional anticoagulants significantly increased the rate of PVT recanalization (71% vs. 42%) with the OR of 4.16 (95% CI: 1.88–9.20) and lower the rate of PVT progression (9% vs. 33%) compared with no anticoagulation therapy.[98,99] Both LMWH and warfarin were effective in preventing the progression of thrombosis. However, LMWH, not warfarin, was significantly associated with complete PVT resolution.[98] Recurrence after discontinuation of anticoagulation therapy following clot resolution was found to be up to 38%.[79] The most feared consequence of anticoagulation is bleeding. However, major and minor bleeding risk related to anticoagulation therapy for PVT in cirrhosis ranges from 3.3% to 11%, which is not different from that of no treatment.[98,99]

DOACs are more widely used in clinical practice for treatment and prevention of venous thromboembolic events due to an acceptable safety profile and availability of antidotes without the need for drug monitoring. Studies examining the pharmacodynamics of DOAC in patients with cirrhosis showed that the anticoagulant effect might be altered in advanced cirrhosis.[100,101] Data regarding the efficacy and safety of DOACs for treatment of PVT in cirrhosis are emerging but remain limited, as shown in Table 1.[85,86,96,97] Nagaoki *et al.*[86] randomized 50 cirrhotic patients with variable CTP scores and PVT to receive either warfarin or edoxaban for 6 months after 2 weeks of daparinoid sodium therapy. They reported a significantly higher rate of complete resolution of PVT with the slower progression of PVT in patients receiving edoxaban and no difference in adverse effects among both treatment groups.

Furthermore, Hanafy *et al.*[85] reported a randomized controlled trial of rivaroxaban versus warfarin for the management of acute PVT in 80 patients with hepatitis C cirrhosis who had undergone splenectomy due to symptomatic hypersplenism. Patients receiving rivaroxaban achieved a higher frequency of recanalization of the portal vein with better short-term survival rates than patients receiving warfarin. Complications such as major bleeding, abnormal liver functions, or death did not occur in the rivaroxaban group, while the warfarin group experienced ascites, gastrointestinal bleeding, encephalopathy, and death. Although the results are promising, rivaroxaban is not the ideal DOAC for patients with cirrhosis due to higher reported rates of hepatotoxicity with rivaroxaban than other DOACs.[102] Given the small sample size and heterogeneous population of each study, the safety and efficacy of DOACs for PVT in patients with cirrhosis need to be further ascertained.

Transjugular portosystemic shunt

The advantages of TIPS for the treatment of PVT in patients with cirrhosis are to recanalize the thrombosed portal vein using endovascular techniques effectively and simultaneously resolve symptomatic portal hypertension and prevent thrombus recurrence or extension by the creation of a portosystemic shunt.[103] Nowadays, TIPS represents an effective adjunctive therapy for PVT if anticoagulation is ineffective or inappropriate. Transplenic TIPS placement is feasible in patients with complete obliterative PVT to recanalize the portal vein in anticipation of transplantation.[90,93] The technical success rate for TIPS is relatively high in experienced centers.[9,90–93] In a recent meta-analysis of 13 studies including 399 patients (92% cirrhosis; PVT: complete 46%, chronic 87%, portal cavernoma 15%), TIPS was technically feasible in 95% of cases, carried a moderate risk of significant complication (10%), and was highly effective in achieving sustained recanalization of PVT (79%), even in cases with the cavernous transformation.[89] This result means that TIPS can be effective in maintaining long-term portal vein patency, allowing avoidance of anticoagulation therapy. Regarding the clinical outcome of this procedure in the management of PVT, the pooled 12-month survival rate was 89%. This finding supports previous reports suggesting that TIPS likely confers survival benefit in patients with advanced liver cirrhosis.[104–106] A retrospective analysis of 57 cirrhotic patients with nontumoral PVT undergoing TIPS and subsequent systemic anticoagulation showed that the independent factors associated with technical success were SMV involvement (OR: 42.8; 95% CI: 1.43-1282) and presence of portal cavernoma (OR: 37.5; 95% CI: 1.96-720).[92] Therefore, careful consideration is needed, especially in patients with these negative predictive factors. Given the heterogeneity of published data, adequately powered clinical trials comparing TIPS to anticoagulation are required to guide clinical decision-making in this field.

Challenges of liver transplantation in cirrhotic patients with nontumoral PVT

Currently, the presence of PVT is no longer an absolute contraindication for liver transplantation. The first successful liver transplantation in a patient with PVT was reported in 1985.[107] Since then, the advancement of surgical techniques has allowed end-to-end anastomosis to be performed in the majority of cases.[26] Physiological portal inflow is defined when splanchnic venous blood from splanchnic vessels or large portosystemic shunt can be redirected to the liver graft.[108] Previous studies showed no significant differences in survival between patients with complete and partial PVT, given that physiological portal flow was established.[65] However, liver transplantation in patients with extensive thrombosis remains technically challenging.[31] A recent

Table 1. Efficacy and safety of anticoagulation therapy for nontumoral portal vein thrombosis in patients with cirrhosis

Author, year	*n*	Characteristics of nontumoral PVT	Type of anticoagulation	Duration of follow-up	Recanalization and progression of thrombosis	Risk of anticoagulation
Francoz *et al.*, 2005[25]	19	18 partial PVT 1 complete PVT	LMWH followed by VKA (*n* = 19)	Mean 8.1 months	42% complete 53% unchanged 5% progression	1 upper gastrointestinal bleeding (not related to anticoagulation therapy)
Amitrano *et al.*, 2010[84]	28	23 partial PVT 5 complete PVT	Enoxaparin (*n* = 28)	Median 6.5 months (range 1-17)	33% after 6-month 75% after 12-month 2 of 5 nonresponders had progression	2 mild anemia form portal hypertensive gastropathy (not related to anticoagulation therapy)
Senzolo *et al.*, 2012[83]	33	24 partial PVT 11 complete PVT	Nadroparin (*n* = 33)	Mean 21.6±8.5 months	36% complete 27% partial 21% unchanged 15% progression	1 epistaxis, 1 hematuria, 1 symptomatic cerebral hemorrhage and 1 heparin-induced thrombocytopenia
Delgado *et al.*, 2012[79]	55	41 partial PVT 14 complete PVT	LMWH (*n* = 47) VKA (*n* = 8)	Median 6.8 months (range 1-56)	45% complete 15% partial 40% unchanged	5 non-variceal bleeding 6 variceal bleeding
Werner *et al.*, 2013[81]	28	All transplant candidates with PVT	VKA (*n* = 28)	Mean 302 days (range 54-1,213)	39% complete 43% partial 18% unchanged	1 significant vaginal bleeding
Cui *et al.*, 2015[94]	34	30 partial PVT 4 complete PVT	LMWH 1.5 mg/kg daily (*n* = 34)	6 months	23.5% complete 53% partial 23.5% unchanged	8 non-variceal bleeding (injection site, epistaxis or hematuria)
Cui *et al.*, 2015[94]	31	24 partial PVT 7 complete PVT	LMWH 1 mg/kg bid (*n* = 31)	6 months	29% complete 52% partial 19% unchanged	2 nonvariceal bleeding (injection site, epistaxis or hematuria)
Chen *et al.*, 2016[57]	30	10 partial PVT 20 complete PVT	VKA (*n* = 30)	Median 7.6 months	50% complete/partial 13% stable 10% progression	4 gastrointestinal bleeding 4 epistaxis or gingival bleeding
Kwon *et al.*, 2018[95]	91	77 partial PVT 14 complete PVT	Dalteparin (*n* = 82) enoxaparin (*n* = 9)	Median 5.7 months (range 1-34.6)	22% complete 40% partial 31% unchanged 4.4% progression	13 clinically relevant bleeding 2 fatal bleeding
Rodriguez-Castro *et al.*, 2019[46]	65	47 partial PVT 18 complete PVT	Enoxaparin (*n* = 65)	Median 4.4 months	43% complete 29% partial 28% unchanged	1 portal hypertensive gastropathy 1 intracranial bleeding
La Mura *et al.*, 2018[80]	63	48 partial PVT 15 complete PVT	VKA (*n* = 63)	Mean 23.3±16.2 months	49% complete 21% partial 30% unchanged	24% major bleeding event 29% minor bleeding event

(*continued*)

Table 1. (*continued*)

Author, year	*n*	Characteristics of nontumoral PVT	Type of anticoagulation	Duration of follow-up	Recanalization and progression of thrombosis	Risk of anticoagulation
Pettinari *et al.*, 2019[1]	81	51 partial PVT 8 complete PVT 19 extension to superior mesenteric vein 4 cavernoma	LMWH (*n* = 56) Fondaparinox (*n* = 15) VKA (*n* =10)	Mean 13.4±14.0 months	38.3% complete	21.8% bleeding complication (only 4 related to anticoagulation therapy)
Noronha Ferreira *et al.*, 2019[82]	37	66.3% portal vein trunk 33.7% extension to splenic or superior mesenteric veins 12.5% cavernoma	VKA (*n* = 22) LMWH (*n* = 15)	Mean 20.6±11.9 months	51.4% complete/partial	10.8% bleeding complication
Nagaoki *et al.*, 2018[86]	50	75% portal vein trunk 15% intrahepatic portal branch 10% superior mesenteric or splenic vein	Danaparoid sodium for 2 weeks followed by edoxaban (*n* = 20) or warfarin (*n* = 30)	6 months	Edoxaban had more complete resolution (70% vs. 20%) and less progression (5% vs. 47%) compared with warfarin	3 gastrointestinal bleeding with edoxaban 2 gastrointestinal bleeding with warfarin
Scheiner *et al.*, 2018[96]	10	53% partial PVT 47% complete PVT	Edoxaban (*n* = 4) Apixaban (*n* = 3) Rivaroxaban (*n* = 2) Dabigatran (*n* = 1)	Median 12.0 months (range 8.7-29.0)	20% with resolution of PVT 80% with stable of cavernoma	1 portal hypertensive gastropathy bleeding
Janczak *et al.*, 2018[97]	26	Splanchnic vein thrombosis	Rivaroxaban or apixaban (*n* = 26) Enoxaparin (*n* = 23)	Mean follow-up 0.9 year (range 0.01-4.18)	No difference in the recurrence rate of thrombosis between groups	No difference in bleeding rates between DOAC and LMW
Hanafy *et al.*, 2019[85]	80	Acute PVT after splenectomy or portal pyemia	Enoxaparin for 3 days followed by rivaroxaban (*n* = 40) or warfarin (*n* = 40)	Mean 20.4±2.2 months	85% recanalization with rivaroxaban 45% recanalization with warfarin	8 deaths with warfarin No death with rivaroxaban

Abbreviations: LMWH, low molecular weight heparin; PVT, portal vein thrombosis; VKA, vitamin K antagonists.

meta-analysis showed that 30-day mortality was higher in recipients with complete PVT than in those with partial thrombosis.[109] Of note, the survival rate is decreased in those with nonphysiologic portal anastomosis.[35,65] In patients with grade I-III PVT, according to Yerdel classification,[6] the thrombus was removed by eversion thrombectomy or thromboendovenectomy (removal of clot and attached intimal layer). If the portal flow is insufficient, various surgical options can be considered to increase the inflow, including ligation of the portosystemic collaterals, portal vein arterialization, interposition graft between patent splanchnic vessels, and portal vein or a jump graft from SMV to donor portal vein.[31] In grade IV PVT with the presence of portosystemic shunt, using systemic veins as the inflow vessels including reno-portal anastomosis, left gastric vein to portal vein anastomosis and pericholedochal varix to portal vein anastomosis allows restoration of physiologic portal hemodynamic.[108] In the absence of portosystemic collaterals, surgical alternatives are reno-portal anastomosis, cavoportal hemitransposition, and multivisceral transplantation.[108] Cautiously, these nonphysiologic anastomoses, except reno-portal anastomosis in patients with patent surgical splenorenal shunt, do not reverse portal hypertension.[35] Multivisceral transplantation, including liver and small bowel, was theoretically the best option to restore physiologic portal flow and reverse portal hypertension in a patient with extensive PVT. However, the experience is very limited. The initial report of 25 patients with grade IV PVT who underwent multivisceral transplantation showed the relatively favorable 1-, 3- and 5-survival rates of 80%, 72%, and 72%, respectively.[110]

PVT is not considered a MELD exception; therefore, patients with PVT do not receive additional points for organ allocation.[35] However, cirrhotic patients with PVT should be transplanted before reaching a MELD score of 30.[111] The living donor liver transplantation in patients with PVT poses characteristic obstacles. The restricted availability of a vein graft is the main technical challenge. In addition, the safety of the donor is of paramount importance. Contrarily, considering living donor liver transplantation in patients with grade I-III PVT may be reasonable in highly experienced centers.[112,113]

After liver transplantation, the hemodynamic alteration of splanchnic circulation was restored, resulting in a low rate of rethrombosis (less than 5%); therefore, long term anticoagulation is not justified.[35,112] However, the consideration of systemic anticoagulation therapy patients with extensive thrombosis and nonphysiologic reconstruction who carry a high risk of rethrombosis needs to be done on a case-by-case basis.

Potential algorithm for the management of PVT in cirrhosis

Based on existing data and international society recommendations, we propose a potential algorithm for the management of PVT in liver cirrhosis (Fig. 3). First, patients with cirrhosis awaiting liver transplantation should be screened for PVT at least every 6 months with Doppler ultrasound. Detection of PVT before transplantation would help in surgical planning and allow potential preoperative therapy to recanalize the portal vein. It seems logical that cirrhotic patients with risk factors for PVT (especially those with portal flow velocity <15 cm/s or decompensated cirrhosis) should be screened for the development of PVT every 6 months. Second, patients with cirrhosis diagnosed with PVT by Doppler ultrasound should be assessed with contrast-enhanced imaging to confirm and stage

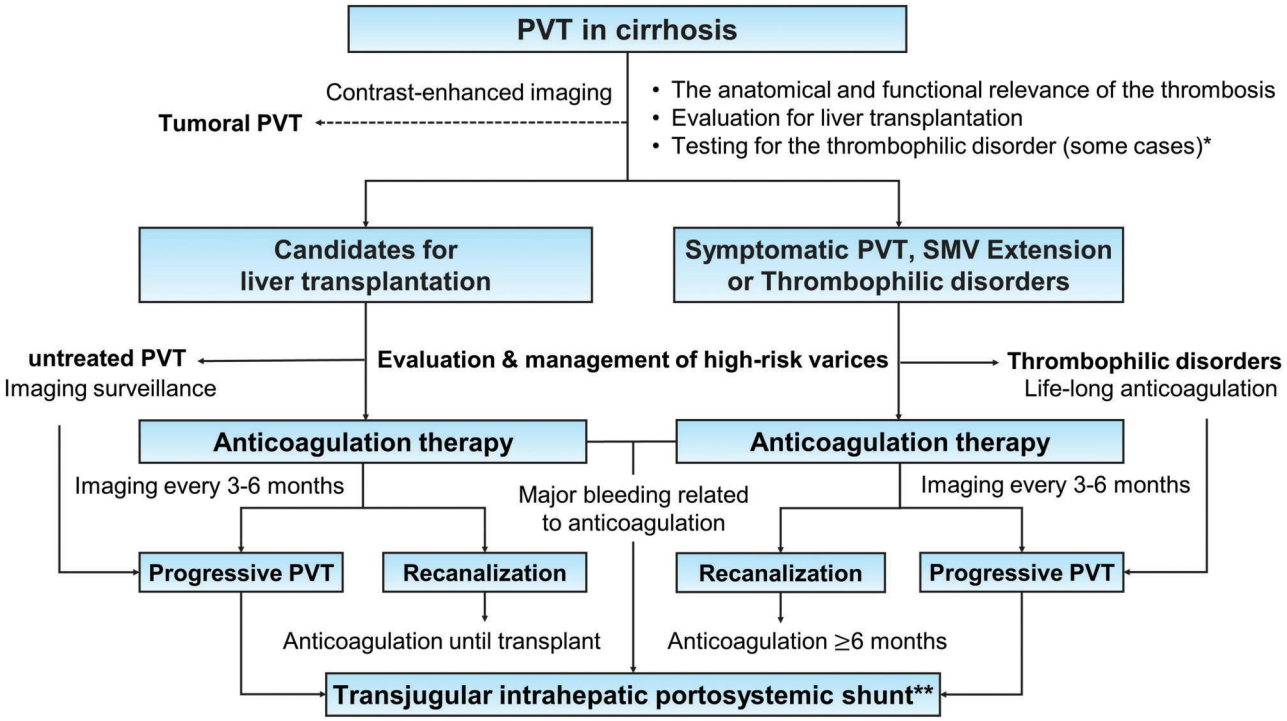

Fig. 3. Potential algorithm for the management of nontumoral portal vein thrombosis (PVT) in liver cirrhosis. *Lupus anticoagulant, anticardiolipin, anti-β2-glycoprotein 1 antibody, factor V Leiden, 20210A prothrombin gene mutation, methylene tetrahydrofolate reductase gene mutation, JAK2 V617F mutation and work-up for paroxysmal nocturnal hemoglobinuria. **Limited technical feasibility in low-volume center, superior mesenteric vein (SMV) thrombosis and portal cavernoma.

the extent of nontumoral thrombosis. Third, evaluation for liver transplantation should be considered once cirrhotic patients have experienced an index complication, such as ascites, hepatic encephalopathy, or variceal hemorrhage or hepatocellular dysfunction resulting in a MELD score ≥15. Fourth, testing for acquired and inherited thrombophilic disorders can be considered in cirrhotic patients with PVT on an individual basis, but universal screening is not currently recommended. Fifth, the assessment of bleeding risk and the benefit of anticoagulation therapy is crucial. Patients should undergo an upper endoscopy to assess for portal hypertension or other mucosal lesions. Subsequent prophylaxis with endoscopic band ligation or pharmacotherapy with nonselective β blockers should be utilized for high-risk varices. Sixth, anticoagulation therapy should be considered for liver transplantation candidates, patients with symptomatic acute PVT, or progression of PVT or extension into the SMV. In cirrhotic patients with nonocclusive thrombosis of the trunk or a single branch of portal vein left untreated, imaging surveillance should be carried out every 3-6 months to evaluate for thrombosis progression. Seventh, the selection of the type of anticoagulation should be individualized. The limitation and benefits of each medication (LMWH, VKA, or DOACs) should be reviewed with the patients. Eighth, the optimal duration of anticoagulation may be at least 6 months to achieve the successful recanalization of the portal vein. In cases of underlying hypercoagulability or liver transplantation candidates, indefinite anticoagulation or treatment until liver transplantation may be considered. If anticoagulation treatment is stopped, close follow-up with abdominal imaging every 3-6 months is advised to evaluate for PVT recurrence. Lastly, TIPS should be considered for the treatment of PVT in patients with cirrhosis requiring treatment for clinically significant portal hypertension, patients with symptomatic and complete occlusion of the main portal vein, or those with progressive PVT despite adequate anticoagulation.

Conclusions

Nontumoral PVT is a challenging consequence of cirrhosis. Existing data have greatly expanded our knowledge of pathophysiology, natural history, and treatment of PVT in cirrhosis. Several case series have shown the efficacy and safety of the anticoagulation treatment and TIPS for the management of PVT in cirrhosis. However, research remains limited to mainly retrospective cohort studies so that any firm conclusions for clinical practice cannot be achieved. The potential risk and benefit of various treatment modalities should be evaluated in prospective and randomized trials. Treatment for nontumoral PVT in liver cirrhosis must be decided on a case-by-case basis.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Drafted the first version of the manuscript (MR), edited and revised the manuscript, and contributed to conceptual development of the study (PC).

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Review Article

Elastography for Longitudinal Assessment of Liver Fibrosis after Antiviral Therapy: A Review

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Abstract

Chronic hepatitis B or C viral infection is a common cause of liver cirrhosis and hepatocellular carcinoma. Fibrosis regression can be achieved after long-term antiviral therapy (AVT). Monitoring of dynamic changes in liver fibrosis after treatment is essential for establishing prognosis and formulation of a follow-up surveillance program. Routine surveillance of fibrosis after AVT by liver biopsy, the gold standard for fibrosis assessment, is hindered by its invasive nature, sampling error and observer variability. Elastography is a noninvasive quantitative alternative that has been widely used and validated for the staging of liver fibrosis prior to treatment. Recently, increasing research interest has been focused on the role of elastography in longitudinal assessment of liver fibrosis after AVT. In this review, the basic principles, acquisition techniques, diagnostic performances, and strengths and limitations of ultrasound elastography and magnetic resonance elastography are presented. Emerging evidence regarding the use of elastography techniques for the monitoring of liver fibrosis after AVT is summarized. Current challenges and future directions are also discussed, designed to optimize the application of these techniques in clinical practice.

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Introduction

Liver fibrosis is a progressive disease that can evolve into cirrhosis, ultimately resulting in liver failure or the development of hepatocellular carcinoma.[1,2] The main etiologies of liver fibrosis include chronic hepatitis B or C (CHB or CHC) viral infection, alcoholic steatohepatitis, nonalcoholic steatohepatitis, and autoimmune and biliary diseases.[3] Increasing evidence indicates that liver fibrosis, even at the cirrhotic stage, is reversible if the major liver diseases and stimulus of liver injury are eliminated.[4,5] This has been shown in both CHB and CHC populations who underwent long-term antiviral therapy (AVT) with virus suppression or clearance,[6–9] and in nonalcoholic steatohepatitis patients after lifestyle changes, predominantly loss of weight.[10] The beneficial effects, particularly of cirrhosis regression, can partly reduce the increased risk of liver-related events, yet, notably, may not eliminate the high risk of hepatocellular carcinoma development.[9,11] Hence, monitoring of liver fibrosis status after treatment is of clinical significance for establishing prognosis and formulating a follow-up surveillance program.

To date, liver biopsy has been the gold standard for fibrosis assessment. However, routine assessment and surveillance of fibrosis after treatment by liver biopsy are hampered by its invasive nature, sampling error, and observer variability.[12,13] Therefore, noninvasive alternatives to liver biopsy are being developed, such as serum markers and imaging examinations, among which elastography has emerged as the leading candidate in clinical development. Quantitative elastography modalities include ultrasound (US) elastography and magnetic resonance elastography (MRE); the US elastography can be further divided into vibration controlled transient elastography (VCTE), point shear-wave elastography (pSWE) and two-dimensional shear-wave elastography (2D SWE).[14,15] Assessment of fibrosis stage prior to treatment by elastography techniques has been a common practice in the clinic setting. More recently, increasing research attention has been put on the role of elastography in longitudinal assessment of liver fibrosis in patients who underwent AVT.

Here, the authors review the current knowledge on US elastography and MRE in terms of their basic principles, acquisition techniques, diagnostic performances, and strengths and weaknesses, highlighting the utility of elastography techniques in monitoring of liver fibrosis among CHB and CHC populations who received AVT and discussing current challenges and future directions to explore the optimization of elastography techniques in practice.

Basic concepts of elastography

Elastography provides a quantitative method to assess liver stiffness, which is a mechanical property of tissue related to the degree of liver fibrosis. In general, liver stiffness values increase with higher fibrosis stages.[16] Hence, liver stiffness is regarded as an "indirect" marker of fibrosis. Notably, despite hepatic fibrosis being the predominant element influencing stiffness of the liver, there are numerous factors that may

Keywords: Liver fibrosis; Elastography; Chronic hepatitis B; Chronic hepatitis C; Antiviral therapy.

Abbreviations: ALT, alanine aminotransferase; ARFI, acoustic radiation force impulse; AST, aspartate aminotransferase; AUCs, area under curves; AVT, antiviral therapy; CHB, chronic hepatitis B; CHC, chronic hepatitis C; FIB-4, fibrosis-4 score; GRE, gradient-recalled echo-based; ICC, intraclass correlation coefficient; LSM, liver stiffness measurement; MR, magnetic resonance; MRE, magnetic resonance elastography; pSWE, point shear-wave elastography; ROI, region of interest; SE-EPI, spin-echo-based echo-planar imaging; SVR, sustained virological response; 2D SWE, two-dimensional shear-wave elastography; US, ultrasound; VCTE, vibration controlled transient elastography.

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exert an impact on liver stiffness, e.g. inflammation, blood flow, and portal pressure.[17] Therefore, interpretation of liver stiffness measurement (LSM) should take into account potential confounding factors. A comparison of quantitative elastography techniques is presented in Table 1.

Ultrasound elastography

VCTE

Principles

One-dimensional VCTE (Fibroscan; Echosens), introduced in France in 2003, is the first Food and Drug Administration-approved elastography technique. For VCTE, three different probes are available, namely, a 3.5-MHz "M" probe (for standard examinations), a 2.5-MHz "XL" probe (for obese patients), and a 5.0-MHz "S" probe (for children). Using a US transducer probe, a low-frequency (50-Hz) mechanical impulse is transmitted to the skin surface, inducing an elastic shear wave that traverses the liver. A pulse echo measures the velocity of shear wave through the liver. Higher shear wave speed indicates greater liver fibrosis. Results are typically recorded as the Young' modulus (E, in kilopascals).[15,16]

Reliability and failure rate

In general, a valid estimation of VCTE encompasses the following three points: (a) at least 10 valid shots; (b) the success rate (number of valid shots of the total number of shots) greater than 60%; and, (c) the interquartile range-to-median LSM ratio less than 30%.[15] In a study of 13,369 patients with chronic liver diseases, the largest prospective study of VCTE to date, technical failure occurred in 3.1% of cases, whereas unreliable measurements were acquired in 15.8% of cases.[18] Obesity (body mass index >30 kg/m^2) and ascites are major factors contributing to failed measurements of VCTE.[14] In obese patients, low-frequency shear waves can be attenuated by the thickened body wall, resulting in a poor signal-to-noise ratio that influences the elasticity measurement algorithm. In these cases, hence, region of interest (ROI) requires being moved deeper below the skin surface so as to avoid fatty tissue. Additionally, in patients with ascites, low-frequency shear waves are unable to propagate through liquids, leading to failed LSM.[16]

Diagnostic performance for the staging of liver fibrosis

Previous meta-analyses have confirmed the excellent diagnostic performance of VCTE for the detection of cirrhosis (area under curves [AUCs], 0.92–0.96), superior to that for diagnosing significant liver fibrosis (AUCs, 0.83–0.88).[19–27] In a study of 916 patients with chronic viral hepatitis (567 CHB and 349 CHC), the accuracy of VCTE to predict significant fibrosis, advanced fibrosis and cirrhosis was 0.79, 0.86 and 0.90, respectively.[28] These results indicate that VCTE is more useful for ruling-out instead of ruling-in cirrhosis, with negative predictive value higher than 90%.[14] Considering the low cost and wide availability, VCTE can be used as a cost-effective technique for liver fibrosis screening.

Longitudinal assessment of liver fibrosis after AVT

Screening of advanced fibrosis and cirrhosis. In contrast with the setting of treatment-naïve CHB and CHC populations, in whom the performance of VCTE for the staging of liver fibrosis has been widely validated,[14,29] data on the use of this method for screening of advanced fibrosis or cirrhosis after AVT are still lacking. According to the data available currently, VCTE has shown approximately good-to-excellent accuracy in diagnosing advanced fibrosis and cirrhosis after AVT, with AUCs of 0.78–0.94 for advanced fibrosis and of 0.86–0.92 for cirrhosis.[30–33] These findings are of clinical significance given that VCTE can be used as a reliable tool to identify patients who should be monitored for liver-related complications after sustained virological response (SVR). The best cutoff values of LSM, however, varied across published studies, which need to be further determined.

Monitoring of dynamic changes of liver stiffness measurement. It has been demonstrated that liver stiffness values decrease during ongoing AVT (Table 2).[34–40] However, it remains to be illuminated whether the improvement of liver stiffness after AVT indicates the regression of fibrosis or merely the alleviation of necroinflammation due to virus suppression or clearance.[31,34] As assumed by some researchers, it might reflect both necroinflammation alleviation and fibrosis regression, as supported by the findings that improvements of liver stiffness values were in concordance with that of biochemical markers and serum fibrosis scores, such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase, AST-platelet ratio index score, and fibrosis-4 (commonly known as FIB-4) score.[34,35] Furthermore, it was considered that the stiffness decline during AVT might be more a result of necroinflammation alleviation than a consequence of fibrosis regression, given that the regression of fibrosis is a relatively slower process as compared with the remission of inflammation.[35] To further clarify the clinical implication of the decrease in liver stiffness values, a rapid-to-slow pattern of LSM kinetics during 2-year AVT was proposed by a multicenter, randomized and controlled trial of 534 CHB patients, which may reflect a mixed remission of both necroinflammation and fibrosis during the initial 24 weeks and the regression of fibrosis during long-term AVT, particularly, following ALT normalization.[11] To be specific, from baseline to week 24 after the initiation of AVT, liver stiffness manifested as rapid decrease (-2.2 kPa/24 weeks) in parallel with ALT; intriguingly, from week 24 to week 104, liver stiffness displayed slow but persisting declination (-0.3 kPa/24 weeks), whereas ALT levels remained stable within the normal range.[11] In other words, significant correlation between the decline in ALT and LSM showed in the first 24 weeks but diminished thereafter. Similar findings were reported in another prospective study of 120 CHB patients, in which a rapid-to-slow pattern of LSM kinetics during 78 weeks of entecavir treatment was noted.[31]

Predicting of fibrosis regression. Correlations between dynamic changes in LSM and histologically-proven fibrosis regression have been assessed in a few studies (Table 3).[11,30,31] In a cohort of 112 HCV-infected liver transplantation recipients who achieved SVR after long-term AVT, a decrease of 50% in baseline LSM could correctly predict 55% of patients achieving fibrosis regression, with a positive predictive value of 78% and a negative predictive value of 44%. Moreover, baseline LSM seems to be useful to predict the possibilities of fibrosis regression after treatment. A LSM

Table 1. Comparison of quantitative elastography techniques[14,15,29]

Modality	Cost	Availability	Evidence	Anatomic imaging	ROI		Reported parameter	Major causes for failure of LSM	Accuracy		
					Size	Placement			SF	AF	Cirrhosis
VCTE	Low	Widespread	++++	None	~3 cm^3	No image guidance	Young modulus (kPa)	Obesity; ascites	Good	Good to excellent	Excellent
pSWE	Low	Moderate	+++-++	Yes (B-mode US)	~1 cm^3	US guidance	Wave speed (m/s) or Young modulus (kPa)	Obesity	Good	Excellent	Excellent
2D SWE	Low	Moderate	+++-++	Yes (B-mode US)	Flexible (≥20 cm^3)	US guidance	Young modulus (kPa)	Obesity	Good to excellent	Excellent	Excellent
MRE	High	Limited	+	Yes (MRI)	≥250 $cm^{3\#}$	MRI guidance	Complex shear modulus (kPa)	Hepatic iron overload; large ascites; obesity*; 3 T (2D GRE)	Good to excellent	Excellent	Excellent

* Conflicting results reported regarding magnetic resonance elastography failure in patients with obesity;
# Up to 1/3 of the total liver volume.
Abbreviations: 2D GRE, two-dimensional gradient recalled echo; 2D SWE, two-dimensional shear wave elastography; AF, advanced fibrosis; LSM, liver stiffness measurement; MRE, magnetic resonance elastography; MRI, magnetic resonance imaging; pSWE, point shear wave elastography; ROI, region of interest; SF, significant fibrosis; US, ultrasound; VCTE, vibration-controlled transient elastography.

cutoff of 21 kPa can be used to accurately predict the probability of cirrhosis regression, with a regression rate of 23% and 57% for patients with baseline LSM ≥21 kPa and <21 kPa, respectively (p=0.005).[30] Similar findings have been reported by other studies on CHB populations.[11,31] For instance, a decline of 40% in liver stiffness from baseline to week 78 has been suggested as a significant determinant of fibrosis regression in CHB patients after AVT, with an AUC of 0.69, a sensitivity of 69% and a specificity of 68%.[31] These promising results indicate that VCTE may be useful for predicting fibrosis regression after AVT. Likewise, further studies are warranted to standardize cutoff values in different etiologies.

Strengths and weaknesses

VCTE is a well validated technique with excellent repeatability and reproducibility, which has been widely used in clinical practice for its portability, cost-effectiveness and patient acceptance.[14,29] However, the application of VCTE is limited by the following: (a) the lack of gray-scale image guidance to determine the ROI placement; (b) the incapacity to identify and avoid large vessels and masses; (c) the difficulty of application in obese patients and the inability to be performed in patients with ascites; (d) the difficulty in imaging between narrow intercostal spaces; (e) the relatively high technical failure rate and limited precision; and, (f) the requirement for recalibration of the spring in the device every 6~12 months.[14,15,29]

pSWE

Principles

Unlike VCTE, which adopts A-mode imaging, pSWE is incorporated into a standard B-mode US imaging that enables the operator to visualize the liver tissue and define the best area for reliable measurements. In pSWE, an acoustic radiation force impulse (ARFI) method is used to generate shear waves in a small ROI (~1 cm^3) within the liver. Tracking US pulses are then used to measure the velocity of shear waves, which is proportional to the square root of the liver stiffness or elasticity. The "stiffness" values are reported as shear-wave speed (in m/s) or converted into Young's modulus (E, in kilopascals) by using the following mathematical equation: $E = 3\rho c^2$, where c is the shear wave speed and ρ is the density of the tissue in homogeneous.[14,29]

Reliability and failure rate

pSWE has shown excellent repeatability and reproducibility, with both reported intraobserver and interobserver intraclass correlation coefficients (ICCs) higher than 0.85.[41,42] The technique failure rate is low (1–2%).[43] Obesity is the main cause of failed or unreliable measurements of pSWE.[14] As mentioned previously, the low-frequency elastic waves can be attenuated by the fatty tissue, leading to a poor signal-to-noise ratio that influences the LSM.

Diagnostic performance for the staging of liver fibrosis

pSWE performs well for the diagnosis of advanced fibrosis stages (F3-4).[29] A meta-analysis comprising 21 studies with 2691 CHB or CHC patients reported the AUCs of pSWE for

Table 2. Recent studies of elastography for monitoring of dynamic changes of fibrosis after antiviral therapy

Study	Region	Study design	Method	Examination time	Etiology	No. of patients	Fibrosis markers	Biochemical or other markers	Predictors for improvement in LSM
Bachofner *et al.* 2017[34]	Switzerland	NA	VCTE	At baseline, within 18 months after therapy	HCV	392	LSM values ↓ FIB-4, APRI ↓	NA	NA
Knop *et al.* 2020[35]	Germany	Prospective	VCTE	At baseline, EOT, 24 weeks after EOT	HCV	260	LSM values ↓	NA	High PLT at baseline
Persico *et al.* 2018[36]	Italy	Prospective	VCTE	At baseline, EOT, SVR12†	HCV	749	LSM values ↓	NA	Low ALT, low PLT count, diabetes**
Singh *et al.* 2017[37]	USA	Systematic review and meta-analysis	VCTE	At baseline, different timepoints after AVT depending on the included studies	HCV	2934	LSM values ↓	NA	NA
Stasi *et al.* 2020[38]	Italy	Prospective	VCTE	At baseline, EOT, 1 and 2 years after EOT	HCV	294	LSM values ↓	AST, ALT, GGT↓	NA
Yada *et al.* 2014[39]	Japan	NA	VCTE	At baseline, 2 years after treatment	HCV	26	LSM values ↓ LFI ↓	NA	NA
Alem *et al.* 2018[40]	Egypt	Prospective	VCTE pSWE	At baseline, SVR24*	HCV	58	LSM values ↓	AST, ALT, PLT ↓	Baseline viral load
Kohla *et al.* 2020[45]	Egypt	Prospective	pSWE	At baseline, EOT, week 24 and week 36	HCV	165	LSM values↓ FIB-4, APRI ↓	AST, ALT ↓	NA
Osakabe *et al.* 2015[46]	Japan	NA	pSWE	At baseline, EOT, 1 and 2 years after EOT	HCV	87	LSM values ↓	NA	NA
Tachi *et al.* 2018[47]	Japan	Prospective	pSWE	At baseline, EOT, 24 weeks after EOT	HCV	176	LSM values ↓	NA	Higher inflammatory activity at baseline;‡ significant fibrosis at baseline‡‡

(*continued*)

Table 2. (*continued*)

Study	Region	Study design	Method	Examination time	Etiology	No. of patients	Fibrosis markers	Biochemical or other markers	Predictors for improvement in LSM
Korda *et al.* 2019[64]	Hungary	Prospective	2D SWE	At baseline, EOT	HCV	23	LSM values ↓ FIB-4, APRI ↓	AST, ALT, GGT ↓ INR ↑	NA
Tada *et al.* 2017[53]	Japan	Retrospective	2D SWE	At baseline, EOT, SVR24*	HCV	210	LSM values ↓	ALT ↓	NA
Tada *et al.* 2018[60]	Japan	Prospective	MRE	At baseline, SVR24*	HCV	198	LSM values ↓	PDFF values ↓	NA

*24 weeks after EOT; †12 weeks after EOT; **Low ALT, low platelet count, diabetes were inversely associated with the LSM improvement; ‡Higher inflammatory activity at baseline was associated with an improvement in liver stiffness at the EOT; ‡‡Significant fibrosis at baseline was associated with an improvement in liver stiffness at 24 weeks after the EOT.

Abbreviations: 2D SWE, two-dimensional shear-wave elastography; ALT, alanine aminotransferase; APRI, aspartate aminotransferase-platelet ratio index; AST, aspartate aminotransferase; EOT, end-of-treatment; FIB-4, fibrosis-4 score; GGT, gamma-glutamyl transferase; HBV, hepatitis B virus; HCV, hepatitis C virus; INR, international normalized ratio; LFI, liver fibrosis index; LSM, liver stiffness measurement; MRE, magnetic resonance elastography; NA, not available; PDFF, proton density fat fraction; PLT, platelet; pSWE, point shear-wave elastography; SVR, sustained virological response.

detecting significant fibrosis, advanced fibrosis and cirrhosis were 0.88, 0.94, and 0.91, respectively.[44] Therefore, pSWE is recommended for differentiating patients with advanced fibrosis to cirrhosis from those with no to minimal fibrosis.[29,44]

Longitudinal assessment of liver fibrosis after antiviral treatment

pSWE represents a reliable and reproducible ARFI method for assessing liver fibrosis, however, available data on pSWE for fibrosis surveillance after AVT are still lacking. Similar to VCTE, significant decrease in LSM by pSWE after AVT have been reported, yet, merely in few CHC patients.[40,45,46] It was considered that reduction of pSWE values indicates not only the improvement of fibrosis but also the resolution of liver inflammation,[40] as an early decline in liver stiffness after SVR was associated with the grade of histological inflammation at baseline.[47]

Strengths and weaknesses

As compared with VCTE, strengths of pSWE include the following: (a) it is incorporated into a standard B-mode US that can achieve the real-time imaging and guide the ROI placement; (b) large vessels and masses can be detected and avoided; (c) it allows for sampling at different segments of the liver; and, (d) ascites is not a limitation for pSWE, enabling its performance in decompensated liver cirrhosis for fibrosis assessment.[29]

Limitations of pSWE include the following: (a) difficulty in delineating intermediate fibrosis stages, owing to prominent overlap in shear wave speeds; (b) susceptibility to liver motion (e.g. deep breath or using the Valsalva maneuver) or physiologic motion (e.g. vascular pulsatility), which may influence the LSM; and, (c) measurement dependence upon the operator's expertise, necessitating operators being properly trained.[14,29,48]

2D SWE

Principles

2D-SWE, similar to pSWE, induces shear waves by using the ARFI to deform hepatic tissues. Nevertheless, in contrast to pSWE, which emits a single push pulse to a focal point, 2D SWE generates shear waves at multiple points, producing a cone-shaped shear wave front. The shear wave propagation is tracked by conventional compressive US waves and depicted as a color-coded elasticity map – elastogram. Using the B-mode US image, a flexible ROI is delineated within the elastogram. The mean shear wave speed (in m/s) within the ROI is obtained from multiple measurements, which can be converted into the Young modulus and reported in kPa.[14,29,48]

Reliability and failure rate

2D SWE has demonstrated excellent repeatability and reproducibility, with reported intraobserver ICC greater than 0.90 and interobserver ICC of 0.88.[49] The failure rate of 2D SWE is low (~5%).[50] Failed measurement is predominantly attributed to obesity.[14] The mechanism underlying the relationship between high body mass index and failed LSM has been discussed.

Table 3. Recent studies of elastography for the prediction of histologically-proven fibrosis regression after antiviral therapy

Study	Region	Study design	Method	Examination time	Etiology	No. of patients	Fibrosis regression*		
							Rate % (*n*/N)	Reference standard	Predictors
Liang *et al.* 2018[11]	China	Prospective	VCTE	At baseline and every 24-28 weeks during follow-up of 104 weeks	HBV	534	60% (98/164)	LB (Ishak score)	Baseline Ishak score; percentage change of LSM values from baseline to week 52
Mauro *et al.* 2018[30]	Spain	NA	VCTE	At baseline and 12 months post-SVR	HCV	112	67% (75/112)	LB (METAVIR system)	Baseline HVPG; LSM; decompensations at baseline
Wu *et al.* 2018[31]	China	Prospective	VCTE	At baseline, 26 week, 52 week and 78 week of treatment	HBV	120	45% (54/120)	LB (METAVIR system)	Percentage decline of LSM values from baseline to week 52 and week 78

*fibrosis regression was defined as ≥ 1 stage decrease in the METAVIR score or ≥ 1-point decrease in Ishak at follow-up biopsy score.

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; HVPG, hepatic venous pressure gradient; LB, liver biopsy; LSM, liver stiffness measurement; NA, not available; SVR, sustained virological response; VCTE, vibration-controlled transient elastography.

Diagnostic performance for the staging of liver fibrosis

2D SWE has shown good-to-excellent performance for the diagnosis of significant fibrosis stages (F2-4). In a previous meta-analysis based on 13 studies with 2303 patients, the reported AUCs of 2D SWE for detecting significant fibrosis, advanced fibrosis, and cirrhosis were 0.87 (95% CI: 0.84–0.90), 0.93 (95% CI: 0.91–0.95), and 0.94 (95% CI: 0.92–0.96), respectively.[51] In addition, our recent meta-analysis involving 1977 CHB patients found AUC of 0.92 (95 % CI: 0.89–0.94) for detecting significant fibrosis.[52] Hence, diagnostic accuracy of 2D SWE for fibrosis assessment might be equivalent or possibly superior to that of VCTE or pSWE. However, further validations regarding the diagnostic performance of 2D SWE are warranted. In addition, thresholds for the staging of liver fibrosis remain to be established.

Longitudinal assessment of liver fibrosis after antiviral treatment

2D SWE is a highly accurate ARFI method for fibrosis estimation in CHB and CHC populations; yet, it is less well investigated than either pSWE or VCTE.[14] In a preliminary study of 210 hepatitis C virus-infected patients undergoing AVT, an early decline of LSM by 2D SWE occurred in those who achieved SVR, and a pronounced decrease in LSM was found particularly in those with progressive liver fibrosis.[53] Evidence from this study indicates that the improvement of liver fibrosis may be a gradual process that initiated at the end of AVT. Concretely, it was considered that the significant decline of ALT levels from baseline to end-of-treatment was strongly correlated with improvement of liver stiffness. Intriguingly, despite ALT levels having decreased to low levels at both end-of-treatment and SVR at week 24, suggesting the remission of liver inflammation, hepatic stiffness decreased persistently and significantly from baseline to end-of-treatment and from end-of-treatment to SVR at week 24.[53]

Strengths and weaknesses

2D SWE, as a new US elastography technique, has the following strengths. First, 2D SWE incorporates conventional B-mode US image with colorized elastogram, which can provide real-time imaging and enables accurate ROI placement for high-quality measurements. In addition, under the guidance of B-mode US, 2D SWE can also be used to depict liver masses, estimate hepatic morphological alterations and monitor changes in blood flow. Similar to pSWE, 2D-SWE is insusceptible to ascites.[29]

2D SWE also has several limitations. Compared with VCTE and pSWE, the sampling time of 2D SWE may be extended since shear waves are slow-moving and 2D SWE makes more measurements over a larger tissue volume. Moreover, like pSWE, 2D SWE is susceptible to motion and therefore requires breath-holding. Additionally, LSM values of 2D SWE derived from different manufacturers are not directly comparable, which complicates the disease-tracking process if machines from different vendors were used. This is because not only tissue stiffness but the applied frequency of the shear waves would exert an influence on the inferred stiffness. On the assumption that all other parameters are equal, the LSM values are larger when the shear waves are employed at higher frequency. Furthermore, similar to pSWE, 2D SWE should be performed by trained sonographers since the technique is operator-dependent.[14,29,48]

MRE

Principles

MRE, approved by the Food and Drug Administration in 2009, is currently considered the most accurate noninvasive elastography technique for fibrosis assessment.[48] In general, during an MRE scan, 60 Hz (ranging from 20-200 Hz) mechanical vibrations generated by an active driver [located outside the MR scanner room] are transmitted via flexible

plastic tubing to a passive driver (placed on the patient's body wall), which then transmits the acoustic pressure into the liver as shear waves. The shear wave propagation is imaged by a MR phase-contrast sequence modified with motion-encoding gradients. The common MRE sequences include the 2D gradient-recalled echo-based (GRE) sequence and 2D spin-echo-based echo-planar imaging (SE-EPI) sequence. Raw data on shear waves acquired from the MRE sequence are postprocessed by an automated inversion algorithm into a color-coded map of liver stiffness, known as an elastogram. Calculating liver stiffness from the elastogram requires delineating ROIs. During this process, anatomical regions that may disrupt the propagation of shear wave, such as lesions, large (>3 mm) vessels, edge artifact and fossae or fissures, need be avoided.[29,48]

Reliability and failure rate

MRE can provide reliable examinations even in pediatric patients and in those with obesity or hepatic steatosis.[14] MRE has shown high repeatability and excellent reproducibility.[54,55] The technical failure rate of MRE is low. In a study of 1377 consecutive MRE examinations, technical failure occurred in 5.6% of cases when using a 2D GRE sequence.[56] The most frequent reason for failed measurement in MRE is hepatic iron deposition, which decreases the liver signal intensity and results in a poor signal-to-noise ratio that influences the elastographic calculation.[56]

Diagnostic performance for the staging of liver fibrosis

MRE has shown good-to-excellent performance for the staging of liver fibrosis in chronic liver diseases. A meta-analysis comprising 12 studies (697 patients) with mixed chronic liver diseases reported AUCs of 2D MRE for detecting any fibrosis, significant fibrosis, advanced fibrosis, and cirrhosis were 0.84 (95% CI: 0.76-0.92), 0.88 (95% CI: 0.84–0.91), 0.93 (95% CI: 0.90–0.95), and 0.92 (95% CI: 0.90–0.94), respectively.[57] In addition, a recent meta-analysis based on 26 studies (3200 patients) with mixed chronic liver diseases found that there were no significant differences between the GRE sequence and SE-EPI sequence in terms of the pooled sensitivity and specificity for the staging of liver fibrosis; the reported AUCs of GRE-MRE and SE-EPI-MRE for diagnosing any fibrosis, significant fibrosis, advanced fibrosis, and cirrhosis were 0.93 vs. 0.94, 0.95 vs. 0.94, 0.94 vs. 0.95, and 0.92 vs. 0.93, respectively.[58] Similar diagnostic accuracy as that with 2D MRE and 3D MRE have been reported in a few prospective studies with mixed chronic liver diseases.[54,59] Based on these observations, MRE is recommended for asymptomatic patients who may have mild fibrosis to accurately define fibrosis stages and guide therapeutic interventions. In addition, for symptomatic patients with advanced fibrosis or cirrhosis, MRE combined with routine magnetic resonance imaging scan can help to establish the fibrosis stages, assess morphologic alterations of the liver, and detect intra- or extra-hepatic complications.

Longitudinal assessment of liver fibrosis after antiviral treatment

Given its limited availability and recent clinical introduction, data on MRE for longitudinal assessment of fibrosis after AVT are scarce. In a prospective cohort of 198 CHC patients, liver stiffness values assessed by MRE significantly decreased from baseline to SVR at week 24.[60] Likewise, it was considered that the reduction of liver stiffness after SVR was associated with both fibrosis regression and inflammation remission, given that elevated ALT levels, corresponding to the presence of necroinflammation, also declined significantly from baseline to SVR at week 24.[60] MRE holds promise to illuminate the underlying mechanisms of liver stiffness improvement following AVT, as the use of MR T1 mapping of diffusion and perfusion may be able to differentiate a real fibrosis regression from a mere reduction of interstitial edema.[34]

Strengths and weaknesses

Unlike US elastography with localized spot measurements at limited depth in the liver, MRE provides a quantitative map of tissue stiffness over a large area of coverage of the liver, which can produce a more reliable LSM and higher accuracy for fibrosis assessment. In addition, MRE is much less operator-dependent and has a lower technical failure rate than US elastography. More importantly, MRE can be incorporated into a routine abdominal magnetic resonance imaging scan protocol, providing a comprehensive estimation of the liver, such as evaluation of liver fat content, diagnosis of focal liver diseases, and detection of complications of cirrhosis, like hepatocellular carcinoma, splenomegaly, varices, and ascites.[61]

Despite these advantages, MRE also has several limitations. First, the presence of hepatic iron overload and motion artifacts result in failed examinations. In addition, a minority of patients cannot tolerate MR examinations, owing to claustrophobia. Moreover, MRE might be contraindicated in patients with incompatible implantable devices, or those who cannot fit into the MR scanner bore.[14,29] Finally, MRE is costlier and less available compared with US elastography,[14] which may limit its clinical use to a certain extent.

Current challenges and future directions

To date, available data on the use of elastography-based methods, particularly of MRE or AFRI methods, for longitudinal assessment of liver fibrosis after AVT are limited. However, it is apparent that only when sufficient evidence has been obtained to validate these novel techniques will they be recommended for monitoring strategies. Moreover, prospective studies comparing the performance of MRE and US elastography for fibrosis evaluation in patients with AVT, particularly for detecting those with advanced fibrosis after SVR, are warranted.

It is still controversial whether a decline in LSM after AVT reflects a real regression of fibrosis, or merely a resolution of hepatic necroinflammation due to virus eradication, or mixed remission of both fibrosis and inflammation. Therefore, robust evidence remains to be provided that will elucidate the correlation of a decline in liver stiffness values with histological changes after SVR.

Despite emerging lines of evidences showing the potential of changes in LSM for the prediction of histological fibrosis regression after long-term AVT,[11,30,31] further validations in different populations are required. More importantly, standardization of cutoff values for these promising biomarkers is urgently needed.

It is clear that liver stiffness is an "indirect" marker of fibrosis; thus, LSM may not be sensitive enough to monitor

subtle changes in fibrosis after AVT or antifibrotic treatment. Recently, molecular imaging probes targeting fibrosis-specific cells or molecules (e.g. hepatic stellate cells, collagen and elastin) might become novel, noninvasive, promising biomarkers for fibrosis.[62,63] These "direct" markers hold promise for a reliable assessment of fibrosis and monitoring of its dynamics during a long-term follow-up period, which can be used to predict the antifibrotic potential of new drugs and to select responders to antifibrotic therapies. These molecular markers could serve as a complementary method to elastography in the future. The combination of these techniques may produce increased accuracy for fibrosis evaluation.

Conclusions

Liver fibrosis is a dynamic process with potential for regression if the underlying causes of chronic liver injury are removed. Fibrosis regression can be achieved after long-term AVT. Monitoring of dynamic changes in liver fibrosis after AVT is of strategic importance for the prediction of prognosis and the surveillance of liver-related events. Routine surveillance of liver fibrosis after AVT by liver biopsy, the gold standard for fibrosis assessment, is hindered by its invasive nature, sampling error, and observer variability. Elastography represents an noninvasive alternative that has been widely used and validated for fibrosis assessment prior to treatment. Emerging evidence indicates that quantitative elastography methods can be used to monitor fibrosis status after long-term AVT, with great potential for screening advanced fibrosis and cirrhosis, monitoring dynamic changes in LSM and predicting histologically-proven fibrosis regression. Future research on elastography is required to elucidate the correlations between liver stiffness improvement and histological changes after AVT, to standardize the cutoffs for both screening and predicting strategies, and to develop noninvasive molecular markers as complementary tools to LSM.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study concept and design (BS), literature research (HW), drafting of the manuscript (HW), critical revision of the manuscript for important intellectual content (BS, HW), and approval of final version of submitted manuscript (BS, HW).

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Review Article

Chronic Liver Disease and Silymarin: A Biochemical and Clinical Review

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Abstract

Chronic liver disease (CLD) is an under-recognized epidemic that continues to increase in prevalence and is a major health concern. Silymarin, the active compound of *Silybum marianum* (Milk thistle), has historically been used in CLD. A significant barrier to silymarin use is its poor bioavailability. Attempts at improving the bioavailability of silymarin have led to a better understanding of formulation methods, pharmacokinetics, dosing, and associated drug interactions. Clinically, silymarin exerts its hepatoprotective effects through antioxidative, antifibrotic, anti-inflammatory, antitoxin, and anticancerous mechanisms of actions. Despite the use of silymarin being extensively studied in alcoholic liver disease, metabolic-associated fatty liver disease, viral hepatitis, and drug-induced liver injury, the overall efficacy of silymarin remains unclear and more research is warranted to better elucidate the role of silymarin in CLD, specifically regarding its anti-inflammatory effects. Here, we review the current biochemical and clinical evidence regarding silymarin in CLD.

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Introduction

Over the last decade, there has been a significant increase in the burden of chronic liver disease (CLD) due to the growing prevalence of metabolic-associated fatty liver disease (MAFLD), with CLD now a major cause of morbidity and mortality worldwide.[1] The increasing numbers of patients at risk for cirrhosis and in need of liver transplantation have become important economic and health concerns, with studies showing hospitalizations due to CLD having doubled in number over the last decade.[2,3] Therefore, there is a need for affordable and effective treatment modalities to reduce the morbidity and mortality associated with CLD.[4] Certain medicinal plants, such as *Silybum marianum*, more commonly known as milk thistle, have historically been used for the treatment and prevention of liver disorders. Specifically, silymarin has shown promising protective effects in preclinical studies using a number of formulations, including Legalon which contains the Eurosil 85 formulation.[5–8] The aim of this article is elaborate on the biochemistry of silymarin pertaining to its formulation, pharmacokinetics, dosing, drug interactions, mechanism of action, while also reviewing the current evidence of silymarin use in chronic liver disease.

Biochemistry

Formulation

Silymarin is a complex mixture that includes an array of different flavonolignan isomers. Silybin, one of these isomers, composes up to 50% of the silymarin mixture and plays an important role in the antioxidative effects of silymarin. These antioxidant effects are a result of silybin diastereomers that undergo biotransformation, leading to the formation of glucuronide derivatives (Fig. 1).[9]

A significant barrier to the clinical use of silymarin is its poor bioavailability, due to its lipophilic nature, and subsequent poor solubility.[9] Attempts at improving the solubility of silymarin formulations has led to the development of many different commercially tested forms of silymarin, which differ in their composition of silybin. Moreover, approximately 75 silymarin brands have been developed in various dosage forms, such as tablets like Carsil, syrups like Alrin-B, and capsules like

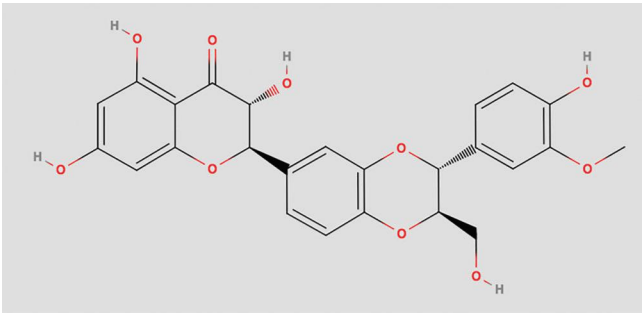

Fig. 1. Chemical structure of silybin.

Keywords: Silymarin; Chronic liver disease; *Silybum marianum*; MAFLD; ALD; Pharmacokinetics.

Abbreviations: ALD, alcoholic liver disease; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CLD, chronic liver disease; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; MAFLD, metabolic-associated fatty liver disease; RCT, randomized control trials.

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Legalon.[9] Legalon contains Eurosil 85Ò, which is a standardized preparation method that contains 60% of silybin and has been used in studies examining the effects of silymarin.

Despite standardized preparation methods, the bioavailability of silymarin can further be affected by genetic polymorphisms and the presence of liver disease. Specifically, the plasma half-life of silybin, the bioactive component in silymarin formulations, is 6 hours, while peak plasma concentrations are usually reached 2-4 hours after administration. Genetic polymorphisms, such as ABCB1 C3435T, however, can affect silybin bioavailability, with one study in healthy patients illustrating varying peak and half times for oral doses of 80 mg of silybin equivalents when compared to plain silymarin capsules.[10] In addition to genetic polymorphisms, the bioavailability of silymarin can also be significantly affected by the presence of liver disease due to alterations in liver metabolism. Studies have also shown that the effectiveness of silymarin varies between MAFLD and hepatitis C virus (HCV)-infected patients. This is a result of higher flavonolignan plasma concentrations and more extensive enterohepatic cycling.[11]

Dosing

Presently, silymarin is available in a variety of different forms, including capsules and tablets of different strength, with a recommended daily dosage between 420 mg to 600 mg. Clinical studies have been conducted with varying doses, ranging as low as 80 mg and as high as 1600 mg. One study tested various amounts (160, 240, and 360 mg/day) and found statistically significant decreases in liver enzymes in the 240 and 360 mg/day groups in patients with alcoholic liver disease (ALD) and chronic viral hepatitis.[12] Another study on HCV-decompensated cirrhotic patients concluded that higher doses of silymarin (1.05 g/day) is superior to a standard dose (420 mg/day).[13] Although the data from some studies point towards a link between higher concentrations of silymarin and better treatment results and patient outcomes, certain patient populations, such as those with hepatocellular carcinoma (HCC), have not shown the same signal.[14] Dosage adjustments may, therefore, be necessary to exert a similar effect in patients with liver disease.

Drug interactions

The drug interaction profile of silymarin has been studied extensively both in laboratory/animal models and clinical trials.[15,16] Studies with human hepatocytes demonstrate negligible inhibition of CYP450 enzymes at supratherapeutic silymarin concentrations, suggesting that at therapeutic doses silymarin is unlikely to cause hepatocyte related drug-drug interactions.[15] Silymarin may be indirectly implicated in this reduction, as an additive effect secondary to interactions with antihyperglycemic agents. Furthermore, studies have shown that silymarin can theoretically interfere and effect the clearance of other drugs, such as statins, glucorinidated drugs, and immunosuppressants, such as sirolimus.

Mechanism of action

The hepatoprotective effects of silymarin are due to its antioxidative, antifibrotic, regenerative, choleretic, immunomodulatory, and anti-inflammatory properties, as illustrated in Fig. 2.[17]

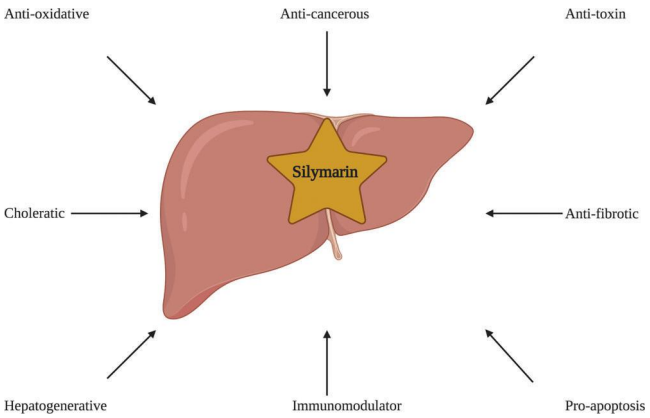

Fig. 2. Hepatoprotective mechanisms of action of silymarin.

Antioxidative effect

The antioxidant properties of silymarin arise from its ability to utilize scavengers, allowing for the elimination of free radicals. Silymarin's antioxidant activities have different potential mechanisms. These include the inhibition of reactive oxygen species-producing enzymes that prevent free radical formation, scavenging of said free radicals, intestinal ion chelation, promoting protective molecule synthesis, and antioxidant enzyme activation.[18] The antioxidant properties of silymarin have been demonstrated to restore NAD+ homeostasis, sirtuin 1 activity, and the AMP-activated protein kinase α pathway to improve poly-(ADP-ribose)-polymerase function (all-important regulatory pathways linked with oxidative stress).[19] Furthermore, the antioxidant capabilities of silymarin improve the hepatic lipid homeostasis by decreasing *de novo* lipogenesis via the down-regulation of peroxisome proliferator-activated receptor γ, acetyl-CoA carboxylase, and fatty acid synthase.[19–22]

Antifibrotic effect

The antifibrotic activity of silymarin is primarily due to its ability to inhibit the conversion of hepatic stellate cells into myofibroblasts through the inhibition of fibrogenic pathways, such as those implicated in cytoskeletal formation, profibrogenic collagen, and electron transfer chains. Specifically, silymarin down-regulates TGF-ß1 mRNA, inhibits NF-kB, and prevents the stimulation of hepatic stellate cells. These findings are supported by studies in animal models, whereby silymarin was shown to slow down the progression of early fibrosis.[23,24]

Anti-inflammatory effect

The immunomodulatory activity of silymarin exerts an anti-inflammatory effect by preventing the activation of the inflammasomes, and NF-κB, which are important in regulating the immune response in inflammatory states.[17] Silymarin can also restore a pathway known as insulin receptor substrate-1/PI3K/Akt, which can reduce MAFLD-induced insulin resistance and steatosis, as well as activate the farnesyl X receptor, which in turn can diminish hepatic inflammation.[20,25,26] Silymarin's anti-inflammatory and antioxidant capabilities have also been shown to reduce virus-related damage to the liver in chronic HCV infection.[27]

Antitoxin effect

In cases of drug/toxin-related hepatic injury, the primary mechanisms by which silymarin protects against further damage is through the regulation of membrane permeability and the competitive inhibition of toxins at specific binding sites. This prevents the absorption of these harmful substances, particularly in the hepatic phalloidin-transporting system.[28,29]

Anticancerous effect

Silymarin also demonstrates anticancerous effects believed to be linked to the inhibition of oxidative stress, promotion of apoptosis, cell cycle arresting, and mitochondrial pathway inhibition.[14] *In vitro* and *in vivo* assays, as well as animal models with HCC treated with silymarin, have showcased the antitumoral effects at varying stages of hepatocarcinogenesis (initiation, promotion, and progression).[17] Silymarin's ability to aid in hepatic regeneration is also an important characteristic that makes it well suited as a potential therapy in patients with CLD. Specifically, there is an association with ribosomal RNA synthesis, possibly through the stimulation of polymerase I.[17]

Current evidence of silymarin in CLD

The clinical applications of silymarin encompass a broad range of CLD. These include ALD, MAFLD, drug-and toxin-induced liver disease, cholestasis (both pregnancy and nonpregnancy related), primary liver malignancies (including both cholangiocarcinoma and HCC), and viral hepatitis.[17]

ALD

Alcohol is a key risk factor for liver diseases and is responsible for about half of all liver-related cirrhosis. Significant alcohol consumption initially leads to a fatty liver, which can progress to cirrhosis.[30] The use of silymarin in ALD is limited, due to the poor design of initial studies. A recent systematic review assessing the role of silymarin in patients with ALD suggested that better clinical trials are indicated in order to determine whether or not there is a role for silymarin in ALD management.[31]

MAFLD

On the contrary, the use of silymarin for MAFLD, appears to better supported with multiple randomized control trials (commonly referred to as RCTs) showing benefit from silymarin use.[32] MAFLD is a clinical spectrum that can manifest with hepatic fibrosis and inflammation. This is due to the associations between MAFLD and the aforementioned, "anti-inflammatory, antioxidant, antifibrotic, and pro-regenerative effects of silymarin, in conjunction with its metabolic actions on insulin resistance and hyperlipidemia".[17,33,34] With a recent meta-analysis involving 587 patients suggesting an improvement in liver function in patients with MAFLD, the use of silymarin for the treatment of MAFLD is promising but still requires further analysis with larger standardized RCTs.[32]

Viral hepatitis

The advent of antiviral therapy has dramatically changed the landscape of viral hepatitis management. Despite this, high treatment costs and issues with accessibility has created a possible niche for other treatment modalities, such as silymarin. Unfortunately, the efficacy of silymarin when compared to placebo in a well-designed double-blinded trial by Fried *et al.*[35] did not significantly reduce serum alanine aminotransferase (ALT) levels. A systematic meta-analysis further demonstrated slightly reduced ALT and aspartate aminotransferase (AST) levels in HCV patients taking silymarin, but these effects were proven to be too variable to provide any concrete clinical significance.[36]

Drug-induced liver injury

Drug- and toxin-induced liver injury can result from harmful increases in oxidative stress from exposure to various chemicals.[37] Normally, a balance exists between free radical production and the human body's ability to produce counter-acting antioxidants as a corresponding defense mechanism. However, in drug- and toxin-induced liver injury, the pathogenesis is associated with an imbalance between these two, which explains why silymarin, with its antioxidant effects, is a considerable treatment option for CLDs including jaundice, cirrhosis, and hepatitis.[38] Studies examining CCl_4-hepatotoxicity in rats showed the prevention of hepatic dysfunction and the restoration of normal liver functionality with silymarin use.[39,40–44] Silymarin aids as an antioxidant, not only by scavenging for free radicals but also by preventing the loss of the antioxidant glutathione.[18] One study known as Hep573 demonstrated that an intervention of silymarin and antioxidants in a complex naturopathic mixture might lead to normal ALT levels in HCV patients as well as an overall improved quality of life, which includes treating comorbidities that are not always properly treated with the antiviral 'cure'.[45] These results were mostly found in patients with the specific HCV genotype 1, which can be explained by these individuals lacking endogenous antioxidants, like glutathione.[46] These findings could be applied to any of the aforementioned liver diseases that result from antioxidant deficiencies and/or free-radical surpluses. Although, various studies have shed a positive light on the beneficial effects of silymarin in different liver diseases, these studies are limited by the volume of data and thus are not adequate for robust conclusions. This, therefore, warrants the need for more quality studies examining silymarin before it can be used as an official clinical treatment in CLD.

Conclusions

Silymarin is a potent inhibitor of inflammation, fibrosis and oxidative stress that is safe and has low risks of drug interactions. Although some evidence of efficacy exists in a subset of patients with CLD, such as those with ALD and MAFLD, the overall efficacy of silymarin remains unclear, with a recent multiple meta-analysis showing no clinically substantial benefit in the management of CLD. Silymarin may have a role in combination with antioxidants; however, more research is warranted to better elucidate the role of silymarin in the management of CLD.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study concept and design (AA, ST, DA and UI), literature search and drafting of the manuscript (ST, UI and DA), critical revision of manuscript for important intellectual content, senior authorship guidance and supervision (AA). All authors agreed with the final version of the manuscript

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Case Report

A Case Report of Alloimmune Hepatitis after Direct-acting Antiviral Treatment in a Liver Transplant Patient

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Abstract

Direct-acting antiviral (DAA) therapy is often well-tolerated, and adverse events from DAA therapy are uncommon. We report a case of a woman who underwent orthotopic liver transplant for chronic hepatitis C infection and later developed alloimmune hepatitis shortly after starting DAA therapy for recurrent hepatitis C infection. The patient developed acute alloimmune hepatitis approximately 2 weeks after starting treatment with sofosbuvir, velpatasvir, and voxilaprevir. This case report proposes a dysregulation of immune surveillance due to the DAA stimulation of host immunity and rapid elimination of hepatitis C viral load as a precipitating factor for the alloimmune process, leading to alloimmune hepatitis in a post-transplant patient who starts on DAA.

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Introduction

Previous studies have shown the link between hepatitis C infection and autoimmune diseases[1], and its presumed mechanism is an excessive immune response against hepatitis C virus (HCV). Direct-acting antiviral (DAA) therapy is well tolerated in liver transplant patients, and serious adverse events from DAA are uncommon. *De novo* autoimmune hepatitis or alloimmune hepatitis are clinical entities that occur in patients who have undergone transplantation and are characterized by biochemical hepatitis, circulating autoantibodies, elevated IgG levels, and an inflammatory infiltration with interface hepatitis.[2] Cases of alloimmune hepatitis induced after DAA in patients undergoing treatment for HCV infection have been reported in the literature. This is a case report of alloimmune hepatitis in a post-liver transplant patient shortly after starting DAA treatment for recurrent hepatitis C in the graft.

Case report

The patient is a woman of Arabic background in her mid to late 50s with a history of vitiligo and end-stage liver disease (induced by chronic HCV infection, genotype 4; treatment-experienced), complicated with hepatocellular carcinoma, who presented to the emergency department with jaundice and elevated liver enzymes. She underwent liver transplantation (LT) 3 months prior.

The patient has a 14-year history of HCV infection and had been diagnosed with cirrhosis 3 years prior to the LT. She had received treatment for HCV 2 years prior to the LT with ombitasvir, paritaprevir, ritonavir, and ribavirin, but the treatment was discontinued after 2 months due to the development of hemolytic anemia. Unfortunately, she failed to achieve a sustained virologic response. She underwent orthotopic LT with a hepatitis B core antibody-positive and hepatitis C antibody-positive graft. Prior to the current admission, the patient had been admitted to the hospital for investigation of elevated serum aminotransferase levels (aspartate aminotransferase of 227 U/L, alanine aminotransferase of 219 U/L) and HCV viral load of 734,703 IU/mL (Table 1). A liver biopsy showed portal inflammatory infiltrates, compatible with severe hepatitis, and features that were suggestive of recurrent HCV infection. DAA treatment was started with sofosbuvir, velpatasvir, and voxilaprevir. The patient responded to treatment with DAA, and the biochemical profile was significantly improved (Table 1).

Two weeks later, the patient complained of a 2-day experience of jaundice, nausea, vomiting, and diarrhea. The review of systems revealed subjective fevers, chills, and fatigue. She denied consumption of alcohol or use of other medications. Her vital signs were unremarkable. On examination, scleral icterus, jaundice, and moderate tenderness in the left upper quadrant and right upper quadrant, without guarding, were noted. The laboratory evaluation revealed a white blood cell count of 3,300 cells/mm^3 with a normal differential, aspartate aminotransferase of 1,395 U/L, alanine aminotransferase of 1,359 U/L, alkaline phosphatase of

Keywords: Autoimmune hepatitis; Hepatitis C; Liver transplant.
Abbreviations: AIH, autoimmune hepatitis; DAA, direct-acting antiviral; HCV, hepatitis C virus; IgG, immunoglobulin; LT, liver transplantation; Tregs, regulatory T cells.
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Table 1. Laboratory findings prior to and after DAA treatment

Lab finding	Prior to DAA treatment	At week 1 of DAA treatment	Post-DAA treatment
Alkaline phosphatase, U/L	84	579	79
Aspartate aminotransferase, U/L	227	1,201	24
Alanine aminotransferase, U/L	490	1,215	24
Total bilirubin, mg/dL	0.3	11.5	0.5
Direct bilirubin, mg/dL	0.1		0.2
Hepatitis C viral load, IU/mL	734,703	537	Not detected
Hepatitis B viral load, IU/mL	Not detected	Not detected	Not detected
Tacrolimus level, ng/mL	8	9	7

Elevated aminotransferase levels and other biochemical profiles improved after the treatment of DAA.

601 U/L, total bilirubin of 12.2 mg/dL, direct bilirubin of 9.6 mg/dL, and international normalized ratio of 1.5. The tacrolimus level was 7 ng/mL. Anti-nuclear antibody test was negative, but an elevated immunoglobulin G (IgG) level of 2,477 mg/dL was noted (Table 2). Other autoimmune serologies were unremarkable, including rheumatoid factor, anti-centromere antibody, anti-double stranded DNA, cyclic citrullinated peptide, liver-kidney microsomal antibody, Sjogren's antibodies SS-A and SS-B, anti-smooth muscle antibody, and anti-mitochondrial antibody.

A liver biopsy showed severe hepatitis with abundant plasma cells and interface activity, compatible with an alloimmune process (Figs. 1 and 2). C4D immunostaining was negative. The immunosuppressive and prophylactic therapies consisted of tacrolimus, mycophenolate, atovaquone, and entecavir. The patient was placed on 1 g of IV methylprednisolone and N-acetyl cysteine infusion, which elicited subsequent clinical improvement. The patient was discharged on a steroid taper, in addition to her immunosuppression regimen. Hepatitis B infection was ruled out as hepatitis B surface antigen, hepatitis B surface antibody, hepatitis B core antibody tests were all negative, and hepatitis B viral load was not detected by PCR. Human immunodeficiency virus screening test was also negative. Hepatitis E IgM antibody test was negative.

Table 2. Laboratory tests for the evaluation of alloimmune hepatitis

Test	Normal range	Result
Anti-nuclear antibody	No range	Not detected
Anti-mitochondrial antibody	Negative <20 U	5
Anti-smooth muscle antibody	Negative <20 U	8.7
IgG	700-1,600 mg/dL	2,477
Immunoglobulin M	40-230 mg/dL	113
Liver-kidney microsomal antibody	Negative <20 U	Not detected

Serological testing for alloimmune hepatitis performed includes elevated IgG level, but anti-nuclear antibody, anti-mitochondrial antibody, and anti-smooth muscle antibody tests were negative. Of note, rheumatoid factor was negative initially but became positive approximately 16 months after the diagnosis of alloimmune hepatitis.

The patient ultimately completed a 12-week course of DAA treatment. At her 3-month follow-up visit, the hepatic enzymes were found to be normalized by the maintenance steroid therapy with methylprednisolone 8 mg PO daily. The patient was determined to have achieved a sustained virological response (Table 1). Repeat liver biopsy was conducted for elevated liver enzymes at 5 months after the initial liver biopsy (approximately 3 months after the completion of DAA treatment).The histopathologic findings of repeat liver biopsy again showed marked portal and lobular mixed inflammatory infiltrate comprised of lymphocytes, abundant plasma cells, and numerous eosinophils and neutrophils with interface activity, consistent with alloimmune hepatitis rather than acute cellular rejection. Hepatitis C viral load was again undetectable. Of note, a repeat testing for rheumatoid factor was positive, approximately 16 months after the diagnosis of alloimmune hepatitis.

Discussion

Alloimmune hepatitis is a rare cause for graft dysfunction that can occur among patients who have undergone LT for reasons

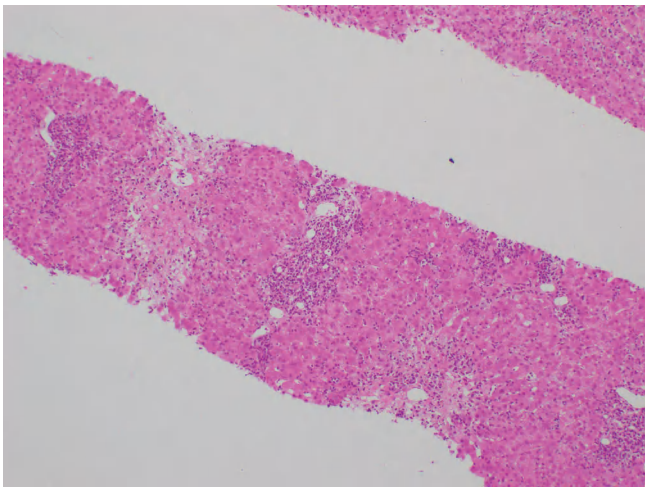

Fig. 1. Both portal and lobular regions demonstrate a mixed inflammatory infiltrate with abundant plasma cells.

Interface activity is present. The portal tract demonstrates extensive necrosis.

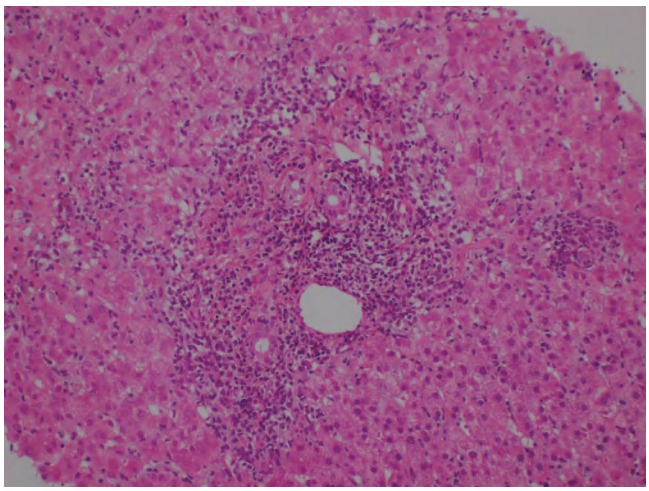

Fig. 2. High-power microscopy shows parenchymal collapse and necrosis. Plasmacytic infiltration is demonstrated.

other than autoimmune hepatitis (AIH).[3–5] Several studies have demonstrated that DAA treatments are well tolerated in liver transplant patients and are successful at achieving sustained virologic response in more than 95% of patients.[6] Serious adverse events can occur, however, including rashes, cytopenia, allograft rejection, severe anemia, and mortality, but these were only reported in 4% of cases[7] and thus our case is an unusual presentation following DAA therapy. The exact pathogenesis is not fully understood but believed to share similar histological and clinical findings with classical AIH.[3] Interferon-based antiviral treatments have been described to precipitate or exacerbate alloimmune hepatitis in the liver transplant population.[8,9] Apart from its antiviral effects, interferon is a potent immunomodulator, and therefore it is not surprising that immune-mediated phenomena, including alloimmune hepatitis, is reported with interferon-based therapy of HCV infection.[10] A possible mechanism for such immune-mediated complications is based on interferon therapy's effects on T-cell activation, which ultimately intensifies pro-inflammatory activity and enhances the presentation and release of antigens.[10]

We made our diagnosis of alloimmune hepatitis based on both histological and immunopathological findings from the liver biopsy, which showed marked portal and lobular mixed inflammatory infiltrate with interface activity. On the liver biopsy, C4D immunostaining was negative, and findings on repeat liver biopsy obtained 5 months after the completion of DAA were again consistent with alloimmune hepatitis rather than acute cellular rejection. We ruled out other causes that may mimic alloimmune hepatitis, such as rejection and viral hepatitis. The patient had a history of vitiligo, which is considered an autoimmune disease. Given the acute onset of the patient's presentation and the lack of evidence of graft rejection or co-infection on liver biopsy, the occurrence of alloimmune hepatitis was likely related to the immune system imbalance induced by a DAA regimen the occurrence of alloimmune hepatitis was likely related to the immune system imbalance induced by a DAA regimen. HCV viral load at the time of diagnosis of alloimmune hepatitis was 537 IU/mL, and the mechanism appeared to be different from an HCV infection-induced autoimmune reaction or drug-induced liver injury. Repeat testing for hepatitis B and C viral loads were all negative. The ImmuKnow™ assay is an immune monitoring test, and the higher test value indicates a greater risk of rejection.[11] In our patient, the ImmuKnow™ assay result was negative.

As the anti-HCV activity of DAA therapy has the potential to stimulate host immunity, activation of the immune system in the setting of rapid elimination of HCV might favor a dysregulation of immune surveillance.[12–14] Although the exact pathogenesis of classic AIHs is not clear, one possible etiology is an imbalance between regulatory T cells and pro-inflammatory cells.[15–18] Regulatory T cells (Tregs) are a subpopulation of T cells that have been demonstrated to suppress pathology in multiple autoimmune diseases. Reduction in frequency and function of Tregs has been reported in peripheral blood in patients with AIH in several studies.[16,17] On the contrary, there is a parallel increase in Treg frequency in the inflamed liver tissue[19] but a decline in the functional capacity of the Tregs in AIH.[21] The functional capacity of Tregs in the liver is essential to control ongoing hepatitis by suppressing the effector cells in the inflamed liver in AIH.[21]

Previous studies showed that IgG4 can down-modulate the immune system, and IgG4-rich infiltrates were more elevated in allograft recipients with *de novo* AIH compared with AIH in native liver or kidney allograft recipients with plasma-cell-rich rejection.[22] In our patient, serological IgG (2,447 mg/dL) was elevated but an IgG4 level was not tested. With a better understanding of the underlying mechanism behind Treg activity in alloimmune hepatitis, further advancements in targeting the Tregs may become alternative treatments to immunosuppressive therapies for treating alloimmune hepatitis. In summary, we report a case of alloimmune hepatitis in a liver transplant patient with recurrent HCV responding to DAA therapy. A thorough follow up of patients undergoing DAA therapy after LT for alloimmune hepatitis might be warranted.

Funding

None to declare.

Conflict of interest

Dr. Pyrsopoulos is a recipient of research grants from Bayer, Beigene, Intercept, Mallinckrodt, Eisai, Novartis, Resusix, Saro, Valeant, Gilead, Genfit, Grifols, Prometheus, and Zydus. Dr. Pyrsopoulos has also served as a consultant for Bayer and Eisai. Dr. Guarrera is a recipient of research grants from and has served a consultant for Organ Recovery Systems Inc for work in Organ Preservation. The other authors have no conflict of interests related to this publication.

Author contributions

Review of the literature (CC, YB, JS, PX), drafting of the manuscript (CC, YB, PX), review of the manuscript (RO, MN, FP, JG), providing figures and descriptions (AJ, MG), critical revision of the manuscript (NTP).

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Case Report

Alcoholic Liver Disease and COVID-19 Pneumonia: A Case Series

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Abstract

The novel coronavirus 2019 (COVID-19) was reported by the World Health Organization in December 2019, and since then it has progressed into a worldwide pandemic, causing significant morbidity and mortality. Gastrointestinal symptoms of COVID-19 and elevated liver chemistries are seen in up to 50% of infected patients. Recent reports have suggested a high mortality rate for COVID-19 in patients with pre-existing liver disease, having an associated mortality of 39.8%. Alcoholic liver disease is a significant cause of morbidity and mortality in New Mexico (USA), and we report here the clinical course and characteristics of three cases of patients with alcoholic cirrhosis who were admitted to our hospital with COVID-19.

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Introduction

The first case of novel coronavirus 2019 (COVID-19) in the USA was reported on January 20, 2020.[1] The USA now has the largest number of confirmed cases in the world; as of July 15, 2020, there were 3,448,625 cases of coronavirus diagnosed in the country, with 136,699 deaths.[2] While liver abnormalities have been reported in patients with coronavirus, most of the related biomarker elevations are mild, with a predominantly hepatocellular elevation ranging from 14-53% and slightly elevated bilirubin in 14-53% of patients.[3,4]

There is limited evidence on the outcomes of COVID-19 in patients with alcoholic liver disease. A meta-analysis showed 3% prevalence of chronic liver disease in patients with COVID-19.[5] Bangash *et al.*[6] considered that abnormal liver biochemistries do not necessarily arise from the liver alone; in fact, several other reasons, such as COVID-19-induced myositis as well as collateral liver damage from induction of a dysregulated immune response and drug-related liver injury, are more likely to explain deranged liver biochemistries in COVID-19.

In data collected from seven Chinese studies, mortality occurred in only 0-2% of patients with chronic liver disease; however, the nature and severity of the liver disease were unknown.[6] The SECURE-Cirrhosis registry and COVID-HEP reported on 334 patients with cirrhosis, out of which 102 (31%) patients had alcoholic cirrhosis; however, outcomes were not defined by etiology of cirrhosis.[7] Our hospital is a tertiary care center in a state with disproportionately excessive alcohol use and alcohol-related liver disease deaths (22 per 100,000 population)[8] compared with other USA states. In this case series, we describe the clinical presentation, management, and outcomes of patients with alcoholic cirrhosis and COVID-19.

Case report

A retrospective chart search was performed (under institutional IRB 20-186) for patients with a past medical history of alcoholic cirrhosis and diagnosis of COVID-19. The patients had been consecutively admitted to the University of New Mexico Hospital from December 1, 2019, to April 23, 2020. Informed consent was waived as part of the institutional IRB. Only patients with a laboratory-confirmed (reverse transcription-PCR-positive) diagnosis for COVID-19 were included.

Information was collected regarding comorbidities, social history, vital signs, demographics, clinical characteristics, symptomatology, alcohol use, lab results, imaging characteristics, and clinical management details. The authors manually analyzed the data.

Case 1

A 32-year-old male with a past medical history of alcoholic cirrhosis, and class I obesity [body mass index (BMI) of 30.5], presented intubated and sedated after he was found unresponsive at home. Baseline model for end-stage liver disease (MELD) labs were not available; however, per his family, the patient was a heavy drinker and was actively drinking alcohol up to 2 days before.

On evaluation, the patient was in multiorgan failure, attributed to septic shock due to COVID pneumonia. He required significant vasopressor support, acute hypoxemic respiratory failure due to acute respiratory distress syndrome (ARDS), requiring mechanical ventilation, and acute kidney injury (AKI) requiring continuous renal replacement therapy (CRRT). His physical exam was significant for bruising on his

Keywords: COVID-19; Alcoholic liver disease; ARDS; Acute kidney injury.
Abbreviations: AKI, acute kidney injury; ALP, alkaline phosphatase; ALT, alanine aminotransferase; ARDS, adult respiratory distress syndrome; AST, aspartate aminotransferase; BMI, body mass index; CK, creatinine kinase; COVID-19, novel coronavirus 2019; CRRT, continuous renal replacement therapy; Fio2, fraction of inspired oxygen; INR, international normalized ratio; MELD-Na, model for end-stage liver disease, sodium; Na, sodium; PEEP, positive end-expiratory pressure; PT, prothrombin time.
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extremities, diffuse anasarca, and jaundice. Liver biochemistries on presentation were an aspartate aminotransferase (AST) level of 276 U/L, alanine aminotransferase (ALT) level of 60 U/L, and alkaline phosphatase (ALP) level of 269 U/L. Total bilirubin was elevated to 15 mg/dL and direct bilirubin to 10.4 mg/dL. MELD-sodium (MELD-Na) on presentation was 36, and Child-Pugh class was C. Coagulation profile was deranged as well, with a prothrombin time (PT) 29s and international normalized ratio (INR) of 2.4. D-dimer was elevated at 6951 μg/mL, and platelet count was 92,000/mL. He was also severely hyponatremic, with serum sodium of 116 mg/dL, and anuric, with serum creatinine of 4.81 mg/dL. Lactate dehydrogenase was elevated to 609 U/L. A nasopharyngeal swab returned positive for COVID-19. Serologies for hepatitis A, B, and C were performed and were negative.

The elevated INR, D-dimer, ferritin, lactate dehydrogenase and C-reactive protein were thought to be due the increased inflammatory response seen with COVID-19, and the patient's respiratory deterioration was thought to be due to the effects of COVID-19. Therapeutic-dose heparin was, therefore, not started. Computed tomography scans of the chest and abdomen were performed at day 12 of his hospitalization, and while this was not arterially gated, no large pulmonary embolus was noted. A bedside echogram was also performed, which did not show evidence of right heart strain. A peripheral blood smear did not show any evidence of schistocytes.

On day 3 of hospitalization, the patient's cardiopulmonary status began to improve, with decreased fraction of inspired oxygen (FiO2) and positive end-expiratory pressure (PEEP) requirements. Unfortunately, he continued to be oliguric and in hepatic failure, with an AST of 317 U/L, ALT of 110 U/L, bilirubin of 17.8 mg/dL, and INR of 1.64 (Table 1). Also, he experienced an acute gastrointestinal bleed, with a drop in hemoglobin from 12.4 g/dL on admission to 7 g/dL, with coffee-ground contents in his nasogastric tube on suction and melena requiring 3 U of packed red blood cells as well as vitamin K administration. Octreotide and proton pump inhibitor infusions were started, and on day 2, overt bleeding had stopped, and no endoscopic intervention was performed. He also received a 10-day course of azithromycin and hydroxychloroquine, according to an ongoing hospital-based clinical trial (unpublished data). A computed tomography scan of his abdomen showed extensive ascites and a nodular-appearing liver as well as dilated bowel loops suggestive of shock bowel. Unfortunately, his septic shock continued to worsen with leukocytosis, poor oxygenation, and elevated lactate levels, and care was withdrawn per family wishes on day 13.

Case 2

A 34-year-old male with a known history of alcoholic cirrhosis, Class II obesity (BMI of 35) and active alcohol use was brought into the hospital intubated and sedated, after

Table 1. Characteristics of cases

	Case 1	Case 2	Case 3
Age in years	32	34	44
Sex	Male	Male	Male
BMI in kg/m^2	30.5	35	41.7
Symptoms reported	Chills, myalgias, and encephalopathy	Dyspnea, abdominal pain, and encephalopathy	Dyspnea, cough
Comorbidities	Alcohol use disorder Obesity Tobacco abuse disorder	Alcohol use disorder Obesity	Alcohol use disorder History of spontaneous bacterial peritonitis Hypertension Pulmonary hypertension Obesity
Anticoagulation administered	Heparin (deep vein thrombosis prophylaxis)	None	Heparin (deep vein thrombosis prophylaxis)
Length of stay in days	13	2	6
Na, 134-144 mmol/L	116	132	134
PT, 9.4- 15.4 s/INR, 0.8 - 1.3 ratio	29.2/2.43	16.8/1.41	15.3/1.29
Total protein/albumin, 6.1-8.1/ 3.4-4.7 g/dL	5.6/2.3	4.8/1.8	5.1/1.0
Cr, 0.62-1.66 mg/dL	4.81	4.25	1.71
AST, 6-58 U/L	276	4969	73
ALT, 14-67 U/L	60	6350	39
ALP, 38-150 U/L	269	160	147
Total bilirubin/direct bilirubin, 0.3-1.2/0.1-0.4 mg/dL	15.1/10.4	5.1/3.6	2.7/ 1.4
Procalcitonin, <0.10 ng/mL/ lactate, 0.4-2.0 mmol/L	1.59/2.2	1.58/13.3	0.12/1.7

presenting to a peripheral hospital with abdominal pain and shortness of breath. His physical exam was unremarkable. He had severely deranged liver biochemistries, with an AST of 4969 U/L, ALT of 6350 U/L, ALP of 160 U/L, and bilirubin of 5.1 mg/dL. MELD-Na on presentation was 32 and Child-Pugh class was C. Creatinine kinase (CK) was elevated to 2095 U/L and lactate was elevated to 13 m/g/dL, suggesting ischemic hepatitis in the setting of shock. Platelet count was low at 54,000/dL, and his coagulation panel was abnormal, with an INR of 1.4. A nasopharyngeal swab was positive for COVID-19. Tests for markers of acute and chronic hepatitis A, B and C were negative. During the day, his hemodynamic instability worsened, requiring the addition of a fourth pressor and stress-dose hydrocortisone. CRRT was initiated, and inhaled nitric oxide was administered due to persistent hypoxemia. Despite all interventions, he continued to deteriorate and died of septic shock, presumed due to COVID-19 pneumonia.

Case 3

A 44-year-old man with Class III obesity (BMI of 41.7) and alcoholic cirrhosis complicated by esophageal varices, as well as a history of spontaneous bacterial peritonitis and pulmonary hypertension was transferred to our hospital for acute hypoxemic respiratory failure secondary to COVID-19, requiring intubation and vasopressor support. His physical exam was significant for diffuse anasarca and jaundice.

Liver biochemistries on admission showed an AST of 73 U/L, ALT of 19 U/L, ALP of 147 U/L, and bilirubin of 2.7 mg/dL. MELD-Na on presentation was 21 and Child-Pugh class was C. The patient's INR was 1.29, with a PT of 15.3 s and creatinine of 1.71 mg/dL. Platelet count was low at 88,000 /mL. He was started on CRRT due to oliguric AKI, azithromycin, and hydroxychloroquine were administered per an ongoing clinical trial (unpublished data). While his liver biochemistries remained stable, he developed severe encephalopathy, and his cardiopulmonary status continued to worsen. Due to his poor prognosis and lack of improvement, his family decided to withdraw care, and he died 6 days after admission.

Discussion

Our cases represent the only patients presenting with alcoholic cirrhosis and COVID-19 to our tertiary hospital through the study period,[9] representing a 100% mortality rate. The severity of liver disease at baseline was not known in cases 1 and 2, as they were transferred from an outside facility with no available medical records; however, case 3 had decompensated cirrhosis with varices as well as a history of spontaneous bacterial peritonitis. All patients were obese, with an average BMI of 35.7 (BMI >30 in all) and were actively drinking before symptom onset. All patients required critical care with aggressive cardiopulmonary resuscitation. All three patients experienced a profound cardiopulmonary collapse, suggestive of septic shock due to COVID-19 pneumonia; however, with the presence of elevated inflammatory markers, COVID-19-induced cytokine storm cannot be ruled out.

Two patients had elevated hepatocellular biochemistries, up to 10 times the upper limit of normal (case 2 likely due to ischemic hepatitis); however, all three patients had disproportional direct hyperbilirubinemia, likely due to the hepatic decompensation in the setting of alcoholic cirrhosis. Two out of the three patients (case 1 and case 3) were started on prophylactic heparin for increased thromboembolic risk. No gastrointestinal bleeding was seen in case 3, although he had known esophageal varices. Case 1 had coffee ground-like material in his nasogastric tube as well as melena, and required blood transfusions. An endoscopy was deferred due to his clinical condition. His bleeding stopped with octreotide administration.

The severity of illness in these cases (three men below the age of 45 (with alcoholic cirrhosis and COVID-19 corroborates with the high percentage of deaths reported by Moon *et al.*[7] In addition to obesity, which is already a known risk factor for poor outcomes,[10] a history of alcoholic cirrhosis and active drinking points towards hepatic decompensation worsening morbidity. Direct kidney involvement in COVID-19 was reported to be low, at only 4.5%;[11] however, subsequent analyses from Italy showed the prevalence to be as high as 15%,[12] especially in patients with severe disease in the Intensive Care Unit setting. All three of our patients required CRRT to address oliguric AKI. While other reasons, including cytokine damage, organ crosstalk and systemic effects, have been cited,[13] dysfunction of the hepatorenal axis should also be considered. The requirement of mechanical ventilation is yet another poor prognostic factor in patients with COVID-19, which may have also contributed to the outcomes in our case series.[14]

We believe these cases are an example of a unique set of problems faced by hepatologists and critical care teams managing COVID-19 patients with decompensated liver disease. Recognition and discussion of a poor prognosis should be considered early on in these patients. Gastrointestinal bleeding, as well as secondary infections, should be recognized and managed promptly. Finally, as a preventative measure, hepatologists should reinforce the importance of abstinence during outpatient visits in patients with alcoholic liver disease.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Conception of the study, writing of the paper (DK, RS), data collection (SU, RS), critical review and writing of the paper (ET, DK).

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Case Report

COVID-19 in Liver Transplant Recipients - A Series with Successful Recovery

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Abstract

The severe acute respiratory syndrome corona virus-2 (referred to as SARS-CoV-2) pandemic had a great impact on public life in general as well as on populations with pre-existing disease and co-morbidities. Liver transplant and immunosuppressant medication predisposes to more severe disease and is often associated with poor outcome. The clinical features, disease course, treatment and process of modulating the immunosuppression is challenging. Here, we describe the clinical presentation, treatment and outcomes in six liver transplant recipients. Out of those six patients, three had mild, one had moderate and one had severe COVID-19, and one was asymptomatic. The immunosuppression minimization or withdrawal was done based upon the clinical severity. Consideration of tocilizumab and/or convalescent plasma as well as antivirals i.e. remdesvir done in severe cases. The routine practice of prophylactic anti-coagulation, consideration of repurposed drugs (i.e. teicoplanin and doxycycline), and watchful monitoring of asymptomatic recipients helped to achieve an uneventful recovery.

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Introduction

A cumulative total of nearly 25 million cases and 800 000 deaths have been reported since the start of the outbreak of severe acute respiratory syndrome corona virus-2 (SARS-CoV-2).[1] The majority of cases often present with mild symptoms, like fever, cough and shortness of breath; however, the severity of symptoms increases with presence of co-morbidities and pre-existing diseases, such as the presence of chronic liver disease.[2] The data on immunosuppression therapy, post-transplant status and impact of SARS-CoV-2 infection on a liver graft as well as the overall survival in liver graft recipients is largely inadequate. A similar lack of information is present regarding the treatment, drug interaction and overall outcome with solid organ transplant and corona virus disease 2019 (COVID-19).

Recently published data from the European Liver Transplant Registry (ELTR) of 103 cases showed high mortality (i.e. 16%), with a much higher rate among those on ventilator support (44%), above 60 years of age, and transplanted at 2 years or more before the COVID-19 disease.[3] To publication, more than 540,000 confirmed cases of COVID-19, with 16,475 deaths, have been reported from India.[1] Every year, on average more than 1500 liver transplants (LTs) are carried out in India, but the effects of infection with SARS-CoV-2 and the implications of such are not yet reported.[4] One study was conducted on patients admitted to Institute of Liver and Biliary Sciences (ILBS) New Delhi, India, which is a high-volume transplant center (>100 cases per year), working with live donors mainly. Today, there is a pandemic situation, with nearly 150,000 active cases and a seroprevalence of COVID-19 at 23% in the population.[5]

This present series of six LT recipients, all within the spectrum of presentation (i.e. asymptomatic, mild, moderate and severe cases), were managed well and recovered successfully.

Case series

The present series of six cases include one severe, one moderate and three mild COVID-19 diseases, and one asymptomatic SARS-CoV-2 infection. The clinical characteristics (Table 1) and laboratory data (Table 2), along with the timeline for maintenance immunosuppression and COVID-19-specific treatments and outcome are shown. Diagnosis was performed by nasopharyngeal swab test with RT-PCR. Chest radiography and high-resolution computed tomography were performed selectively, upon diagnosis of moderate

Keywords: Liver transplant; COVID-19; SARS-CoV-2; Liver injury; Remdesivir; Convalescent plasma.

Abbreviations: COVID-19, corona virus disease 2019; LDLT, live donor liver transplant; LT, liver transplant; SARS-CoV-2, severe acute respiratory syndrome corona virus-2.

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and severe COVID-19 disease as per World Health Organization definition. All the patients were managed in a dedicated COVID-19 ward with intensive care unit facility at Institute of Liver and Biliary Sciences.

Severe COVID-19 in a post-LT recipient

A 52 year-old gentleman, post-live donor (LD)LT for decompensated nonalcoholic steatohepatitis cirrhosis (8 months back) presented with high-grade fever, cough (for 4 days), and increasing shortness of breath (for 1 day). At admission, he was febrile with temperature of 99°F, oxygen saturation of 92% at room air, respiratory rate of 22 breaths/m, blood pressure of 120/80 mmHg, and pulse rate of 112/min. Chest X-ray showed bilateral lower lobe infiltrations. Chest computed tomography revealed mixed diffuse ground-glass opacities with multifocal patchy consolidations involving both lungs, consistent with atypical infection likely due to SARS-CoV-2 (Fig. 1). The laboratory results (Table 2) reflected total normal leukocyte count with lymphopenia (8%), high neutrophil-to-lymphocyte ratio 10 and thrombocytopenia (82×10^3/cc) with nearly normal bilirubin, aspartate transaminase, Alanine transaminase, serum alkaline phosphatase and gamma-glutamyl transpeptidase. After admission to the intensive care unit, his hypoxia required support by noninvasive ventilation and he was started on dexamethasone (6 mg once daily), loading dosage of tocilizumab (8 mg/kg, 400 mg), hydroxychloroquine (400 mg twice daily), antibiotics (piperacillin-tazobactam and teicoplanin), and prophylactic enoxaparin. Tacrolimus as well as mycophenolate was kept on-hold, temporarily. The dexamethasone at low dose helped in low-dose maintenance for the LT status, in addition to its role in COVID-19. In view of persistent hypoxia (oxygen saturation of <94%), tachypnoea and fever, remdesivir was started (200 mg bolus, followed by 100 mg once daily) on the third day with a second dose of tocilizumab, and hydroxychloroquine was stopped. However, on day 7, he again experienced respiratory distress (oxygen saturation of 90%), requiring10 L/m of oxygen on high-flow nasal cannula. The convalescent plasma was planned at admission but deferred due to resource constraint; however, it was able to be transfused on day 8 (delayed, but considered as rescue therapy), along with remdesivir being continued for 10 days. The patient improved over time, his oxygen requirement was maintained with 2 L of oxygen. X-ray showed improvement, and the fever, neutrophil-to-lymphocyte ratio, and thrombocytopenia improved by the 10th day (Fig. 2). As of day 14, he was maintaining oxygen saturation at room air, and the tacrolimus with prednisolone treatment was reintroduced without mycophenolate.

Moderate COVID-19 in post-LT recipient

A 39 year-old gentleman, 6 years from LDLT for decompensated ethanol-related cirrhosis with diabetes, hypertension, obesity and biliary anastomotic stricture (stent-free for 2 years), presented with 3-day history of fever and dry cough. At presentation, he was febrile with temperature of 100°F, oxygen saturation of 96% under ambient air, respiratory rate of 22 breaths/m, blood pressure of 136/84 mmHg, and pulse rate of 102/m. Chest X-ray showed bilateral lower lobe infiltrations. Chest computed tomography revealed features atypical of pneumonia or viral pneumonia (Fig. 3). The previous immunosuppression regimen (i.e. tacrolimus and mycophenolate) was stopped and the patient was maintained on prednisolone (20 mg once daily) and 2 L/m oxygen by nasal canula. He received hydroxychloroquine (400 mg twice daily) and prophylactic anticoagulation. However on day 8 of the hospital stay, he had a spiking fever [up to 102°F, worsening leukopenia (of 2.4×10^3/cc), increasing neutrophil-to-lymphocyte ratio (to 4.8), and respiratory distress with oxygen saturation of 90%, requiring 8 L/m of oxygen by high-flow nasal cannula]. Therapy with remdesivir, tocilizumab or convalescent plasma was unavailable, so he was started on teicoplanin and doxycycline (100 mg twice daily), with antibiotics upgraded upon suspicion of nosocomial pneumonia, despite culture and procalcitonin showing non-contributory roles. The patient became afebrile with improvement in clinical symptoms in the next 72 h. He was discharged with low-dose tacrolimus (0.5 mg twice daily) and prednisolone (5 mg, once daily starting on the 15th day), and with a plan for resuming previous immunosuppression on follow-up.

Mild COVID-19 in post-LT recipients

We successfully managed three mild COVID-19 patients with mild disease, and all had a separate background of disease in addition to their post-transplant status. The first one was a 48 year-old male with pre-transplant hypertension, hypothyroidism and receipt of a deceased donor graft 6 years back. He presented with a 2-day history of fever and cough. At admission, he was febrile, oxygen saturation of 98% under ambient air, respiratory rate of 18/m, with normal chest X-ray findings. The second was a 50 year-old gentleman, who was 7 months post-LDLT with biliary anastomotic stricture and a Percutaneous Transhepatic Billiary Drainage (PTBD) catheter *in situ*. He presented with a 2-day history of fever and a sore throat. At presentation, his temperature was 99.6°F, oxygen saturation was 95% under ambient air, and respiratory rate was 22 breaths/m. Initial suspicion of cholangitis was kept; however, in view of the current pandemic as well his sore throat, we tested for SARS-CoV-2 and obtained a positive result. Antibiotic coverage with meropenem and fluconazole continued, due to deranged Liver function parameters (cholestatic pattern, prolonged PTBD, but a negative bile culture). The tacrolimus was stopped, and the patient was maintained on prednisolone (20 mg once daily). The third patient was a 38 year-old gentleman, being 5 years' post-LDLT, who presented with a 2-day history fever and dry cough and a 1-day history of shortness of breath. He had a bad post-transplant course with recidivism, graft failure, development of cirrhosis after transplant and chronic kidney disease due to immunoglobulin A nephropathy and maintenance dialysis. At presentation, he was febrile (99.4°F), oxygen saturation of 92% under ambient air, respiratory rate of 22/m, and blood pressure of 170/90 mmHg. Chest X-ray was suggestive of central prominence suggestive of fluid overload, and testing for SARS-CoV-2 gave positive result. Urgent hemodialysis was performed to address metabolic acidosis, high creatinine, anuria and respiratory distress.

All these patients, managed in the COVID-19 ward, received hydroxychloroquine and prophylactic enoxaparin, stoppage of immunosuppression, and were maintained on low-dose prednisolone (10-20 mg per day). The previous immunosuppression was started on day 14 and all had a negative throat swab result for SARS-CoV-2 at discharge.

Table 1. Baseline characteristics

	Patient 1, Severe	Patient 2, Moderate	Patient 3, Mild	Patient 4, Mild	Patient 5, Mild	Patient 6, Asymptomatic
Age in year	52	39	48	50	38	48
Gender	Male	Male	Male	Male	Male	Male
Co-morbidity	Diabetes	Diabetes	Hypertension Diabetes, Hypothyroid	None	Hypertension Chronic Kidney Disease	Diabetes, Hypertension
Time from LT in months	16	72	72	17	60	24
Etiology	Nonalcoholic steatohepatitis	Ethanol	Nonalcoholic steatohepatitis	Ethanol	Ethanol	Ethanol
Post-LT events, if any	Moderate acute cellular rejection (>5 months back)	Biliary anastomotic stricture (stent-free), early chronic Rejection	Severe acute cellular rejection	Biliary anastomotic stricture with PTBD *in situ*	Recidivism with cirrhosis	None
Immunosuppressive regimen	Tacrolimus 2 mg/day and mycophenolate 2 g/day	Tacrolimus 1 mg/day and mycophenolate 2 g/day	Tacrolimus 2 mg/day	Tacrolimus 2 mg/day	Steroids only, maintenance dialysis	Everolimus 2 mg/day
Symptoms of COVID-19	Fever, cough, shortness of breath	Fever, cough	Fever, cough	Fever, sore throat	Fever, diarrhea	Asymptomatic
Anticoagulation prophylaxis	LMWH	LMWH	LMWH	LMWH	LMWH	No
Antibiotics	Meropenem, teicoplanin, fluconazole	Meropenem, teicoplanin, doxycycline	Cefepime	Meropenem, teicoplanin, fluconazole	Meropenem, teicoplanin, fluconazole	No
Respiratory support	NIV	High-flow nasal cannula	No	2L/min O2	No	No
Antiviral	Remdesivir	No	No	No	No	No
Duration of stay in days	14, hospitalized	14	10	10	10	Home isolation

Abbreviations: LMWH, low molecular weight heparin; PTBD, percutaneous transhepatic billiary disease; NIV, noninvasive ventilation.

Table 2. Laboratory parameters

Investigations	Patient 1	Patient 2		Patient 3		Patient 4		Patient 5		Patient 6	
	Baseline 24/06/20	Baseline 29/05/20	Discharge 12/06/20	Baseline 20/06/20	Discharge 30/06/20	Baseline 18/06/20	Discharge 29/06/20	baseline 09/06/20	Discharge 19/06/20	Baseline 14/06/20	Discharge 24/06/20
Total white blood cell count as 10^3/cc	7000	3300	5300	8800	8700	9300	7200	7300	6800	11100	9700
Neutrophil, %	88	82	60	53	67	85	67	82	80	72	70
Lymphocyte, %	10	25	36	30	31	9	30	16	18	25	28
Neutrophil-to-lymphocyte ratio	7.5	4.2	2.5	1.5	2.5	9.1	2.5	5.1	4.2	2.9	2.9
Platelet count as 10^3/cc	82	42	130	145	179	202	150	83	75	423	317
Bilirubin, mg/dL	0.6	1.6	0.9	0.6	0.4	1.2	0.8	0.9	0.8	0.5	0.3
Aspartate transaminase, IU/L	37	40	40	52	29	195	18	48	26	46	32
Alanine aminotransferase, IU/L	42	44	47	66	42	108	34	34	33	50	36
SAP, IU/L	74	221	347	63	68	378	454	296	180	148	159
GGT, IU/L	212	503	682	52	58	571	522	467	267	66	70
INR	1.03	1.1	1.05	1.1	1.2	1.1	1.15	1.32	1.22	1.32	1.33
Serum albumin, g/dL	2.83	2.6	3.2	2.67	3.37	2.7	3.9	3.02	3.39	3.3	3.39
Serum creatinine, mg/dL	0.66	0.76	0.4	1.13	0.8	0.6		19.2	5.00	1.2	1.19
FDP, mcg/mL	<5	<5		<5		<5					
D-dimer (ng/ml)	<0.5	<0.5		<0.5		<0.5					
TNF-alpha, pg/mL	20	17		13.7		30					
IL-6, pg/mL	296/175	113		2.0		91					
Ferritin, mcg/L	101	123		353		121					
LDH, IU/L	615	335		131		225					

Abbreviations: FDP, fibrin degradation products; GGT, gamma-glutamyl transferase; IL-6, interleukin 6; LDH, lactate dehydrogenase; SAP, serum alkaline phosphatase; TNF, tumor necrosis factor.

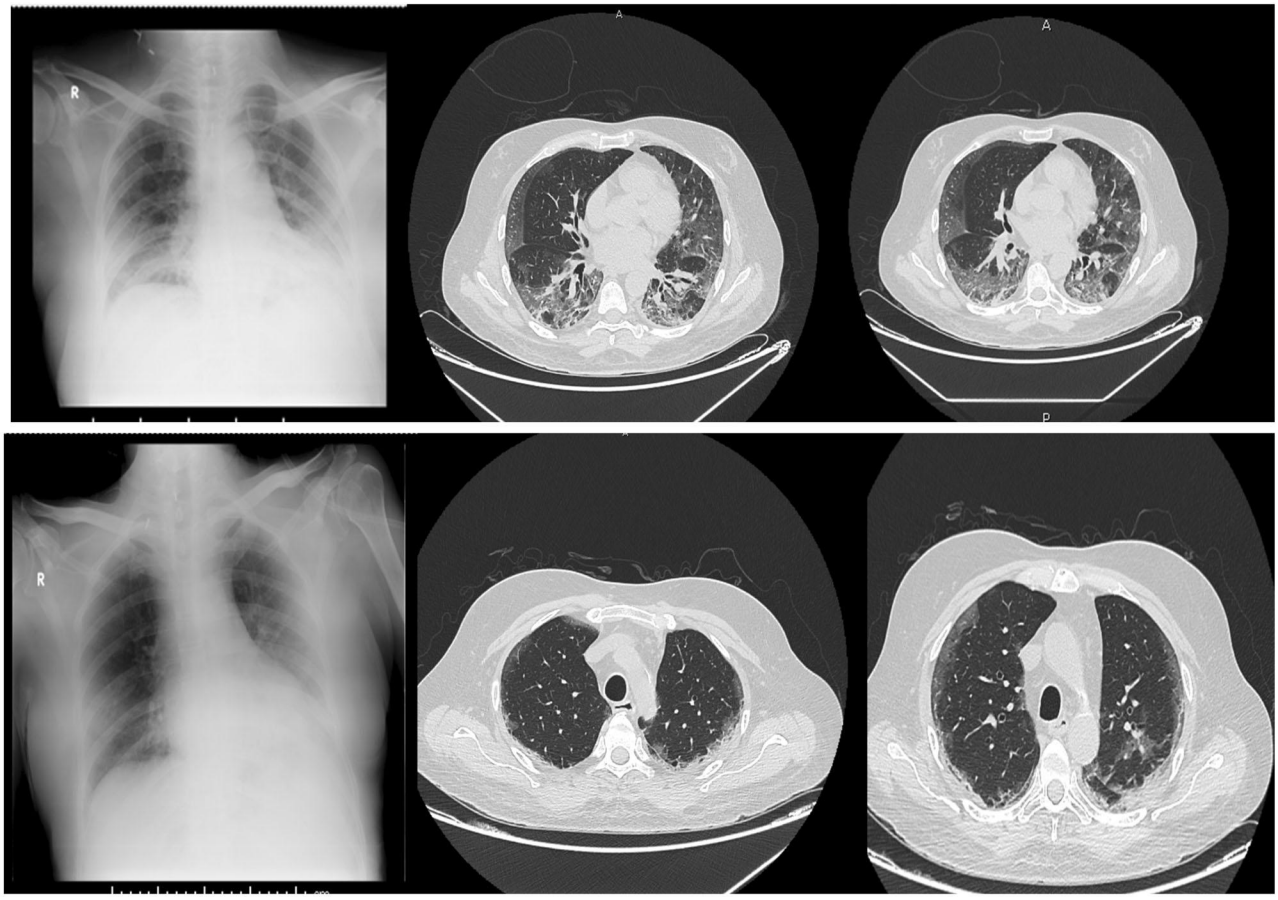

Fig. 1. X ray of chest and CT Thorax at admission (upper panel) and on day 10 (lower panel) which showed improvement, post tocilizumab and convalescent plasma of COVID-19.

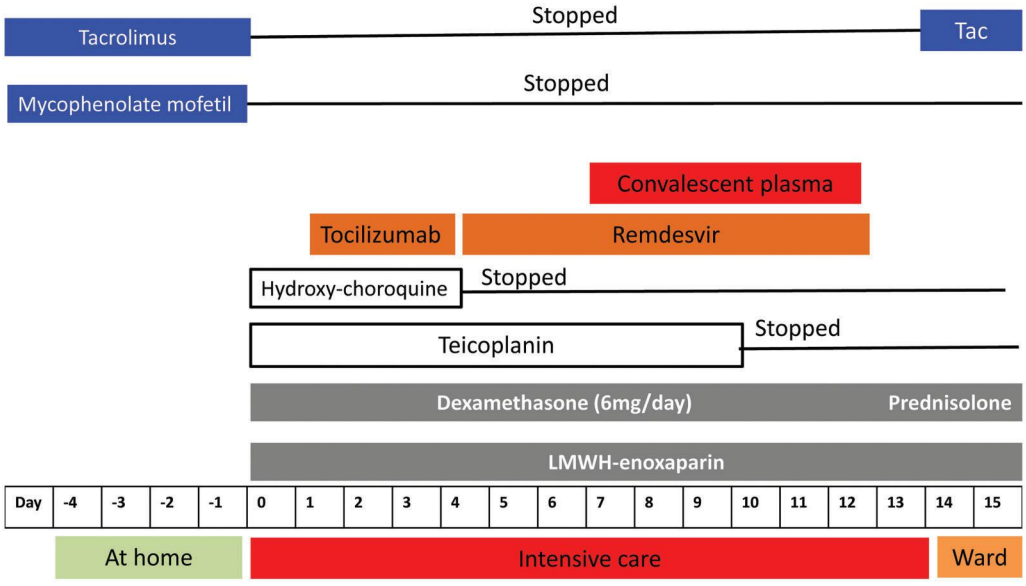

Fig. 2. Treatment timeline of severe COVID-19 case.

None had deranged liver function, due to immunosuppression minimization for 2 weeks during the COVID-19 infection.

Asymptomatic COVID-19 disease in a post-LT recipient

This 48 year-old male, 18 months post-LDLT, had a close family contact with a symptomatic COVID-19 case and was asymptomatic positive for SARS-CoV-2 upon testing. He had multiple co-morbidities (i.e. diabetes, hypertension, obesity and had calcineurin inhibitor nephrotoxicity-induced kidney dysfunction, maintained on a calcineurin inhibitor nephrotoxicity-free regimen with everolimus). He was comfortable, afebrile and monitored from home. His everolimus was stopped, and he was given prednisolone (10 mg once daily) without hydroxychloroquine or any COVID-19-specific drug. In view of no symptoms or disease progression, low-dose tacrolimus (0.5 mg twice daily) and prednisolone (5 mg once daily) was started on day 5, instead of the everolimus. His subsequent SARS-CoV-2 tests, given at two occasions on day 12 and day 14, were negative. The previous calcineurin inhibitor nephrotoxicity-free regimen (i.e. everolimus) was started on day 15 and there was no derangement of graft function during these period.

Discussion

The SARS-CoV-2 infection and outcome among solid organ transplant recipients is variable. Whether immunosuppression therapy is a risk is largely unknown, but the severity of disease and outcome has been generally poorer than observed in others. The dosage of immunosuppression at infection and COVID-19 disease severity is poorly correlated.[6] Most of the recommendations have been for minimization or temporary withdrawal and balance of risk for rejection. However, this modification is individualized but mostly agrees for stopping the antiproliferative drug, reducing or stopping calcineurin inhibitor nephrotoxicity drugs and maintaining on a low dose of steroid.[7] The same course was followed in the present series and none experienced a rejection.

Severe or moderate COVID-19 often has poor outcome among transplant recipients, with mortality in 12-18% of cases.[8,9] The multimodal approach with combination of an antiviral, tocilizumab, has been reported with good outcome.[9] In our series, the severe case (despite early use of tocilizumab and remdesivir) had a protracted course and addition of convalescent plasma likely helped in recovery for this patient. Convalescent plasma action occurs through binding of the transfused antibodies to the pathogen, resulting in cellular cytotoxicity, phagocytosis, or direct neutralization of the pathogen.[10] One large study showed that early administration of antibodies led to an optimal clinical effect, as compared to later administration.[11] However, the data on transplant recipients need to be studied in larger cohorts to determine a routine recommendation.

In the moderate cases of our series, the limited availability of remdesivir or plasma represented a real-world scenario. Consideration of teicoplanin and doxycycline as repurposed drugs helped in the infection's resolution. Teicoplanin is a glycopeptide antibiotic, found to be active *in vitro* against SARS-CoV in the early stage of the viral life cycle, working by inhibiting the low-pH cleavage of the viral spike protein by cathepsin L and in the late phase by action on the endosomes, thereby preventing the release of genomic viral RNA and causing disruption of the virus replication.[12] Doxycycline, often used for atypical pneumonia, has been studied recently for SARS-CoV-2 chemoprophylaxis[13] but regarding its use for therapy the data is scanty. The antiviral effects are secondary to transcriptional up-regulation of the intracellular zinc finger antiviral protein ZAP and repression of RNA translation.[13] In patients with moderate disease who are transplant recipients with multiple co-morbidities, out more than 2 years after the transplantation, leucopoenia and thrombocytopenia remain challenging scenarios to be managed without tocilizumab or antivirals, like remdesivir.[14–16]

Another important consideration is the natural course of asymptomatic SARS-CoV-2 in a transplant recipient with multiple co morbidities (i.e. diabetes, hypertension and overweight). It was recently shown that 11 of 96 asymptomatic patients developed symptoms, and it was suggested that this could occur more with increasing age.[17] Kumar *et al.*[18] showed the most frequent abnormality in liver functions was hypoalbuminemia, followed by derangements in gamma-glutamyl transferase and aminotransferases, and these abnormalities were more frequent in severe disease.[18] The results were the same in our study. The consensus is lacking for this group of patients; few have suggested close monitoring, while others in favor of early antiviral therapy to prevent prolong shedding.[19] Data are limited for treatment in an immunocompromised person or transplant recipients.[20] The asymptomatic case was managed well by immunosuppression modulation only, with no disease progression or development of symptoms; however, large series are needed to support or refute our findings.

To summarize, we reported six cases of COVID-19 disease in LT recipients with co-morbidities, who were successfully managed. These cases included one severe and one moderate case. Stoppage of antiproliferative or antimetabolites, temporary tacrolimus withdrawal and low-dose maintenance steroid was followed. Severe cases, those with COVID pneumonia, thrombocytopenia or high neutrophil-to-lymphocyte ratio with lymphopenia should be considered for remdesivir and tocilizumab. Convalescent plasma therapy is usually preserved for severe cases in the absence of evidence, mostly for compassionate use or under clinical trial. Therapeutically repurposed drugs with minimal adverse effects, like teicoplanin and doxycycline, can be considered in resource-poor settings.

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Conflict of interest

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Author contributions

Conceptualized the study (AC and SKS); drafted the manuscript (AC), collected the data (GSR, SV), revised the manuscript and generated the figure (VP, SMS, VSTP, RPM, AT, LGM, DB), provided final correction of the manuscript and all logistic support (SKS).

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Case Report

Transaminitis in a Three-year-old Boy with Duchenne Muscular Dystrophy

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Abstract

Duchenne muscular dystrophy (DMD) is a fatal X-linked genetic disease of the neuromuscular system and is the most serious type of muscular dystrophy in humans. The disease is characterized by progressive muscular atrophy and a poor prognosis. The incidence rate is 1/3500, and symptoms appear at age of 5 years-old. Some patients present with abnormal aminotransferases as the first symptom. In addition to the clinical characteristics and genetic history, electromyography examination, muscle biopsy, serum enzyme examination, and measures of creatine kinase (CK), CK isoenzyme, and serum lactate dehydrogenase are important features of auxiliary examination. Clinicians who encounter unknown causes of transaminitis should consider the possibility of DMD. We describe here a 3 year-old pediatric patient with increased aminotransferases who had elevated CK and a family genetic history but without liver damage on computed tomography. He was suspected as having inherited the disorder and was finally diagnosed as having DMD by next-generation sequencing.

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Case report

On August 22, 2019, a 3 year-old male received an annual physical examination at his local hospital. His blood tests showed alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels of 588 U/L and 492 U/L, respectively, negativity for serum hepatitis B surface antigen and positivity for hepatitis B surface antibody, and normal serum total bilirubin. Thereafter, on October 1, 2019, blood ALT and AST levels were 580 U/L and 302 U/L, respectively, and B ultrasonic examination revealed thickening of the intra-hepatic bile-duct wall and echo. He was admitted to the Department of Infectious Diseases, Shunde Hospital of Southern Medical University on October 1, 2019.

Since onset of the disease, he has had a good appetite and normal mood, no headaches or dizziness, no chest tightness or pain, no shortness of breath, no abdominal pain or distension, no skin itching, no bleeding from the mouth or nose, normal sleep patterns, normal urination and defecation, no weight loss, and has been able to carry out daily activities as normal. An investigation of the boy's family history revealed that his grandfather had chronic hepatitis B. The patient's uncle became weak and a little unsteady on his feet at 7 years-old, then became paralyzed and died at the age of 17 (unknown cause of death). Another of the boy's uncles and the uncle's son had "congenital lameness" (no specific information). The second child of the patient's mother died when she was 30-weeks pregnant.

Physical examinations, including of the nerve and muscular systems, found no abnormalities. Biochemical tests showed his ALT and AST to be 526 U/L and 408 U/L, respectively. All hepatitis viral markers, including cytomegalovirus and Epstein-Barr virus, were negative on PCR and immunoblot assay. Ceruloplasmin, blood copper, r-glutamyl transpeptidase, and alkaline phosphatase levels were normal. Autoimmune liver disease antibodies were negative. Creatine kinase (CK) and CK isoenzyme (CK-MB) were 42320 U/L and 700 U/L, respectively, and 3-hydroxybutyrate dehydrogenase was 1840 U/L. Serum lactate dehydrogenase was 2496 U/L and free fatty acids levels were 63 μmol/L. His upper abdomen was normal on computed tomography scan, chest x-ray and electrocardiograph findings were normal, and the heart was normal on ultrasound examination.

The primary diagnosis was liver impairment (description of cause pending). After admission, the boy was treated with vitamins and other regimens to decrease blood aminotransferases. Blood tests were repeated on October 22, 2019, and they showed his CK to be 21755 U/L, CK-MB to be 311.7 U/L, ALT to be 430 U/L, AST to be 319 U/L, and lactate dehydrogenase to be 1207 U/L. Liver and muscle biopsies were refused by his parents. His blood sample was sent to MyGenostics (Beijing, China) for genetic disorder testing, and gene sequencing showed a DMD gene exon 8-43 hemizygous deletion (Fig. 1). To ensure the best treatment, the boy was transferred to Guangzhou Children's Hospital for further medical care.

Discussion

DMD is the most common type of progressive muscular dystrophy.[1,2] The disease is characterized by progressive muscular atrophy and a poor prognosis. The gastrocnemius muscles of patients undergo pseudohypertrophy, tendon reflex weakening or disappearance, and proximal

Keywords: Transaminitis; Duchenne muscular dystrophy; Gene sequencing.
Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatine kinase; CK-MB, CK isoenzyme; DMD, Duchenne muscular dystrophy.
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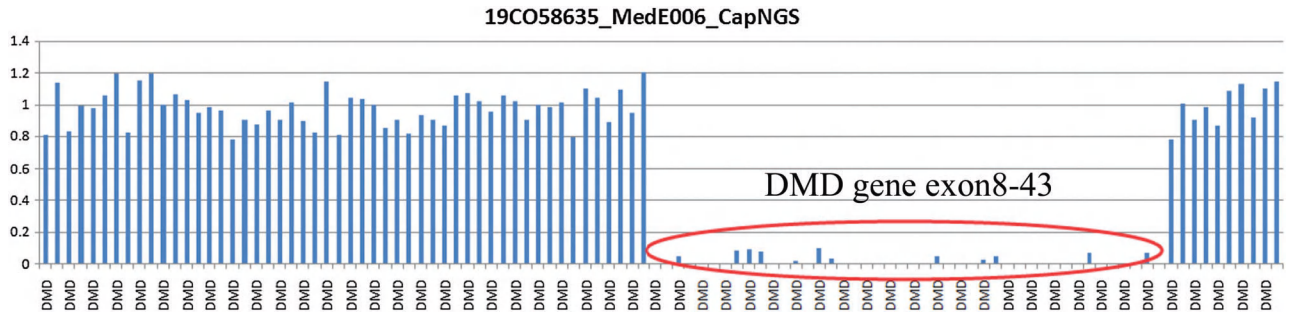

Fig. 1. Next-generation sequencing results.
DMD gene deletion of exon 8-43 was hemizygous.

myasthenia. As the disease progresses, the patients may lose their ability to walk before or around 12 years of age and die of respiratory failure or heart failure when approximately 20 years-old. The incidence rate of DMD is about 1 in 3500 newborn boys, and symptoms start to appear at 5 years of age. Patients are usually male but some female carriers are affected by partial inactivation of the X chromosome; DMD is an X-linked-recessive inherited disease.[3,4] At present, approximately 60-70% of the known pathogenic genes of DMD are deletion mutations of one or more exons, 5-10% are repetitive mutations, and 25-35% are point mutations (single base mutations or small base insertion/deletion mutations). Currently, there are no specific treatments available clinically.[2,5] Traditional methods, including acupuncture, massage, functional training, and traditional Chinese medicine, can maintain and enhance muscle strength, and some later-stage cases need orthopedic treatment. Glycine, glutamic acid, vitamin E, and hormones have no positive effect. Muscle strength increases within half a year after cell transplantation[6–8] but the long-term effects are not ideal. Additionally, the potential use of gene therapy is still under exploration.[9,10]

In clinical practice, it is difficult to explain the phenomena of liver injury and myocardial damage using routine biochemical tests and liver protection treatment. When there are no other reasons for auxiliary examination, CK levels can be further investigated.[11] It is easy to ignore the early-stage symptoms of the disease, which could be misdiagnosed as viral hepatitis, drug-induced liver injury, etc. Detailed physical examination is very important in the diagnosis of DMD. The extensive application of next-generation gene sequencing provides promise for the early diagnosis of many diseases, including DMD.[12,13] Early intervention is highly advantageous for maintaining the normal functions of organs, and delaying disease progression.

Conclusions

DMD is a serious genetic disease of childhood. Gene sequencing is crucial for early diagnosis.

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Conflict of interest

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Author contributions

Study design (JD), analysis and interpretation of data (QX, YF, JL,QC), manuscript writing and critical revision (JD).

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Impact of Liver Injury in COVID-19 Patients: Single-center Retrospective Cohort Analysis

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Introduction

Coronavirus disease 2019 (COVID-19) has become a serious threat to global public health. Unfortunately, to date, there are no specific vaccines or targeted drugs, and the number of patients with positivity for systemic acute respiratory syndrome-novel coronavirus-2 infection is growing worldwide.[1] Patients with COVID-19 may be at risk for liver injury, but the mechanism and clinical significance of injury remains unclear. Proposed mechanisms include direct virus-induced insults, immune-mediated damage (due to excessive inflammatory response), and drug-induced injury. COVID-19-related liver dysfunction is now gaining widespread attention; however, liver injury's impact on the outcome of COVID-19 patients is not clearly understood. We have evaluated the impact of liver insults on the hospitalization outcome of COVID-19 patients admitted to a suburban New York safety-net hospital and would like to share our preliminary results in a *Letter to the Editor* instead of an *Original Article* for rapid dissemination to the worldwide audience.

In our retrospective, unmatched, single-center analysis, we have identified the first 639 confirmed COVID-19 patients (ages ≥18 years) admitted to our facility from March 2020 to May 2020. Elevated liver-related enzymes [serum alanine aminotransferase (commonly referred to as ALT) >40 U/L, aspartate aminotransferase (commonly referred to as AST) >40 U/L, or alkaline phosphatase (commonly referred to as ALP) >120] were used to stratify patients with or without liver injury. The primary outcome was all-cause in-hospital mortality; other in-patient outcomes, including cardiac arrest, acute respiratory distress syndrome, arrhythmia, shock, and intubation rate, were also measured. The Pearson's chi-square test and Student's *t*-test were used for evaluating categorical and continuous variables, respectively. A two-step hierarchical multivariate regression model was performed to assess the risk of in-patient mortality and other hospitalization outcomes after adjusting for baseline characteristics and comorbidities. All statistical analyses were performed using SPSS© Corp. Version 22 (Armonk, NY, USA). This analysis was approved by the Institutional Review Board (IRB) of Nassau Health Care Corporation (NHCC) at Nassau University Medical Center (NUMC), under IRB reference # 20-277.

Out of the total 639 COVID-19 patients, 476 (74.5%) [mean age of 58.89±15.61 years, 63.0% male] had evidence of liver injury. COVID-19 liver injury cohorts had statistically significant higher rates of all cause in-patient mortality [35.5% vs. 22.7%; adjusted odds ratio (aOR): 2.84; 95% confidence interval (CI): 1.71-4.71; $p<0.001$). COVID-19 liver injury was observed more often in our Hispanic patient population (38.2%). The COVID-19 liver injury group showed higher risk of other in-patient outcomes, such as cardiac arrest (26.1% vs. 14.1%; aOR: 2.65; 95% CI: 1.52-4.59; $p\leq0.001$), requirement of intubation (30% vs. 14.7%; aOR: 2.87; 95% CI: 1.70-4.85; $p<0.001$), acute respiratory distress syndrome (43.1% vs. 30.7%; aOR: 1.89; 95% CI: 1.23-2.91; $p=0.004$), arrhythmia (5.2% vs. 0.6%; aOR: 3.16; 95% CI: 0.95-10.33; $p=0.05$) and shock (15% vs. 2.8%; aOR: 2.06; 95% CI: 1.15-3.70; $p=0.016$) compared to COVID-19 patients without evidence of liver injury (Tables 1 and 2).

The infection of liver cells with the systemic acute respiratory syndrome-novel coronavirus-2 (SARS-CoV-2) may directly cause liver dysfunction. Other indirect mechanisms of liver injury are also plausible. Chen *et al.*[2] showed that more than one-third of COVID-19 patients have some liver dysfunction; in most cases, patients had mild-to-moderate elevations of ALT or AST levels. Our analysis has shown that approximately every fourth patient presented evidence of liver injury.

It is postulated that SARS-CoV-2 binds to host ACE-2 receptors (ACE2) on target cells to gain entry. Interestingly, ACE2 receptors are also highly expressed within the biliary tree. However, the cholestatic liver disease is not a common feature of COVID-19.[3] Chau *et al.*[4] demonstrated in their study that liver biopsies of SARS patients showed a significant increase in mitotic cells, eosinophils and balloon-like liver cells, which indicated that SARS-CoV-2 might induce liver cell apoptosis and thus lead to liver damage. The study by Tan *et al.*[5] showed that SARS-CoV-2 specific protein 7a could induce cell apoptosis in different organs (including lung, kidney, and liver) through the caspase-dependent

Abbreviations: ACE2, ACE-2 receptors; ALP, alkaline phosphatase; ALT, alanine aminotransferase; aOR, adjusted odds ratio; AST, aspartate aminotransferase; CI, confidence interval; COVID-19, coronavirus disease 2019.

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Table 1. Baseline characteristics of COVID-19 hospitalizations with vs. without liver injury

Variable	With liver injury, *n*=476 (74.5%)	Without liver injury, *n*=163 (25.5%)	*p*∗
Age in years at admission			
Mean age±standard deviation	58.89±15.61	61.92±17.32	0.038
Sex			0.041
Male	63.0%	54.0%	
Female	37.0%	46.0%	
Race			0.26
White	22.1%	21.5%	
African American	24.6%	30.7%	
Hispanic	38.2%	30.7%	
Asian or Pacific Islander	2.9%	6.1%	
Other	11.1%	9.8%	
Insurance status			0.23
Uninsured/self-pay	16.0%	16.0%	
Medicare/Medicaid	40.1%	45.4%	
Private insurance	41.8%	34.4%	
Unknown	1.9%	4.3%	
Comorbidities			
Alcohol abuse	14.9%	10.4%	0.268
Asthma/COPD	10.5%	11.0%	0.713
HIV/AIDS	1.1%	1.8%	0.789
Congestive heart failure	4.8%	8.6%	0.247
Diabetes mellitus	34.5%	43.6%	0.078
OSA/OHS	1.9%	0.6%	0.521
Hypertension	50.6%	58.9%	0.221
Malignancy	5.0%	3.7%	0.321
Chronic kidney disease	7.6%	15.3%	0.010
Coronary artery disease	8.0%	14.1%	0.05
In- hospital Outcomes			
All cause in-hospital mortality	35.5%	22.7%	<0.001
Cardiac arrest	26.1%	14.1%	<0.001
Intubation	30%	14.7%	<0.001
ARDS	43.1%	30.7%	0.004
Arrhythmias	5.2%	0.6%	0.05
Shock	15%	2.8%	0.016

∗ $p \leq 0.05$ at 95% confidence interval indicates statistical significance.

Abbreviations: ARDS, acute respiratory response syndrome; COPD, chronic obstructive pulmonary disease; OSA/OHS, obstructive sleep apnea/obesity hypoventilation syndrome; HIV/AIDS, human immunodeficiency virus/acquired immunodeficiency syndrome.

pathway, further confirming the possibility of SARS-CoV-2 directly attacking liver tissues and causing liver damage. In our analysis, COVID-19 patients with liver injury demonstrated nearly a three times higher risk of in-patient mortality and other poor hospital outcomes, including cardiac arrest, acute respiratory distress syndrome, the requirement of intubation, arrhythmia, and shock. Our analysis also showed that male and Hispanic patients were more likely to develop COVID-19-associated liver dysfunction than infected female patients and those of other ethnicities. We recommend studies be designed on a large scale, to better understand the sex and race-related difference that our data suggest.

Studies are also needed to assess the outcome of COVID-19 patients with preexisting liver disease. Our analysis could not differentiate direct COVID-19-induced hepatotoxicity, drug-induced liver injury, or 'bystander effects' of systemic inflammatory response syndrome caused by the virus, which could be a major limitation of our analysis. Regardless of the mechanisms intricated in the liver injury of patients with COVID-19, worse hospitalization outcomes were noted in our analysis, requiring close monitoring. In the meantime, we

Table 2. Hospitalization outcomes among patients with COVID-19 with liver injury

Hospitalization outcome	aOR	95% CI	p*
All cause in-hospital mortality	2.84	1.71-4.71	<0.001
Cardiac arrest	2.65	1.52-4.59	<0.001
Intubation	2.87	1.70-4.85	<0.001
ARDS	1.89	1.23-2.91	0.004
Arrhythmias	3.16	0.95-10.33	0.05
Shock	2.06	1.15-3.70	0.016

*$p \leq 0.05$ at 95% CI indicates statistical significance.

Abbreviations: aOR, adjusted odds ratio; ARDS, acute respiratory response syndrome; CI, confidence interval.

believe that the front-line medical staff should pay attention to liver-related tests in patients infected with COVID-19. We recommend using appropriate hepatoprotective therapies, especially in patients with preexisting liver disease, to attenuate the potentially deleterious impact of COVID-19-related liver dysfunction.[6]

Thus, in our preliminary observations, we noted that COVID-19 patients with liver injury demonstrated nearly a three times higher risk of in-patient mortality and other poor hospitalization outcomes.

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Conflict of interest

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Author contributions

Conception and design (JD, UP, SA, PM), acquisition of data (JD, UP, SA, KF, KY, SGP, JI, AC, RR), analysis and interpretation of data (JD, UP, SA, PM), drafting of the manuscript (KF, KY, SGP, JI, AC, RR), critical revision of the manuscript (JD, UP, PM).

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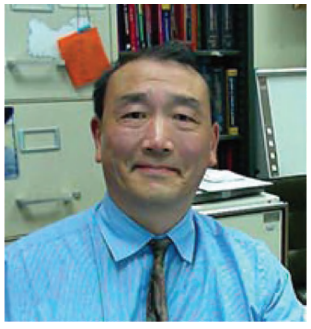

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