<table>
<thead>
<tr>
<th>Page</th>
<th>Title</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1459</td>
<td>Role of endoscopic ultrasound in the field of hepatology: Recent advances and future trends</td>
<td>Dhar J, Samanta J</td>
</tr>
<tr>
<td>1512</td>
<td>Fatty acid metabolism and acyl-CoA synthetases in the liver-gut axis</td>
<td>Ma Y, Nenkov M, Chen Y, Press AT, Kaemmerer E, Gassler N</td>
</tr>
<tr>
<td>1552</td>
<td>Chelation therapy in liver diseases of childhood: Current status and response</td>
<td>Seetharaman J, Sarma MS</td>
</tr>
<tr>
<td>1568</td>
<td>Hepatocellular carcinoma: Understanding molecular mechanisms for defining potential clinical modalities</td>
<td>Natu A, Singh A, Gupta S</td>
</tr>
<tr>
<td>1584</td>
<td>Heterogeneity of non-alcoholic fatty liver disease: Implications for clinical practice and research activity</td>
<td>Pal P, Palui R, Ray S</td>
</tr>
<tr>
<td>1611</td>
<td>Newly discovered endocrine functions of the liver</td>
<td>Rhyu J, Yu R</td>
</tr>
<tr>
<td>1642</td>
<td>Health-related quality of life in autoimmune hepatitis</td>
<td>Snijders RJ, Milkiewicz P, Schramm C, Gevers TJ</td>
</tr>
</tbody>
</table>
### Contents

**World Journal of Hepatology**  

**Monthly Volume 13 Number 11 November 27, 2021**

<table>
<thead>
<tr>
<th>Page</th>
<th>Title</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1653</td>
<td>Fungal infections following liver transplantation</td>
<td>Khalid M, Neupane R, Anjum H, Surani S</td>
</tr>
<tr>
<td>1663</td>
<td>Elastography as a predictor of liver cirrhosis complications after hepatitis C virus eradication in the era of direct-acting antivirals</td>
<td>Cerrito L, Ainora ME, Nicoletti A, Gargovich M, Riccardi L, Pompili M, Gasbarrini A, Zocco MA</td>
</tr>
<tr>
<td>1677</td>
<td>Role of immune dysfunction in drug induced liver injury</td>
<td>Girish C, Sanjay S</td>
</tr>
<tr>
<td>1688</td>
<td>Abnormal liver enzymes: A review for clinicians</td>
<td>Kalas MA, Chavez L, Leon M, Taweesedt PT, Surani S</td>
</tr>
<tr>
<td>1699</td>
<td>Hepatopulmonary syndrome: An update</td>
<td>Gandhi KD, Taweesedt PT, Sharma M, Surani S</td>
</tr>
<tr>
<td>1707</td>
<td>Mitochondrial hepatopathy: Respiratory chain disorders- ’breathing in and out of the liver’</td>
<td>Gopan A, Sarma MS</td>
</tr>
<tr>
<td>1727</td>
<td>Cystic fibrosis associated liver disease in children</td>
<td>Valamparampil JJ, Gupte GL</td>
</tr>
</tbody>
</table>

**ORIGINAL ARTICLE**

**Case Control Study**

<table>
<thead>
<tr>
<th>Page</th>
<th>Title</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1743</td>
<td>Tumor characteristics of hepatocellular carcinoma after direct-acting antiviral treatment for hepatitis C: Comparative analysis with antiviral therapy-naive patients</td>
<td>Fouad M, El Kassas M, Ahmed E, El Sheemy R</td>
</tr>
<tr>
<td>1753</td>
<td>Circulating microRNA 9-3p and serum endocan as potential biomarkers for hepatitis C virus-related hepatocellular carcinoma</td>
<td>Wahb AMSE, El Kassas M, Khamis AK, Elhelbawy M, Elhelbawy N, Habieb MSE</td>
</tr>
</tbody>
</table>

**Retrospective Cohort Study**

<table>
<thead>
<tr>
<th>Page</th>
<th>Title</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1766</td>
<td>Do peripartum and postmenopausal women with primary liver cancer have a worse prognosis? A nationwide cohort in Taiwan</td>
<td>Tseng GW, Lin MC, Lai SW, Peng CY, Chuang PH, Su WP, Kao JT, Lai HC</td>
</tr>
<tr>
<td>1777</td>
<td>Nonalcoholic fatty liver disease is associated with worse intestinal complications in patients hospitalized for <em>Clostridoides difficile</em> infection</td>
<td>Jiang Y, Chowdhury S, Xu BH, Meybodi MA, Damiris K, Devalaraju S, Pyrsopoulos N</td>
</tr>
</tbody>
</table>

**Observational Study**

<table>
<thead>
<tr>
<th>Page</th>
<th>Title</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1791</td>
<td>Six-minute walking test performance is associated with survival in cirrhotic patients</td>
<td>Pimentel CFMG, Amaral ACC, Gonzalez AM, Lai M, Mota DO, Ferraz MLG, Junior WM, Kondo M</td>
</tr>
</tbody>
</table>
1802  Incidence of umbilical vein catheter-associated thrombosis of the portal system: A systematic review and meta-analysis

ABOUT COVER
Editorial Board Member of World Journal of Hepatology, Igor Skrypnyk, MD, MDS, PhD, Professor, Internal Medicine #1, Poltava State Medical University, Poltava 36011, Ukraine. inskrypnyk@gmail.com

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Role of endoscopic ultrasound in the field of hepatology: Recent advances and future trends

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Abstract

The role of endoscopic ultrasound (EUS) as a diagnostic and therapeutic modality for the management of various gastrointestinal diseases has been expanding. The imaging or intervention for various liver diseases has primarily been the domain of radiologists. With the advances in EUS, the domain of endosonologists is rapidly expanding in the field of hepatology. The ability to combine endoscopy and sonography in one hybrid device is a unique property of EUS, together with the ability to bring its probe/transducer near the liver, the area of interest. Its excellent spatial resolution and ability to provide real-time images coupled with several enhancement techniques, such as contrast-enhanced (CE) EUS, have facilitated the growth of EUS. The concept of “Endo-hepatology” encompasses the wide range of diagnostic and therapeutic procedures that are now gradually becoming feasible for managing various liver diseases. Diagnostic advancements can enable a wide array of techniques from elastography and liver biopsy for liver parenchymal diseases, to CE-EUS for focal liver lesions to portal pressure measurements for managing various liver conditions. Similarly, therapeutic advancements range from EUS-guided eradication of varices, drainage of bilomas and abscesses to various EUS-guided modalities of liver tumor management. We provide a comprehensive review of all the different diagnostic and therapeutic EUS modalities available for the management of various liver diseases. A synopsis of all the technical details involving each procedure and the available data has been tabulated, and the future trends in this area have been highlighted.

Key Words: Endoscopic ultrasound; Liver disease; Elastography; Varices; Liver tumor; Liver biopsy

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Core Tip: The advancements in the field of endoscopic ultrasound (EUS) have enabled endosonologists to rapidly expand their wings in the field of hepatology. “Endo-hepatology” encompasses the wide range of diagnostic and therapeutic endoscopic procedures that can be used for the management of various liver diseases. Diagnostic advancements range from elastography for liver parenchymal diseases, contrast-enhanced EUS for a focal liver lesion to portal pressure measurements. Therapeutic advancements range from EUS-guided eradication of varices to drainage of abscesses to liver tumor ablation. In this comprehensive review, all the various diagnostic and therapeutic EUS modalities available for the management of liver diseases have been detailed.

INTRODUCTION

The armamentarium of endoscopic ultrasound (EUS) has grown considerably in recent years, both as an investigative and a therapeutic modality. The established diagnostic tools for the study of liver diseases include trans-abdominal ultrasound (USG), computed tomography (CT) scan and magnetic resonance imaging (MRI). While in the past, interventions in liver disease have predominantly been performed by the percutaneous or vascular route, EUS is now more and more being used for both diagnostic and therapeutic purposes. The ability to combine endoscopy and sonography in one hybrid device is a unique property of EUS, together with the ability to bring its probe/transducer in close proximity to the liver, the area of interest. In addition, its excellent spatial resolution and ability to provide real-time images, along with additional techniques, such as contrast-enhanced (CE) EUS, have facilitated the growth of EUS.

Furthermore, EUS guided intervention is also used as a rescue modality when the percutaneous approach is not favorable. EUS has opened doors to a variety of other procedures which are being explored, such as portal vein (PV) sampling for cancer cells, delivery of chemotherapy in the PV, measurement of portosystemic pressure gradient, and EUS guided transjugular intrahepatic portosystemic shunt (TIPS) creation. Harnessing its use in various liver-related interventions paves the way for a new zone of specialty, “Endo-hepatology.” Herein we provide a comprehensive review on the use of EUS in the field of hepatology, both diagnostic and therapeutic, discussing the various recent advances and future trends (Figure 1).

LITERATURE SEARCH

A search was performed in PubMed and Embase and the search strategy is outlined in Supplementary Doc 1. All studies such as case reports, series, clinical studies, animal models and reviews regarding EUS applications in liver disorders, including portal hypertension (PHTN), were reviewed. Non-English language literature was not included in the review. EUS applications for extrahepatic bile duct obstruction, gallbladder, etc., including their interventions, are beyond the scope of this review and have been excluded.

EUS FOR LIVER PARENCHYMA ASSOCIATED DISEASES

EUS can be used for the diagnosis, assessment and therapeutic management of ascites, liver parenchymal pathologies, space-occupying lesions (SOLs), liver biopsy, drainage of liver abscesses, bilomas and the management of hepatic tumors.
Ascites: Assessment and paracentesis

Ascites can be due to benign or malignant diseases. Although the differential diagnosis is broad, around 80%-90% of cases are attributed to underlying cirrhosis and PHTN \[1\]. Traditionally, routine paracentesis is performed bedside and sometimes with abdominal ultrasound guidance. However, abdominal paracentesis may become difficult in the presence of multiple abdominal scars, previous puncture marks, obesity, dilated bowel loops, dilated/tortuous veins, or the presence of omental or peritoneal nodules\[1-3\]. EUS guided paracentesis (EUS-P) is more sensitive than CT in detecting ascites\[2,4\]. The presence of ascites not visualized on imaging (CT/USG) as well as compartmentalization of fluid (such as benign etiologies like tuberculosis or tumor implants in peritoneal carcinomatosis) makes EUS-P a very promising tool in these areas\[4,5\]. With EUS-P, even small amounts of fluid (as little as 2.7 mL) can be aspirated and provide valuable diagnostic information\[6\]. In addition, EUS-P can be used as a rescue procedure in the case of previously failed percutaneous paracentesis or part of diagnostic workup during diagnostic EUS (Figure 2).

Additionally, EUS guided fine needle aspiration (EUS-FNA) of suspicious nodules in the omentum/peritoneum can be performed simultaneously while performing paracentesis for targeted cytological diagnosis\[7\]. Contrast-enhanced EUS (CE-EUS) has also been evaluated to identify enhancement patterns of peritoneal nodules or omental caking and differentiate benign or malignant causes of undiagnosed ascites \[8\].

The technique of EUS-P: The technique of EUS-P is detailed in Table 1.

Future trends: Since the first report of EUS-FNA of ascites and pleural fluid performed in 1995, various reports of EUS-P with/out FNA of peritoneal deposits have been published subsequently with excellent diagnostic capability and correlation with intraoperative findings\[12\]. Some cases of development of infectious complications (attributed to traversing the contaminated gastrointestinal wall) such as self-limited fever (3.3%) and bacterial peritonitis (4%) have been reported\[5,10\]. Recent developments include the deployment of double plastic stents in loculated ascites (benign/malignant), leading to internal drainage causing significant improvement in quality of life\[13,14\] (Figure 3). A clinical trial is also recruiting patients for EUS guided placement of a plastic prosthesis for refractory malignant ascites\[15\]. The various studies on EUS-P are summarized in Table 2.

Thus, EUS-P is an excellent tool (sensitivity 94%, specificity 100%) to detect a small quantity of ascites\[10\] and therapeutic drainage where the percutaneous approach is
Table 1 | Technique of endoscopic ultrasound guided paracentesis [1-3,9-11]

<table>
<thead>
<tr>
<th>Pre-procedure requirements</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) No recommendations exist for EUS-P, although most studies have been performed under the cover of pre/peri-procedural antibiotics; and (2) Patient is usually fasted for 4-6 h before the procedure</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Technical aspects</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) EUS-P is usually performed using a 22 G/25 G FNA needle. A specialized spring-loaded 22 G FNA needle can also be used for the same; (2) The approach can be transgastric or transduodenal. The tip of the needle is visualized under EUS guidance in the ascites; (3) At the time of puncture, care is taken to avoid a trajectory involving any tumor/vessels to avoid peritoneal seeding or bleeding; (4) For therapeutic paracentesis, a suction tube attached to a vacuum canister can be used; (5) Repositioning of the needle is carried out in case it gets blocked by the tumor or omentum; (6) Two and fro motion is usually not needed; (7) CE-EUS followed by FNA of the peritoneal/omental nodules can also be done for added diagnostic value; and (8) The sample aspirated is sent for routine cytological assessment and for any additional tests that might be needed</td>
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</table>

<table>
<thead>
<tr>
<th>Post procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>The administration of albumin post 5 L of paracentesis and post procedure observation are carried out as per standard recommendations (EASL, AASLD)</td>
</tr>
</tbody>
</table>


**Figure 2 | Endoscopic ultrasound guided paracentesis.** Needle is visualized in the ascitic fluid.

not amenable. Furthermore, FNA of peritoneal/omental nodules is an added advantage that can increase the diagnostic yield.

**Assessment of liver parenchyma/SOLs: Anatomy of the liver, its segments and surrounding structures**

The requirement for three-dimensional conceptualization of the liver parenchyma makes EUS assessment of the liver and surrounding structures different from the conventional methods of USG/CT/MRI. Depending on the position of the EUS scope, either in the stomach or duodenum, various structures can be identified (Table 3 and Figure 4) such as [17]: (1) From the gastric end: Segments I (caudate lobe), left lobe segments (II, III, IV), right lobe (V, VIII), umbilical part of the left PV and ligamentum teres, ligamentum venosum, inferior vena cava, and hilum; and (2) From the duodenal bulb: Segments VI, VII; the hepatoduodenal ligament structures and PV and hepatic artery branches, the liver hilum and the segmental divisions of the right PV and hepatic artery.

Although transabdominal USG or CT scan is the first-line approach for evaluation of liver parenchyma or focal lesions, EUS has additional features which can add to its diagnostic/therapeutic potential [18,19]: (1) Transducer proximity enables better identification of the structures; (2) Combination of real-time images with elastography enables semi-quantitative measurements of liver parenchymal stiffness; (3) Newer generation EUS machines with color, power and pulsed Doppler systems helps easy assessment of the vasculature; (4) CE-EUS or harmonic EUS increases the diagnostic
Table 2: Studies on endoscopic ultrasound guided paracentesis

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Study design</th>
<th>Patient population</th>
<th>Imaging</th>
<th>Age (yr)</th>
<th>Gender (M/F)</th>
<th>Needle</th>
<th>Route (TG/TD)</th>
<th>Amount of fluid aspirated</th>
<th>Diagnosis on EUS</th>
<th>Actual diagnosis</th>
<th>Complications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chang et al[12], 1995</td>
<td>Case report</td>
<td>2 cases</td>
<td>CT (pleural effusion and ascites)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Malignant effusion and ascites</td>
<td>-</td>
</tr>
<tr>
<td>Romero-Castro et al[14], 2017</td>
<td>Case series</td>
<td>3 cases</td>
<td>DLBCL (1 case), HCC (2 cases)</td>
<td>60/74/55</td>
<td>3/-</td>
<td>19 G FNA (all cases)</td>
<td>TG (3 cases)</td>
<td>Double Pigtail placement (3 cases)</td>
<td>-</td>
<td>Malignant ascites (3 cases)</td>
<td>None</td>
</tr>
<tr>
<td>Wardeh et al[16], 2011</td>
<td>Retrospective study</td>
<td>101</td>
<td>Ascites not detected in 6/9 cases on CT</td>
<td>68.3</td>
<td>54/47</td>
<td>19 G FNA</td>
<td>NA</td>
<td>10 mL (max) in 90 cases, 2 smears in 11 cases</td>
<td>74 negative</td>
<td>84 malignant</td>
<td>None</td>
</tr>
<tr>
<td>Suzuki et al[11], 2014</td>
<td>Retrospective study</td>
<td>11 cases</td>
<td>CT (no ascites in 4)</td>
<td>66.4</td>
<td>7/4</td>
<td>22 G (automated spring-loaded)</td>
<td>NA</td>
<td>14.1 mL (range 0.5-38 mL)</td>
<td>Benign 5; malignant 6</td>
<td>NA</td>
<td>None</td>
</tr>
<tr>
<td>Kaushik et al[10], 2006</td>
<td>Retrospective study</td>
<td>25</td>
<td>NA</td>
<td>66-70</td>
<td>16/9</td>
<td>22/25 G FNA</td>
<td>Both</td>
<td>6.8 mL (range, 1-20 mL)</td>
<td>64% malignant (benign 9; malignant 16)</td>
<td>Benign 8; malignant 17</td>
<td>1 cases (4%) (bacterial peritonitis)</td>
</tr>
<tr>
<td>Lee et al[4], 2005</td>
<td>Retrospective study</td>
<td>250 cases</td>
<td>CT in all</td>
<td>60.3</td>
<td>160/90</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>37% ascites, 28% peritoneal metastasis</td>
<td>All malignant</td>
<td>None</td>
</tr>
<tr>
<td>Dewitt et al[5], 2007</td>
<td>Retrospective study</td>
<td>60</td>
<td>CT/MRI/USG in all (ascites 31 cases (51%))</td>
<td>67</td>
<td>33/27</td>
<td>22 G</td>
<td>55 (TG), 5 (TD)</td>
<td>8.9 (1-40) mL</td>
<td>Benign 42; malignant/ atypical 18</td>
<td>Benign 15; malignant 45</td>
<td>2 cases fever</td>
</tr>
<tr>
<td>Köck et al[13], 2018</td>
<td>Case report</td>
<td>2 cases</td>
<td>Rectal cancer, ovarian cancer</td>
<td>36, 56</td>
<td>-/2</td>
<td>19 G</td>
<td>Both TG</td>
<td>Pigtail (plastic) placed</td>
<td>-</td>
<td>-</td>
<td>None</td>
</tr>
<tr>
<td>Nguyen and Chang[2], 2001</td>
<td>Retrospective study</td>
<td>31 cases (of 85) (18%)</td>
<td>CT had ascites in 14/79</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>7.9 (1-40 mL)</td>
<td>Malignant 5; benign 26</td>
<td>NA</td>
<td>None</td>
</tr>
<tr>
<td>Varadarajulu and Drelichman[3], 2008</td>
<td>Case report</td>
<td>1</td>
<td>SCC anus</td>
<td>31</td>
<td>-/1</td>
<td>19 G</td>
<td>TG (l)</td>
<td>10 mL (diagnostic); 5 L (therapeutic)</td>
<td>Malignant ascites</td>
<td>NA</td>
<td>None</td>
</tr>
</tbody>
</table>

DLBCL: Diffuse large B cell lymphoma; TG: Transgastric; TD: Transduodenal; M: Male; F: Female; EUS: Endoscopic ultrasound; CT: Computed tomography; FNA: Fine needle aspiration; SCC: Squamous cell carcinoma; USG: Ultrasound; MRI: Magnetic resonance imaging.

The performance of focal liver lesions; and (5) Simultaneous assessment and interventions such as management of varices and liver biopsy can be performed in a single setting.

**Techniques of assessment: Elastography and contrast enhancement techniques**

Real-time elastography (RTE) has been developed for the assessment and quantification of liver tissue stiffness. Qualitative RTE uses the degree of deformation by the compression of structures as an indicator of tissue stiffness and is depicted using a color map wherein hard tissue is blue, intermediate stiffness is green and soft tissue is red. Quantitative RTE, on the other hand, uses hue histogram and strain ratio. While
Table 3 Structures visualized with endoscopic ultrasound in the liver

<table>
<thead>
<tr>
<th>Structure</th>
<th>Features</th>
<th>Doppler</th>
</tr>
</thead>
<tbody>
<tr>
<td>Portal vein branches</td>
<td>Thick and hyperechoic walls</td>
<td>Positive signal</td>
</tr>
<tr>
<td>Hepatic vein branches</td>
<td>Thin, non-reflective walls, straight course</td>
<td>Positive signal</td>
</tr>
<tr>
<td>Biliary radical</td>
<td>Hyperechoic walls, irregular course</td>
<td>Negative signal</td>
</tr>
<tr>
<td>Ligaments (teres and venosum)</td>
<td>Thick, hyperechoic (no lumen) between vessels and Glisson’s capsule</td>
<td>Negative signal</td>
</tr>
<tr>
<td>Gallbladder</td>
<td>Cystic structure, hyperechoic walls, anechoic content</td>
<td>Negative signal</td>
</tr>
<tr>
<td>Falciform ligament</td>
<td>Thick, hyperechoic (no lumen); on the left anterior to segment III, on the right anterior to segment IVa and IVb</td>
<td>Negative signal</td>
</tr>
<tr>
<td>Hepatic artery</td>
<td>Thick with reflective walls</td>
<td>Positive signal</td>
</tr>
</tbody>
</table>

Figure 3 Endoscopic ultrasound-guided internal drainage of loculated ascites. A: Puncture of the loculated ascites with 19-G aspiration needle; B: Guidewire negotiated across as visualized on endoscopic ultrasound; C: Fluoroscopic view of guidewire coiled inside the loculated ascites; D: Naso-cystic drain placed inside the loculated ascites.

CE-EUS is a more valuable technique to improve the diagnostic performance of focal liver lesions. It is of 2 types: CE-EUS with the Doppler method (CE-EUS-D) and CE-EUS with harmonic imaging (CE-EUS-H). The former helps distinguish vascular-rich and hypovascular areas of a liver SOL, whereas the latter helps provide a detailed roadmap of the vasculature of the same. Of the contrast agents available, Sonovue and Sonazoid are more commonly used[21].

The concept of CE-EUS depends on the dual blood supply of the liver and has 3 phases: arterial phase (20-45 s), portal venous phase (lasting up to 120 s), and the late phase (contrast agent clearance, around 6 min)[21].

The advantages of CE-EUS over CT and MRI are that: (1) It provides real-time imaging; (2) Contrast is not excreted by the kidneys, and thus can be used in cases with renal insufficiency; (3) Contrast is confined to the vascular space only and so has prolonged enhancement of vascular system; (4) Higher resolution helps in targeted biopsies; and (5) Can characterize lesions less than 1 cm.
**Figure 4 Endoscopic ultrasound anatomy of liver segments.** A: Anatomy of the left lobe with S2 and S3 segments; B: Ligamentum teres with umbilical portion of the left portal vein; C: Middle hepatic vein with segments of the liver; D: Anatomy of the bifurcation of portal vein from the duodenal bulb. PV: Portal vein; MHV: Middle hepatic vein; LHV: Left hepatic vein; RPV: Right portal vein; LPV: Left portal vein.

**Figure 5 Endoscopic ultrasound elastography of the liver parenchyma.**

**EUS imaging in chronic liver diseases**

Certain tests such as transient elastography (TE), Fibroscan, and RTE can aid in the diagnosis of the degree of liver fibrosis. However, these tests are fraught with limitations in people with obesity and ascites. EUS can be used similarly with probably better diagnostic sensitivity for the same. Schulman et al.[22] reported that liver fibrosis index (LFI) correlated with abdominal imaging (LFI in normal, fatty liver and cirrhosis patients were 0.8, 1.4 and 3.2, respectively). Similar findings were replicated in liver fibrosis assessment for chronic hepatitis C cases (LFI of 2.38 had an area under the receiver operating characteristic curve of 0.73) compared with the gold standard of
liver biopsy. Histogram acquisition was successful in 82% of patients[23]. A recent study by Tu et al[24] in early-stage cirrhosis showed that the accuracy of a combination of EUS, EUS-RTE, acoustic radiation force impulse (AFRI) and aspartate aminotransferase-to-platelet ratio (APRI) had the highest diagnostic rate (sensitivity 87%). Thus, EUS can provide a one-stop diagnostic modality to screen and rule out a host of conditions in patients with liver disease, from the screening of varices, pancreatobiliary pathology to hepatic parenchymal/SOL assessment.

EUS imaging in focal liver lesions

The diagnostic accuracy of EUS in detecting focal liver lesions, mostly less than 1 cm, exceeds that of USG, CT, and MRI[25,26]. Singh et al[27] addressed the diagnostic yield of EUS vs CT for hepatic metastasis (98% vs 92%), wherein EUS identified a significantly greater number of metastatic lesions (40 vs 19). Diagnostic criteria proposed by Fujii-Lau et al[28] can be used to differentiate between benign and malignant metastatic hepatic lesions based on EUS findings with a positive predictive value of 82%. Lesion shape, borders, echogenicity, homogeneity, and size are used to delineate malignant lesions. It is said to be neoplastic if it meets at least three criteria: (1) Lack of isoechoic/slightly hyperechoic center; (2) Post-acoustic enhancement; (3) Adjacent structures distortion; (4) Hypoechochogenicity (slightly or distinctly); and (5) At least 10 mm in size.

With the advent of EUS-RTE, the characterization of liver SOLs and their biopsies have become better (Figure 6). A study reported a hue histogram cutoff of 170 to discriminate between benign and malignant tumors (sensitivity 92.5%, accuracy 88.6%) [29]. In addition, the use of contrast agents in CE-EUS helps in differentiating primary tumors and metastasis[30]. CE-EUS has also been utilized for the assessment of treatment response in hepatocellular carcinoma (HCC) post-trans-arterial catheter embolization[31]. Hence, EUS with RTE, CE-EUS and CE-EUS-H might be a promising tool for diagnosing focal liver lesions and targeted intervention.

EUS-FNA of focal liver lesions

Several studies exist on the use of EUS-FNA/FNB (fine needle biopsy) for solid liver lesions with a complication rate of 0%-6% (Table 4). A recent systematic review by Ichim et al[42] showed the diagnostic yield of EUS-FNA to be 80%-100%.

Future trends

Studies have reported additional assessment of KRAS mutation in inconclusive cytological samples, which has resulted in an improved diagnostic yield from 89.3% to 96.4%[43]. Similarly, an animal study has evaluated the art of in vivo cytological observation using a high-resolution micro-endoscopy (HRME) system under EUS guidance[44] to decrease the number of needle-passes and subsequent adverse events. Recently, Minaga et al[45] have reported the additive role of CE-EUS-H in the detection of left lobe liver metastasis from pancreatic ductal adenocarcinoma. The diagnostic accuracy of CH-EUS was 98.4% compared to 90.6% with CECT.

EUS guided liver biopsy

Despite the advances in various non-invasive testing available to determine the degree of fibrosis, liver biopsy remains the gold standard method for accurate assessment in diagnosis and staging. As first described in 1883 by Dr. Paul Ehrlich, percutaneous liver biopsy (PC-LB) has evolved from a mere percussion method to an "image-guided" technique in the last ten years using ultrasound/CT imaging to accomplish it. However, despite image guidance, the risk of bleeding persists, occurring in up to 0.6% of cases, including other adverse events like pneumothorax and gallbladder puncture and even death in a few cases[46]. The transjugular technique of liver biopsy, introduced in 1973, can help reduce this risk, especially in patients with underlying coagulopathy. However, this method also carried added risks of local site hematoma, intraperitoneal bleeding, arrhythmia and carotid puncture[47].

EUS guided liver biopsy (EUS-LB) initiated as early as 2007 is currently emerging as a cost-effective, safe and well-tolerated procedure and helps in more representative sampling. The American Association for the Study of Liver Diseases recommends a tissue length of at least 2-3 cm with ≥ 11 or more complete portal tracts (CPTs) for determining the adequacy of liver biopsy samples[48]. The mean tissue length and CPTs for EUS-LB, PC-LB and TJLB, as shown in various studies is 36.9, 9 and 17.7 mm, and 7.7, 13.5 and 6.8 mm, respectively[49,50]. This can be achieved with a regular 19 G EUS-FNA needle (71). Similarly, a meta-analysis on EUS-LB revealed that pooled successful histological diagnosis was achieved in 93.9% of cases. Adverse event rates
### Table 4 Studies on endoscopic ultrasound guided fine needle aspiration/fine needle biopsy of focal liver lesions

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Design</th>
<th>Patients</th>
<th>Diagnostic yield (%)</th>
<th>Needle passes (median)</th>
<th>Complications</th>
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<tbody>
<tr>
<td>Nguyen et al [32]</td>
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<td>14</td>
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<td>2</td>
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</tr>
<tr>
<td>TenBerge et al [33]</td>
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<td>26</td>
<td>88.6</td>
<td>-</td>
<td>3.8% (fever)</td>
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<td>77</td>
<td>91</td>
<td>3.4 (mean)</td>
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<tr>
<td>Hollerbach et al [35]</td>
<td>Prospective</td>
<td>33</td>
<td>94</td>
<td>1.4 ± 0.6</td>
<td>6.1% (self-limited bleeding)</td>
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<td>96</td>
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<td>Retrospective</td>
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<td>75</td>
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<td>Lee et al [40]</td>
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<td>90.5</td>
<td>2</td>
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<tr>
<td>Chon et al [41]</td>
<td>Retrospective</td>
<td>58</td>
<td>89.7</td>
<td>2</td>
<td>1.7% (bleed)</td>
</tr>
</tbody>
</table>


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**Figure 6** Endoscopic ultrasound elastography of a focal liver lesion with strain ratio calculation.

with EUS-LB, PC-LC and TJLB were 2.3%, 0.09%-3.1% and 0.56%-6.5%, respectively [48, 51, 52]. A recent meta-analysis between the three techniques revealed that EUS-LB was comparable to PC-LB in terms of CPT, but tissue length was better with the former with no complication rates [53].

EUS-LB has been used in the setting where patients undergo other endoscopic procedures such as screening of the biliary tree, assessment of surrounding structures and lymph nodes and variceal screening in those not affected with ascites and obesity [50], thereby saving time and resources. Furthermore, EUS-LB is theoretically less painful as it does not require skin puncture, eliminates the need for breath-hold and allows visualization and avoidance of blood vessels even 1 mm in size and is suitable for anxious patients by using adequate sedation (Figure 7). Moreover, bilobar biopsy can be achieved, reducing sampling error and helping in better assessment of disease activity and fibrosis [54].
Figure 7 Endoscopic ultrasound-guided liver biopsy.

**Technique:** The technique of EUS-LB is described in Table 5.

**Future trends:** In attempts to acquire better quality and quantity of specimens, various studies have been published on different needles and methods of executing a EUS-LB procedure. A recent RCT comparing a 19 G FNB needle (fork-tip) vs 19 G standard FNA needle yielded better results with the former (pre-processing length 2.09 cm vs 1.47 cm and more CPTs)[55]. In contrast, a recent meta-analysis showed the superiority of FNA needles over core biopsy needles in terms of better tissue acquisition[51]. Thus, 19 G FNA needle may be used for EUS-LB procedures except for the cases where immunohistochemistry and architecture characterization are warranted, in whom core biopsy needle may be used.

Mok et al[56] showed that the “wet heparin” suction technique had greater tissue yield compared to “dry suction” (aggregate specimen length 49.2 mm vs 23.9 mm; mean CPT count 7 vs 4). Thus, the combination of wet-heparinized suction and a 19-G second-generation (FNA/FNB) needle might help achieve better specimens with minimal fragmentation.

The various studies using EUS-LB (FNA/FNB) in patients with chronic liver disease are highlighted in Table 6. The average technical success and diagnostic yield for EUS-FNA and EUS-FNB-guided liver biopsy are 100% and 89.8%, respectively, with a complication rate of 3.3%, consisting entirely of minor events[70]. In addition, studies reporting the use of EUS-LB in patients with NAFLD (overall technical success rate 100%, yield 96.8% with 7.7% complication rate) are reported in Supplementary Table 1.

**EUS guided therapeutic management of liver cysts, liver abscess and biloma**

Symptomatic liver cysts, abscesses and bilomas may require drainage. Traditionally, these were approached through surgical or interventional radiology using percutaneous catheter drainage (PCD). Recently, EUS guidance has been used to drain simple intrahepatic cysts of varied etiologies, liver abscesses and bilomas. EUS guided drainage may be superior to PCD as it enables a one-step approach, leading to internal drainage and thus avoiding the complications of catheter dislodgement, pericatheter leak, multiple interventions and movement restrictions.

**EUS guided treatment of hepatic cysts:** The most frequent liver cysts encountered for drainage via EUS include simple hepatic cysts and intrahepatic pancreatic pseudocysts. Those located in the left lobe of the liver or the caudate lobe can be drained via EUS guidance. PCD would be preferred for right lobe cysts as it is difficult to access the right lobe in the duodenal bulb with an unstable scope position. Therapies offered by EUS include fine-needle aspiration, ethanol lavage and transmural stent placement.

In a retrospective study by Lee et al[71], 19 cases of hepatic cysts were treated by PCD and EUS guided ethanol lavage and reported a 97.5% reduction in cyst volume at 11.5 mo of follow-up in the PCD group and a 100% reduction at 15 mo in the EUS arm. The studies on EUS guided treatment of hepatic cysts are outlined in Supplementary-
Pre-biopsy: The following workup is needed in all cases of liver biopsy

(1) Coagulation work up including platelet count, PT/INR and BT/CT; (2) Prior to the biopsy, the medications should be stopped as follows: anti-platelet medications 7 d, warfarin 5 d, heparin and related products discontinued 12-24 h prior to biopsy; and (3) Use of conscious sedation such as midazolam and nallbuphine or propofol as per operator’s preference or patient comfort

Procedural details of EUS-LB

(1) A linear array echoendoscope (Olympus GF-UCT180, Center Valley, United States) is generally used for the procedure; (2) Prior to the procedure, Doppler imaging is done to ensure that no vascular structures are present along the expected trajectory of the needle; (3) The EUS-LB can be performed using a 19 G EUS-FNA/FNB needle; (4) The left lobe is identified first, as that liver parenchyma which is a few centimeters below the gastro-esophageal junction; (5) The left lobe is identified first, as that liver parenchyma which is a few centimeters below the gastro-esophageal junction. The right lobe if needed to be biopsied, is accessed from the duodenal bulb. Two site biopsy can be undertaken at the discretion of the endosonographer; (5) A preferably long vessel free trajectory allowing free passage of the needle to a depth of at least 3 cm or more is usually selected; (6) For wet heparin suction, the stylet is removed and the needle is primed with a heparin flush; and (8) Once liver parenchymal penetration is achieved with the needle (around 1-2 cm), full suction is applied with the 20 mL vacuum syringe with fluid column; (9) One pass consists of a total of 4-5 to-and-fro needle motions using the fanning technique under direct EUS guided visualization of the tip of the needle. Two such passes are usually taken (maximum 10 actuations); and (10) The specimen is pushed from the needle directly into the formalin solution using the stylet or saline flush

Post-biopsy: The following instructions are to be followed in all cases post liver biopsy

(1) The patient post biopsy, irrespective of the type of procedure, is transferred to the post procedure recovery room and monitored as per the AASLD protocol[60]; (2) The minimum observation period is 2-4 h; (3) Post-procedure pain and need for analgesics to be noted and provided; and (4) Patient is asked to report adverse events at specific time intervals (as per institute policy)

**EUS guided drainage of liver abscess**: Traditionally, pyogenic and amoebic liver abscesses have been drained by PCD with a high technical success rate. However, EUS guided drainage of liver abscesses is a promising new approach, especially for difficult-to-reach locations. Additionally, the advantage of internal drainage with a single-step procedure and easy access from the stomach makes transmural drainage of left and caudate lobe abscess convenient.

The technique was first described by Seewald et al[72], who reported complete resolution 4 weeks post-procedure. Literature on EUS guided drainage is limited to retrospective case series only in which the majority have been drained with double pigtail plastic stents[73-75]. Recently, data are emerging on the use of fully covered self-expandable metal stents (SEMS)[76] for the same. Ogura et al[77] reported retrospective comparative data on EUS vs PCD guided abscess drainage wherein EUS guided abscess drainage (EUS-AD) cases showed greater clinical success (100% vs 89%) with shorter hospital stay (21 d vs 41 d). Studies on EUS-AD are listed in Supplementary Table 3.

**EUS guided drainage of biloma**: Biloma is defined as a well-demarcated collection of bile outside the biliary tree, which can be extrahepatic or intrahepatic, encapsulated or without a capsule[78]. It is most frequently caused by iatrogenic biliary tree injury during cholecystectomy. It has been traditionally managed with PCD or surgery. However, large bilomas in opposition to the gastric wall can be taken up for transmural drainage (Figure 8). Similar to EUS-AD, earlier plastic stents were utilized for the same, but now SEMS has been in vogue for biloma drainage with excellent results. Post drainage, such patients should be evaluated to determine the need for endoscopic retrograde cholangiopancreatography, or sphincterotomy with or without stenting or surgery[79]. Studies on EUS guided drainage of bilomas are described in Supplementary Table 4.

Despite it being a point of contention, EUS guided drainage of intrahepatic lesions (cysts, abscesses and bilomas) is an upcoming promising technique and may be considered in conditions where PCD is not amenable or has failed.

**EUS guided treatment of liver tumors**

A thrilling offshoot of EUS guided therapeutic interventions has been EUS guided local treatment of tumor lesions (both pancreatic and hepatic tumors)[80]. EUS-guided tumor management is a new experimental application that has shown promise in reaching difficult lesions (left lobe, caudate lobe), provided a rescue option in refractory cases, and has potential to improve quality of life by minimizing systemic side effects[81,82]. This procedure has been extensively studied in cases of pancreatic...
neoplasm, but its role in hepatic tumors (primary or metastatic) is still in its infancy. Various techniques of EUS guided liver tumor management have been described.

**Fine needle injection therapy: Ethanol ablation**

Percutaneous injection of ablative injections is most commonly used worldwide to manage HCC, although EUS guided fine needle injection can be performed using acetic acid or ethanol (pure alcohol 95%-99%)\[83\]. Its advantage is that it enables real-time imaging during delivery of ethanol to the tumorous lesion and thus can help avoid collateral damage.
Initial case reports using 22 G and 25 G FNA needles have been reported with excellent technical success and complete resolution of HCC\cite{84-87}. For example, Nakaji et al\cite{87} reported a high-resolution rate at 31 mo in 12 cases of caudate lobe HCC, whereas Jiang et al\cite{88} only showed 30% complete resolution at 12 mo. This technique has also been evaluated for the treatment of hepatic metastasis from pancreatic adenocarcinoma\cite{89}.

**Thermal ablative therapy**

**Radiofrequency ablation:** Radiofrequency ablation (RFA) uses a high-frequency alternating current (375 kHz to 500 kHz) and is minimally invasive with good tolerability\cite{90}. It can be delivered percutaneously, intraoperatively, via an endoluminal approach or endosonographic (transmural) route. Emerging data on the latter have resulted in its application in cases wherein the percutaneous approach fails. Obesity, tumor nodules in the left lobe or caudate lobe, deep-seated and subcapsular/sub-diaphragmatic lesions that carry an inherent risk of hemothorax or pleural effusion are some of the conditions where it has been applied\cite{81,90}. A specifically designed needle tip electrode for performing EUS-RFA (EUSRA RFA Electrode, STARmed, Koyang, Korea) with a designed internally cooled needle electrode is the most extensively studied. Only a few case reports exist on EUS-RFA using EUSRA in HCC\cite{91-93}. Also, hybrid models combining EUS-RFA with cryoablation in the bovine liver have demonstrated better efficacy of the combination treatment\cite{94}.

**Laser ablation by neodymium:yttrium-aluminum-garnet:** Neodymium:yttrium-aluminum-garnet (Nd-YAG) is a type of LITT (laser interstitial thermotherapy) wherein laser waves are introduced through the EUS needle directly into the tumor tissue leading to cell apoptosis and eventual necrosis. Only two human studies have been published so far for the treatment of HCC. Di Matteo et al\cite{95} reported complete HCC resolution in 2 mo in a case of previously failed caudate lobe HCC. Similarly, Jiang et al\cite{96} reported resolution at 3 mo with an encouraging safety profile.

**Cryotherapy ablation:** Cryotherapy ablation (CYA) destroys tissue through multiple freezing-thawing cycles leading to osmotic dehydration and injury to the intracellular structures and cell death\cite{90}. No human study exists for its use in liver lesions. However, a single animal study showed the efficacy of a hybrid EUS-RFA and cryoderm device in a porcine model\cite{97}.

**High-intensity focused ultrasound:** This is a non-invasive technique that causes tissue necrosis via heat generation and acoustic cavitation by the formation and collapse of bubbles produced by intense USG waves\cite{90}. Its use in EUS has only been tested in animal models\cite{98,99}, showing complete necrosis of the lesions with no immediate

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**Figure 8** Endoscopic ultrasound-guided drainage of biloma. A: Post-operative biloma noted on endoscopic ultrasound (EUS) with internal echoes; B: EUS-guided puncture of the biloma; C: Guidewire negotiated into the collection followed by placement of naso-cystic drain; D: Endoscopic view of the cavity entered with catheter noted in situ.
side effects.

**Brachytherapy**

This treatment modality has been used for various cancers with the advantage of less toxicity to surrounding tissues over external beam radiotherapy\cite{81,90}. For example, EUS guided brachytherapy with permanent seed placement of Iodine (I125) or palladium (Pd103) has been performed for head-neck, esophageal, and pancreatic cancer\cite{100-102}. In addition, Jiang et al\cite{88} have used EUS guided I125 seed implantation for liver tumors with high efficacy and safety.

Studies on EUS guided liver tumor treatments are outlined in Table 7.

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**EUS GUIDED VASCULAR INTERVENTIONS**

The presence of real-time, high-resolution sonographic imaging with Doppler, along with the relative proximity of the gastrointestinal tract to the major blood vessels in the abdomen and the mediastinum, has led to a growing interest to explore the role of EUS in the field of vascular interventions. EUS may be preferred over the percutaneous route, especially in obesity, ascites and overlying distended bowel\cite{104}.

**Esophageal and gastric varices: diagnosis and management**

EUS guided vascular intervention in patients with PHTN has been well established in managing varices (esophageal, gastric, duodenal, and ectopic).

**Management of esophageal varices**: Endoscopic variceal band ligation (EVL) has been the standard treatment of esophageal varices (EV) (both primary and secondary prophylaxis). However, re-bleeding rates of 15%-65% have been reported due to the failure to obliterate perforating veins and collaterals feeding the varices\cite{105}. Lahoti et al\cite{106} described the first report of EUS guided sclerotherapy in 5 cases, wherein sclerosant (sodium morrhuate) was injected under EUS guidance (2-4 mL per injection site) directed at the perforating vessels as determined by color Doppler with complete eradication of the varices. An RCT comparing EUS vs direct sclerotherapy revealed no difference in both arms\cite{107}. Thus, although EUS carries a theoretical advantage for identifying the feeders, more studies are needed to assess its practical clinical benefit.

**Management of gastric varices**: In patients with PHTN, gastric varices (GV) are present in up to 20% with a 50%-65% re-bleeding rate\cite{108}. Endoscopic injection of CYA glue for GV has been the treatment of choice since its first description in 1986 but is still prone to a re-bleeding rate of 40%-109. In the current era of EUS guided vascular interventions, management of GV by EUS has many conceptual advantages, both diagnostic and therapeutic such as\cite{110,111}: (1) A higher detection rate (6 times) over conventional endoscopy; (2) Greater success in differentiating varices from thick gastric folds; (3) Confirmation of the cessation of blood flow post-treatment; (4) Real-time varix visualization and hence accurate delivery of hemostatic agent to the varix; and (5) Targeted treatment for feeder vessels.

The first description of EUS guided CYA injection in GVs was given by Romero-Castro et al\cite{111} and Lee et al\cite{112}. To reduce the chances of embolization with CYA, stainless steel coils alone or in combination with CYA glue have been introduced. The advantage is three-pronged: additive hemostasis and varix obliteration, reducing the volume of glue needed and acting as a scaffold to retain the glue within the varix, thereby decreasing embolization. Various studies, including RCTs, have favored coil over glue. Bhat et al\cite{113} reported a complete obliteration in 93% with only 3% re-bleeding rates using coils and glue combination. Similarly, two RCTs and a meta-analysis have reported the combination therapy of coil with glue to be superior to either agent alone\cite{114-116}. Newer treatments of utilizing coils with gelatin sponge and sclerotherapy or isolated thrombin injection have been reported in various case series and have shown good results\cite{117-119}.

The technical steps of the EUS guided coil and glue placement for the obliteration of GV are outlined in Table 8 and Figure 9.

**Use of EUS in the prediction of re-bleeding from EV/GV**: EUS with Doppler has a higher sensitivity for detecting esophageal and GV than upper GI endoscopy and can also be used to predict re-bleeding. Certain parameters can help guide us in this direction\cite{120,121}: (1) EUS can help in demonstrating collaterals or feeders, a strong indicator to a future occurrence of a re-bleed; (2) Hematocystic spots on EVs identified
Table 7 Studies in humans demonstrating the role of endoscopic ultrasound guided therapies for liver lesions

<table>
<thead>
<tr>
<th>EUS guided treatment</th>
<th>Study design</th>
<th>Patients</th>
<th>Location of the lesion</th>
<th>Technical success (%)</th>
<th>Response to therapy</th>
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<tr>
<td>Jiang et al[88]</td>
<td>RCT</td>
<td>13</td>
<td>Left lobe</td>
<td>92</td>
<td>Near complete</td>
<td>0</td>
</tr>
</tbody>
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EUS: Endoscopic ultrasound; HCC: Hepatocellular carcinoma; RCT: Randomized controlled trial; Nd-YAG: Neodymium:yttrium-aluminum-garnet; RFA: Radiofrequency ablation.

Table 8 Steps of endoscopic ultrasound guided coil and glue placement for gastric varices obliteration

<table>
<thead>
<tr>
<th>Pre-procedure requirements</th>
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<tbody>
<tr>
<td>(1) All procedures are done under the cover of pre/peri-procedural antibiotics; (2) Patient is usually fasted for 4-6 h before the procedure; and (3) Adequate resuscitation of the patient, in case of active bleeding is ensured, prior to the procedure</td>
<td></td>
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<table>
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<tr>
<th>Technical aspects</th>
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<tr>
<td>(1) The echoendoscope is usually positioned either in the distal esophagus or the gastric fundus; (2) Water is filled intra-luminally in the fundus. This enables a good acoustic coupling for better visualization of the gastric varices. Adequate examination of the fundus, the intramural varices and the feeder vessels is carried out; (3) The approach can be trans-esophageal or transgastric, wherein the trans-esophageal route is given preference; (4) EUS-guided coil and glue embolization is usually performed using a 22 G/19 G (gauge) FNA needle. The size of the coil is determined by the short axis of the diameter of the varix; (5) After puncture of the varix, blood is aspirated to confirm the location. This is followed by flushing of the needle with saline; (6) The coils are then deployed into the varix using the stylet as a pusher. Once the coils are deployed, flushing of the needle is done with normal saline; (7) After coil deployment, 1-2 mL of cyanoacrylate glue is injected over 30-45 s followed by rapid flushing with saline; and (8) Once, the varix is obliterated, visualized by absence of flow on color Doppler, the sheath of the needle is advanced beyond the endoscope tip for 2-3 cm before withdrawing the scope. This avoids contact of glue with the endoscope tip. The sample aspirated is sent for routine cytological assessment as well as for any additional tests that might be needed</td>
<td></td>
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<table>
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<tr>
<th>Post procedure</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>(1) The patients are kept under observation for 12 h; (2) Repeat EUS can be done after 2 d to look for residual varices; and (3) Follow-up EUS can be performed at 1- and 3-mo intervals</td>
<td></td>
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EUS: Endoscopic ultrasound; FNA: Fine needle aspiration; G: Gauge.
Figure 9 Endoscopic ultrasound-guided coil embolization of fundal varix. A: Endoscopic view of the fundal varix; B: Endoscopic ultrasound (EUS) view of the fundal varix; C: EUS guided puncture of the varix with a 22-G needle; D: Coil deployment inside the varix. GV: Gastric varices.

Thus, there is a huge prospect for using EUS in PHTN, namely in the evaluation of vascular changes of the digestive wall, hemodynamic assessment by measurement of PV pressure gradient, management of variceal bleeding and re-bleeding prediction and currently liquid biopsy via PV sampling. Nonetheless, despite the diversity of possible uses, more data on efficacy and safety are warranted.

EUS guided PV access
The PV can be accessed from both the stomach and duodenum and is in very close juxtaposition with the tip of the echoendoscope. The most frequent location to target is the intrahepatic PV through the hepatic parenchyma. The other less commonly used technique is the extrahepatic PV via the duodenum[122,123].

Technique of the procedure: After confirming the vascular structure with color Doppler and pulse-wave verification, PV puncture is done via the EUS-FNA needle. Studies have shown that 25 G needle causes the least trauma. The trans-gastric, trans-hepatic approach is safer than the trans-duodenal approach. CO2 is better than using iodine as a contrast (allows better PV visualization and easier intravascular administration through the small-caliber FNA needle). After PV puncture, on withdrawal of the needle, the track is monitored with color Doppler to check for bleeding. In cases of blood flow being identified, the needle is kept in place until the flow has stopped[122,123].

Animal studies: The first case of PV access was reported in 2004 by Lai et al[124], wherein a EUS guided trans-duodenal access to extrahepatic PV was adopted with a 22 G FNA needle in 21 swine models, proving the technical feasibility of the procedure. Thereafter, PV angiography was reported for the first time in 2007 by Magno et al[125], wherein autopsies revealed no injuries with a 25 G needle and a hematoma with 19 G needle. Subsequently, Giday et al[123,126] reported trans-hepatic access to the PV with a 25 G needle.

EUS guided portal pressure gradient measurement
Measurement of PHTN is useful in determining the stage, progression, prognosis and complications of cirrhosis. Currently, the standard practice of measuring the portal pressure gradient (PPG) is the percutaneous route. However, both direct PV access
and hepatic venous pressure gradient (HVPG) measurement are invasive procedures and have high complication rates. Moreover, HVPG correlated poorly in presinusoidal PHTN cases. Therefore, EUS guided PPG can be performed to overcome these difficulties. Moreover, additional analyses such as assessment of varices and liver biopsy can be carried out in the same sitting. The technique of PPG measurement and the studies (human and animal models) on the same are shown in Supplementary Tables 5 and 6.

**EUS guided TIPS**

TIPS has an established role in managing PHTN-related complications like variceal bleeding (pre-emptive or rescue) and refractory ascites. EUS-guided TIPS creation in a live porcine model (8 cases) was first described by Buscaglia et al[127], wherein the hepatic vein (HV) and PV were sequentially punctured, and a metal stent was inserted with the distal end in the PV and proximal end in the HV. In addition, Binmoeller and Shah[128], and Schulman et al[129] have both reported using a lumen apposing metal stent (LAMS) in porcine models for the same purpose.

**EUS guided PV sampling**

“Liquid biopsy” for hepatobiliary malignancies is gaining momentum in view of the PV harboring circulating tumor cells (CTCs) from the primary tumor. These CTCs are the forerunners of future metastasis of solid organ cancers and help predict the development of liver metastasis[130]. They have been inconsistently found in the peripheral blood due to hepatic sequestration. They reflect tumor signature, help in prognostic stratification, and potentially form organoids for future tumor study. Catenacci et al[131] reported the first human study of PV sampling wherein a 19 G FNA needle was used to sample the PV as four 7.5 mL aliquots of blood. CTCs were detected in 100% cases from the PV vs 4 (22.2%) cases from peripheral blood. Liu et al [132] reported similar findings in cases of advanced pancreatic cancer (100% detection of CTCs in PV vs 54% in peripheral blood). Besides these, further studies are needed to establish the clinical utility of EUS guided liquid biopsies.

**EUS guided PV thrombosis**

The presence of malignant PV thrombosis (PVT) usually portends a poor prognosis. Therefore, differentiating bland and malignant thrombus needs FNA confirmation. Various case reports have suggested the use of EUS guided FNA of the PVT by overcoming the complications encountered via the percutaneous route[133-135] with excellent results.

**EUS guided PV injection chemotherapy**

Both systemic palliative chemotherapy and transarterial microbead injection into the hepatic artery for diffuse liver metastasis are fraught with complications. However, Faigel et al[136] reported the feasibility of EUS guided PV injection chemotherapy in 24 porcine models using drug-eluting microbeads and nanoparticles. In comparison with systemic injection, systemic levels were halved, but the hepatic concentration of drugs was doubled. Human studies are warranted for the same.

**EUS guided PV embolization**

Preoperative PV embolization before liver resection in hepatobiliary malignancies induces affected lobe atrophy and ultimately hypertrophy in the functional liver[137]. However, preliminary studies in the animal model by Matthes et al[138] and Park et al [139] using EUS guided PV embolization using ethylene-vinyl alcohol copolymer and coil with CYA glue embolization, respectively, reported high success rates.

**EUS guided PV stent placement**

EUS directed PV access has opened up avenues for stent placement via this route in PV occlusion or thrombosis. Park et al[140] reported 100% technical success (all uncovered stents) in 6 swine models.

**FUTURE ADVANCES**

**Photodynamic therapy**

Photodynamic therapy (PDT) is a commonly used modality for treating malignant biliary obstruction, requiring pretreatment with a photosensitizer followed by
exposure to selective tissue wavelength of light-generating singlet oxygen species (tissue necrosis from 6-40 mm depth) [141]. Preliminary animal studies exist on the use of EUS guided PDT on the porcine pancreas [141,142] and pancreaticobiliary malignancies (with lesions in the caudate lobe) [143].

**EUS guided fiducial marker placement**

Stereotactic body radiation therapy demands high targeting accuracy to minimize toxicity to surrounding organs. Placement of fiducial markers can help localize and track the target and can be placed via a percutaneous or surgical approach. EUS guided fiducial marker placement has come into the forefront for targeting even deeper abdominal lesions not amenable via standard means [144,145]. However, no studies exist on its use in liver malignancies.

**Artificial intelligence**

Artificial intelligence (AI) is a prediction technique using mathematical algorithms to create automated learning and recognize patterns in the fed data. Artificial neural network (ANN) and deep learning (DL) are powerful machine-learning-based tools used to provide high yield predictions and are being used more and more in the medical field to aid in diagnosis. Just like its widespread use in the field of endoscopic diagnosis of polyps and other lesions, AI has also found its place in the arena of diagnostic EUS. Studies have used ANN for the interpretation of EUS-elastography and CE-EUS [146]. However, to date, only two studies have used DL for EUS image analysis. With the availability of additional studies, AI can add to the diagnostic armamentarium of EUS and lead to much better accuracy.

**CONCLUSION**

Hepatologists have always turned to radiologists for imaging and intervention of various liver-related conditions. However, with the expansion of this intersection of endoscopy in EUS and hepatology, the field of “Endo-hepatology” may soon evolve into a sub-specialty with hepatologists trained in interventional EUS. Starting from EUS-guided liver biopsy to PV interventions, the merger of EUS and hepatology seems to show invigorating scope in the future. However, more studies are needed to establish the safety and efficacy of these newer modalities in regular mainstream practice.

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Porta-caval fibrous connections — the lesser-known structure of intrahepatic connective-tissue framework: A unified view of liver extracellular matrix

Leila Patarashvili, Salome Gvidiani, Elza Azmaipharashvili, Keti Tsomaia, Marom Sareli, Dimitri Kordzaia, Ilia Chanukvadze

Abstract

Knowledge about the connective-tissue framework of the liver is not systematized, the terminology is inconsistent and some perspectives on the construction of the hepatic matrix components are contradictory. In addition, until the last two decades of the 20th century, the connective-tissue sheaths of the portal tracts and the hepatic veins were considered to be independent from each other and that they do not make contact with each other. The results of the research carried out by Professor Shalva Toidze and his colleagues started in the 1970s in the Department of Operative Surgery and Topographic Anatomy at the Tbilisi State Medical Institute have changed this perception. In particular, Chanukvadze I showed that in some regions where they intersect with each other, the connective tissue sheaths of the large portal complexes and hepatic veins fuse. The areas of such fusion are called porta-caval fibrous connections (PCFCs). This opinion review aims to promote a systematic understanding of the hepatic connective-tissue skeleton and to demonstrate the hitherto underappreciated PCFC as a genuine structure with high biological and clinical significance. The components of the liver connective-tissue framework — the capsules, plates,
sheaths, covers — are described, and their intercommunication is discussed. The analysis of the essence of the PCFC and a description of its various forms are provided. It is also mentioned that analogs of different forms of PCFC are found in different mammals.

Key Words: Hepatic capsule; Hilar plate; Perivascular fibrous sheath; Glissonean pedicle; Portal tract; Caval port

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Core Tip: In the places of spatial intersection of the Glissonean pedicles with the main hepatic veins, the fusion of their connective tissue sheaths is described. The sites of the above-mentioned fusion are called porta-caval fibrous connections. Various forms of porta-caval fibrous connections are discussed as well as their clinical and scientific implications.

INTRODUCTION

The extracellular matrix — the connective-tissue framework of the liver — determines the shape of the organ and creates specialized compartments for the liver cell populations and blood and lymph circulations, the synergy of which determines the diverse functioning of the organ. The structure and components of the human liver extracellular matrix were comprehensively analyzed in a series of studies performed in the 1980s and the 90s[1,2].

The last five years saw a new wave of studies on hepatic connective-tissue structures. This "revisiting" is thanks to the introduction of new methods and computer technologies in morphological studies[3] and includes studies not only of the human liver but also of the liver of various animals and birds[4-7].

The emergence of endoscopic anatomic liver resections strengthened the need to specify the anatomy and interrelationship of the connective-tissue structures within the liver[8-11]. Additionally, the prospects for the use of human and animal liver matrices as scaffolds for the creation of bioartificial livers (thanks to the development of stem cells and bioengineering technologies)[11-14] also contribute to the resurgence of interests in the hepatic connective-tissue structures.

However, upon reviewing these studies, we noticed that knowledge on the connective-tissue skeleton of the liver were not systematized, the terminology was inconsistent, and the literature concerning the construction of one or another component of the hepatic matrix were sometimes contradictory[15].

Until the last two decades of the 20th century, the branches of the portal vein and the hepatic veins were considered to be independent from each other in the liver and that their connective-tissue sheaths did not make contact with each other[16-18]. Modern hepatology textbooks usually perpetuate this notion that the Glissonean portal pedicles and the main hepatic veins intersect spatially, but some liver parenchyma always remains between them. Thus, it was believed that they are anatomically independent of each other[19].

The results of the research carried out by Professor Shalva Toidze and his colleagues started in the 1970s in the Department of Operative Surgery, and the Topographic Anatomy of Tbilisi State Medical Institute changed this perception. In particular, Chanukavadze[20] showed that in some regions where they intersect with each other, the connective-tissue sheaths of the main portal complex and a hepatic vein fuse. The regions of such fusion he called porta-caval fibrous connections (PCFCs). Several forms of PCFC have been described. It has also been revealed that PCFC, as an
anatomical formation, develops in the 11th-12th weeks of gestation. Despite numerous publications, these data have not yet received proper acknowledgement in scientific discourse and, as a result, in clinical hepatology. This opinion review aims to promote a systematic understanding of the connective-tissue skeleton of the liver, standardize the definition and the nomenclature of its structural components, and highlight the importance of the hitherto underappreciated PCFC as a genuine structure.

Since the same connective-tissue structure of the liver is often referred to by different names, we have tried to standardize the terms used throughout this article. The following terms will be used in the ensuing discussion: (1) Liver capsule is the same as Laennec’s capsule (but not Glisson’s capsule); (2) Hilar plate is the same as Waleaeus vasculo-biliary sheath (but not Glisson’s plate); (3) Perivascular fibrous capsule is the same as Glisson’s capsule; (4) Proper hepatic capsule (PHC) is the same as the intrahepatic part of Laennec’s capsule covering the liver parenchyma; (5) Portal hilus is the same as portal port; (6) Caval port is the same as hepatic venous port (where the inferior vena cava adjoins to the liver and incorporates the hepatic veins); and (7) Glissonean pedicle is the same as the portal tract surrounded by Glisson’s capsule.

**DISCUSSION**

**Liver capsule and its derivatives**

Laennec’s capsule (liver capsule) covers the entire liver surface, including its bare area (aperitoneal area). In the portal hilus and venous port of the liver, Laennec’s capsule around the Glissonean pedicles and the hepatic veins enters the hepatic parenchyma, covers it, and separates it from the portal tracts and hepatic vein tributaries[21].

In the hepatic hilus, the liver capsule directly touches the hilar plate (also known as Waleaeus vascular-biliary sheath) covering the portal vein, the hepatic artery, and the bile ducts, while within the liver, the intrahepatic part of the liver capsule — PHC — covering the parenchyma, sets against the perivascular fibrous capsule (Glisson’s capsule), which is a direct extension of the Waleaeus sheath and envelops the lobar, sectoral, and segmental portal tracts[15,22]. These two fibrous fascial structures — PHC and Glisson’s capsule — are separated by a narrow fissure[10] (Figure 1A and C). The individual fibers of the connective tissue (or their bundles) are located in this fissure and connect the outer side of Gleason’s capsule with the PHC. On the other hand, soft collagen fibers (type I and III collagen) separate from the internal side of PHC and extend within the liver lobule (Disse’s spaces), fusing to the intralobular matrix[3].

In the region of the thinner portal tracts (subsegmental, zonal), Glisson’s capsule tapers off, and cross-banded collagen fibers from portal spaces are in continuity with similar fibers in the immediately adjacent lobular interstitium, which in turn are in continuity with those in central spaces; in this manner, collagen type I fibers and bundles form the structural scaffold of the liver lobule[2]. Meanwhile, the portal, extralobular and intralobular matrices of the liver are united by creating a complex labyrinth that represents the circulation area for tissue fluid and prelymph[23,24].

Laennec’s capsule covering the liver parenchyma is related to the adventitia of the hepatic veins and their tributaries, represented by type I and type III collagen fibers and single muscle fibers, mainly running along the veins. Thick collagen fibers were found external to thin elastic fibers, which were intimately related to smooth muscle. The above-mentioned features are consistent with the observation that all veins of the infracardiac region in humans are mainly propulsive veins[25]. The increase in collagen content on the adventitial side of the interface may strengthen it and prevent rupture of the vein during extreme liver movements[26].

The PHC is often separated from the adventitia of the hepatic veins and their large tributaries by a narrow slit (similar to that described in relation to Glissonean pedicles), in which the tissue fluid and prelymph circulate[23,24] (Figure 1C). The average distance between the PHC and the Glissonean pedicle is 32 ± 8.7 μm, while that between the PHC and the hepatic veins is 26 ± 6.3 μm[8]. Some authors suggest that Laennec’s capsule, Glisson’s capsule and the sheath for the hepatic vein tributaries can be characterized by a high content of thin, wavy elastic fibers. The Waleaeus vasculo-biliary sheath of the thick vessels and ducts does not contain elastic fibers[15]. However, some researchers believe that there is no fibrous sheath around the hepatic veins and that the adventitia of the hepatic veins is in direct contact with the PHC covering the liver parenchyma[27]. With the reduction of the diameter (caliber) of the tributaries of the hepatic veins, the adventitia of these veins thins out, PHC tapers off,
and intralobular connective-tissue fibers connect directly to the connective-tissue fibers of adventitia of the small tributaries of the hepatic veins[2,28]. Such a relationship further reinforces the notion that the merger of the intralobular and extralobular connective-tissue fibers and that of the capsule covering the organ create a complex, yet well-regulated, structure of the extracellular matrix, which is the connective-tissue skeleton of the liver, coordinating the synergy between the cell populations and the neural and circulatory tubular structures. The PHC is mainly composed of reticular fibers (RFs) that cover the hepatic lobules. The ring of hepatocytes abutting the connective tissue of the portal region is called the periportal limiting plate. The RF bordering the hepatocytes constituting the limiting plate forms a capsule. This capsule covers the hepatic lobule from one side and abuts the perivascular fibrous capsule (Glisson's capsule) enveloping the portal tract, from another side[3].
Based on computer software analysis of liver specimens (histotopograms), the same authors distinguish loose fiber construction (and not the fissure described above) between Glisson’s capsule and the PHC and called it the private hepatic ligament (PHL). The PHL is a structure in which collagen fibers have invaded from the portal region into the lattice-like or mesh-like RF that originally surrounded the lost hepatocytes[3]. However, it should be noted that the existence of such a formation has to be confirmed by additional studies.

There is a system of connective-tissue plates in the area of the hepatic port, whose origin and structure continue to be the subject of debate. This system includes a cystic plate, a round ligament plate, an Arantial plate, and a hilar plate (Walaeus vasculo-biliary sheath)[27,29].

The names of the plates are determined by their location: the gallbladder bed, round ligament gutter, Arantial ligament (obliterated venous duct) gutter, and hilus of the liver[30,31]. Several researchers have further described the caval plate, the connective-tissue sheath situated between the hepatic parenchyma and the adventitia of the hepatic part of the inferior vena cava[26,32]. Some researchers believe that these plates are derivatives of Laennec’s capsule, which is attached to the liver capsule as an additional outer layer in the above-mentioned areas[30]. Other researchers indicate that the plate complexes, especially the hilar plate (which has special functional and clinical significance), is not an embryological derivative of Laennec’s capsule and is connected with the fibrous part of the hepatoduodenal ligament and the connective tissues surrounding the blood vessels and bile ducts located in the portal area[27]. However, another group of researchers believes that the hilar plate does not exist at all as an independent entity; it is part of the liver capsule, which thickens in the area of the hepatic port due to a large number of thin-walled bile ducts (so-called “vaginal ductuli”). During surgery and dissection, it should be kept in mind that the hilar plate is likely to be artificially generated when, the surgeon unintentionally bundles collagenous fibers around the vaginal ductuli[15,29,33]. Taken together, the origin of the plates located on the visceral surface of the liver requires additional studies. Furthermore, we can state with confidence that the hilar plate (Walaeus vasculo-biliary sheath) covers the structures entering or exiting the liver at the hepatic port — the branches of the portal vein, hepatic artery, and bile ducts and accompanying lymphatic vessels and nerve cords. In combination with the accompanying connective-tissue fibers, afore-mentioned structures form the portal tracts that branch inside the liver. Large portal tracts, such as lobar, sectoral, segmental, and sometimes subsegmental tracts, are enveloped by a perivascular fibrous capsule (Glisson’s capsule), which forms the so-called Glissonian pedicle[30]. Glisson’s capsule is an intrahepatic extension of the hilar plate (Walaeus sheath). Thus, the portal tracts at the hepatic port are surrounded by the Walaeus sheath and inside the liver with Glisson’s capsule. As mentioned above, Glisson’s capsule is prominent around the large-caliber portal tracts but tapers off or completely disappears in thinner tracts[7].

Taking all of the above into consideration, Hu et al[8] concluded that the plate system represented a fibrous, thickened part of the Walaeus vasculo-biliary sheath and that Laennec’s capsule had no continuity with the Glissonian pedicle. However, Laennec’s capsule, which is dissociated from the main Glissonian capsule, extends to the peripheral portal tracts, where the structural integrity loosens and directly continues into the intralobular connective tissue fibers.

Laennec’s capsule is the critical structure for understanding the comprehensive surgical anatomy of the liver and standardizing extrahepatic Glissonian pedicle isolation in anatomical liver resection[21]. Its precise understanding may rewrite the descriptions in the hepatology textbooks on the relationship between the hepatic capsule and intrahepatic and extrahepatic portal pedicle sheaths as follows: the connective tissue that constitutes the hepatic capsule wraps around the portal vein, hepatic artery, bile duct, lymphatics, and nerves that enter and exit the liver from the hilar part and then enters the liver where it is distributed as a skeleton in the parenchyma[34].

Portal tracts and their connective-tissue structures
The blood vessels, bile ducts and nerves located in the portal tracts are covered by their own fascial connective tissue. These structures are individually encased by a typical membrane containing laminin, collagen type IV, entactin, and heparan sulfate proteoglycan. The surrounding portal interstitium contains collagen types I, III, V, and VI, fibronectin and tenascin[2]. The fibrous covers are separated from the blood vessel walls by a space called the conceptual paravascular body[35].
In the liver hilus and adjacent proximal part of the hepatoduodenal ligament, the connective tissue cover of the portal vein is well distinguished. It surrounds the blood vessel in the form of a sheath, inside of which there is the aforementioned paravascular fissure, which contains connective tissue fibers running in different directions, connecting the portal vein adventitia with the inner surface of its fibrous cover. Likewise, in the same regions, the hepatic artery is also surrounded by a layer of fibrous connective tissue called the fibrous cover. It is separated from the blood vessel wall by a well-defined fissure containing the bundles of connective-tissue fibers connecting the inner wall of the fibrous cover with the adventitia of the artery.[20,32]

The Brisbane Meeting of the International Society of Hepatobiliary-Pancreatic Surgery in 2000 formed a consensus on the uniform anatomical term/terminology classification to remedy the confusion that was present at that time. Their consensus was that first-order divisions of the elements of the portal triad were those that supplied the right and left halves of the liver, second-order divisions were those that supplied the liver sectors, and third-order divisions were those that supplied the segments.[36]

The perivascular fibrous capsule abruptly appears in the area of the sectoral portal tract. It is dense and easily separates from the liver tissue, which in turn is covered by the PHC (the intrahepatic part of Laennec’s capsule).[3]

The perivascular fibrous capsule is formed by collagen fibers running in various directions (elastic fibers are relatively rare). In addition, the outer layer of the capsule is denser. The relatively loose inner layer is contiguous to the connective tissue that surrounds the covers of individual elements of the portal triad. The thickness of the perivascular fibrous capsule is 45-110 μm (average 70-75 μm). Gradually, with the decrease in the caliber of the portal tract, the perivascular fibrous capsule also becomes thinner. The perivascular fibrous capsule of the 2-3 mm caliber subsegmental portal tract loses its sheath-like structure and transforms into loose connective tissue located between the individual elements of the portal triad.

The thickness of the proper cover of sectoral and segmental branches of the portal vein ranges from 50 μm to 150 μm (on average 90-100 μm) and it is directly proportional to the caliber of the blood vessel. The portal vein cover, within the subsegmental tract, gradually becomes thinner and looser. In addition, studies have shown that in 15% of cases, the identification of the connective tissue cover of the portal vein is hampered, even around the sectoral and segmental branches.[32,37]

The number of bile ducts in sectoral and segmental portal tracts always exceeds three. Bile ducts are enveloped by the fibrous parabiliary sheath. The sheath has circularly oriented internal bundles, while the external bundles form septa oriented in various directions and connect closely to both the adjacent bile duct wall and the perivascular fibrous capsule. Bile ducts are accompanied by the peribiliary glands, which are connected to the ducts along their opposite edges. The glands can be distinguished between intramural and extramural parts. The extramural part of the glands is several times larger in size than the intramural part. It is covered by the fibers of the fibrous parabiliary sheath, extends a considerable distance from the duct wall, is closely related to the connective tissue sheaths of other elements of the portal complex, and sometimes directly attaches to the perivascular fibrous capsule. Occasionally, the fibers covering the peribiliary glands and that of the internal surface of the perivascular capsule are so intertwined that no border can be identified between them.[32,38-40]

The number of branches of the hepatic artery with a caliber larger than 1 mm varies from 2 to 5 in each sectoral and/or segmental portal tract. They are located more centrally (closer to the portal vein branch) than the bile ducts. The covers of the hepatic artery are not as distinct in sectoral and segmental tracts as in the hepatic hilus or hepatoduodenal ligament. The paravascular fissure is invisible as the adventitia is virtually contiguous with its own cover. The covers of the arteries at the peripheral edges of the blood vessels extend into the septa, which often interconnect and create the circular layer of para-arterial connective tissue located between the portal vein and the parabiliary fibrous sheath (Figure 1C). The degree of differentiation of the connective tissue covers of the arteries strongly depends on the caliber of the portal tract. In the small (subsegmental and thinner) portal tracts, the arteries have no connective tissue covers at all, and they are surrounded only by loose connective tissue that forms a bed for all elements of the portal triad[32,37]. Therefore, a combination of paravascular and parabiliary connective-tissue formations concentrated around the portal vein makes the skeletons of the hepatic portal tracts. The perivascular fibrous capsule, with adjacent parabiliary tissue with bile ducts and peribiliary glands, is located on the periphery of Glissonian pedicles[32,37].
PCFCs
In the liver, at the site of the spatial intersection of the main portal tracts and the hepatic veins, there is a little-known anatomical formation generated by the fusion of the connective-tissue fibrous sheaths of the portal tracts and the hepatic veins where these two structures come into contact with each other. The perivascular fibrous capsule extends from the portal complex to the wall of the hepatic vein and it becomes an additional element (Figure 1A, B, D and E). An anatomical formation created by the fusion of the sheaths of portal tracts and hepatic veins is called the intrahepatic PCFC [20,32].

Anatomical classification of PCFCs
Various forms of PCFC are distinguished.

Complete fusion: This type of porta-caval connection is characterized by the complete fusion of the surfaces of connective tissue sheaths of the portal tract and hepatic vein directed towards each other (Figure 1D and 2B). This type of connection is mainly found in segments II and III of the liver. The connective tissue sheaths of the hepatic veins are highly developed in the PCFC area, and its thickness reaches 90 μm. It represents a thick network of the collagen fibers running in various directions and the spiral bundles of elastic fibers and separate cellular elements are located between them. At the same time, irrespective of the density of the elements of the portal triad that merge with the hepatic veins in the area of the PCFC, there is always a narrow gap between them, filled with loose connective tissue. Small blood vessels (up to 1.5 mm in diameter), which are separated from the branches of the hepatic artery located in the portal tract, might pass through this place. They extend to the wall of the hepatic vein and supply it with blood.

Touching connection: This type of PCFC occurs when the perivascular fibrous capsule and the sheath of the hepatic vein merge only with the parts of the surface facing each other, while the rest of the space between them is filled with liver tissue. Similar to complete fusion, this form of PCFC also contains small blood vessels, but rarely the nerves or lymphatics. Touching PCFCs are often found within segments II, III, VI, and VII of the liver.

Fan-shaped connection: The fan-shaped connection, a special form of connection, is formed when the 2-5 mm caliber portal tract touches the wall of the inferior vena cava or large hepatic vein and immediately splits into thinner branches. The fan-shaped PCFC is constantly found within segment I (caudal lobe), including the inferior vena cava wall. The branches feeding the wall of the inferior vena cava or large caliber hepatic veins are separated from the arteries of the portal tract within this connection [20,32]. Within the complete fusion, touching and fan-shaped PCFCs, the hepatic vein is most often bordered by the bile ducts and their peribiliary glands. Such direct contacts may facilitate the spread of the inflammatory process from the bile ducts to the liver[32].

Plate and thread-shaped connections: The plate or thread-shaped PCFCs are represented by a fibrous plate or a cone that stretches between the perivascular fibrous capsule and the hepatic venous sheath. The plate may contain small blood and lymphatic vessels[20,32] (Figure 1E and 2A).

It should also be noted that the presence of various forms of PCFC has been confirmed in other mammals (pigs, sheep, dogs, rats). In the histological liver specimens of these animals, the sites of the crossing of different size portal tracts and hepatic vein tributaries with integration (fusion) of their connective-tissue sheaths were described. At the same time, in rat livers, the translocation of biliary structures from the portal tract toward hepatic veins was shown. This translocation causes the appearance of ductular profiles accompanying hepatic veins and their tributaries on histological specimens[32] (Figure 2C and D).

Clinical significance of PCFCs
Today, among the modern methods of surgical treatment of portal hypertension complicated by bleeding from varicose veins, the transjugular method of intrahepatic porta-caval anastomosis, which has a palliative effect, is widely used[41]. However, this method is often accompanied by complications; the most common ones are thrombotic or proliferative occlusion of the endoprosthetic shunt implanted between the branches of portal and hepatic veins, as well as stent migration-transposition[20]. This is exacerbated by the fact that the tubular shunt-prosthesis is often placed between the right branch of the portal vein and the right hepatic vein, which are
Figure 2 Porta-caval fibrous connections in humans and animals. A: Plate-shaped porta-caval fibrous connection (PCFC) (histotopogram of liver tissue): 1: Lumen of the portal vein; 2: Lumen of hepatic vein tributary; 3: Bile ducts and biliary glands (filled with Indian ink); 4: Liver parenchyma; Red arrow: Proper hepatic capsule (PHC); Blue arrow: Perivascular fibrous capsule (Glisson’s capsule); Green arrowhead: Fissure among the PHC and Glisson’s capsule; Yellow arrowhead: Fissure among the PHC and perivenous connective-tissue sheath. Large portal tract is surrounded by a yellow ellipse; 1: Lumen of the portal vein; 2: Lumen of hepatic vein tributaries; 3: Small portal tract; 4: Liver parenchyma; 5: Bile ducts (filled with Indian ink); Green arrowhead: The area of complete fusion; Red arrowhead: Thread-shaped PCFC (preparation from the private archive of Professor Ilya Chanukvadze); C: PCFC in rat liver (surrounded by a red ellipse). 1: Lumen of the portal vein; 2: Lumen of a hepatic vein; Red arrow: Bile ductule abutted to hepatic vein connective tissue sheath (preparation from the private archive of Professor Dimitri Kordzaia). Hematoxylin-eosin, Ob × 10, Oc × 10; D: Fragment of Figure C. Hematoxylin-eosin, Ob × 40, Oc × 10. C and D: Citation: Kordzaia D, Jangavadze M. Unknown bile ductuli accompanying hepatic vein tributaries (experimental study). Georgian Med News 2014: 121-129. Copyright ©Georgian Medical News 2014. Published by Georgian Medical News.

CONCLUSION

In the human liver where the portal tracts and hepatic veins spatially intersect (spatial crossing), the fusion of their connective-tissue sheaths develops an anatomical structural element in the form of a nodal fibrous connection — “porta-caval fibrous connections” — significantly separated from each other (from 2 cm to 9 cm). The longer the shunting prosthesis is, the higher the likelihood of thrombosis, suppression and/or transposition. It is quite probable that the endovascular method may be more successful in developing portocaval anastomoses in the area of PCFCs, where parenchyma-free areas of direct contact between the walls of large branches (5 mm to 20 mm) of the hepatic and portal veins already exist. It is preferable to perform endovascular intervention on liver segments II and III, where the left hepatic vein passes below the main portal complex and is in direct contact with the portal vein branch, as well as between the right hepatic vein and the portal vein branch of segment VII. The various types of branching of the portal and caval veins determine a large variation in the number of PCFCs — from 4 to 20; however, despite this, the above-mentioned PCFCs in segments III and VII are characterized by high stability. In addition, the sites of integration within the connective-tissue sheaths of the large portal tracts and hepatic veins with the standard topography can be visualized by magnetic resonance imaging.
connection” — allowing the hepatic vein to interact closely with the elements of the portal complex. The PCFC is a stable structure, whose formation begins at the 11th–12th week of embryogenic development. Based on the above discussion, intrahepatic PCFC can be considered an independent anatomical element of the liver, which deserves to be reflected in international anatomical nomenclature. Knowledge of the existence and features of PCFC enhance our understanding of the liver connective tissue framework and support the development of new surgical approaches for the treatment of various liver pathologies.

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Promising diagnostic biomarkers of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis: From clinical proteomics to microbiome

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Abstract

Fatty liver has been present in the lives of patients and physicians for almost two centuries. Vast knowledge has been generated regarding its etiology and consequences, although a long path seeking novel and innovative diagnostic biomarkers for nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) is still envisioned. On the one hand, proteomics and lipidomics have emerged as potential noninvasive resources for NAFLD diagnosis. In contrast, metabolomics has been able to distinguish between NAFLD and NASH, even detecting degrees of fibrosis. On the other hand, genetic and epigenetic markers have been useful in monitoring disease progression, eventually functioning as target therapies. Other markers involved in immune dysregulation, oxidative stress, and inflammation are involved in the instauration and evolution of the disease. Finally, the fascinating gut microbiome is significantly involved in NAFLD and NASH. This review presents state-of-the-art biomarkers related to NAFLD and NASH and new promises that could eventually be positioned as diagnostic resources for this disease. As is evident, despite great advances in studying these biomarkers, there is still a long path before they translate into clinical benefits.
INTRODUCTION

Thomas Addison first described “fatty liver” in 1836 in England; however, it was not until 1885 when Bartholow made an association between obesity and fatty liver. In 1938, Charles Connor demonstrated a link between fatty liver and progression to cirrhosis in diabetic patients. Throughout the 1950s and up to the 1970s, pathologists reported similarities between alcoholic liver disease and hepatic histological changes in obese and diabetic patients. In 1980, Jurgen Ludwig[1] described patients who denied excessive alcohol consumption yet still had chronic liver disease and histological characteristics of alcoholic fatty liver disease. There was no name for the disease, so Ludwig coined the terms nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH)[1].

As reported in the most recent guidelines, NAFLD is defined as the presence of steatosis in > 5% of hepatocytes in the absence of significant ongoing or recent alcohol consumption and other known causes of liver disease. While in 2005 it had a global prevalence of 15%, a rapid increase in sedentarism and excessive calorie intake independent of diet has pushed it to 24%, with the highest rates in South America (31%) and the Middle East (32%), followed by Asia (27%), the United States (24%), and Europe (23%)[2]. In persons with obesity or type 2 diabetes, it increases up to 70%-90% [3]. Although there is a significant difference between ethnicities within these populations, the exact explanation remains unknown[2].

NAFLD is a necessary and opportune diagnosis, given that 59% progress to NASH. From this stage, 41% continue to fibrosis, with 40% ending with cirrhosis, increasing their risk of a liver transplant, cardiovascular disease, and mortality if there are no interventions[4]. In our country, the Mexican population has several risk factors for the disease because there is a high incidence of overweight and obesity[5], making the NAFLD prevalence likely to surpass 50%. Up to 82% of obese patients who have undergone bariatric surgery present NAFLD, alongside 36% of women with obesity[6].

An international panel has now proposed to rename the disease metabolic dysfunction-associated fatty liver disease to represent the hepatic manifestation of a multisystemic disorder. Until now, the diagnosis was reached by the exclusion of other liver diseases; however, as the pathogenesis is better understood, it is now perceived as a distinct disease and requires a positive diagnosis, which is why it is proposed that the criteria be based on histological, imaging, or blood biomarker evidence of fat accumulation in the liver in addition to one of the following three: Overweight/obesity, type 2 diabetes mellitus, or evidence of metabolic dysregulation (at least two metabolic risk abnormalities)[1].
Today, the liver biopsy remains the gold standard for diagnosing and monitoring liver disease, with the disadvantage of being a costly and invasive procedure[7], which is why it is important to look into possible new noninvasive diagnostic tools, such as biomarkers, use of transcriptomics, proteomics, metabolomics, and now “glycomics”[8]. These should aid in predicting liver disease severity, progression, and response to lifestyle changes and pharmacological treatment[9]. The objective of this article is to review concisely and present the potential diagnostic biomarkers for NAFLD and NASH (Figure 1).

PROTEOMICS

The concentrations of several plasma components are determined in routine clinical practice, including electrolytes, molecules, and proteins. Plasma proteins, which constitute the plasma proteome, are released as a result of inflammation, apoptosis, and oxidative stress (OS)[10]. Mass spectrometry-based proteomics[9] and two-dimensional electrophoresis are powerful tools for studying differences[11] in the plasma proteome. There are differences in protein expression among patients with NAFLD and healthy controls. Proteomics technologies have gained relevance as potential non-invasive diagnostic methods for NAFLD.

Plasma proteomics

Plasma proteomics may be secreted by the liver or as a result of the response of the host to steatosis. Hemoglobin is currently the most replicated proteomic biomarker in NAFLD[12]. Studies have found that higher hemoglobin levels are associated with a higher incidence of NAFLD[12]. Circulating aminotransferase [aspartate aminotransferase (AST) and alanine aminotransferase (ALT)] levels are markers of several liver diseases, including NASH. Changes in these enzymes are one of the most commonly observed abnormalities[10].

Fibroblast growth factor 21 is another protein secreted in response to peroxisome proliferator-activated receptor (PPAR)-α activation, and several studies support its potential use as a biomarker for NAFLD[13,14]. The elevation of retinol-binding protein 4 has also been associated with liver fat accumulation[15]. Some glycoproteins like serum fucosylated haptoglobin and Mac-2 binding protein are predictors of hepatocyte ballooning and liver fibrosis[16].

Cytokeratin-18 fragments, such as CK18Asp396, are other proteins that have been extensively studied. These are produced during apoptosis (M30) or cell death (M65). CK18 is the most reviewed biomarker to evaluate liver inflammation[15], but current knowledge does not support its use in clinical practice[17] because of its modest accuracy[8].

Increased cytokeratin-18 levels have good predictive value for NASH vs normal livers but do not differentiate NASH vs simple steatosis[18,19]. Cytokeratin-18 serum levels decrease parallel with histological improvement, but its predictive value is not better than ALT in identifying histological responders[20].

Circulating concentrations of cytokeratin-18 fragments were proposed as the most reliable predictors of NASH in patients with NAFLD[21].

Circulating extracellular vesicles

Another important plasma component includes circulating extracellular vesicles (EVs), which are small cell-derived membrane-surrounded structures enclosed by a phospholipid bilayer, with a specific cargo of bioactive molecules of cell origin. There are three types according to their size: Exosomes (40-100 nm), microvesicles or microparticles (0.1-1 μm), and apoptotic bodies (1-4 μm)[22].

They can be detected in several body fluids and can serve several functions by delivering a variety of bioactive molecules, including non-coding RNAs, proteins, lipids, and nucleic acids[23]. Recent studies have provided insight on the bioavailability of circulating EVs in various fluids and, as a consequence, on their potential use as biomarkers for various diseases such as cancer[20,24,25], cardiovascular disease[26], renal disease[27], and liver disease[28,29].

Some authors consider them noninvasive “liquid biopsies” for NASH diagnosis, and studies suggest they can assess disease severity[30]. Serum levels of total and hepatocyte-derived EVs correlate with NASH clinical characteristics, and disease severity in experimental models of NASH, liver and blood levels of EVs are increased and correlate positively with changes in liver histology[31].
Figure 1 Although liver biopsy remains as the gold standard for the diagnosis of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis, other current imaging studies are shown, along with promising diagnostic and/or monitoring biomarkers that may be present in each of the stages of hepatic pathology, ranging from reversible steatosis and inflammation to irreversible fibrosis and eventually cirrhosis (Figure 1 created with BioRender.com). US: Ultrasound; TE: Transient elastography; BMI: Body mass index; Hb: Hemoglobin; FGF-21: Fibroblast growth factor 21; RBP4: Retinol binding protein 4; CK18Asp396: Caspase cleaved cytokeratin-18 fragment; TMAO: Trimethylamine N-oxide; LDL-c: Low density lipoprotein cholesterol; Fecal SCFAs: Fecal Short chain fatty acids; fCh: Ferrochelatase; IL-17: Interleukin-17; IL-22: Interleukin-22; PPARα: Peroxisome proliferator-activated receptor α; DR5: Death receptor 5; miRNA-122: MicroRNA 122; miR-192: MicroRNA 192; N/L ratio: Neutrophil/lymphocyte ratio; Th17/Treg imbalance: T helper 17/T regulatory cells imbalance; IL-1: Interleukin-1; IL-6: Interleukin-6; TNFα: Tumor necrosis factor alpha; ASGPR1+: Asialoglycoprotein receptor 1; CNN2: Calponin 2; miRNA-214; MicroRNA 214; miR-34a: MicroRNA 34a; Hifβ1: Hepatic fibrosis 1; N/L ratio: Neutrophil/lymphocyte ratio; IFNγ: Interferon γ; IL-4: Interleukin-4; IL-13: Interleukin-13.

Povero et al[30] performed a study isolating EVs from controls with histologically confirmed NASH without cirrhosis and patients with cirrhotic NASH[30]. After the characterization of EV structural features, they found that differences in the quantity and protein components of circulating EVs could be potentially useful for differentiating patients with NASH from controls and patients with pre-cirrhotic NASH from patients with cirrhotic NASH[30]. Notably, asialoglycoprotein receptor 1-positive hepatocyte-specific EVs may represent a surrogate noninvasive biomarker of portal hypertension in patients with cirrhotic NASH. If confirmed, these findings may support the clinical utility of asialoglycoprotein receptor 1-positive EVs (hepatocyte-specific EVs) as a potential alternative to an invasive hepatic venous pressure gradient[30].

Patients with NAFLD or NASH secrete increased levels of microvesicles derived from macrophages/monocytes [CD14(+) and natural killer (NK) T cells; these levels correlate with NASH severity based on histology[28]. Hirsova et al[32] have demonstrated that lipids that stimulate death receptor 5 on hepatocytes also induce the release of hepatocyte EVs that activate an inflammatory phenotype in macrophages that lead to NASH[32]. However, a major problem in translating this research into clinically useful information is a lack of reproducibility and rigorous criteria for reporting these biomarkers. Proteomics analysis of EVs from patients with advanced NASH is currently limited.

Exosomes
Exosomes are a type of EVs secreted in most cells[22]. These nanovesicles of endocytic origin are present in nearly all-human fluids. Exosomes have several bioactive
molecules, including proteins, lipids, and genetic materials[33]. They are conduits for intracellular transfer, and their signals can induce fibrosis, macrophage activation, cytokine secretion, and remodeling extracellular matrix (ECM) production and inactivate hepatic stellate cells (HSC)[34]. Hepatocytes are exosome-secreting cells that are also regulated by hepatic and extrahepatic exosomes[33].

Koeck et al[35] found that exosomes from visceral adipose tissue were involved in the progression of NAFLD by inducing dysregulation of the transforming growth factor-beta (TGF-β) pathway in hepatocytes and HSCs in vitro[35]. Another study by Seo et al[36] detected that during liver injury, damaged hepatocytes produce exosomes that activate toll-like receptor 3, which exacerbates liver fibrosis by enhancing interleukin-17A (IL-17A) production by γδ T cells[36].

Liver fibrogenic pathways are primarily controlled by HSC, which produces and responds to fibrotic mediators such as connective tissue growth factor (CCN2)[37]. Tadokoro et al[29] found that CCN2 upregulation in fibrotic or steatotic livers is associated with the downregulation of microRNA-214 (miRNA-214). miR-214 levels increased in quiescent HSC-secreted exosomes compared with active HSC-released exosomes[29]. On the other hand, exosomal CCN2 may amplify fibrogenic signaling and might be useful for assessing hepatic fibrosis[37].

Chen et al[38] found that the miR-214 promoter binds to the basic helix-loop-helix transcription factor (Twist1), which drives miR-214 expression and results in CCN2 suppression. Twist 1 expression was suppressed during HSC activation. The amounts of Twist1, miR-214, or CCN2 in circulating exosomes from fibrotic mice reflected fibrosis-induced changes in the liver[38]. These findings suggest that during liver fibrosis, exosomes contain specific types of biomarkers, which could be helpful in the diagnosis and progression of liver diseases.

miRNA
Circulating microRNAs (miRNA) are RNA molecules that do not encode proteins but regulate gene expression in the body, binding to target miRNAs and interfering with their translation[22]. They are expressed in several liver cell types and may offer a biologically stable blood-based biomarker tool for the detection and stratification of liver disease[29].

Tadokoro et al[29] have suggested that serum/plasma miR-122 correlates with liver damage. They have also identified that miR-155 might serve as a liver inflammation biomarker. The one limitation found is that this miRNA cannot differentiate different liver damage etiologies[29].

Another study reported that miRNA-122 and miR-192 levels are dynamic and increase over time, closely correlating with the histopathological severity of NASH[31]. The miR-29 family (miR-29a, miR-29b, miR-29c) mediates the regulation of liver fibrosis through several cellular signaling pathways such as the nuclear transcription factor-kappa B pathway, TGF, and phosphatidylinositol 3-kinase/ AKT signaling in HSC with upregulation of ECM genes for the progression of liver fibrosis[39].

Members of the miR-34 family (miR-34a, miR-34b, miR-34c) have pleiotropic roles in the cell cycle and promote the progression of hepatic fibrosis by activation of HSC[39]. miR-34a appears to have an important role in liver fibrosis by regulating the deposition of ECM[40]. miR-30c and miR-193 are also involved in fibrotic remodeling processes that modify the TGF-β-dependent regulation of ECM-related genes in HSCs[41].

The miR-15 family mainly regulates the TGF-β pathway. The activation of HSCs relates to miR15a and miR15b, and they are thought to be essential for apoptosis by targeting Bcl-2 and the caspase signaling pathway[42]. The miR-378 family (specially miR-378a-3p) suppresses the activation of HSCs by directly targeting Gli3[43]. miR-571 closely correlates with the liver cirrhosis stage, and it is upregulated in human hepatocytes and HSC[44]. miR-503 also acts on HSC activation and hepatic fibrosis through the TGF-β/SMAD pathway[45].

The miR-199 family and miR-200 family are responsible for ECM deposition and the release of profibrotic cytokines, which might play profibrotic or anti-fibrotic roles[39]. HSCs also have anti-fibrotic miRNAs, and these include miR-19b, miR-29, miR-30, miR-101, miR-122, miR-133a, miR-144, miR-146a, miR-150-5p, miR-155, miR-195, miR-200a, miR-214, miR-335, miR-370, miR-454, miR-483, etc. The latter are responsible for the maintenance of the quiescent phenotype of normal HSCs[46]. Thus, these studies evidence the role of microRNAs as potential biomarkers of liver damage in NAFLD.
**METABOLOMICS**

Technological advances in metabolomic analyses on feces, serum, plasma, urine, or liver biopsies led to identifying different metabolites in patients with NAFLD or NASH[47]. Recent studies have found that the severity of fibrosis is associated with serum metabolite changes[48-50]. Remarkably, some metabolites come from the host or the diet, but most need the participation of gut microbes. Notably, inosine and hypoxanthine are enriched in serum samples from patients with mild or moderate NAFLD[47]. Another study found that liver steatosis correlates with phenylacetic acid levels in humans[51]. Glutathione plasma concentration is significantly lower in subjects with liver steatosis, while in subjects with NASH, homocysteine and cysteine concentrations in plasma are higher [52].

**Gut microbially-derived metabolomics**

**Choline, betaine, and circulating methylamines:** Choline is an essential component of phosphatidylcholine (a precursor of acetylcholine), mostly obtained from the diet[53]. It is known that a reduction in dietary choline is related to an increase in liver fat. Mice fed with a choline deficient diet are identified as a characteristic model of NAFLD[54]. Choline can be oxidized to betaine, and it has been found that patients with increasing severity of NAFLD have a decreased betaine to choline ratio[55]. The gut microbiota metabolizes choline into trimethylamine (TMA), which is further metabolized into trimethylamine-N-oxide (TMAO) in the liver[56]. Studies suggest that NAFLD severity is associated with increased urinary levels of TMA and TMAO, while TMAO seems to be associated with NAFLD severity[47].

**TMAO and bile acids:** Gut microbiota regulates secondary bile acid metabolism and inhibits the liver synthesis of lipids by alleviating farnesoid X-activated receptor inhibition[57]. TMAO is a gut-dependent metabolite of choline. A decreased level of bile acids could be associated with TMAO production and NAFLD since it induces a decrease in the bile acid pool by inhibiting two key enzymes of bile acid metabolism: Cytochrome P450 (CYP)7A1 and CYP27A1[55]. Some studies have found adverse associations between the circulating TMAO levels and the presence and severity of NAFLD and a favorable betaine-NAFLD relationship in participants[55].

**Three-(4-hydroxyphenyl) lactate:** Three-(4-hydroxyphenyl) lactate is a derived product of amino acid metabolism. It was consistently associated with increased liver fibrosis severity in a test and validation cohort[48].

**Ethanol:** Gut microbiota leads to endogenous ethanol production, which might be a liver toxin involved in NAFLD and NASH development[47]. A study showed that *Klebsiella pneumoniae* can produce ethanol from glucose in the absence of alcohol consumption, and it might be associated with NAFLD[58].

**LIPIDOMICS AND LIPOTOXICITY**

Human serum and plasma are composed of lipids that play important roles in energy storage, metabolic regulation, signaling, etc.[10]. Technological advances have made possible the identification of specific alterations in lipids and metabolites in the feces, serum, plasma, urine, and liver of patients with NAFLD[47]. Choline is a dietary component metabolized in the liver, necessary for cell function. Epidemiological studies suggest that increased free choline levels are related to the degree of hepatic steatosis fibrosis[59]. Kalhan et al[60] have shown that plasma levels of triglycerides[60] and low-density lipoprotein cholesterol are higher in patients with NAFLD[52]; however, differences in this lipidomic profile are also observed in obesity. Therefore, this lack of specificity remains a limitation for their use. Barr et al[61] described a lipidomic signature associated with NAFLD progression to distinguish NASH from steatosis, depending on the body mass index in a large cohort of samples[61].

Gorden et al[62] described a panel of 20 lipids that differentiate patients with NASH and liver steatosis[62]. Later, Kimberly et al[63] identified the association between anandamide (endocannabinoid derived from arachidonic acid metabolism) and NAFLD severity[63]. Tokushige et al[64] reported 28 metabolites associated with liver fibrosis, showing a decrease of dehydroepiandrosterone sulfate and etiocholanolone-S with the progression of fibrosis[64].

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*Castillo-Castro C et al. Promising biomarkers for liver disease*
Puri et al.[65] analyzed plasma lipids and eicosanoid metabolites in NAFLD and NASH patients. They reported increased plasma monounsaturated fatty acids and primary palmitoleic and oleic acids and decreased linoleic acid. Plasma levels were significantly decreased in NASH, and 11-HETE (a nonenzymatic product of arachidonic acid) was increased in NASH[65]. Loomba et al.[66] assessed the lipidomic profile in NAFLD and NASH patients and reported that 11,12-dihydroxy- eicosatetraenoic acid (11,12-diHETE) was the best biomarker for differentiating NAFLD from NASH[66].

Short-chain fatty acids (SCFAs) are comprised of butyrate, acetate, and propionate. They are produced in the colon through microbial fermentation of dietary fiber and are a substrate that increases liver triglyceride levels[67]. They are also involved in fatty acid synthesis and gluconeogenesis[68]. Human studies have observed an increased fecal concentration of SCFAs in patients with NAFLD and/or NASH[69].

In NAFLD, lipid metabolism is disrupted, and lipotoxicity is a key mechanism for NAFLD progression. Lipidomic profiling might provide a novel biomarker for the noninvasive prediction of NASH.

**GENETIC MARKERS**

The role of genetic and epigenetic factors in the progression of liver fibrosis is well documented. It is known that key regulatory genes partially control the cell phenotype. Several genes are involved in the pathogenesis and histological stage of liver fibrosis, although the mechanisms underlying gene regulation are highly complex and need additional research[70].

Chromosome 15, designated Hfib1 (hepatic fibrogenic gene 1), affects the stage of liver fibrosis[71]. The core of risk genes that control fibrosis progression has been defined by quantitative trait locus analysis in mouse strains by genome-wide interval mapping, which identified several genomic loci related to fibrosis phenotypes on chromosomes 4, 5, 7, 12, and 17[72].

Bruschi et al.[73] reported that PLPNA3 quantification correlates with the liver fibrosis stage. Expression of PLPNA3 in biopsies from NASH patients is increased during progression from mild to severe liver fibrosis. Carriers of the I148M single-nucleotide polymorphism (C>G) had higher PLPNA3 and serum liver enzyme (ALT/AST) levels, along with steatosis grade inflammation ballooning and NAFLD activity score, compared with non-polymorphism carriers[73]. On the other hand, Sharma et al.[74] stated that neurocan is associated with NASH and liver fibrosis in patients of European ancestry. Another study found that patients of Indian descent with neurocan variations had higher ALT levels[74].

**EPIGENETIC MARKERS**

Epigenetics describes reversible gene expression changes that do not imply changes in the DNA sequence and are entirely cell type-specific. Epigenetic mechanisms initiate and sustain chromatin modifications by facilitating gene transcription, cell phenotype, and consequently, organ function. These mechanisms include DNA methylation, histone modifications, and noncoding RNAs mediating gene silencing[75].

Aberrant DNA methylation is associated with fibrosis. Komatsu et al.[76] suggested that DNA hypomethylation in fibrogenic genes is crucial for the onset and progression of liver fibrosis[76]. Mann et al.[77] confirmed this functional association of DNA methylation with liver fibrosis. The transdifferentiation of HSC to profibrogenic myofibroblast phenotype was suppressed in vitro by the DNMT inhibitor 5′-aza-deoxycytidine[77]. The development of fibrosis is also related to changes in the expression of enzymes that regulate DNA methylation and hydroxymethylation[78].

Epigenetic modulation on the PPAR-γ gene promoter is involved in HSC differentiation. Aberrant expression of a series of chemokines in HSCs aggravate inflammation and OS[79].

Small non-coding RNAs contribute to various pathologic states of liver disease, but miRNA has been previously reviewed. The detection of genetic and epigenetic markers may be helpful in the recognition and monitoring of disease evolution and can eventually be applied for targeted therapies.
NASH pathology encompasses an intricate network of mechanisms. OS activates Kupffer cells (KC), and KC activation triggers an innate and adaptive immune response, including the release of cytokines and chemokines that activate NK T (NKT) cells and HSCs[80]. Besides, there is augmented infiltration of different immune cells, such as monocytes, T lymphocytes, and neutrophils, in the activation and in situ expansion of liver cells, like KC or stellate cells. Activated KC and NKT cells promote additional fat accumulation in the liver. KC, neutrophils, NKT cells, and inflammatory T cells [T helper (Th)1, Th17, CD8+ T cells] enhance liver inflammation and contribute to the development of fibrosis[81].

The neutrophil to lymphocyte ratio (N:L ratio) has been proposed as a novel noninvasive marker to predict NASH and advanced fibrosis in patients with NAFLD [82]. In patients with cirrhosis, these cells are functionally deficient, with impaired chemotaxis, phagocytosis, and intracellular killing. Their function correlates with 90-d survival[83].

On the other hand, monocytes are myeloid-derived cells that migrate to inflammation sites, phagocytose microbes, and secrete cytokotoxins. They are spontaneously activated in patients with liver fibrosis. Cirrhotic patients have an increased peripheral frequency of monocytes, impaired phagocytosis, and reduced responses to stimulation [84].

Studies have reported that NK cells are dysregulated in liver diseases. One study found that IL-17- and IL-22- secreting iNKT cells are dominant at the beginning of liver steatosis, and IFNy/IL-4/IL-13-secreting iNKT cells are prevalent at the most advanced course of the disease[85].

Notably, CD4+ T cells are reduced in patients with liver fibrosis. This finding could explain the increased risk of spontaneous bacterial peritonitis in these patients[86]. CD8+ T cells isolated from mice hepatic cells expressed an increased cytotoxic IL-10 phenotype and CD8+ T cell depletion[87].

Th17 cells and T regulatory cells (Treg) originate from naïve T cell precursors. Th17 cells are important for pathogen clearance and inflammation. Treg cells in patients with liver fibrosis are significant[88]. There is a Th17/Treg imbalance that positively correlates with NASH histological progression[89].

Innate lymphoid cells are lymphocytes that secrete cytokines and chemokines in response to pathogenic tissue damage. They have a role in inflammation and fibrogenesis that progresses with advancing chronic liver disease[90].

**OS AND INFLAMMATION**

Detoxification is a crucial hepatic activity. It is vulnerable to OS and inflammation. An increase in free fatty acids is critical for the elevation of reactive oxygen species (ROS). A balance between the ROS and antioxidant systems is necessary for adequate cell function[80]. OS causes liver damage by altering DNA molecules, proteins, and lipids and modulating pathways associated with gene transcription, protein expression, cell apoptosis, and HSC activation. Inflammation is manifested as inflammatory cell infiltration in the liver to fight pathogen invasion. When the stimuli are persistent, it can lead to cell injury and lipid accumulation associated with an increased risk of severe liver disease, including steatohepatitis and fibrosis[91].

In NASH, ROS are generated in several ways that can alter signaling pathways, such as cell kinases, phosphatases, and transcription factors, which impact cell proliferation, differentiation, and apoptosis. They can lead to cirrhosis via the rebuilding of stellate cells and ECM within the liver. Substantial hepatic ROS is produced by excessive angiotensin II and activated CYP2E1, resulting in impaired beta-oxidation and eventually fatty liver[91].

Lipotoxicity in NAFLD causes OS and induces organelle damage due to decreased antioxidant systems, mitochondrial dysfunction, and an increase in unfolded protein response by endoplasmic reticulum stress[80]. On the other hand, there is an impairment of α-oxidation due to a decrease in PPARα activity, which upturns hepatic lipid levels. Fatty acid overload is the major source of reducing equivalents responsible for increased ROS production. Also, TNF-α and lipid peroxidation products could induce mitochondrial dysfunction. Mitochondrial damage will result in secondary lipid α-oxidation inhibition and a further increase in the degree of steatosis[80].

Furthermore, inflammatory cytokines such as IL-1-β, TNF-α, and IL-17/20/33, chemokines, like monocyte chemoattractant protein-1 and C-X-C chemokine ligand 10,
and the toll-like receptor pathway are intensively involved in the regulation of hepatic fibrogenesis[91]. Macrophage activation and influx in the liver are important for the progression of NAFLD since hepatic macrophages promote NASH development via cytokines IL-1, IL-6, and TNF-α[92]. Liver failure causes an increase of TNF-α, IL-6, and angiotensin II[80].

OTHER NOVEL MARKERS

Gut permeability markers
The intestinal barrier is composed of chemical, physical, and immunological barriers. Maintaining a healthy barrier is essential to prevent microbial translocation and keep the liver safe to prevent systemic inflammation[93].

Differences in the taxonomic composition of the intestinal microbiome in NAFLD (an increased proportion of Firmicutes and a reduced proportion of Bacteroidetes) change metabolic function. The availability of bile acids, endogenous alcohols, and volatile organic compounds increases. When these changes are combined with reduced SCFAs and choline, the integrity of the intestinal barrier is reduced[93].

Gut barrier disruption is recognized in patients with cirrhosis. The epithelial layers show structural abnormalities related to increased intestinal permeability or bacterial translocation[94]. Permeability can be measured by the urinary excretion of radiolabeled chromium-ethylenediamine tetraacetic acid or by measuring volatile organic compounds formed by the fermentation of some dietary polysaccharides[95].

CTC—cardiotonic steroids
Cardiotonic steroids (CTS) are part of a group of specific ligands of Na⁺, K⁺-ATPase, a ubiquitously expressed enzyme responsible for the maintenance of electrochemical gradients across the cell membrane through active transport[96] that provokes a variety of cell signals[70]. In the last decades, studies have revealed the role of Na⁺, K⁺-ATPase and its signaling in various diseases, including inflammation and fibrosis[97].

CTS increase cholesterol synthesis in liver HepG2 cells, which augments the activity and expression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase[98]. Disturbed cholesterol balance underlies cardiovascular disease and an increasing number of other diseases, such as neurodegenerative diseases, cancers, and liver disease[99].

Elevated CTS might encourage increased cholesterol levels in the liver and worsen liver fibrosis by activating HSCs[100] and other redox-inflammatory pathways[101]. This increase in cholesterol levels could precipitate hepatocyte injury and macrophage activation that could lead to liver fibrosis progression. However, even CTS seem to have an important role in hepatocyte lipotoxicity and fibrosis; to our knowledge, they have not been studied as biomarkers for liver disease progression.

GUT MICROBIOTA

A large community of viruses, bacteria, archaea, and fungi live in the gastrointestinal tract and composes the gut microbiota[102]. It has critical roles in digestion, immunity, and metabolism[103]. Recently, the characterization of gut microbiota has evolved rapidly due to the advances in sequencing technology, permitting the creation of a gut microbiota gene catalogue[102]. The collective genetic material of the microbiota is often referred to as the “gut microbiome”. It encodes pathways that produce small bioactive molecules derived from dietary or metabolic precursors and may alter human health[104].

Thus, knowledge of microbiome characteristics in different metabolic diseases has increased in the past years. There has been great interest in dysbiosis (alterations in the composition and balance of microbiota[104]). Microbiota alterations are being studied as possible diagnostic biomarkers to improve personalized care. Animal studies have demonstrated a potential causal role of gut microbiota in NAFLD development[105]. However, extrapolating mouse model experimental information to humans has several limitations[106]. Consequently, signatures specific to liver alterations would be useful as NAFLD diagnostic biomarkers. However, discrepant microbiome signatures might be linked to the heterogeneity of diet, drugs, infections, environmental exposures, among others[104].
**Bacterial microbiome**

Alterations in the gut microbiome have been associated with the progression and severity of NAFLD\[^{107}\]. Proteobacteria are enriched in steatosis\[^{103,108,109}\]. Patients with NAFLD, compared with healthy individuals, also have significant changes at the phylum (increased Enterobacteriaceae\[^{109}\] and decreased Rikenellaceae and Ruminococcaceae\[^{109}\]) and genera level (increased *Escherichia*\[^{109}\], *Dorea*, and *Peptostreptococcus*, and decreased *Anaerostipes*, *Coprobiacterium*, *Faellobacterium* and *Prevotella*\[^{103}\]).

When comparing people with NASH vs healthy controls, some patterns are observed that also overlap with the NAFLD microbiome: Phylum (increased Proteobacteria\[^{50,109-111}\]), family (increased Enterobacteriaceae\[^{109,110}\] and decreased Ruminococcaceae\[^{110-113}\] and Rikenellaceae\[^{110}\]), and genera (increased *Dorea*\[^{111}\] and decreased *Faellobacterium*\[^{110,113,114}\], *Coprobiacterium*\[^{110,112,113}\], and *Anaerostipes*\[^{112,114}\]). Few projects have studied microbial composition as a function of fibrosis progression. *Bacteroides vulgatus* and *Escherichia coli* are the most abundant species in advanced fibrosis (F3–F4)\[^{50}\]. Models have been proposed to use the microbiome as a reservoir for diagnostic signatures of NAFLD fibrosis\[^{50}\], but further confirmation in independent cohorts and across geographical regions is necessary to assess their clinical relevance.

Microbial signatures of liver fibrosis are related to a severe shift in taxa conformation, leading to a growth in pathogenic taxa and a decline in metabolically beneficial taxa\[^{115}\]. However, the evaluation of gut microbiota contribution to liver disease progression (from steatosis to NASH and NASH cirrhosis) is limited and bacterial markers are frequently identified in a given study yet not confirmed in independent cohorts.

Although some studies consider gut bacterial groups as promising markers of different stages of liver disease, if the microbiota is a causal factor and how it interacts with the complex pathophysiological processes driving disease progression from mild fibrosis to severe fibrosis is still under investigation\[^{50,109}\].

**Virome**

Dense and complex populations of intestinal viruses reside in the gut and interact with other microorganisms and the human host\[^{116,117}\]. Most intestinal viruses are bacteriophages (phages), viruses that can specifically infect bacteria\[^{118}\]. Phages may serve as important microbiota genetic diversity reservoirs by acting as vehicles for the horizontal transfer of virulence, antibiotic resistance, and metabolic determinants among bacteria\[^{119}\].

Lang *et al.*\[^{120}\] studied the fecal viromes from NAFLD patients and controls. They found associated histologic markers of NAFLD severity with significant decreases in viral diversity and proportion of bacteriophages\[^{120}\]. The intestinal virome is specific for every individual, and viral diversity measures were the third and fifth most important variables following a higher AST and higher age. The most important viral species belonged to *Lactococcus* phages, and several *Lactococcus* phages were less present in patients with NAFLD and NASH.

**Protozoa and fungi**

Fungi and archaea are important components of the human microbiota. Recent findings have revealed that mycobiome (commensals fungi at barrier surfaces) can influence host immunity and the development and progression of human inflammatory diseases\[^{121}\]. The human gut mycobiome is dominated by *Saccharomyces*, *Malassezia*, *Candida*, and *Cladosporium* and are an important modulator for local and peripheral immune responses. Patients with liver fibrosis have decreased fungal diversity and increased *Candida*\[^{122}\]. Gut mycobiota disturbance might produce metabolites called mycotoxins (trichotheccenes, zearalenone, fumonisins, ochratoxins, aflatoxins) that can alter gut health by compromising intestinal epithelia\[^{123,124}\].

**LIMITATION**

The increasing burden of NAFLD worldwide has encouraged the search for novel biomarkers to detect liver diseases. Liver biopsy is currently the gold standard for diagnosis and staging, but it has several limitations, including sampling errors, invasiveness, inter-observer variability, and related procedure risks. Researchers have faced the challenge of developing novel biomarkers in past decades, and significant advances have been made. A promising biomarker should be liver-specific, accessible...
Figure 2 Potential biomarkers involved in hepatic pathophysiology. Hb: Hemoglobin; FGF-21: Fibroblast growth factor 21; RBP4: Retinol binding protein 4; CK18Asp396: Caspase cleaved cytokeratin-18 fragment (M30); Fuc-Hpt: Fucosylated haptoglobin; Mac2bp: Mac-2-binding protein; DR5: Death receptor 5; miRNA-122: MicroRNA 122; miR-192: MicroRNA 192; ASGPR1+: Asialoglycoprotein receptor 1; CNN2: Calponin 2; miRNA-214: MicroRNA 214; miR-34a: MicroRNA 34a; TMAO: Trimethylamine N-oxide; LDL-c: Low density lipoprotein cholesterol; Fecal SCFAs: Fecal Short chain fatty acids; Fch: Ferrochelatase; 11-HETE: 11-Hydroxyeicosatetraenoic Acid; 11,12-diHETrE: 11,12-dihydroxyicosatrienoic acid; DHEA-S: Dehydroepiandrosterone sulphate; PPAR-γ: Peroxisome proliferator-activated receptor γ; IFNγ: Interferon gamma; IL-4: Interleukin-4; IL-13: Interleukin-13; CD4+T: Cluster of differentiation 4, T helper cells; T reg: Regulatory T cells; ILCs: Innate lymphoid cells.

and accurate, replicable, and available in clinical laboratories. As summarized in this article, most studies have focused on proteomics, metabolomics, genome-wide association studies, microbiome, and inflammation markers. Still, some may be more specific for NAFLD while others for NASH, although the challenge for determining the etiology and staging the degree of severity remains a limitation (Figure 2).

The evaluation of future biomarkers for the assessment of liver fibrosis could greatly impact the health system. There is a priority for non-invasive diagnostic tools to fulfill medical needs, differentiate patients with steatosis from those with NASH and fibrosis, predict disease progression, and monitor patients to evaluate the therapeutic response. In the following years, it would be expected that a physician who faces a hepatic patient could suspect hepatic disease, perform imaging studies, and from there have a set of potential biomarkers that they may request to have a concrete and specific diagnosis. Some of these biomarkers have strong diagnostic performance, but current evidence shows a lack of reproducibility. Besides, the analytical, clinical validity of the methodology is lacking. Validity is necessary to translate basic research into real clinical application. Even if we perform this validation, it is unlikely that a single biomarker could fulfill this necessity. A combination of these biomarkers could soon be used to create a diagnostic panel. This panel, combined with the patient’s clinical history and clinical data, could certainly lead to a medical decision that results in an accurate diagnosis and treatment. This result must be the goal in the following years.
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Promising biomarkers for liver disease


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Fatty acid metabolism and acyl-CoA synthetases in the liver-gut axis

Yunxia Ma, Miljana Nenkov, Yuan Chen, Adrian T Press, Elke Kaemmerer, Nikolaus Gassler

Abstract

Fatty acids are energy substrates and cell components which participate in regulating signal transduction, transcription factor activity and secretion of bioactive lipid mediators. The acyl-CoA synthetases (ACSs) family containing 26 family members exhibits tissue-specific distribution, distinct fatty acid substrate preferences and diverse biological functions. Increasing evidence indicates that dysregulation of fatty acid metabolism in the liver-gut axis, designated as the bidirectional relationship between the gut, microbiome and liver, is closely associated with a range of human diseases including metabolic disorders, inflammatory disease and carcinoma in the gastrointestinal tract and liver. In this review, we depict the role of ACSs in fatty acid metabolism, possible molecular mechanisms through which they exert functions, and their involvement in hepatocellular and colorectal carcinoma, with particular attention paid to long-chain fatty acids and short-chain fatty acids. Additionally, the liver-gut communication and the liver and gut intersection with the microbiome as well as diseases related to microbiota imbalance in the liver-gut axis are addressed. Moreover, the development of potentially therapeutic small molecules, proteins and compounds targeting ACSs in cancer treatment is summarized.

Key Words: Long-chain fatty acids; Short-chain fatty acids; Acyl-CoA synthetases; Microbiota; Liver-gut axis

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INTRODUCTION

Lipids, one of three main nutrients, are mainly composed of fatty acids (FAs), triglycerides (TGs), phospholipid and cholesterol. Lipid metabolites are involved in various biological functions and physiological processes, ranging from energy storage and degradation and structural composition to molecule signaling as well as signal transduction cascade[1].

The liver-gut axis plays a critical role in the homeostasis of lipid metabolism in the human body during the feed-fast cycle. Free FAs are absorbed by enterocyte and intestine-derived products released into portal blood which is directed to the liver; in turn, the liver responds by secreting bile acids (BAs) to the intestine via the biliary tract. BAs are transported back to the liver via enterohepatic circulation. Since the Volta group identified the important role of microorganisms in the liver-gut axis for the first time[2], a number of studies have confirmed that gut microbiota, described as an invisible metabolic ‘organ’, has a tight and coordinated connection with the gut and liver[3,4]. The intestinal mucosal barrier either acts as a physical barrier or lives in symbiosis with microbiota. Once the balance of symbiosis is disrupted, microbiota responds to this imbalance, microbiota metabolites (short-chain fatty acids, SCFAs) are modified and circulated into the liver. Aberrant lipid metabolism in the liver-gut axis has been linked with intestinal bowel diseases and diverse liver diseases[5].

Around 95% of dietary lipids absorbed are TGs, mainly composed of long-chain fatty acids (LCFAs)[6]. Fatty acid metabolism takes place mainly in intestinal enterocytes and hepatocytes, further assisted by adipocytes and other cell types. To become further involved in both anabolic and catabolic pathways, FAs must be taken up and activated by thioesterification. This ATP-mediated coupling reaction of FAs with coenzyme A is catalyzed by the enzymes called acyl-CoA synthetases (ACSs). ACSs are classified into five groups according to the fatty acid chain length: short-chain, medium-chain, bubblegum-chain, long-chain and very-long-chain acyl-CoA synthetases (ACSVLs)[7]. ACSVLs as membrane channel proteins have been identified as a major enzyme responsible for LCFA uptake and activation[8]. Long-chain acyl-CoA synthetases (ACSLs) are responsible for the catalyzation of intracellular free LCFAs which are transported by other transport proteins, such as fatty acid translocase (CD36) and fatty acid binding proteins (FABPs)[9]. Short-chain acyl-CoA synthetases (ACSSs) are involved in the activation of microbiota-derived SCFAs, such as acetate and propionate[10] (Table 1).

In this review, we will summarize the functional role of ACSs in fatty acid metabolism, focusing on LCFAs and SCFAs, as well as potential therapeutic targets of ACSs. Furthermore, we will explore the influence of dietary diversity on microbiota and the microbial metabolites, and their bidirectional communication in the liver-gut axis.

Core Tip: To understand the role of acyl-CoA synthetases (ACSs) in the fatty acid metabolism, it is necessary to explore the biological function, gene interactions/ regulations and signal pathways in physiological and pathological conditions. Growing evidence demonstrates that the control of microbial balance plays an important role in maintaining homeostasis and normal functions of the liver-gut axis, and the bidirectional communication in turn affects microbial communities. As novel therapeutic targets, miRNAs are receiving more and more attention, together with other compounds targeting ACSs.
Table 1 miRNA and compounds targeting acyl-CoA synthetases

<table>
<thead>
<tr>
<th>Type</th>
<th>Name</th>
<th>Target</th>
<th>Mechanism</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>miRNA</td>
<td>miR-205</td>
<td>ACSL4/ACSL1</td>
<td>Inhibition of ACSL4/ACSL1 in hepatocellular carcinoma</td>
<td>[155,171]</td>
</tr>
<tr>
<td></td>
<td>miR-211-5p</td>
<td>ACSL4</td>
<td>Inhibition of ACSL4 in hepatocellular carcinoma</td>
<td>[172]</td>
</tr>
<tr>
<td></td>
<td>miR-196-1</td>
<td>ACSL1/ACSL4/SCD1</td>
<td>Inhibition of ACSL1/ACSL4/SCD1 axis in colorectal cancer</td>
<td>[173]</td>
</tr>
<tr>
<td></td>
<td>miR-142-3p</td>
<td>ACSL1/ACSL4/SCD1</td>
<td>Inhibition of ACSL1/ACSL4/SCD1 axis in colorectal cancer</td>
<td>[173,174]</td>
</tr>
<tr>
<td></td>
<td>miR-34c</td>
<td>ACSL1</td>
<td>Inhibition of ACSL1 and induction of liver fibrogenesis</td>
<td>[175]</td>
</tr>
<tr>
<td></td>
<td>miR-497-5p</td>
<td>ACSL5</td>
<td>Inhibition of ACSL5 in colon cancer</td>
<td>[170]</td>
</tr>
<tr>
<td>Compounds</td>
<td>Triacsin C</td>
<td>ACSL1/ACSL3/ACSL4 and ACSL5</td>
<td>Inhibition of ACSL1/ACSL3/ACSL4 and ACSL5</td>
<td>[177,178]</td>
</tr>
<tr>
<td></td>
<td>Roglitazone</td>
<td>ACSL4</td>
<td>Inhibition of ACSL4</td>
<td>[179-181]</td>
</tr>
<tr>
<td></td>
<td>Pioglitazone</td>
<td>ACSL4</td>
<td>Inhibition of ACSL4</td>
<td>[179-181]</td>
</tr>
<tr>
<td></td>
<td>Troglitazone</td>
<td>ACSL4</td>
<td>Inhibition of ACSL4</td>
<td>[179-181]</td>
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<tr>
<td></td>
<td>Lipofermata</td>
<td>FATP2</td>
<td>Inhibition of FATP2</td>
<td>[191,192]</td>
</tr>
<tr>
<td></td>
<td>Grassofermata</td>
<td>FATP2</td>
<td>Inhibition of FATP2</td>
<td>[191,193,194]</td>
</tr>
<tr>
<td></td>
<td>Ursodiol</td>
<td>FATP5</td>
<td>Inhibition of FATP5 in liver</td>
<td>[195]</td>
</tr>
<tr>
<td></td>
<td>chenodiol</td>
<td>FATP5</td>
<td>Inhibition of FATP5 in liver</td>
<td>[195]</td>
</tr>
<tr>
<td></td>
<td>Fenofibrate</td>
<td>PPARα</td>
<td>Indirect activation of FATP in liver predominantly</td>
<td>[196,197]</td>
</tr>
</tbody>
</table>

Triacsin C is also competitive inhibitor of ACSL5 when used in higher concentration.

FATTY ACID METABOLISM MEDIATED BY ACYL-COA SYNTHETASES IN THE LIVER-GUT AXIS

Circulation of fatty acids and bile acids in the liver-gut axis
Intestinal absorption of FAs is a multistep process that includes digestion, uptake and absorption and needs to cooperate with large numbers of enzymes secreted by series of organs in the gastrointestinal tract[11]. TGs are first released from a fatty diet after digestion with lingual and gastric lipase in the stomach, and released TGs are further hydrolyzed by pancreatic lipase to produce 2-monoacylglycerides and free FAs[12]. Sequentially those digested FAs mix with BAs and emulsify to form spherical water-soluble droplets, called micelles (MCs). With intestinal peristalsis, MCs are transported to the small intestinal lumen and further translocated into the apical membrane of enterocytes.

In intestinal enterocytes, absorbed LCFAs experience a series of catabolic metabolisms for energy supply for massive biological activities, and anabolic metabolism to reconstitute lipids. Newly synthesized lipids are incorporated into transport vehicles, chylomicrons (CMs), that are later liberated from enterocytes, and then transported to the liver through the hepatic portal vein. The liver is the major processing factory of FAs and regulates and balances lipid homeostasis systemically in the liver-gut axis. Fatty acid uptake and metabolism occur in hepatocytes. During feeding, hepatocytes take up the influx of FAs and get rid of FAs via β-oxidation to produce energy, and reformed TGs integrated into CMs partition into two pathways: (1) Secreted into bloodstream; and (2) transported and stored in adipose tissue. During fasting or starvation, hepatocytes recycle TGs from lipid droplets and adipose tissue, and initiate de novo lipogenesis by using other energy sources in the liver, such as carbohydrates [13,14]. Therefore, the pool of FAs is always in dynamic equilibrium between dietary absorption in the enterocytes, process and lipogenesis in the liver and liver feedback regulation via BAs during the feed-fast cycle.

As previously mentioned, BAs are involved not only in facilitating MC formation, but also as signaling molecules and metabolic regulators of lipid/glucose metabolism, energy homeostasis and inflammation in the liver-gut axis[15]. It has been demonstrated that a higher level of BAs can be detected in the tissues of the liver-gut axis compared to peripheral blood[16]. Primary BAs are synthesized in the hepatocytes and secreted into the small intestine; most of them are reabsorbed in the ileum. A small
number of unabsorbed BAs are taken up by microbiota and metabolized into secondary BAs[17]. In enterocytes BAs are reabsorbed through the apical sodium-dependent BA transporter (ASBT), carried by the intestinal bile acid-binding protein (FABP6) and released into portal blood via heterodimeric transporter OStα/OStβ. BA activation of the nuclear farnesoid X receptor (FXR) also upregulates FABP6, OStα/OStβ and fibroblast growth factor 19 (FGF19), which further inhibits BAs synthesis. In hepatocytes, the transport of BAs is mediated by sodium-taurocholate cotransporting polypeptide (NTCP) and organic anion transporters (OATPs). BAs acting as an activator of hepatic FXR regulate the expression of genes involved in bile acid transport and synthesis. This enterohepatic circulation of BAs plays a critical role in maintaining the BAs pool in the liver-gut axis[18,19].

Long-chain fatty acid transport to enterocytes and hepatocytes

Free fatty acid uptake is requested across the phospholipid bilayer in the mammalian intestine. It is widely known that LCFAAs can be taken up into cells via flip-flop diffusion with rate limiting[20,21]. High permeability of LCFA transport is mediated by several membrane-associated transport proteins including FA transport proteins (FATPs), FABPs, CD36 and caveolin (CAV)[9].

FATP1-6 (fatp in mice, also called ACSVL1-6) is a group of enzymatic proteins with double capabilities of transport and activation. FATP can trap and activate a broad range of LCFA and VLCFA to form acyl-CoA[9,22]. Different FATP family members have tissue-specific expression patterns[23]. In the intestine, FATP4 (ACSVL5) is strongly expressed in intestinal villi but not in crypts, which plays an important role in fatty acid absorption[24]. Fap4-null mice display an embryonic lethality with a defective epidermal barrier. Fap4 depletion alters the ceramide fatty acid composition significantly, especially in saturated VLCFA substitutes C26:0 and C26:0-OH[25]. FATP5 (ACSVL6) mainly transports BAs but also LCFAAs, is only expressed in the liver and particularly in the basal membrane of hepatocytes[6,26]. Fap5 knockout mice showed this defective bile acid conjugation, indicating that Fapt5 is essential for fatty acid uptake by hepatocytes and maintains balance lipid weight[27]. With the discovery of the topological structure of murine FAP1 containing one transmembrane domain and a large cytoplasm domain[28], different mechanisms of FATP1 transporting exogenous FAs into cells have been proposed, one of which is vectorial transport or flipase function[29]. Moreover, BAs acting as a FATP5 antagonist dramatically decrease hepatic fatty acid uptake as well as liver triglyceride synthesis[30].

FABP 1-9 (fapb in mice) are a fatty acid binding protein superfamilly that binds to FAs, cholesterol or other non-esterified FAs, facilitat fatty acid uptake and lipid metabolism[31]. FABP appears in two distinct forms depending on localization; one is peripheral membrane protein (FABPpm) and the other is intracellular/cytoplasmic protein (FABPc)[32]. Like FATP, different family members of FABPs exhibit organ-specific expression. FABP2, 4, 5, 6 (intestinal-FABP, I-FABP) encode the intestinal form which is only expressed in small intestine, and FABP-1 (Liver-FABP, L-FABP) is only expressed in the liver[33]. L-FABP and L-FABP are all cytoplasmic proteins, but it is reported that they deliver FAs through different mechanisms of L-FABP in diffusion and L-FABP in collision[34]. L-fabp-null mice showed a reduced uptake of LCFAAs as well as new biosynthesis for lipid storage or secretion, suggesting the important role of L-fabp in fatty acid esterification at endoplasmic reticulum (ER)[35]. Furthermore, L-FABP depletion suppresses lipid catabolism in mitochondria and downregulates the transcription of oxidative enzymes through inhibition of peroxisome proliferator-activated receptor (PPARα) transcriptor in the nucleus[36,37].

CD36, officially designated as scavenger receptor B2 (SR-B2), is a transmembrane glycoprotein which has a broad range of binding profiles including LCFAAs, plasma lipoproteins, phospholipids, collagen[38]. CD36 whole body knockout mice showed significantly decreased fatty acid uptake in the heart and skeletal muscle[39]. In the intestine, CD36 is only detected in the duodenal and jejunal parts and plays a critical role for fatty acid and cholesterol uptake in the small intestine[40]. Although CD36 has a very low expression level in the liver, CD36 liver-specific knockout in the steatosis model indicated that CD36 deletion reduces lipid content and inflammation and improves insulin sensitivity[41].

CAV 1-3 (cav in mice) are intramembrane proteins which are responsible for caveola formation. CAV1 as a cholesterol-binding protein is implicated in cholesterol trafficking and absorption[42]. However, Cav1 knockout mice did not show a compensatory mechanism to increase other family members, such as Cav2 and Cav3, and cholesterol absorption and sterol excretion were also not changed in the intestine[43]. Additionally, CAV1 also acts as a cytosolic intermediate form involved in
lipogenesis and lipid body formation during liver regeneration[44]. It is widely recognized that several fatty acid transport proteins cooperate synergistically to accomplish the process of fatty acid transport (Figure 1). Due to the tissue-specific expression pattern, FATP4, FABPpm, FABP-I, CD36 are main types in the intestine and FATP5, FABPpm, FABP-L, CD36 are major types in the liver. Partial LCFAs are activated during transport via FATP. The rest of the LCFAs are grabbed by FABPpm and presented to CD36. Free cytosolic LCFAs is not only activated by ACSLs for esterification of acyl-CoA but also trapped by FABPc for subcellular function. Generated acyl-CoA as a raw material initiates the subsequent metabolism pathway to produce energy or synthesize diverse complex lipids. In addition, acyl-CoA can be deactivated to free FAs and CoA, and this process is mediated by acyl-CoA thioesterases (ACOTs). ACSLs and ACOTs are two critical enzymes helping to control the dynamic balance between acyl-CoA and free FAs.

**Long-chain fatty acid activation in enterocytes and hepatocytes**

As mentioned previously, most of the abundant dietary FAs are LCFAs so ACSLs are addressed in more details here. In humans and rodents there are five existing ACSL isoforms namely ACSL1, ACSL3, ACSL4, ACSL5 and ACSL6 (acsl in mice), each one coded by the different gene containing several splice variants[45]. Due to the differences in the 5'UTRs, the first coding exon, alternative coding exons and exchangeable motifs, different variants of each ACSL isoform are available[46]. The ACSL isoforms have two motifs: ATP binding and fatty acid binding[47]. The fatty acid binding tunnel located at the N-terminal domain has been linked to the substrate specificity of each ACSL isoform[48]. Since the N-terminal domain varies between the different ACSL isoforms, it contributes to the substrate preference of each family member and its different subcellular localization which is essential for vectorial acylation[49].

ACSL1 is predominantly located in the liver. Knockout of ACSL1 in the liver demonstrated a reduction in total ACSL activity of up to 50%, together with a decrease in the hepatic amount of acyl-CoA and a decreased level of oleic acid-derived TG[1, 50]. Acsl1 deficient mice showed a 50% reduction in the amount of long-chain acyl-carnitines, leading to the conclusion that the loss of Acsl1 impaired partitioning of its products into TG synthesis and oxidation pathways[41]. Due to its both endoplasmic and mitochondrial localization, ACSL1 directs its metabolites to both the anabolic (TG synthesis) and catabolic (β-oxidation) pathway[1].

ACSL3 Localization is linked to the lipid droplets and ER in the liver and other tissue. The increase in fatty acid uptake causes a transition of ACSL3 from ER to the lipid droplets, suggesting its role in neutral lipid synthesis[1]. Knockdown of ACSL3 reduced the activity of transcription factors including PPARγ, ChREBP, SREBP1C and Liver X receptor and their target genes involved in hepatic lipogenesis[1]. ACSL3 activates FAs incorporated into phospholipids, which are used for very-low density lipoprotein (VLDL) production[50]. As revealed by Yan et al, ACSL3 knockdown decreased the level of VLDL in hepatic cells[50]. Besides its role in the activation of FAs, overexpression of ACSL3 was found to be able to induce cellular fatty acid uptake[51].

ACSL4 is mostly expressed in adrenal glands and steroid-producing organs[52,53]. The role of ACSL4 is related to the activation of polyunsaturated FAs in steroidogenic tissue. ACSL4 has a preference for the arachidonic acid which is involved in the eicosanoid synthesis.

The nuclear-coded ACSL5 is prominent in both the mitochondria and ER of the intestinal mucosa and liver[50]. Highest expression was detected in the jejenum and acsl5 was assumed to be involved in dietary fatty acid absorption. However, studies in acsl5 null mice showed no alteration in dietary fatty acid absorption but a significant decrease in total ACSL activity[1]. In the liver, ACSL5 activates LCFAs mostly of C18 carbon atoms, which are further incorporated into TGs, phospholipids and cholesterol esters. According to previous reports, ACSL5 plays a role in the metabolism of dietary FAs, but not in de novo synthetized ones[50,54,55]. Since ACSL5 is localized on the mitochondrial outer membrane, the activity was initially attributed to β-oxidation. Some studies with ectopic expression of ACSL5 failed to prove this, but the increased synthesis of TGs and diglycerides was observed in the liver[54]. ACSL5 is a dominant activator of dietary LCFA and displayed an 80% lower activity in total acsl of the jejenum in acsl5 knockout mice[56]. ACSL5 is strongly expressed by enterocytes in an ascending gradient along the crypt-villus axis with the highest expression level at the villus tip; however, nuclear β-catenin, a hallmark of Wnt activation, is expressed in a descending gradient along the crypt-villus axis[57], suggesting an interplay between ACSL5 and Wnt activity during enterocyte differen-
Ma Y et al. Acyl-CoA synthetases

Figure 1 Mechanism of long-chain fatty acid transport across the lipid raft. LCFA are taken up into cell in two different ways. One is passive transport by a flip-flop with rate limiting. The other is active transport, which is mediated with transport-associated proteins (FATPs, CD36, FABPs and Caveolin). FATPs with tissue-specific distribution integrating both transport and activation functions are responsible for LCFA uptake. Free FAs trapped by the FABPpm present to CD36 and are transported into cells. Consequently released free FAs bind with FABPc and CAV channel into different organelles and are activated by different subcellular expression of ACSLs into acyl-CoA. In addition, acyl-CoA can be deactivated to free FA and CoA which is mediated by ACOTs. Liver-specific proteins: FATP5, FABP-L, ACSL1; Intestine-specific proteins: FATP4, FABP-I, ACSL5; ACSL: Acyl-CoA synthetase, ACOT: Acyl-CoA thioesterase; MCs: Micelles, CMs: Chylomicrons.

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ACSL6 is highly expressed in the brain where it plays a role in phospholipid synthesis during neurite outgrowth. ACSL expression is controlled by the level of intracellular FAs in physiological conditions[1].

Short-chain fatty acid transport and activation in enterocytes and hepatocytes

Microbiota-derived SCFAs cross the lipid membrane via different mechanisms: non-ionized diffusion, Na+/H+-dependent gradient exchange[59,60]. Intracellular SCFAs can shuttle between cytosol, nucleus and mitochondria via a diffusion mechanism[10, 60]. SCFA activation by ACSSs is the first step in utilizing the energy source. ACSS 1-3 (acss in mice) are encoded and designated in humans. ACSS1 and ACSS3 are localized at the mitochondria matrix, while ACSS2 is a nuclear-cytosolic enzyme. ACSS1 and ACSS2 activate acetate to thioester into acetyl-CoA, but ACSS3 favors propionate[10].

In humans, mitochondrial ACSS1 is most highly expressed in the brain, blood, testis and intestine, also to a certain level in the heart, muscle and kidney, but not in the liver or spleen[61]. In mice, ACSS1 is strongly expressed in the heart, kidney, skeletal muscle and brown adipose tissue, which all need high energy expenditure[62]. Acss1 knockout mice showed a remarkably decreased acetate oxidation in the whole body during fasting compared with the wild type, however, no histological changes were detected in multiple tissues including the intestine and liver[63]. ACSS3 displays the character of propionyl-CoA synthetase as well as the highest expression in the liver. Knockdown of ACSS3 in hepG2 significantly decreases the activity of propionyl-CoA synthetase. During fasting, ACSS3 is upregulated, which is probably linked to ketogenesis, and ACSS2 is downregulated[64].

ACSS2 is most highly expressed in the liver and kidney[64,65]. Moffet et al[10] introduced the concept that the expression of ACSS2 in different cell types is based on the different physiological conditions to utilize acetate. Therefore, the liver is supposed to be the main organ for processing acetate. With the feature of localization, ACSS2 catalyzes acetate into acetyl-CoA which is correlated with fatty acid biosynthesis in cytosol, and retains acetate released from histone in the nucleus[66]. Acss2-deficient mice with high-fat feeding can lighten fat deposition in the liver by regulating many genes involved in lipid metabolism, suggesting that Acss2 acts as a transcription regulator during lipogenesis[67].

The expression and localization pattern of ACSS 1-3 suggests that ACSS1 and ACSS3 are responsible for energy production by using acetate in the intestine and liver respectively. The majority of acetate is taken up by the liver, ACSS2 in cytoplasm is involved in lipogenesis and is distributed to other organs in ketone bodies through systemic circulation. Acetyl-CoA as a central metabolite can go into either energy...
production or lipid biosynthesis. ACSS1-3 plays a key role in regulating the level of acetyl-CoA in the nucleus, mitochondria and cytoplasm (Figure 2).

**MICROBIOTA UTILIZATION OF DIET, MICROBIOTA METABOLITES AND THE ROLE OF MICROBIOTA IN THE LIVER-GUT AXIS**

**Dietary structure shapes the composition of microbiota**

Gut microbiota, a diverse microbial community with approximately 100 trillion microorganisms, is colonized in the gastrointestinal tract. In human adults, five families microbiota are mainly Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria and Verrucomicrobia, while phylum Firmicutes and Bacteroidetes make up approximately 80% of all species [68].

A high-fiber intake population has higher diversity microbiota and more SCFAs production than a high-calorie diet population, and two populations showed distinct diet favor microbiota [69]. Bacteroides and Prevotella are two dominant groups which are highly enriched in a high-protein/fat diet population and high-fiber population respectively [70,71]. Moreover, the composition of fecal microbiota varies by age, geography and lifestyle due to the behavior of microbiota dietary preferences [72]. The term microbiota-accessible carbohydrates (MACs) introduced by Sonnenburg et al refers to microbiota favorable-carbohydrates that cannot be digested by the host. Mice feeding on a long-term low-MACs diet display a remarkably reduced diversity of microflora containing mostly Bacteroidales and Clostridiales. Although the microbiota composition cannot be restored after refeeding with a high-MAC diet, it increases again mainly in Bacteroidales upon reintroduction of fecal microbiota [73].

SCFAs are metabolic end-products from specialized bacteria utilizing with undigested dietary polysaccharides in human small intestine. The most abundant SCFAs in the intestine are acetate (C2), propionate (C3) and butyrate (C4). The phylum Bacteroidetes, the most abundant gram-negative bacteria with a high flexibility to adapt the environment, are associated with acetate production [74]. Phylum Bacteroidetes and Negativicutes (Akkermansia muciniphila, family Veillonellaceae and phylum Firmicutes) are dominantly responsible for production of propionate by the succinate pathway, small bacterial genera from phylum Firmicutes have been identified to form propionate through the acrylate pathway, and distant Lachnospiraceae are known to produce propionate by utilizing the propanediol pathway [75]. Several species from families Lachnospiraceae, Ruminococcaceae and Erysipelotrichaceae (Phylum Firmicutes) produce butyrate via butyrate kinase route and butyryl-CoA:acetate CoA-transferase route [76]. Diverse composition of microbiota has distinct SCFAs profiles, and additionally, SCFAs-metabolic network is a cross-feeding microbial system between different bacterial species [77].

In all, a high intake of MACs is pivotal in shaping the diversity and composition of microbiota. Diverse microbiota-generated SCFAs reversely influence the microbial communities and further act as a mediator is strongly involved in host-microbiota cross-talk.

**Utilization of long-chain fatty acid in microbiota**

Microbiota can also employ luminal unabsorbed LCFAs directly as energy source once there is a fermentable fiber deficiency [78]. LCFAs cross the cellular envelope in bacteria and yeast, unlike in mammalian cells. In bacteria, FadL transports exogenous LCFAs from outer membrane to periplasm, FadD (role as ACSLs) extracts LCFAs into the cytoplasmic membrane and activates to form acyl-CoA. In yeast, Fat1p and Faa1p/Faa4p are required for LCFAs transport and activation respectively [28]. Moreover, LCFAs can also permeate the bilayers via the TolC channel in E. coli [79,80]. Several species from families Lachnospiraceae, Ruminococcaceae and Erysipelotrichaceae (Phylum Firmicutes) produce butyrate via butyrate kinase route and butyryl-CoA:acetate CoA-transferase route [76]. Diverse composition of microbiota has distinct SCFAs profiles, and additionally, SCFAs-metabolic network is a cross-feeding microbial system between different bacterial species [77]. During bacterial overgrowth, acetyl-CoA is not only necessary only for energy generation via entering citric acid cycle and respiratory chain, but also synthesizes new cell material via the glyoxylate cycle. Moreover, the conversion from acetyl-CoA to acetate and ethanol takes place through anaerobic fermentation due to oxidant deficiency [82].

In addition to being a nutrient, LCFAs serve as an environmental factor which guides a series of gram-negative bacteria to colonize and invade intestinal lumen by repressing the expression of the strain-specific pathogenicity island. A pathogenicity island has been reported as a transcriptional activator which is mandatory for tissue invasion, such as Salmonella P11/hilA [80], the Vibrio cholerae AraC/Xyls family ToxT...
Figure 2 The crosslink between acyl-CoA synthetases and short-chain fatty acids. In mitochondria, acetyl-CoA is generated either from fatty acid β-oxidation and glucose via pyruvate or SCFAs through ACSS1 and ACSS3; acetyl-CoA is directed into energy production through the TCA cycle and electron respiration chain, and as reflux into cytosol via citrate and again synthesizes acetyl-CoA. In addition, excessive acetate and butyrate synthesize into ketone bodies and are released into cytosol. In cytosol, acetyl-CoA is produced from pyruvate which is from both glucose and propionate; the source of acetyl-CoA can be converted from butyrate and acetate via butyryl-CoA/acetate CoA-transferase and ACSS2 respectively; cytosolic ketone bodies can also either produce acetyl-CoA or enter the blood circulation in the whole body. On the other hand, acetyl-CoA is involved in ketone body synthesis. In the nucleus, acetate synthesizes acetyl-CoA via ACSS2 which is responsible for chromosome stability through histone acylation regulation.

Microbiota-derived short-chain fatty acids

Microbiota-derived SCFAs make up almost all SCFAs due to the lower level of SCFAs in human blood[85]. SCFAs as the basic substance sources play an important role in regulating lipid metabolism as well as maintaining the host energy homeostasis. In part, SCFAs can be absorbed directly as an energy source by enterocytes or transported to the liver via the portal vein; in part, SCFAs are reassigned by the liver and released into bloodstream for the systemic circulation through the whole body[10, 86]. SCFAs are mainly composed of acetate, butyrate and propionate which comprise 60%, 20% and 20% respectively[87]. SCFAs are transported and taken up into cells via non-ionized and ionized diffusion. The liver-gut axis plays a key role in the absorption, metabolism and systemic circulation of SCFAs[88].

Acetate, which is produced from pyruvate via acetyl-CoA and the wood-Ljungdahl pathway in microbiota, is the most abundant SCFA. Acetate is activated by ACSS1-3 to form acetyl-CoA and metabolized for energy production. However, the majority of acetate reaches and is processed in the liver. In cytosol, acetyl-CoA can synthesize cholesterol[89]; in the nucleus, acetate and acetyl-CoA are involved in regulating DNA histone acetylation and deacetylation[90]; in mitochondria, acetyl-CoA can be either for energy supply or ketogenesis in case of glucose deficiency, ketone bodies enter blood circulation for peripheral tissues usages[91]. Moreover, acetate can cross the blood-brain barrier freely and is an energy source for glial cells[92]. Acetate has a direct role in appetite regulation. Acetate is metabolized to generate more adenosine triphosphate, and inhibits adenosine monophosphate-active protein kinase (AMPK), as well as upregulating anorectic neuropeptide POMC and downregulating orexigenic neuropeptide AgRP[93].

Of the SCFAs which are mainly composed of acetate, butyrate and propionate, butyrate is the most widely studied. Butyrate is generated through the butyrate kinase or butyryl-CoA/acetate CoA-transferase route. Butyrate is a major SCFA in the large intestine. In enterocytes, the majority of butyrate is converted into acetyl-CoA that further participates in catabolism for host energy supply[94]; a small amount of butyrate is delivered to the liver and incorporated into ketone bodies (β-hydroxybutyrate) in mitochondrial for ATP production[95]. Butyrate plays a key role in maturing the intestinal barrier function in premature infants[96].
showed that butyrate administration has favorable therapeutic effects on normal colonic health in a safe dose\cite{86}. In a mouse model with globin chain synthesis disorder, the application of a high dose of butyrate resulted in striking neuropathological changes and multiorgan system failure due to harmful systemic concentrations \cite{97}. Therefore, mechanisms underlying the dosage-dependent effects on the intestinal barrier are controversial, but reasonable. A low dose promotes restitution of intestinal epithelial lumen and a high dose impairs the intestinal barrier function with regulation of permeability by inducing apoptosis\cite{98}. The selective paracellular permeability is determined by junction proteins including tight junction, adherence junction and desmosomes\cite{99}. Excessive SCFA accumulation downregulates the expression of junction protein and further impairs the integrity of the membrane, leading to a leaky gut\cite{100}. Moreover, increased intestinal permeability has been linked to inflammatory bowel disease\cite{101}.

Propionate is produced via the succinate, acrylate and propanediol pathway in microbiota. Propionate is activated by ACSS3 in mitochondria of hepatocytes. The concentration of dietary propionate regulates the balance between lipid and glucose metabolism\cite{102}. Propionate reduces cancer cell proliferation through activation of G-protein-coupled receptors 43 GPR43\cite{103} in mice liver\cite{103}.

In view of the biosynthesis of SCFAs, acetate, butyrate and propionate have crosslinks through acetyl-CoA, pyruvate, oxaloacetate, some of which can be converted between them to meet the physiological need of microbiota\cite{104}. SCFAs as key microbiota metabolites are closely correlated with host health and disease conditions through regulation of diverse physiological processes. Two major signaling pathways related to SCFAs including G-protein-coupled receptors (GPCRs) and histone deacetylases have been characterized\cite{105}. GPCRs, also named free fatty acid receptors (FFAR) are activated by SCFAs. Two SCFA receptors, GPR41 (FFAR3) and GPR43 (FFAR2) have been reported. FFAR2 has preference to acetate and propionate, and FFAR3 has a specificity in butyrate\cite{106}. FFAR2 is expressed along the entire gastrointestinal tract. FFAR2 can be upregulated by propionate during adipocyte differentiation\cite{107}. In addition, FFAR2 activated by SCFAs releases glucagon-like peptide 1(GLP-1) and peptide YY (PYY) in enteroendocrine L cells, GLP-1 and PYY, are involved in gut motility, glucose tolerance and regulation of appetite\cite{108}. Moreover, Butyrate plays a role in anti-inflammation through inhibition of pro-inflammatory mediators/adipokines, adhesion molecules, metalloproteinase production as well as inflammatory signaling pathways (NFkB, MAPKase, AMPK-α, and PI3K/Akt). However, the anti-inflammatory activity of butyrate was eliminated by FFA43 knockdown\cite{109}. Supplementation of SCFAs significantly improved hepatic metabolic activity in FFAR3-deficient mice, but not FFAR-2 deficient mice\cite{110}.

SCFAs are also considered a promising supplementary treatment for active intestinal bowel disease\cite{111}. Moreover, SCFAs, as inhibitors of histone deacetylases, show potential anti-inflammatory activity\cite{112,113}. It is demonstrated that three SCFAs alone or in combination protect the intestinal barrier \textit{via} stimulation of tight junction formation and repression of NLRP3 inflammasome and autophagy in the colon cancer cell model\cite{114}. Apart from this, a high-fiber intake, fecal microbiota transplantation, prebiotics and probiotics are suggested to have a beneficial effect on colonic health by increasing the level of SCFAs.

**Microbiota-imbalance-related diseases in the liver-gut axis**

Gut microbiota exert multifunction in maintaining the host homeostasis, including defending against pathogens, affecting immune system, mediating digestion and metabolism, involving in insulin regulation and maintaining the intestinal epithelial cell renewal\cite{115}. Gut microbiota interact with host through producing a serial of metabolites, particularly SCFAs. Imbalance in diversity and composition as well as alterations in the function of gut microbiota is associated with the pathogenesis of diverse gastrointestinal tract diseases, such as small intestinal bacterial overgrowth (SIBO), intestinal bowel disease (IBD), and a serial of liver diseases\cite{116}.

SIBO takes place in short bowel syndrome (SBS) and causes variable signs and symptoms resulting in nutrient malabsorption\cite{117}. SIBO is characterized with the small intestinal excessive numbers and types of bacteria overgrowth exceeding 10$^5$ organisms/mL, which are mainly colonic type with predominantly gram-negative aerobic species (\textit{Streptococcus}, \textit{Escherichia coli}, \textit{staphylococcus}) and anaerobic species (\textit{Lactobacillus}, \textit{bacteroides}, \textit{clostridium and veillonella})\cite{118}. Enterotoxins expressing in the outer membrane of germ-negative species can damage the intestinal mucosa barrier by stimulation of fluid secretion in enterocytes, and further affect the absorptive function\cite{119}. SIBO is associated with irritable bowel syndrome (IBS), celiac disease (CD) as well as IBD\cite{120}, and also involved in the development of nonalcoholic fatty liver disease\cite{121}. 

\textit{Lactobacillus}, \textit{bacteroides}, \textit{clostridium and veillonella})\cite{118}. Enterotoxins expressing in the outer membrane of germ-negative species can damage the intestinal mucosa barrier by stimulation of fluid secretion in enterocytes, and further affect the absorptive function\cite{119}. SIBO is associated with irritable bowel syndrome (IBS), celiac disease (CD) as well as IBD\cite{120}, and also involved in the development of nonalcoholic fatty liver
and glutamine metabolism in cancers, abnormal lipid metabolism was also found in the Warburg effect, synthetize ATP by oxidative phosphorylation, which has been called the reverse of the classical glycolysis. Recently, several studies have shown that some cancers, including colon cancer, rather than relying on oxygen, can convert glucose into lactate even in the presence of oxygen. This Warburg effect, one of the hallmarks of cancer, first introduced by Otto Warburg, has been used to describe the deregulated metabolism of cancer cells characterized by increased conversion of glucose into lactate even in the presence of oxygen. Many cancer cells are highly dependent on aerobic glycolysis for their growth and division. In addition to previously reported abnormalities of glucose and glutamine metabolism in cancers, abnormal lipid metabolism was also found in diseases.

IBD occurs due to the imbalance between the host immune system and gut microbiota in digestive tract and is becoming an increasing health problem. Crohn’s disease and ulcerative colitis are the two prevailing types. The worldwide epidemiologic data shows that the higher incidence and prevalence of IBD is associated with industrialization. Differences in dietary habits highly influence the composition of microbiota; a high-fat diet induces microbiota dysbiosis which alters the intestinal permeability.

Additionally, the disruption of bacterial colonization with dysbiosis and an exaggerated inflammatory response has been linked with the pathological process of necrotizing enterocolitis (NEC) in preterm infants. In NEC cases, an increased proportion of Proteobacteria and Actinobacteria, a decreased numbers of Bifidobacteria and Bacteroidetes were detected before NEC diagnosis. Moreover, a type of bacteria related to Klebsiella pneumoniae has been strongly correlated with the NEC development later stage.

Although the mechanism involved in diverse gastrointestinal tract diseases is still not completely understood, an impaired intestinal mucosal barrier is common feature among them. In addition, Paneth cells located in the crypts of the small intestine are very important for providing a sterile inner mucus layer and maintaining mucosal barrier integrity against microbiota by secreting antibiotic peptides containing α-defensin, angiogenin, lysozyme and lectins. α-defensin 5/6 are the most abundant components. α-defensin 5 can be digested into fragments which exert specific antibiotic activity. However, α-defensin 6 prevents invasion by bacterial pathogens through self-assembly to form fibronectin and components. Diminished expression of Paneth cell defensins regulated by the Wnt factor is associated with Crohn’s disease (also called Paneth’s disease). Paneth-cell-deficient mice showed a dysbiosis in favor of an E. coli expansion and further weakening of the intestinal mucosal barrier with a visceral hypersensitivity. Moreover, active Crohn’s disease is accompanied by bile acid malabsorption due to altered expression of the major bile acid transporter.

As a consequence of intestinal mucosal barrier disruption, microbial/pathogen-associated molecular patterns (MAMPs/PAMPs) pass through lumen and mucosa to induce the inflammatory signaling nuclear factor kappa B (NFκB) via toll-like receptors (TLRs) and nod-like receptors (NLRs). Activation of this signaling induces the release of cytokines and chemokines into portal circulation.

Both bacterial components and metabolites reach the liver via the portal vein to induce hepatocytes damage. Additionally, if dysbiosis occurs, secondary BAs including deoxycholic and lithocholic acid, which are toxic for both intestine and liver, are produced more than usual in microbiota. Hepatocytes are damaged due a high level of secondary BAs, bacterial components and metabolites. High lipid peroxides and PAMPs derived from damaged hepatocytes induce liver microphase activation and initiate an immune response through NFκB, p38/c-Jun-N-terminal kinase, TGF-β 1 and other inflammation cytokines. A macrophage-mediated immune response is a major player in liver fibrogenesis. Chronic liver injury leads to hepatic stellate cells to transition into myofibroblast-like cells which produce an extracellular matrix and further contribute to the progression of fibrosis. Moreover, chronic liver inflammation is significantly involved in the pathogenesis of liver fibrosis/cirrhosis and probably contributes to carcinogenesis.

POTENTIAL THERAPEUTIC APPLICATION TARGETING ACYL-COA SYNTHETASES

Long-chain acyl-CoA synthetases and cancer

Alteration in a fatty acid metabolism with a higher fatty acid synthesis and lipid deposition is a major player in the pathogenesis of metabolic disorders and cancer. Deregulation of metabolism is known as a hallmark of cancer. The Warburg effect, one of the hallmarks of cancer, was first described by Otto Warburg, has been used to describe the deregulated metabolism of cancer cells characterized by increased conversion of glucose into lactate even in the presence of oxygen. Many cancer cells are highly dependent on aerobic glycolysis for their growth and division. Recently, several studies have shown that some cancers, including colon cancer, rather synthetize ATP by oxidative phosphorylation, which has been called the reverse Warburg effect. In addition to previously reported abnormalities of glucose and glutamine metabolism in cancers, abnormal lipid metabolism was also found in...
different cancer types[143]. Highly proliferative cancer cells are dependent not only on glucose but also on other metabolites including glutamine, serine and FAs[147-151]. It was reported that many cancer cells are characterized by an increased level of de novo fatty acid synthesis[152,153]. Upregulation of processes as fatty acid synthesis and FA release from lipid storage on the one hand, and downregulation of β-oxidation of FAs and their reesterification on the other, leads to an increased level of fatty acid in cancer cells. The fatty acid level was reported as a prognostic marker in several types of cancers including colorectal carcinoma (CRC)[7]. A high level of FA is considered a cancer biomarker and is associated with a worse prognosis and survival[7].

There is some evidence from mice with genetic inactivation of the Muc2 gene that in adenocarcinoma arising in both the small and large intestine, alterations of the glucose metabolism induce expression of genes linked to de novo lipogenesis[154]. However, a systematic comparative analysis of adenocarcinomas arising in different locations of the intestinal tract with lipidomics is not available at present. Increased expression of ACSL1 was reported in several cancers, including colon[155,156] and liver[157,158], related to a poor clinical outcome[159]; ACSL4 was also upregulated in multiple cancer types, including colon[155,160] and liver[161-163]. Poorer patient survival in stage II colon cancer was correlated with the expression of ACSL4 and expression of stearoyl CoA desaturase 1 (SCD1)[156]. Concomitant overexpression of ACSL1, ACSL4 and SCD1 was found to induce epithelial-mesenchymal transition in colorectal cancer [155]. ACSL3 and ACSL4 were upregulated in hepatocellular carcinoma (HCC)[164]. Deregulated expression of both ACSL3 and ACSL4 is associated with disease and especially with cancer[7]. ACSL3 drives tumor growth by increasing both fatty acid β-oxidation[165] and arachidonic acid conversion into prostaglandins[166]. As previously reported, ACSL4 indirectly stabilizes c-Myc by acting on the ERK/FB/ NFκB axis and driving oncogenesis via c-Myc-oncogenic signaling in HCC[167]. ACSL4 expression is highly linked to the cell sensitivity for ferroptosis, known as an iron-mediated non-apoptotic cell death[168]. Reported roles of ACSL4 include metabolic signaling resulting in drug resistance and the activation of intracellular, pro-oncogenic signaling pathways[139]. Impaired expression of ACSL5 is associated with coeliac disease and sporadic colorectal adenocarcinomas[169] and overexpression of ACSL5 induces apoptosis[170] and suppresses proliferation by inhibiting the activation of the Wnt/β-catenin signaling pathway in colon cancer[57].

ACSS1 and ACSS2 are overexpressed in HCC[171]. Both are key players in acetate metabolism which is shown to be highly taken up by several types of cancers, including liver. Gao et al[171] reported a role of acetate in epigenetic regulation (Histone acetylation) of a promoter region of FASN. Induction of lipid synthesis driven by increased FASN expression supports tumor cell survival and growth[171].

miRNAs targeting of long-chain acyl-CoA synthetases
Micro RNAs (miRNAs) are non-coding single stranded RNAs which regulate transcription of messenger RNA via binding to their 3'-untranslated region[172]. Cancer cells evolved a regulatory mechanism to control the mRNA stability of ACSs by targeting their 3'-untranslated regions (3'UTR). For example, it was reported that miR-205 was decreased in liver cancer[173]. Negative correlation between miR-205 and ACSL4 expression was reported in human HCC patients[173]. The miR-205 targeting site is reported at the 3'UTR region of ACSL4-mRNA[173]. In addition, it is known that miR-205 binds to the 3'UTR of ACSL1 and induces its degradation[157]. The role of miR-211-5p as a tumor suppressor was reported in HCC[174]. This tumor-suppressive role was accomplished by downregulation of ACSL4 which is highly expressed in HCC[174]. miR-19b-1 showed an inhibitory effect on the ACSLI/ACSL4/SCD1 axis by downregulating the Wnt/β-catenin pathway[175]. ACSL/SCD increases GSK3β phosphorylation, activating Wnt signaling and EMT, therefore, downregulation of β-catenin signaling by miR-19b-1 can be beneficial in colon cancer[175]. miR-142-3p has been reported to target cancer stem cell markers, such as the Wnt target and LGR5 in colorectal cancer cells[176], in agreement with its action on the ACSL/SCD network cancer stem cell feature generation[175,176]. miR-34c was reported to be involved in hepatic fibrogenesis, miR-34c increases lipid droplet formation and hepatic stellate cell activation by downregulating ACSL1 in the liver[177]. miR-497-5p was reported to induce death in colon cancer by targeting ACSL5, suggesting its therapeutic potential in colon cancer[172].

Pharmacological targeting of long-chain acyl-CoA synthetases
Triacsin C, a fungal metabolite and a potent competitive inhibitor of ACSs activity[178,179], competes with FAs for the catalytic domain. It inhibits ACSL1, ACSL3 and ACSL4, and in higher concentration proves effective against ACSL5[179,180]. It is
worth highlighting that triacsin C has a high toxicity (IC50) and consequently normal cells can be damaged[7].

Thiazolidinediones, also known as glitazones, are used for the therapy of diabetes II. Troglitazone and rosiglitazone are PPARγ agonists; interestingly they inhibit ACSL4 via PPARγ indirect mechanism[181,182]. Some of these drugs (Troglitazone, Ciglitazone) showed a protective effect against diabetes-promoted cancer[183].

Pharmacological targeting of very-long-chain acyl-CoA synthetases

FATP1 and FATP4 inhibitors were detected using high-throughput screening[184-186]. However, these compounds were not effective as revealed by in vivo studies. Screening compounds that specifically target domains involved in fatty acid transport, rather than the ACSL activity domain, might help to discover more effective compounds which could inhibit fatty acid transport. FATP2/ACSVL1, expressed mostly in the liver and intestine, acts as a transport protein and ACS[187]. FATP2 might be considered as an early marker for the development of overweight disorder after a high-fat diet[188]. A high-fat diet significantly upregulated fatp2 expression in the intestine of mice[188,189]. It has a role in hepatic long-chain fatty acid uptake[190].

Due to its important role in fatty acid transport, FATP2 can be a promising pharmacological target in diseases which are characterized by an abnormal accumulation of intracellular FAs and lipids which may eventually result in irreversible hepatic cirrhosis[191,192]. Lipofermata and Grassofermata are selected FATP2 inhibitors which show specificity toward attenuating transport of LCFAs and VLCFAs. Lipofermata (5'-bromo-5-phenyl-spiro[3H-1,3,4-thiadiazole-2,3'-indoline]-2-one) inhibits the function of FATP2 as a transport protein, without compromising its function as an ACS[193,194]. Grassofermata (2-benzyl-3-(4-chlorophenyl)-5-(4-nitrophenyl) pyrazolo[1,5-a] pyrimidin-7(4H)-one) suppresses palmitic acid mediated lipotoxicity[193,195,196]. Both of them reduce intestinal fat absorption of 13C labeled oleate[186]. In addition to its contribution to the development of metabolic liver diseases, FATP2 promotes the growth of cancer cells and induces their resistance to targeted therapies[190]. A study by Veglia et al[194] demonstrated that lipofermata abrogated the activity of polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) and substantially delayed tumor progression in colon cancer cell line CT26 tumor-bearing mice. STAT3 signaling induced by granulocyte-macrophage colony stimulating factor (GM-CSF) upregulated the FATP2 in these cells. FATP2 overexpression in these PMN-MDSCs cells induced PGE2 synthesis and its immunosuppressive effect on CD8+ T cell[194]. Interestingly in this study, it was found that lipofermata elevated the therapeutical effect of immune checkpoint inhibitor therapy (anti-PD-1 and anti-CTLA-4) as well as macrophage targeted therapy (anti CSF-1R) [194].

FATP5 can be exclusively found in liver, at the basal plasma membrane of hepatocytes[197]. Both its location and role in long-chain fatty acid uptake make it an attractive target for treatment of metabolic disorders. Interestingly, screening of potential compounds revealed the potential of BAs including the primary BAs produced by the liver and the secondary BA secreted by intestinal bacteria (microbiota) to attenuate specifically FATP5 function without affecting FATP4[197]. The following BAs showed potential for FATP5 inhibition: chenodiol, primary BA, produced by the liver and ursodiol, secondary BA, which is metabolically produced by intestinal bacteria[197].

Experimental in vivo studies in rats showed induction of FATP mRNA expression, finding the highest upregulation in the liver. In the intestine, there was an increase in the FATP mRNA level but two times less than in the liver[198], suggesting that fenofibrates show specificity towards liver FATPs. Fibrate are known as PPARα activators, their hypolipidemic effect is accomplished via FATP activation, induction of β-oxidation and consequently reduction in triglyceride synthesis[198]. The indirect activation of FATP by the fenofibrate is mediated via PPARα[199].

Targeting of short-chain acyl-CoA synthetases

As reported by Bjorson et al[200], mitochondrial acetate appears to be the main metabolic energy source under hypoxia in HCC patients. Upregulation of ACS1 led to an enhanced level of mitochondrial acetate in HCC, which is associated with several metabolic alterations including decreased fatty acid oxidation, glutamine utilization, gluconeogenesis and increased glycolysis[200] This finding suggests a potential of ACS1 as a target in cancer treatment. Indeed, the ACS1 inhibitor showed a growth inhibitory effect on glioma[201].
CONCLUSION

LCFAs and SCFAs are the most abundant energy sources from dietary lipid intake and microbiota-derived fermentation products. Members of ACSs play a critical role in lipid metabolism, participating in fatty acid transport and activation. Abnormal expression of ACSs is closely associated with lipid metabolic disorders and carcinogenesis. Research on ACSs will shed further light on their biological functions and molecular mechanisms in fatty acid metabolism and eventually lead to the development of therapeutic drugs targeting ACSs in the treatment of human metabolic diseases.

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Liver involvement in inflammatory bowel disease: What should the clinician know?

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Abstract

Inflammatory bowel disease (IBD) may show a wide range of extraintestinal manifestations. In this context, liver involvement is a focal point for both an adequate management of the disease and its prognosis, due to possible serious comorbidity. The association between IBD and primary sclerosing cholangitis is the most known example. This association is relevant because it implies an increased risk of both colorectal cancer and cholangiocarcinoma. Additionally, drugs such as thiopurines or biologic agents can cause drug-induced liver damage; therefore, this event should be considered when planning IBD treatment. Additionally, particular consideration should be given to the evidence that IBD patients may have concomitant chronic viral hepatitis, such as hepatitis B and hepatitis C. Chronic immunosuppressive regimens may cause a hepatitis flare or reactivation of a healthy carrier state, therefore careful monitoring of these patients is necessary. Finally, the spread of obesity has involved even IBD patients, thus increasing the risk of non-alcoholic fatty liver disease, which has already proven to be more common in IBD patients than in the non-IBD population. This phenomenon is considered an emerging issue, as it will become the leading cause of liver cirrhosis.

Key Words: Inflammatory bowel disease; Liver; Primary sclerosing cholangitis; Viral hepatitis; Immunosuppression; Non-alcoholic fatty liver disease

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Cholangitis is one of the most well-known comorbidities and deserves more attention by the clinician. Liver damage due to drugs used to cure IBD is also a relevant issue. Finally, some emerging topics such as the spread of liver steatosis or the implications of chronic viral hepatitis have been analyzed.

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INTRODUCTION

Inflammatory bowel disease (IBD) consists of two separate disease entities, ulcerative colitis (UC) and Crohn’s disease (CD), affecting the gastrointestinal tract[1]. However, IBD does not exclusively affect the gut. The gut-liver axis refers to the bidirectional relationship between the gut and its microbiota, and the liver, resulting from the integration of signals generated by dietary, genetic and environmental factors[2]. Therefore, a perturbation of this axis may mirror pathologic conditions both in the gut and the liver. Based on this consideration, the relationships between IBD and liver disorders are noteworthy and should always be considered by the clinician. The association between IBD and primary sclerosing cholangitis (PSC) is the most known and studied model, as it has several implications, the most important ones are the increased risk of both colorectal cancer and cholangiocarcinoma. Additionally, hepatotoxicity due to drugs such as thiopurines or biologic drugs is a relevant issue that should also be taken into account when planning IBD treatment[3]. It should not be forgotten that IBD patients may have concomitant chronic viral hepatitis, such as hepatitis B (HBV) and hepatitis C (HCV)[3]. Chronic immunosuppressive regimens may cause a hepatitis flare or reactivation of a healthy carrier state; therefore, careful monitoring of these patients is necessary. Finally, the obesity epidemic has involved even IBD patients, thus increasing the risk of non-alcoholic fatty liver disease (NAFLD), which has already proven to be higher than the control population in IBD patients[3]. This phenomenon is considered an emerging issue, as it will become the leading cause of liver cirrhosis.

Therefore, we aimed to perform a narrative review describing the main interactions between IBD and corresponding liver involvement, with a particular focus on PSC and other autoimmune liver disorders, drug-induced hepatitis, HBV, HCV and NAFLD (Table 1).

IBD AND PRIMARY SCLEROSING CHOLANGITIS

IBD and PSC are two pathologic entities that can occur alone or in combination. In this case they create a phenotypically different disease known as PSC-IBD. PSC-IBD prevalence is uncertain and differs in several studies, but it is agreed that it is very low (0.024%-0.041%)[4-6]. PSC and IBD may occur simultaneously or sequentially. Indeed, PSC patients develop IBD in 20%-70% of cases, with a stronger association with UC (80%) than with CD (10%) and indeterminate colitis (IC) (10%)[7]. Conversely only 5% of patients with UC show concomitant PSC.

Primary Sclerosing Cholangitis and Ulcerative Colitis

UC represents the underlying IBD in most cases of PSC-IBD. In patients with PSC and UC (PSC-UC), UC characteristically tends to be mild, quiescent and may even appear endoscopically normal (in this case, the diagnosis is based simply on histological analysis)[8]. Therefore, random biopsies during the first colonoscopy should always be performed to reveal an underlying UC in patients with PSC. Similarly, PSC may be underdiagnosed in patients with UC, as it can be asymptomatic. Thus, liver function tests, including cholestatic and hepatocellular damage markers, should always be recommended in the follow-up of UC. If a patient with UC is found to have hepatocellular injury or a cholestatic pattern, magnetic resonance cholangiopancreatography
Table 1 Main liver comorbidities associated with inflammatory bowel disease

<table>
<thead>
<tr>
<th>Associated diseases</th>
<th>Prevalence in IBD (%)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSC</td>
<td>0.024-0.041</td>
<td>Higher risk of cholangiocarcinoma and colorectal cancer; IBD shows less severe lesions than IBD alone</td>
</tr>
<tr>
<td>NAFLD</td>
<td>20-30</td>
<td>Associated with the use of corticosteroids, long disease duration, severe disease course; Associated with metabolic syndrome</td>
</tr>
<tr>
<td>Viral hepatitis</td>
<td>1-9</td>
<td>More common in the elderly; Association with advanced liver fibrosis; Need for anti-viral treatment before starting immunosuppressive drugs; HBV vaccine recommended</td>
</tr>
</tbody>
</table>

HBV: Hepatitis B virus; NAFLD: Non-alcoholic fatty liver disease; PSC: Primary sclerosing cholangitis; IBD: Inflammatory bowel disease.

(MRCP) should be performed to confirm the diagnosis[9]. The onset of the two disorders may vary. Typically, UC occurs first, with a median time interval of 10 years [10]. Nevertheless, in a minority of cases, UC may appear some years after the diagnosis of PSC, even after orthotopic liver transplantation[11]. The degree and the extension of colorectal inflammation in PSC-UC differ from UC alone. Indeed, the incidence of pancolitis appears increased in PSC-UC patients when compared with UC-only patients, as shown by Boonstra et al[12]. In their series, PSC-UC patients were affected by pancolitis in 94% of cases, while pancolitis was demonstrated only in 62% of patients affected by UC alone. Patients with PSC-UC usually have a greater prevalence of backwash ileitis and rectal sparing (51% and 52%, respectively) than controls with UC alone (7% and 6%, respectively)[13]. However, the mild degree of colitis and the low rate of endoscopically visible inflammation may overestimate rectal sparing, when random biopsies are not performed[12,14]. Even though, the extension of colitis tends to be more diffuse, and in PSC-UC the severity of the mucosal inflammation seems less pronounced. Patients with PSC-UC have less significant bowel symptoms, a lower need for steroids and undergo fewer hospitalizations than patients with UC alone[15].

Primary Sclerosing Cholangitis and Crohn’s Disease

Similar to patients affected by PSC-UC, patients with PSC and CD (PSC-CD) have a phenotypical and clinical pattern that sharply differs from patients with CD alone. Indeed, isolated ileal involvement, which occurs in about 30% of patients affected with CD, is rare in patients with PSC-CD (2%-5%)[12,16]. As shown with PSC-UC, the degree of endoscopically visible inflammation is milder in patient with PSC-CD than in those affected by UC. Likewise, the incidence of CD complications seems low in PSC-CD[12,16,17].

Main characteristics of PSC in IBD

While IBD in PSC-IBD has specific phenotypical patterns as listed above, PSC does not show significant differences in terms of histologic findings such as periductal fibrosis, inflammation and portal edema or fibrosis[18]. From a clinical point of view, according to Yanai et al[19] PSC outcomes, including cirrhosis incidence and transplant-free survival, did not differ in PSC-IBD compared with PSC alone patients. Conversely, Fevery et al[20] reported higher rates of liver-related death and malignancies in patients with PSC-UC when compared to patients with PSC-CD. Interestingly, Nordenvall et al[21] found that patients with PSC-UC who underwent colectomy, seemed to have a lower risk of mortality, morbidity and the need for liver transplantation.

Risk of colorectal cancer (CRC) and hepatobiliary carcinomas in PSC-IBD

Although both PSC and IBD patients do not have a general higher risk of malignancies than the general population, patients with PSC-IBD show a significantly more marked risk of developing colorectal carcinoma (CRC) and cholangiocarcinoma (CCA), and hepatocellular carcinoma (HCC). In a meta-analysis, Zengh et al[22] found that patients with PSC-IBD have a strikingly higher risk for the development of CRC than patients with IBD alone. In detail, the stratification by IBD type showed a three-fold increased risk for the development of CRC and colorectal dysplasia in patients with PSC-UC compared to those with UC alone. A non-significant increase in the risk of neoplasia was shown in patients with PSC-CD, in contrast to that found in patients with CD alone. For these reasons, patients with PSC-IBD (especially those with PSC-
UC) require close colorectal neoplasia endoscopic surveillance. Major American and European Societies recommend that annual CRC screening should be started at the time of PSC-IBD diagnosis. In PSC-IBD patients an increased risk of hepatobiliary malignancies such as CCA, gallbladder carcinoma (GBC), and HCC has been demonstrated. Gulamhusein et al[23] demonstrated that prolonged duration of IBD is associated with an increased risk of CCA in patients with PSC-IBD. They also observed that the risk of CCA was not modified after colectomy, thus suggesting that colonic resection itself does not reduce the risk of CCA. European and American Societies recommend that CA 19-9 and biliary imaging should be completed every year for these patients[24,25]. IBD could be an additional risk factor that further increases the hazard of CCA in PSC. In particular, a long duration of IBD is associated with CCA with a hazard ratio of 1.37[23].

There are no studies demonstrating an increased risk of GBC in PSC-IBD patients, even if that risk is demonstrated in PSC-alone patients[26]. Said et al[27] found in their cohort of patients affected with PSC, that 6% had gallbladder masses, of which 56% were malignant. The American Association for the Study of Liver Disease (AASLD) guidelines support cholecystectomy for polyps of any size in these patients, given the high likelihood of malignancy[28]. HCC seems to be a rare malignancy in PSC-IBD. Zanouzi et al[29] analyzed a cohort of PSC-cirrhosis patients and found no cases of HCC. However, in the same cohort of patients, IBD was found in 65%.

As both CCA and CRC are likely to occur in PSC-IBD patients, a chemopreventive strategy could be proposed. A meta-analysis[30] showed that low dose ursodeoxycholic acid may have a protective effect on both CRC and colonic dysplastic lesions, with an odds ratio of 0.19. However, the studies were performed on small populations in tertiary centers, and the strength of evidence is not high[31]. Even mesalazine has demonstrated, in vitro and in animal models, an anti-proliferative effect as well as the ability to inhibit the Wnt/β-Catenin pathway and epithelial growth factor receptor activation; therefore, it may be a promising agent for CRC prevention, despite the chemopreventive effect of mesalazine only being documented for patients with UC alone so far[32]. Unfortunately, no effective approach for CCA chemoprevention has emerged, therefore surveillance remains the mainstay for early CCA detection in PSC patients.

**Therapeutic perspectives**

The pathogenetic mechanisms underlying PSC-IBD remain unknown, even though many hypotheses have been proposed. Understanding the basis of the disease could lead to the identification of a new targeted therapy. One of the most interesting assumptions suggests that intestinal mucosal lymphocytes may migrate to the liver following activation in the bowel of IBD patients, thus promoting liver inflammation[33]. It has been shown that adhesion molecules and chemokine receptors normally expressed only in the gut can be aberrantly expressed within the liver to promote the homing of gut-associated lymphocytes. One of these adhesion molecules is α4β7 integrin. A monoclonal antibody directed against α4β7, vedolizumab, has been approved for the treatment of IBD. It was hypothesized that vedolizumab could provide hepatic anti-inflammatory benefits. Nevertheless, Christensen et al found that, after treatment with vedolizumab, symptoms and intestinal clinical activity were significantly decreased, but the Mayo PSC Risk Score and liver damage biomarkers were only slightly improved[34].

Aberrant microbiota epitope recognition and gut dysbiosis seem to have a role in the pathogenesis of PSC-IBD, while genetics, gut mucosal permeability and autoimmune mechanisms have a controversial role[35]. Further studies are needed to improve our knowledge on the pathogenesis of PSC-IBD in order to provide new and efficient therapeutic strategies.

When PSC causes end-stage liver disease, liver transplantation is the only curative treatment. Regarding this point, some studies found that IBD does not worsen survival in patients who undergo liver transplantation for PSC. Only exposure to azathioprine seems to increase post-transplant mortality, while IBD per se increases the risk of cytomegalovirus infection[36].

**PRIMARY BILIARY CHOLANGITIS AND AUTOIMMUNE HEPATITIS IN IBD**

PBC is an autoimmune liver disease characterized by inflammatory cell infiltration of intralobular biliary ducts, with consequent biliary duct damage, which can progress towards fibrosis. Currently, there is no solid link between IBD and PBC, as only a few
HEPATIC STEATOSIS IN IBD

Hepatic steatosis is defined as intrahepatic fat accumulation of at least 5% of liver weight. Prolonged hepatic lipid storage may lead to liver metabolic dysfunction, inflammation, and advanced forms of NAFLD. Non-alcoholic hepatic steatosis is associated with obesity, type 2 diabetes and dyslipidemia. Several mechanisms are involved in the accumulation of intrahepatic fat, including increased flux of fatty acids to the liver, increased de novo lipogenesis, and/or reduced clearance through β-oxidation or very-low-density lipoprotein secretion in the absence of secondary causes of lipid overload such as significant alcohol intake.

A link between hepatic steatosis and IBD has been studied since 1873, when Thomas described for the first time the association between “ulceration of the colon” and a “much enlarged fatty liver”. In recent years, due to the spread of obesity in the context of IBD, fatty liver disease has been increasingly recognized in IBD. The intestinal inflammatory state and gut barrier perturbation secondary to IBD might increase toxin and bacterial constituents translocation from the gut to the portal vein; this event has been recognized as a possible pathophysiologic mechanism underlying NAFLD. Moreover, diets poor in high fiber foods, such as fruits and vegetables, frequently consumed by IBD subjects to avoid intestinal symptoms, could lead to a great prevalence of NAFLD. Moreover, food components and alimentary habits with high proteins and fats, excessive sugar intake and less vegetables and fiber can influence the composition of the intestinal microbiome, and play a role in driving IBD pathogenesis and fat metabolism leading to NAFLD onset.

A recent meta-analysis showed that the overall pooled prevalence of NAFLD in IBD patients was 27.5%. NAFLD, in particular, was more common among patients with features of severe IBD, such as longer disease duration or a history of abdominal surgery.

Another study by Bessisow et al. showed a frequency of NAFLD in IBD of 33.6% and demonstrated that disease activity, duration of IBD and prior surgery were predictors of NAFLD development.

Conversely, in a Japanese study, the ultrasonographic prevalence of NAFLD in CD was 21.8% and this was the only study in which NAFLD was identified as an independent predictor of a negative C-reactive protein level and higher rate of remission, so NAFLD might offer a protective effect in patients with CD.

Nevertheless, most studies did not include non-IBD patients as a control group. Glassner et al. examined 3 groups of patients: IBD + NAFLD, IBD alone, and NAFLD alone. A total of 168 patients were evaluated, 56 patients in each group. They found an overall NAFLD prevalence of 13.3% in IBD patients. IBD patients with NAFLD had longer IBD disease duration and developed NAFLD even in the absence of metabolic risk factors when compared to patients with NAFLD alone.

A study performed in 2018 by Principi et al. included 465 IBD patients and 223 non-IBD patients. The prevalence of NAFLD was higher in IBD than in non-IBD patients (28.0% vs 20.1% respectively, \( P = 0.04 \)); furthermore, younger age was observed in NAFLD-IBD than in non-IBD individuals, whereas no other differences were found between these two subgroups. Regarding risk factors, diabetes and fasting blood glucose were associated with development of NAFLD in IBD, without any difference in the populations without IBD, with only a higher waist circumference in IBD compared to non-IBD patients. No IBD-related variable was associated with NAFLD.

There are no studies on the progression of NASH in IBD. However, since IBD may induce gut barrier perturbation and an increase in toxin and bacterial translocation, it...
is possible that in patients with NAFLD, the coexistence of IBD can trigger the progression from simple steatosis to NASH. A single study, on the other hand, has shown that progression of fibrosis, estimated by the NAFLD fibrosis score, is quite rare in IBD[53].

In conclusion, NAFLD is common in patients with IBD. Screening, prevention, and early treatment of NAFLD might be recommended in IBD patients. However, a better understanding of the underlying mechanism of the coexistence of IBD and NAFLD is necessary to improve management. The treatment of NAFLD in IBD does not differ from other cases. In particular, so far only diet and physical exercise have been proved to be effective[54].

### CHRONIC VIRAL HEPATITIS IN IBD

Chronic viral hepatitis, in particular HBV and HCV-related, is a very common infection and a worldwide health issue. It is estimated that over 350 million people in the world have chronic HBV infection and over 250 million people have chronic HCV infection, with a mean prevalence of 5% and 2% for HBV and HCV, respectively[55, 56].

With regard to the prevalence of chronic hepatitis B (CHB) and chronic hepatitis C (CHC) in IBD, recent evidence[57-61] shows that it was comparable to a control population, ranging from 1% to 9%. A recent Italian study by Losurdo et al[62] on 807 IBD patients and 189 controls, found a prevalence of 3.4% for CHC and 0.9% for CHB, a result which agrees with recent literature reports[57,58,61]. This analysis demonstrated that advanced age was independently associated with increased risk of CHB/CHC. It is possible that surgery performed before the diffusion of presurgical hepatitis screening could explain this result, also taking into account that CHC was more common in patients operated before 1990. Indeed, the introduction of the HBV vaccine and HCV routine detection led to an improvement in the prevention measures against viral hepatitis transmission during surgery or blood donation, thus reducing the risk of infection in young generations[62].

As the treatment of IBD is based in selected cases on immunosuppressive agents (thiopurines and biologic drugs such as monoclonal antibodies), an accurate clinical and laboratory assessment is preliminarily required to look for chronic infections that may have a severe flare under biologic drugs[57,63]. Among these, chronic viral hepatitis and in particular CHB and CHC, are advised to be investigated by the guidelines before starting immunosuppressive treatment[64].

According to the guidelines, all IBD patients should be tested for HBV (HBsAg, anti-HBs, anti-HBc) at diagnosis of IBD to determine HBV status. In patients with positive HBsAg, viremia (HBV-DNA) should also be quantified. Moreover, HBV vaccination is recommended in all HBV anti-HBc seronegative patients with IBD. All HBsAg positive subjects should start anti-viral agents before undergoing biologic treatment to prevent potentially serious hepatitis B flares[64,65]. A number of case series and study cohorts suggest that nucleotide/nucleoside analogues are safe and effective in IBD patients on immunomodulator treatment[66]. Entecavir and tenofovir are preferred for IBD patients due to their rapid onset of action, high anti-viral potency and low incidence of resistance. On the other hand, patients with HBsAg positive (chronic HBV infection) should receive anti-viral agents before, during and for at least 12 mo after immunomodulator treatment has ceased[64]. Additionally, HBV vaccination is strongly advised by the guidelines, possibly before starting any immunosuppressive treatment and preferably at the moment of diagnosis, if anti-HBs level is not protective. This approach should be followed in any region, irrespective of HBV prevalence.

With regard to CHC, present knowledge shows in some cases mild liver dysfunction and an amplified detrimental effect by the simultaneous presence of other viruses (HBV/HIV) in relation to immunomodulator assumption[67,68]; therefore, HCV antibody testing and HCV-RNA should be investigated. Immunomodulators are not contraindicated but should be used with caution. The decision depends on the severity of IBD and the stage of liver disease. In the past years, an interferon-based treatment for HCV infection in CD has generally not been recommended, as it could worsened the intestinal disorder; however, this aspect remains controversial[69]. Conversely, in UC, interferon therapy did not appear to have an adverse effect[70]. In addition, the administration of ribavirin plus interferon or triple anti-viral therapy (interferon, ribavirin and protease inhibitors) could have increased the toxicity of drugs used for IBD maintenance (for example azathioprine, methotrexate)[64]. Therefore, the risk that anti-viral therapy or drug interactions with IBD therapy might
exacerbate IBD should assessed cautiously when considering the need for HCV treatment[64]. However, over the last years, concomitant IBD and HCV infection management has completely changed due to the recent introduction of direct-acting anti-virals (DAAs). Recently published data on DAAs are very encouraging also in IBD patients[71]. There are three possible timing strategies for administration in patients requiring biological therapies: (1) Sequential strategy, meaning the choice of treating firstly the active IBD with biologics and then, once the acute phase has been controlled, treating the HCV infection; (2) Concomitant strategy, that is the contemporaneous initiation of DAAs and biologic drug administration; and (3) Inverted sequential strategy, i.e., the administration of anti-viral therapy before biologics. The timing strategy could depend on several factors, including IBD activity and patient comorbidity. This means that a case-by-case decision could be the best choice[72]. The opportunity to eradicate HCV should always be taken into account, as it has demonstrated that a sustained viral response may reduce liver stiffness in these patients[73].

### IBD AND DRUG-INDUCED LIVER INJURY

In the last decade, treatment options for IBD have included new molecules acting at different target levels. Usually, as new drugs are introduced, their side effects should also be considered, and liver toxicity is one of the most meaningful among these.

Drug-induced liver injury (DILI) caused by these drugs can be classified into three forms: hepatocellular, cholestatic or a mixed pattern. Moreover, some forms of drug-induced AIH should also be considered. This issue leads to a schedule of specific screening before starting therapy for IBD, and a follow-up to monitor liver enzymes is necessary[74,75].

In Table 2, we summarize the main knowledge on DILI in IBD patients.

#### Thiopurines

Thiopurines, in particular azathioprine (AZA) and 6-mercaptopurine (6-MP) are used for induction and maintenance of remission in IBD. Studies have shown that AZA/6-MP as add-on to infliximab can reduce the development of antibodies against infliximab. Thiopurines act as DNA synthesis inhibitors by incorporating purine analogues into DNA with cytotoxic and immunosuppressive effects. AZA is metabolized in the liver to 6-MP, which is metabolized by three enzymes, including thiopurine S-methyltransferase (TMPT) to 6-methylmercaptopurine (6-MMP). AZA and 6-MP are prodrugs of 6-thioguanine (6-TGN), the real effective metabolite. Some studies have suggested that some TMPT polymorphisms could cause a rise in 6-MMP level, thereby amplifying hepatotoxicity. In a cohort study of 270 patients treated with 6-MP, 47 patients showed evidence of altered liver function tests (LFT) in the first 20 weeks of treatment and > 80% of these patients had elevated levels of 6-MMP in the first week[76]. Another study proved that patients with high concentrations of 6-MMP had not only a strong risk of side effects but also a reduction in therapeutic response[77]. Conversely, Dong et al[78] found that the presence of TMPT polymorphisms increased bone marrow toxicity but not hepatotoxicity. A recent meta-analysis of 10 studies (recruiting 1875 patients) proved that TMPT polymorphisms were not linked with liver injury. The physiopathology of liver injury due to thiopurine is still unclear.

The prevalence of thiopurine-induced liver toxicity can vary between 0% and 17%. In a systematic review of 34 studies with 3485 patients, the prevalence of hepatotoxicity induced by AZA/6-MP was 3.4% with no differences between the two drugs[79]. Additionally, Chaparro et al[80] in a study of 3931 patients with IBD treated with thiopurine reported that hepatotoxicity was one of the most common side effects, with a prevalence of 4%. CD, smoking and preexisting NAFLD seemed to be risk factors, while the prevalence was lower in females. In a study by Shroder, who analyzed 259 patients undergoing immunosuppressive treatment with AZA, 6MP and MTX, liver steatosis was found in 28.2% of them, and patients with steatosis also had a higher risk of having elevated alanine transaminase (ALT) blood levels[81].

On the other hand, dose independent, idiosyncratic liver reactions have been described for thiopurines. Acute dose-independent toxicity is caused by an idiosyncratic cholestatic reaction accompanied by fever, rash, lymphadenopathy and hepatomegaly with increased alkaline phosphatase level. The median onset time of hepatotoxicity is 110 days, and in most cases is self-limiting with a good prognosis.

Another atypical, long-term liver injury caused by thiopurines is characterized by vascular endothelial lesions. Nodular regenerative hyperplasia (NRH), is the most
Table 2 Main features of drug-induced liver injury in inflammatory bowel disease

<table>
<thead>
<tr>
<th>Drug</th>
<th>Characteristics of drug-induced liver injury</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminosalicylates</td>
<td>Increases in LFT; Cholestatic pattern; Rarely eosinophilia</td>
</tr>
<tr>
<td>Thiopurines</td>
<td>Influenced by TMPT polymorphisms &gt; increase in 6-MMP, the hepatotoxic molecule; Increases in LFT; Idiosyncratic cholestatic reaction; Fever, rash, lymphadenopathy and hepatomegaly; Nodular regenerative hyperplasia</td>
</tr>
<tr>
<td>Anti-TNF</td>
<td>Idiosyncratic reaction &gt; dose-dependent mechanism; Hepatocellular injury &gt; cholestasis; Autoimmune phenomena</td>
</tr>
<tr>
<td>Anti-integrins</td>
<td>Rare; Asymptomatic LFT increase</td>
</tr>
<tr>
<td>Anti IL12/23</td>
<td>Mild LFT increase</td>
</tr>
</tbody>
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LFT: Liver function test; TMPT: Thiopurine S-methyltransferase; TNF: Tumor necrosis factor.

Frequent of these lesions, while pelvicis hepati and sinusoidol obstruction syndrome (SOS) are less common. NRH is frequently asymptomatic. The mechanism underlying NRH is still unknown, it is possible that hepatocyte atrophy and portal venules destruction could be involved; risk factors seem to be male sex, CD with stricturing behavior and previous small bowel resection. In a large French study, NRH was found in 37 cases, with a cumulative risk of 0.5% at five years and a median onset time of 48 mo[82]. A recent study observed a similar prevalence of NRH between patients treated with thiopurines and patients thiopurine-naive[83]. On the other hand, it was found that thiopurines are associated with NRH when the dose is high (tioguanine > 40 mg/day) or in male patients with small bowel resection > 50 cm[84,85]. The evolution of NRH after stopping thiopurine therapy is still unclear.

There is no agreement on thiopurine toxicity management. In a large study with a long-term follow-up only 3.6% of patients needed to discontinue therapy[86]. In another study, 90% of patients had normalization of LFT by reducing thiopurine doses[87]. It is unclear whether the frequency of hepatotoxicity is the same for AZA and 6-MP treatment: a study of 135 patients reported that 6-MP was well tolerated in 71% patients who had shown liver toxicity with AZA[88]. Coadministration of allopurinol (a xanthine-oxidase inhibitor) seems to reduce 6-MMP levels as it leads to a higher concentration of 6-MMP converted to 6-TGN. However, since allopurinol is a xanthine-oxidase inhibitor, the AZA dose should be reduced. A retrospective cohort study of 105 patients reported that coadministration of allopurinol allowed long-lasting therapy and transaminase normalization[89]. Also, in another study by Krejineof, among 211 patients with liver toxicity, 86% experienced an improvement by lowering the dose of thiopurines in association with allopurinol[90]. A larger study by Vasuvedan analyzed 767 patients on thiopurine therapy and demonstrated that allopurinol should be started to reduce side effects, as 94% of patients who had hepatotoxicity achieved resolution by changing to co-therapy[91]. As TMPT polymorphisms are likely to be involved in hepatotoxicity, some authors have proposed that these polymorphisms should be identified before starting therapy, but a review by the American Gastroenterological Association Institute stated that the benefits of these tests were low[92]. On the contrary, a consensus guideline by the British Society of Gastroenterology focused on TMPT activity and recommended the administration of a half-dose of thiopurines to patients with low TMPT activity[93].

LFT should be monitored routinely, but there is no agreement on their timing. Mottet et al.[93] recommended LFT every wk for the first mo, then twice a mo during the second mo and then once every 3 mo.

**Sulfasalazine and mesalamine**

Sulfasalazine is used for mild UC. It has been associated with acute hepatitis, cholestatic hepatitis, granulomatous hepatitis and rarely with acute liver failure[94]. The incidence of hepatotoxicity is low: A review by Ransford et al who analyzed 4.7 million prescriptions in the period from 1991 and 1998, reported only 9 cases of hepatitis caused by sulfasalazine[95].

Mesalamine (oral and rectal) is approved for mild UC. Authors in the last three years have demonstrated that the prevalence of liver toxicity caused by mesalamine is low, between 0% and 4%. The use of mesalamine may be associated with asymptomatic elevations in LFT, hepatitis and cholestatic hepatitis[96]. A recent review reported that LFT should be monitored every year and therapy should be stopped in the case of abnormal increases, while treatment with corticosteroids should be considered if fever, rash, or eosinophilia are observed. The same review
demonstrated that most cases of hepatotoxicity quickly reversed with drug withdrawal[97].

**Methotrexate**

Low doses of methotrexate (MTX) are used for mild CD, and it is widely used for rheumatologic disease; therefore, in this field its hepatotoxicity has been more extensively studied. The underlying mechanism is still not clear; several polymorphisms of enzymes involved in folic acid metabolism are thought to be involved. Two systematic reviews on this topic reported opposite results: the first review found an association between MTX hepatotoxicity and C677T polymorphism of methylenetetrahydrofolate reductase (MTHFR) gene, while the second review did not confirm this result[98,99]. MTX can cause different histological liver findings according to the Roenigk’s classification including: (1) Normal; (2) Mild fatty infiltration, nuclear alterations or portal inflammation; (3) Moderate to severe fatty infiltration, nuclear alterations, or portal infiltration and mild fibrosis; (4) Moderate to severe fibrosis; and (5) cirrhosis[100].

Some studies reported that the prevalence of abnormal LTF in these patients ranged from 15 to 50%, while most recent evidence demonstrated a lower prevalence. A meta-analysis of patients with IBD treated with MTX reported a rate of abnormal LTF (defined as ALT higher than normal values but less than x2 upper normal limit (ULN)) of 1.4 per 100 person-month and a rate of hepatotoxicity (defined as ALT higher than two times normal values) of 0.9 per 100 person-month[101]. It should be noted that, in CD, methotrexate is given i.m., with a dose of 25 mg/wk at induction and 15 mg/wk for maintaining remission. Considering that this dose is higher than in rheumatologic patients, this could explain the more frequent liver adverse events.

Before starting MTX treatment, patients should be screened for preexisting medical conditions, such as alcohol intake, viral hepatitis, steatosis and family history of liver disease. Rheumatological consensus guidelines recommend monitoring LFT every two wk for the first 2 mo, then every 2 or 3 mo[102]. Liver biopsy should be considered in some cases, such as when liver laboratory tests remain abnormal despite dose reduction or when there are high blood levels of drug in patients with known risk factors for hepatotoxicity. Treatment should be stopped in the case of severe fibrosis or cirrhosis and daily doses should be reduced in the case of LFT elevation. Co-administration with folic acid or folinic acid seems to reduce the frequency of serum transaminase elevation[103]. Elastography (Fibroscan) and laboratory tests are emerging tools to diagnose fibrosis as reported by Labadie et al[104]. Furthermore, in a case control study of 518 patients treated with MTX, 8.5% showed Fibroscan and FibroTest abnormalities, i.e., severe fibrosis[105]. A multivariate analysis reported that elastography should be used mainly in patients with an alcohol habit or obesity, or affected by NAFLD. Similar results were reported in a study by Herfath et al[106].

**Tumor necrosis factor alpha inhibiting agents**

Currently several molecules belonging to this class have been approved to treat IBD: infliximab (IFX), adalimumab (ADA), golimumab and certolizumab pegol. Few data are available on the hepatotoxicity of golimumab and certolizumab, while most of the literature reports DILI by IFX and ADA.

The Food and Drug Administration (FDA) in 2004 after 130 cases of liver injury in patients treated with IFX and etanercept (which has no indication in IBD), issued an alarm statement of severe hepatic adverse reactions, including acute liver failure, autoimmune hepatitis (AIH) and cholestatic hepatitis during IFX therapy[107]. In an Icelandic study by Bjarnsson that included patients with IBD, rheumatological and dermatological disorders, the occurrence of DILI in patients treated with IFX or ADA was 1:120 and 1:270, respectively[108], Shelton et al[109] in a retrospective study analyzed 1753 patients under anti-TNF therapy (1170 IFX, 575 ADA, 8 certolizumab), and found that 102 patients had high blood levels of ALT, but in 54 of these patients, additional risk factors for liver injury were found and, of the remaining 48 patients (45 IFX, 3 ADA), only 4 were considered to be affected by anti-TNF induced liver injury. Koller et al[110] in a recent observational study of 251 patients with IBD, monitored liver injury in 163 receiving IFX. Twenty-six patients (16%) showed a grade 1 liver injury (ALT < x3 ULN), 4 patients (2.5%) a grade 2 (ALT > x3 ULN); grade 1 alkaline phosphatase elevation was seen in 11 patients (6.7%) and grade 2 alkaline phosphatase elevation (> x2.5 ULN) in none. Liver injury in these patients was associated with high BMI, hepatic steatosis and longer duration of IBD[110]. In an Australian retrospective cohort study of adult patients with IBD treated with IFX (IDLE STUDY), out of 175 patients (149 with CD and 26 with UC), 57 showed abnormal liver laboratory tests. In this study, the authors used the Roussel Uclaf Causality Assessment Method...
(RUCAM) score to predict the risk of hepatic injury caused by drugs. A score of 0 rules out DILI, 1-2 means unlikely DILI, 3-5 possible DILI, 6-8 probable DILI, and > 8 highly probable DILI. Eleven patients had a RUCAM score > 3, but just one patient had a score > 8. Usually, liver injury due to IFX occurs after multiple infusions and a mean latency of 14-18 wk from induction. In this context, the RUCAM score is not a diagnostic test, but it is useful to predict DILI relying on LFT, timing of drug initiation and cessation, and on liver biopsy, when performed[111].

Although IFX, ADA and etanercept are anti-TNF drugs, they are structurally different. This explains the different responses to these agents and the different capacity to induce liver injury. Some authors have described how patients tolerate successful treatment with another molecule after a prior DILI episode induced by an anti-TNF agent. This suggests a lack of cross-toxicity within this class of drugs.

The pathogenetic mechanism underlying anti-TNF hepatotoxicity is still unknown. As liver injury can occur after a singular infusion it seems more an idiosyncratic injury rather than a dose-dependent one[107]. A genetic predisposition may be considered. Another hypothesis is that anti-TNF agents may trigger a pre-existing autoimmune disorder or generate autoantibodies: the binding of IFX to the transmembrane TNF-alpha can lead to apoptosis of monocytes and T-lymphocytes with exposure of nucleosomal autoantigens and the production of autoantibodies[112,114]. Another possibility is that anti-TNF drugs inhibit T-lymphocytes activity, thus suppressing auto-reactive B cells; this may lead to increased humoral autoimmunity[114].

However, there are several cases without evidence of autoimmunity, in which direct liver injury is involved. DILI caused by anti-TNF agents can show different patterns: Hepatocellular injury in 75% cases, but also a mixed pattern, most rarely with cholestasis, while few cases of acute liver failure have been described. Colina et al[115] reported histological necroinflammation caused by IFX, with bridging and massive necrosis in the most severe cases and some features of autoimmune injury with piecemeal necrosis in the periportal interface and prominent plasma cells infiltration. Liver injury caused by anti-TNF drugs is associated with the presence of autoimmunity markers in some patients: anti-nucleus, anti-DeDNA and anti-smooth muscle actin positivity and/or histologic features of AIH are described for IFX, ADA and etanercept. In a study analyzing 34 patients undergoing anti-TNF treatment with DILI, 22 were positive for such antibodies and showed higher levels of ALT than seronegative patients. Fifteen out of 22 subjects underwent liver biopsy that revealed clear features of autoimmunity [116]. Indeed, it is difficult to distinguish between AIH and drug-induced AIH, since these conditions may have similar clinical, biochemical, serological and histological features. Actually, IFX-induced AIH is rare in IBD patients and is described more often in rheumatology patients. In several studies, autoimmunity features were treated with corticosteroids, achieving in some cases a reduction or disappearance of autoantibodies titer; this suggests an immune-mediated DILI rather than an anti-TNF induced AIH. Ierardi et al[117] reported a case of acute liver injury after a single IFX administration. Analogously, Adar et al[118] described the first case of AIH caused by ADA that resolved after treatment cessation and corticosteroid therapy.

There is still a lack of consensus on the management of DILI induced by anti-TNF agents. The prognosis is usually favorable with normalization of LFT without cessation of anti-TNF therapy. Liver enzymes should be monitored before starting treatment and then monitored periodically, especially during the first 3 mo. If ALT remains < x3 ULN, anti-TNF can be continued until resolution; if ALT is persistently elevated > x3 ULN or in the case of jaundice, corticosteroids and liver biopsy should be considered. If a DILI is documented, anti-TNF withdrawal is still controversial. Also, the necessity to obtain an autoimmune panel before starting anti-TNF treatment is debated: several studies demonstrated that this practice does not predict the risk of developing drug-induced AIH and that anti-TNF therapy could be continued in the presence of asymptomatic anti-nucleus positivity[102].

**Anti-Integrins**

Natalizumab and vedolizumab were approved some years ago for the treatment of IBD. Both drugs have shown a good safety profile, but in the post-marketing phase, 6 cases of significant DILI associated with natalizumab were reported to the FDA[119].

Liver injury caused by natalizumab is rare with a 5% rate of asymptomatic liver enzymes elevation and it can manifest with both the hepatocellular and cholestatic pattern and can be associated with jaundice. Some cases with autoimmune features (autoantibodies positive) have also been described[120]. The guidelines recommend monitoring LFT before starting the treatment and then every 3 or 6 mo[121]. Nevertheless, the use of natalizumab is quite rare in IBD due to possible severe
neurologic complications such as progressive multifocal leukoencephalopathy\cite{122}.

Similar to natalizumab, liver injury associated with vedolizumab is rare, less than 2% in clinical trials, with both the hepatocellular or cholestatic pattern\cite{123}. Similar to natalizumab, the guidelines recommend monitoring liver enzymes every 3-6 mo.

**Anti IL12/23**

Ustekinumab was approved for CD treatment in 2016 and UC treatment in 2019. Most of the data regarding hepatotoxicity induced by ustekinumab comes from dermatologic studies. In PHOENIX 1 and 2, both studies evaluated the efficacy and safety of ustekinumab in patients with psoriasis, and the rate of liver enzymes abnormalities was low (between 0.5% and 2%) and similar between the case and control group\cite{124,125}. A small retrospective study including 44 patients with psoriasis treated with ustekinumab described cases of mild elevation of liver enzymes and no cases of severe DILI\cite{126}. Some case reports described spontaneous regression of liver injury after ustekinumab withdrawal\cite{127}.

**Small molecules**

Tofacitinib was approved for UC treatment in 2018. Liver enzymes elevation with a hepatocellular pattern has been rarely described\cite{128}. One case of possible AIH was reported, but liver injury due to other drugs could not be excluded\cite{129}. Monitoring liver enzymes periodically during tofacitinib treatment is recommended.

Ozanimod is a new molecule introduced for IBD treatment. Aspartate transaminase increases 32 wk after drug exposure were described in 2% and 1% of patients treated with 0.5 mg and 1 mg of ozanimod, respectively. Preliminary data suggest a low rate of hepatotoxicity associated with these new therapeutic approaches\cite{102}.

**PORTAL VEIN THROMBOSIS**

Portal vein thrombosis (PVT) is a common event in IBD. Indeed, IBD patients have a high risk of thromboembolism due to systemic inflammation and alterations in the concentrations of some coagulation factors, such as high factor V and VIII or low antithrombin III\cite{130}.

In a retrospective study, the incidence of thromboembolic events in patients with IBD rose from 5.65% in 2000 to 7.17% by 2009\cite{131}. In particular, the prevalence of PVT in IBD has been estimated to be about 0.17%\cite{132}. There are several causes of PVT, including inflammation, immobilization, major extent of colon disease, disease severity, surgery, use of corticosteroids and smoking. For that reason, the guidelines recommend starting heparin when facing an acute flare of UC, for PVT prophylaxis\cite{133}.

After the onset of PVT, complications such as portal hypertension, bleeding or even death are not common, but early anticoagulation is safe and associated with a better outcome, and the use of novel direct oral anticoagulants was associated with particularly favorable outcomes in this setting\cite{134}.

**CONCLUSIONS**

In conclusion, the scenario of liver involvement of IBD patients is quite extensive. The relationship between IBD and PSC is the most studied. PSC is a disease that currently has no effective medical therapy; therefore, research on drugs that may be effective for both hepatic and intestinal disorders is required. Moreover, the strategies for early neoplasia screening (both CCA and CCR) in these patients are not sufficiently efficient at present, and this is a pitfall that needs to be resolved.

NAFLD in IBD is another focal issue, as this novel comorbidity may complicate the management of IBD patients due to its multifaceted aspects.

As viral hepatitis may soon become a thing of the past, due to the advent of drugs with very high success rates, some patients will still require careful monitoring, especially when immunosuppression for IBD is required.

Among the drugs currently in use to treat IBD, thiopurines, mesalazine derivatives and methotrexate are the most studied, and periodic assessment of LFT is still required. However, the field of DILI is expected to expand quickly, as several novel molecules for the treatment of IBD (tyrosine kinase inhibitors, small molecules and others) have been developed, and their possible hepatotoxicity will be a matter of
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Chelation therapy in liver diseases of childhood: Current status and response

Jayendra Seetharaman, Moinak Sen Sarma

Abstract

Chelation is the mainstay of therapy in certain pediatric liver diseases. Copper and iron related disorders require chelation. Wilson’s disease (WD), one of the common causes of cirrhosis in children is treated primarily with copper chelating agents like D-penicillamine and trientine. D-Penicillamine though widely used due its high efficacy in hepatic WD is fraught with frequent adverse effects resulting discontinuation. Trientine, an alternative drug has comparable efficacy in hepatic WD but has lower frequency of adverse effects. The role of ammonium tetra-thiomolybdate is presently experimental in hepatic WD. Indian childhood cirrhosis is related to excessive copper ingestion, rarely seen in present era. D-Penicillamine is effective in the early part of this disease with reversal of clinical status. Iron chelators are commonly used in secondary hemochromatosis of liver in hemolytic anemias. There are strict chelation protocols during bone marrow transplant. The role of iron chelation in neonatal hemochromatosis is presently not in vogue due to its poor efficacy and availability of other modalities of therapy. Hereditary hemochromatosis is rare in children and the use of iron chelators in this condition is limited.

Key Words: Wilson’s disease; D-Penicillamine; Trientine; Indian childhood cirrhosis; Deferoxamine; Deferasirox; Hemochromatosis

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Core Tip: Chelation forms the most important part of management of certain liver diseases in children. In Wilson's disease and secondary hemochromatosis related to transfusion, chelation is well established treatment modality with proven efficacy. In other diseases like copper associated childhood cirrhosis and neonatal hemochromatosis the role of chelation is doubtful. In hereditary hemochromatosis, chelation is...
Chelation therapy in pediatric liver diseases: Current status and response

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INTRODUCTION

Chelation is a process in which a synthetic compound is administered to remove an excess mineral or heavy metal from the body. There are various liver diseases that are caused by excess deposition of various heavy metals such as copper, iron, and arsenic. Some of these are genetic-metabolic, others are due to environmental exposure. In the landmarks of chelation therapy in hepatology, Walshe documented cupriuresis after administering dimethyl cysteine (penicillamine) in Wilson’s disease (WD) in 1956[1]. Chelation was thereafter used in non-Wilsonian liver diseases. In the subsequent years newer chelators such as trientine and ammonium tetra thiomolybdate were identified for WD. From the 1970s, transfusion-related liver siderosis of hemolytic anemias was revolutionized by the use of deferoxamine[2]. The use of iron chelators was attempted in gestational alloimmune liver disease and hereditary hemochromatosis. This review explores the rationale and outcome of chelation therapy in various pediatric liver diseases.

MECHANISM OF CHELATION

Metal ion (M) complexes with chelating agent (L) through an equilibrium reaction to form metal-ligand complex (ML) or chelate. The concentration of the chelate in the solution is directly proportional to the concentration of metal ion [M] and the ligand [L]:

\[ M + L \rightleftharpoons ML \]

\[ [M][L] \propto [ML] \]

\[ [M][L]k = [ML] \]

Where k is the effective stability constant. Value k denotes the affinity of the chelating agent. High k values suggest high affinity of the chelating agent. The value of k depends on the nature of the chelating agent, temperature, pH of the solution[3]. The in-vitro milieu is not similar to the in-vitro chemical reaction. The presence of weak acids in the body fluids like glutamate, sulfate, citrate, amino acids, albumin, macroglobulin etc. affect the chelation. These are called biological ligands. Chelating agent binds to the biological ligands and the effective concentration in the body fluid is lowered. Hence the equation becomes:

\[ [M][L]k = [ML] \]

Where Mt, Lt is the total concentration of the metal ion and chelating agent respectively which is very difficult to assess in the clinical setting[4]. Effective chelation occurs when concentration of M and/or L is high, when affinity of the chelator (k) is high or when the concentration of the chelate [ML] is low. The metal ion concentration [M] in the body depends on the severity of the disease. For example, in a WD presenting as acute liver failure, serum copper (Cu) levels are usually very high. The concentration of chelating agent [L] is increased by increasing the dosing and/or frequency as tolerated by the patient. For the chelation to progress, urinary excretion of chelate [ML] is very important as it effectively reduces the concentration[3]. Ideal chelating agents must have good oral absorption, acceptable bioavailability, high affinity to metal ions, low toxicity at appropriate plasma concentration, undergo rapid elimination or detoxification after combining with metal ions and more...
Seetharaman J et al. Chelation in pediatric liver diseases

importantly should be available in affordable price[5].

**CHELATION IN WD**

WD is an autosomal recessive disorder caused by mutation of ATP7B gene that encodes for a protein P-type ATPase which transports copper into trans Golgi network and for biliary excretion of copper. In lysosomes, copper is incorporated into ceruloplasmin. In WD, due to defect in ATPase transport protein, ceruloplasmin formation is defective and biliary excretion of copper is impaired[6,7]. This causes excess accumulation of intracellular copper subsequently increasing the levels in blood causing accumulation in extra-hepatic organs (Figure 1).

**Chelating drugs**

D-Penicillamine (3, 3-dimethylcysteine) is the most commonly used medication for WD worldwide. The L-isomer of this drug is not advised for treatment due to its neurotoxicity. The chelation property of DPA is due to the presence of thiol (-SH), which is responsible for its high affinity towards divalent metal ions such as copper. The mechanism of action of D-Penicillamine (DPA) is by inducing cupriuresis, inducing hepatic metallothionine synthesis, reducing fibrosis (by preventing collagen formation). DPA also has an anti-inflammatory property[8]. It is rapidly absorbed in proximal intestine but only 40%-70% are absorbed[9]. The peak plasma concentration occurs after 1-3 h after ingestion. It circulates in the plasma predominantly by binding to albumin (80%), while the rest of the compound is present as free or disulphide forms. DPA is metabolized in the liver by conjugation with sulfide or by methylation (phase II reaction) and excreted in urine with almost 80% being eliminated within 10 h of ingestion. After discontinuation of therapy, the drug is eliminated in about 3-6 d [10]. Food, antacids, iron and zinc preparations reduce the bioavailability by almost 50%. Plasma concentration reduces significantly when the drug is taken with food[11]. It is recommended to give the drug either 1- hour before or 2- h after food. The drug is given in the dose of 20 mg/kg per day (up to 1500 mg) rounded to nearest 250 mg in 2-4 divided doses and can be maintained at 1000 mg/d once the disease is in remission[12]. As DPA causes pyridoxine deficiency, pyridoxine should be supplemented at 25-50 mg/d. In case of neurological WD, to prevent paradoxical neurological worsening, the drug is started at low dose (125-250 mg) and slowly increased (125-250 mg every week) to reach the desired dose by 4-6 wk[13].

Trientine (triethylenetetramine) is an alternative chelating agent in WD. It is a derivative of spermine and putrescine and binds to copper in the ratio 1:1 to form a stable complex, which is eliminated in the urine. Trientine dihydrochloride is the oral ingestible form requiring storage at 2-8 degree Celsius to maintain stability. 10% of the trientine is absorbed in the proximal small intestine and achieves its peak concentration 1.5-4 h after ingestion. Trientine is extensively metabolized in tissues by acetylation but the enzyme responsible for it is not identified. 1% of ingested trientine and 8% trientine metabolite acetyltrien, appears in the urine. Plasma concentration of the trientine significantly reduces when given with food due to its affinity to dietary copper in the lumen thereby compromising the removal of tissue copper and the other reason could be due to the physiological polyamines secreted during food intake inhibits effective trientine absorption[14]. Trientine is not to be given with iron as it forms toxic complexes. The dose recommended is 20 mg/kg per day with the maximum of 1500 mg/d rounded to nearest 250 mg (300 mg capsules in North America) and maintenance dose of 1000 mg/d. Similar to DPA, trientine also should be ingested 1 h before or 2 h after food intake[12,15]. The decoppering efficacy of any chelating agent is evident from the effective stability constant (k) which denotes copper affinity. The comparison of k-value of DPA (2.38 × 10⁻¹⁶) and trientine (1.74 × 10⁻¹⁸) suggests the decoppering efficacy of DPA is much higher than trientine[16].

**Efficacy of chelation**

Improvement in symptoms and biochemical parameters in WD takes around 2-6 mo in hepatic forms whereas in isolated neurological forms it may take up to 12-24 mo[12]. DPA in WD children shows an efficacy of almost 70%-90%[17-20]. The response depends on whether it is hepatic or neurological form and severity of the disease at presentation. Long term of follow up of WD (median duration: 15.1 years) studied by Bruha et al[19] showed the response to DPA to hepatic forms is 82% compared to 69% for neurological forms. One of the largest series of WD patients (n = 327) from Euro Wilson consortium, showed hepatic forms had 91% response compared to only 68% in
Due to mutation in ATP 7B gene, P type ATPase is defective and copper is not incorporated in ceruloplasmin. Free copper increases in blood and is deposited in liver and extrahepatic sites (brain, kidneys, bones, cornea, RBC).

In most series, trientine is used as a second line either due to poor response or due to toxicity to DPA. Hence, there are no head-to-head randomized trials comparing the efficacy of DPA and trientine. Overall efficacy of trientine is reported to be 80%-92% [21,22]. Retrospective analysis of efficacy of the two drugs by Hölscher et al [23] showed response in hepatic forms with DPA was 92% compared to 84% response with trientine after a median follow up duration of 13.3 years. In neurological forms, DPA fares significantly better (68%) than trientine (48%, \( P = 0.008 \)) [23]. In Euro Wilson consortium, the response of both the DPA and trientine were comparable when used as a first line in both hepatic (90.7% vs 92.6%, \( P = 0.98 \)) and neurological forms (67.5% vs 55%, \( P = 0.76 \)). However when used as a second line therapy, trientine vs DPA showed similar response in hepatic form (75% vs 68.9%, \( P = 0.76 \)) but better response in neurological form (51% vs 23.1%, \( P = 0.01 \)) [20].

**Adverse effects of copper chelators**

Adverse effects of DPA are always a major concern with up to 30% of the patients develop one or more adverse effects (Table 1) [20,24,25]. Adverse effect can be early onset (less than 3 wk of therapy) or late (more than 3 wk to up to 2-3 years of initiation of therapy). Early adverse effects like fever, rash, arthralgia, lymphadenopathy, pancytopenia are predominantly immune mediated [26]. Nephropathy, the most common late adverse effect of DPA is seen in 5%-30%. Presentations include proteinuria, glomerulonephritis, nephrotic syndrome less commonly as Good Pasture’s syndrome [27-29]. More than 90% of the nephropathy occurs within 12 mo of therapy. High doses of DPA, decompensated liver disease, intrinsic renal diseases or presence of HLA-B8/DR3 are probable risk factors of nephropathy [30]. Eighty percent are membranous glomerulonephritis on renal biopsy. In a study by Hall et al [27] of 33 patients with DPA nephropathy, one-third each showed resolution at 6, 12 and 18 mo respectively, after drug discontinuation. There are no clear recommendations as to whether the drug can be rechallenged after resolution of nephropathy. However, in such situations, it is prudent to continue the patient on an alternative drug such as trientine or zinc. DPA related myelotoxicity occur in up to 7% patients undergoing chelation with DPA [31-33]. Two types of myelotoxicity are known to occur, idiosyncratic (usually with in 1 year of therapy) or dose dependent (more than after 1 year therapy) [34]. Though, there are no definite guidelines for monitoring and treatment of myelotoxicity, European society of Pediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) suggests weekly blood counts initially, 1-3 mo till remission and 3-6 monthly thereafter [35]. If two or more values of total leukocyte count less than 3.5 \( \times 10^{9} \) per cubic mm, drug is to be discontinued. Bone marrow examination and reticulocyte counts differentiates this condition if concomitant hypersplenism is present [36,37]. Blood products, colony stimulating factor and anti-thymocyte globulin may improve the counts. Usual time of spontaneous recovery is 4-12 wk. Rarely hematopoietic stem cell transplantation may be required in refractory and prolonged cases. Once bone marrow toxicity has ensued, the drug should not be re-challenged. Adverse effects of DPA related to skin may be due to either acute hypersensitivity
Table 1: Adverse effects of copper chelating drugs

<table>
<thead>
<tr>
<th>Name of the drug</th>
<th>Side effects</th>
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<tbody>
<tr>
<td>D-Penicillamine</td>
<td>Early (1-3 wk): Fever, rash, arthralgia, cytopenia, proteinuria. Late: (1) Skin: degenerative dermatoses elastosis perforans serpingosa, cutis laxa, pseudoxanthoma elasticum, bullous dermatoses, psoriasiform dermatoses, lichen planus, seborrheic dermatitis alopecia, aphthous ulcers, hair loss; (2) Connective tissue disorders: Lupus like syndrome, arthralgia, Rheumatoid arthritis, polymyositis; (3) Renal: proteinuria, hematuria, glomerulonephritis, nephrotic syndrome, renal vasculitis, Goodpasture’s syndrome; (4) Nervous system: paradoxical neurological worsening, neuropsychopathies, myasthenia, hearing abnormalities, serous retinitis; (5) Gastrointestinal: Nausea, vomiting, diarrhea, elevated transaminases, cholestasis, hepatic siderosis; (6) Respiratory: pneumonitis, pulmonary fibrosis, pleural effusion; (7) Hematological: cytopenia, agranulocytosis, aplastic anemia, hemolytic anemia; and (8) Others: Immunoglobulin deficiency, breast enlargement, pyridoxine deficiency.</td>
</tr>
<tr>
<td>Trientine</td>
<td>Paradoxical neurological worsening (10%-50%), sideroblastic anemia, bone marrow suppression, gastritis, skin rash, arthralgia, myalgia, hirsutism.</td>
</tr>
<tr>
<td>Ammonium tetra thiomolybdate</td>
<td>Neurological dysfunction (rare), hepatotoxicity, bone marrow suppression.</td>
</tr>
</tbody>
</table>

reaction presenting as morbilliform rash, urticaria, degenerative dermatoses (cutis laxa or elastosis perforans serpingosa) or an autoimmune phenomenon (pemphigus, scleroderma or lichen planus). Rare muscular adverse effects of DPA include myasthenia (1%-2%) and ptosis. Anti-nicotinic acetyl choline receptor or Anti-MuSK (Anti-Muscle Specific tyrosine Kinase) is present in up to 70%. Systemic lupus erythematosus can occur within 6-12 mo after the onset of DPA therapy presenting as pleurisy, arthritis, rash with or without presence of anti-nuclear antibody. Deutscher et al noted 3 out of 50 WD children with elevated transaminases within 6 wk of DPA therapy who resolved subsequently following discontinuation. Trientine also present with similar adverse effects as DPA like nausea, vomiting, arthralgia, myalgia, leukopenia, elevation in anti-nuclear antibody (ANA), nephropathy but adverse effects requiring discontinuation of trientine is significantly lower compared to DPA.

In hepatic WD, paradoxical neurological worsening occurs commonly within 6 mo of therapy, in patients with an underlying overt or occult neurosympathetic feature. Paradoxical neurological worsening occurs even when dosing and compliance is good. It occurs due to the sudden release of Cu from the liver following chelation therapy causing oxidative brain injury. Overall incidence of paradoxical neurological worsening ranges from 7%-26%. Those with previous known neurological WD, the incidence of worsening is up to 75%. Both DPA and TA have shown to cause neurological worsening. In series from Euro Wilson consortium, paradoxical neurological worsening occurred significantly more with TA compared to DPA.

Litwin et al studied natural history of 143 WD (70 Neuro/Neurohepatic WD and 73 hepatic WD), of whom 23% neurological cohort and none of the hepatic cohort developed early neurological worsening on chelation. In this series, median time of onset of neurological worsening was 2.3 mo. Fifty-three percent were completely reversible and 13% were partially reversible on drug discontinuation with median time of reversibility of 9.2 mo. Prior neurological involvement, lesions in brain stem or thalamus and concomitant anti-dopaminergic drugs had higher chances of neurological worsening. Treatment consists of drug discontinuation and addition of zinc for a transition period. Chelators can be restarted in lower doses with gradual increment once the symptoms improve.

**Assessment of adequacy of chelation: Clinical parameters**

Currently there is no fool-proof, gold standard yardstick to assess chelation adequacy. All have fallacies in assessment and hence multiple parameters are considered. Chelation adequacy can be assessed firstly by assessing compliance to drug intake. Compliance is assessed by having a pill count, self-reporting by patients themselves or by checking empty blister packs during follow up outpatient visits. There are various scales being developed assessing medication adherence (MAQ: Medication adherence questionnaire, MARS: Medication adherence Rating scale) but none have been validated in children. More objective way of assessing compliance is by measuring drug levels but it is not routinely available under clinical setting. Secondly, follow up of clinical parameters assess the adequacy of chelation like improvement in jaundice, ascites, encephalopathy which usually take 2-6 mo post therapy. Resolution of neurological symptoms may take longer than 2-3 years. The resolution of Kayser-Fleischer ring on de-coppering therapy has considerable controversies to the
same. Studies have heterogeneity in their assessment and reports. It appears to be independent on type of presentation (neurologic vs hepatic), stage of disease (pre-symptomatic vs symptomatic) and choice of chelator and compliance. Initial reports showed, Kayser-Fleischer (KF) ring disappearance in 81% of the patients (completely in 41% and incompletely in 59%), more in pre-symptomatic stage (60%) than those in symptomatic phase with ongoing therapy (2%) over 22 years of follow-up on DPA (90%) and zinc or trientine (10%). Conversely one-third of asymptomatic patients the rings did not reabsorb even after therapy of > 10 years. In this study, the fading of KF rings seemed to be independent of the stage of the disease and effectiveness of the decopperizing treatment[45]. In a study by Fenu et al[46] where 66% were hepatic and 31% were neuro-hepatic (90% on DPA ± zinc therapy), partial or total KF ring resolution was observed in 28%, deterioration in 6% and static in the rest of the cohort over 1-3 years of therapy. Other smaller cohorts report reduction of KF ring in neuropsychiatric manifestation or disappearance over 10 years on maintenance zinc and molybdate therapy in pediatric hepatic WD[47,48]. KF rings may reappear with non-compliance, and occasionally even with successful maintenance therapy[49].

Liver status can be appropriately assessed by Pediatric end-stage liver disease or Child-Turcotte-Pugh score. Biochemical parameters like serum albumin, total bilirubin and prothrombin time normalize by 6 mo but liver enzymes might take longer[12]. In the author’s experience it takes 9-12 mo for complete normalization of Liver function tests in majority of the cases[50]. In patients who have additional neurological involvement, neurological response is monitored by indices such as Global assessment scale (GAS)[51]. Even with neurological WD with significant MRI changes, 50% show improvement with long term chelation[52].

**Assessment of adequacy of chelation: Biochemical parameters**

Presently the most widely acceptable way to assess adequacy of chelation is by 24-h urine copper and non-ceruloplasmin copper. Twenty-four hours urine copper (UCu) increases immediately following chelation and takes around 12-18 mo to reach a stable level[53]. European Association for the Study of the Liver (EASL) and American Association for the Study of Liver diseases (AALSD) recommends targeting 24-h urine copper between 200-500 mg/d for adequate chelation[12,13]. Values > 500 mg/d suggest under chelation as lot of unchelated copper remains in the body. Values < 200 mg/d may be either due to over chelation or poor compliance (Table 2). This can be differentiated by non-ceruloplasmin copper (NCC) levels calculated by the formula (serum copper (mg/L) - 0.3 x serum ceruloplasmin(mg/L))[54]. NCC has a few fallacies. Firstly, almost 20% of NCC are negative values, seen mostly when immunoassay method was used to measure ceruloplasmin as it measures both holoceruloplasmin and apoceruloplasmin. NCC calculation becomes inappropriate when inactive apoceruloplasmin is included. Secondly, there are variabilities in reference ranges in ceruloplasmin values between various laboratories across the world creating disparities in NCC cut-offs[55]. According to EASL guidelines, NCC > 15 mg/d/L suggest poor compliance and < 5 mg/d/L suggest over chelation. Additionally, 24-h urine copper after 48-h cessation of therapy has been recommended by EASL. Values > 100 mg/d is suggestive of under chelation or poor compliance while values < 100 mg/d suggest adequate treatment[15].

A novel and upcoming modality to assess chelation is the use of exchangeable copper. Exchangeable copper is the fraction of copper bound to albumin, peptide and amino acids which are easily chelated by chelating agents. It denotes a direct estimation of non-ceruloplasmin copper (NCC)[56]. On WD with chelation for long time, exchangeable copper values tend to reduce comparable to non-Wilson children. In a pilot study by the authors, the role of exchangeable copper was assessed in a cohort of 96 children with hepatic WD. Exchangeable copper was significantly higher in newly diagnosed WD compared to WD on chelation for more than 1 year (3 ± 7 μmol/L vs 0.9 ± 0.6 μmol/L, P = 0.03). Exchangeable copper values were lower in stable liver disease compared to unstable liver disease (0.86 ± 0.5mmol/L vs 1.3 ± 0.6 mmol/L, P = 0.01). Exchangeable copper values showed excellent correlation with non-ceruloplasmin copper (r = 0.92, P < 0.001). Predictive model incorporating exchangeable copper into standard monitoring tools improved the yield of disease control assessment by 21%[57].

**Comparison of single vs dual chelation: Which is better in hepatic WD?**

Strictly zinc is not considered as a systemic chelator. Oral zinc (Zn) induces metallothionein in enterocyte. Metallothioneine is an endogenous chelator that has high affinity to copper. Hence induced metallothionein combines with luminal Cu, preventing its entry into circulation. This Cu is removed through feces when enterocyte is shed. Zn
also induces hepatic metallothionine\[58\]. Hence, Zn is used in pre-symptomatic WD, stable well chelated WD on maintenance therapy, severe neurological WD. It is also used as a last resort in those with DPA or trientine intolerance. In severe hepatic disease, many centers consider giving a trial of dual chelation DPA and zinc for rapid chelation and quick stabilization. In a study conducted by the authors, 65 children with > 9 mo chelation were followed up for long term outcome. Majority had advanced disease at presentation. 83% of children were treated with DPA monotherapy and 17% treated with DPA and zinc combination. Trientine was started in 4 children due to DPA toxicity. 77% of children responded to DPA monotherapy even when the disease is severe at presentation and 50% responded when DPA and zinc combination was started. The overall response to oral chelation is 71%\[50\]. Hence, DPA should be the first line of therapy for any hepatic WD and zinc is added in those who failed to show optimal response with DPA in desperate circumstances with the hope of rapid synergistic chelation and quicker liver recuperation\[50\]. Though there are no comparative trials of dual or single chelation therapy, there are limited case series that have used DPA or trientine with zinc for WD presenting with ascites, coagulopathy and encephalopathy\[59-61\]. Though the efficacy of dual therapy in these studies were 91%-100%, sample sizes were small. Systematic review of 17 studies that assessed the efficacy of dual therapy (DPA/ Trientine with zinc) showed pooled efficacy rate (60.4%, 95%CI: 55.8-65.0) compared to DPA (73.7%, 95%CI: 65.1-85.4) and trientine monotherapy (82.6%, 95%CI: 75.4-89.5). Adverse effects following monotherapy is also lesser with either DPA or trientine compared to combination therapy\[62\]. Another retrospective study assessed 30 of 313 patients on dual chelator therapy, showed long term discontinuation and non-adherence was higher as compared to monotherapy (P = 0.006). Combination therapy, may fare better in neurological WD compared to exclusive hepatic forms\[63\]. Compliance and adequate spacing with chelating agent need careful consideration in the treatment schedule. If consumed together, chelator can combine with zinc in the lumen and effective absorption of both the medication gets reduced. Animal studies have shown that hepatic zinc stores is also significantly reduced during decoppering\[64\]. Hence, when chelator is combined with zinc, a proportion of chelator is used up in removing the body zinc thereby compromising the efficacy.

**Efficacy of ammonium tetra thiomolybdate**

Ammonium tetra thiomolybdate is a strong decoppering agent used in limited trials. It prevents intestinal absorption of copper if given with meals but also reduces serum copper when given in between meals. Ammonium tetra thiomolybdate (ATM) is predominantly advised for neurological forms due to it low risk of neurological worsening\[65\]. In the comparative study of ATM with trientine in neurological WD, paradoxical neurological worsening is significantly lower with ATM (4%) compared to trientine (26.1%, P = 0.01)\[66\]. At larger doses, ATM can form toxic insoluble complex that gets deposited in liver causing hepatotoxicity\[67\]. Hence the role of ATM in hepatic WD is precarious. Up to 10% of patients receiving ATM might develop bone marrow toxicity also\[68\]. Bis-choline tetra thiomolybdate (W101) is an investigational derivative of ATM being studied recently in neurological WD with better stability and lower toxicity\[69\]. Twenty-four weeks treatment of the drug caused improvement in 71% of neurological WD. Seven percent developed leukopenia and almost 39% developed elevated liver enzymes post therapy\[69\]. Robust experience in exclusive hepatic WD is not yet available.

**Table 2 Twenty-four hours urine copper and non-ceruloplasmin copper in various stages of Wilson’s disease treatment**

<table>
<thead>
<tr>
<th>Stages of treatment</th>
<th>UCu &gt; 500 μg/d</th>
<th>NCC &gt; 25 μg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early stages of treatment (&lt; 1 yr)</td>
<td>UCu &gt; 500 μg/d</td>
<td>NCC &gt; 25 μg/dL</td>
</tr>
<tr>
<td>Good control (treatment &gt; 1 yr)</td>
<td>UCu 200-500 μg/d</td>
<td>NCC &lt; 15 μg/dL</td>
</tr>
<tr>
<td>Poor compliance/uncontrolled disease</td>
<td>UCu &gt; 500 μg/d</td>
<td>NCC &gt; 15 μg/dL</td>
</tr>
<tr>
<td>Inadequate dose</td>
<td>UCu &lt; 200 μg/d</td>
<td>NCC &gt; 15 μg/dL</td>
</tr>
<tr>
<td>Over-treatment</td>
<td>UCu &lt; 200 μg/d</td>
<td>NCC &lt; 5 μg/dL</td>
</tr>
</tbody>
</table>

UCu: Twenty-four hours urinary copper; NCC: Non ceruloplasmin copper.
CHELATION IN INDIAN CHILDHOOD CIRRHOSIS

Indian childhood cirrhosis is commonly seen in children between 6 mo and 5 years of age in Indian subcontinent with its peak incidence seen during 1970-1990[70]. Presently this entity is seen to be waning in the Indian subcontinent. Predominant etiology advocated was excessive copper ingestion with use of copper utensils[71]. There was also a possibility of genetic predisposition affecting copper metabolism[70]. Clinical features consist of nonspecific symptoms to start with like fever, lethargy, easy fatigability, palpable liver with leafy edges in stage I, splenomegaly and ascites in stage II and jaundice, coagulopathy and encephalopathy in stage III. Histopathological examination of liver shows diffuse hepatocyte necrosis, presence of Mallory bodies and granular orcein staining. Treatment monitoring is by liver function tests (LFT), serum copper and in many studies, by repeat hepatic copper and liver histology, while on treatment. Mortality is almost 60% in stage II but reaching almost 90% in stage III[72]. In the study by Bavdekar et al[73] 65 children with Indian childhood cirrhosis (ICC) on treatment with DPA were followed up for the mean duration of 3.5 years, showed response in 60% of the children in pre-icteric phase compared to only 6% response (P < 0.01) in icteric phase (Table 3). Another study in ICC children who received DPA or DPA with steroids showed 50% survival as compared to10% in placebo group (P = 0.002)[74]. In a pediatric study, DPA therapy has showed better response compared to DPA with intravenous immunoglobulin (P = 0.018)[75]. Chelation may improve symptoms if given early as prognosis is poor in advanced disease despite treatment[75].

CHELATION IN NON-WILSONIAN COPPER RELATED DISORDERS

Non-Wilsonian copper related diseases termed by Baker et al[76] as copper associated childhood cirrhosis includes ICC from India and ICC-like illness from western countries. This ICC like illnesses is otherwise called idiopathic copper toxicity. Type I copper associated childhood cirrhosis (CACC) resembles ICC, with an early onset of disease and related to increased copper intake. Type II CACC has onset later than 4 years of age and possibly has an autosomal recessive inheritance without an obvious increase in copper intake[77]. Although there are few case reports of ICC-like illnesses, meagre number of reports use chelation therapy probably due to its conflicting results. One child from Bangladeshi origin, presented with jaundice, anorexia, weight loss at 7 years, with normal serum ceruloplasmin, and elevated hepatic copper 2319 mg/g. Improvement in symptoms and decrease in liver copper (35 mg/g) was noted after 19 mo of DPA therapy (Table 3)[77]. In contrast, a 10 year old Italian child with ascites and hepatomegaly, normal ceruloplasmin levels and liver copper of 1970 mg/g did not show any improvement clinically and biochemically even after 2 years of DPA[78]. Largest cohort of endemic Tyrolean infantile cirrhosis studied by Muller et al[79] showed both genetics and copper contamination were responsible for the disease. However there is paucity of chelation therapy experience in this condition.

IRON CHELATION IN GESTATIONAL ALLOIMMUNE LIVER DISEASES

In Gestational alloimmune liver disease alloimmunization of fetal liver antigen occurs in maternal blood resulting in IgG fetal liver antibody causing complement activation in fetal liver and significant impairment in hepcidin production (Figure 2)[80]. This causes iron storage in various organs like liver, heart, gonads, pancreas etc. Gestational alloimmune liver disease (GALD) causes liver failure as a result of hemochromatosis in newborn period and has high mortality if not intervened earlier. The liver injury causes reduced production of hepcidin resulting in uncontrolled iron absorption through placenta. This excess iron might further aggravate liver injury and also result in extra-hepatic iron deposition[81,82]. There have been few studies of GALD being treated with iron chelators (intravenous deferoxamine) and antioxidants with no clear-cut benefit. In the series by Flynn et al[83] five infants with neonatal hemochromatosis received intravenous deferoxamine but only one survived without liver transplantation. In the study by Rodrigues et al[84] 10 infants received iron chelation but only one survived without transplantation. In another series by Sigurdsson et al[85] six infants with neonatal hemochromatosis received supportive measures whereas eight infants received combination of deferoxamine and antioxidants. Two out of six who
<table>
<thead>
<tr>
<th>Ref.</th>
<th>Disease</th>
<th>Drug</th>
<th>Follow up duration</th>
<th>Response</th>
<th>Adverse effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dhawan et al [60]</td>
<td>WD</td>
<td>DPA (n = 32)</td>
<td>Median: 11.78 (1.45-34.2) yr</td>
<td>20/32 (62.5%)</td>
<td>Minor: 6.3%; Major: 21.9%</td>
</tr>
<tr>
<td>Wang et al [105]</td>
<td>WD</td>
<td>DPA/TA (n = 9)</td>
<td>Mean: 5.1 4.1 yr</td>
<td>All responded</td>
<td>Not mentioned</td>
</tr>
<tr>
<td>Das et al [50]</td>
<td>WD</td>
<td>DPA (n = 65), TA(n = 4)</td>
<td>Median: 3.6 (0.8-12) yr</td>
<td>DPA (42/65) 64.6%, TA (3/4) 75%</td>
<td>DPA 10.8%</td>
</tr>
<tr>
<td>Arnon et al [107]</td>
<td>WD</td>
<td>TA (n = 10)</td>
<td>Treatment duration: 18 mo. Follow up: 12-60 mo</td>
<td>All responded</td>
<td>1/10 (10%) reported hepatotoxicity</td>
</tr>
<tr>
<td>Taylor et al [108]</td>
<td>WD</td>
<td>TA (n = 16)</td>
<td>6.4 (0.78-18.6) yr</td>
<td>14/16 (87.5%)</td>
<td>1 had allergic reaction</td>
</tr>
<tr>
<td>Santos Silva et al [59]</td>
<td>WD All decompensated liver disease</td>
<td>DPA (n = 1)TA (n = 4)</td>
<td>18-60 mo</td>
<td>All responded one still had raised transaminase</td>
<td>3/4 (75%) on DPA developed cytopenia</td>
</tr>
<tr>
<td>Bavdekar et al [73]</td>
<td>ICC</td>
<td>DPA (n = 68)</td>
<td>3.5 (1-7) yr</td>
<td>29/68 (42.6%) alive after follow up</td>
<td>5 children had proteinuria</td>
</tr>
<tr>
<td>Tomnar et al [75]</td>
<td>ICC</td>
<td>DPA (n = 60)</td>
<td>12 mo duration</td>
<td>13/17 (76.5%) of grade III survived</td>
<td>11.8% drug rash, 5.9% liver</td>
</tr>
<tr>
<td>Tanner et al [74]</td>
<td>ICC (15 children treated with DPA in both trials together)</td>
<td>DPA (n = 15)</td>
<td>6 yr</td>
<td>Trial I: 1/15 (6.7%) survived in 6 yr, Trial II: 5/10 (50%) survived in 6 yr</td>
<td>Not mentioned</td>
</tr>
<tr>
<td>Horseelen et al [77]</td>
<td>Case report CACC (age 7 yr)</td>
<td>DPA</td>
<td>19 mo</td>
<td>Hepatic copper normalized</td>
<td>None</td>
</tr>
<tr>
<td>Maggiore et al [78]</td>
<td>Case report CACC (age 10 yr)</td>
<td>DPA</td>
<td>24 mo</td>
<td>No improvement</td>
<td>Not mentioned</td>
</tr>
<tr>
<td>Rodeck et al [109]</td>
<td>CACC (age 6 and 10 mo)</td>
<td>DPA</td>
<td>18 mo, other child deteriorated immediately following DPA initiation</td>
<td>One child improved and other developed acute liver failure requiring liver transplantation</td>
<td>None</td>
</tr>
<tr>
<td>Flynn et al [83]</td>
<td>NH</td>
<td>DFO (n = 5) with antioxidant</td>
<td>Follow up at 48 mo</td>
<td>2/5 (40%) survived without transplantation</td>
<td>Not mentioned</td>
</tr>
<tr>
<td>Rodrigues et al [84]</td>
<td>NH</td>
<td>DFO with antioxidant (n = 9)</td>
<td>Follow up at 3.9-8 yr</td>
<td>1/9 (11.1%) survived without transplantation</td>
<td>Not mentioned</td>
</tr>
<tr>
<td>Sigurrdsson et al [85]</td>
<td>NH</td>
<td>DFO with antioxidant (n = 8)</td>
<td>Not mentioned</td>
<td>None survived without transplantation</td>
<td>Not mentioned</td>
</tr>
<tr>
<td>Masera et al [110]</td>
<td>HJV hemochromatosis Case report (7/1)</td>
<td>DFX</td>
<td>12 mo of treatment</td>
<td>Iron indices improved on 12 mo treatment</td>
<td>Not mentioned</td>
</tr>
</tbody>
</table>


received supportive measures survived compared to only one who received chelation. It is not clear if the small proportion of response to chelation is due to efficacy of the drug in already advanced disease or due to natural history. In the recent years, it now clear that intravenous immunoglobulin has a superior role than chelation therapy in GALD.

**IRON CHELATION IN HEREDITARY HEMOCHROMATOSIS**

Hemochromatosis is due to iron accumulation in various organs with secondary causes being commoner in children than hereditary hemochromatosis. Secondary causes of hemochromatosis are commonly related to repeated transfusions in hemolytic anemia especially thalassemia major. In normal individuals, increased plasma iron induces the genes like HFE, TFR2 and HJV. This causes release in hepcidin, binding with ferroportin in enterocytes and macrophages, reducing iron absorption. Hereditary hemochromatosis (HH), most commonly due to mutation in
Figure 2 Pathogenesis of gestational alloimmune liver disease. Alloimmunization of fetal liver antigen by maternal blood produces IgG antibody passively transferred through the placenta to cause fetal liver injury by complement activation. Liver injury reduces the hepatic synthesis of hepcidin resulting in uncontrolled placental iron absorption. Excess iron is deposited in liver, pancreas, heart, gonads, etc.

HFE, cause impaired production of hepcidin making checkpoint for iron absorption defective[86]. Animal studies showed excessive fat intake causes impaired hepcidin production and increased transferrin receptor 1 and divalent metal transporter 1 Levels by altering mRNA expression. Hence, increased iron absorption and iron related liver injury may be responsible for development of non-alcoholic steatohepatitis[87]. Hereditary hemochromatosis (HH) is extremely rare in children. Excess iron in the serum causes liver cirrhosis, skin pigmentation, pancreatic insufficiency, cardiac dysfunction and hypothyroidism[88]. Iron chelation forms the mainstay of therapy in transfusion related siderosis in various hemolytic anemias in children. In a few studies, iron chelators have been implicated in treatment of HH also. Deferoxamine is parenteral iron chelator, given either as subcutaneous or intravenous infusion (20-50 mg/kg per day) over 8-24 h. Adverse effects seen are local reaction in injection site, hearing abnormalities, bone abnormalities etc. Deferasirox is an oral chelator with a similar efficacy as deferoxamine in removing hepatic iron but prone for its gastrointestinal side effects. Deferiprone, also an oral chelator is prone for gastrointestinal side effects and agranulocytosis and is highly effective in removing cardiac iron compared to other chelators (Table 4)[89]. Phatak et al[90] from Italy studied multiple doses of deferoxamine in HH, showed 10 mg/kg is the dose with optimal response and lower side effects. Nagler et al[91] analyzed 2 patients treated for 6 mo and 10 mo respectively who showed significant reduction in serum ferritin in the follow up. EASL and AASLD guidelines on HH recommend phlebotomy as the treatment of choice in HH[92,93]. Chelation may be considered in HH when phlebotomy is not tolerated due to severe congestive cardiac failure, anemia and in case of difficult venous access.

**IRON CHELATION IN SECONDARY HEMOCHROMATOSIS**

In children, secondary hemochromatosis is more common than HH and is usually caused by transfusion related iron overload seen in chronic hemolytic anemia especially beta thalassemia[94]. Each milliliter of packed RBC adds 1mg of iron to the body stores. Iron is usually bound to transferrin in plasma. However when the iron load increases, transferrin sites saturate and excess iron spills as labile plasma iron causing free radical injury to heart, liver and endocrine organs[95]. Multiple transfusion causes liver injury by various mechanisms such as siderosis causing hepatitis eventually progressing to fibrosis and cirrhosis. Hepatic foci of hemopoiesis and transfusion related hepatitis B and C infection are also seen[96].

Iron overload related liver injury can be assessed by various modalities. Serum ferritin is easily available and an inexpensive method to assess iron overload but its utility is limited in the presence of infection and inflammation. Liver iron concentration > 15 mg/g dry weight of liver is associated with significant mortality and morbidity[97]. The superconducting quantum interface device (SQUID) measures liver iron stores non-invasively but the SQUID scanners are not available in many centers.
Table 4 Properties of iron-chelators

<table>
<thead>
<tr>
<th>Properties</th>
<th>Deferoxamine (DFO)</th>
<th>Deferasirox (DFX)</th>
<th>Deferiprone (DFP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chelator: Iron ratio</td>
<td>1:1</td>
<td>2:1</td>
<td>3:1</td>
</tr>
<tr>
<td>Plasma $t_{1/2}$</td>
<td>30 min</td>
<td>12-16 h</td>
<td>2-3 h</td>
</tr>
<tr>
<td>Usual dose</td>
<td>20-50 mg/kg per day over 8-24 h</td>
<td>20-40 mg/kg per day once daily</td>
<td>75-100 mg/kg per day in 3 divided doses</td>
</tr>
<tr>
<td>Route of administration</td>
<td>Subcutaneous, intravenous</td>
<td>Oral</td>
<td>Oral</td>
</tr>
<tr>
<td>Clearance</td>
<td>Renal, hepatic</td>
<td>Hepatic</td>
<td>Renal</td>
</tr>
<tr>
<td>Efficacy in removing liver iron stores</td>
<td>Good</td>
<td>Good</td>
<td>Moderate</td>
</tr>
<tr>
<td>Efficacy in removing cardiac iron</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Good</td>
</tr>
<tr>
<td>Advantages</td>
<td>Long safety data available, strongest chelator on molar basis</td>
<td>Oral once daily dose is sufficient</td>
<td>Oral, effective in removing cardiac iron</td>
</tr>
<tr>
<td>Adverse effects</td>
<td>Local reactions</td>
<td>Gastric intolerance</td>
<td>Nausea</td>
</tr>
<tr>
<td></td>
<td>Sensorineural hearing loss</td>
<td>Rash</td>
<td>Vomiting</td>
</tr>
<tr>
<td></td>
<td>Bone abnormalities</td>
<td>Diarrhea</td>
<td>Diarrhea</td>
</tr>
<tr>
<td></td>
<td>Retinopathy</td>
<td>Elevation in creatinine</td>
<td>Arthralgia</td>
</tr>
<tr>
<td></td>
<td>Pulmonary disease</td>
<td>Elevation in transaminases</td>
<td>Elevated liver enzymes</td>
</tr>
<tr>
<td></td>
<td>Allergic reaction</td>
<td>Peptic ulcer</td>
<td>Agranulocytosis</td>
</tr>
<tr>
<td></td>
<td>Bacterial infections (e.g., Listeria, Klebsiella)</td>
<td>Renal dysfunction</td>
<td>Hepatic dysfunction</td>
</tr>
</tbody>
</table>

Magnetic resonance imaging estimates liver iron by R2 and R2* techniques and it correlates well with liver iron concentration attained from biopsy. Magnetic resonance imaging (MRI) has now become the primary monitoring tool for both liver and cardiac iron[99].

Liver injury due to iron overload was common in children in the pre-chelation era. Liver biopsies obtained in 80 children with beta thalassemia during splenectomy showed cirrhosis in 40% of children > 11 years with risk of cirrhosis increasing with age. 60% of the children showed hypoalbuminemia and 70% showed elevated transaminases[96]. Iron-chelators are well established treatment modality to prevent iron overload related liver injury. In a retrospective study by Maira et al[100] deferasirox for a duration of 4 ± 1.5 years showed significant improvement in liver stiffness measurement by transient elastography (7.4 ± 3.2 kPa vs 6.6 ± 3.2 kPa, $P = 0.017$) and liver iron concentration (LIC) (4.81 ± 3.82 mg/g vs 3.65 ± 3.45 mg/g, $P = 0.001$). Thus, iron chelation not only prevents progression of liver injury but also reverses inflammation and fibrosis. In the multicentric cross-sectional study from Italy, 924 beta-thalassemia patients were evaluated for iron overload assessment and management. The study showed serum ferritin had an excellent correlation with liver iron concentration. Deferasirox (38.3%) was most preferred chelator, especially in children because of its safety and easy administration[101]. Deferiprone was less commonly used when transaminases were elevated due to its concern of hepatic fibrosis[97]. Combination of two chelators were used whenever serum ferritin > 2500 ng/mL or MRI R2* values < 20 ms. Guidelines suggest that LIC assessment should be done at 1-2 yearly intervals[102]. Iron overload needs to be monitored and treated pre- and post-alloimmune hematopoietic stem cell transplantation (HSCT) for hemolytic anemia. Pre-transplant serum ferritin > 1000 ng/mL is associated with increased risk of post-transplant complications such as chronic liver disease, graft vs host disease (GVHD), sinusoidal obstruction syndrome and infection[103,104]. Hence it is mandatory to rapidly reduce ferritin levels before HSCT. Gruppo Italiano Trapianto di Midollo Osseo (GITMO) study group recommends switching to intravenous deferoxamine for rapid lowering of serum ferritin pre-transplant. From 6 mo post-transplant, iron overload is to be assessed by serum ferritin and MRI R2*. If LIC in MRI > 7 mg/g phlebotomy is preferred, but when LIC > 15 mg/g phlebotomy along with iron chelators are required to prevent complications[105].
CONCLUSION

Copper chelation by D-penicillamine and trientine forms the mainstay of treatment in childhood WD. Appropriate dosing, compliance to medications and scheduled monitoring with liver function tests, 24-h urine copper and non-ceruloplasmin copper are required for better control of the disease. D-penicillamine is a promising treatment for Indian childhood cirrhosis especially in early stages. The role in other non-Wilsonian copper diseases is doubtful. The use of iron chelator in Gestational alloimmune liver disease is waning due to its poor efficacy. Iron chelator may be considered as an alternative therapy in hereditary hemochromatosis when the primary treatment fails or not feasible but in case of secondary hemochromatosis chelation forms the main treatment.

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Hepatocellular carcinoma: Understanding molecular mechanisms for defining potential clinical modalities

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Abstract
Liver cancer is the sixth most commonly occurring cancer and costs millions of lives per year. The diagnosis of hepatocellular carcinoma (HCC) has relied on scanning techniques and serum-based markers such as α-fetoprotein. These measures have limitations due to their detection limits and asymptomatic conditions during the early stages, resulting in late-stage cancer diagnosis where targeted chemotherapy or systemic treatment with sorafenib is offered. However, the aid of conventional therapy for patients in the advanced stage of HCC has limited outcomes. Thus, it is essential to seek a new treatment strategy and improve the diagnostic techniques to manage the disease. Researchers have used the omics profile of HCC patients for sub-classification of tissues into different groups, which has helped us with prognosis. Despite these efforts, a promising target for treatment has not been identified. The hurdle in this situation is genetic and epigenetic variations in the tumor, leading to disparities in response to treatment. Understanding reversible epigenetic changes along with clinical traits help to define new markers for patient categorization and design personalized therapy. Many clinical trials of inhibitors of epigenetic modifiers (also known as epi-drugs) are in progress. Epi-drugs like azacytidine or belinostat are already approved for other cancer treatments. Furthermore, epigenetic changes have also been observed in drug-resistant HCC tumors. In such cases, combinatorial treatment of epi-drugs with systemic therapy or trans-arterial chemoembolization might re-sensitize resistant cells.

Key Words: Hepatocellular carcinoma; Diagnosis; Treatment; Epigenetics; Epi-drugs; Drug resistance
INTRODUCTION

Liver cancer ranks sixth in cancer incidence globally and accounts for 8.2% of total cancer deaths. The different categories of primary liver cancer are intrahepatic cholangiocarcinoma, hepatocellular carcinoma (HCC), fibrolamellar carcinoma, and hepatoblastoma. These categories have distinct changes in their molecular, histological, and pathological features. HCC alone accounts for 85%-90% of liver cancer cases[1]. Almost 2/3 of the population affected by HCC is found in east Asian and south-east Asian countries, making this disease endemic to the region[2]. Globally, 5-year median survival is below 20% for HCC[3]. Major risk factors for HCC include chronic infection with hepatitis B virus and hepatitis C virus, excessive consumption of alcohol, exposure to aflatoxin, physiological state such as non-alcoholic fatty liver disease, and diabetes[4]. According to the Barcelona Clinic Cancer Liver Classification (BCLC) algorithm, curative care for HCC involves tumor resection, ablation, and liver transplantation[5]. However, this mode of treatment is offered to patients diagnosed in an early stage of the disease. Current research suggests that only 20% of patients are diagnosed in the early stage[6]. The lacunae in diagnosis are the unavailability of promising liquid-based biomarkers and detection limits of scanning techniques. Palliative care involving chemo/radiation-based treatment is given to patients with intermediate and advanced stage disease. Following this, 70% of patients come back with a relapse of disease and suffer treatment side effects[7,8].

A new approach should be considered to identify diagnostic markers and achieve better therapy response to overcome disease management challenges. Recent advances in the omics field shed light on the pathogenesis and molecular classification of HCC[9-11]. The omics approach can help to investigate new markers to improve the therapeutic outcome. Liver carcinogenesis involves both genetic and epigenetic changes. It is impossible to target all genetic variations due to tumor heterogeneity, but gene signature can be manipulated as epigenetic changes are reversible[12]. Therefore, epi-drug-based treatment may act as an alternate treatment strategy instead of targeting a single protein or molecular pathway. Epi-drugs can be beneficial not only for the treatment of HCC but also for dealing with cancer resistance[13,14].

This article focuses on the existing approach for diagnosis and treatment in the management of HCC. We also review transcriptomic-based signatures of HCC for patient sub-categorization and their potential implications for diagnosis and therapy. Finally, we propose an epi-drug based treatment strategy based on the epigenetic landscape of HCC.

DIAGNOSIS OF LIVER CANCER

Five standard WHO-approved guidelines include the European Association for the Study of Liver Disease (EASLD)[15], American Association for the Study of Liver Diseases (AASLD)[16], Asia-Pacific Association Study of the Liver[17], EASL-EORTC Clinical Practice Guidelines[18], and the updated AASLD guidelines are used for diagnosis of liver cancer. The diagnosis is primarily based on imaging techniques such
as ultrasound, computed tomography (CT) scan, and conventional magnetic resonance imaging (MRI)[19]. Invasive biopsies are not helpful for the diagnosis of liver tumors. The myriad risk factors involved in biopsy are the local spread of HCC along the needle track and different complications observed in individual patients[20]. The early-stage diagnosis of HCC continues to be crucial due to reduced sensitivity and specificity of the diagnostic methods, due to which an ample number of tumors are undetected. The complete list of diagnostic methods with detection limits is shown in Table 1. The various factors responsible for undetectable tumors involve a lack of specific markers and an asymptomatic condition during the early stages of HCC[21]. Thus, the diagnosis of tumor occurs when it has spread and has reached an advanced stage.

The diagnostic marker used most frequently is serum α-fetoprotein (AFP)[22]. AFP level increases beyond 20 ng/mL in more than 70% of patients with HCC. However, AFP elevations are not explicitly associated with HCC as AFP levels from 10-500 ng/mL and even occasionally to 1000 ng/mL may be seen in patients with a high degree of necro-inflammatory activity such as chronic viral hepatitis[23]. Chan et al[24] in 2008 have shown that AFP could be better used as a prognostic marker to evaluate response to treatment and detection of recurrence instead of diagnosis[25]. Studies have shown that multiple combinations of markers provide more appropriate results in diagnosis than a single marker. A recent study investigated the use of HSP90α (heat shock protein 90) combined with AFP and thymidine kinase 1 to diagnose HCC with more efficiency[26]. A study from Beijing YouAn Hospital found that for early diagnosis of HBV-related HCC, a combination of AFP, GPC3, and GP73 had the highest diagnostic value[27]. Ghosh et al[28] have shown that the exosome encapsulated microRNAs could be used as a circulating diagnostic marker for HCC with low AFP levels.

Another marker, α-L-fucosidase (AFU), is expressed in liver cirrhosis patients[29]. However, limited research is available regarding the utility of AFU in the diagnosis of HCC. In the liver and gallbladder, cell membrane protein 5’-nucleotidase (5’-NT) is released into the blood during hepatic injury or obstruction[30]. It has been observed that 5’-NT levels also increase with age and during pregnancy[31]. Other markers such as AFP-L3, glypican-3, and des-γ-carboxy prothrombin also show inconsistent data due to low sensitivity and specificity. Hence, the discovery of putative liquid biomarkers is required, which can associate with tumor progression, recurrence, and effectiveness of therapeutic programs.

### TREATMENT REGIME AND LIMITATIONS OF CHEMOTHERAPY IN LIVER CANCER

Treatment of HCC is decided based on different stages of tumor detection[32]. The BCLC algorithm is widely used for treatment as it considers tumor stage, liver function, performance status, and treatment impact (Figure 1). Early-stage cases are treated with surgery, ablation, or liver transplantation. The patients undergoing surgery showed 70% recurrence within five years[33]. The currently used methods for tumor ablation in HCC are percutaneous ethanol injection (PEI) and radiofrequency ablation (RFA). PEI consists of the direct injection of absolute ethanol into HCC nodules[34]. RFA is responsive in tumors > 4 cm in size. It involves necrosis of the tumor using a needle tip electrode that reaches temperatures up to 100°C[35]. Microwave ablation and irreversible electroperation have shown more promising results than tumor removal with PEI[36].

Patients with an intermediate stage having a tumor size greater than 5 cm or multinodular HCC with no vascular invasion are treated with trans-arterial chemoembolization (TACE). TACE is used to obstruct the nutrient supply to the tumor using the occlusion of arterial blood vessels[37]. Chemotherapeutic drugs such as doxorubicin or cisplatin are given during embolization, allowing prolonged exposure of the drug to tumor cells, resulting in tumor reduction. Yeo et al[38] showed that the overall response rate for doxorubicin-treated patients was 10.5%. Moreover, doxorubicin alone and combined with PIAF had no significant difference in response rate but showed treatment-associated toxicity in patients. Another study showed that combinatorial treatment of fluorouracil, leucovorin, and oxaliplatin failed to improve survival compared to doxorubicin[39]. In a multicohort study involving patients with unresectable tumors treated with TACE, overall survival (OS) was approximately 26-40 mo, with only 52% of patients achieving treatment benefits[40,41]. In some cases, selective internal radiation therapy is used in patients with intermediate-stage HCC.
Table 1 Utility and detection limits of existing diagnostic measures of hepatocellular carcinoma

<table>
<thead>
<tr>
<th>Diagnostic methods</th>
<th>Definition/concept</th>
<th>Diagnostic limit/range</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contrast-enhanced ultrasound</td>
<td>Inexpensive, non-invasive, first choice for screening HCC; Real time dynamic of blood supply.</td>
<td>Small HCC less than 1 cm</td>
<td>[101]</td>
</tr>
<tr>
<td>Multi phasic enhanced computed tomography</td>
<td>3 dimensional reconstructions, high sensitivity 3D; Diffusion weighted imaging-functional imaging</td>
<td>1-2 cm HCC lesion</td>
<td>[102]</td>
</tr>
<tr>
<td>Magnetic resonance imaging</td>
<td>High resolution anatomic details, pre-contrast and multi-phasic enhanced 3D; Diffusion weighted imaging-functional imaging</td>
<td>2-3 cm HCC lesion</td>
<td>[103]</td>
</tr>
<tr>
<td>Positron emission tomography</td>
<td>Hepatocyte-specific PET tracer, 2-[18F] fluoro-2-deoxy-D-galactose, is used which accumulates in the liver compared with other tissues</td>
<td>Detection of small intrahepatic; HCC lesions</td>
<td>[104]</td>
</tr>
<tr>
<td>AFP</td>
<td>Elevated in HCC, non-specific</td>
<td>Range: &gt; 500 ng/mL</td>
<td>[23]</td>
</tr>
<tr>
<td>α-L-fucosidase</td>
<td>Expressed in liver cirrhosis</td>
<td>Cut-off: 870 nmol/L</td>
<td>[105]</td>
</tr>
<tr>
<td>Des-γ-carboxy prothrombin</td>
<td>Sensitive; Not expressed in other liver disease</td>
<td>Cut-off: 40 mAU/mL</td>
<td>[105, 106]</td>
</tr>
<tr>
<td>HSP90α + AFP + TKI</td>
<td>Combination of markers have improved diagnostic value</td>
<td>HSP90- (76.65-144.00); AFP- (5.33-2000.00); TK1- (0.57-2.30)</td>
<td>[26]</td>
</tr>
<tr>
<td>AFP, GPC3, and GF73</td>
<td>Useful markers for early diagnosis and prognosis</td>
<td>Upregulated</td>
<td>[27, 107]</td>
</tr>
<tr>
<td>microRNA: miR-21, miR-199, and miR-122, miR-23a</td>
<td>Specific for diagnosis of HCC; Extremely sensitive</td>
<td>Cut-off value of ≥ 210</td>
<td>[108, 109]</td>
</tr>
</tbody>
</table>

HCC: Hepatocellular carcinoma; AFP: α-fetoprotein.

Figure 1 Treatment modalities for hepatocellular carcinoma based on tumor-node-metastasis staging. HCC: Hepatocellular carcinoma.

Intraarterial infusion of radioisotope labeled microspheres is carried out in this modality. Another radiation-based technique known as stereotactic body radiation is used for patients with > 3 cm of the tumor.

Systemic chemotherapy is given for advanced stages of HCC. NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines) have recommended sorafenib and lenvatinib as first-line systemic therapy for patients with unresectable HCC[42]. Brivanib, sunitinib, erlotinib, and regorafenib are other preferred drugs for late-stage HCC treatment. Kudo et al[43] observed that treatment with lenvatinib results in significantly higher OS than sorafenib and improvement in all secondary efficacy endpoints. This trial further results in FDA approval of lenvatinib as the first line of therapy for HCC[43]. Sorafenib and sunitinib are protein kinase inhibitors targeting VEGFR, PDGFR, and the Raf kinase pathway. However, a study suggested that sunitinib had an adverse effect in these patients and had no advantage over sorafenib[44]. Moreover, sorafenib has been extensively explored in the systemic treatment of
advanced stage HCC and combination with TACE, but it provided contradictory results.[45,46] Brivanib is an inhibitor of FGFR1 and VEGFR2. Phase II clinical trials of brivanib showed the ineffectiveness of the drug compared to sorafenib for improving OS.[47,48] The EGFR inhibitor erlotinib or cetuximab was administered in phase II clinical trials of advanced stages of HCC. However, the trial results did not show the anti-tumor effect of cetuximab in HCC patients[49]. Interestingly, erlotinib showed a positive response in treatment by increasing OS to 13 mo and a response rate of 59% [50].

As discussed earlier, ablation treatment is possible in less than 40% of patients due to late diagnosis, and only 20% are treated with TACE. For the patients with advanced stages of HCC, treatment modalities are limited to systemic therapy, and response rates are also significantly less due to resistance towards available chemotherapy. Multimodal treatment involving more than one therapeutic drug has also failed in different combinations due to cytotoxicity and poor trial outcomes. Despite the significant research in targeted therapy of HCC management, a promising drug is yet to be identified. Thus, the hunt for combinatorial treatment with different therapeutic agents continues (Figure 2).

MOLECULAR LANDSCAPE OF LIVER TUMOR TISSUE FOR PATIENT STRATIFICATION AND IDENTIFICATION OF ALTERNATE TARGETS

Over the past years, HCC classification has mainly focused on histological analysis of tumor tissues. However, the molecular profile and clinical attributes have a significant impact on the prognosis of the disease, thereby redefining HCC into several subgroups. Boyault et al[51] published molecular classification systems for HCC composed of 6 groups. The groups were based on mutation profile, disease prognosis, and transcription landscape. The first group included patients with hepatitis B infection and low viral load, increased AFP levels, and high IGF2 expression, whereas the second group included patients with a high viral titer and associated microvascular invasion (MVI) and satellitosis. However, the difference in groups 3 and 4 was based on histological parameters. The third group consisted of poorly differentiated tumors with the worst prognosis; on the other hand, group 4 had well-differentiated tumors. Group 5 and 6 had a low proliferation rate and activated Wnt-signaling pathway. Moreover, pathways are differentially activated in different groups. Another group classified HCC into three subtypes based on histology and expression analysis of the tumor[52]. In this study, the first group showed the presence of satellitosis and MVI. Group 2 had high AFP expression, and the third group consisted of well-differentiated tumors with a low proliferation rate.

Tumor morphology-based classification has been proposed by Murakata et al[53]. The nodal status of the tumor was correlated with survival and recurrence of the disease. Moreover, the miRNA profile of HCC patients has been used to classify sorafenib responders[54]. c-myc signaling and EB-1 protein were functionally linked with HCC[55]. Similar findings were observed by Lee et al[56] in progenitor-like HCC, which correlated with poor prognosis. In another study, HCC progenitor-like signature consisting of CK-19, Ep-CAM, and CD133 was seen by Woo et al[57]. Morofuji et al[58] identified the gene signature of early recurrent HCC, including ERK1, PKG, ApaF1, and Bcl-X. Furthermore, ERK1 and Bcl-X were identified as genes associated with the poor prognosis of HCC[58]. However, these studies did not consider the survival status of an individual while proposing subtypes.

Jiang et al[59] showed that heterogeneity exists in proteomic profiling of paired early-stage HCC patients. The tumors were segregated into three subtypes: S-I, S-II, and S-III. S-I tumors had increased expression of liver-associated functional proteins. In contrast, S-II and S-III had a more proliferative nature due to overexpression of cell-cycle-related proteins. Furthermore, S-III were more aggressive and had a high expression of KRT19 and MMP9, associated with poor prognosis. Gao et al[60] sub-grouped 159 HBV infected patients based on survival, tumor thrombus, and omics profile. These sub-groups were classified based on metabolic rewiring, alterations in the microenvironment, and cellular proliferation. Moreover, the study proposed two prognostic markers PYCR2 and ADHIA.

In the past decade, data generated under the TCGA consortium can be used to understand the gene expression profile of patients and obtain correlations with clinical attributes[9]. Machine learning algorithms are necessary to analyze such multivariate data. The molecular alterations obtained from the cancer genome atlas liver hepatocellular carcinoma (TCGA-LIHC) cohort (423 patients) can be explored to predict new
Figure 2 Challenges in the treatment of hepatocellular carcinoma.

targets and rationally the combinatorial therapy. Transcriptome data generated from TCGA-LIHC identified over 13000 differentially expressed genes compared to cut-margin samples, and around 3330 genes correlated with poor survival ($P$ value < 0.05). Furthermore, 1730 genes overlapped between the DE gene list and genes correlated with patient survival. The majority of overlapped genes showed more than 30% alteration compared to adjacent normal in this cohort and had a significant association with OS. Patients were categorized into different groups using clustering analysis of gene expression. It was observed that these genes belong to metabolism-related pathways and the cellular proliferation-related family (Figure 3). Deep learning computational framework on the TCGA-LIHC dataset suggested that aggressive subtype has TP53 inactivation with high expression of KRT10, EPCAM, and active AKT, WNT signaling [61]. Furthermore, drugs and small molecular compounds are available to target these genes. Schulze et al [62] reported that potential gene targets have FDA-approved drugs in 28% of liver tumors. Therefore, these genes can be used for prognosis of the disease, and targeting them may improve patient survival.

Gene expression analysis of liver cancer samples can also be utilized to identify new markers for diagnostic purposes. For example, SPP2 is downregulated at the transcript level in HCC. This gene is deregulated in multiple HCC cohorts. Moreover, a stage-wise decrease at the transcript level was observed in HCC TCGA data. Also, the downregulation of SPP2 leads to a significant decrease in patient survival (Figure 4). This observation indicates that SPP2 level is associated with normal liver function, and a change in levels can be a measure of liver carcinogenesis.

**EPI-DRUG BASED TREATMENT FOR IMPROVEMENT OF THERAPEUTIC OUTCOME**

The lack of success in disease management can be explained by the multifactorial nature of carcinogenesis involving multiple mutations and global level epigenome alterations [63-65]. Epigenetic changes being reversible can be useful to understand the relationship between tumor biology and help in redefining therapeutic response [12]. Epigenetics deals with changes in gene expression without change in the DNA sequences [66]. Despite all cells having the same DNA sequence, the epigenome decides cell fate regarding differentiation, cell proliferation, and cell death [67,68]. The widely studied epigenetic marks are DNA methylation, histone post-translational modifications, and non-coding RNAs. DNA methylation is the most characterized heritable epigenetic mark. This is where a methyl group is transferred onto the cytosine of the CpG di-nucleotide-rich region in DNA by DNMT enzymes [69]. DNA methylation plays a vital role in gene inactivation, genomic imprinting, attaining
Figure 3 The cancer genome atlas liver hepatocellular carcinoma data analysis. A: Volcano plot representing differential gene expression between 373 tumor samples and 50 normal samples. Genes colored with red or green are most significantly altered; B: Venn diagram showing overlap between differentially expressed gene list and genes affecting survival of patients upon alteration (survival); C: Normalized expression of top 300 genes associated with overall survival represented using heatmap. Patients with overall survival below the median are marked with a red bar while those above the median are marked with a green bar; D: Altered biological process from overlap gene. Upregulated processes highlighted with red and downregulated processes are depicted as blue; E: Pathways analysis for overlap genes. Deregulated KEGG pathways shown by yellow bars and reactome pathways displayed using green bars. DE: Differentially expressed gene list.

tissue-specific gene expression, and X chromosome inactivation [69].

Similar to DNA modification, histone proteins also undergo post-translational modifications carried out by chromatin modifiers, namely writers, readers, and erasers [70]. The well-studied modifications include methylation, acetylation, phosphorylation, and ubiquitination. Histone methylation involves the addition of a methyl group at the lysine or arginine residue on the protruding histone tails. Histone methylation marks can result in repression of transcription or gene activation [71]. A typical example of gene suppression is trimethylation at H3K9, and H3K27 whereas methylation at H3K4, H3K36, and H3K79 enhance transcriptional activity [71]. Histone acetylation is the transfer of an acetyl group from acetyl CoA. This reaction leads to a change in electrostatic interaction between DNA and histones, resulting in the unwinding of chromatin and enhances gene transcription [72]. Histone phosphorylation has an essential role in DNA damage repair, gene transcription, and
chromatin condensation during mitosis\cite{72}. An illustration of chromatin-associated modifications and the role of epigenetic modifiers is shown in Figure 5. Non-coding RNAs are the transcribed intragenic regions of the DNA that are not translated into proteins. These entities govern gene silencing via RISC and RNA-induced transcriptional silencing complex formation\cite{73}.

Different research groups have extensively studied the epigenetic landscape of liver carcinogenesis. Moreover, in the past few years, researchers are investigating the epigenetic basis of chemoresistance in HCC. Lie \textit{et al}\cite{74} showed that lysine-specific demethylase 1 (LSD1) is upregulated in LGR5+ cells contributing to stemness and chemoresistance properties. Mechanistically, LSD1 removes the H3K4 methylation mark from the promoter of genes which inhibit Wnt-signaling. Thus, promoting pathway activation, which is essential for stemness and chemoresistance\cite{74}. EpCAM+ liver cancer cells have high expression of chromodomain helicase DNA binding protein (CHD4), a DNA damage response protein. The abundance of CHD4 in liver cancer cells leads to epirubicin resistance\cite{75}. Zinc-fingers and homeoboxes 2 (ZHX2) is one of the signature proteins which is downregulated in liver CSCs and is associated with tumor progression. It has been found that low expression of ZHX2 is correlated with epigenetic regulation of OCT4, SOX4, and NANOG by H3K36 methylation\cite{76}. Oriana Lo Re \textit{et al}\cite{77} observed that low expression of MacroH2A1 leads to paracrine mediated chemoresistance and imparts CSCs properties to the tumor cells. Another study showed that the regulator of chromosome condensation 2 promotes metastasis and cisplatin resistance in HCC\cite{78}. Ling \textit{et al}\cite{79} discovered that USP22 helps to attain chemoresistance by hypoxia-driven p53 mutant tumors. Hypoxia-induced expression of carbonyl reductase 1 leading to chemoresistance in HCC was observed by Tak \textit{et al}\cite{80}. H19 long non-coding (Inc)RNA has been shown to sensitize sorafenib or doxorubicin-resistant liver cancer cells\cite{81}. The IncRNA CRNDE has been shown to interact with histone methyltransferase to enhance their effect on the inhibition of tumor suppressors and induce resistance in tumor cells\cite{82}.

Epigenetic alterations can be targeted by the class of small-molecule inhibitors that specifically inhibit or reverse the changes\cite{83}. This class of inhibitors are referred to as epi-drugs. Different research groups have synthesized epi-drugs for all three prominent families of epigenetic modifiers- readers, writers, and erasers. Many epi-drugs have cleared pre-clinical trials, and initial phase trials have shown promising results. Few epi-drugs are clinically approved for the treatment of hematological malignancies. In some studies, treatment of solid tumors with an epi-drug helps in sensitizing tumor cells to chemotherapy\cite{84,85}. These findings have promoted the research on inhibitors of HDAC, HAT, and DNMTs in combination with chemotherapeutic drugs. In HCC and gastric cancer, the inactive or suppressed state of tumor suppressor genes (TSGs) is mainly attributed to the overexpression of DNMTs and HDACs, leading to heterochromatinization. Reversion of the chromatin state using epi-drugs further leads to activation of TSGs and prevents tumor growth\cite{86}. Ongoing
Figure 5 Schematic illustration of epigenetic modifications observed in hepatocellular carcinoma and chromatin modifiers targeted by epi-drugs. The figure represents general epigenetic alterations observed in hepatocellular carcinoma and different epigenetic modifiers that can be targeted via small molecule inhibitors. Moreover, DNA and chromatin mediated alterations observed in tumors are highlighted. Changes in DNA methylation and histone post-translational modifications levels inside normal cells lead to tumor formation. HCC: Hepatocellular carcinoma; HDM: Histone demethylase; HDAC: Histone deacetylase; HMT: Histone methyltransferase; DNMT: DNA methyltransferase; HAT: Histone acetyltransferase.

pre-clinical trials have been carried out with HDAC and DNMT inhibitors in combination or in comparison with each other to study the anti-tumor effects of the drugs. Guadecitabine (SGI-110), a DNMT inhibitor with sorafenib and oxaliplatin, is in phase II clinical trials for HCC (NCT01752933). Multicenter phase I/II clinical trials using belinostat (HDAC inhibitor) in patients with unresectable HCC showed a tumor stabilization effect\[87\]. One study showed that the combination of panobinostat and sorafenib significantly decreased tumor volume by inducing apoptosis in the tumor \[88\]. A group of researchers observed that the DNMT inhibitor 5-aza-2′ deoxycytidine and HDAC inhibitor SAHA down-regulated DNMT1, DNMT3a, DNMT3b, and HDAC1 and upregulated GSTP1 and SOCS1 gene expression, which further resulted in inhibition of cell viability and induced apoptosis\[89\]. A detailed list of potential epi-drugs is given in Table 2. These findings indicate the ability of epi-drugs, which can restructure the treatment strategy for HCC.

Future perspectives
The most effective way of controlling HCC is preventing the disease by spreading knowledge of etiological agents and hepatitis B vaccination. An increase in surveillance is one of the strategies to achieve better survival. This practice helps in the early diagnosis of HCC, monitors progression-free survival, and improves quality of life. Diagnosis of HCC at an early stage is crucial in order to start treatment at the right time and improve patient survival. Due to the reduced sensitivity of current diagnostic techniques, ultrasound scanning of high-risk individuals should be carried out every three months. Although ultrasound is cost-effective compared to MRI and CT scans, there is scope for developing more advanced MRI or CT versions to detect small lesions in the liver. Similarly, there is a need for an appropriate combination of liquid biomarkers used for the investigation of liver carcinogenesis. From a treatment perspective, upon early diagnosis, liver transplantation is preferred over surgical removal or ablation as it has less than 15% chance of recurrence\[90\].

The primary cause of treatment failure in cancer is resistance to available chemotherapy, which results in relapse. From heterogeneous tumors, cells respond to treatment differently, and a rare small percentage of cells found in the quiescent G0 state of the cell cycle can escape treatment. These cells are inherently resistant to
## Table 2 List of Food and Drug Administration approved/under trial epi-drugs

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Classification</th>
<th>Approved year</th>
<th>Indicated disease</th>
<th>Reference/ clinical trial number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azacytidine</td>
<td>DNMT inhibitor</td>
<td>2004</td>
<td>MDS</td>
<td>NCT01186939</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2009</td>
<td>AML</td>
<td>NCT00897068</td>
</tr>
<tr>
<td>Deoxycytidine</td>
<td>DNMT inhibitor</td>
<td>2006</td>
<td>MDS</td>
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<td>2017</td>
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chemotherapy and involved in relapse. Studies have shown that tumor cells maintain the drug-tolerant state via chromatin-mediated changes after drug treatment\[13\]. The drug-tolerant persistor (DTP) stage is reversible; however, prolonged exposure to chemotherapeutic drugs results in stable drug resistance properties\[91-93\]. DTP cells have non-random differential gene expressions, implicating chromatin-mediated changes leading to hetero-chromatinization of the transposable elements such as LINE1\[94\]. Recent findings suggest that ablation of the DTP cell population with FDA-approved epi-drugs impedes the development of resistance and relapse\[13,94\]. Hangauer et al\[95\] have shown DTP cells dependence on mesenchymal state and GPX4 (lipid hydroperoxide) for survival. Furthermore, inhibition of GPX4 triggers cell death of DTP cells via the ferroptosis pathway, indicating ferroptosis is required for the survival of DTP cells\[95\]. Thus, targeting inherently resistant residual cells could be helpful in reducing relapse in patients. However, more research on the identification and characterization of DTP cells is required to choose the appropriate drug combination for treatment purposes.

Targeted drug delivery is the critical factor in improving treatment outcomes and reducing the drug’s side effects. Currently, researchers are investigating nanoparticle-mediated drug delivery. In addition, modified liposomal formulation showed a successful therapeutic response in HCC due to tumor-directed delivery and low drug load in the system\[96\]. Albumin is also a suitable drug-carrier molecule. An albumin-tagged drug has more potent effects compared to the drug alone\[97\]. Other materials such as dendrimers, micelles, polysaccharides, and silica are also used as carrier molecules\[98-100\]. Still, the hunt for an effective delivery system continues for targeted delivery.

### CONCLUSION

Existing diagnostic methods are inadequate for the early detection of HCC. Similarly, implemented treatment modalities are unsuccessful in improving the survival of patients and result in cytotoxicity in normal cells. The use of credible biomarkers in the prognosis of HCC is essential to reduce mortality due to the disease. In the future, clinicians should focus on patient stratification based on molecular signatures and decide the treatment strategy to achieve maximum therapy outcome. The development of a combinatorial regime consisting of epi-drugs is urgently needed to treat the tumor mass.

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Heterogeneity of non-alcoholic fatty liver disease: Implications for clinical practice and research activity

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is a heterogeneous condition with a wide spectrum of clinical presentations and natural history and disease severity. There is also substantial inter-individual variation and variable response to a different therapy. This heterogeneity of NAFLD is in turn influenced by various factors primarily demographic/dietary factors, metabolic status, gut microbiome, genetic predisposition together with epigenetic factors. The differential impact of these factors over a variable period of time influences the clinical phenotype and natural history. Failure to address heterogeneity partly explains the sub-optimal response to current and emerging therapies for fatty liver disease. Consequently, leading experts across the globe have recently suggested a change in nomenclature of NAFLD to metabolic-associated fatty liver disease (MAFLD) which can better reflect current knowledge of heterogeneity and does not exclude concomitant factors for fatty liver disease (e.g. alcohol, viral hepatitis, etc.). Precise identification of disease phenotypes is likely to facilitate clinical trial recruitment and expedite translational research for the development of novel and effective therapies for NAFLD/MAFLD.

Key Words: Non-alcoholic fatty liver disease; Metabolic-associated fatty liver disease; Heterogeneity; Phenotypes; nomenclature; Clinical trial; Effective therapies

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HETEROGENEITY OF NAFLD: NEED FOR A NEW TERMINOLOGY

NAFLD represents an umbrella term with considerable heterogeneity among its subtypes. This is evidenced by variable disease severity and progression (disease phenotype) among patients with NAFLD[4]. The disease phenotype in NAFLD is in turn influenced by primary drivers of the disease and dynamic interaction between various disease modifiers (age, sex, ethnicity, co-existing disease, diet, alcohol consumption, smoking, hormonal status, genetic and epigenetic factors, gut microbiota, and metabolic risk factors)[2]. Although steatosis is highly prevalent, progression to steatohepatitis or other liver-related complications like cirrhosis and hepatocellular carcinoma (HCC) is highly unpredictable. The rate of fibrosis progression can also vary widely among patients. Moreover, there is growing evidence that the heterogeneity of NAFLD and principal driving factors between individuals. This becomes gradually more obvious that this term is inherently complicated, chiefly due to the presence of multiple pathophysiological pathways intrinsic to its progression suggest that the nomenclature should be revised and NAFLD may be classified in a way that takes into account the various underlying processes[3]. However, a change of name of any disease has considerable implications for both clinical practice as well as public health policy. Based on these evolving paradigms, this review will explore the factors contributing to NAFLD heterogeneity and its clinical and therapeutic implications. Besides, proposed changes in the current nomenclature and definition of NAFLD are discussed along with future perspectives.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is increasing in both developed and developing countries, in parallel with the global obesity epidemic. Nevertheless, much is still unknown on the NAFLD phenotype. Moreover, since the term NAFLD was coined by Ludwig et al[1] in 1980, the nomenclature and diagnostic criteria have not been revisited. With a deeper understanding of the natural history of NAFLD, it has become gradually more obvious that this term is inherently complicated, chiefly due to the heterogeneity of NAFLD and principal driving factors between individuals. This heterogeneity in clinical presentation and the course of NAFLD is probably influenced by several factors which include age, gender, ethnicity, diet, alcohol consumption, genetic predisposition, microbiota, and metabolic milieu[2]. The combined effect of the dynamic and complex systems-level interactions of these drivers is probably reflected in the phenotypic manifestations of NAFLD. Therefore, comprehensive phenotyping will translate into individual-level risk prediction and preventive strategies, and improvements in the design of clinical trials[2]. The heterogeneity of NAFLD and the presence of multiple pathophysiological pathways intrinsic to its progression suggest that the nomenclature should be revised and NAFLD may be classified in a way that takes into account the various underlying processes[3]. However, a change of name of any disease has considerable implications for both clinical practice as well as public health policy. Based on these evolving paradigms, this review will explore the factors contributing to NAFLD heterogeneity and its clinical and therapeutic implications. Besides, proposed changes in the current nomenclature and definition of NAFLD are discussed along with future perspectives.

Core Tip: It is being increasingly recognized that non-alcoholic fatty liver disease (NAFLD) is a heterogeneous condition with wide variability in clinical presentation and natural history. This heterogeneity is driven by genetic predisposition, metabolic factors, gut microbiota, diet and demographic factors. The suboptimal response to current pharmacotherapy in NAFLD highlights the failure to recognize this heterogeneity. Experts believe that updating NAFLD nomenclature is the first step towards this. Identification of disease subtypes can help development of preclinical model evaluating novel targets. This would in turn help clinical trial design by comparing and pooling results and thus improve disease outcomes.

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1980 by Ludwig et al[1] and it was used to describe fatty liver disease without a history of significant alcohol intake. Although the prevalence of NAFLD has grown to epidemic proportions involving one-fourth of the population, the nomenclature and the diagnostic criteria have not been reevaluated[2]. The term NAFLD does not consider the heterogeneity of the disease and hence does not reflect current knowledge.

Based on recent epidemiological studies, it has been increasingly recognized that there is no cut-off for safe drinking in so-called NAFLD as there is frequent coexistence of at-risk drinking and dysmetabolism[7]. Moreover, accurate assessment of alcohol intake is often challenging especially in subpopulations like children and women due to cultural interdiction[8]. To further confuse the issue, there is evidence that an altered gut microbiome can lead to excess production of endogenous alcohol in non-drinkers[9]. Hence, the dichotomy between alcoholic liver disease and NAFLD should be abandoned. Until now, diagnosis of NAFLD was based on the exclusion of excess alcohol intake, concomitant viral hepatitis/other liver diseases, and secondary cause of fatty liver (e.g. drug-induced). With the increasing prevalence of NAFLD and the high prevalence of other liver diseases such as viral hepatitis particularly in countries like Middle East and north Africa, dual causes of liver disease should be considered[8]. The current definition of metabolic-associated fatty liver disease (MAFLD) does not require the exclusion of the above, considering the co-existence of different pathology for fatty liver disease (Figure 1). However, it requires the presence of overweight/obesity, type 2 diabetes mellitus (T2DM), or 2 metabolic risk factors. The term “non” in “nonalcoholic fatty liver disease” trivializes a disease that has major hepatic, cardiovascular (CV), and oncological sequelae[2,10]. Due to the “non”-rubric, it could be misinterpreted as something not serious and even encourage alcohol consumption. The term “alcohol” makes the nomenclature derogatory and thus stigmatizing the condition blaming the patient for their condition[2]. This has profound implications on recognition of the disease as a major public health problem and resource allocation by regulatory authorities to intercept this potentially deadly disease.

Due to the aforementioned reasons, the term MAFLD was proposed by Lonardo and Carulli 16 years back[11]. However, NAFLD nomenclature remained unchanged until now. For the same reasons, Polyzos and Mantzoros[12] have proposed the term dysmetabolism associated fatty liver disease (DAFLD). Recently two consensus guidelines have proposed a change in the nomenclature of NAFLD to MAFLD and have redefined the condition based on the presence of hepatic steatosis and metabolic risk factors[2,13] (Figure 2). The impact of such change was reflected in the identification of patients with hepatic steatosis with a higher risk of disease progression in a cross-sectional study of more than 13000 patients based on data from the third National Health and Nutrition Examination Surveys of the United States[14]. Another study from Hong Kong has shown that MAFLD definition reduces the incidence of fatty liver disease by 25% [more so in patients with low body mass index (BMI)], while the prevalence remains unchanged. Patients with a fatty liver disease not fulfilling the criteria of MAFLD were unlikely to have significant liver disease.

However, the future implications of change in the nomenclature are still unknown. Hence, Younossi et al[15], on behalf of the American Association for the Study of Liver Disease[15] have cautioned about the impact of premature change in terminology to MAFLD. While there are still existing challenges in widespread disease awareness, identification of treatment endpoints, and biomarkers for risk stratification, changing terminology may negatively impact the field[15]. Moreover, international consensus involving all scientific societies, regulatory bodies, pharmacological industry, and patient organizations is required before a change in terminology. No matter what is the terminology for fatty liver disease, it is clear that it is a heterogeneous disease with varying manifestations.

**NAFLD AND CARDIOVASCULAR RISK**

Patients with NAFLD are more likely to have morbidity and mortality from cardiovascular disease (CVD). Currently proposed term MAFLD is closely linked to DM, dyslipidemia, hypertension, systemic inflammation which are known to increase CVD risk. A higher risk of CVD and CVD associated events have been noted in epidemiological and observational studies in NAFLD[16,17]. NAFLD not only damages the coronary arteries (atherosclerosis and ischemic heart disease), but also the other cardiac structures like myocardium (heart failure), cardiac valves (aortic stenosis,
Hepatic steatosis based on imaging /histology/biomarkers

Overweight or obesity (BMI > 25 kg/m² in Caucasians or BMI > 23 kg/m² in Asians)

or

Type 2 diabetes mellitus

With or without additional metabolic risk factors

MAFLD as compared to NAFLD

Does not require exclusion of

Excessive alcohol consumption

Viral hepatitis or other liver diseases

Secondary causes of fatty liver (e.g. Tamoxifen/Methotrexate)

Lean or normal weight (BMI < 25 kg/m² in Caucasians or BMI < 23 kg/m² in Asians)

Presence of at least 2 metabolic risk factors

Figure 1 Proposed diagnostic criteria of metabolic associated fatty liver disease and key differences with non-alcoholic fatty liver disease definition. *Metabolic risk factors include (1) Waist circumference ≥ 102/88 cm in Caucasian men and women (≥ 90/80 cm for Asian men and women); (2) Blood pressure ≥ 130/85 mmHg or on drug treatment; (3) Triglyceride levels ≥ 150 mg/dL (≥ 1.70 mmol/L) or on drug treatment; (4) Plasma high density lipoprotein [HDL < 40 mg/dL (< 1.0 mmol/L) for men and < 50 mg/dL (< 1.3 mmol/L) for women or on drug treatment; (5) Pre-diabetes [i.e., fasting glucose levels 100 to 125 mg/dL (5.6 to 6.9 mmol/L), or 2-h post-load glucose levels 140 to 199 mg/dL (7.8 to 11.0 mmol/L) or HbA1c 5.7% to 6.4% (39 to 47 mmol/moL)]; (6) Homeostasis model assessment of insulin resistance score ≥ 2.5; and (7) Plasma high-sensitivity C-reactive protein level > 2 mg/L. BMI: Body mass index; MAFLD: Metabolic-associated fatty liver disease; NAFLD: Non-alcoholic fatty liver disease.

mitral annular calcification), and conduction system (atrial fibrillation, conduction defects)[18]. CV disease in NAFLD can be subclinical (coronary and courted atherosclerosis) or clinical (myocardial infarction, stroke). Pathophysiological factors include dyslipidemia, oxidative stress, systemic inflammation, endothelial dysfunction, and a pro-thrombotic state leading to structural and functional cardiac changes including arterial stiffness, atherogenic plaque formation, and coronary calcification[19]. Among genetic factors related to NAFLD, MBOAT7 may promote venous thromboembolism whereas Transmembrane 6 superfamily 2 (TM6SF2) appears to be protective and PNPLA3 seems not to be associated with the risk of CVD. Other pathogenetic mechanisms of NAFLD such as environmental factors (diet, obesity, etc.), gut microbiota (through the gut liver axis and altered intestinal permeability), and epigenetic alterations also influence the CV risk[16].

Lifestyle modification and weight loss help in primary and secondary prevention of CVD in NAFLD. Aspirin and statins may be considered for primary and secondary prevention in individuals with NAFLD who are at high risk of CVD. Newer anti-diabetic medications such as SGLT2 inhibitors and GLP-1 receptor agonists are known to reduce CV events in T2DM and may be useful in this regard. Additional data are required on CV risk modification by farnesoid X receptor (FXR) agonists such as obeticholic acid. Future studies will likely address the predictive factors responsible for elevated CVD risk in NAFLD as there is a lack of targeted pharmacological therapy. Hence, CV endpoints should be included in clinical trials in NAFLD/MAFLD [16,19].
Figure 2 Key drivers of metabolic-associated fatty liver disease, resulting in disease heterogeneity and its clinical implications. Genetic predisposition, metabolic health, and environmental factors influence molecular and phenotypical heterogeneity of metabolic-associated fatty liver disease leading to various disease subtypes, variable disease progression, and response to therapy. MAFLD: Metabolic-associated fatty liver disease; NAFLD: Non-alcoholic fatty liver disease.

FACTORS FOR HETEROGENEITY

Age
The prevalence, risk of hepatic/extra-hepatic complications, and all-cause mortality of NAFLD increase with age. This is due to multiple factors like reduction in hepatic blood flow/volume, decrease in bile acid synthesis, altered cholesterol metabolism, increase in oxidative respiration due to decrease in mitochondria numbers, cellular aging, increased exposure to disease drivers over a prolonged period, and progressive increase in insulin resistance (IR) due to change in body composition (sarcopenia, abdominal and visceral adiposity with ectopic fat deposition)[20-23].

Gender and menopause effect
The prevalence of NAFLD and degree of hepatic fibrosis are lower in pre-menopausal women compared to men and postmenopausal women with better overall survival rates in the former[24]. Changes in body fat distribution (abdominal obesity after menopause), differences in metabolic risk factors, sexual dimorphism of key metabolic pathways (lipid metabolism, insulin signaling, and inflammation), and differences in hepatic gene expression of various metabolic pathways (e.g. FXR, liver X receptor) are likely mechanisms for the difference[25-27]. The prevalence of NAFLD and fibrosis risk is lower in postmenopausal women on hormone replacement therapy (HRT) compared to those who are not on HRT[28]. The extent of hepatic fibrosis increases with the prolonged duration of estrogen deficiency in postmenopausal women[29]. Hence, risk stratification in NAFLD should be based on gender and menopausal status.

Ethnicity
The prevalence of NAFLD and risk of nonalcoholic steatohepatitis (NASH) are seen in
Among the multiple variant genes associated with NAFLD identified on genome-wide genetics and epigenetics profiling may thus help in predicting the risk of progression, cardio-metabolic, and treatment outcomes. Increasingly various metabolomic signatures leading to hepatic steatosis are being recognized based on RNA-sequencing analysis study of NASH and HCC is increased. Increasingly various metabolomic signatures leading to hepatic steatosis are being recognized. In type 2/PLNPLA3 NAFLD (with rs738409 risk allele), there is increased intra-hepatic lipogenesis and impaired lipolysis leading to accumulated liver fat is composed of monounsaturated triacylglycerols and free fatty acids enriched with ceramides. In type 2/PLNPLA3 NAFLD (with rs738409 risk allele), there is increased intra-hepatic lipogenesis and impaired lipolysis leading to hepatic pro-inflammatory cells and stellate cells leading to inflammation and fibrosis which is in turn sensed by hepatic Toll-like receptors which induce activation of hepatic pro-inflammatory cells and stellate cells leading to inflammation and fibrosis progression. Apart from dietary factors, genetic makeup and ethnicity influence gut microbiome composition.

Metabolic health

Obese vs lean NASH: Although intra-hepatic fat content is closely influenced by obesity, 45% of the obese are said to be metabolically healthy as they don’t have any components of metabolic syndrome (MetS). It is not clear whether these individuals have a lower risk of CV complications compared to normal-weight, metabolically healthy individuals. On the other hand, 30% of normal-weight individuals have MetS and higher cardiometabolic risk. This is because the distribution and nature of fat are more important than the amount of fat in predicting metabolic risk. Visceral fat is associated with higher metabolic risk compared to peripheral and subcutaneous fat. Fat distribution is influenced by ethnicity (higher visceral adiposity in Asians) and genetic makeup. 5%-45% of NAFLD (20% among Europeans) are also lean NAFLD as defined by the presence of hepatic steatosis with normal BMI in the absence of significant alcohol intake. Lean NAFLD has distinct genetic predisposition, metabolic and microbial profiles. Increased prevalence of TM6SF2 risk allele, increased bile acids/Farnesoid receptor activity due to intact metabolic adaptation, and gut microbial profile which facilitates liver fat generation have been seen in lean NAFLD. Individuals with lean NAFLD have a better metabolic profile compared to their obese counterparts. The data on the natural history of disease progression in lean NAFLD have shown variable outcomes. Distinct pathways of liver fat accumulation are being recognized. In type 1/metabolic NAFLD, calorie excess due to dietary intake and physical inactivity leads to increased hepatic fatty acid supply by peripheral lipolysis and hepatic lipogenesis. This is associated with IR and other components of MetS thus leading to increased cardiometabolic risk. The accumulated liver fat is composed of monounsaturated triacylglycerols and free fatty acids enriched with ceramides. In type 2/PLNPLA3 NAFLD (with rs738409 risk allele), there is increased intra-hepatic lipogenesis and impaired lipolysis leading to steatosis. The fat composition is predominantly polyunsaturated triacylglycerols. This is not associated with IR and adverse cardiometabolic outcomes although the risk of NASH and HCC is increased. Increasingly various metabolomic signatures leading to hepatic steatosis are being recognized based on RNA-sequencing analysis study. Identification of the key pathway for hepatic steatosis by genetic and molecular profiling may thus help in predicting the risk of progression, cardio-metabolic, and treatment outcomes.

Genetics and epigenetics

Among the multiple variant genes associated with NAFLD identified on genome-wide
Pal P et al. Nonalcoholic fatty liver disease heterogeneity

association studies, few common variants (PNPLA3, TM6SF2, GCKR, MBOAT7, HSD17B13) are worth mentioning which have divergent metabolic effects[49]. PNPLA3 and TM6SF2 variants increase the risk of NAFLD and advanced fibrosis[50, 51]. PLPLA3, TM6SF2, and GCKR variants are associated with T2DM[52]. MBOAT7 and HSD17B13 variants do not affect serum lipid or glucose levels and do not increase cardiometabolic risk[53,54]. These variants explain only a minority of NAFLD. That is why it is important to consider the effect of other variants, gene-environment interactions (described with the PNPLA3 gene), and epigenetics. Epigenetic alterations of key regulators of metabolic, inflammatory, and fibrotic pathways represent a bridge between variant genes and the environment in NAFLD. Micro-RNAs such as miRNA-122, miRNA-192, and miRNA-34a are unregulated in NAFLD[55], miRNA-34A also correlates with disease activity. The role of long non-coding RNAs (incRNAs) in NAFLD is limited requiring further elucidation[56]. Reversible alteration of methylation signatures of key regulatory pathways is seen in NAFLD which reverses following weight reduction surgery[57]. Methylation signatures can help identify patients with advanced fibrosis [e.g. hyper-methylation of peroxisome proliferator-activated receptor gamma (PPARγ)][58]. Epigenetic alterations can alter the expression of PNPLA3 explaining the gene-environment link[59]. There is increasing evidence that maternal high fat diet leads to epigenetic alterations in fetal liver and increasing the possibility of NAFLD in adolescence in the offspring[60,61]. Higher maternal BMI is associated with hypermethylation of the PPARγ coactivator 1 (PGC1) gene which regulates energy metabolism in the newborn[62].

**Familial risk**

Twin studies, prospective and retrospective family studies have shown heritable factors in hepatic steatosis and fibrosis. In a prospective study, the risk of advanced fibrosis in first-degree relatives of patients with NAFLD-cirrhosis was 18% which is significantly higher than the general population risk[63,64]. Hence family history also should be considered while doing risk stratification of NAFLD patients.

**Alcohol intake**

The effect of alcohol use in fatty liver disease has a dose-dependent response which synergistically increases in the presence of metabolic risk factors[65]. This is contrary to the earlier belief that alcohol consumption has a “J” shaped effect on fatty liver disease progression with a beneficial effect on light to moderate use and deleterious effect on excessive use[66]. Hence, it is being increasingly revealed that there is no safe cutoff of alcohol consumption in fatty liver disease.

**CLINICAL IMPLICATIONS OF NAFLD HETEROGENEITY**

**NAFLD sub-classification**

The heterogeneity in NAFLD due to its multifactorial etiology, pathophysiological diversity, genetic polymorphisms, and on the other side, the ultimate unifying fate of steatosis and its progression, made NAFLD more like an umbrella disease with multiple subtypes. The proposed change of nomenclature as MAFLD, will not truly represent the full spectrum of the disease pathophysiology and thus this overgeneralized new nomenclature has been criticized. Singh et al[33] had proposed the ‘MEGA-D’ classification representing the ‘Mega-diversity’ of the NAFLD. They had proposed five sub-types of the disease, each representing a major pathophysiological hypothesis behind each subtype. The subtypes are as follows: M-Metabolic syndrome, E-Environmental stressor, G-Genetic Factor, A-Bile Acid dysregulation, and D-Gut dysbiosis related NAFLD. Moreover, it is also suggested to consider fatty liver disease as an umbrella term to include the whole spectrum of cryptogenic to classic to alcohol-associated fatty liver disease. Till any consensus-driven widely accepted terminology and sub-classification of NAFLD comes into place, it is prudent to consider fatty liver disease as common outcome pathology with different etiological triggers.

Alteration of lipid metabolism is one of the major pathophysiological factors behind the development and progression of NAFLD. Lipidomics based sub-classification of patients with NAFLD had been proposed which depends upon the signature patterns of alteration in the fatty acid homeostasis pathway[67]. ‘M-subtype’ is characterized by increased hepatic fatty acid uptake and reduced hepatic glutathione and S-adenosine methionine (SAM) content. On the other hand, the ‘non-M subtype’ occurs due to increased de novo hepatic lipogenesis and is characterized by normal hepatic SAM levels. Gut microbiota composition-based sub-classification of NAFLD had also been
proposed. However, till now no studies had been able to reveal any signature gut microbiota profile suitable for phenotypical classification of NAFLD patients.

Automated algorithm-driven cluster sub-classification, based on demographic factors (age, gender, ethnicity), clinical and laboratory findings, had been evaluated in a cohort of 13,290 NAFLD patients in the United States. The whole cohort had been divided into 5 subtypes and evaluated for disease outcomes including survival rates. In subtype 1, there were mostly female Hispanics with mild metabolic comorbidities with minimal fibrosis, but on the other hand subtype 2 had mostly patients with MetS with signs of developing liver dysfunction. Subtype 3 was a mostly young and healthy population with mild disease and minimal abnormalities. Subtype 4 patients were predominantly elderly male Caucasians who had more severe disease at baseline with features of fibrosis and also showed features of progression to cirrhosis stage. Subtype 5 patients were the oldest with more severe cirrhosis and associated with significant co-morbidities. Among the disease outcome, subtype 5 was at the highest risk mortality and subtype 4 had the highest risk of cirrhosis and HCC. Although this type of cluster-based subtyping of the disease needs to be validated clinically it can help to identify relevant disease subtypes in future studies.

In a gene expression study by Hoang et al., the disease progression score of individual genes had been evaluated and it showed a strong correlation with histological manifestations of disease severity. In this study, the authors proposed NAS (gene-level NAFLD activity score) and gene-level fibrosis stage (gFib) scores. These score-based subtypes of NAFLD not only can assess the risk of disease progression but also can predict the response to therapy. This molecular-based cluster classification either can be the forerunner of different clinical subtypes of NAFLD or can represent different phases of a dynamic spectrum of the disease.

Though genetic, clinical cluster, and pathophysiological based sub-classification of NAFLD had been proposed as discussed above, none of them are universally accepted. Moreover, detailed literature is mainly limited to disease phenotypes depending upon demographic factors, obesity, and clinical outcomes.

Inter-individual variation
Demography (Asian vs Western countries): The prevalence of NAFLD is now showing an increasing trend in Asian countries. A meta-analysis done in 2016 showed a higher prevalence in Asia (27.4%) than North America (24%) or European Union (23.7%). In a recent meta-analysis, the prevalence in Asia was found to have increased further (29.62%) and a secular trend of the rising prevalence in the last few decades had been reported. The increase in prevalence in Asia is likely due to an increase in obesity, sedentary lifestyle, changing westernized eating habits, and various socio-economic factors. The prevalence in the rural area was significantly lower than in the urban areas, suggesting the detrimental effect of urbanization on obesity and the consequent NAFLD. In both Asian and western countries, the prevalence increases with age. Prevalence is higher in males as well as among elderly women indicating protective effects of estrogen in females in the reproductive age group. Apart from the increased prevalence of metabolically unhealthy obesity and excessive visceral obesity, alteration of gut microbiota and bile acid profiles has also been postulated as possible contributing factors behind the development of steatosis. Among the genetic factors, PNPLA3 polymorphism (rs738409) had been strongly associated with hepatic steatosis in both western and eastern studies. However, a higher prevalence of PNPLA3 risk allele had been reported in Asia than in African or European countries. Genetic polymorphisms of other genes like TM6SF2, HSD17B13, GCKR, AGTR1, TM6SF2, and GCKR genes had also been linked with increased susceptibility of NAFLD in Asian subjects. Sarcopenia and hypovitaminosis D also was associated with NAFLD development. One of the major differences in Asian countries from their western counterpart is the increased prevalence of lean NAFLD (discussed later) in the former. Though the overall prevalence of NAFLD is almost similar in eastern and western countries, however, the rate of complications is still lesser in Asian countries. In a retrospective study from Japan with a median follow-up of 5.8 years, only 0.25% of patients developed HCC with an annual incidence of 0.043%. In contrast to western countries, NAFLD still contributes only to a minor proportion of liver-related complications requiring liver transplantation in Asia. In a Japanese nationwide survey, only 2.1% of patients with cirrhosis had NASH and almost two-thirds of the patients had viral hepatitis. The indolent course of NAFLD in Asian countries is likely due to relatively short disease duration in the majority of the patients in this part of the world. As there is a considerable lag in economic growth and consequent obesity epidemic in Asian countries, the rise in NAFLD and its complications are likely to follow the western trend in the coming years.
years. Moreover, the relatively higher chance of co-existence of viral hepatitis and NAFLD in Asian countries increases the risk of hepatic complications further.

**Ethnicity:** Irrespective of ethnic variability, a trend of overall increased prevalence of NAFLD had been seen globally. In the world, Middle East had the highest prevalence of NAFLD, and in Africa; it is the lowest. Studies from the United States reported that Hispanics had shown the highest risk of NAFLD and on the other hand, the risk is much less in the Alaskan Native. Among Asians, the prevalence is highest among Indonesians and lowest in Japanese. Interestingly, people of South Asian origin who are living in the United Kingdom, also showed higher risk. In a recent meta-analysis, which evaluated ethnic heterogeneity of NAFLD in the United States, both higher overall prevalence of NAFLD and risk of progression to NASH had been reported in Hispanics and the risks were lowest among Blacks. Although there was no significant difference in patients with fibrosis among different ethnicities. The reasons behind the ethnic variation are multifactorial. A significantly high risk of NAFLD among American Japanese than the native Japanese suggests the impact of socio-economic development and differences in lifestyles in the pathogenesis. With the advent of Genome Wise Association studies, the role of predisposing polymorphisms of other candidate genes like TM6SF2 and GCKR gene had been explored further. The rs58542926 variants of the TM6SF2 gene were significantly associated with intra-hepatic fat (triglyceride) accumulation in White and African-American but not among Hispanic individuals. Different polymorphisms in the AGTR1 gene were protective among Indians but not in Chinese and Malay subjects. Recently, polygenic gene scores had been developed to evaluate the cumulative effects of multiple candidate genes in the development and progression of NAFLD. Further studies are needed in the future to explore the complex interaction of different genetic polymorphisms which can explain disease heterogeneity across different ethnic populations.

**Age (Children and adolescents):** With the increasing prevalence of pediatric obesity, the prevalence of NAFLD in children and adolescents is ever rising. The pooled prevalence of pediatric NAFLD in general population and obesity clinic were 7.6% (95% CI: 5.5%-10.3%) and 34.2% (95% CI: 27.8%-41.2%) respectively. The factors which can influence the intrauterine metabolic milieu of the developing fetus, like maternal obesity and diabetes, had been postulated to increase the future risk of NAFLD. Increased consumption of fructose-rich beverages, processed food, saturated fat along with decreased intake of dietary fibers (westernized dietary habits) had been strongly associated with the development of NAFLD in children. On the other hand, breastfeeding was protective against the development of NAFLD. The genes which had been shown to increase the risk of pediatric NAFLD are similar to the adults. Genetic variants of PNPLA3 (rs738409), TM6SF2 (rs58542926), and GCKR gene had been shown to increase the susceptibility of development of NAFLD in pediatric patients. Though histological diagnosis of NAFLD remains ideal, diagnosis by imaging (ultrasound/MRI) is the most practical one in the pediatric population. As the prevalence of obesity in children is ever-increasing, the chance of co-existence of other secondary causes of hepatic steatosis should also be carefully evaluated before confirming the diagnosis of NAFLD. Histological pattern in pediatric NAFLD (periportal distribution-Type 2 NASH) differs from that of their adult counter-part (pericentral distribution-Type 1 NASH). Both fibrosis and steatosis are mainly present in the periportal region in type 2 NASH and are seen more in younger children. Moreover, the classical ‘ballooning’ change is also seen less frequently in children. On the other hand, type 1 NASH of the adult pattern can be seen in the older adolescent.
There is a paucity of longitudinal studies evaluating the natural history of pediatric NAFLD. Around 10%-25% of patients had advanced fibrosis and almost half of the patients had NASH at the time of diagnosis[97]. Though the incidence of HCC in the pediatric age group is extremely rare, a large number of pediatric patients with NAFLD are at increased risk of developing HCC in early adulthood. Weight loss and lifestyle changes were effective in the reversal of steatosis in pediatric patients[98].

**BMI (lean/non-obese NAFLD):** Lean and non-obese NAFLD is defined as NAFLD in a person with BMI < 25 kg/m² (< 23 for Asian subjects) and < 30 kg/m² ( < 25 for Asian subjects) respectively. In a meta-analysis that included 93 studies from 24 countries, the prevalence of lean and non-obese NAFLD in the general population was reported as 5.1% and 12.1% respectively[99]. Globally, the prevalence of non-obese NAFLD among the whole NAFLD group was 40% and in countries like India, it is as high as 47%, indicating that a large proportion of fatty liver disease is now developing in the non-obese population. Though non-obese NAFLD initially was more common in Asian countries, now almost similar prevalence of NAFLD is being reported from the western part of the world (United States 43.2%). Globally the prevalence of lean/non-obese NAFLD is showing an increasing trend over the last 3 decades[100]. Though Shi et al[101] had reported a lower prevalence of hypertension, hyperuricemia, and fasting blood glucose in lean/non-obese NAFLD patients compared to obese NAFLD, these lean patients are not necessarily metabolically healthy. Rather lean NAFLD patients are more likely to have visceral obesity, metabolic syndrome, dyslipidemia, hypertension, and DM as co-morbidities than the lean controls[101]. The pathophysiologic basis of the development of NAFLD in lean/non-obese individuals is complex and multi-factorial. Increased prevalence of the PNPLA3 G allele had been found in lean NAFLD patients[102]. Other genetic factors like TM6SF2 (T)[46], cholesteryl ester transfer protein, and interferon lambda 3 (IFNL3)/IFNL4(C) had also been found to increase the risk of lean/non-obese NAFLD[103,104]. On the other hand, possible roles of distinct gut microbiota, bile acid profile[46,105], increased lysine, tyrosine, lysophosphatidylcholines, and phosphatidylcholines, had also been implicated in the development of NAFLD among lean individuals[106]. The progression of NAFLD in the lean population can be conceptualized as a state of gradual attenuation of metabolic adaptation. Pathophysiologically, this can be divided into 3 stages: stage of susceptibility, stage of adaptation, and stage of failure[107]. Studies evaluating the true natural history of lean NAFLD are sparse in the literature. In the largest meta-analysis Ye et al[99] reported that among lean/non-obese NAFLD patients, NASH and fibrosis (> stage 2) were present in 39% and 29% of patients respectively, which was less than the prevalence among obese NAFLD population. However, liver-related mortality was reported as almost twice in lean/non-obese NAFLD patients than in the obese NAFLD group. In another study with a mean longitudinal follow-up of almost 20 years, lean NAFLD patients did not show any significantly increased risk of overall mortality but the risk of progression to severe hepatic diseases was significantly higher (HR 2.69) than the obese NAFLD population[108]. Like obese NAFLD, lifestyle modification in the form of dietary modifications and increased physical activity remains the main therapeutic approach in lean NAFLD patients[109].

**Variable natural history**

**Classic and dynamic model:** Previously, the natural history of NAFLD had been conceptualized as a disease spectrum that follows a linear model of disease progression. This classic model hypothesized that there is a gradual progression of the disease from NAFL to NASH to cirrhosis and HCC. However, this progressive worsening of the disease does not occur in all of the patients of NAFLD and significant heterogeneity in the natural history of NAFLD had been observed. Recent literature had identified that not all the patients with NAFLD follow this ‘classic linear model’ of natural history. A study by Pais et al[110], which systemically evaluated serial liver biopsy in NAFLD patients, had shown that 60% of NAFL patients had progressed to NASH and around 25% of patients of NAFL had directly progressed to the fibrotic stage. Various factors like DM, obesity, old age, and a higher degree of baseline abnormality were identified as possible risk factors for disease progression. In another longitudinal follow-up study by McPherson et al[111], no significant difference in the rate of fibrosis progression between NAFL and NASH patients was found. In an excellent systematic review by Singh et al[112], serial liver biopsy data of 411 biopsy-proven NAFLD from 11 cohort studies were analyzed. They had also re-emphasized that both NAFL and NASH can progress to the fibrotic stage. However, it takes much
Pal P et al. Nonalcoholic fatty liver disease heterogeneity

longer (14 years) time to progress one fibrosis stage in NAFL than in NASH (7 years). The annual fibrosis progression rate was slower in NAFL (0.07 stage) than in NASH (0.14 stage). Moreover, NAFL and NASH had a comparable rate of CV mortality (OR 0.9) though all-cause and liver-related mortality are higher in NASH[113]. To summarize, NAFL can progress both to the NASH and fibrosis stage directly and on the other hand, NASH can also regress to NAFL or progress to the fibrotic stage. Thus, in the ‘dynamic model’ of NAFLD, it has been conceptualized that in early NAFLD, there is dynamic cycling between NAFL and NASH[114] (Figure 3).

Slow and rapid progressor: In the same meta-analysis discussed above, Singh et al [112] also had identified significant heterogeneity among disease progression in NAFLD. They reported 2 subtypes of NAFLD patients according to fibrosis progression rate - rapid and slow progressor. The rapid progressors were around 20% of the NAFLD group who progressed rapidly from baseline (stage 0 fibrosis) to advanced (stage 3 or 4 fibrosis). On the other hand, the majority of NAFLD patients are slow progressors who only progressed 1 or 2 stage fibrosis in a similar time frame. Older age, low ASL: Alanine aminotransferase (ALT) ratio, co-morbidities like diabetes mellitus or hypertension, and genetic polymorphisms are probable risk factors for rapid progressors[103,115] (Figure 3).

HCC: With the progressive increase in the prevalence of NAFLD worldwide, the risk of HCC and liver-related mortality are likely to rise as a consequence. Viral hepatitis-related HCC usually occurs in the background of the advanced stage of cirrhosis. Though classically HCC usually occurs in the advanced stage of cirrhosis in the NAFLD spectrum, this is not true for all the cases of NAFLD-related HCC[116]. Rather one of the most common causes of chronic liver disease-related HCC without evidence of cirrhosis is NAFLD[5]. Leung et al[117] had reported 15% percent of NAFLD-related HCC as non-cirrhotic and they usually had larger hepatic tumor diameter at diagnosis. In a retrospective analysis, Mohamad et al[118] also reported that HCC in NAFLD patients without cirrhosis are likely to present in the older age group with a larger tumor size with a high recurrence rate in comparison to those with cirrhosis (Figure 3).

THERAPEUTIC AND RESEARCH IMPLICATIONS

NAFLD progression and prognostication

Many factors may influence the progression of NAFLD to the more advanced stage but are not routinely or easily assessed in day-to-day practice (e.g. genotype, gut microbiome, mitochondrial function, immunological response)[119]. Consequently, we need to consider the natural history studies to help provide clinical, biochemical, and histological variables that can be utilized to decipher which patients will develop severe disease with worse outcomes. With regard to clinical features, a paired biopsy study by McPherson et al[111] underscores the impact of IR with 80% of patients with NAFL and progression of fibrosis developing diabetes by the time of follow-up biopsy compared with 25% of nonprogressors. Other studies have also shown that weight gain and worsening IR are associated with fibrosis progression in NAFLD[110]. Data for biochemical predictors are somewhat deficient. However, a study found that in patients with biopsy-proven NASH and compensated cirrhosis; lower levels of serum cholesterol, ALT, and platelets are independently associated with hepatic complications and higher aspartate aminotransferase (AST)/ALT ratio with overall mortality[120]. In NAFLD, baseline histology can provide a good prognostic value. According to a systemic review and meta-analysis of paired-biopsy studies, a third of individuals with NAFLD will have progression of fibrosis with a mean progression rate of 0.14 stages per annum for NASH, corresponding to one stage of fibrosis progression over a median of 7.1 years[112]. Nevertheless, many epidemiological studies have de-emphasized the presence of NASH and confirmed the presence and degree of fibrosis as the most important histologic predictor of liver-related morbidity and mortality[121,122].

It is now widely accepted that the severity of fibrosis is the only significant predictor of outcomes in NAFLD. The histological differentiation between NAFL and NASH is unlikely to predict fibrosis progression and carries very little prognostic value. Thus, it is better to consider the diagnosis of patients with advanced fibrosis (F3 and F4) because this stage is a predictor for hepatic and extrahepatic morbidity and mortality[123]. This strategy identifies those with liver disease sufficient to call for specific interventions to prevent complications of cirrhosis and the development of HCC. People with NAFL or NASH with early F0-F2 don’t need to be considered as having
Figure 3 Natural history of non-alcoholic fatty liver disease (classic and dynamic model). HCC: Hepatocellular carcinoma; NAFL: Nonalcoholic fatty liver; NASH: Nonalcoholic steatohepatitis.

As progressive fibrosis indicates a poor prognosis with unfavorable CV and adverse hepatic outcomes, the approach should now focus on the risk stratification of patients and identify those needing liver-specific intervention.

Non-invasive tests of hepatic fibrosis

As the severity of fibrosis is the major driver for the long-term prognosis of NAFLD patients, it is, therefore, critical to identify patients at higher risk of advanced fibrosis to optimize their management[124]. Although required to detect patients with NASH and early fibrosis, liver biopsy is an invasive procedure. Patient acceptability is low, and it is not desirable to perform liver biopsy repetitively to assess disease progression and response to treatment. Moreover, as only a small proportion of the patients would develop liver-related complications, performing non-invasive tests (NITs) as the primary assessment is preferable[125]. This section focuses on the confounding factors that can affect the performance and accuracy of NITs of liver fibrosis in patients with NAFLD.

Impact of confounding factors

Non-invasive fibrosis scores are usually used to detect or exclude advanced fibrosis in individuals with NAFLD. A few studies purposely looked at reasons for imprecise prediction by these scores. In a multicentric European study in subjects with biopsy-proven NAFLD, the AST-to-ALT ratio, NAFLD fibrosis score (NFS) and Fibrosis-4 (FIB-4) index performed poorly for the detection of significant fibrosis in persons aged 35 years or below[126]. The specificity of the FIB-4 index and NFS reduced to unacceptable levels in those aged 65 years and older in the same study. This reason is that age is a component of both the fibrosis scores. The performance of NITs and the used transient elastography (TE) liver stiffness cutoffs in different ethnic populations...
Pal P et al. Nonalcoholic fatty liver disease heterogeneity

and special subpopulations such as individuals with diabetes and obesity also need to be taken into account. For example, depending on the ethnicity, the diagnostic accuracy of the NITs may be altered. Compared to Western populations, South Asians develop more metabolic complications at lower body mass indices. The accuracy of the NFS, AST-to-platelet ratio index, FIB-4, AST/ALT ratio, and BARD score is found to be lower in the South Asian population in comparison with the Caucasian population [127]. In addition, the NFS has a lower sensitivity in individuals of South Asian descent, as the majority had a lower BMI and were younger than Caucasian counterparts with a comparable disease stage, and therefore had a lower score[125]. Serum markers of liver fibrosis and possible confounding factors are summarized in Table 1.

With regards to imaging modalities that estimate liver stiffness as a potential surrogate of hepatic fibrosis, vibration-controlled transient elastography (VCTE) has been widely validated against liver histology[128] and shows correlation with clinical outcomes in longitudinal studies[129]. However, there are a number of factors to be considered while using this modality. Pathologies that increase liver stiffness can lead to a false-positive diagnosis of advanced fibrosis. Besides, high BMI and severe hepatic steatosis have been reported to increase the false positive rate of VCTE[130]. A recent study suggests that when using the XL probe in obese patients, steatosis does not augment liver stiffness independent of fibrosis[128]. Magnetic resonance elastography (MRE) can surmount many of these barriers, except for iron overload and acute inflammation; nonetheless, restricted availability at most centers and cost are the limiting factors. MRE has higher applicability and accuracy than VCTE when compared head-to-head[131].

While it is expected that blood-based parameters or imaging modalities will replace liver biopsy for the diagnosis in people who would benefit from treatment, equally it indicates that validation of any future marker should be done in more specifically defined cohorts. A recent International Consensus Panel suggested that the factors that shape the NAFLD heterogeneity should be taken into account when devising risk-stratification scores and algorithms[2]. Caution should be exercised by clinicians during the interpretation of test results when the tests are applied in patients with potential confounding factors.

Considerations for best practice

Early detection of advanced fibrosis is essential in the efforts to halt the NASH progression. Therefore, screening is vital to ensure that patients, mainly those with advanced F3–F4, are identified and linked to care before they develop end-stage liver disease. With the development of reliable NITs to identify patients with advanced fibrosis, there is now potential to put management strategies earlier in place[132]. Clinicians need to be more proactive in detecting patients with advanced fibrosis due to NASH. Figure 4 shows a diagnostic algorithm that targets screening of patients with characteristics of MetS who are at risk of progressive fibrosis. This is in accordance with guideline recommendations to screen this high-risk group[133]. This pathway includes sequential use of NITs (preferably a serum biomarker and an imaging technique) and can decrease secondary and tertiary referral rates and achieve larger cost savings.

In the Asia–Pacific region, quite a few studies have assessed the cross-sectional accuracy of non-invasive surrogates of liver biopsy among NAFLD patients[134,135]. It has been suggested that the serum tests and physical tools when used in combinations can yield more reliable data than that provided by either method alone[136]. Nevertheless, concerns are there regarding the definition of threshold values in Asian patients and Asia-Pacific Working Party stated that “at the present time, the clinical use of such tools to avoid liver biopsy remains undefined”[137].

Newsome et al[138] recently published the FibroScan-AST (FAST) score for the non-invasive identification of patients with significant fibrosis (≥ F2) and a NAFLD activity score (NAS) of ≥ 4 to detect those at increased risk of disease progression. This could reduce unnecessary liver biopsies in patients unlikely to have significant disease. The incorporation of VCTE values in the score enhanced the diagnostic performance. This prospective study was validated in multiple global cohorts from North America, Europe, and Asia. Discrimination was considerably higher for the FAST score when compared with FIB-4 and NFS. Now, further research on the performance of the FAST score is required to transition the use of such predictive models to clinical practice. The diagnostic accuracy of the sequential combination of FIB-4 and VCTE has been evaluated recently in an individual participant data meta-analysis that included 5735 patients. Depending upon the different cut-offs used, this combined algorithm can diagnose cirrhosis with a specificity of 95%-98%, obviating the need for liver biopsy.
Table 1 Non-invasive tests of hepatic fibrosis and potential confounding factors

<table>
<thead>
<tr>
<th>Biomarker panel</th>
<th>Parameters</th>
<th>Validation</th>
<th>Prognostic ability</th>
<th>Confounding factors/limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>APRI</td>
<td>AST, platelet</td>
<td>Good</td>
<td>Fair</td>
<td>Large number of individuals fall in the indeterminate range</td>
</tr>
<tr>
<td>Fibrosis-4 index</td>
<td>Age, AST, ALT, platelet</td>
<td>Very good</td>
<td>Very good</td>
<td>Poor performance in patients aged ≤ 35 yr, Low specificity in patients aged ≥ 65 yr, Less sensitive in South Asian Population</td>
</tr>
<tr>
<td>NAFLD fibrosis score</td>
<td>Age, BMI, IFG or diabetes, AST, ALT, platelet, albumin</td>
<td>Very good</td>
<td>Good</td>
<td>Different cutoff values needed for younger or older participants, Albumin may decrease in chronic illnesses, malnutrition, nephrotic syndrome and protein-losing enteropathy, Less sensitive in South Asian Population</td>
</tr>
<tr>
<td>Enhanced liver fibrosis panel</td>
<td>PIIINP, HA, TIMP1</td>
<td>Good</td>
<td>Very good</td>
<td>PIIINP is increased in other fibrotic diseases or bone fracture, TIMP1 is increased in cancer and inflammation, Not as widely available as non-patented scores and more expensive</td>
</tr>
<tr>
<td>FibroMeter NAFLD</td>
<td>Age, weight, prothrombin index, ALT, AST, ferritin, fasting glucose</td>
<td>Fair</td>
<td>NA</td>
<td>Prothrombin index affected by anti-coagulants, Ferritin is an acute phase protein, Glucose is affected by anti-diabetic treatment, More validation needed</td>
</tr>
<tr>
<td>NIS4</td>
<td>miR-34a-5p, n2-M, YKL-40, and glycated hemoglobin</td>
<td>Fair</td>
<td>NA</td>
<td>Not as widely available as non-patented scores and more expensive, More validation is needed</td>
</tr>
</tbody>
</table>

ALT: Alanine aminotransferase; APRI: AST-to platelet ratio index; AST: Aspartate aminotransferase; BMI: Body mass index; HA: Hyaluronic acid; IFG: Impaired fasting glucose; α2-M: α2 macroglobulin; NA: Not applicable; NAFLD: Non-alcoholic fatty liver disease; PIIINP: Procollagen type III N-terminal peptide; PTI: Prothrombin index; TIMP-1: Tissue inhibitor of matrix metalloproteinase 1.

Identification of novel therapeutic targets

As the burden of NAFLD has become increasingly evident, so also have hurdles to developing effective therapeutic points of action. The development of progressive steatohepatitis is connected to excess metabolic substrate delivery to the liver that, in turn, induces cell stress, which can activate inflammatory and apoptotic signaling. Eventually, inflammation triggers a fibrogenic response that can lead to cirrhosis in the end[140]. This simplified model facilitates the evaluation of precise mechanisms underlying each of these factors and targeting them for treatment. Table 2 summarizes proposed ‘druggable’ pathophysiologic targets in NAFLD[141-153].

Quite a few of the recently carried out phase 2 and 3 studies failed to reproduce the encouraging antifibrotic or NASH-resolving effects observed in animal models. Reasons for this discrepancy between preclinical models and clinical settings are likely diverse. Most importantly, no model can ever assess compounds in the actual physiological settings of heterogeneous human populations. This aspect may become further relevant if mechanisms are not entirely translatable between two different species[154]. Additionally, none of the available NASH models used for preclinical trials adequately represents all the human disease aspects from the macroscopic to the molecular level. Moreover, only a few models reflect linked extrahepatic diseases (such as atherosclerosis, obesity, or IR). Finally, a higher heterogeneity in humans in relation to genetics, the gut microbiota, gender, and existing comorbidities leads to even more complications. It is, therefore, critical to recognize the drawbacks of preclinical models to improve clinical trial outcomes in drug development.

There is significant interindividual variability in the NAFLD susceptibility and for progression to liver-related complications[49]. It is becoming more and more apparent that there is substantial heterogeneity in the molecular and cellular processes
Table 2 Liver-targeted therapies in development for the treatment of nonalcoholic fatty liver disease

<table>
<thead>
<tr>
<th>Treatment targets</th>
<th>Mechanism of action</th>
<th>Agent (oral/injectable)</th>
<th>Current status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolism</td>
<td>FXR agonism</td>
<td>Obeticholic acid</td>
<td>Interim analysis of a phase 3 RCT (REGENERATE) showed significant histological improvement[141]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tropifexor (LJN452)</td>
<td>A phase 2 study recently completed (NCT02855164)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cilofexor</td>
<td>A phase 2 study in patients with NASH showed a decrease in hepatic fat[142]</td>
</tr>
<tr>
<td>PPAR agonism</td>
<td>Elafibranor</td>
<td></td>
<td>Interim analysis a phase 3 trial (RESOLVE-IT) failed to show any treatment effect</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lanifibranor (IVA337)</td>
<td>A phase 2 study in patients with T2DM and NAFLD is actively recruiting (NCT03459079)</td>
</tr>
<tr>
<td></td>
<td>Saroglitazar</td>
<td></td>
<td>A phase 2 RCT (EVIDENCES IV) in participants with NAFLD/NASH has shown significant improvement in ALT, LFC, and IR[143]</td>
</tr>
<tr>
<td>Acetyl-CoA Carboxylase inhibition</td>
<td>PF-05221304</td>
<td>Improved liver chemistry and liver fat in an RCT[144]</td>
<td></td>
</tr>
<tr>
<td>GLP-1 agonism</td>
<td>Liraglutide</td>
<td></td>
<td>Only data from small studies have been published and the relative contribution of weight loss and improvement in glycemic control to the observed benefits in NASH are yet to be determined[145-147]</td>
</tr>
<tr>
<td></td>
<td>Semaglutide</td>
<td></td>
<td>In a phase 2 trial, the primary endpoint (resolution of NASH with no worsening in fibrosis), was met[148]</td>
</tr>
<tr>
<td>FGF21 agonism</td>
<td>Pegbelfermin (BMS-986036)</td>
<td>A series of phase 2b trials of pegbelfermin are underway</td>
<td></td>
</tr>
<tr>
<td>MCP2 antagonism</td>
<td>MSDC-0622 K</td>
<td></td>
<td>The EMINENCE phase 2b trial didn’t meet the primary end point[149]</td>
</tr>
<tr>
<td>THRβ agonism</td>
<td>Resmetirom (MGL-3196)</td>
<td>A phase 3 study is actively recruiting (NCT03900429)</td>
<td></td>
</tr>
<tr>
<td>Cell stress and apoptosis</td>
<td>Antioxidant</td>
<td>Vitamin E</td>
<td>Resolution of NASH in some studies, but not all; no impact on fibrosis[150]</td>
</tr>
<tr>
<td></td>
<td>Pan-caspase inhibition</td>
<td>Emricasan</td>
<td>Phase 2b clinical trials for NASH failed to meet their primary efficacy end points[151]</td>
</tr>
<tr>
<td></td>
<td>ASK1 inhibition</td>
<td>Selonsertib</td>
<td>Phase 3 STELLAR trials discontinued due to lack of efficacy</td>
</tr>
<tr>
<td>Inflammation</td>
<td>CCR2/CCR5 inhibition</td>
<td>Cenicriviroc</td>
<td>Phase 3 trial AURORA terminated due to lack of efficacy</td>
</tr>
<tr>
<td></td>
<td>Inflammmasome inhibition</td>
<td>SGM-1019</td>
<td>A phase 2 study is terminated due to a safety event (NCT03676231)</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>LOXL2 inhibition</td>
<td>Sintuzumab</td>
<td>No benefit on histological analysis or on clinical outcomes[152]</td>
</tr>
<tr>
<td>Gut-liver signaling axis</td>
<td>FGF19 agonism</td>
<td>Aldafermin (NGM282)</td>
<td>In a phase 2 trial of patients with NASH, aldafermin reduced liver fat and produced a trend toward fibrosis improvement[153]</td>
</tr>
</tbody>
</table>

ACC: Acetyl-CoA carboxylase; ALT: Alanine aminotransferase; ASK1: Apoptosis signal-regulating kinase; CCR: C–C motif chemokine receptor; FGF: Fibroblast growth factor; FXR: Farnesoid X receptor; GLP1: Glucagon-like peptide 1; IR: Insulin resistance; LFC: Liver fat content; LOXL2: Lysyl oxidase homolog 2; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; PPAR: Peroxisome proliferator-activated receptor; THRβ: Thyroid hormone receptor β.

propelling the disease from one patient to the next. This understanding raises the possibility of matching specific therapeutic strategies to the particular disease drivers in a given patient. The development of such personalized approaches and the detection of subpopulations with distinctive disease drivers will need a combination of phenotypic, genetic, and molecular data[140]. Furthermore, genetic insights present a powerful approach to deduce and prioritize candidate drugs. Such selection can avoid numerous drawbacks while defining likely benefits[155]. However, drug discovery based on genetics is still in its infancy, and this area will present its challenges. NAFLD is associated with several metabolic disturbances. As many circadian clock-controlled genes are fundamental in the metabolic processes of the body, it is not unexpected that some of these genes can be potential therapeutic targets[156]. Thus, by considering the circadian cycling of their targets, new drugs for NAFLD can be administered in a way that optimizes the benefits and minimizes the side effects.
Figure 4 A suggested algorithm for the use of non-invasive tests for risk stratification of patients with suspected non-alcoholic fatty liver disease in clinical practice. Obesity, type 2 diabetes, or metabolic syndrome; Estimated prevalence for low, intermediate, and high risks groups; Patented serum biomarkers (FibroTest, Fibrometer, or ELF) could be considered in patients with intermediate-risk. ARFI: Acoustic radiation force imaging; LSM: Liver stiffness measurement; MRE: Magnetic resonance elastography; NPV: Negative predictive value; PPV: Positive predictive value; SWE: Shear wave elastography.

Impact on clinical trials and endpoints

Given the rising disease burden associated with NAFLD, the development of outcome measures to assess the at-risk population and validate clinically relevant study endpoints is vital. Nevertheless, the natural history of NAFLD is highly variable, often nonlinear in progression. In addition, NAFLD itself is a heterogeneous disease that is shaped by the dynamic interaction between genetic predisposition, environmental factors, and several modifiable risk factors[157]. This pathogenetic background provides numerous potential targets for therapeutic intervention, however, this same complexity limits defining clear, measurable, and objective clinical endpoints[158]. Considering these factors, surrogate endpoints, which can be used to predict outcomes on clinically relevant endpoints, are expected to be beneficial in most patients. Furthermore, NAFLD is a slowly progressive disease, with a gap of many years between onset and development of “hard” clinical outcomes, such as liver-related and all-cause mortality. As stated earlier, the fibrosis stage is the most important predictor of liver-related outcomes. Unfortunately, the progression of fibrosis itself is also slow, with a median of 7.1 years in subjects with NASH[112]. Thus, selecting meaningful clinical endpoints has been a major challenge in drug development and validation. At present, before enrolling patients into NASH clinical trials, identifying which patients with NAFLD have NASH, particularly those with advanced fibrosis, is one of the major stumbling blocks. Once these at-risk patients have been selected, monitoring for fibrosis regression in individuals with advanced fibrosis appears to be the optimal endpoint in clinical trials and should supplant NASH-based endpoints[158]. Surrogate measures of liver-related outcomes also seem reliable. Although important, to assess for all-cause mortality (primarily CV death) and liver-related mortality will require
longer-term follow-up.

Liver biopsy is essentially prone to sampling error and interobserver variability; its invasive nature also makes it a barrier for large clinical trials. Given these limitations, the development of accurate, robust, and reproducible noninvasive surrogate endpoints which may ultimately replace biopsy in trials are eagerly sought in NAFLD research[159]. Algorithms such as NFS and FIB-4 may be useful tools for prescreening, in order to enrich the patient group with an appropriate spectrum of NASH and fibrosis for enrollment. Noninvasive imaging methods such as VCTE and MRE are likely to play a future role but presently lack the ability to differentiate between closely related fibrosis stages[160].

To summarize, a combination of the slow nature of disease progression in NAFLD, heterogeneity of therapeutic targets, and inherent limitations of serial liver biopsy to evaluate effects of intervention have considerably hampered clinical trial design as well as the development of new and effective therapies[158]. Thus, the standard trial design that does not consider the disease heterogeneity may not be the best approach for learning this complex disease. Future clinical trials need to target patients with specific characteristics (gender, hormonal status, genetic susceptibility, metabolic and microbiota signatures, and the presence or absence of comorbidities) once the connections between these characteristics and the therapeutic targets are clearly understood[2].

**FUTURE PERSPECTIVES**

With increasing recognition of heterogeneous molecular and genetic drivers of NAFLD, there is a possibility of precision medicine based on the identification of specific drivers of the disease. An integrated model of NAFLD development based on genetic, molecular, histology, “omics” based data (transcriptome, metabolite, proteome, microbiome), and disease phenotype to identify disease subpopulations is required for such personalized approaches[140]. Critical data on molecular heterogeneity and its relation to clinical outcomes of NAFLD to going to explore new horizons in the management of this global pandemic[161]. A better understanding of bidirectional and dynamic disease progression and regression (e.g. fibrosis), the influence of behavioral factors, and establishing a correlation with end-organ damage is warranted. Prospective follow-up data on the evolution of pediatric NAFLD into adulthood shall shed light on pediatric disease evolution[162]. Identification and validation of non-invasive methods of disease assessment and biomarkers will accelerate the development of pharmacotherapy and testing of combination therapies. Seamless phase II-IV trial designs, virtual placebo cohort analysis, master clinical trials testing multiple agents and multiple disease types, use of effectiveness trials in real-world settings, and patient-reported outcomes would revolutionize clinical trials for NAFLD. Precise terminology, characterization of disease heterogeneity (both molecular and clinical), novel translational models to identify new therapeutic target, and thus better designed clinical trials would help reduce the burden of the disease[2].

**CONCLUSION**

The impact of the upsurge in NAFLD patients and a rising proportion with advanced disease will be reflected in higher rates of hepatic and extrahepatic morbidity and mortality, which will continue to burden the health care system heavily. On the other hand, a lack of enough consideration of heterogeneity in risk profiles and responsiveness to treatment posing impediments that hampers progress to effective treatments. It is anticipated that a more robust understanding of pathophysiology will result in better characterization and subphenotyping of the disease and its drivers. In turn, this understanding of disease variability may help the introduction of appropriate noninvasive biomarkers for each subtype, thus promoting more individualized interventions. In this regard, any discussions on the update of nomenclature or more appropriate terminology are in the right direction. However, the proposed redefining of the disease should increase the prioritization of research activity on NAFLD to fill current knowledge gaps and find new tools to overcome the challenges. It appears to be important to place NAFLD/MAFLD/DAFLD under the same umbrella with significant comorbidities and approach NAFLD/MAFLD/DAFLD holistically rather than facing NAFLD as a separate entity. Future studies are likely to provide us the necessary prerequisites for designing more appropriate clinical trials to
identify finely tailored diagnostic and treatment strategies for our patients.

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Newly discovered endocrine functions of the liver

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Abstract

The liver, the largest solid visceral organ of the body, has numerous endocrine functions, such as direct hormone and hepatokine production, hormone metabolism, synthesis of binding proteins, and processing and redistribution of metabolic fuels. In the last 10 years, many new endocrine functions of the liver have been discovered. Advances in the classical endocrine functions include delineation of mechanisms of liver production of endocrine hormones [including 25-hydroxyvitamin D, insulin-like growth factor 1 (IGF-1), and angiotensinogen], hepatic metabolism of hormones (including thyroid hormones, glucagon-like peptide-1, and steroid hormones), and actions of specific binding proteins to glucocorticoids, sex steroids, and thyroid hormones. These studies have furthered insight into cirrhosis-associated endocrinopathies, such as hypogonadism, osteoporosis, IGF-1 deficiency, vitamin D deficiency, alterations in glucose and lipid homeostasis, and controversially relative adrenal insufficiency. Several novel endocrine functions of the liver have also been unraveled, elucidating the liver’s key negative feedback regulatory role in the pancreatic α-cell-liver axis, which regulates pancreatic α cell mass, glucagon secretion, and circulating amino acid levels. Betatrophin and other hepatokines, such as fetuin-A and fibroblast growth factor 21, have also been discovered to play important endocrine roles in modulating insulin sensitivity, lipid metabolism, and body weight. It is expected that more endocrine functions of the liver will be revealed in the near future.

Key Words: Liver; Endocrine function; Hormone; Amino acids; Hepatokine; Fibroblast growth factor 21

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Core Tip: The liver has many newly discovered endocrine functions, most of which are in regulating metabolism, underscoring the functioning of the liver as a major metabolic organ. Convincing evidence has shown that the liver regulates endocrine
functions in mineral and fuel metabolism, especially in the metabolism of glucose and lipids via hepatokines and amino acids via negative feedback on pancreatic α cells. As research into the endocrine function of the liver is a rapidly evolving field, controversial findings often exist; caution needs to be taken when interpreting novel findings to avoid over-simplification of complex metabolic processes and premature allocation of research resources.

INTRODUCTION

The liver is a dynamic endocrine organ and mediates critical metabolic pathways via roles in direct hormone and hepatokine production, hormone metabolism, synthesis of binding proteins, detoxification, and processing and redistribution of metabolic fuels [1-4]. It participates in multiple signaling pathways with other endocrine organs, including the pituitary, pancreas, gut, thyroid, adrenal glands, and bone, with hormones in turn modulating the liver’s metabolic and synthetic functions [1,5]. Diseases that affect the liver lead to a variety of endocrine manifestations, including hypogonadism, osteoporosis, effects on glucose metabolism and growth hormone (GH), and controversial effects on cortisol [1,5].

The liver, with its vascularity, is well-positioned to provide and receive endocrine signals, including those from pancreatic and gut hormones [6]. It also receives exposure to antigen-rich blood systemically and from the gastrointestinal system as a lymphoid organ [7] and serves as a principal organ in drug metabolism and clearance [8]. Despite only representing 2.5% of the body weight, the liver receives up to 25% of the total cardiac output at rest [9]. It also receives a unique double afferent blood flow from the hepatic artery and partially deoxygenated portal vein, with around 75% of the blood flow from the latter [9]. The portal vein, in turn, receives blood from the stomach, small and large intestines, pancreas, spleen, and gallbladder [9], with direct physiological implications on the regulation of metabolism by endocrine liver functions [6]. Great progress has been made in the understanding of the endocrine functions of the liver in the last 10 years.

ADVANCES IN CLASSIC ENDOCRINE FUNCTIONS OF THE LIVER

We will first briefly summarize the advances in the understanding of the liver classic endocrine functions (Table 1).

Direct hormone production

The liver directly synthesizes multiple hormones, including 25-hydroxyvitamin D, insulin-like growth factor 1 (IGF-1), and angiotensinogen. Given roles in direct hormone production, the liver also has permissive roles of normal hormone function, in particular with effects on bone health, the GH-IGF-1 axis, and renin-angiotensin-aldosterone (RAA) pathway.

Vitamin D: The liver is the primary site of 25-hydroxylation of vitamin D to 25-hydroxyvitamin D (calcidiol), the main storage form of vitamin D [10]. Vitamin D is a secosteroid hormone well known for its role in calcium and bone homeostasis, with pleiotropic effects on cellular proliferation, differentiation, and immunomodulation [11-13]. 25-hydroxyvitamin D (calcidiol) then undergoes 1-alpha-hydroxylation in the kidney to the activated form 1,25-dihydroxyvitamin D (calcitriol) [10], which provides the active hormonal effects of vitamin D. The hydroxylation of vitamin D to produce calcidiol is mainly carried out in the liver by multiple cytochrome P450 mixed-function oxidases (CYPs) located in the mitochondria, endoplasmic reticulum (ER), and microsomes, though studies also show presence of these CYPs in extrahepatic tissues [10,11].
### Table 1 Classic endocrine functions of the liver

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Liver function</th>
<th>Target organ</th>
<th>Action on target organ</th>
<th>Alteration in liver diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-hydroxyvitamin D</td>
<td>Direct production</td>
<td>Gut</td>
<td>Prohormone of calcitriol which stimulates gut calcium absorption</td>
<td>Decreased production resulting in low bone density</td>
</tr>
<tr>
<td>Insulin-like growth factor 1</td>
<td>Direct production</td>
<td>Ubiquitous</td>
<td>Promoting growth and differentiation and regulating nutrients metabolism</td>
<td>Decreased production resulting in dysmetabolism</td>
</tr>
<tr>
<td>Angiotensinogen</td>
<td>Direct production</td>
<td>Cardiovascular system</td>
<td>Precursor of angiotensin II which regulates aldosterone level. Both regulate vascular tone, sodium retention, and cardiac remodeling</td>
<td>Near-normal function</td>
</tr>
<tr>
<td>Thyroid hormone</td>
<td>Activation through T4 to T3 conversion; inactivation through degradation; TBG production</td>
<td>Ubiquitous</td>
<td>Increasing metabolism and energy expenditure</td>
<td>Low T3 syndrome</td>
</tr>
<tr>
<td>Glucagon-like peptide 1 (GLP-1)</td>
<td>Metabolism of GLP-1 via dipeptidyl peptidase-4 (DPP4)</td>
<td>Pancreas, gut, and brain</td>
<td>Stimulating insulin production, decreasing gut motility, and suppressing appetite</td>
<td>Increased DPP4 expression resulting in higher risk of diabetes</td>
</tr>
<tr>
<td>Sex hormones</td>
<td>Hormone metabolism and SHBG production</td>
<td>Ubiquitous</td>
<td>Numerous (details beyond this review)</td>
<td>Hypogonadism</td>
</tr>
<tr>
<td>Glucocorticoids</td>
<td>Hormone metabolism and CBG production</td>
<td>Ubiquitous</td>
<td>Numerous (details beyond this review)</td>
<td>Relative adrenal insufficiency</td>
</tr>
<tr>
<td>Mineralocorticoids</td>
<td>Hormone metabolism</td>
<td>Cardiovascular system</td>
<td>Maintaining electrolyte balance and blood pressure</td>
<td>Largely intact</td>
</tr>
</tbody>
</table>

TBG: Thyroxine binding globulin; CBG: Cortisol binding globulin; SHBG: Sex hormone binding globulin.

IGF-1: The liver is the primary source of IGF-1, a 70-amino acid polypeptide hormone with endocrine, paracrine, and autocrine effects[14]. IGF-1 affects almost every tissue and organ[15], and its receptors are ubiquitously expressed[16]. Besides mediating the actions of GH, more recently, non-growth-related actions of IGF-1 are found. IGF-1 binds to the insulin receptor and the hybrid IGF-1/insulin receptors, with implications on the metabolic effects of IGF-1[14]. IGF-1, GH, and insulin are hypothesized to constitute a regulated axis to inform cells about nutritional status, helping direct cells grow and differentiate vs induce a state of quiescence, senescence or apoptosis[14]. The IGF-1 receptor also participates in a crosstalk with the thyrotropin receptor by forming heterodimers[17], with implications on cellular growth and pathological implications in Graves’ eye disease.

Angiotensinogen: The liver is the primary source of angiotensinogen, which is involved in the RAA system[18]. The RAA system is vital for maintaining blood pressure homeostasis, via effects on sodium balance, intra- and extra-vascular volume, and systemic vascular tone[19]. Angiotensinogen, an alpha-globulin, is the only known substrate for renin and the main precursor molecule for angiotensin II (AngII), the major biologically active peptide in the RAA pathway[19]. Despite local tissue production of AngII, liver angiotensinogen is the primary source of renal AngII[18]. Hepatocytes tonically secrete angiotensinogen and primarily determine plasma angiotensinogen levels, with small increases in angiotensinogen levels increasing blood pressure and AngII levels[20].

**Hormone metabolism**

The liver is involved in the metabolism of multiple endocrine hormones, including thyroid hormones, glucagon-like peptide-1, and steroid hormones, with roles in both activation and inactivation of the hormones.

**Thyroid hormone:** Hepatic metabolism has roles in both activation and inactivation of thyroid hormones. The biologic activity of thyroid hormone is mainly mediated through the active thyroid hormone T3. The thyroid only secretes 20% of the daily T3 requirement, with the remainder 80% converted from T4 by peripheral selenium-containing deiodinase enzymes (DIO), of which three primary deiodinases (type 1, 2, and 3) have been identified[21]. The liver expresses DIO1, along with the kidney and thyroid, which converts T4 to T3, though with less kinetic efficiency compared to
DIO2, which is expressed by brown adipose tissue and the pituitary. Subsequently, the thyroid hormone is metabolized by conjugation with sulfate or glucuronic acid, which occurs prominently in the liver[22].

Glucagon-like peptide 1: With the discovery of glucagon-like peptide 1 (GLP-1), increasing research has been studying the gut-pancreas-liver axis, and the liver has been shown to play a key role in the hormone’s metabolism[23]. GLP-1 is an incretin hormone produced by the intestinal L-cells in response to ingestion of nutrients, including carbohydrates, fatty acids, and fiber[24]. It stimulates insulin secretion in a glucose-dependent manner, with associated inhibition of hepatic gluconeogenesis, and promotes insulin gene transcription and growth and proliferation of islet cells[24]. GLP-1 is inactivated by dipeptidyl peptidase-4 (DPP IV), also known as CD26, a ubiquitous membrane-associated peptidase[25]. DPP IV has pleiotropic effects and widespread tissue distribution in all organs, with expression in capillary endothelial cells and high expression in the liver[25].

Steroid hormone metabolism: The liver participates in most steps of steroid hormone regulation, starting from being the primary site of cholesterol biosynthesis[26, 27]. At the liver, steroid hormones undergo phase I metabolism by cytochrome P450 enzymes (CYPs), via multiple pathways including hydroxylation or reduction, and phase II metabolism, also via various processes including glucuronidation, sulfation, or methylation[27], ultimately leading to excretion of their conjugates in urine or bile.

Steroid hormone metabolism: Sex hormones: The liver is the main site for metabolic conversion of estrogens, progesterone, and androgens to their metabolites via CYPs, which are abundantly expressed in the liver[28]. In particular, as part of the first phase of metabolism, estrogens undergo hydroxylation by numerous CYPs, including 2-hydroxylation to 2-hydroxyestradiol and 4-hydroxylation to 4-hydroxestradiol, which represent 80% and 20% of biotransformation of estradiol in the liver, respectively. 2-hydroxylation is mainly catalyzed by CYP1A2 and CYP3A4, which are expressed in the liver, and CYP1A1 in extrahepatic tissues[28]. 4-hydroxestradiol, unlike 2-hydroxestradiol, is associated with free radical generation and cellular damage, with associated increased risk of carcinogenesis in the breast and endometrium. Subsequent phase II metabolism of sex hormones, via O-methylation by catechol O-methyltransferase (COMT), glucuronidation, or sulfation, occurs at high levels at the liver, with subsequent elimination in the urine or stool[28-30].

Steroid hormone metabolism: Glucocorticoids and mineralocorticoids: The liver is also the primary site of glucocorticoid and mineralocorticoid metabolism[27]. Cortisol is converted to and from its inactive metabolite cortisone by two isozymes of 11-beta hydroxysteroid dehydrogenase (11-beta-HSD)[31]. 11-beta-HSD type 1 (11-beta-HSD1) is widely distributed, though most abundantly located in the liver and adipose tissue, and is responsible for converting cortisone back to cortisol[31], with in vitro activity being greater in omental than subcutaneous adipose tissue[32]. In healthy individuals, local splanchnic cortisol production, including from the liver, can equal or even exceed that produced by extra-splanchnic tissues, including the adrenal gland[32]. In obese, non-diabetic individuals, the liver has been shown to account for virtually all splanchnic cortisol production[32]. Though primarily secreted from the adrenal glands under the regulation of the RAA axis, animal studies suggest possibility of local hepatic aldosterone production during liver injury, which may contribute to fibrogenesis[33]. Glucocorticoids and mineralocorticoids, like other steroid hormones, undergo phase I and phase II metabolism in the liver, with excretion of their conjugates in urine or bile[27].

Binding protein production
Lipophilic hormones, including steroid hormones, are not water soluble and need to be carried in the blood stream by binding proteins[2, 24]. The liver is the primary source of binding proteins for many hormones. The liver produces specific binding proteins to multiple lipophilic hormones, including glucocorticoids, mineralocorticoids, sex steroids, thyroid hormones (T3 and T4), and vitamin D metabolites[2, 24]. Binding globulins for these lipophilic hormones include cortisol binding globulin (CBG, which binds cortisol, aldosterone, and progesterone), sex hormone binding globulin (SHBG, which binds estradiol, testosterone, and other sex hormones), thyroxine binding globulin (TBG, which binds T3 and T4), and vitamin D binding globulin (DBG, which binds vitamin D metabolites)[2, 24]. Binding proteins that are produced by the liver also include transthyretin (which binds thyroid hormone and
retinol), IGF-1 binding proteins (IGFBP, which binds IGF, including IGF-1), and nonspecific binding proteins including albumin and lipoproteins. Binding proteins serve as a circulating reservoir for hormones, potentially regulating tissue distribution and target destination in a manner that can be highly selective and targeted[2,35]. Binding protein expression and production, which occur primarily at the liver, is complex and under the regulation and influence of multiple factors[2]. Most binding protein expression increase in response to estrogens, including physiologically with pregnancy or with oral contraceptives[2,34]. Hepatic failure and protein-losing nephropathies lead to decrease of binding proteins in general[2,34].

**Endocrine dysregulation in liver disease**

The liver mediates the effects of numerous hormonal pathways, whether directly or indirectly; thus, not surprisingly, derangements affecting the liver lead to disruptions of various hormonal pathways. Patients with cirrhosis are characterized by various endocrinopathies, including relative increase in estrogen compared to androgens, hypogonadism, osteoporosis, IGF-1 deficiency, vitamin D deficiency, alterations in glucose and lipid homeostasis, and perhaps more controversially a relative adrenal insufficiency.

**Sex hormones:** Cirrhosis is characterized by symptoms of estrogen-androgen imbalance, with relatively higher estradiol and lower testosterone concentrations[36]. The etiology of estrogen-testosterone imbalance is at least in part due to conversion of androgens to estrogens in cirrhosis, which in large part occurs peripherally[36]. The pathophysiology of hypogonadism is complex, including potential contribution from hypothalamic-pituitary suppression from a relatively increased estrogen circulation. SHBG is elevated in compensated cirrhotic patients, with subsequent decreases with decompensated cirrhosis, leading to concern for potential underestimation of hypogonadism in cirrhosis[34].

**Cortisol:** Patients with cirrhosis have relatively lower cortisol levels, also in the setting of lower production of cortisol binding globulin[37]. Some studies suggest the presence of a relative adrenal insufficiency in cirrhosis, also termed critical illness-associated corticosteroid insufficiency[38]. These studies suggest a potential hepatoadrenal syndrome in advanced liver disease, with associated inadequate cortisol production during stress response[38]. The decrease in cortisol binding globulin makes the diagnosis more difficult, though some studies suggest that free cortisol levels are decreased in relative adrenal insufficiency[37]. Hepatoadrenal syndrome and associated low free cortisol are attributed to decreased formation of HDL precursors and formation of proinflammatory cytokines and endotoxins[38].

**RAA system:** In liver disease, the systemic RAA pathway is upregulated due to systemic and splanchnic arterial vasodilation and associated hypoperfusion of the renal system[39]. Notably, the cirrhotic liver is able to produce angiotensinogen to near-normal plasma levels until the end stages[40].

**DPPIV and GLP-1:** DPPIV may play a role in linking type 2 diabetes with chronic liver disease. Type 2 diabetes has been associated with a greater than 2-fold increased risk of liver disease[41], and *in vitro* studies have suggested that elevated glucose can induce DPPIV expression in liver cells[42]. The increased DPPIV activity, which degrades the incretin hormone GLP-1, may contribute towards development of IGT, insulin resistance, lipogenesis, and hepatic injury in liver disease[25,43]. Serum DPPIV levels are notably increased in cirrhosis[25], and increased DPPIV expression in the liver has been observed in hepatitis C, NAFLD, experimental liver regeneration, and cirrhosis[25,43]. Cirrhotic nodules show diffuse and uniform staining of DPPIV, with loss of usual zonal expression of DPPIV[43], and degree of hepatic expression of DPPIV has also been shown to correlate with NAFLD grading[25]. Increased DPPIV expression has also been seen in various malignant tumors, including hepatocellular carcinoma, with DPPIV noted to promote resistance to anticancer agents[25].

**Thyroid hormone:** Given the liver’s role in thyroid hormone metabolism, including local conversion of T4 to T3 by DIO1[21], patients with cirrhosis may present with abnormalities in thyroid hormone levels[44]. Though a variety of patterns are seen, the most common pattern is a low total T3 (TT3), low free T3 (FT3), elevated reverse T3 (rT3), low total T4 (TT4), variable literature on elevated vs low free T4 (FT4) levels, and possible elevations in TSH[44,45]. The low total hormone levels are attributable to low TBG[44]. The pattern is consistent with low T3 syndrome, which occurs in systemic illnesses, and represents non-thyroidal illness syndrome, previously known as
euthyroid sick syndrome\[44\].

IGF-1: Systemic IGF-1 deficiency in cirrhosis has been associated with an altered metabolic profile, including diabetes, deregulated lipid profile, and cardiovascular disease\[14\]. Lack of liver-derived IGF-1, in particular, has been associated with resultant insulin insensitivity in the liver, skeletal muscle, and adipose tissue, and corresponding hyperinsulinemia\[46\]. In NAFLD, the severity of steatosis has been correlated with a decrease in IGF-1 levels, with statistically significant differences in IGF-1 levels between mild-moderate vs severe steatosis\[14,47\].

**Bone health and vitamin D:** Chronic liver disease, including cirrhosis regardless of etiology, is associated with osteomalacia, osteopenia, and osteoporosis, and up to 40% of patients with chronic liver disease may develop an osteoporotic fracture\[48\]. The etiology of hepatic osteodystrophy is not well understood, though potential contributing factors include hypogonadism, and decreased hepatic production of IGF-1 and fibronectin\[48\]. There is a shift in cytokine production with changes in the receptor activator of nuclear factor kappa-B ligand (RANKL)/osteoprotegerin (OPG) system and an up-regulation of IL-6, which stimulates osteoclasts\[48\]. Decreased vitamin D synthesis, which is more marked in severely compromised liver function or in cholestatic liver disease, can further contribute to increased osteoporotic risk\[49\]. History of steroid treatment in chronic liver disease may be a risk factor for osteoporosis as well\[48,49\]. Different etiologies of liver disease may differ in their pathogenesis of osteoporosis, and in particular, diseases such as hemochromatosis and Wilson’s may also directly impact bone health\[48\].

**NOVEL ENDOCRINE FUNCTIONS OF THE LIVER**

Besides the advances in the understanding of classic endocrine functions of the liver, novel endocrine functions have been unraveled in the last several years (Table 2), including endocrine regulation of pancreatic α cells, adipose tissue, and insulin sensitivity.

**Feedback regulation of pancreatic α cells and glucagon**

A major novel endocrine function of the liver is its critical role in a pancreatic α cell-liver axis that regulates pancreatic α cell proliferation and circulating glucagon and amino acid levels\[50,51\]. The pancreatic α cells, unlike the insulin-secreting β cells, have been considered a mysterious cell type until recently\[52,53\]. The α cells appear first during embryogenesis\[54\]. The main known function of the α cells is to produce and secrete the hormone glucagon\[55\]. Glucagon raises circulating glucose levels directly by stimulating gluconeogenesis and glycogenolysis, and indirectly by inhibiting insulin secretion\[55,56\].

Recently, a new α cell-liver axis has been discovered, endowing the liver with new endocrine functions\[50,51\]. The first clue of the α cell-liver axis came from glucagon receptor (GCGR) knockout mice\[57,58\]. The GCGR knockout mice harbor diffusely enlarged pancreas and exhibit extremely high glucagon levels\[57-59\]. Histologically, the pancreas of GCGR knockout mice contain numerous islets at various sizes, which are composed of mostly α cells as demonstrated by immunohistochemistry\[57-59\]. Normally the number of islets is quite small, and the islets are mostly composed of β cells. Mahvash disease, a human autosomal recessive hereditary disease discovered by our group, is caused by biallelic inactivating GCGR mutations, and its universal features are also α cell hyperplasia and hyperglucagonemia\[60-62\]. GCGR inactivation in zebra fish and non-human primates also result in α cell hyperplasia and hyperglucagonemia\[63-66\]. Thus, preservation of glucagon function is conserved throughout evolution.

Although a physiological compensation of hyperglucagonemia in animals and humans with inactive GCGR is quite intuitive, the specific mechanism of the compensation was initially not clear\[67\]. The liver-specific GCGR knockout mice interestingly have similar α cell hyperplasia and hyperglucagonemia, as those in global GCGR knockout mice\[57,58,68\], suggesting that the liver is the only target organ of glucagon that sends feedback signals to α cells, and that loss of the usual negative feedback mechanism stimulates α cell hyperplasia and glucagon secretion. This theory is also supported by the liver-specific stimulatory G protein α subunit (Gsa) knockout mice, which also exhibit α cell hyperplasia and hyperglucagonemia\[69\]. As glucagon antagonists were a promising anti-diabetes medication, both academia and pharmaco-
logical companies became interested in the α cell-liver axis due to potential applications in diabetes drug development. Some of the key original large-scale experiments leading to the discovery of the role of amino acids in regulating α cells were performed by pharmaceutical companies.

The liver may regulate α cells via neural or humoral mechanisms. Islet transplantation experiments demonstrate that the liver uses a humoral mechanism. Wild-type islets transplanted into the kidney of GCGR knockout mice undergo α cell hyperplasia, while GCGR knockout islets transplanted into wild-type kidney undergo reduced α cell proliferation. Thus, it is assumed that the liver sends a humoral factor (hormone) to stimulate pancreatic α cells, a phenomenon that is pronounced in diseases where the usual negative feedback mechanism is affected.

Initially, it was hoped that a single liver hormone would be isolated from differential liver gene expression patterns of wild-type and GCGR knockout mice. Several groups, including ours, performed liver mRNA arrays of GCGR knockout mice and in wild-type mice treated with inhibitory GCGR antibodies, using wild-type mice as control. Not surprisingly, many genes are overexpressed (potential stimulatory hormones) or underexpressed (potential inhibitory hormones) in the GCGR knockout liver. Genes involved in gluconeogenesis are downregulated in the GCGR knockout liver. On the other hand, genes involved in amino acid synthesis (e.g., asparagine synthetase, Asns) are upregulated, and genes involved in amino acid catabolism (e.g., glutaminase 2, Gls2) are downregulated. Genes regulating lipid metabolism are also differentially expressed. Most of the genes with significant differential expression were not bona fide hormone candidates because they were not secreted proteins. InhβA and DefB1 were the only 2 overexpressed secreted proteins by both the GCGR knockout liver and wild-type liver treated with inhibitory GCGR antibodies; however, these two proteins were also upregulated by glucagon in primary hepatocytes and thus unlikely the pursued liver hormone.

Another possibility was that the liver hormone may not be a direct gene product such as a protein or polypeptide; rather, the hormone may be a small molecule or metabolite. Metabolomes of the GCGR knockout and wild-type mice were compared. Many differences exist but most notable differences were in glucose, amino acid, nucleotide, and bile acid levels. The GCGR knockout mice have lower glucose levels (70% of wild-type value) and higher levels of most amino acids (up to 15-fold for alanine, glutamine, glycine, lysine, and threonine) and 2 bile acids (cholic acid and glycocholic acid, both about 200-fold) in humans with Mahvash disease, glucose levels are generally normal, but the levels of amino acids, especially alanine and glutamine, are clearly elevated.

Pinpointing the identity of the novel liver hormone requires tremendous amount of work. Parabiosis of GCGR knockout and wild-type mice was considered, but no such models were published. A more practical in vitro islet culture assay was adopted by most groups to screen for the liver hormone that stimulates α cells hyperplasia and hyperglucagonemia. With the islet culture assay, it is shown that a < 10 kDa

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Table 2 Novel endocrine functions of the liver

<table>
<thead>
<tr>
<th>Liver hormone</th>
<th>Target organ</th>
<th>Action on target organ</th>
<th>Alteration in liver diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acids</td>
<td>Pancreatic α cells</td>
<td>Stimulate cell proliferation and glucagon secretion</td>
<td>Not studied yet</td>
</tr>
<tr>
<td>Betatrophin</td>
<td>Pancreatic β cells (?)</td>
<td>Stimulate cell proliferation (?)</td>
<td>Increased in cirrhosis</td>
</tr>
<tr>
<td>Fetuin</td>
<td>Skeletal muscle; Adipose tissue</td>
<td>Decrease insulin sensitivity; Reduce adiponectin expression</td>
<td>Elevated in nonalcoholic fatty liver disease</td>
</tr>
<tr>
<td>FGF21</td>
<td>Adipose tissue; Brain</td>
<td>Increase insulin sensitivity; Reduce food intake</td>
<td>Elevated in nonalcoholic fatty liver disease</td>
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<tr>
<td>Activin E</td>
<td>Adipose tissue</td>
<td>Increase fat oxidation</td>
<td>Increased in nonalcoholic fatty liver disease</td>
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<tr>
<td>Tsukushi</td>
<td>Adipose tissue</td>
<td>Increase thermogenesis</td>
<td>Increased in nonalcoholic fatty liver disease</td>
</tr>
<tr>
<td>GPNMB</td>
<td>Adipose tissue</td>
<td>Increase lipogenesis</td>
<td>Increased in nonalcoholic fatty liver disease</td>
</tr>
</tbody>
</table>

FGF21: Fibroblast growth factor 21; GPNMB: Glycoprotein nonmetastatic melanoma protein B.
fraction of serum from GCGR knockout mice sufficiently stimulates α cell proliferation [75]. This fraction contains small proteins or peptides, lipids, amino acids, and metabolites [75]. We have discussed earlier that most proteins or peptides are unlikely the liver hormone. Eliminating lipids from the fraction does not change the activity of the fraction in stimulating α cell proliferation [75]. Finally, as amino acids levels are much higher in GCGR knockout serum, cocktails that mimic the amino acids levels in GCGR knockout mice serum have been tested for their ability to stimulate α cell proliferation, and indeed they do [73-75].

Individual amino acids were further tested to see if a particular amino acid is sufficient to stimulate α cell proliferation [73-75, 79]. So far, the data on individual amino acids are still somewhat controversial. Most individual amino acid do not stimulate α cell proliferation or glucagon secretion [73-75, 79]. Glutamine alone stimulated α cell proliferation in 2 studies, but it did not stimulate glucagon secretion in another, which is intriguing as α cell hyperplasia and hyperglucagonemia coexist in all models of GCGR inhibition [74, 75, 79]. Alanine alone stimulated α cell proliferation in one study, but not in another, albeit acutely stimulating glucagon release [75, 79]. Experimental conditions may explain some of the different results. It is also possible that α cell proliferation and acute glucagon release may be separate processes.

The α cell receptor for amino acids is under active research. In GCCR knockout mice and in wild-type type mice treated with inhibitory GCCR antibodies, the most upregulated α cell gene is the amino acid transporter Slc38a5 (20-80-fold increase) [74, 75]. Slc38a5 preferentially transports glutamine and several other amino acids, which is concordant with the stimulatory effect of glutamine on α cell proliferation [74, 75]. Slc38a5 knockout mice treated with inhibitory glucagon antibodies and Slc38a5 and GCCR double knockout mice exhibited less prominent α cell hyperplasia (~50% less) but similar hyperglucagonemia [74]; this data suggested that Slc38a5 is at least partially responsible for amino acid-stimulated α cell hyperplasia and that α cell hyperplasia and hyperglucagonemia may be regulated separately. Slc38a5, however, is not expressed in human α cells [74]. Another amino acid transporter Slc38a4 is enriched in human α cells when mice with human islet implants are treated with inhibitory GCCR antibodies [80]. In humans with Mahvash disease, Slc38a4 is expressed in the α cells [80], supporting a role of the amino acid transporter in mediating amino acid-stimulated α cell hyperplasia in humans as well. The mTOR pathway in α cells is activated by amino acids as well, contributing to α cell hyperplasia [73-75].

As a result of these studies, the α cell-liver axis has largely been clarified (Figure 1). The α cells secrete glucagon, which signals the liver to increase hepatic amino acid breakdown and reduce amino acid synthesis, consequently leading to desirable amino acid levels in the circulation. After glucagon signaling is inhibited, the liver decreases amino acid breakdown and increases amino acid synthesis, thus raising circulating amino acid levels. The amino acid levels, in turn, act on the α cell amino acid transporters to stimulate α cell proliferation. The evolutionarily conserved α cell-liver axis suggests that glucagon’s primary role may be regulating amino acid levels.

**Betatrophin**

Betatrophin (also known as angiopoietin-like protein 8, ANGPTL8) is a 22-kD protein produced and secreted by the liver and adipose tissue [81, 82]. Several years ago, betatrophin was touted as the long sought-after liver hormone that stimulates pancreatic β cell proliferation and insulin production in conditions with insulin resistance [83, 84]. An insulin resistance mouse model based on insulin receptor antagonist (S961) infusion exhibits remarkable hyperinsulinemia and beta cell hyperproliferation [83]. As S961 does not directly stimulate β cell proliferation, it was hypothesized that a humoral factor mediates the stimulation of β cell proliferation in this mouse model [83]. Screening of liver genes that were differentially expressed as a result of S961 infusion suggested that betatrophin, a secreted protein that is upregulated by S961 infusion, could be the humoral factor [83]. Betatrophin expression correlated well with β cell proliferation rates. The original report found that liver overexpression of betatrophin stimulated β cell proliferation [83].

The potential of betatrophin as the Holy Grail for diabetes treatment attracted much attention, but later experiments strongly argue against this function of betatrophin [85-87]. Betatrophin knockout mice exhibited normal glucose metabolism and similar hyperinsulinemia and β cell hyperproliferation in response to S961 infusion [85, 86]. Detailed analysis of pancreas morphometry by several laboratories definitively showed that betatrophin overexpression does not stimulate β cell proliferation [88]. The only exception was that direct delivery of betatrophin to pancreas does stimulate β cell proliferation in rats [89]. In some mouse models of diabetes, betatrophin lowered
Figure 1 Schematic drawing of regulation of pancreatic α cell number and glucagon secretion by amino acid levels controlled by the liver. The numbers indicate specific ways to disrupt glucagon signaling. (1) Glucagon deletion; (2) Prohormone convertase 2 deletion (with no mature glucagon secretion); (3) Glucagon receptor (GCGR) global deletion; (4) GCGR liver-specific deletion; (5) GCGR inactivating mutation; (6) GCGR antisense RNA; (7) GCGR antibodies; and (8) GCGR antagonists; (9) Gs secretion); (3) Glucagon receptor (GCGR) global deletion; (4) GCGR liver-specific deletion; (5) GCGR inactivating mutation; (6) GCGR antisense RNA; (7) GCGR antibodies; and (9) Gs deletion. See text for details. Citation: Yu R, Zheng Y, Lucas MB, Tong YG. Elusive liver factor that causes pancreatic α cell hyperplasia: A review of literature. World J Gastrointest Pathophysiol 2015; 6(4): 131-139. Copyright ©The Author(s) 2015. Published by Baishideng Publishing Group Inc[87]. GCGR: Glucagon receptor.

Betatrophin, however, could be a circulating marker of insulin resistance[82]. Early studies of betatrophin levels in various forms of human insulin resistance were quite conflicting, partly due to the differences in measurement methods[82]. Later studies using more standardized methods for measuring betatrophin were summarized by several meta-analyses on the correlation of circulating betatrophin levels and type 2 diabetes, gestational diabetes, polycystic ovary syndrome (PCOS), and obesity — all conditions with insulin resistance[91-95]. Xu et al[91] analyzed 25 such studies and showed a positive and significant correlation between circulating betatrophin levels and insulin resistance. Yue et al[92] analyzed 11 studies on betatrophin in type 2 diabetes and found that betatrophin is significantly elevated in type 2 diabetes. Kong et al[93] analyzed 8 studies on betatrophin in gestational diabetes and concluded that betatrophin is significantly elevated in gestational diabetes. Varikasvu et al[94] analyzed 11 studies on betatrophin in PCOS and concluded that betatrophin is significantly elevated in PCOS. Similarly, Ye et al[95] analyzed 6 studies on betatrophin in obesity and concluded that betatrophin is significantly elevated in obesity. Thus, overall, circulating betatrophin is likely a marker of insulin resistance in humans. The high betatrophin liver expression in mice treated with S961, in retrospect, could simply be a sign of insulin resistance caused by S961[83]. It is, however, not clear how insulin resistance upregulates betatrophin. In humans, hyperinsulinemia, often associated with insulin resistance, and metformin, an insulin sensitizer, both decrease betatrophin levels, suggesting that insulin resistance per se upregulates betatrophin levels[96]. Betatrophin overexpression could further worsen hepatocyte sensitivity to insulin, the significance of which needs to be further explored[97].

Betatrophin also has a role in lipids regulation[98]. Betatrophin knockout mice exhibit much reduced triglyceride levels due to reduction in liver VLDL secretion[96]; betatrophin also forms a complex with ANGPTL3, which inhibits lipoprotein lipase (LPL) activity[86]. The increased production of VLDL and decreased LPL activity both contribute to hypertriglyceridemia. Betatrophin overexpression doubles triglyceride levels in mice[86]. In humans, circulating betatrophin levels are positively correlated with triglyceride levels in the general population[99]. In people with dyslipidemia, however, betatrophin levels were lower than in controls[100]. Betatrophin may potentially be a target in dyslipidemia treatment[101].

Hepatokines

Hepatokines are metabolism-regulating proteins produced and secreted by the liver [102,103]. Several hepatokines have been reported and studied. Five of the most studied hepatokines are discussed in this review: Fetuin-A, fibroblast growth factor 21 (FGF21), activin E, Tsukushi, and glycoprotein nonmetastatic melanoma protein B (GPNMB).

Fetuin-A: Fetuin-A, also known as α-2-Heremans-Schmid glycoprotein in humans, is one of the first discovered hepatokines[104]. A 52-kD glycoprotein, fetuin-A has diverse metabolic functions[104]. Under physiological conditions, fetuin-A mostly functions as a carrier protein and regulates osteogenesis and inhibits extra-skeletal...
calification[105]. Fetuin-A’s role in regulating insulin sensitivity has also been studied in detail[106,107]. Fetuin-A knockout mice exhibit higher insulin sensitivity and have less tendency to develop obesity[106]. At the molecular level, fetuin-A inhibits insulin receptor phosphorylation in myocytes and adipocytes and adiponectin expression in adipocytes[107]. Fetuin-A levels are elevated in patients with insulin resistance or type 2 diabetes, likely mediated by high free fatty acid levels, and high fetuin-A levels are a risk factor for type 2 diabetes[108,109]. The thiazolidinedione-type diabetes medication pioglitazone directly inhibits hepatic production of fetuin-A, partly contributing to its action in improving insulin sensitivity[110].

**FGF21:** FGF21 is a hepatokine that was first discovered in 2000, but its metabolic regulation functions were not characterized until recently[111,112]. Although FGF21 is also expressed in adipose tissue and the pancreas, circulating FGF21 is predominantly derived from the liver[113]. Hepatic FGF21 expression is regulated by a number of physiological conditions and factors[114]. Prolonged starvation (> 7 d) and overnutrition both upregulate FGF21 expression[115,116]. Glucagon and the thyroid hormone triiodothyronine (T3) both stimulate FGF21 expression, while insulin may inhibit FGF21 expression in liver[117,118]. High-carbohydrate, high-fat diet, and low protein diets stimulate FGF21 expression as well[119,120]. The microRNAs miR-577 and miR-212 target FGF21 mRNA for degradation, thus suppressing FGF21 expression[121,122]. FGF21 is also upregulated by ER stress[123]. At the molecular level, at least some of the above actions are mediated by the nuclear hormone receptor peroxisome proliferation-activated receptor α (PPARα), which binds to regions of the FGF21 promoter and simulates FGF21 expression[124-126].

The human pre-FGF21 (precursor of mature FGF21) includes a 28-amino-acid signaling peptide and a 181-amino-acid FGF21 proper as the circulating form[127]. FGF21 signals through its transmembrane tyrosine kinase receptors, FGFR1c and FGFR3c, and its transmembrane co-receptor, Klotho-β (KLB)[128]. FGF21 downstream signaling is tissue-specific but generally leads to metabolic benefits such as increased insulin sensitivity and weight loss[129]. In the adipose tissue, FGF21 stimulates the Ras/Raf/MAPK pathway, with phosphorylation of ERK1 and ERK2, and the mTOR pathway, contributing to higher insulin sensitivity[130-132]. Other FGF21 metabolic benefits such as weight loss is mediated by non-adipose tissue such as the brain[133]. FGF21 has been a major interest of metabolic drug development. As the native FGF21 is not stable in the usual formulation, re-engineered FGF21 analogues and PEGylated FGF21 have been developed to be more stable[134]. Activating monoclonal antibodies targeting FGFR1-β-klotho have also been developed[135]. Preclinical and clinical studies have demonstrated clear metabolic benefits of the FGF21 analogs and activating antibodies, such as appetite suppression, weight loss, improved glycemia, and favorable lipid profile[134,135].

**Activin E:** Activin E belongs to the family of transforming growth factor-β (TGF-β) proteins[136]. Activin E is a secreted homodimer of inhibin-βE, which is mainly expressed in the liver[137]. Each mature inhibin-βE monomer has 113 amino acids [137]. In both mice and humans, inhibin-βE is upregulated by obesity and insulin resistance[138]. In mice, hepatic overexpression of inhibin-βE prevents excess weight gain and improves insulin sensitivity by promoting energy expenditure via increased fat oxidation[139,140]. Inhibin-βE ablation in mice gives conflicting results[138,139]. In one study using the transcriptional activator-like effector nucleases (TALENs) to remove liver specific inhibin-βE expression, inhibin-βE-deficient mice exhibited normal weight but had impaired thermogenesis during cold exposure[139]. In another study, however, use of small interfering RNA (siRNA) to silence Inhibin-βE expression in the liver reduced weight gain in obese mice[138]. Thus, the roles of Activin E in metabolic regulation are still controversial.

**Tsukushi:** Tsukushi belongs to the family of small leucine-rich proteoglycan (SLRP) extracellular matrix proteins[141]. The secreted human Tsukushi protein has 337 amino acids. Besides its role in regulating embryonic development, Tsukushi is found to be a hepatokine, potentially regulating adipose tissue, weight, and energy expenditure[142]. In both mice and humans, Tsukushi is upregulated by thyroid hormone[142,143]; in mice, Tsukushi is induced by obesity and cold exposure[142]. Tsukushi deficiency in mice protects them from diet-induced obesity by increasing adipose tissue thermogenesis and energy expenditure[142]. Using mice from a different genetic background, another group could not reproduce the metabolic benefits of Tsukushi deficiency[144]. Furthermore, studies have also failed to show deleterious metabolic effects from Tsukushi overexpression[144]. The roles of
Tsukushi in metabolic regulation thus also remain controversial.

**GPNMB:** GPNMB is a transmembrane glycoprotein expressed in the liver and other organs\[145]. The cleaved extracellular domain of GPNMB (a glycosylated 480-amino-acid protein) is a hepatokine targeting adipose tissue\[146,147]. In 2 obese mouse models, GPNMB expression was upregulated in the liver and secreted GPNMB levels were higher as well\[148]. Secreted GPNMB stimulates lipogenesis in vitro and in vivo\[147]. A neutralizing antibody targeting GPNMB reduces obesity and improves insulin sensitivity\[147]. In both mice and humans, GPNMB levels are positively correlated with obesity and insulin resistance\[147]. GPNMB is thus a promising therapeutic target for treatments of obesity and diabetes.

**CONCLUSION**

The liver has numerous endocrine functions such as direct hormone and hepatokine production, hormone metabolism, synthesis of binding proteins, and processing and redistribution of metabolic fuels. In the last 10 years, many new endocrine functions of the liver have been discovered (Figure 2). Several novel endocrine functions of the liver have been unraveled. The liver plays a key negative feedback regulatory role in the pancreatic α cell-liver axis which regulates pancreatic α cell mass, glucagon secretion, and circulating amino acid levels. Betatrophin and other hepatokines such as fetuin-A and FGF21 play important endocrine roles in modulating insulin sensitivity, lipid metabolism, and body fat weight. It is expected that more endocrine functions of the liver will be discovered in the near future. As endocrine function of the liver is a rapidly evolving field, controversial findings often exist; caution needs to be taken when interpreting novel findings to avoid over-simplification of complex metabolic processes and premature allocation of research resources.

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Liver endocrine functions

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Rhyu J et al. Liver endocrine functions


MINIREVIEWS

Current strategies to induce liver remnant hypertrophy before major liver resection

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Abstract

Hepatic resection is the gold standard for patients affected by primary or metastatic liver tumors but is hampered by the risk of post-hepatectomy liver failure. Despite recent improvements, liver surgery still requires excellent clinical judgement in selecting patients for surgery and, above all, efficient pre-operative strategies to provide adequate future liver remnant. The aim of this article is to review the literature on the rational, the preliminary assessment, the advantages as well as the limits of each existing technique for preparing the liver for major hepatectomy.

Key Words: Liver regeneration; Major hepatectomy; Liver insufficiency; Future liver remnant; Portal vein embolization

Core Tip: Hepatic resection is the gold standard for patients affected by liver tumors but is hampered by the risk of post-hepatectomy liver failure. We herein review the literature on the rational, the preliminary assessment, the advantages as well as the limits of each existing technique for preparing the liver for major hepatectomy.
LIVER REGENERATION

The liver’s unique capacity for regeneration was first recorded in the legend of Prometheus in Greek mythology and it represents the basis of the treatment of many liver diseases. Regeneration of the liver is a pathophysiological process, embracing both hypertrophy (increase in cell size or protein content in the prereplicative phase) and hyperplasia (increase in cell numbers). Both events can take place independently [9]. The mechanisms of liver regeneration have mainly been studied after extensive hepatectomy. The players of regeneration following the different techniques exposed in this article are thought to be similar to those after hepatectomy, but the precise mechanism remains unknown. Basically, the regeneration process is a cytokine- and growth-factor-mediated pathway. The main cytokine-mediated pathways include members of the innate immune system, tumor necrosis factor (TNF)α and interleukin (IL)-6, and growth-factor-mediated pathways are regulated by HGF and transforming growth factor (TGF)-β[10]. It is a multi-step process, starting from the “priming” of hepatocytes, the moment they acquire replicative capacity, followed by the proliferative step in which an adequate cell mass is attained, and a termination stage in which liver cell proliferation is ended once the necessary functional mass has been reached[11]. Proliferation of hepatocytes advances from periportal to pericentral areas of the lobule, as a wave of mitoses[12]. Proliferation of biliary epithelial cells occurs a
little later than hepatocytes. The particularity of liver regeneration is that replacement of the lost hepatic mass is not mediated by selected stem cells proliferation but it entirely depends on mature adult hepatocytes and other hepatic cell types. Concerning the time interval, as far as we know, normal liver weight is reestablished within 8-15 d in humans[13].

**POST-HEPATECTOMY LIVER FAILURE**

Although morbidity and mortality after liver surgery have improved over the past 10 years, PHLF is still reported in up to 8%, ranging from 1.2% to 32%, and depends on the patient’s condition and functional reserve of the liver before resection[1]. Different definitions of PHLF are available. In 2011, the International Study Group of Liver Surgery (ISGLS) defined PHLF as “a post-operatively acquired deterioration in the ability of the liver to maintain its synthetic, excretory, and detoxifying functions, which are characterized by an increased International Normalized Ratio (INR) and concomitant hyperbilirubinemia on or after postoperative day 5”[14]. It is worth pointing out that severe PHLF is associated with a mortality rate of 54%.

A related syndrome that results in a transient but sometimes fatal form of liver failure has been described following liver transplantation (LT) but also after extensive liver resection. This is the so-called Small For Size Syndrome (SFSS). In 2005, Dahm et al[15] defined SFSS as a graft to recipient weight ratio < 0.8% alongside two of the following for three consecutive days; bilirubin > 100 mmol/L, INR > 2 and encephalopathy grade 3 or 4. In this definition, SFSS is a clinical syndrome characterized by post-operative liver dysfunction, prolonged cholestasis and coagulopathy, portal hypertension and ascites. It can lead to a higher rate of hemorrhage, sepsis and gastrointestinal bleeding[16]. The key point of SFSS is the presence of portal hypertension and intra-hepatic portal congestion as the underlying cause of liver failure[17].

**PREDICTION OF PHLF RISK**

Despite improvements in surgical and postoperative management, parameters determining the degree of possible hepatectomy remain largely uncertain. Different patient related and surgical factors have to be considered to decrease PHLF incidence. Surgical factors include the extent of resection and volume of FLR, duration of intraoperative liver ischemia during portal pedicle clamping, duration of surgery and the need for blood transfusion. The risk of PHLF is highly influenced by the quality of underlying liver parenchyma. The type of underlying liver parenchyma is frequently assessed by preoperative liver biopsy, but noninvasive methods, such as liver stiffness, are now available. For example, liver stiffness measurement by transient elastography (Fibroscan) predicts persistent hepatic decompensation in patients undergoing resection for hepatocellular carcinoma[18].

It is generally thought that the minimal functional liver mass needed for adequate postoperative liver function is estimated to be 20%-25% in patients with normal liver parenchyma, whereas those with chemotherapy-induced liver injury require a FLR volume of approximately 30%, while those with cirrhosis at least a 40% minimal functional liver mass[19]. Therefore, standardized FLR volume can be easily evaluated by a tridimensional computed tomography (CT) reconstruction method, as FLR/estimated total liver volume[20]. Estimated total liver volume is generally calculated using a formula based on body surface area[21].

In addition to volume, estimation of FLR function is an important factor. Typical biochemical parameters, such as liver function tests, albumin, and clotting factors must be evaluated. The old but effective Child-Turcotte-Pugh score, which was introduced in 1964, still represents a simple system for grading liver function[22]. The model for end-stage liver disease score, which is mainly used in liver transplantation, can also predict the survival rate of cirrhotic patients to better select ideal candidates for surgery[23]. A recent study also showed that mean serum level of hyaluronic acid can be a useful tool, especially when liver biopsy is not feasible[24].

Dynamic tests of liver function can also be used. The most well-known is indocyanine green (ICG) clearance. ICG is a water soluble, inert, fluorescent tricarbocyanine dye with protein binding close to 95% (mainly, alpha1- and beta-lipoproteins and albumin), a hepatic extraction rate above 70%, and is almost completely excreted in its unchanged form by the liver. ICG elimination can be
expressed as ICG plasma disappearance rate (ICGPDR) or retention rate at 15 min (ICGR15), reflecting liver function. Use of the ICG test for patient selection has been shown to decrease postoperative mortality[25].

In recent years, there have been several attempts to assess hepatobiliary magnetic resonance imaging (MRI) as a tool to predict liver dysfunction. Since it was first described in 1991 by Weinmann et al[26], MRI has been showed to provide both global and segmental liver function information, and postoperative remnant liver function thanks to the measurement of liver signal intensity in the hepatobiliary phase.

Liver function evaluation by nuclear medicine techniques is also more and more used. Dynamic 99mTc-macrovoidin hepatobiliary scintigraphy has been used to provide quantitative information on total and regional liver function. The hepatic uptake of 99mTc-macrovoidin is similar to the uptake of organic anions such as bilirubin[27]. This technique efficiently estimates the risk of postoperative liver failure especially in patients with uncertain quality of liver parenchyma[28]. The 99mTc-GSA is another recently proposed agent that is not affected by hyperbilirubinemia and can be used for liver function assessment in cholestatic patients[29]. Finally, the LiMAx test allows real-time in vivo determination of liver Cytochrome P450 1A2 (CYP1A2) activity. The CYP1A2 is not influenced by cholestasis or drugs and is ubiquitous in liver parenchyma. Intravenous administration of 13C methacetin, a substance exclusively metabolized by CYP1A2, with continuous real-time breath analysis represents the basis of the LiMAx test[30].

PORTAL VEIN EMBOLIZATION

Since the first report in 1986, PVE has progressively become the gold standard for inducing liver hypertrophy with satisfying safety and efficacy[31]. Initially described by laparotomy, the portal system access is now obtained by percutaneous puncture of the portal vein. According to the operator’s preference, an ipsilateral or contralateral approach can be chosen, in reference to the segment bearing the tumor. The ipsilateral approach has the main advantage of protecting the FLR from injury[2] whereas the contralateral approach facilitates embolization[32]. Irrespective of the approach chosen, PVE is performed in a retrograde manner (Figure 1). Many embolic materials have been used for PVE without significant differences in terms of hypertrophy. Embolic materials include fibrin glue, N-butyl-2-cyanoacrylate and ethiodized oil, gelatin sponge and thrombin, coils, microparticles e.g., polyvinyl alcohol (PVA) particles or tris-acyrl gelatin microspheres and absolute alcohol[33]. A non-absorbable material is generally used. However, interesting results were reported with the use of an absorbable powder material (Gelfoam® powder, Pfizer, New York, USA) that lasts approximately 2 wk, leading to temporary PVE. In an animal model, this method showed efficient and stable liver regeneration[34]. These results were confirmed in a limited preliminary series in clinical practice[35] and a prospective study is undergoing (EMBORES study, NCT02945059). One of the advantages of temporary PVE is that it can theoretically be repeated several times to boost more liver hypertrophy, as has been suggested in an animal model[36].

PVE is successfully performed in more than 90% of cases[37]. A computed tomography scan with volumetric evaluation is generally performed between 4 and 8 wk after embolization. PVE induces a FLR hypertrophy than can reach 40%[37], with a low 2% morbidity rate and no mortality in the vast majority of studies[37-39]. PVE is considered an efficient method, allowing successful hepatectomy in more than 70% of cases[37,38,40].

Contraindications to PVE are extensive portal thrombus and important portal hypertension[41]. Another potential limit of PVE is the risk of tumor growth during the 4 to 8 wk separating PVE and liver surgery. In addition, several authors have suggested that PVE itself could promote tumor growth within the emboled liver[42-45]. Among others, these reasons have led to the development of alternative strategies.

PORTAL VEIN LIGATION (PVL) AND TWO-STAGE HEPATECTOMY

As it requires a surgical procedure with portal pedicles dissection, PVL is nowadays mainly indicated in the setting of two-stage hepatectomy (TSH) for the treatment of bilobar liver disease[46,47]. In the TSH strategy, the first surgical step includes tumoral clearance of the FLR (usually by parenchymal sparing resections or locoregional treatment like radiofrequency ablation) and concomitant PVL that allows FLR growth.
In the second step, after liver regeneration (approximately 4 to 8 wk later), major liver resection is performed (usually right or right extended hepatectomy). Similarly, PVL can be performed for the management of patients presenting synchronous colorectal metastases or neuroendocrine tumors [47]. The first surgical step associates colorectal resection with PVL, followed by major liver surgery in the second procedure. However, many centers have adopted PVE (performed by the percutaneous approach after FLR clearance or colorectal resection) for two-step procedures, avoiding portal pedicle dissection and facilitating the second procedure [48].

It was initially suggested that PVE resulted in superior FLR growth compared to PVL [49] as in theory PVE allows distal portal obstruction which decreases the possibility of intrahepatic collateral development. Several studies demonstrated that the results are globally similar [50, 51]. In fact, the debate concerning the efficiency of PVL compared to PVE is no longer relevant. PVL requires a surgical procedure and can appear as an alternative to PVE only when a two-step surgery is planned. In other cases, percutaneous PVE is clearly a simpler and better tolerated approach.

ASSOCIATING LIVER PARTITION AND PORTAL VEIN LIGATION FOR STAGED HEPATECTOMY

The aim of this alternative strategy, described by Schnizbauer et al. [52] in 2012, is to induce rapid and massive liver hypertrophy, to allow liver surgery in a short period of time in patients with initially very limited FLR volume. The first step of the associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) procedure consists of performing PVL and an in situ splitting of the liver parenchyma, leaving the hepatic artery, bile duct, and hepatic vein intact until the subsequent operation. This first surgical step can be associated with tumoral clearance of the FRL. During the second operation (that can be performed one to two weeks later) the remaining hepatic artery, bile duct, and hepatic vein are divided and the liver specimen is extracted (Figure 3).

The first report demonstrated a morbidity rate of 44% and a mortality rate of 12% [52], and triggered an intense debate on the safety of this procedure, limiting its promotion worldwide. The morbi-mortality rate decreased with experience but remains high, with approximately 40% of major postoperative complications and 9% of mortality [53]. Nevertheless, the ALPPS technique induces more than 65% of FLR growth in approximately 7 days [52-55] and the second procedure is feasible in more than 90% of cases [56]. The main advantage of the ALPPS procedure is the rapid increase in FLR volume in a short interval and therefore a shorter interval between the two stages. Although the volumetric results of this technique are impressive, several authors suggested that FLR volume hypertrophy is not correlated to functional improvement [57, 58] which could partly explain the high morbidity of the procedure. Besides, concerns have been raised by some authors regarding potentially poorer oncological results comparing to the classical TSH [59]. The results of a meta-analysis comparing ALPPS to TSH showed that the extent of FLR increase was not different.
between the two groups[60]. The time needed to reach final liver volume was shorter in ALPPS than in the TSH approach[60]. In this meta-analysis, ALPPS was associated with a higher incidence of major and overall morbidity and mortality compared to TSH[60]. However, in a recent randomized controlled trial, Hasselgren et al[61] observed similar morbidity between ALPPS and classical TSH and an improved survival in the ALPPS group.

To decrease complication rate, a variety of technical modifications have been proposed such as partial-ALPPS, mini-ALPPS, tourniquet-ALPPS, hybrid-ALPPS, microwave ablation-assisted ALPPS and radiofrequency ablation-assisted ALPPS. Huang et al[62] suggested in a systematic review that a partial ALPPS technique in which only partial parenchymal sparing is performed during the first surgical step could achieve lower morbidity and mortality rates, reaching the same FLR hypertrophy rate as ALPPS in non-cirrhotic patients.

**SEQUENTIAL TRANS-ARTERIAL EMBOLIZATION (TAE) AND PORTAL VEIN EMBOLIZATION**

Although PVE remains the gold standard for FLR hypertrophy, two concerns persist with this approach: An insufficient contralateral hypertrophy, particularly in patients with underlying liver disease (steatosis, fibrosis or cirrhosis), and the eventuality of tumor progression while waiting for the non-embolized liver to hypertrophy. In particular, portal flow interruption may induce a compensatory increase in arterial blood flow of embolized segments and result in a paradoxical growth of tumors vascularized by arterial blood flow. In this context, it has been postulated that the addition of trans-arterial embolization (TAE) or trans-arterial chemoembolization (TACE) would produce more rapid and extensive FLR growth (by obtaining...
obliteration of intrahepatic arterioporal shunts) and may help to counteract the stimulating effect on tumor growth[63]. Therefore, hepatocellular carcinomas, which are tumors particularly vascularized by arterial blood flow and develop generally in underlying pathological liver parenchyma, are the main target of this combined strategy[64].

During TAE, a catheter is directly inserted via either the common femoral or left radial artery and an intra-arterial injection of a combination of microspheres and PVA particles is performed in the arterial branches of the segments to be resected. During TACE, an intra-arterial injection of a cytotoxic drug is performed such as doxorubicin, epirubicin, idarubicin, mitomycin C, or cisplatin, that is emulsified in ethiodized oil (Lipiodol®Ultra-Fluid, Guerbet). This is followed by intra-arterial injection of an embolic agent, such as gelatin sponge, PVA particles, or microspheres[65] (Figure 4). TACE can also be performed using recently developed drug-eluting beads (DEB) that allow the slow release of chemotherapeutic agents, and increase ischemia intensity and duration[65].

A sequential approach, with a time interval of a few days, is recommended to limit the risk of nontumoral liver ischemic necrosis[66] and TAE is mostly performed before PVE[66,67]. Although the number of patients reported in studies that evaluated this approach is limited, observed FLR hypertrophy is generally superior to that observed after isolated PVE. For example, Yoo et al[68] reported a statistically significant increase of 7.3% and 5.8% in FLR (over the total liver volume) for sequential TACE/PVE and isolated PVE, respectively.

An important elevation of transaminases is generally observed after this sequential approach without important clinical consequences. In the largest series reporting this approach, Peng et al[64] reported 29 procedures without deaths and only one complication and 27 patients (93%) underwent subsequent hepatectomy. Post-hepatectomy morbidity and mortality among these patients was 27.5% and 6.9%, respectively.

Theoretical contraindications of this method include extensive portal thrombus, important portal hypertension or previous biliary surgery (biliodigestive anastomosis) which exposes the patient to hepatic abscess formation after arterial embolization.

**LIVER VENOUS DEPRIVATION**

This technique consists of performing conventional PVE and ipsilateral hepatic vein obstruction (Figure 5). By associating hepatic vein embolization, the aim is to eliminate any residual portal vein flow and reduce hepatic artery inflow which can further encourage liver regeneration. Initially described as a sequential approach in which hepatic vein embolization is secondarily performed in case of insufficient FLR growth after PVE, it was demonstrated that both procedures (portal and hepatic vein embolization) can be performed simultaneously[69,70]. This novel approach is particularly interesting as it allows important liver regeneration with good tolerance. Although no study comparing ALPPS to LVD is available, it has been suggested that LVD could overcome the limits of ALPPS, abolishing the necessity of two major surgical interventions in close sequence.

Firstly, PVE is performed as previously described. For hepatic vein embolization, a vascular plug is placed in the proximal part of the hepatic vein to avoid migration of embolization agent. The vein is then embolized with a mixture of ethiodized oil and N- butyl cyanoacrylate[71]. The term “extended LVD” is used for concomitant embolization of the right and middle hepatic vein with the right portal branch[57].

The results of this approach on FLR increase are superior to those observed after isolated PVE. In a recent large comparative study, Laurent et al[71] observed a FLR volume increase of 28.9% after PVE compared to 61.2% after LVD (P < 0.0001). In this study, LVD allowed surgery in 86.4% of patients and no PHLF was reported. Kobayashi et al[72] observed similar results with a superior FLR hypertrophy after LVD compared to PVE (35% vs 24%, P = 0.034). In addition, the tolerance of LVD seems to be similar to the tolerance of isolated PVE[71,72].

**RADIATION LOBECTOMY**

This recent approach is derived from trans-arterial radioembolization with yttrium-90 [73]. In radiation lobectomy (RL), radioembolization of both the tumor and the non-tumoral liver parenchyma that will be secondarily resected is performed, which
Figure 4 Sequential embolization. A: Trans-arterial embolization; B: Portal vein embolization of the right liver (authors’ own work).

Figure 5 Right liver venous derivation associates in a sequential or concomitant approach. A: Right portal vein embolization; B: Ipsilateral hepatic vein embolization (authors’ own work).

requires higher radiation doses[74,75]. This technique allows concomitant tumoral control and FLR increase. One major advantage of this approach is that it could be carried out in patients with portal vein thrombosis[75].

The procedure is well-tolerated[74] with transient moderate adverse events. Results in terms of FLR volume growth are very similar to those observed after PVE. Vouche et al.[74] reported 45% of FLR hypertrophy and observed a correlation between the presence of a portal vein thrombosis and FLR growth. However, series reporting major liver resection after RL are scarce[76,77]. Andel et al.[77] recently reported 10 major hepatectomies in patients that were initially treated with RL for insufficient functional FLR. The RL allowed a 41% increase in FLR volume with 84% of FLR function increase (evaluated on scintigraphy). All resections were performed without major intraoperative problems. Only one patient developed a serious complication not directly related to the liver surgery and other complications were mild.
Table 1 Indication, advantages, and disadvantages of existing approaches to induce liver remnant hypertrophy before major liver resection

<table>
<thead>
<tr>
<th>Approach</th>
<th>Indication</th>
<th>Advantage</th>
<th>Disadvantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVE</td>
<td>Insufficient FLR volume</td>
<td>Percutaneous approach</td>
<td>Contraindicated in patients with extensive portal thrombus and important portal hypertension; Could promote tumoral growth within the embolized liver</td>
</tr>
<tr>
<td>PVL and two-stage heptectomy</td>
<td>Insufficient FLR volume and treatment of bilobar liver disease</td>
<td>PVL is performed during the first surgical step (tumoral clearance of the FLR)</td>
<td>Surgical procedure; Morbidity</td>
</tr>
<tr>
<td>Associating liver partition and PVL for staged heptectomy</td>
<td>Insufficient FLR volume +/- treatment of bilobar liver disease</td>
<td>Liver surgery is performed in a short period of time (15d); First surgical step (PVL and in situ splitting of the liver parenchyma) can be associated with tumoral clearance of the FLR</td>
<td>Surgical procedure; Morbidity</td>
</tr>
<tr>
<td>Sequential trans arterial embolization and PVE</td>
<td>Insufficient FLR volume in patients with hepatocellular carcinoma</td>
<td>Percutaneous approachMay help to counteract the stimulating effect of PVE on tumor growth</td>
<td>Sequential approach (two procedures) is recommended to limit the risk of nontumoral liver ischemic necrosis; Contraindicated in patients with extensive portal thrombus, important portal hypertension or previous biliary surgery (biliodigestive anastomosis)</td>
</tr>
<tr>
<td>Liver venous deprivation</td>
<td>Insufficient FLR volume</td>
<td>Percutaneous approach</td>
<td>Contraindicated in patients with extensive portal thrombus and important portal hypertension; Could promote tumoral growth within the embolized liver</td>
</tr>
<tr>
<td>RL</td>
<td>Insufficient FLR volume</td>
<td>Percutaneous approachConcomitant tumoral control and FLR increaseCan be carried out in patients with portal vein thrombosis</td>
<td>Data reporting liver resection after RL is scarce</td>
</tr>
</tbody>
</table>

PVE: Portal vein embolization; FLR: Future liver remnant; PVL: Portal vein ligation; RL: Radiation lobectomy.

CONCLUSION

Careful initial evaluation of FLR volume and function is crucial before planning major liver resection. When required, several approaches are now available to decrease the risk of PHLF (Table 1) and thus postoperative mortality. Although PVE remains the gold standard, recent techniques that are derived from PVE might play an increasingly important role in future years.

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Health-related quality of life in autoimmune hepatitis

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Abstract
Autoimmune hepatitis (AIH) is a severe chronic autoimmune disease and has a significant impact on the patient’s quality of life, in particular regarding psychological problems such as anxiety and depression. Consistent evidence on which patient-related, disease-related or physician-related factors cause health-related quality of life (HRQoL) impairment in patients with AIH is lacking. Current studies on HRQoL in AIH are mainly single-centered, comprising small numbers of patients, and difficult to compare because of the use of different questionnaires, patient populations, and cutoff values. Literature in the pediatric field is sparse, but suggests that children/adolescents with AIH have a lower HRQoL. Knowledge of HRQoL and cohesive factors in AIH are important to improve healthcare for AIH patients, for example by developing an AIH-specific chronic healthcare model. By recognizing the importance of quality of life beyond the concept of biochemical and histological remission, clinicians allow us to seek enhancements and possible interventions in the management of AIH, aiming at
Autoimmune hepatitis (AIH) is a severe chronic autoimmune disease that occurs mainly in women and affects health-related quality of life (HRQoL) worldwide. The diagnosis of AIH is based on the presence of autoantibodies, typical features on liver histology, and increased immunoglobulin G (IgG) levels[1]. The presentation of AIH is variable, ranging from mild and asymptomatic disease to fulminant hepatic failure. Nonspecific symptoms at presentation are fatigue, anorexia, jaundice, and abdominal pain, whereas others are asymptomatic at disease onset[1]. The majority of patients need lifelong treatment to prevent disease progression to cirrhosis and/or decompensation[2]. Current treatment strategies in AIH include administering corticosteroids (mainly prednisolone) and a long-term corticosteroid-sparing regime, including azathioprine (AZA) as first-line treatment[3,4]. Second-line immunosuppressants include mycophenolate mofetil (MMF), calcineurin inhibitors (CNIs), and mercaptopurine and have proven to be effective in mainly uncontrolled studies[5].

The main goal of AIH treatment is to achieve complete biochemical and histological remission without the occurrence of side effects. Alanine aminotransferase, aspartate aminotransferase and IgG serum levels are used as parameters to monitor biochemical response, and current guidelines advocate pursuit of normalization of those parameters as the aim of treatment. As a result, treatment failure, defined as absence of normalization of transaminases, triggers clinical actions such as increase of drug dose or change in drug class. A sole focus on biochemical response is insufficient when managing AIH. From a patient perspective, other aspects that affect HRQoL, including but not limited to side effects, psychological health, and implications of the disease, are just as important.

One of the main objectives relating to AIH according to the International Autoimmune Hepatitis Group (IAIHG), is better assessment of HRQoL in patients. However, literature or guidelines on that topic in AIH are scarce and inconsistent. An update on current literature on HRQoL in AIH, is warranted to reveal the most important research gaps[6]. Understanding which potentially treatable factors are associated with reduced quality of life in patients with AIH is essential for development of interventions targeting well-being. The focus of this paper is to review the current knowledge of HRQoL and associated factors in AIH, to comment on the current status, and to identify future perspectives that may influence and benefit disease management of adult patients with AIH.

Key Words: Autoimmune hepatitis; Quality of life; Depression; Anxiety; Corticosteroids

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METHODOLOGY

We searched the titles, abstracts, and MeSH terms of articles indexed in PubMed using the keywords “autoimmune hepatitis,” “AIH,” “health-related quality of life,” and “quality of life.” The search was limited to articles published before January 27, 2021. We included articles based on the following criteria: (1) Full-text articles published in peer-reviewed journals; (2) English or Dutch articles; (3) Publication dates within the last 20 years at the time of the search; and (4) Either adult or pediatric AIH. The search retrieved 116 publications; 39 were evaluated in full-text after screening the titles and abstracts (Figure 1). We also checked the reference lists of the included articles to identify other articles. For the purpose of this review, we primarily focused on articles addressing the role of HRQoL in AIH.

HRQOL IN ADULT PATIENTS WITH AIH

Several studies have reported reduced general or liver-specific HRQoL in AIH patients (Tables 1 and 2)[7-15]. The first study published was conducted in the Netherlands and showed a reduced quality of life in 141 patients with AIH compared with healthy controls, using three instruments, the SF-36 for generic HRQoL, the Multidimensional Fatigue Index-20, and the Liver Disease Symptom Index 2.0, which is a liver-specific questionnaire addressing nine topics. In particular, patients had lower scores in subscales measuring physical problems or general health. Patients with AIH mentioned fatigue more often than healthy controls did[13]. A landmark study performed in Germany compared 102 AIH patients to the German general population and to published data of patients with arthritis using the SF-12[12]. They reported lower mental well-being in patients with AIH compared with both groups, but the physical component score (PCS) was unaffected[12]. A Polish single-center study showed that patients with AIH (n = 140) scored significantly worse in all subscales of the SF-36, except for one measuring the impact of emotional problems on work and daily activities[15]. The majority of the AIH patients in that cohort had cirrhosis (55%), and as in the previously mentioned study, that did not have a significant effect on well-being. A recent Italian multicenter study of chronic liver disease reported that of a total of seven different chronic liver diseases without cirrhosis, patients with AIH had a lower quality of life measured with the EQ-5D VAS score, and experienced difficulties in the self-care domain, even after adjusting for multiple possible confounders, including age, sex, education, and professional status[10]. That was confirmed in a Cuban study in which AIH patients had lower quality of life scores than hepatitis B patients using the disease-specific Chronic Liver Disease Questionnaire (CLDQ)[7]. Only one meta-analysis was performed, including three studies that evaluated HRQoL measured with the SF-36. The analysis confirmed reduction of the PCS and mild reduction of the mental component score in patients with AIH. However, they included only older studies and compared all AIH patients (including Dutch and German patients) to the United States general population norm [16]. Finally, the largest study conducted so far involved multiple health centers in the United Kingdom and confirmed previous results by finding that the HRQoL of patients with AIH (n = 990) was worse than it was in the general population, adjusted for age and gender and using the EQ-5D-5L[14]. Although these studies consistently report a lower HRQoL in AIH, albeit in varying domains, it remains difficult to compare the studies because of the use of different questionnaires (EQ-5D-5L vs SF-12 or SF-36 vs CLDQ), cutoff values, methodology, and patient populations. Moreover, most studies were conducted at single centers and included small numbers of participants, thereby introducing bias based on the heterogeneity in study populations (e.g., remission status and demographic differences).

HRQOL IN PEDIATRIC PATIENTS WITH AIH

A lower HRQoL was also found in children and adolescents with AIH, although literature in the pediatric field is sparse[17-19]. A study performed in Portugal compared 43 children with AIH to 62 healthy children using the Pediatric Quality of Life Inventory 4.0 (PedsQL 4.0)[17]. They found that especially children with associated comorbidities (e.g., inflammatory bowel disease, hemolytic anemia, and hypothyroidism) had a lower quality of life. That was confirmed in a Brazilian cohort using the same questionnaire[18]. Interestingly, the evaluation of HRQoL in the
Table 1 Overview of the studies assessing aspects of health-related quality of life in autoimmune hepatitis

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Country</th>
<th>Population (n)</th>
<th>Biochemical remission (%)</th>
<th>Cirrhosis (%)</th>
<th>Questionnaire</th>
<th>Factors/results</th>
</tr>
</thead>
<tbody>
<tr>
<td>van der Plas et al [13], 2007</td>
<td>The Netherlands</td>
<td>AIH (142), other liver diseases (776)</td>
<td>-</td>
<td>-</td>
<td>SF-36, MFI-20, LDSI</td>
<td>HRQoL impairment; Association with: Fatigue</td>
</tr>
<tr>
<td>Afsandy et al [6], 2009</td>
<td>United States, Italy</td>
<td>AIH (13), other chronic liver diseases (1090)</td>
<td>-</td>
<td>84.6</td>
<td>SF-36</td>
<td>HRQoL impairment; Negative correlation: Age (every scale), female gender (primary predictor of mental health), cirrhosis (every scale, primary predictor of physical health)</td>
</tr>
<tr>
<td>Schramm et al [12], 2014</td>
<td>Germany</td>
<td>AIH (103)</td>
<td>77</td>
<td>27</td>
<td>SF-12, PHQ-9, GAD-7</td>
<td>HRQoL impairment (total mental score/mental well-being); Association with: depression and anxiety (positive correlation with female gender, corticosteroid use, and concerns about progression of the liver disease)</td>
</tr>
<tr>
<td>Takahashi et al [11], 2018</td>
<td>Japan</td>
<td>AIH (265), chronic hepatitis C (88)</td>
<td>-</td>
<td>10.6</td>
<td>CLDQ, SF-36</td>
<td>HRQoL impairment; Negative correlation: Age, cirrhosis, comorbid diseases, corticosteroid use (worry domain), disease duration, AST; Positive correlation: platelet count</td>
</tr>
<tr>
<td>Wong et al [14], 2018</td>
<td>United Kingdom</td>
<td>AIH (990)</td>
<td>56</td>
<td>33</td>
<td>EQ-5D-5L, FIS, CFQ, HADS</td>
<td>HRQoL impairment; Positive correlation: Biochemical remission; Negative correlation: overlap syndromes, corticosteroid use, and calcineurin inhibitor use</td>
</tr>
<tr>
<td>Janik et al [15], 2019</td>
<td>Poland</td>
<td>AIH (140)</td>
<td>-</td>
<td>55</td>
<td>SF-36, MFIS, PHQ-9, STAI</td>
<td>HRQoL impairment (every scale, except role emotional); Negative correlation: Female gender, depression, trend toward better HRQoL (physical health) with budesonide vs prednisone; Association with: Anxiety, depression, and fatigue</td>
</tr>
<tr>
<td>Dirks et al [9], 2019</td>
<td>Germany</td>
<td>AIH (27), AIH/PBC (8), other liver diseases (97)</td>
<td>-</td>
<td>0</td>
<td>SF-36, FIS, HADS</td>
<td>HRQoL impairment; Association with: Anxiety, depression, and fatigue</td>
</tr>
<tr>
<td>Castellanos-Fernández et al [7], 2021</td>
<td>Cuba</td>
<td>AIH (22), overlap syndrome of AIH and PBC (7), PBC (14), other liver diseases (500)</td>
<td>-</td>
<td>43.9</td>
<td>FACT-F, WPAI:SHP, CLDQ</td>
<td>HRQoL impairment; Positive correlation: Male gender, exercising &gt; 90 min/wk; Negative correlation: Fatigue, abdominal pain, anxiety, depression, and extrahepatic comorbidity (diabetes mellitus type 2, sleep apnea)</td>
</tr>
<tr>
<td>Cortesi et al [16], 2020</td>
<td>Italy</td>
<td>AIH (51), other chronic liver diseases (2911)</td>
<td>-</td>
<td>0</td>
<td>EQ-5D-3L</td>
<td>HRQoL impairment in AIH</td>
</tr>
</tbody>
</table>

1Eight patients with Child-Pugh class A and three patients with Child-Pugh class C.
2Cirrhosis in patients with autoimmune liver diseases (n = 43). AIH: Autoimmune hepatitis; HRQoL: Health-related quality of life; PBC: Primary biliary cholangitis; AST: Aspartate aminotransferase; CFQ: Cognitive failure questionnaire; CLDQ: Chronic liver disease questionnaire; ECR: Experiences in close relationship scale; EQ-5D-5L/3L: European quality of life 5-dimension 5-level/3-level; FACT-F: Functional assessment of chronic illness therapy-fatigue; FIS: Fatigue impact scale; GAD-7: Generalized anxiety disorder screener; HADS: Hospital anxiety depression scale; LDSI: Liver disease symptom index 2.0; MFI-20: Multidimensional fatigue index-20; PHQ-9: Patient health questionnaire; SF-12: Short-form 12; SF-36: Short-form 36; STAI: State-trait anxiety inventory; WPAI:SHP: Work productivity and activity-specific health problem.

parents differed from the children’s self-reports[18]. Only the physical and total scores were significantly lower in patients with AIH based on the parental reports, whereas in the children’s reports the emotional, school, physical, and total scores were significantly lower.
Determinants of HRQoL in AIH

The HRQoL of patients with chronic diseases can be affected by various patient-related, disease-related, and physician-related factors. We have summarized the patient-, disease- and physician-related factors that are liable to influence HRQoL in AIH in Figure 2.

Patient-related factors

Patients with AIH are more often diagnosed with symptoms of depression and anxiety compared with the general population or healthy controls[7,9,10,12,15]. Studies by Schramm and Janik et al[15] showed a significantly higher percentage of depression...
and anxiety symptoms, measured with the PHQ-9, GAD-7, or State-Trait Anxiety Inventory [12,15]. Depression was strongly correlated with both physical and mental components of SF-36. Despite biochemical remission in 77% of the patients (n = 103), the occurrence of severe depressive symptoms within the German cohort appeared to be five times as frequent compared with the general population. In addition, even AIH patients without cirrhosis revealed more problems with regard to depression and anxiety compared with the general population [10]. It is interesting to note that psychological stress was also associated with relapses in patients with AIH type 1 [20].

Other patient-related factors, particularly age and sex, have been described often in previous studies [7,11,12,14]. Studies in the United Kingdom and Japan reported a negative correlation between age and HRQoL [11,14], but Polish and Cuban studies did not find such a correlation [7,15]. With respect to sex differences, female patients experience more symptoms of depression [12,15] and have a worse quality of life than their male counterparts [7,15]. In our experience, women experience weight increase and other cosmetic changes associated with corticosteroids as a great inconvenience in
In contrast, a study in the United Kingdom study found that the female sex was associated with a higher quality of life, albeit in an unadjusted regression analysis. These inconsistent correlations highlight that we still do not know which patient factors are important when assessing HRQoL in patients with AIH.

For all chronic liver diseases, it holds that lifestyle changes are part of the treatment. While tackling lifestyle is a hot topic in chronic disease, it is infrequently addressed in AIH. However, patients should still be informed about the risk of specific lifestyles, such as overweight, alcohol misuse, and sedentary behavior. Losing weight, more exercise, and a healthier diet contribute to successful management of chronic liver diseases and cirrhosis. Indeed, exercising for more than 90 min/wk is a predictor of a better quality of life in patients with chronic liver diseases (e.g., AIH). Other study confirmed that an increased body mass index was associated with a lower quality of life in patients with AIH. In addition, alcohol consumption presents a clear risk of the progression of liver fibrosis in chronic liver diseases. Other factors, such as education level, socioeconomic data, smoking, or losing weight, were not frequently mentioned in the described studies. It follows that physicians need to communicate with patients about lifestyle adaptations through motivational interviews.

Coping with chronic conditions and taking medication daily goes hand in hand with discomfort, which potentially results in reduced HRQoL. Patients with more than one chronic disease that take daily medication have a lower quality of life. Adherence to treatment is rarely discussed with patients but has a great impact on well-being and treatment response. A high psychosocial burden has been shown to significantly decrease adherence to treatment and to be associated with poor treatment response. Therefore, prompt recognition of symptoms of depression and anxiety is important to improve patient adherence and lead to better response to treatment. Various factors may influence adherence to drug treatment in adolescents with AIH, particularly depression, anxiety, younger age, sex, prednisone dose, and long-term therapy have been found in previous studies. In liver transplant recipients, marital status (if the patient is divorced) and having mental distress are associated with reduced self-reported adherence to immunotherapy. However, information on demographic factors or socioeconomic data, including the status of a relationship, were not explicitly examined in all previous studies, which would be necessary for more detailed conclusions.

### Disease-related factors

As mentioned previously, the main objective in treating AIH is to achieve complete biochemical and histological remission without side effects. While it is plausible that achieving biochemical remission results in better HRQoL, the association has not been studied often. One study found that patients with biochemical remission had a significantly higher quality of life. One could speculate that incomplete biochemical remission causes uncertainty about, and possibly fear of, a relapse, which is understandable given that every relapse increases the risk of decompensated liver failure or the necessity of liver transplantation. Whether this has a role in AIH is unknown at present.

Liver cirrhosis, or an advanced stage of fibrosis in patients with chronic liver disease is a known cause for reduced HRQoL, independent of the underlying liver disease. However, studies in patients with AIH demonstrate significant variability regarding the relation between fibrosis and HRQoL. Most studies describe that having liver fibrosis or compensated cirrhosis does not affect patient well-being in general. In contrast, another study did find an impaired physical condition in patients with AIH using the same SF-36 questionnaire and an overall lower quality of life using the CLDQ. Plausible explanations for the discrepancy are the use of different general vs. disease-specific, SF-36 vs. SF-12 vs. EQ-5D-5L questionnaires and the inclusion of different AIH populations regarding biochemical remission status and disease duration. Interestingly, none of the cited studies included AIH patients with decompensated cirrhosis in their cohort, which is known to be a major factor for reduced HRQoL in cirrhosis with other etiologies.

Patients with an overlap syndrome or a variant syndrome of AIH and primary biliary cholangitis (PBC) or primary sclerosing cholangitis (PSC), had a worse quality of life than patients not reporting those comorbidities. In addition, fatigue is a typical symptom in patients with characteristics of PBC, and is expected to have a negative impact on HRQoL. In that context, it is not only essential to treat both AIH and the overlapping syndrome (i.e., PBC or PSC), but also to address associated symptoms (i.e., IBD in PSC, itch in PBC) in the patients. Interestingly, such a correlation was not found in a study in children with autoimmune liver diseases.
found no differences in HRQoL scores in children with AIH vs overlap syndrome or variant syndrome with PSC[19]. Extrahepatic manifestations, for example thyroid disease, insulin-dependent diabetes mellitus, connective tissue disorders, and autoimmune skin disease, are common in AIH and can affect well-being, including fatigue, but the effect on HRQoL is unstudied so far[32].

A large proportion of patients with AIH receive corticosteroid therapy[11,33]. All treatments have specific side effects[34,35], but long-term use of corticosteroids is well-known for its undesirable effects, including osteoporosis, mood swings, depression, obesity, cognitive dysfunction, chronic fatigue, and reduced physical activity[1,5]. The negative impact of the use of corticosteroids on HRQoL was demonstrated in several studies[12,14]. In the United Kingdom cohort, corticosteroids were extensively linked to impaired HRQoL. Even patients who received low-dose of corticosteroids, and independent of their biochemical status, had a lower HRQoL[14]. Schramm et al[12] found a significant correlation between corticosteroids and depression. Sockalingam et al[23] found that patients with a moderate or high PHQ-9 score of > 10 were administered a significantly higher dose of prednisone compared with patients with a score of < 10. These data give additional support for steroid well-being or HRQoL, such as markers of disease activity or disease duration, are so far unknown[12,15].

Currently, AZA is still the primary choice for maintenance therapy, and was not directly associated with a lower quality of life or health utility in a large cross-sectional analysis[14]. It is important to note that the use of AZA is associated with an increased risk of lymphoma and nonmelanoma skin cancer[36,37]. Although lymphoma in the long term is rare, it has to be taken into account that the occurrence of these side effects, or even the patient’s concerns, might affect their quality of life. AZA may also cause hair loss that leads to alopecia. The possibility is frequently raised by the female patients and may affect various aspects of quality of life and lead to incompliance. The effect of other prescribed therapies on improving psychosocial outcomes, such as mycophenolate mofetil and mercaptopurine, is unknown. However, calcineurin inhibitors that have undesirable effects may be associated with lower health utility[14].

**Physician-related factors**

Physician-related factors are usually not addressed in studies and are thus difficult to take into account. Schramm et al[12] found that patient concerns about the severity of their disease, and being fearful of cirrhosis (mostly unnecessary) were factors associated with depression and anxiety symptoms. Providing the patient with information on his/her illness or medications and involving the patient in treatment options, can contribute to the patient’s well-being. Whether the location of care (i.e. transplant vs nontransplant center) matters is uncertain. One study showed that there was no difference in health utility between transplant and nontransplant centers[14], and another found that biochemical remission rates were higher in transplant centers compared with nontransplant centers[33]. Both were conducted in the United Kingdom. Extrapolation of the results to other countries is difficult given the differences in health care management among countries.

**CONCLUSION**

It is clear that patients with AIH experience a lower quality of life and have more psychological problems, such as anxiety and depression, compared with the general population. Consistent evidence on which patient-related, disease-related, or physician-related factors cause HRQoL impairment in patients with AIH is lacking. Most studies did not include information on important socioeconomic, disease behavior, maintenance treatment, or even geographical factors, whereas they are known to affect patient well-being and HRQoL in other chronic liver diseases. In addition, some aspects of AIH are unexplored so far, for example the effect of lifestyle changes, extrahepatic manifestations, and patient counseling on HRQoL. Studies addressing HRQoL in pediatric AIH and their parents/support team are scarce and are desperately needed as a first step to improve their well-being.

Knowledge of HRQoL and associated factors in AIH are important to improve healthcare for AIH patients, for example by incorporating the factors in a chronic healthcare model (CCM). A CCM provides a clear approach for managing chronic diseases, with focus on assessment of the modifiable factors affecting the disease in
order to improve patient well-being. While no studies mentioned a CCM for AIH so far, some studies discussed elements that could be part of a model. For example, Janik et al.[38] screened AIH patients for moderately severe depression and redirected them to a psychiatrist and psychiatric therapeutic interventions in case of a PHQ ≥ 15 points. Another example are lifestyle interventions for overweight patients. There is also a role for the development of a disease-specific questionnaire for AIH patients, similar to the PBC-40 questionnaire, to measure the patient’s perspective of the disease.[40]. In what way, a CCM can be developed and implemented that would probably differ from country to country because of differences in health care. However, it is paramount that the AIH-specific CCM incorporate the most important factors of HRQoL in AIH, as discussed in this review.

Finally, HRQoL should not only be targeted in everyday clinical treatment approaches, but also as an important outcome of clinical trials and a research objective per se. Most studies of HRQoL in AIH have been conducted at a single center and comprised small numbers of patients, which underlines the need for collaboration between healthcare centers in different countries. Currently, there is an ongoing multicenter, cross-sectional study of HRQoL in patients with AIH within the European Network for Rare Liver Diseases. Recognizing the importance that quality of life has for the patient beyond the concept of biochemical and histological remission allows us to strive for significant improvements in management of adult and pediatric AIH.

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Sockalingam S, Blank D, Abdelhamid N, Abbey SE, Hirschfield GM. Identifying opportunities to improve management of autoimmune hepatitis: evaluation of drug adherence and psychosocial factors.
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MINIREVIEWS

Fungal infections following liver transplantation

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Abstract

With increasing morbidity and mortality from chronic liver disease and acute liver failure, the need for liver transplantation is on the rise. Most of these patients are extremely vulnerable to infections as they are immune-compromised and have other chronic co-morbid conditions. Despite the recent advances in practice and improvement in diagnostic surveillance and treatment modalities, a major portion of these patients continue to be affected by post-transplant infections. Of these, fungal infections are particularly notorious given their vague and insidious onset and are very challenging to diagnose. This mini-review aims to discuss the incidence of fungal infections following liver transplantation, the different fungi involved, the risk factors, which predispose these patients to such infections, associated diagnostic challenges, and the role of prophylaxis. The population at risk is increasingly old and frail, suffering from various other co-morbid conditions, and needs special attention. To improve care and to decrease the burden of such infections, we need to identify the at-risk population with more robust clinical and diagnostic parameters. A more robust global consensus and stringent guidelines are needed to fight against resistant microbes and maintain the longevity of current antimicrobial therapies.

Key Words: Invasive fungal infections; Liver transplantation; Candidiasis; Antifungal prophylaxis; Aspergillosis; Cryptococcus

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INTRODUCTION

Liver transplantation is one of the principal treatment modalities for the treatment of many hepatic diseases, mainly but not limited to chronic and end-stage liver disease. Despite advances in the field of transplantation, invasive fungal infections remain a major source of morbidity and mortality. This is attributed to delay in diagnosis, nonspecific clinical features[1], fastidious nature of these organisms, lack of consensus on prophylactic regimens, and rise of antifungal resistant species.

Moreover, with an increase in the number of grafts being offered, there is a trend towards recipients being older, debilitated, and having more non hepatic comorbidities which contributes to the burden and subsequently leads to a higher rate of fungal infections[2].

In this article, we aim to discuss the incidence and trend of invasive fungal infections (IFI) in liver transplant (LT) patients, associated risk factors, diagnostic challenges, and data on prophylaxis.

IFI DEFINITION

IFIs, according to the Invasive Fungal Infections Cooperative Group in Europe and the Mycoses Study Group in the United States, are divided into 3 categories: proven, probable and possible.

Proven IFI is defined as a positive fungal culture or histological proof of fungal or hyphal elements in a sterile site biopsy. This also includes positive cryptococcal antigen in cerebrospinal fluid.

Probable and possible IFIs have a wider definition and inclusion criteria. This is based on several host factors along with various clinical and mycological criteria[3].

Some studies evaluating prophylactic regimens, in this regard have been a focus of criticism as their IFI’s were considered colonization rather than infection[4].

INCIDENCE AND RESPONSIBLE FUNGI

The incidence of IFI after LT has decreased in recent years and this is attributable to advancement and improvement in surgical techniques along with more aggressive post-operative care. Previously, in one study by Fung et al[5], the incidence of IFI after LT was reported to be 6.6% with a mortality of 54.5%. The ninety-day cumulative mortality after invasive candidiasis has been reported to be 26% and 1-year survival after invasive aspergillosis is about 59% according to TRANSNET in 2010[6].

More recently, according to some cohort studies, the overall incidence of IFI after solid organ transplant is about 1%-4%[7-9]. 1-year cumulative probability of IFI in LT was 1.8%[7]. This shows a promising trend and is related to improvise surgical techniques and timely recognition of risk factors that make certain patients more susceptible to IFIs.
However, in underdeveloped nations, it remains higher at 14.7% with an in-hospital mortality rate of 77%[10]. A future streamlined approach to the problem with specific guidelines might be one of the ways to improve these numbers.

The three major fungi involved are Candida spp., Cryptococcus, and Aspergillus spp. Candida predominates with 81% followed by Aspergillus (16%) and Cryptococcus (3%). Non-Albicans Candida accounted for 68% of all Candida infections[11]. The rise of resistant non-Albicans Candida especially C. parapsilosis was felt to coincide with the increased use of fluconazole[11]. C. parapsilosis is associated with increased mortality in these patients. This increase in resistant fungal species indicates a dire need for a patient-specific prophylactic regimen based on risk factors vs a universal approach.

The distribution of the fungal species remains similar in the East with Candida representing 64.1% and Aspergillus 35.8% of the IFIs in LT patients.

Despite the highly variable clinical presentation, these pathogens most commonly affect the respiratory system followed by renal and gastrointestinal tract[10]. According to a retrospective study in 2015 by Eschenauer and colleagues, intra-abdominal candidiasis (73%) was the most common IFI[12]. The common clinical manifestations of various fungal organisms are shown in Table 1.

**TIMING FROM TRANSPLANT TO INFECTION**

There has been a shift in the time duration between the developments of IFIs after LT. It was initially thought to occur in the early post-operative phase most commonly within the first couple of months.

Grauhan et al[13] in 1994 reported a median time from LT to IFI of 2 mo.

According to Husain et al[14] in 2003, the median time to infection for invasive candidiasis was 13.5 d with 72% of the IFIs happening within the first month after LT.

Aspergillus tends to present later as compared to Candida. Results from one study by Singh and colleagues in 2003 reported 55% of their Aspergillus IFI occurring after 90 d[15] and Gravalda et al[16] also described 43% of their IFIs as late onset Aspergillus.

In transplant centers with a higher risk of Aspergillus based on epidemiology, this delayed time to presentation is important to consider while deciding on the length of prophylactic regimen in high-risk patients. Moreover, clinicians need to be mindful of this time frame while diagnosing an already difficult-to-diagnose disease.

**RISK FACTORS**

Multiple factors have been observed over time to be associated with the development of fungal infections in LT. Identifying patients that are at high risk for developing IFI can be of immense help as that can aide in decreasing the diagnostic delay and assure appropriate prophylaxis. By adopting this targeted method of prophylaxis vs universal approach, we can also potentially reduce the incidence of drug-resistant fungi, lower the morbidity due to side effects and interactions of these medications particularly with immunosuppressants, and mitigate the overall cost.

Many scientists over the past few decades have worked on identifying these attributes. These can be categorized into pre-operative, operative, and post-operative factors as shown in Table 2. Risk factors for Aspergillus specifically seem to depend more on post-operative factors as highlighted in Figure 1.

Collins et al[17] in 1994 identified the following as potential risk factors: renal insufficiency, length of transplant operation, rate of re-transplantation, abdominal or intra-thoracic reoperation, and cytomegalovirus infection.

Other studies showed that model for end-stage liver disease (MELD) scores > 25, post-transplant acute kidney injury (Cr > 2 or risk, injury, failure, loss of kidney function, and end-stage n criteria I- or F-) and pre-transplant fungal colonization seem to be the culprits identified with IFIs[11,18].

One of these was an important and common risk factor of daily prophylactic fluconazole dose of < 200 mg, which was thought to cause a rise in drug-resistant non-Albicans Candida spp[11].

Although very rare, a French study also identified contamination during organ procurement as a risk factor with a 1.33% prevalence of Candida spp. in preservation fluid. This was associated with a higher rate of IFI and impaired survival[19].

Alongside predictable risk factors like diabetes and hemodialysis dependence, Verma et al[10] pointed out prior antibiotic use, cerebral and respiratory organ failures, chronic liver failure (CLIF) organ failure/CLIF-consortium acute-on-chronic liver
Table 1 Common clinical manifestations of invasive fungal infection

<table>
<thead>
<tr>
<th>Clinical manifestations</th>
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<tbody>
<tr>
<td><strong>Candida</strong></td>
</tr>
<tr>
<td>Intra-abdominal abscesses</td>
</tr>
<tr>
<td>Recurrent cholangitis</td>
</tr>
<tr>
<td>Peritonitis</td>
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<tr>
<td>Fungemia</td>
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<tr>
<td><strong>Aspergillus</strong></td>
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<tr>
<td>Invasive pulmonary <em>Aspergilosis</em></td>
</tr>
<tr>
<td>Brain abscess</td>
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<tr>
<td>Endophthalmitis</td>
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<tr>
<td>Osteomyelitis</td>
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<tr>
<td>Endocarditis</td>
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<tr>
<td><strong>Cryptococcus</strong></td>
</tr>
<tr>
<td>CNS infection</td>
</tr>
<tr>
<td>Focal lesions on imaging</td>
</tr>
<tr>
<td>Meningeal enhancement</td>
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</table>

CNS: Central nervous system.

Table 2 Risk factors for invasive fungal infections

<table>
<thead>
<tr>
<th>Risk factors</th>
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<tbody>
<tr>
<td><strong>Pre-operative</strong></td>
</tr>
<tr>
<td>SBP prophylaxis with fluoroquinolone</td>
</tr>
<tr>
<td><strong>Operative</strong></td>
</tr>
<tr>
<td>Retransplantation</td>
</tr>
<tr>
<td>Long transplantation time</td>
</tr>
<tr>
<td>Long transplantation time</td>
</tr>
<tr>
<td>Class 2 partial or complete match</td>
</tr>
<tr>
<td>Donor from male</td>
</tr>
<tr>
<td><strong>Post-operative</strong></td>
</tr>
<tr>
<td>Post-transplant HD</td>
</tr>
<tr>
<td>High number of RBC units transfused</td>
</tr>
<tr>
<td>Post-transplant bacterial infection</td>
</tr>
<tr>
<td>Cytomegalovirus infection</td>
</tr>
<tr>
<td>Use of muromonab-CD3</td>
</tr>
<tr>
<td>Aspergillus antigenemia</td>
</tr>
</tbody>
</table>

SBP: Spontaneous bacterial prophylaxis; HD: Hemodialysis; RBC: Red blood cells.

failure as predictors of IFIs. Non-survivors in their study also had higher levels of 1.3-beta D glucan (BDG) levels. BDG levels have been studied as a diagnostic marker and look promising.

There has been a general shift in the trend of risk factors over the last 2 decades, which is attributable to better surgical techniques. Singh *et al* [20] studied 190 liver transplants during 1990 and 2000 and demonstrated improvement in length of operation, intraoperative transfusion requirements, use of roux-en-Y biliary anastomosis, re-transplantation, rate of rejection over time, and cold ischemic time. This led to a decrease in the incidence of invasive candidiasis in this study population from 9%-1.7% without any use of antifungal prophylaxis.

In 2015, Eschenauer and colleagues identified bile leaks within the first 30 d post-transplant and living donor liver transplants as new independent risk factors for IFIs. This is because *Candida* has an affinity for growth in the biliary tract. Moreover, living donor liver transplants are highly technical procedures that are not commonly
performed in the United States. The increased length and complexity of these procedures along with higher disruption of the biliary tract is responsible for these findings. The authors recommended instituting antifungal prophylaxis in all living donor liver transplants [12].

A small study recently in 2020 by Jorgenson et al [21] studied the effects of pre-transplant roux-en Y gastric bypass on liver transplant outcomes. There were increased rates of fungal infection in patients with bariatric surgery before transplant and might be associated with loss of defense provided by gastric acid. This study is limited by its retrospective nature and its size.

**DIAGNOSTIC CHALLENGES**

In general, fungal infections do not present themselves vividly and are increasingly difficult to grow in culture media. It makes it even more challenging in patients who have chronic liver disease, are immunosuppressed, or have other underlying comorbidities. They are difficult to detect clinically and also objectively in laboratories. Hence, prevention becomes essential, and it has significantly improved in the last decade with the advancement in surgical techniques, intense pre-operative evaluation, and appropriate use of antifungal prophylactic agents in high-risk patients.

Distinguishing between colonization and true infection can be challenging for the clinician. Apart from proven IFI as discussed above, the other two categories are vague and have plenty of variable factors. In these clinical scenarios, the use of newer diagnostic tools like BDG and galactomannan (GM) can be helpful. Polymerase chain reaction fungal assays are promising but not yet approved by the Food and Drug Administration.

BDG has been studied and looks promising as a diagnostic marker in serum. In a study from 2017, with 271 transplant patients, weekly BDG was tested and monitored for IFIs. 95% of the patients with IFI had positive BDG and a very promising negative predictive value of 96% was seen. The sensitivity of BDG was 75% and specificity was 65%, making it a very good tool to rule out IFIs [22].

The GM test is an enzyme-linked immunosorbent assay that detects the GM antigen released by *Aspergillus* hyphae when they invade host cells.

The patient’s epidemiological risk factors should be considered strongly which would help guide better towards increasing clinical suspicion and ordering appropriate tests and guided treatments. Objective risk factors such as the MELD score, the overall duration of need for total parenteral nutrition, length of the operative procedure, and removal of abdominal drains and other catheters or lines should be evaluated [23].
PROPHYLAXIS

Fungal infections in liver transplant recipients are mostly attributed to Aspergillus and Candida. Three agents are mainly used in prophylaxis—fluconazole, liposomal amphotericin B, and itraconazole. The studies involving these agents have been confounded by the difficulty of differentiating colonization and a true infection, the variability between patient selection, therapeutic agent(s) used in comparison with placebo or each other, and variable duration of treatment.

Data on the effectiveness of antifungal prophylaxis in LT over the past 10 years have been summarized in the Table 3 below.

There have been three meta-analyses as summarized in Table 3. Playford et al[24] and Cruciani et al[25] published two in 2006, with 10 and 6 studies respectively. These summarized that universal fungal prophylaxis leads to a reduction in proven IFIs without any mortality benefit. This universal approach leads to a significantly higher proportion of episodes of non-Albicans Candida infection.

In 2014, Evans et al[26] published a meta-analysis of studies on prophylaxis to prevent IFIs after LT and concluded that the odds of proven IFI and IFI related mortality were lower in patients receiving antifungal prophylaxis, even if the overall mortality did not change. It was also demonstrated that the efficacy of fluconazole compared to liposomal amphotericin was similar with the latter having the benefit of not altering the cytochrome P450 system and therefore not affecting the calci-neurin inhibitor levels. However, fluconazole is favored because of its cost-effectiveness and safety profile. This meta-analysis did not reveal any information on echinocandins, however, it was different from their counterparts in that they did a mixed treatment comparison and was more recent of the few meta-analyses already on the subject matter.

Studies since 2014 (after the last meta-analysis) on prophylaxis are summarized in Table 4.

In 2015, Eschenauer and colleagues performed a retrospective study involving liver transplant patients that were divided into three main groups. Group 1 included 145 patients who received targeted prophylaxis with either voriconazole in 54%, fluconazole in 5% or no antifungal which was the case of 38% of these patients. This was compared to a group of 237 patients, who received universal prophylaxis with voriconazole. These regimens were continued for a median time of 11 d in the targeted group and for 6 d in the universal group, with a significant P value. There was no statistical difference between incidence of IFI between both groups (6.8% in targeted and 4.2% in universal). Similarly, the P value was not statistically significant for the mortality rates over 100 d from IFIs in both groups (10% for targeted and 7% for universal group). They, therefore concluded that targeted approach to antifungal use in liver transplant patients was a safe, cost effective strategy and prevented unnecessary side effects[12].

With regards to echinocandins, Saliba et al[27] in 2015 compared micafungin vs standard treatment and found them equally effective. Standard therapy was center-specific and included IV fluconazole, liposomal amphotericin, or IV caspofungin.

Similarly, in a study from Spain in 2016, caspofungin was compared to fluconazole in high-risk patients and similar efficacy was reported to prevent global IFIs. In this study caspofungin was related to decrease in breakthrough IFIs and also led to a lower rate of invasive aspergillosis[28].

Echinocandins should be considered as prophylactic agents, where appropriate, especially in areas of increased prevalence of drug-resistant non-Albicans Candida. Unfortunately, these too come with a higher price tag compared to fluconazole which can affect their use, especially in non-affluent countries.

According to the Infectious Disease Society of America guidelines, patients who meet 2 or more of the following risk factors to be considered for prophylaxis: creatinine more than 2 mg/dL, need for re-transplantation, choledochojunostomy, more than 11 h of operative time, need to transfuse with ≥ 40 units of blood products, evidence of fungal colonization in immediate pre and post-operative days. Suggested duration of antifungal use is 14-21 d.

However, since the current data suggest that the incidence and risk of fungal infection overall in the general liver transplantation population is low, these agents should be utilized for higher-risk patients as unguided use is associated with drug-resistant non-Albicans Candida infection and higher mortality in these patients[23].
Table 3 Effectiveness of antifungal prophylaxis in liver transplant

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Trials</th>
<th>Patients</th>
<th>Regimens</th>
<th>Infection reduction (95%CI)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cruciani et al [25], 2006</td>
<td>6</td>
<td>698</td>
<td>AmB vs Pla (1)</td>
<td>Total proven fungal infections RR 0.31 (0.21-0.46), IFI RR 0.33 (0.18-0.59)</td>
<td>Patients receiving prophylaxis had higher number of non-Albicans proven fungal infections. Mostly C. glabrata.</td>
</tr>
<tr>
<td></td>
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<td>Flu vs nonsystemic AF (1)</td>
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<td>Flu vs Pla (2)</td>
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<td>Itra vs Pla (1)</td>
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<td>Amb-Itra vs Flu-Itra vs Pla (1)</td>
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<tr>
<td>Playford et al [24], 2006</td>
<td>7</td>
<td>793</td>
<td>Flu vs Pla (2)</td>
<td>Proven IFI RR 0.39 (0.18-0.85), fungal colonization RR 0.51 (0.41-0.62), fungal colonization with C. glabrata/C. krusei, RR 1.57 (0.76-3.24)</td>
<td>Formulated algorithm in which patients with &lt; 2 RF deemed low risk (4% incidence) for IFI and those with ≥ 2 at high risk (25% incidence) for IFI.</td>
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<td>Flu vs nonsystemic AF (2)</td>
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<td>Itra vs Pla (2)</td>
<td></td>
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<td></td>
<td></td>
<td>AmB vs Pla (1)</td>
<td></td>
</tr>
<tr>
<td>Evans et al [26], 2014</td>
<td>14</td>
<td>1633</td>
<td>Flu vs Pla/nonabs AF (4)</td>
<td>Proven IFI OR 0.37 (0.19-0.72), P = 0.003, Bayesian MTC, AmB vs Pla OR 0.21 (0.05-0.71), Flu vs Pla OR 0.21 (0.06-0.57)</td>
<td>Benefit of AmB is of similar magnitude to that previously described for fluconazole.</td>
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<td></td>
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<td>Itra vs Pla (1)</td>
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<td>AmB vs Pla (1)</td>
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<td></td>
<td>3 arm study with Pla/AmB/Flu (1)</td>
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<td>Flu vs AmB (3)</td>
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<td>Liposomal + Flu vs standard AmB + Flu</td>
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<td>Itra vs Flu (2)</td>
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<td>Micafungin vs standard care (1)</td>
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<td></td>
<td>Clo vs Nys (1)</td>
<td></td>
</tr>
</tbody>
</table>

AmB: Amphotericin-B; Pla: Placebo; Flu: Fluconazole; AF: Antifungal; Itra: Itraconazole; Nonabs AF: Nonabsorbable antifungal; Nys: Nystatin; Clo: Clofotrimazole.

**CONCLUSION**

Fungal infections following liver transplantation remain an influential cause of morbidity and mortality in these patients, despite the low incidence. Identification of high-risk patients based on risk factors discussed above and starting an appropriate prophylactic antifungal regimen based on epidemiology, calcineurin inhibitor use, and renal function is the first step in avoiding dealing with this evasive disease.

Prophylactic antifungals are generally well tolerated but can lead to drug-resistant *Candida* spp., hence the importance of selecting the appropriate patient and agent. Using BDG as a negative predictive tool and having a high degree of suspicion, even if the time from transplant exceeds 2 mo, can prevent diagnostic delays.

Further randomized controlled trials comparing azoles, amphotericin, and echinocandins are needed to develop an updated standard of care.
**Table 4 Studies since 2014 (after the last meta-analysis) on prophylaxis for liver transplant**

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Design</th>
<th>Regimen</th>
<th>Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antunes et al</td>
<td>Single center. Retrospective</td>
<td>High risk group: AmB vs nystatin; Low risk group: nystatin</td>
<td>Higher IFI in high risk patients who did not receive AmB</td>
</tr>
<tr>
<td>[29], 2014</td>
<td>(n = 461)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Winston et al</td>
<td>Randomized, double-blind.</td>
<td>Group 1: Andulafugin; Group 2: Flu</td>
<td>1:1 randomized. Similar cumulative IFI occurrence and equal 3 mo mortality</td>
</tr>
<tr>
<td>[30], 2014</td>
<td>(n = 200)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saliba et al</td>
<td>Randomized, open label.</td>
<td>Micafungin vs center specific standard care (Flu/AmB/Caspo)</td>
<td>Micafungin was non-inferior to standard of care</td>
</tr>
<tr>
<td>[27], 2015</td>
<td>(n = 347)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Giannella et al</td>
<td>Prospective, non-randomized.</td>
<td>Amb 10 mg/kg Q weekly until hospital discharge for a minimum of 2 wk</td>
<td>10 patients discontinued therapy. (6 for AmB related AEs and 4 for IFI)</td>
</tr>
<tr>
<td>[31], 2015</td>
<td>2009-2013 Safety of high dose AmB (n = 76)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[12], 2015</td>
<td>2008-2012. Effectiveness of targeted prophylaxis (n = 381)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Balogh et al</td>
<td>Single center study.</td>
<td>Voriconazole vs oral nystatin or Flu</td>
<td>No episodes of IA occurred. No difference in graft and patient survival curves between the two groups</td>
</tr>
<tr>
<td>[32], 2016</td>
<td>2008-2014 (n = 314)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perrella et al</td>
<td>Single center study.</td>
<td>Group 1: AmB 3 mg/kg/day; Group2: Caspofungin 70 mg loading→50 mg/day</td>
<td>No episodes of IFI in both groups</td>
</tr>
<tr>
<td>[33], 2016</td>
<td>2006-2012. Comparative observational study for targeted prophylaxis (n = 54)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fortian et al</td>
<td>Multicenter. 2005-2012.</td>
<td>Group 1: Caspofungin 50 mg/d; Group 2: Flu median 200 mg/day</td>
<td>Similar 6 m IFI occurrence 5.2% (G1) vs 12.2% (G2)). Reduced risk of IA in LT receiving caspofungin. Similar overall mortality</td>
</tr>
<tr>
<td>[28], 2016</td>
<td>Comparative observational study for targeted prophylaxis (n = 195)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chen et al</td>
<td>Single center study.</td>
<td>Group 1: Anidulafugin 100 mg/day or micafungin 100 mg/day; Group 2: No prophylaxis</td>
<td>High risk patients MELD &gt; 20; Similar IFI occurrence lower cumulative mortality in group 1 (P = 0.001)</td>
</tr>
<tr>
<td>[34], 2016</td>
<td>2005-2014. Effectiveness of targeted prophylaxis (n = 402)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Giannella et al</td>
<td>Retrospective, single center.</td>
<td>Group 1: No RF: No prophylaxis; Group 2: 1RF IC, Flu; Group3: High risk, anti mould agent</td>
<td>Antifungal prophylaxis administered to 45.9% patients. Cumulative IFI prevalence 6.3%. Flu independently associated with IFI development</td>
</tr>
<tr>
<td>[35], 2016</td>
<td>2010-2014. Evaluation of RF for a targeted prophylaxis (n = 303)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lavezzo et al</td>
<td>Single center study.</td>
<td>Group 1 high risk: AmB; Group 2 low risk: No prophylaxis</td>
<td>Overall IFI prevalence 2.8%. 1 yr mortality higher in prophylaxis group (P = 0.001). 1 yr mortality higher in IFI patients (P &lt; 0.001)</td>
</tr>
<tr>
<td>[36], 2018</td>
<td>2011-2015. Effectiveness of targeted prophylaxis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jorgenson et al</td>
<td>Single center study.</td>
<td>Group 1: Flu 400 mg/d for 14 d for high risk patients; Group 2: unsupervised antifungal protocols</td>
<td>Reduction in 1 yr IFI among high risk group (12.5% vs 26.6%). Similar 1 yr patient and graft survival</td>
</tr>
<tr>
<td>[37], 2019</td>
<td>2009-2016. Effectiveness of fixed dose prophylaxis (n = 189)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kang et al</td>
<td>Multicenter, randomized,</td>
<td>Group 1: Micafungin</td>
<td>Group 1 vs Group 2: 69 vs 75 pts. IFI occurrence in 3 wk: 1/69 vs 0/75. Micafungin was noninferior to Flu</td>
</tr>
<tr>
<td>[38], 2020</td>
<td>open label. Living donor LT.</td>
<td>100 mg/d; Group 2: Flu 100-200 mg/day</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2012-2015 (n = 144)</td>
<td></td>
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</tr>
</tbody>
</table>

AmB: Amphotericin-b; Flu: Fluconazole; Caspo: Caspofungin; AE: Adverse effects; Vori: Voriconazole; ppx: Prophylaxis; gp: Group; IA: Invasive aspergillosis; IC: Invasive candidiasis.

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Elastography as a predictor of liver cirrhosis complications after hepatitis C virus eradication in the era of direct-acting antivirals

Lucia Cerrito, Maria Elena Ainora, Alberto Nicoletti, Matteo Garcovich, Laura Riccardi, Maurizio Pompili, Antonio Gasbarrini, Maria Assunta Zocco

Abstract

Chronic inflammation due to hepatitis C virus (HCV) infection leads to liver fibrosis and rearrangement of liver tissue, which is responsible for the development of portal hypertension (PH) and hepatocellular carcinoma (HCC). The advent of direct-acting antiviral drugs has revolutionized the natural history of HCV infection, providing an overall eradication rate of over 90%. Despite a significant decrease after sustained virological response (SVR), the rate of HCC and liver-related complications is not completely eliminated in patients with advanced liver disease. Although the reasons are still unclear, cirrhosis itself has a residual risk for the development of HCC and other PH-related complications. Ultrasound elastography is a recently developed non-invasive technique for the assessment of liver fibrosis. Following the achievement of SVR, liver stiffness (LS) usually decreases, as a consequence of reduced inflammation and, possibly, fibrosis. Recent studies emphasized the application of LS assessment in the management of patients with SVR in order to define the risk for developing the complications of chronic liver disease (functional decompensation, gastrointestinal bleeding, HCC) and to optimize long-term prognostic outcomes in clinical practice.

Key Words: Direct-acting antiviral agents; Liver stiffness; Portal hypertension; Hepatocellular carcinoma

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

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L-Editor: Webster JR
P-Editor: Yu HG

Core Tip: Direct-acting antiviral agents lead to hepatitis C virus eradication and to the regression of liver inflammation. However, they do not eliminate the risk of possible portal hypertension-related complications and hepatocellular carcinoma (HCC), increasing the necessity for post-sustained virological response surveillance and the development of non-invasive predictive models to detect the categories of patients requiring more intensive follow-up. Many studies reported a significant reduction in liver fibrosis markers after treatment with direct-acting antiviral drugs. Ultrasound elastography is gaining growing importance as a predictive element in the assessment of the risk of developing esophageal varices or gastrointestinal bleeding, liver functional decompensation and HCC.

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INTRODUCTION

Hepatitis C virus (HCV) infection is one of the major causes of chronic liver disease and a significant cause of morbidity and mortality worldwide[1]. In 2015, it was estimated that over 70 million people were affected, most of whom were unaware of the infection[2]. Chronic inflammation due to HCV infection leads to liver fibrosis and rearrangement of liver tissue, which is responsible for the development of portal hypertension (PH) and other complications. Moreover, inflammation and microenvironmental changes are known risk factors for the occurrence of hepatocellular carcinoma (HCC)[3].

The advent of direct-acting antiviral drugs (DAAs) has revolutionized the natural history of HCV infection, providing an overall eradication rate of over 90% associated with a remarkable safety profile in all stages of chronic liver disease[1].

The achievement of sustained virological response (SVR) prevents the development of cirrhosis in the early stages of the disease and significantly reduces the risk of HCC and PH-related events, such as ascites, hepatic encephalopathy, hepatorenal syndrome, infections and gastrointestinal bleeding, in patients with advanced liver disease[4-6]. However, initial reports have warned of an increased risk of HCC in patients who achieved SVR after treatments with DAAs[7,8]. On the other hand, other studies have shown a protective effect on the development of HCC[9,10]. More recently, a meta-analysis analyzing 41 studies concluded that there is no evidence for increased occurrence or recurrence of HCC in patients treated with DAAs compared with interferon-based therapies[11].

Despite a significant decrease after SVR, the rate of HCC and liver-related complications is not completely eliminated in patients with advanced liver disease. Although the reasons are still unclear, cirrhosis itself has a residual risk for the development of HCC and other PH-related complications[12]. At present, there are no validated predictors to estimate the risk of HCC and PH-related events after HCV eradication.

Ultrasound elastography is a recently developed non-invasive technique for the assessment of liver fibrosis. Vibration controlled transient elastography (VCTE), is the oldest share-wave-based method and the reference standard in this field. The device is equipped with a one-dimensional probe, where a vibrator sends low frequency shear waves through the liver. Wave propagation, evaluated by an ultrasound receiver inside the probe, is directly related to liver tissue elasticity. Since its emergence, this technique has provided a fast point-of-care estimate of liver fibrosis in daily clinical practice, avoiding the complications of liver biopsy[13]. Indeed, several studies using histology as the reference standard defined accurate thresholds that are able to distinguish the different stages of liver fibrosis[14]. In the last few years, new ultrasound based elastographic techniques have been developed. They are embedded into conventional ultrasound devices, allowing visualization of the sampling area. The two main categories are the point shear wave elastography (pSWE) and bidimensional SWE (2D-SWE)[13]. All these devices are able to evaluate the elastic properties of the
liver during real-time B mode imaging. In particular, the ultrasound probe generates short-duration acoustic impulses in a small region of interest that causes soft tissue displacement and shear waves running in the perpendicular plane. Shear wave travelling speed can then be quantified and interpreted as a measurement for liver stiffness (LS) [13].

To date, LS measurement (LSM) is recommended by the European Association for the Study of Liver Disease (EASL) and the American Association for the Study of Liver Disease (AASLD) guidelines for the assessment of liver disease severity in patients with HCV infection eligible for DAAs [1, 15]. Following the achievement of SVR, LS usually decreases, as a consequence of reduced inflammation and, possibly, fibrosis [16-19]. Recent studies evaluated the usefulness of LS assessment after HCV eradication and the prediction of HCC and other PH-related complications in patients with advanced liver disease.

In this review, we summarize the current evidence on the role of ultrasound elastography in the prediction of liver-related outcomes of patients with HCV infection treated with DAAs.

**DIRECT-ACTING ANTIVIRAL AGENTS AND LIVER FIBROSIS**

Despite DAAs being pharmacologically designed only for the eradication of HCV infection and since HCV is directly responsible for liver injury and consequent parenchymal fibrosis, the achievement of both SVR and anti-fibrotic effect results in advantages in terms of prevention of chronic liver disease complications (Table 1).

Different non-invasive methods traditionally used to assess liver fibrosis such as VCTE and the Fibrosis-4 (FIB-4) score (based on patient’s age, transaminases levels and platelet count) and aspartate aminotransferase to platelet ratio index (APRI score) have been evaluated for staging chronic liver disease and predicting hepatic fibrosis in patients with HCV infection.

It has been demonstrated that baseline LSM by VCTE together with FIB-4 and APRI score have an important role in the prediction of treatment outcome in the new era of DAAs and could be integrated in pre-treatment assessment as a guide for treatment decisions and optimization of patient management [20, 21].

Many authors have documented the improvement of VCTE, FIB-4 and APRI score after DAAs treatment. However, it is not clear if this finding is a true recovery of liver fibrosis or represents only an epiphenomenon of the reduction in liver inflammation resulting in the normalization of blood tests and decrease of LS values [22-25]. The retrospective study by Elsharkawy et al. [26] analyzed a group of 337 Egyptian patients with chronic genotype 4 HCV infection who underwent sofosbuvir-based treatments. Among the patients evaluated, 29.1% had non-relevant fibrosis (F0-I; VCTE < 7.1 kPa), 17.2% were included in the F2 group (7.1 kPa ≤ VCTE < 9.5 kPa), 8.6% in the F3 group (VCTE ≥ 9.5 kPa) and 45.1% were classified as cirrhotic (F4; ≥ 12.5 kPa). One year after treatment, 77% of responders (with any stage fibrosis) and 81.8% of cirrhotic patients had a valuable recovery in liver fibrosis parameters (measured with FIB-4 and APRI score), due to the increase in platelet count and decrease in transaminase levels together with a reduction in LS values (11.8 ± 8.8 kPa vs 14.8 ± 10.7 kPa, *P* = 0.000). A higher number of patients with poor LS improvement after DAAs-therapy was observed in cases with low baseline LS values and infection relapse.

In a group of 42 patients treated with DAAs, Chekuri et al. [27] demonstrated a significant decrease in LS values at SVR 24 wk after the end of treatment (median values: 10.40 kPa vs 7.60 kPa, *P* < 0.01), without significant improvement in the follow-up.

Abdel Alem et al. [28] used pre-treatment liver fibrosis (measured by VCTE and FIB-4 score) as a predictor of treatment outcome after sofosbuvir-based regimens in 7256 HCV patients (46.6% cirrhotic, 91.4% with SVR12). Both, baseline FIB-4 and VCTE were significantly lower in the group with SVR (2.66 ± 1.98 kPa and 17.8 ± 11.5 kPa, respectively) compared to relapers (4.02 ± 3.3 kPa and 24.5 ± 13.9 kPa, respectively). Based on these results, the authors concluded that fibrosis stage is a crucial element in the evaluation of treatment outcome and disease prognosis. In particular, a LS value higher than 16.7 kPa resulted as an unfavorable prognostic factor for treatment response (relapse rate 13%), probably related to an impaired immune-mediated HCV clearance that is worsened in advanced liver fibrosis. Similar considerations were drawn by Neukam et al. [29] in patients treated with pegylated interferon/ribavirin-based therapy associated with NS3/4A protease inhibitor (PR-PI) and patients under DAAs therapy. In the PR-PI group, SVR12 was obtained in 59.6% of patients with LS < 21 kPa and in 46.5% of subjects with LS ≥ 21 kPa (*P* = 0.064); in the DAAs group,
Table 1 Liver stiffness improvement after treatment with direct acting antivirals

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Study design</th>
<th>Number of Patients</th>
<th>Drugs</th>
<th>Patients with LS improvement (%)</th>
<th>Pre-treatment LS</th>
<th>Post-treatment LS</th>
<th>P value</th>
<th>Measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elsharkawy et al [26], 2017</td>
<td>Retrospective</td>
<td>337</td>
<td>DAA</td>
<td>81.8% (cirrhotic) 71.7% (non-cirrhotic)</td>
<td>14.8 ± 10.7 kPa</td>
<td>11.8 ± 8.8 kPa</td>
<td>0.000</td>
<td>Fibroscan</td>
</tr>
<tr>
<td>Chekuri et al [27], 2016</td>
<td>Observational</td>
<td>100</td>
<td>IFN-based and DAA</td>
<td>NA</td>
<td>10.40 kPa</td>
<td>7.60 kPa</td>
<td>&lt; 0.01</td>
<td>Fibroscan</td>
</tr>
<tr>
<td>Bachofner et al [30], 2017</td>
<td>Multicenter, observational</td>
<td>392</td>
<td>DAA</td>
<td>93%</td>
<td>12.65 kPa</td>
<td>8.55 kPa</td>
<td>&lt; 0.001</td>
<td>Fibroscan</td>
</tr>
<tr>
<td>Afddhal et al [59], 2017</td>
<td>Prospective</td>
<td>52</td>
<td>DAA</td>
<td>59.6%</td>
<td>15.2 kPa</td>
<td>9.3 kPa (6.7–16.8 kPa)</td>
<td>&lt; 0.0001</td>
<td>Fibroscan</td>
</tr>
<tr>
<td>Ravaioli et al [68], 2018</td>
<td>Retrospective</td>
<td>139</td>
<td>DAA</td>
<td>44.6% (LS reduction &gt; 30%)</td>
<td>18.6 kPa (15-26.3 kPa)</td>
<td>13.8 kPa (10.4-20.4 kPa)</td>
<td>&lt; 0.001</td>
<td>Fibroscan</td>
</tr>
<tr>
<td>Pan et al [70], 2018</td>
<td>Retrospective</td>
<td>84</td>
<td>DAA</td>
<td>62%</td>
<td></td>
<td>Fibrosis regression by at least two stages: Cirrhosis group (48%); F3 fibrosis group (39%)</td>
<td></td>
<td>Fibroscan</td>
</tr>
</tbody>
</table>

DAA: Direct acting antivirals; IFN: Interferon; LS: Liver stiffness; NA: Not applicable.

SVR12 was reached by 95.3% of patients with LS < 21 kPa and 87.4% of patients with ≥ 21 kPa. Relapse rates after an apparent end-of-treatment response were 4.8% vs 17.9% in patients treated with PR-PI and 2.4% vs 8.2% in the DAAs group, respectively, for LS < 21 kPa and ≥ 21 kPa. These results suggest that LS evaluation might be useful to avoid HCV-relapse in cirrhotic patients by choosing both the appropriate composition and duration of DAAs-therapy.

Many studies reported a significant reduction in liver fibrosis markers after treatment with DAAs. In particular, Bachofner et al [30] highlighted a 32.4% drop in VCTE values from 12.65 kPa to 8.55 kPa (P < 0.001), a reduction of FIB-4 from 2.54 to 1.80 (P < 0.001) and a decrease of APRI from 1.10 to 0.43 (P < 0.001).

DIRECT-ACTING ANTIVIRAL AGENTS AND LIVER CIRRHOSIS RELATED EVENTS

Even though DAA-therapy leads to HCV eradication and to the regression of liver inflammation, it does not eliminate the risk of possible PH-related complications and HCC, increasing the necessity for post-SVR surveillance and the development of non-invasive predictive models to detect the categories of patients requiring more intensive follow-up (Table 2).

To this purpose, Trivedi et al [31] suggested a VCTE-based algorithm in order to schedule the controls of patients with SVR after HCV eradication: In the case of mild fibrosis (F1) without liver-related comorbidities, regular monitoring with the primary care physician is indicated; for advanced fibrosis/cirrhosis (F3-4), routine HCC and variceal surveillance is prescribed (six-monthly ultrasound, upper endoscopy every 2-3 years, annual non-invasive fibrosis assessment); for moderate fibrosis (F2) or in the case of concomitant liver-related comorbidities an annual non-invasive fibrosis measurement should be performed.

The importance of liver fibrosis stage in the development of liver-related complications was confirmed by Kozbial et al [32], who analyzed 551 patients treated with DAAs for a median period of 65.6 wk: No complications were registered in patients with severe fibrosis, whereas 9.1% of subjects with compensated cirrhosis developed liver-associated complications including HCC (4.1%). Furthermore, the presence of decompensated cirrhosis was markedly associated with the development of complications and mortality.

Even though histology remains the gold standard in evaluating fibrosis, liver biopsy presents some potential obstacles such as patient compliance, severe post-procedural complications, and sampling errors. For this reason, elastography has been proposed as a possible non-invasive alternative to biopsy for patient surveillance after SVR [33-35].
Table 2 Direct-acting antiviral agents and liver cirrhosis related events

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Study design</th>
<th>Number of patients</th>
<th>Drugs</th>
<th>HCC</th>
<th>Portal hypertension-related complications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Koebel et al [32], 2018</td>
<td>Prospective</td>
<td>551</td>
<td>DAA</td>
<td>16 (4.1%)</td>
<td>Ascites: 3.1%; variceal hemorrhage: 1%; hepatic encephalopathy: 0%</td>
</tr>
<tr>
<td>Masuzaki et al [34], 2009</td>
<td>Prospective</td>
<td>984</td>
<td>DAA</td>
<td>77 (2.9% per 1 person-year); HCC risk: 43.5 times higher in LS &gt; 25 kPa</td>
<td>NA</td>
</tr>
<tr>
<td>Afshari et al [39], 2017</td>
<td>Prospective</td>
<td>50</td>
<td>DAA</td>
<td>LS improvement in patients who did not develop HCC during follow-up (42.6% reduction in patients without HCC vs 13.6% in HCC group)</td>
<td>24% patients had ≥ 20% decreases in HVPG during treatment (89% subjects with baseline HVPG ≥ 12 mmHg had a ≥ 20% reduction in HVPG after SVR)</td>
</tr>
<tr>
<td>Giannini et al [51], 2019</td>
<td>Prospective</td>
<td>52</td>
<td>DAA</td>
<td>4 (7.7%)</td>
<td>Clinical decompensation: 0%</td>
</tr>
<tr>
<td>Tachi et al [58], 2017</td>
<td>Prospective</td>
<td>263</td>
<td>DAA</td>
<td>19 (7.2%)</td>
<td>NA</td>
</tr>
<tr>
<td>Foster et al [60], 2016</td>
<td>Retrospective, observational</td>
<td>467</td>
<td>DAA</td>
<td>NA</td>
<td>MELD improvement (0.85, SD 2.54); composite adverse outcome in 52.0% (treated) vs 61.7% (untreated)</td>
</tr>
<tr>
<td>Rinaldi et al [63], 2019</td>
<td>Multicenter, prospective</td>
<td>258</td>
<td>DAA</td>
<td>35 (13.6%)</td>
<td>NA</td>
</tr>
<tr>
<td>Ravaioli et al [68], 2018</td>
<td>Retrospective</td>
<td>139</td>
<td>DAA</td>
<td>20 (14.4%)</td>
<td>NA</td>
</tr>
<tr>
<td>Pan et al [70], 2018</td>
<td>Retrospective</td>
<td>84</td>
<td>DAA</td>
<td>4 (4.8%)</td>
<td>NA</td>
</tr>
<tr>
<td>Toyoda et al [75], 2015</td>
<td>Retrospective/prospective</td>
<td>522</td>
<td>IFN-based</td>
<td>18 (1.2% after five yr; 4.3% after ten yr)</td>
<td>NA</td>
</tr>
<tr>
<td>D’Ambrosio et al [77], 2018</td>
<td>Prospective</td>
<td>38</td>
<td>DAA</td>
<td>5 (13%)</td>
<td>Clinical decompensation: 0%</td>
</tr>
<tr>
<td>Lleo et al [78], 2019</td>
<td>Prospective</td>
<td>1927</td>
<td>DAA</td>
<td>Previous HCC: 38/161 (recurrence rate: 24.8 per 100-yr); No previous HCC: 50/1766 (incidence rate: 2.4 per 100-yr)</td>
<td>NA</td>
</tr>
<tr>
<td>Hamada et al [79], 2018</td>
<td>Retrospective</td>
<td>196</td>
<td>DAA</td>
<td>8 (4.1%)</td>
<td>NA</td>
</tr>
</tbody>
</table>

DAA: Direct acting antivirals; HCC: Hepatocellular carcinoma; HVPG: Hepatic venous pressure gradient; IFN: Interferon; LS: Liver stiffness; MELD: Model for end-stage liver disease; NA: Not applicable; SD: Standard deviation; SVR: Sustained virological response.

VCTE is gaining growing importance as a predictive element in the assessment of the risk of developing esophageal varices or gastrointestinal bleeding, liver functional decompensation and HCC [36]. The retrospective study by Mandorfer et al [37] was the first to compare Hepatic Venous Pressure Gradient (HVPG) measurement with VCTE for the assessment of PH and showed a good agreement between the techniques. The authors also observed that a PH decrease after SVR was less likely in subjects with baseline HVPG higher than 16 mmHg and severe liver function impairment.

The review by Garbuzenko et al [38] confirmed that staging the severity of PH in cirrhotic subjects and personalized preventive therapy could lead to an increase in both patient survival and treatment effectiveness; particularly, DAAs achieve the amelioration of subclinical PH. In a recent study by Afshari et al [39] of 50 patients with clinically significant PH (presence of esophageal varices, HVPG > 6 mmHg) from different international centers, 89% obtained a HVPG reduction of > 20% and only 3 patients obtained a reduction of portal pressure to less than 12 mmHg.

Paternostro et al [40] endorsed spleen stiffness measurement (SSM) through elastography (especially pSWE and 2D-SWE) as an effective tool for high-risk varices assessment in chronic liver disease, especially in distinguishing between small and large varices as confirmed by Sharma et al [41]. Previously, both Colecchia et al [42] and Fraquelli et al [43] had underlined the efficacy of LSM and SSM association in the assessment of HVPG and prediction of gastroesophageal varices in cirrhotic patients, showing a very high sensitivity (98% and 100% in the two studies, respectively), and economic advantages following the implementation of endoscopic screening progr-
ams. However, there are some important limitations related to SSM: It is an operator-dependent measurement and the upper limit of VCTE is fixed to a fibrosis value of 75 kPa that, in the case of severe PH, could be widely exceeded by SSM unlike LSM. Concerning the latter issue, Calvaruso et al.[44] demonstrated the superior predictive value of SSM for high-risk varices, adopting a modified VCTE unit with a maximum stiffness value of 150 kPa (AUC: 0.80 for SSM vs 0.71 for LSM).

It has been demonstrated that the association of LSM with other non-invasive items (e.g. platelets, SSM) has a powerful positive predictive value in the detection of esophageal varices: Stefanescu et al.[45] created a simple diagnostic algorithm with the combination of LSM and SSM (cut-off: 19 kPa and 55 kPa, respectively), thus reaching a 93% sensibility and a 95% positive predictive value.

Wang et al.[46] observed that the combination of Baveno VI criteria with SSM (with 46 kPa cut-off) might help to avoid 61.6% of esophagogastroduodenoscopies in HBV-related cirrhosis with persistent viral suppression due to antiviral therapy, missing less than 5% high-risk varices.

An interesting analysis by Fofiu et al.[47] evaluated a score based on the combination of LSM, SSM and spleen size as non-invasive predictors of high-risk varices in compensated cirrhosis, proving a better performance of the association of the three elements compared to each parameter alone. However, a meta-analysis by Ma et al.[48] found that SSM alone is superior to LSM in predicting any grade esophageal varices, thus turning out to be useful in clinical practice, especially in the case of non-measurable LSM (multifocal HCC, biliary obstruction or liver metastasis).

Semmler et al.[49] underlined the predictive value of LSM by VCTE included in a non-invasive algorithm together with von Willebrand factor-platelet count ratio as a useful method to define PH, stratify risk categories and predict liver decompensation and HCC development in patients with HCV-related advanced chronic liver disease treated with DAAs. These results could be very interesting in introducing the concept of a tailored follow-up strategy.

It is still not clear if the improvement in non-invasive markers after SVR could be associated to a decline in PH itself. However, in a recent study, Thabut et al.[50] noted that subjects with previous unfavorable Baveno VI status (LS > 20 kPa, platelets < 150000/mm3) who experienced platelets increase and/or LS reduction after SVR reached a favorable Baveno VI class, with a subsequent reduction in the probability of PH progression and development of esophageal varices. A decrease of PH has also been demonstrated by Giannini et al.[51] in a group of 52 patients with advanced fibrosis/cirrhosis at baseline followed for approximately 60 wk after SVR with DAAs. A significant improvement in HVPG was detected, together with a decrease in LS values (from 15.2 kPa at baseline to 9.3 kPa at the end of follow-up), APRI and FIB-4 score, spleen bipolar diameter and an increase in platelet count[37].

As the role of these indices is quite limited, other non-invasive methods have been proposed to detect varices at high risk of bleeding: Considering the worldwide low availability of TE, Jangouk et al.[52] demonstrated the effectiveness of Baveno VI consensus criteria as a non-invasive method to identify patients with compensated liver cirrhosis and low-risk of varices requiring endoscopic treatment. In particular, the authors highlight the uppermost role of both platelet count (> 150000/mm3) and MELD score (< 6) in defining a low probability of high-risk varices.

Chen et al.[53] demonstrated the efficacy and extremely high negative predictive value (97.1% in the study group and 98.1% in the validation cohort) of the association of albumin-bilirubin grade with platelet count (ALBI-PLT score) in the screening of high-risk esophageal varices in subjects with HCC. The 5-year variceal hemorrhage rate was 9.7% in patients with ALBI-PLT score > 2 (decompensated liver disease) as compared to 1.7% in those with a score of 2 (P = 0.007).

Baveno VI guidelines indicate platelet count and VCTE as effective elements in the identification of cirrhotic patients who are at high-risk of developing esophageal varices: Due to the not-always easy access to VCTE (for example, in the case of in-mates) or to the unavailability of adequate instrumentation in all hepatological centers, Calvaruso et al.[54] proposed the “Rete Sicilia Selezione Terapia–HCV” algorithm as an effective and simple tool (based only on blood tests: Platelet count and serum albumin level) that could substitute Baveno VI criteria in the identification of HCV-cirrhotic patients with medium/large varices, thus simplifying the diagnosis of the complications of PH, with a reduction of more than 30% of useless endoscopic exams and diminishing the risk of false-negative results.

The implications of HCV eradication on HCC development are even more complex. Despite the widely demonstrated efficacy of DAAs in both achieving SVR and a reduction in liver fibrosis, there is no corresponding decrease in HCC development risk. These data led to an initial alert claiming the possibility of a DAAs-driven
oncogenic mechanism[7], even if this theory was subsequently proved wrong by other studies[11]. The mechanism of HCC development post SVR is probably sustained by a “point of no-return” in HCV pathogenesis that determines the loss of the potential benefits brought by viral eradication[55]. This evidence highlights the necessity for optimizing regular HCC surveillance with a particular focus on patients with advanced fibrosis or cirrhosis[56]. In fact, even though a decrease in LS values from cirrhosis to advanced fibrosis was observed in some cases after DAAs therapy, patients with SVR maintained an elevated HCC risk[57,58].

Whether the HCC risk of patients with SVR coincides with that of viremic subjects is still a matter of debate. In the case of precariously compensated or decompensated liver function, the achievement of SVR could be useful to reduce the risk of HCC because of the decrease in intrahepatic inflammatory processes, despite the persistence of PH and decompensated liver function (that increase the risk of liver cancer in cirrhotic patients)[59,60].

Both EASL and AASLD guidelines recommend continuing ultrasound surveillance in subjects with advanced fibrosis/cirrhosis despite histological response to treatment and suggest accurate definition of the additional baseline risk-factors profile[61,62].

Rinaldi et al[63] assessed the importance of both baseline LS evaluation and ultrasound liver surveillance for the risk of HCC in patients with HCV-related cirrhosis, treated with DAAs: Among 258 subjects enrolled, divided into three groups according to liver fibrosis stage (< 20 kPa, from 20 kPa to 30 kPa, > 30 kPa), 35 developed HCC during follow-up. The group with LS higher than 30 kPa had a statistically significant increase in HCC risk [HR (95%CI): 0.329 (0.131-0.830); P = 0.019].

Even though the mechanisms directly involving HCV in both fibrogenesis and oncogenesis have not yet been completely explained, it seems crucial to define the degree of liver fibrosis through VCTE and FIB-4, in order to set appropriate HCC screening and the subsequent therapeutic strategy[64,65].

Many attempts have been made to create prognostic scores to evaluate the risk of HCC development in chronic liver diseases, considering other criteria than PH alone [66]. An interesting example is represented by the King score that includes laboratory parameters (platelet count and bilirubin levels) and gene signature, and classifies cirrhotic patients with HCV infection into three risk categories for functional decompensation, HCC and death. However, it is not clear if this score maintains its predictive efficacy in patients with SVR[67].

Ravaioliet al[68] studied 139 cirrhotic patients treated with DAAs, analyzing the difference between LS at baseline and at the end of treatment: They found a lower reduction of LS in patients who developed HCC compared to patients who did not (-18.0% vs -28.9%, P = 0.005).

Recent studies demonstrated that LS assessment after SVR could be an inaccurate method to define the grade of fibrosis in patients treated with DAAs. In fact, the fast modifications in LS could be determined by both the reduction of liver inflammatory activity and the narrowing of fibrotic septa, without real histological improvement in fibrosis grading as demonstrated by liver biopsy[69-71]. Notwithstanding, LS evaluation by VCTE remains a cornerstone in the assessment of HCC risk after SVR, especially due to its non-invasiveness.

Masuzaki et al[36] demonstrated that HCC risk was 45.5 times higher in patients with LS values higher than 25 kPa.

However, it becomes important in the association to other elements in a more complete non-invasive score. Among them, we can include: Age, alcohol abuse, pre-treatment advanced fibrosis/cirrhosis, platelet count, steatosis, diabetes, ALF, fetoprotein (AFP), baseline gamma-glutamyltransferase (GGT) levels together with ethnic and environmental factors. All these factors have been studied in patients treated with interferon-based therapies with interesting results[72-76]. During the pre-DAAs era, studies on the complications of liver cirrhosis after HCV-treatment showed that SVR and fibrosis regression did not prevent hepatic carcinogenesis. D’Ambrosio et al[77] found that 13% of patients who responded to interferon-based treatments, developed HCC during an 8-year follow-up (17% cumulative probability and 1.2% annual incidence rate) whereas neither variceal-bleeding nor liver-function decompensation occurred. Higher baseline levels of GGT and glycemia were identified as risk factors for HCC development. Similarly, Toyoda et al[75] demonstrated that diabetes mellitus and FIB-4 index increase represent risk factors for HCC after SVR with interferon-based regimens, thus suggesting continuing active surveillance in these groups of patients.

In a prospective analysis of 1927 patients with HCV-related cirrhosis, receiving DAAs in ten tertiary Italian liver centers, Lleo et al[78] observed a recurrence rate of HCC of 24.8 per 100 patients/year and a de novo occurrence rate of 2.4 per 100...
patients/year. They found that treatment failure and high AFP levels represent independent predictors of HCC development, while SVR and absence of PH are associated with a lower HCC incidence, suggesting that HCC risk stratification should rely on the presence of PH and elevated baseline AFP levels.

It has been suggested that PH as a complication of liver fibrosis (more than fibrosis itself) may represent an independent risk factor for HCC[66]. Alidhal et al.[39] analyzed 50 patients with HCV-related liver cirrhosis treated with DAAs and observed a significant reduction in HVPG values during long-term follow-up after SVR: 24% of all patients and 89% of subjects with baseline HVPG ≥ 12 mmHg who reached SVR had a ≥ 20% reduction in HVPG. With regard to LS, a more evident improvement was observed in patients who did not develop HCC during follow-up (42.6% reduction in patients without HCC vs 13.6% in the HCC group), thus proposing a protective role of HVPG and LS against HCC development.

In a recent retrospective study performed in patients with SVR after DAAs, Hamada et al.[79], identified six variables that could be included in the HCC prediction model: Age, body mass index, platelet count, albumin, AFP, LS and FIB-4 index. Following multivariate analysis they found that age ≥ 75 years, AFP ≥ 6 ng/mL, and LS ≥ 11 kPa were independent risk factors for hepatocarcinogenesis (risk ratio: 35.16, 43.50 and 28.71, respectively; P = 0.001, 0.003 and 0.006, respectively). In particular, patients with LS < 11 kPa had a cumulative HCC incidence of 1.3% at 12 mo, 24 mo, 36 mo and 48 mo, while in the group with LS > 11 kPa the HCC incidence rate was 4.6% at 12 mo and 24 mo, 24.8% at 36 mo and 62.4% at 48 mo.

The role of LSM in the development of a prediction model for HCC has also been emphasized by Feier et al.[80]. They confirmed that high levels of AFP, transaminases and LS are excellent predictors of HCC but underlined the importance of interquartile range (IQR) in LSMs. This led to the hypothesis of “stiffness shadow” that indicated an inhomogeneous shear stress due to the chaotic tumoral growth in the already hard cirrhotic tissue, with relevant diagnostic repercussions[81,82]. The overall prognostic model combining the four variables demonstrated relevant results both in the training and validation phase with a positive relation with tumor size. The four parameters together showed a 64.5% HCC prediction, with LS alone reaching the highest predictive power. The authors concluded that an elevation in LS values and IQR during follow-up could enhance the diagnostic skill towards early HCC[80].

It is interesting to note that some genetic factors also seem to be involved in hepatocarcinogenesis, despite the lack of clear evidence and the need for further prospective studies.

In their cohort of 200 patients with HCV-related cirrhosis with SVR after DAAs, Simili et al.[83] noted a strong association of the single-nucleotide polymorphism of interleukin 28 (IL28B–rs12979860) with HCC development (both de novo and disease recurrence); furthermore, they observed a relation of HCC with lower levels of serum retinol and the presence of another two polymorphisms: Major histocompatibility complex class I polypeptide-related sequence A gene (MICA) and tolloid-like 1. The latter has proven particularly controversial since its oncogenic role was stated by Matsuura et al.[84] but denied by Degasperi et al.[85]: The difference between these studies could be ascribed to the different allele frequency or the presence of still unknown cofactors in the two ethnic groups (Japanese and Caucasian) or to discrepancies in the length of the follow-up period.

**CONCLUSION**

DAAs-therapy has brought about an effective revolution in hepatology resulting in HCV eradication in a wide range of patients and eventually reducing liver fibrosis after SVR. However, these benefits have not erased the risk of developing liver disease-related complications and in particular HCC and PH associated events. For this reason, it is crucial to continue long-term systematic surveillance after HCV eradication focusing on the subjects with a high-risk score.

Due to its accuracy, cost-effectiveness and non-invasiveness, together with specific clinical and laboratory parameters, LSM is gaining a relevant role in the construction of algorithms assessing both liver fibrosis and PH. The potential application of this non-invasive and simple method has been emphasized especially in the management of patients with SVR in order to define the risk to develop the complications of chronic liver disease (functional decompensation, gastrointestinal bleeding, HCC) and optimize long-term prognostic outcomes in clinical practice.
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Role of immune dysfunction in drug induced liver injury

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Core Tip: This review demonstrates the critical role of the immune system in the progression of drug-induced liver injury and also in determining the severity of the damage. Drugs affect the normal functioning of hepatocytes through several direct and indirect mechanisms leading to the dysfunctional immune response. The major effector cells in amplifying liver damage are Kupffer cells, monocytes and neutrophils. Genetic predispositions and environmental factors also make individuals vulnerable to immune dysfunction.

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Abstract

Drug-induced liver injury (DILI) is one of the leading causes of liver failure and withdrawal of drugs from the market. A poor understanding of the precipitating event aetiology and mechanisms of disease progression has rendered the prediction and subsequent treatment intractable. Recent literature suggests that some drugs can alter the liver’s repair systems resulting in injury. The pathophysiology of DILI is complex, and immune dysfunction plays an important role in determining the course and severity of the disease. Immune dysfunction is influenced by the host response to drug toxicity. A deeper understanding of these processes may be beneficial in the management of DILI and aid in drug development. This review provides a structured framework presenting DILI in three progressive stages that summarize the interplay between drugs and the host defence networks.

Key Words: Immune dysfunction; Liver damage; Hepatotoxic drugs; Drug-induced liver injury; High mobility group box 1

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INTRODUCTION

The liver plays a central role in the complex process of metabolism and elimination of drugs from the body. The liver is equipped with a wide array of detoxification systems that have evolved over time with exposure to xenobiotics. The primary role of this system is to convert a drug to a more hydrophilic form so that it can be eliminated through bile or urine. Despite the liver’s detox potential, certain drugs can still cause hepatotoxicity that can range from mild asymptomatic liver damage to liver failure[1, 2].

A study showed that, out of the 462 pharmaceuticals withdrawn due to adverse drug reactions between 1953 and 2013, hepatotoxicity ranked first with 81 cases (18%). It is estimated that over 1000 drugs currently available on the market that cause liver damage[3] despite these drugs passing the safety measures of clinical trials before entering the market. Some drugs that are hepatotoxic at doses higher than the therapeutic range can also cause drug-induced liver injury (DILI) at doses within the therapeutic range[4-6]. This implies that the dose may not be the only contributing factor.

Despite large number of drugs known to cause liver injury, the incidence of DILI is rare. DILI is reported in 1 in every 10000 to 100000 individuals annually. This suggests that drug-host interactions in these susceptible individuals may play an important role in DILI[7-9]. Recent data shows that this interaction can result in an imbalance between damage and repair mechanisms resulting in DILI with immune dysfunction being cited as an important precipitating event in the pathophysiology of DILI[10-12]. This is supported by evidence from experimental studies. Some drugs that are hepatotoxic in humans do not cause liver damage in animal models, but the administration of these drugs along with low doses of lipopolysaccharide (LPS) result in a similar pattern of liver injury as observed in humans. For example, Trovafloxacin (TVX) is a broad-spectrum fluoroquinolone antibiotic, and a study reported that TVX use caused 140 severe hepatic reactions resulting in 14 cases of liver failure. Examination of the case reports suggest that the duration of TVX therapy in patients does not correlate with the toxic response, so TVX hepatotoxicity is classified as idiosyncratic. In rodent models, TVX did not cause liver damage, even at high doses. However, further studies with a normally nontoxic dose of TVX coupled with LPS induced inflammatory stress caused acute liver injury[13,14].

The upcoming sections provide a structured framework presenting DILI in three progressive stages, summarizing the interplay between drugs and the host defence networks that lead to immune system dysfunction.

STAGES OF DILI

Initiation of DILI

Direct initiation: The metabolism of drugs by phase 1 enzymes results in the production of intermediary metabolites and free radicals, in some instances. These intermediary metabolites may also be unstable and reactive, but they are subsequently neutralized by phase 2 conjugation. DILI is initiated when there is an imbalance between the production of reactive metabolites and their subsequent detoxification[2, 5] (Figure 1).

Certain drugs and reactive metabolites can bind to cellular organelles resulting in loss of function and likely cell death. One such case is the damage caused by drugs acting on the endoplasmic reticulum (ER). The ER plays an important role in protein synthesis, folding, assembly, trafficking, and regulation of intracellular calcium homeostasis. Drug related oxidative stress can disturb ER function and lead to the accumulation of unfolded proteins in the ER. This process is termed ER stress. A variety of common drugs cause ER stress, including paracetamol, lopinavir, ritonavir, saquinavir, nelfinavir, atazanavir, and amprenavir[15].

During drug metabolism, free radicals are released that are normally detoxified by cell defence mechanisms. Excessive free radical generation can be caused by enzyme induction or genetic defects in enzyme systems. Free radicals damage the cellular organelles and the lipid bilayer, which results in amplification of damage. Lipid
bilateral damage can lead to the release of cytosolic components and alarmins that attract the liver’s resident immune cells. This initial immune response can amplify the sterile damage. Some of the alarmins associated with DILI are high mobility group box 1, S100 proteins, hepatoma-derived growth factor and heat shock proteins[16-20].

Free radicals can also damage the mitochondrial membrane leading to cell dysfunction and death. Mitochondrial dysfunction includes disruption or disturbance to different metabolic pathways and damage to mitochondrial components. In addition, these mitochondrial alterations can have several deleterious consequences, such as oxidative stress, ATP depletion, triglycerides accumulation, and necrotic cell death[21].

**Indirect initiation of DILI:** There are two main mechanisms of indirect initiation of DILI. Inhibition of efflux transporters. Bile salt export pump (BSEP) is a member of the ABC transporter superfamily located in the canalicular membrane of hepatocytes. BSEP is responsible for the biliary excretion of bile acids. Drug metabolites inhibit BSEP function, resulting in toxicity. One such metabolite, Troglitazone sulphate, a metabolite of troglitazone, inhibits BSEP mediated taurocholate transport which contributes to troglitazone toxicity. Other potent BSEP inhibitors with the potential to cause DILI include cyclosporin A, bosentan, sulindac, rifamycin, and glibenclamide[2,22].

**Enzyme induction:** Paracetamol is known to cause liver injury through enzyme induction due to CYP2E1 induction by ethanol. A minor percentage of ethanol is metabolised by CYP2E1. When ethanol and paracetamol are taken simultaneously, ethanol slows the degradation of the CYP enzyme increasing its half-life from 7 h to 37 h. Until ethanol is present in the body more CYP2E1 is induced and a portion is blocked from paracetamol for ethanol metabolism. Once ethanol is completely removed, CYP2E1 enhances paracetamol metabolism resulting in the excess production of toxic intermediary metabolite, NAPQI, causing liver injury[2,23] (Figure 2).

**PROGRESSION**

The initiation of DILI does not necessarily result in adverse outcomes. In experimental models, the progression of DILI mainly depends on the persistent and recurrent assault by the toxins that deplete the liver’s resources leading to irreversible damage. This is unlikely at the therapeutic dose of most drugs, as the liver has highly developed protective and regenerative mechanisms. Experimental and clinical data suggest that a myriad of host and drug-related factors contribute to the progressive dysfunction of survival mechanisms that lead to DILI. This is further complicated by
the fact that each drug can cause multiple patterns of liver disease, implying an important role for host-drug interactions in the progression of DILI. Immune dysfunction is a major determinant of hepatic cell death and DILI progression[2,4,6,24-26].

This section covers the two main mechanisms of immune reactions induced by drugs and the influence of host factors on them.

**Immune allergic DILI**

A drug or its metabolites alone cannot activate an immune response due to their small size, but a drug’s reactive metabolites or the drug itself can bind to cellular proteins and form protein-drug adducts that elicit an immune response. In normal individuals, this complex is degraded by cellular detoxification but in susceptible individuals, these adducts act as immunogens and are taken up by antigen-presenting cells and presented by major histocompatibility complexes to helper T cells, and further activation by cytokines stimulates an immune response and anti-drug antibodies are also produced, resulting in extensive death of cells where the drug has accumulated[6,27-29] (Figure 3).

It is hypothesized that ER stress is a contributing factor for this type of reaction. Accumulation of drug/metabolite causes ER stress, which results in misfolding of proteins. These misfolded proteins are more susceptible to drug-protein adduct formations that elicit an immune response[15].

An example of this type of reaction is abacavir, a reverse transcriptase inhibitor employed in the treatment of AIDS, which causes a rare, but serious hypersensitivity reaction that resembles an immune allergic drug reaction. Several genetic variants in the HLA regions are identified as risk factors for DILI, the incidence of hypersensitivity reactions to abacavir is markedly elevated in subjects who carry the B*57:01 variant in the human leukocyte antigen B (HLA-B) gene. Furthermore, carriers of this genotype are at increased risk of flucloxacillin-induced DILI. Studies have shown an association between HLA-B1*15:01 and amoxicillin/clavulanate DILI. The HLA-B*35:02 allele is reported to have a significant association with minocycline DILI[10,25,30,31]. DILI caused by other drugs such as amoxicillin-clavulanate, lumiracoxib, ticlopidine, lapatinib, and ximelagatran is also associated with HLA genotypes, suggesting an important role of the immune system in DILI[25,31].

**Autoimmune DILI**

Autoimmune DILI is caused by the release of alarmins from necrotic cells or cells with leaky cell membranes. This results in the activation of innate immune cells. Alarmins are rapidly released following necrotic cell death that are not released by apoptotic cells. The immune system also can be induced to produce and release alarmins to recruit and activate innate immune cells[19,32] (Figure 4).

Mitochondrial dysfunction is reported to play a critical role in the pathogenesis of autoimmune DILI. NSAIDs, such as diclofenac and nimesulide, and other drugs can cause mitochondrial dysfunction that leads to the formation of the mitochondrial...
permeability transition pore (MPTP). MPTP formation is induced by increased oxidative stress that results in a dissipation of membrane potential, uncoupling of oxidative phosphorylation leading to necrotic cell death and the release of alarmins[18, 21,33].

HMGB-1 is an alarmin released by necrotic cells that binds to TLR4 receptors of kupffer cells (KCs) and hepatic stellate cells (HSC), and activates them. Activated KCs produce mediators that directly induce cell death, such as tumor necrosis factor (TNF)-α, Fas ligand and reactive oxygen species, or indirectly cause death through the recruitment of neutrophils by cytokines and chemokines like IL-1β and CXCL2. Production of chemokine, CCL2 (MCP-1) recruits monocytes from the bone marrow to the liver. These infiltrating monocytes produce inflammatory chemokines resulting in the activation of HSCs and the promotion of fibrosis[18,34].

Host sex and sex hormones influence immune response. Studies have shown that female patients with DILI are at higher risk of developing acute liver failure (ALF) with more severe hepatitis and higher levels of pro-inflammatory cytokines. In a halothane-induced experimental DILI model, oestrogen reduced liver injury while progesterone increased liver damage, both hormones influenced immune response. Another important factor affecting DILI is race. A study reported that African-Americans are at a higher risk of developing chronic DILI, while Asian individuals are at increased risk of ALF, liver-related death, or damage that precipitates a need for liver transplantation[4,7,10,24,35].
ADVERSE OUTCOMES

In normal individuals, DILI resolves completely without any residual liver injury. But there are three major exceptions. They are ALF, cirrhosis and acute-on-chronic liver failure (ACLF). These conditions are relatively rare but severe and may result in death or require a liver transplant.

ALF

Even in the absence of pre-existing liver disease, drugs can cause a rapid loss of liver function either directly, as seen in overdoses, or through inflammatory cell mediated mechanisms such as cytokine overproduction. Drug-induced ALF is defined by the signs or symptoms of hepatic failure and encephalopathy during the course of acute DILI. The time to onset of ALF after the start of a medication can vary from a few days to months, but not exceeding six months\[4,24,36-38].

In Western countries, paracetamol overdose is the most common reason behind ALF. In India, anti-TB regimens with isoniazid, rifampicin and pyrazinamide are reported as the leading cause of ALF. Other drugs that are reported to cause ALF include phenytoin, carbamazepine, valproate, nitrofurantoin, propylthiouracil, disulfiram, diclofenac, ketoconazole, flutamide, sulphonamides, terbinafine, fluoroquinolone antibiotics and macrolide antibiotics. Drug-induced ALF is a major cause for withdrawal from the market or restricted use of a medication (troglitazone, bromfenac, nefazodone, halothane, telithromycin). ALF occurs in cases with acute hepatocellular injury with characteristics similar to acute viral hepatitis\[10,23,39-41].

Paracetamol is responsible for more than 50% of drug related ALF and about 20% of liver transplant cases in the United States\[42]. In case of paracetamol overdose, the drug metabolite NAPQ1 depletes GSH and causes organelle damage, the most significant resulting in mitochondrial stress. Thereby the NAPQ1 accumulation triggers necrosis\[43,44]. Hepatocyte necrosis passively releases various DAMPs such as HMGB-1, HSP and DNA fragments. These DAMPs activate the resident immune cells such as Kupffer cells and natural killer (NK) cells. Cytokines and chemokines such as TNF-α, IL-1β and CCL2 produced by the activated immune cells and the DAMPs enter systemic circulation and cause infiltration of neutrophils and monocytes into the liver. In conditions of sterile injury, the immune cells function to clear the dead cells by producing chemokines and free radicals to digest it. Once the cellular debris is cleared the immune cells undergo phenotypic change and support in liver regeneration. However, in case of paracetamol overdose, the overwhelming amount of cellular debris and DAMPs causes excess immune activation, whose products such as superoxide, nitric oxide and peroxynitrite result in further amplification of liver injury leading to massive necrosis and organ failure\[45-48].

Cirrhosis

Cirrhosis is characterized by islands or nodules of regenerative parenchymal cells surrounded by excessive deposition of fibrous tissue and portal hypertension. Cirrhosis is rarely the initial manifestation of DILI and is most often a cumulative response to long-term exposure to hepatotoxic drugs. It usually occurs at least six months after starting the drug treatment. The time to onset of cirrhosis due to medications is typically long; at least 6 month after starting the medication but usually several years afterwards. The drugs that are most commonly cause cirrhosis are vitamin A, amiodarone, statins, tamoxifen, valproic acid, fibrates, and methotrexate\[4,25,26,49-51]. Drugs such as dantrolene, phenytoin, trazadone and nitrofurantoin are also associated with chronic hepatitis with autoimmune features that may lead to cirrhosis\[52-54].

Amiodarone is a benzofuran derivative mainly used in the treatment of arrhythmia. The safety of long-term use of amiodarone is well established however there are several reports of reversible and irreversible liver injury from its long-term use. Even though rare amiodarone can cause asymptomatic continuous liver injury that has histological features similar to alcoholic hepatitis such as nodular formation, fibrosis, steatosis and neutrophil infiltration\[55-61]. Due to its lipophilic nature and long half-life, amiodarone accumulates in the hepatocytes affecting cellular organelles such as ER and mitochondria causing misfolding of proteins. Amiodarone affects the cholesterol metabolism by blocking enzymes emopamil binding protein and dehydrocholesterol reductase 24. As cholesterol plays an important role in maintaining membrane fluidity and composition this affects the function of potassium channels and other membrane proteins resulting in “lipid traffic jam”\[62-67]. The immune cells in the liver get activated in response to cellular debris, misfolded
proteins and accumulating cholesterol precursors such as desmosterol[63,66,68]. Unless diagnosed in an early stage, this leads to irreversible end stage liver disease[62,69].

**Acute-on-chronic liver failure**

Acute-on-chronic liver failure (ACLF) as the name suggests is characterized by ALF due to a different cause in patients with chronic liver disease (compensated) resulting in short term mortality. It consists of two components: a chronic underlying liver disease and an acute trigger[70,71]. Devarbhavi et al[72] reported that drugs contributed to 10.5% cases in the Asia-Pacific region. Among these drugs, the most common culprits were complementary and alternative medications (71.7%), followed by anti-TB drug combination therapies (27.3%). Anti-TB drug isoniazid is also observed to cause severe hepatitis that leads to liver failure[72-74].

Studies suggest that excessive focal liver and systemic inflammatory response play a significant role in the development of ACLF. Reports have shown high levels of cytokines in patients with ACLF. This may be due to the activation of monocytes and macrophages in response to DAMPs, microbial toxins or drug adducts[19,75,76].

Paracetamol induced liver failure in patients with alcoholic hepatitis is a typical example of drug induced ACLF. Alcoholic hepatitis is reported in approximately 25% of the cases of ACLF. The trigger due to paracetamol toxicity can occur in two ways—the first is due to direct toxicity by paracetamol and the second due to immune response that is secondary to the hepatocellular damage due to the direct toxicity. The activation of innate immune response due to the paracetamol acute toxicity results in upregulation of cytokine and chemokine production that initiates severe systemic inflammation, liver damage and mortality[70,75,77,78].

The dysregulation in innate immune response plays important roles in disease progression as well as disease severity. In the liver, systemic inflammation plays a significant role in the development and course of chronic alcoholic hepatitis. Similar to the acute toxicity, immune activation in alcoholic liver disease results in activation of resident Kupffer cells and dendritic cells as well as the infiltrating immune cells—monocytes and neutrophils lead to progression towards fibrosis and cirrhosis. This disrupts the liver architecture and function setting stage for liver failure, that can be actuated by an acute trigger[75,78,79].

**CONCLUSION**

Drugs and their metabolic products can cause liver damage through multiple mechanisms. Under normal conditions, the liver is well equipped to neutralize potential drug-related damage, but in susceptible individuals, this same drug use can result in severe liver injury. This is further amplified by a dysfunctional immune responses that is influenced by host factors like genetics, age and sex. The severe adverse outcomes of DILI are ALF, cirrhosis and acute-on-chronic liver injury. All these injuries are associated with concurrent immune dysfunction. A better understanding of immune mediators may offer new targets for the management of DILI. Individualized therapy that focuses on early detection of risk factors, triggers and stage of the liver injury may play a significant role in effectively attenuating this disorder.

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Abnormal liver enzymes: A review for clinicians

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Abstract

Liver biochemical tests are some of the most commonly ordered routine tests in the inpatient and outpatient setting, especially with the automatization of testing in this technological era. These tests include aminotransferases, alkaline phosphatase, gamma-glutamyl transferase, bilirubin, albumin, prothrombin time and international normalized ratio (INR). Abnormal liver biochemical tests can be categorized based on the pattern and the magnitude of aminotransferase elevation. Generally, abnormalities in aminotransferases can be classified into a hepatocellular pattern or cholestatic pattern and can be further sub-classified based on the magnitude of aminotransferase elevation to mild [< 5 × upper limit of normal (ULN)], moderate (> 5-< 15 × ULN) and severe (> 15 × ULN). Hepatocellular pattern causes include but are not limited to; non-alcoholic fatty liver disease/non-alcoholic steatohepatitis, alcohol use, chronic viral hepatitis, liver cirrhosis (variable), autoimmune hepatitis, hemochromatosis, Wilson’s disease, alpha-1 antitrypsin deficiency, cielac disease, medication-induced and ischemic hepatitis. Cholestatic pattern causes include but are not limited to; biliary pathology (obstruction, autoimmune), other conditions with hyperbilirubinemia (conjugated and unconjugated). It is crucial to interpret these commonly ordered tests accurately as appropriate further workup, treatment and referral can greatly benefit the patient due to prompt treatment which can improve the natural history of several of the diseases mentioned and possibly reduce the risk of progression to the liver cirrhosis.
Liver function tests are some of the most commonly ordered tests in the United States due to the automation of routine laboratory tests. A United States population-based study of 6823 subjects from 1999 to 2002 showed elevated alanine aminotransferase (ALT) in 8.9% of subjects and aspartate aminotransferase (AST) in 4.9% of subjects.

Another population-based study consisting of 15676 subjects was done from 1988 to 1994 which showed elevation in aminotransferases (either ALT or AST) in 7.9%. In that study, 69% of the elevated aminotransferases results were unexplained [1].

Laboratory tests normal ranges are calculated based on the mean value found amongst a group of healthy individuals +/- 2 standard deviations. Hence 5% of healthy individuals' results lie outside the reference range [2].

As a result of the prevalence of liver biochemical tests ordered and abnormal results, we will be writing this review to increase the knowledge about liver tests to clinicians and improve the interpretation of these tests.

Liver biochemical studies include; ALT, AST, ALP, gamma-glutamyl transferase (GGT), 5’nucleotidase, lactate dehydrogenase (LDH), bilirubin, albumin, PT/INR (Table 1).

**Enzymes**

ALT is an enzyme that is found primarily in hepatocytes (lower concentrations in cardiac, renal, and muscle tissue) and thus is specific to the hepatocellular injury. ALT levels often fluctuate throughout the day. ALT facilitates the formation of glutamate and pyruvate in the hepatocyte which is important for energy production[4]. The normal range for ALT in males is between 29-33 IU/L and 19-25 IU/L for females.
ALT levels have been a point of debate recently as newer studies are suggesting the need for a lower ALT cutoff to increase the sensitivity of the test. It’s believed that the current ALT cutoffs were defined by using patients with possible underlying subclinical liver disease and hence decrease the sensitivity of the test. A retrospective study in 2002 evaluated 6835 patients and hypothesized that undiagnosed hepatitis C and non-alcoholic fatty liver disease (NAFLD) are likely to have skewed the studies previously used to determine normal ALT levels based on the 95th or the 97.5th percentile.

Suggested new cut-offs from this study are ALT < 30 in men and < 19 in women. It was found that the sensitivity in detecting hepatitis C virus viremia with the lower cut-offs was higher than that of the traditional cut-offs. Nonetheless these values should be cautiously interpreted as body mass index, cholesterol levels and age can affect ALT levels[5].

It is important to note that the reference ranges for labs differs across countries and sometimes even between different centers in the same country.

AST is an enzyme which like ALT is also found in the liver however has also other sites where its presence is not as minimal as ALT. These sites are primarily skeletal muscle, cardiac muscle, renal tissue, and brain. It occurs as 2 isoenzymes that are not differentiated on standard testing and hold little clinical value. AST facilitates amino acid metabolism[6]. When it comes to AST, caution must be practiced when evaluating abnormal levels due to its presence in other tissues. The normal range for AST is < 35 IU/L[7].

ALP is an enzyme that is primarily found in the hepatobiliary tract, bone, placenta, and to a smaller extent in intestinal tissue. ALP is involved in multiple dephosphorylating reactions. The normal range for ALP is between 30-120 IU/L. ALP is generally higher in children and adolescents due to the increased osteoblastic activity associated with the bone growth[8].

GGT is an enzyme that is found in multiple organs in the body including the pancreas, seminal vesicles, kidneys, biliary tract, and liver. Its elevation is usually considered significant for a hepatobiliary disease when accompanied by an elevation in other liver biochemical tests. It is generally elevated in biliary disease, cytochrome-inducing medications, and alcohol abuse. GGT is involved in the glutathione metabolism and production in multiple tissues in the body. Normal GGT levels range between 0-30 IU/L. GGT levels are generally 6-8 times higher in infants[9].
5’nucleotidase is an enzyme that is present in many organs however its clinical value holds significance primarily in hepatobiliary or cholestatic disease. It is generally used as a test to help in evaluating whether an isolated elevated ALP is from a hepatobiliary source vs an osseous source. Its primary function is in nucleotide hydrolysis reactions. The normal range for 5’nucleotidase 0.3-3.2 Bodansky units (levels need to be corrected with elevated serum ALP)[10].

LDH is an enzyme that is widely present in the body, it has multiple isoenzymes of which one is primarily excreted/taken up by Kupffer cells in the liver[11]. Hence liver disease/injury can result in elevated LDH. This is non-specific and is rarely used as means of evaluating liver disease. Normal LDH ranges between 140-280 U/L (ranges vary slightly between different labs).

Markers of liver synthetic function
Albumin is one of the major protein constituents in the blood and comprises 50%-60% of total protein in the serum. Albumin synthesis occurs in the liver hence it is considered a marker of the liver’s synthetic function. Albumin levels can be influenced by other causes such as systemic inflammation as albumin is a negative inflammatory marker, protein malnutrition, nephrotic syndrome, fluid overload, or protein-losing enteropathy. Albumin has multiple functions such as maintaining serum oncotic pressure and endogenous (i.e., bilirubin) and exogenous (i.e., drugs) substances transport in the blood[12]. Normal albumin levels range between 3.5-5 g/dL.

PT and INR reflect the coagulation cascade and in specific, the extrinsic pathway of the coagulation cascade. The liver is involved in the synthesis of multiple clotting factors including, factors I, II, V, VII, IX, X, XI, and XIII, in addition to protein C, protein S, and anti-thrombin. The reason why PT and INR are primarily elevated rather than activated partial thromboplastin time (aPTT) is due to factor VIII and von Willebrand factor being produced in multiple organs around the body and conceals the aPTT prolongation in vitro. Due to deficiency of both pro-coagulant and anticoagulant factors, PT/INR and aPTT are not reliable measures of bleeding risk in cirrhotic patients. Moreover, PT/INR and aPTT are measures of pro-coagulant activity and do not take into consideration defects in anticoagulant pathways. Besides, patients with chronic liver diseases or cirrhosis are likely to have thrombocytopenia due to splenic sequestration and decreased thrombopoietin levels which further increases the risk of bleeding[13].

Bilirubin itself is not a marker of liver synthetic function per se however its excretion and conjugation are closely linked to the liver’s conjugating and excreting function. Bilirubin is the end product of heme breakdown and is initially bound to albumin in the serum. In the liver, it is conjugated and excreted in the bile. Elevations in bilirubin levels are further classified as direct hyperbilirubinemia and indirect hyperbilirubinemia. Direct hyperbilirubinemia is generally due to an excretion defect in the liver such as cholestasis or Dubin-Johnson and Rotor syndrome. Indirect hyperbilirubinemia can be due to intrinsic liver injury or hemolysis[14].

PATTERN RECOGNITION AND INTERPRETATION
Pattern recognition and interpretation are crucial in the evaluation of abnormal liver biochemical tests. Patterns can be primarily divided into hepatocellular and cholestatic. These can be subdivided further into; acute (< 6 wk), subacute (6 wk-6 mo), or chronic (> 6 mo).

In hepatocellular pattern, there is a disproportionate rise in ALT and AST in contrast to ALP and GGT. In hepatocellular injury, there is release of aminotransferases from the hepatocytes resulting in elevated serum levels. R value is a proposed score aimed to aid physicians in determining the pattern of liver injury based on the upper limit of normal (ULN) of certain enzymes. R value = (ALT ÷ ULN ALT)/(ALP ÷ ULN ALP). R value > 5 is suggestive of hepatocellular pattern, > 2 to < 5 is suggestive of a mixed pattern, and < 2 suggestive of cholestatic pattern (Table 2)[15].

Hepatocellular pattern
Aminotransferase elevations can be divided into mild, moderate, and severe even though the values for this classification are variable, in this review we will be taking mild as > 2 × - < 5 × ULN lab value, moderate > 5 × - < 15 ×, severe as > 15 × ULN and massive > 10000 IU/L[16]. These values are not accurate measures of the extent of liver injury however can aid in initial workup.
Table 2 R-value calculation and interpretation

<table>
<thead>
<tr>
<th>R value</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 5</td>
<td>Hepatocellular pattern</td>
</tr>
<tr>
<td>&gt; 2 but &lt; 5</td>
<td>Mixed pattern</td>
</tr>
<tr>
<td>&lt; 2</td>
<td>Cholestatic pattern</td>
</tr>
</tbody>
</table>

ALT: Alanine aminotransferase; ULN: Upper limit of normal; ALP: Alkaline phosphatase.

One of the most commonly known and used ratios is AST:ALT and is generally helpful only for an alcoholic liver disease where AST:ALT > 2. A study done in 1979 among patients with histologic evidence of liver disease demonstrated that 90% of patients with AST:ALT > 2 had alcoholic liver disease and > 96% of patients with AST:ALT > 3 had alcoholic liver disease[17]. This ratio can be explained due to alcohol being a mitochondrial toxin and low pyridoxal phosphate absorption as a result of heavy alcohol use. AST is found in mitochondria and cytoplasm, while ALT is found in cytoplasm but not mitochondria. ALT synthesis is more dependent on pyridoxal phosphate when compared to AST. In alcoholic liver disease, ALT is generally < 300 IU/L and is rarely > 500 IU/L. In situations where ALT > 500 IU/L, even if AST: ALT > 2, other etiologies should be explored. AST:ALT > 1 can be seen in cases of liver cirrhosis. GGT > 2 × the ULN is suggestive of alcohol abuse specifically when paired with AST:ALT > 2. GGT on its own is not a specific indicator of alcohol abuse[1].

Mild elevations in aminotransferases are common to be seen in clinical practice and are generally caused by medications (nontoxic ingestions), alcohol use, and chronic liver diseases such as liver cirrhosis, NAFLD, chronic hepatitis infections (B and C), hemochromatosis, Wilson’s disease, autoimmune hepatitis, alpha-1 antitrypsin deficiency (AATD) and celiac disease (CD)[16]. It is advisable in patients with a mild increase in AST and ALT to undergo repeat testing in addition to the investigation of the aforementioned causes.

Moderate and severe elevations of aminotransferases are generally attributed to acute exacerbations of chronic liver diseases (such as exacerbations of hepatitis B virus, Wilson’s disease, acute viral hepatitis, autoimmune hepatitis), drug-induced liver injury (DILI), and ischemic liver injury[16]. Also, they can occur in cases of acute biliary obstruction and tend to resolve soon after the obstruction is relieved.

**Cholestatic pattern**

Elevation of ALP and bilirubin levels often indicate a cholestatic pattern[18]. ALP can be elevated in the presence of liver or bone disease, additionally, it can be elevated due to pregnancy (placenta production). GGT is often used to clarify the origin of ALP elevation. Since ALP is produced in the bile duct epithelia, cholestasis or biliary pathology elevates the enzyme. Both anatomic and autoimmune conditions that affect the biliary system cause a cholestatic pattern. When obstruction of the common bile duct (CBD) is the cause of ALP elevation, the aminotransferases can also be elevated[18].

GGT elevation is also caused by biliary or hepatocyte disease but not bone disease. However, other causes may elevate this enzyme such as drugs (anticonvulsants and oral contraceptives), pulmonary and renal disease. As a marker, it has a high sensitivity for liver disease but low specificity[19,20].

Elevations in bilirubin levels are further classified as direct (conjugated) hyperbilirubinemia and indirect (unconjugated) hyperbilirubinemia. Hemolysis is the most common cause of indirect hyperbilirubinemia followed by Gilbert’s syndrome. On the other hand, direct hyperbilirubinemia indicates liver pathology including cholestatic drug reactions, autoimmune cholestatic disease, and biliary obstruction[21].

Further laboratory and imaging studies are essential to work up the causes of a cholestatic pattern[18]. When autoimmune cholestatic liver disease is suspected the presence of anti-neutrophil cytoplasmic antibodies (for primary sclerosing cholangitis) or anti-mitochondrial antibodies (for primary biliary cirrhosis) among other studies help aid in the diagnosis.
**COMMON CONDITIONS ASSOCIATED WITH ABNORMAL LIVER ENZYMES**

NAFLD is one of the most common liver diseases, a meta-analysis was done in 2016 demonstrating the global prevalence of NAFLD to be approximately 25.24%[22]. Common condition associated with abnormal liver enzyme is shown in Table 3.

Nonalcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH) are diseases in the same spectrum where NAFL can progress to NASH and subsequently liver cirrhosis if no intervention or modification of risk factors was done[23]. These terms are often used interchangeably however it is important to note that the management is different and accurate assessment should be made. The difference between the two is primarily seen on histology as NAFL has only fatty infiltration without inflammation whereas NASH has marked inflammation. AST and ALT levels can be normal in NAFL and are generally mildly elevated in NASH (ALT > AST). NAFL and NASH are diseases of exclusion and general risk factors are metabolic, such as obesity, dyslipidemia, and diabetes mellitus[23]. It is important to note that NAFL is generally reversible with lifestyle modifications in contrast to NASH (Table 4).

Viral hepatitis can result in a mild increase in aminotransferases, specifically chronic viral hepatitis. Hepatitis B and Hepatitis C infections can generally cause chronic infections and also have a risk for developing liver cirrhosis. In a study done in 1988, patients with chronic viral hepatitis without liver cirrhosis had an AST:ALT < 1 (0.59 average), however those with chronic viral hepatitis and liver cirrhosis had an AST:ALT > 1. This was found to be significant and is important to identify in cases of chronic viral hepatitis to aid in recognizing possible concomitant liver cirrhosis[24]. Nonetheless, caution must be practiced when looking at AST:ALT specifically when alcohol use cannot be excluded. Acute viral hepatitis on the other hand can result in moderate to severe elevation in aminotransferases, often with ALT elevations higher than that of AST. Acute hepatitis C virus can result in marked elevations in aminotransferases however generally the elevation is modest compared to hepatitis A and B. Acutely, elevation in aminotransferases levels peak before bilirubin levels, however, begins declining gradually after in contrast to bilirubin[25]. Acute hepatitis A and B in adults are associated with elevations in bilirubin resulting in jaundice (more common with hepatitis A infection) and ALP. The risk of progression to chronic hepatitis is approximately 10% in hepatitis B patients above the age of 6, hepatitis A is not associated with chronic infection[26].

Hereditary hemochromatosis is an autosomal recessive disease caused by over absorption of iron secondary to abnormal iron sensing in the gastrointestinal tract resulting in iron overload[27]. The 2 most common mutations identified are C282Y and H63D on the hemochromatosis (HFE) gene. Non-HFE hemochromatosis exists, however in this review we will talk only about HFE hemochromatosis.

Hemochromatosis causes mild elevations in aminotransferases (ALT > AST), elevations in ALP and bilirubin can also be seen however liver biochemical tests are non-specific in cases of hemochromatosis[27]. Bilirubin elevation is thought to be a protective mechanism to help mitigate oxidative damage caused by excess iron in the liver. Moreover, a study done in 2004 demonstrated that bilirubin level elevation was found to have a positive correlation with serum iron level[28]. In cases of elevated aminotransferases without a clear cause, it would be wise to check iron studies including iron level, ferritin level, total iron-binding capacity, and transferrin saturation. If results suggestive of iron overload, genetic testing and liver biopsy should be considered.

Wilson’s disease is an autosomal recessive disease due to mutations in the ATP7B gene with a prevalence of approximately 1:30000 worldwide, studies have suggested higher prevalence based on gene mutation frequency. The difference between the 2 reported prevalence could be related to the disease’s possible low penetrance[29]. Wilson’s disease liver presentation is variable and can be from asymptomatic elevation in aminotransferases to acute liver failure (ALF). Aminotransferase elevation is mild in the majority of cases however can be moderate to severe in patients with Wilson’s presenting with ALF. 6%-12% of emergent liver transplant referrals are due to Wilson’s disease ALF[30]. Markers that aid in the diagnosis of ALF secondary to Wilson’s disease are non-immune hemolytic anemia, acute renal failure, AST:ALT > 2.2, and ALP: Bilirubin < 4. Almost all patients presenting with ALF secondary to Wilson’s have underlying liver fibrosis or cirrhosis[31,32].

AATD is an autosomal co-dominant disease with an expected prevalence of 3.4 million globally with combinations for severe AATD[33]. However, this number is thought to be under-representative of the actual prevalence[33]. A study done in 1989...
### Table 3 Common condition with abnormal liver biochemical tests

<table>
<thead>
<tr>
<th>Condition</th>
<th>AST/ALT</th>
<th>ALP</th>
<th>GGT</th>
<th>Bilirubin</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcoholic hepatitis</td>
<td>↑†</td>
<td>†</td>
<td>↑</td>
<td>↑</td>
<td>AST/ALT &lt; 500</td>
</tr>
<tr>
<td>NAFLD</td>
<td>/↑</td>
<td>†</td>
<td>/Mild↑</td>
<td>↑</td>
<td>If progress to cirrhosis</td>
</tr>
<tr>
<td>Viral hepatitis</td>
<td>↑†</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>AST-ALT &gt; 1 suggestive of cirrhosis</td>
</tr>
<tr>
<td>Hemochromatosis</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>Higher levels = higher iron load</td>
</tr>
<tr>
<td>Wilson’s disease</td>
<td>↑†↑↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>ALP:Bilirubin &lt; 4</td>
</tr>
<tr>
<td>AATD</td>
<td>↑†</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Celiac disease</td>
<td>↑↑</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Autoimmune hepatitis</td>
<td>↑↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>ALP:AST/ALT &lt; 3</td>
</tr>
<tr>
<td>DILI</td>
<td>↑↑↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑PT/INR</td>
</tr>
<tr>
<td>Cholestasis</td>
<td>↑</td>
<td>↑↑</td>
<td>↑</td>
<td>↑</td>
<td>AST-ALT &lt; 1.5 = ExtrahepaticAST-ALT &gt; 1.5 = Intrahepatic</td>
</tr>
</tbody>
</table>

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyl transferase; NAFLD: Non-alcohol fatty liver disease; AATD: Alpha-1 antitrypsin deficiency; DILI: Drug induced liver injury; PT: Prothrombin time; INR: International normalized ratio.

### Table 4 Non-alcoholic fatty liver disease spectrum

<table>
<thead>
<tr>
<th>Non-alcoholic fatty liver disease spectrum</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NAFL</td>
<td>Stratosis changes. No cellular ballooning, hepatocyte inflammation or fibrosis</td>
</tr>
<tr>
<td>NASH</td>
<td>Stratosis changes. Cellular ballooning and hepatocyte inflammation. No fibrosis</td>
</tr>
<tr>
<td>NASH related liver cirrhosis</td>
<td>Hepatocyte destruction and fibrosis</td>
</tr>
<tr>
<td>Healthy liver ←→ NAFL → NASH → NASH related cirrhosis</td>
<td></td>
</tr>
</tbody>
</table>

NAFL: Non-alcoholic fatty liver; NASH: Non-alcoholic steatohepatitis.

in St. Louis examined 20000 blood bank samples, 700 blood samples came back positive for homozygous PI*Z mutation, however, only 28 of those individuals have been diagnosed with AATD[34]. AATD involves multiple alleles however the alleles thought to be contributing to liver disease are M (maltron) and Z allele. In adults with homozygous PI*Z mutation, 40% were found to have evidence of injury and cirrhosis histologically. Aminotransferases are generally mildly elevated with ALT predominance. Bilirubin levels are elevated in later stages (cirrhosis) along with a decrease in albumin[35].

CD is an autoimmune disease characterized by gluten intolerance which often leads to malabsorption. A study was done where 158 adults recently diagnosed with CD were followed, 42% of patients were found to have mild elevations in aminotransferases. Patients were started on a gluten-free diet and in 95% of cases, the aminotransferases levels normalized at 1 year[36]. Another study was done evaluating patients with chronically elevated aminotransferases, workup on those patients revealed that 9.3% of patients had serological evidence of CD and all but one of the 9.3% had duodenal biopsy findings of CD[37]. Aminotransferases elevation is mild with an AST:ALT < 1, bilirubin levels are generally normal. ALP can be slightly elevated in a subset of patients but is generally normal. Albumin and PT/INR values are not very reliable indicators of hepatic synthetic function in cases of CD as CD is an autoimmune disease, and a state of inflammation could cause a decrease in albumin levels. Moreover, PT/INR values can be elevated due to concomitant vitamin K deficiency secondary to malabsorption[38].
Autoimmune hepatitis is an inflammatory disorder with a female predilection and a prevalence of approximately 1:5000-1:10000 in Europe. At the time of diagnosis, almost 50% of patients have jaundice and approximately 30% have cirrhosis[39,40]. Autoimmune hepatitis affects aminotransferases variably depending on acute vs chronic presentations. Acutely, elevations in aminotransferases can be moderate to severe and tend to gradually decline as the disease becomes chronic and/or liver cirrhosis ensues. Bilirubin, ALP, and gamma globulins elevations are also seen in autoimmune hepatitis. ALP:AST or ALT ratio < 3 which is calculated by using the following equation (ALP/ALP ULN)/(AST/AST ULN) (ALT can be used in place of AST for this calculation) and this ratio is thought to be helpful as disproportionate elevation of ALP should prompt exploration of other differentials such as primary biliary cholangitis[41]. Furthermore, it was found that patients with higher elevations in aminotransferases had a better prognosis when compared to those with milder aminotransferase elevations[42].

DILI can cause a multitude of effects on aminotransferases and elevations of aminotransferases can be mild, moderate, or severe. A wide range of medications can cause mild elevations of aminotransferases and those include antibiotics (such as amoxicillin-clavulanic acid, macrolides (cholestatic pattern), ceftriaxone), anti-convulsants (such as Carbamazepine, Phenytoin, Valproic acid, Gabapentin), statins, anti-tuberculosis medications, and herbal supplements. Hence, a thorough history of medication history is crucial in patients with elevated aminotransferases. More commonly, DILI is ALT predominant.

Drugs can also be a cause of moderate to severe aminotransferase elevation with the most commonly implicated drug being acetaminophen. Acetaminophen is advertised as safe with a daily dose < 4000 mg/d[43]. Acetaminophen-induced hepatotoxicity has a prevalence of approximately 30000 cases a year in the United States[44]. Up to 50% of overdoses were found to be unintentional[44]. Studies have been done which showed 6% of acetaminophen prescriptions to be > 4000 mg/d. A study evaluating AST:ALT ratio found that in cases of severe toxicity, an AST:ALT < 0.4 is suggestive of resolving hepatitis and is a positive prognostic marker[45]. Bilirubin, ALP, and PT/INR can all rise in cases of acetaminophen overdose. It is important to note that aminotransferases generally rise 2-3 d after an initial overdose and that an initial normal liver biochemical test does not exclude acetaminophen toxicity[45].

Acute cholecystitis (AC) usually presents as a cholestatic pattern or mixed. The biochemical test abnormalities are associated with obstruction from CBD, reactive hepatitis, fatty liver, direct gallbladder pressure on the biliary tract, or portal tract inflammation[19-21]. Patients with calculous AC may have CBD stones in up to 15%[17]. Gallbladder ultrasound and computed tomography (CT) is not entirely reliable for the diagnosis of CBD stones. Therefore, LFTs may be used for the identification of patients with suspected CBD stones who would benefit from endoscopic retrograde cholangiopancreatography (ERCP) or magnetic resonance cholangiopancreatography (MRCP) which are more sensitive and specific for this condition[18]. Multiple studies have shown mean values of LFTs higher in patients with AC plus CBD stones[18,22]. Bilirubin, AST, ALP, and GGT are the variables mostly studied to predict CBD stones. Ahn et al[18] found GGT to be the most reliable variable for CBD stones prediction with a sensitivity of 80.6% and specificity of 75%. Another study found an elevation in ALP to be the most important predictor for CBD stones[21]. Elevated LFTs in patients with AC without CBD stones are more likely to be transient and resolve within 2-7 d after surgery[18].

Ischemic hepatitis (often also referred to as hypoxic liver injury, shock liver, and hypoxic hepatitis) is a clinical condition characterized by acute liver injury causing severe elevation of aminotransferases secondary to hypoperfusion with a prevalence of approximately 2:1000 admissions and 2.5:100 in intensive care unit admissions. Moreover, it was found that approximately 4 out of 10 admissions with severe elevations in aminotransferases had ischemic hepatitis diagnosis. After further analysis, 78.2% of patients with ischemic hepatitis had a preceding acute cardiac event, 23.4% of patients with ischemic hepatitis had a diagnosis of sepsis and 52.9% of patients had a documented episode of hypotension (unspecified duration)[46].

The aminotransferase elevation is generally severe with level > 75 × ULN being suggestive of ischemic hepatitis. AST:ALT > 1 usually due to the location of AST (zone 3) in the liver and ischemic effect on zone 3. Bilirubin rise is not uncommon yet it can be mild and typically < 3 mg/dL. ALP is usually normal and PT/INR can be mildly elevated[47]. Another ratio that was found to be useful is AST:LDH < 1.5 which helps in differentiating ischemic hepatitis from viral hepatitis[48]. The AST:LDH ratio is thought to be due to the rapid and severe rise of LDH in cases of ischemic hepatitis due to hypoperfusion.
ALF is another potential cause of severe elevation in aminotransferases and cautious identification of this condition is crucial as mortality risk is approximately 40%-80%. ALF is defined as the presence of severe liver injury in addition to clinical and laboratory features of liver failure such as hepatic encephalopathy and elevation in INR specifically in an individual with no prior history of liver cirrhosis or liver disease. Etiologies of ALF include but are not limited to Ischemic hepatitis, Budd Chiari syndrome, Wilson’s disease, autoimmune hepatitis, acute viral hepatitis, and drug-induced liver disease. Biochemical test evaluation in ALF can be hepatocellular initially and progress to cholestatic in later stages. Labs are typically significant for severe elevation in aminotransferases, mild to moderate elevation in bilirubin and ALP in addition to INR ≥ 1.5, and in some cases LDH elevation. While declining aminotransferases can be suggestive of recovery, this is not an accurate measure of recovery as it could be indicative of worsening liver failure and severe loss of liver mass. It is more appropriate to follow bilirubin, INR, and clinical features (hepatic encephalopathy) in patients with ALF for possible recovery.

DIAGNOSTIC TESTS

The initial evaluation of abnormal biochemical tests will be guided by the pattern (hepatocellular, cholestatic, or mixed). As a first step, the clinician should inquire about the use of medication, herbal therapies, drugs, or alcohol consumption. If a hepatocellular pattern is identified, initial serology should be obtained to rule out infectious and autoimmune etiologies. A right upper quadrant ultrasound (RUQ US) is also justified to evaluate for fatty liver. If the previous workup is unrevealing uncommon causes should be worked up (such as Wilson disease, AATD, etc.). If the serologic studies and imaging are unremarkable and ALT/AST is persistently elevated, consider a liver biopsy. When ALP is elevated, GGT and 5’ nucleotidase tests are important to identify the source of ALP elevation. If the latter is elevated ALP likely is elevated from hepatobiliary origin. The RUQ US will help to identify ductal dilation or the absence of it. Further workup includes either an MRCP or an ERCP (when ductal dilation is present) or serological studies including AMA if no dilation is identified. Cholestasis can be further divided into intrahepatic or extrahepatic both usually seen with marked elevation of ALP. The workup for extrahepatic cholestasis should aim to rule out choledocholithiasis, malignant obstruction, and biliary strictures. For intrahepatic cholestasis, laboratory works up should aim to rule out primary biliary cholangitis, primary sclerosing cholangitis, sickle cell disease among other causes. In intrahepatic cholestasis imaging or laboratory, workup may not yield a definitive diagnosis and other causes should be considered (i.e., total parenteral nutrition, drugs associated with cholestasis, ischemic, cholestasis of pregnancy, etc.)

CONCLUSION

The elevation of liver biochemical studies is a common encounter of all clinicians. The multiple markers used to identify liver injury may be also elevated due to other sources (bone, placenta, kidney, muscle, etc.). The biochemical knowledge helps to better understand the behavior of these markers in specific conditions. The proper recognition of hepatocellular or cholestatic pattern prompts further investigations that include imaging and laboratory studies. Other factors highly important to consider when evaluating abnormal liver biochemical patterns are signs and symptoms, medications, degree of liver tests elevation, and other laboratory abnormalities present. Unfortunately, despite the use of additional tests (imaging and laboratory) in some causes the diagnostic is unclear and liver biopsy is recommended.

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LFT interpretation

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Hepatopulmonary syndrome: An update

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Abstract

Hepatopulmonary syndrome (HPS) is characterized by defects in oxygenation caused by intra-pulmonary vasodilation occurring because of chronic liver disease, portal hypertension, or congenital portosystemic shunts. Clinical implications of portal hypertension are very well-known, however, awareness of its effect on multiple organs such as the lungs are less known. The presence of HPS in chronic liver disease is associated with increased mortality. Medical therapies available for HPS have not been proven effective and definitive treatment for HPS is mainly liver transplantation (LT). LT improves mortality for patients with HPS drastically. This article provides a review on the definition, clinical presentation, diagnosis, and management of HPS.

Key Words: Hepatopulmonary syndrome; Chronic liver disease; Hypoxemia; Intrapulmonary vasodilatation; Liver failure

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Core Tip: Hepatopulmonary syndrome (HPS) is a progressive disease, the presence of which in cirrhotic patients worsens their prognosis. Patients with HPS have an increase rate of mortality compared to those without HPS when matched for severity of liver disease, age, sex, and liver transplantation (LT). HPS should be identified in all patients with chronic liver disease and supportive management should be provided until definitive treatment, e.g., LT could be done.
INTRODUCTION

HPS is a progressive disease associated with worsen prognosis in patients with chronic liver disease. Patients with HPS have an increase rate of mortality compared to those without HPS when matched for severity of liver disease, age, sex, and liver transplantation (LT) [1]. Hepatopulmonary syndrome (HPS) was first described in 1884 by Fluckiger based on observation in a woman with cyanosis, clubbing, and cirrhosis. Later, HPS was coined in 1977 after multiple post-mortem studies showing pulmonary vascular dilation in cirrhotic patients. These studies showed marked peripheral dilation of pulmonary arteries at precapillary and capillary levels, without any obvious lung parenchymal disease. These studies were also remarkable for multiple pleural spider naevi [2].

DEFINITION

HPS is defined as hypoxemia due to pulmonary vascular dilation in the setting of liver disease with or without portal hypertension. Definition and staging of HPS are shown in Table 1 and Table 2.

INCIDENCE/PREVALENCE

HPS has been reported in 5%-35% of patients with end-stage liver disease [3,4]. Studies have shown the presence of HPS in various liver etiologies including cirrhosis, non-cirrhotic portal fibrosis, and extra-hepatic portal vein obstruction [5,6]. Studies showed an increasing prevalence of intrapulmonary shunt in patients with increased severity of cirrhotic disease such as pretransplant patients with Child-Pugh Class C when compared with class A or B [7]. It has also been found to be associated with liver disease severity assessed by MELD score [3].

PATHOPHYSIOLOGY

Chronic liver disease can lead to hypoxemia due to a variety of underlying pathologies. Thus, it is imperative to differentiate between them. For example, HPS is caused by pulmonary vasodilation in the setting of liver disease whereas Portal-pulmonary hypertension, which is very similar in clinical presentation, is defined by pulmonary vasoconstriction causing hypoxemia due to resultant pulmonary hypertension.

The hypoxemia associated with HPS is secondary ventilation-perfusion mismatch caused mainly by diffusion defect in the dilated pulmonary bed: (1) Increased blood flow through the intra-pulmonary vasodilatation (IPVD) through the well-ventilated alveoli results in the passage of mixed venous blood in the pulmonary veins; and (2) Diffusion of oxygen is limited through the dilated pulmonary vessels due to their increased diameters resulting in disequilibrium. Supplemental oxygen increases the partial pressure of oxygen by providing the driving pressure for the oxygen to diffuse across the dilated vessels. Thus, IPVDs act as physiologic shunts more than anatomic shunts as oxygenation improves with external supplementation [8].

The unique pathological feature of HPS is dilatation of pulmonary precapillary and capillary vessels (15-100 µm diameter) along with an absolute increase in the number of dilated vessels. Paraumbilical vein and hepatic artery diameters are significant larger in cirrhotic patients with HPS compared to non-HPS [9]. Lungs and pleural spider nevi are the terms used when these vessels are noted in the lungs and along the pleural surface. Intrahepatic vasculature changes which were reported in HPS include thrombosis in intrahepatic portal venules, fibrous septa with vessels proliferation, and
Table 1 Hepatopulmonary syndrome definition

<table>
<thead>
<tr>
<th>Index</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxygenation</td>
<td>PaO2 &lt; 80 mmHg or A-a gradient (corrected for age) &gt; 15 mmHg or 20 mmHg if age &gt; 64 years while breathing room air</td>
</tr>
<tr>
<td>Intrapulmonary vasodilation</td>
<td>Confirmed by contrast-enhance echocardiography or lung perfusion scanning showing brain shunt fraction &gt; 6%</td>
</tr>
<tr>
<td>Liver disease</td>
<td>Cirrhosis and/or portal hypertension</td>
</tr>
</tbody>
</table>

Table 2 Staging based on severity of hepatopulmonary syndrome

<table>
<thead>
<tr>
<th>Stage</th>
<th>Partial pressure of oxygen (mmHg) on room air</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>≥ 80</td>
</tr>
<tr>
<td>Moderate</td>
<td>60 to &lt; 80</td>
</tr>
<tr>
<td>Severe</td>
<td>50 to &lt; 60</td>
</tr>
<tr>
<td>Very severe</td>
<td>&lt; 50 on room air or &lt; 300 while breathing 100% oxygen</td>
</tr>
</tbody>
</table>

centrilobular venous thickening[9]. Doppler ultrasonography in HPS reveals hepatojugular flow and portal blood flow of less than 10 cm/s[9].

The underlying pathophysiology is not fully proven, however, is thought to be caused by loss of pulmonary capillary vessel tone and inhibition of pulmonary vasoconstrictors. Enhanced production of nitric oxide (NO) is the major factor for pulmonary vasodilatation. NO is produced by the action of NO synthase on L-arginine. NO synthase has three isoforms of which endothelial NO synthase (eNOS) produced by pulmonary endothelial cells is the major source of NO production[10].

In experimental rat models of HPS with common bile duct ligation, proliferating cholangiocytes produce endothelin-1 (ET-1) which activates pulmonary vascular endothelin-B (ETB) receptor which in turn mediates eNOS activation and pulmonary macrophages accumulation. These animal models also showed overall increased expression of ETB receptors and increased circulation of ET-1[11,12].

In humans with HPS, exhaled NO is elevated which is a result of pulmonary vascular production and it normalizes after LT[13,14]. Acute administration of methylene blue, an inhibitor of NOS, transiently improves oxygenation[15].

Bacterial translocation from the gut in the setting of portal hypertension results in pulmonary vascular macrophages has been proposed as a mechanism causing pulmonary vasodilatation[16,17]. A study shows the decrease in this bacterial translocation by norfloxacin and thus, decreasing the severity of HPS[18]. Heme-oxygenase-derived carbon monoxide and tumor necrosis factor-alpha are also observed to contribute to pulmonary vasodilatation and angiogenesis[19,20].

**CLINICAL PRESENTATION**

Dyspnea on exertion or rest is the most common presenting symptom of HPS. However, dyspnea is very non-specific given it can be present in chronic liver disease due to ascites, volume overload, anemia, or muscle weakness. The presence of platypnea and orthodeoxia are specific for HPS, but not pathognomonic. Platypnea means dyspnea in an upright position which is relieved in the supine position. Orthodeoxia refers to a decrease in partial pressure of oxygen by greater than 4 mmHg or a decrease in oxygen saturation by more than 5% from a supine to upright position[21]. Both platypnea and orthodeoxia are attributed to the ventilation-perfusion mismatch.

Physical signs such as the presence of spider nevi, clubbing, cyanosis along hypoxia are strongly suggestive of HPS. Of these signs, patients with the chronic liver disease having spider nevi have a higher prevalence of HPS compared to those without spider nevi[22].
**DIAGNOSIS**

Patients with chronic liver disease who has dyspnea, or signs of clubbing, cyanosis, spider nevi should undergo screening and evaluation for HPS. All patients who are candidates for LT are also screened for HPS. Evaluation of HPS includes assessment of hypoxemia and intrapulmonary vasodilation. Exhaled NO is found to be higher in HPS than non-HPS patients which may help with the diagnosis.

**ASSESSMENT FOR HYPOXEMIA**

Pulse oximetry is used for screening purposes in chronic liver diseases to assess for HPS. All the patients with oxygen saturation < 96% should further undergo arterial blood gas analysis (ABG) to evaluate for underlying hypoxemia[23]. ABG should be drawn in the upright position to evaluate for orthodeoxia. A-a gradient > 15 mmHg or PaO2 < 80 mmHg is used for evaluation of hypoxemia. A-a gradient is more reliable than the partial pressure of oxygen as it accounts for hyperventilation, which is common in chronic liver disease[24].

The establishment of hypoxemia alone is not enough for the diagnosis of HPS, as it can be seen in other diseases such as Porto-pulmonary hypertension. Diagnosis requires confirmation of intrapulmonary vasodilation.

**ASSESSMENT FOR INTRAPULMONARY VASCULAR DILATATIONS**

Transthoracic contrast echocardiography (TTCE) is first-line diagnostic tool for IPVDs. IPVDs create a shut wherein 5%-6% of the cardiac output gets shunted. TTCE is performed by injecting the agitated saline into the venous system during the echocardiogram. Agitated saline leads to the formation of bubbles in the right atrium which is then filtered by the pulmonary capillary bed. Pulmonary capillary diameter varies from 8 to 15 μm which does not allow the passage of the microbubbles. The presence of intra-cardiac or intra-pulmonary shunt leads to visualization of microbubbles/contrast in the left heart chambers. The timing of the appearance of these bubbles in the left atrium varies with heart rate, cardiac output, and shunt size. With the intra-pulmonary shunt, the microbubbles or opacification of the left atrium occurs in three to six cardiac cycles after their first appearance in the right atrium. Whereas with the intra-cardiac shunt, this opacification of the left atrium is visualized within the first three cardiac cycles after its first appearance in the right atrium. Thus, TTCE is a sensitive tool for the diagnosis of pulmonary shunt[25].

Transesophageal echocardiography is a more specific alternative to TTCE, however, is generally avoided due to the high risk associated with bleeding from esophageal varices in this patient population[26].

Technetium-99m-labeled macro aggregated albumin is also filtered by the pulmonary capillary bed and can be used to measure shunt fraction by identifying its uptake in the brain and/or kidneys. Under normal circumstances, macro aggregated albumin should not pass the pulmonary capillary bed. However, in presence of right-to-left shunt, the radionuclide is taken up by the brain and kidneys and the percentage uptake can be used to quantify the shunt. In contrast to TTCE, this method does not distinguish between intra-pulmonary and intra-cardiac shunts[27].

Contrast pulmonary angiography is rarely used to visualize the IPVD due to the invasive nature of this procedure. It is generally indicated in patients with suspicion for pulmonary arteriovenous malformations, which rarely occurs in HPS[28]. Contrast-enhanced triple phase multi-detector computed tomography abdominal portosystemic shunts of more than 10 mm in diameter[9].

**MANAGEMENT**

**LT**

The only definitive management for HPS is LT. All the patients with the partial pressure of oxygen less than 60 mmHg should be evaluated for LT. Mortality is significantly higher in patients with HPS who do not undergo LT compared to those who undergo LT. A study showed 78% mortality in HPS patients who did not undergo LT compared to 21% mortality in patients who underwent LT[29]. Thus,
patients with HPS are given higher priority for liver transplants compared to other factors. LT has been shown to improve oxygenation and shunt within the first year of transplant[30,31]. A retrospective study with 74 patients showed improvement in PaO₂ from 89% to 94% and a decrease in A-a gradient from 16 to 8 mmHg after transplantation, without significant change in DLCO[32]. A study showed a 76% 5-year survival rate in HPS who underwent LT, which is similar to liver transplant patients without HPS[33].

Oxygen supplementation
All the patients with mild to moderate HPS should be evaluated every 3 to 6 mo with ABG. All patients with oxygen saturation less than 89% or partial pressure of oxygen less than 55 mmHg at rest, exercise and while sleep should be provided supplemental oxygen.

Investigational therapies
Pentoxifylline, a tumor necrosis factor-alpha inhibitor, vasodilator with anti-angiogenesis, showed variable results in oxygenation improvement in HPS[34-36]. Early-stage HPS patients seem to have a favorable outcome, while patients with advanced-stage HPS had unimproved oxygenation and difficulty tolerating pentoxifylline due to gastrointestinal adverse effects. Randomized placebo-controlled trial is needed to prove its result.

Garlic, has allicin which is a potent vasodilator and anti-angiogenesis. It shows significant improvement in gas exchange in small studies, which include one randomized controlled trial[37,38]. Large trials are still required to prove its benefit. Inhaled NO, a vasodilator, showed an improvement of PaO₂ in a recent physiologic study even though prior findings were contradicting[39,40]. Vascular dilatations, pulmonary capillary arteriovenous communication, and blood flow shunting in HPS are thought to be more prominent in lower lung zones due to gravitation and the vasodilators use in HPS are believed to be more potent in upper and mid lung zones. Therefore, ventilation-perfusion mismatch decreased.

Methylen blue causes vasoconstriction by inhibiting NO and may also decrease angiogenesis. It shows some benefits in improving oxygenation; however, no randomized clinical trial is available to support its use[15]. Another agent that has been shown to reduce pulmonary NO is N(G)-nitro-L-arginine methyl ester. However, it didn’t improve arterial oxygenation or ventilation-perfusion mismatch[41]. Sorafenib is a tyrosine kinase inhibitor that can reduce angiogenesis. It significantly decreased alveolar-arterial oxygen gradient in rat model but failed to show benefit in patients with HPS in a randomized-controlled trial[42]. Octreotide, a somatostatin analogue that can inhibit angiogenesis, also showed no benefit in HPS patients in few studies[43].

Mycophenolate mofetil only showed benefit in one case report[44]. Norfloxacin decreases bacterial translocation and reveals benefit in an animal study and a human case report but not in a randomized controlled trial[45]. Other medications including iloprost (vasodilator), paroxetine (NO synthase inhibitor), almitrine bimesylate (pulmonary vasoconstrictor) have been tried without any clear benefit. Letrozole is undergoing an ongoing phase two trial.

The transjugular intrahepatic portosystemic shunt has been proposed to decrease portal hypertension in HPS. A small prospective study showed improvement in gas exchanged, but limited data are available[46,47]. Few case reports regarding embolization of pulmonary vasodilatation have shown improvement in oxygen[28]. All these studies do not have clear establish benefits.

CONCLUSION
All the patients with chronic liver disease with dyspnea should be screened for HPS using ABG. There is no definitive proven treatment plan for HPS except LT. Thus, all patients with HPS should undergo expedited evaluation of LT.

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Mitochondrial hepatopathy: Respiratory chain disorders—‘breathing in and out of the liver’

Amrit Gopan, Moinak Sen Sarma

Abstract

Mitochondria, the powerhouse of a cell, are closely linked to the pathophysiology of various common as well as not so uncommon disorders of the liver and beyond. Evolution supports a prokaryotic descent, and, unsurprisingly, the organelle is worthy of being labeled an organism in itself. Since highly metabolically active organs require a continuous feed of energy, any dysfunction in the structure and function of mitochondria can have variable impact, with the worse end of the spectrum producing catastrophic consequences with a multisystem predisposition. Though categorized a hepatopathy, mitochondrial respiratory chain defects are not limited to the liver in time and space. The liver involvement is also variable in clinical presentation as well as in age of onset, from acute liver failure, cholestasis, or chronic liver disease. Other organs like eye, muscle, central and peripheral nervous system, gastrointestinal tract, hematological, endocrine, and renal systems are also variably involved. Diagnosis hinges on recognition of subtle clinical clues, screening metabolic investigations, evaluation of the extrahepatic involvement, and role of genetics and tissue diagnosis. Treatment is aimed at both circumventing the acute metabolic crisis and long-term management including nutritional rehabilitation. This review lists and discusses the burden of mitochondrial respiratory chain defects, including various settings when to suspect, their evolution with time, including certain specific disorders, their tiered evaluation with diagnostic algorithms, management dilemmas, role of liver transplantation, and the future research tools.

Key Words: Mitochondrial hepatopathy; Respiratory chain defects; Maternal inheritance; Neonatal liver failure; DNA depletion syndrome; Pearson syndrome

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FULL TEXT

Core Tip: Liver disease with multi-system involvement should arouse the suspicion for mitochondrial respiratory chain hepatopathies. These disorders are predominantly autosomal recessive with some having a maternal inheritance. Presence of lactic acidosis without hypoglycemia is an important clue. A tiered evaluation yields the most data, with the final step being a genetic and enzyme analysis from tissue of interest. Treatment is largely supportive with blood transfusions, correction of acidosis and shock, providing cofactors and salvage therapies, with liver transplantation in a select group. A periodic follow-up is mandatory for monitoring evolution of disease including “migration” to other systems.

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INTRODUCTION

Mitochondria are intracellular organelles, with a double lamellar covering outer membrane serving as a corset that holds the highly convoluted inner membrane in place (Figure 1). The inter-membrane space is the first of two liquid components within the mitochondria mainly participating in the exchange of lipids, proteins, and metal ions and also signaling cascades[1]. The second space is the soluble matrix, lying within the inner membrane and the hub of various metabolically active processes, most notably the tricarboxylic acid cycle, fatty acid oxidation, and urea synthesis. The inner membrane is folded into multiple cristae, which are shelf like projections into the matrix. The number of cristae are reflective of a metabolically active state, with a more active tissue having numerous mitochondria with many cristae, an ideal comparison being striated muscle tissue against adipocytes. This inner membrane of the mitochondria houses the respiratory chain comprised of electron carriers (complexes I, II, III, and IV, cytochrome c, coenzyme Q) and complex V, which is the hydrogen adenosine triphosphates complex (Figure 2). All metabolic processes within the matrix generate reducing equivalents in the form of electrons (carried as NADPH2), which pass through these complexes, entering it at various points. While doing so, from one complex to another, it also results in proton (H+) flow from matrix to intermembrane space leading to its pooling up and a chemical gradient that then flows down the potential via the complex V, which utilizes the energy to generate adenosine triphosphate from adenosine diphosphate, the ultimate objective of this intricately woven complex process called oxidative phosphorylation[2].

MITOCHONDRIAL GENOME AND ITS IMPLICATIONS

From the perspective of evolution, the classical endosymbiont theory proposes that mitochondria are actually prokaryotes within eukaryotic cells and hence have a genome of their own[3]. Mitochondrial genome consists of a circular double stranded DNA made of 16569 base pairs organized to make up 37 genes. Of these, 13 genes are exclusively for synthesis of proteins that are part of the respiratory chain. The other 24 genes are required for mitochondrial DNA (mtDNA) translation process (22 genes for an equal number of transfer RNA and two for ribosomal RNA synthesis). There are three major differences between mitochondrial and Mendelian inheritance: Maternal inheritance, heteroplasy and threshold effect, and mitotic segregation. Maternal inheritance in simple terms means that the mtDNA and its aberrations are transferred from mother (ovum) to its offspring (zygote), as there is hardly any mitochondria left in the sperm, which concentrates itself to fill its entire cytoplasm with the energy dense nucleus. However, there are a few exceptions, as reported in skeletal muscle defects linked to mitochondrial inheritance that are transmitted by father to offspring [4]. It is essential to understand that all characteristics encoded by mtDNA are maternally inherited but all mitochondrial diseases are not maternally inherited.
Gopan A et al. Mitochondrial hepatopathy: Respiratory chain disorders

Figure 1 Diagrammatic representation of structure of mitochondria. mtDNA: Mitochondrial DNA.

Figure 2 The electron transport chain formed by the respiratory chain complexes and process of oxidative phosphorylation. ADP: Adenosine diphosphate; ATP: Adenosine triphosphate; CoQ: Coenzyme Q; Cyt c: Cytochrome c; FAD: Flavin adenine dinucleotide; FADH2: Reduced form of FAD; NAD: Nicotinamide adenine dinucleotide; NADH: Reduced form of NAD.

Nuclear DNA (nuDNA) encodes most of the metabolic processes occurring in the mitochondria. NuDNA also encodes many enzymes and cofactors required for maintenance of mtDNA as well as approximately 70 respiratory chain subunits[5]. Figures 3 and 4 describe the way of inheritance and the mathematics of genetics in mitochondrial diseases. In normal persons, all mtDNA are identical, a state known as homoplasmy. Presence of both mutated and non-mutated wild type mtDNA containing mitochondria together in a cell is cellular heteroplasmy, while having 2 types of mtDNA within a single mitochondrion is organellar heteroplasmy. A particular number of abnormal mtDNA burden should exist for disease phenotype to manifest, a phenomenon known as threshold effect. This effect is seen at different levels of mutated mtDNA in various organs, the lowest threshold (and hence maximum susceptibility) being in organs dependent highly on oxidative metabolism like brain, heart, skeletal muscle, retina, and endocrine organs. Another interesting phenomenon is “skewed heteroplasmy” where some organs selectively have a higher burden of abnormal mitochondria, exemplified by mitochondrial diabetes, cardiomy-
opathies, and deafness [6-8]. Mitotic segregation effect refers to the random distribution of mitochondria at end of cell division, which can segregate mutated and non-mutated mtDNA in a variable manner into the two daughter cells. This may result in a daughter cell phenotype that is diseased (due to presence of more abnormal mitochondria), i.e. more abnormal than the originator cell in the subsequent divisions. With age, abnormal cells may predominate, explaining age related unmasking of diseases.

THE PROBLEM STATEMENT: EPIDEMIOLOGY

Prevalence of respiratory chain defects is variable across geographical lines as well as across eras. A large study examining birth prevalence of mitochondrial respiratory
chain disorders (RCDs) up to 16 years of age puts the figure at 5/100000 births[9]. This would mean that for every 20000 births in a particular time period, 1 child has the probability of getting affected by a respiratory chain defect of any type till he or she reaches the age of 16. The same study extrapolated the prevalence as 13.1/100000 births with onset at any age when seen together in the light of another study by Chinnery et al[10]. According to the Swedish registry, in a population study identifying mitochondrial encephalomyopathies, 20% had liver involvement[11]. In a 5 year French study of 1041 children, 22 (10%) of the 234 patients with respiratory chain defects had hepatopathy[12]. We would, however, add a word of caution that these figures can be an underrepresentation of true values in view of the heterogeneity of presentation and difficulty in diagnosis of mitochondrial respiratory chain defects.

CLASSIFICATION OF MITOCHONDRIAL HEPATOPATHIES AND STATUS OF RESPIRATORY CHAIN DISORDERS

Mitochondrial disorders are characterized by their variability in presentation and predilection for more than one organ system simultaneously or separated in time. Sokol and Treem proposed classifying these disorders as primary and secondary depending on whether defect is inherently present in the mitochondria and leads to liver dysfunction or there is secondary involvement of mitochondria in the form of injury or alteration in non-mitochondrial genetics. There are two broad types of mitochondrial hepatopathies, one which affects the respiratory chain present on the inner mitochondrial membrane and the other includes fatty acid oxidation defects, which are related to the process within the mitochondrial matrix. The RCDs can also be divided into those arising due to defective mtDNA and those due to defect/mutation in nuDNA. Among the diseases affecting mtDNA, the affliction can be in the form of either mutations or an overall depletion of quantity of mtDNA compared to nuDNA in a cell/tissue. Figure 5 shows a simplified way of classification of mitochondrial hepatopathies (MH), and a tabular representation of primary MH individual disorders is shown in Table 1. It is worthwhile to note that usually mitochondrial disorders with primary myopathic involvement have mutations in mtDNA, while those with primary hepatic involvement have mutations in nuDNA affecting mitochondrial processes, with some exceptions[13]. Since we are discussing respiratory chain disorders not confined to liver but to include the gastrointestinal tract, we will include one prototype non-hepatic RCD affecting the gastrointestinal (GI) tract, mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), in our review. This review does not cover non-RCD mitochondrial hepatopathies (fatty acid oxidation disorders and others) and GI manifestations of non-RCD, non-hepatic mitochondrial disorders.

CLINICAL PRESENTATION

Mitochondrial disorders are often called mitochondrial multiorgan disorder syndrome (MIMODS) in view of their heterogenous presentation affecting the nervous system (central and peripheral), eyes, ears, endocrine system, kidneys, heart and blood vessels, bone marrow, lungs, and also the intestinal tract, apart from affecting the liver (hepatopathy). The liver involvement is also variable in clinical presentation as well as in age of onset, from acute liver failure, cholestasis, or as chronic liver disease. A graphical summary of all mitochondrial RCDs affecting the liver is represented in Figure 6. Each of the individual disorders is briefly discussed.

Neonatal liver failure

Neonatal liver failure is a catastrophic event, and there are few disorders that present in the first few months as liver failure. Neonatal acute liver failure (ALF) is distinct from pediatric and adult liver failures in that it can include causes that have underlying cirrhosis. Also, the cut-off for coagulopathy is proposed as an international normalized ratio (INR) of ≥ 3 for newborns, as normal INR can be up to 2 in this age [14]. The four main causes of neonatal liver failure are: (1) Gestational alloimmune liver disease (neonatal hemochromatosis); (2) Viral infections (herpes simplex); (3) Hemophagocytic lymphohistiocytosis (primary-familial/secondary to infections); and (4) Mitochondrial hepatopathies (respiratory chain defects).
Table 1 Various mitochondrial primary respiratory chain disorders

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Mutation/defective gene</th>
<th>Location of defect</th>
<th>Affected proteins/consequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neonatal liver failure: (1) Complex I deficiency; (2) Complex III deficiency; (3) Complex IV deficiency; and (4) Multiple complex deficiencies</td>
<td>ACAD9; BCS1L; SCO1</td>
<td>nuDNA</td>
<td>Respective complexes deficiency as per name</td>
</tr>
<tr>
<td>Delayed onset liver failure: Alper’s Huttenlocher syndrome</td>
<td>POLG mutation</td>
<td>nuDNA</td>
<td>Defective mtDNA polymerase; mtDNA depletion</td>
</tr>
<tr>
<td>MtDNA depletion syndrome</td>
<td>DGUOK; TK-2; MPV 17; POLG</td>
<td>All nuDNA</td>
<td>Decreased deoxyribonucleotide concentrations within mitochondria</td>
</tr>
<tr>
<td>Mitochondrial neuro-gastrointestinal encephalomyelopathy</td>
<td>TYMP</td>
<td>nuDNA</td>
<td>Markedly low levels of thymidine phosphorylase activity</td>
</tr>
<tr>
<td>Pearson marrow pancreas syndrome</td>
<td>4000-5000 bp deletions in mtDNA; tRNA gene of mtDNA</td>
<td>Both mtDNA</td>
<td>Complex I, IV, V</td>
</tr>
<tr>
<td>Navajo neurohepatopathy</td>
<td>MPV 17 mutations</td>
<td>nuDNA</td>
<td>mtDNA depletion</td>
</tr>
<tr>
<td>Villous atrophy with hepatic involvement</td>
<td>Rearrangement defect/deletion-duplications in mtDNA</td>
<td>mtDNA</td>
<td>Complex III deficiency</td>
</tr>
</tbody>
</table>

nuDNA: Nuclear DNA; mtDNA: Mitochondrial DNA.

Figure 5 Simplified way of classification of mitochondrial hepatopathies based on location of defect. IMS: Intermembrane space; mtDNA: Mitochondrial DNA; PEP: Phosphoenolpyruvate.

Apart from these, galactosemia, tyrosinemia, and hereditary fructose intolerance can present as ALF in early infantile period and rarely in neonatal age[15]. The key here is to keep mitochondrial hepatopathy (RCD and non-RCD) as one of the differentials of acute liver failure in a newborn/early infantile period, though it accounts for < 5% in neonatal ALF series[14]. Multi-system involvement, especially with neurological symptoms in form of lethargy, floppy tone, vomiting, poor suck, and seizures, are diagnostic clues. Some patients are apparently normal until a viral illness or an unknown inciting event seems to trigger a downhill course either hepatic or neurological or both. Infants with mitochondrial hepatopathies are seen to have a low birth weight in up to 23%, and associated intrauterine growth retardation is seen in 16%, likely due to insult beginning from intrauterine period[16]. Laboratory findings of metabolic acidosis, elevated lactate levels, high lactate to pyruvate ratio often more than 30 mol/mol, elevated ketone bodies beta-hydroxybutyrate, and beta-hydroxybu-
tyrate to acetoacetate ratio > 2 mol/mol are corroborative, but absence does not rule out the diagnosis. Liver biopsy findings may yield micro or macrovesicular steatosis, which reflects impaired energy metabolism. Liver or muscle tissue respiratory chain analysis shows decreased levels of complexes I, III, or IV. Liver biopsy is often done post-mortem due to the inability to do so percutaneously in view of coagulopathy. Treatment including liver transplant is discussed subsequently.

**Delayed onset liver disease: Alpers Huttenlocher syndrome**

This syndrome presents anywhere from 2 mo to 8 years of age, predominantly in late infancy to childhood (Figure 7 graphical summary). The diagnostic criteria include: (1) Presence of refractory seizures including focal seizures; (2) Infection triggered psychomotor regression that is episodic in nature; and (3) Liver dysfunction with or without liver failure. Liver involvement is in the form of hepatomegaly, jaundice, coagulopathy, and episodes of hypoglycemia. Gastrointestinal involvement mainly due to the muscle impairment results in progressive feeding difficulty and gastroesophageal reflux, progressing to intractable vomiting. One series of 5 patients with Alpers Huttenlocher syndrome (AHS) showed mean age of liver disease presentation of 35 mo, and all died over a mean 4.6 wk period, due to progressive liver failure.

Autopsy findings across series show macrovesicular steatosis, massive hepatocyte dropout, proliferating bile ductular elements replacing hepatocytes, and often cirrhosis. Valproate is known to precipitate liver failure in these patients when given for the frequently associated seizure disorder, which often demands use of more than one anticonvulsant. This is possibly because of depletion of respiratory chain enzyme activity by the drug and inability to increase metabolic rate by the DNA polymerase subunit gamma (POLG) deficient cells. Valproate increases glycolysis, likely an indirect clue of impaired mitochondrial function as shown in yeast and mouse liver models. POLG mutation subtype and zygosity influence outcome, with worst outcomes shown in compound heterozygous mutations for A467T and W748S.

Liver failure management, addressing feeding issues often mandating percutaneous endoscopic gastrostomy tube insertion, seizure control, and use of respiratory aids like continuous positive airway pressure in view of progressive motor impairment are cornerstones of management. Liver transplantation is often contraindicated in view of the multisystem involvement.

**MtDNA depletion syndrome**

DNA depletion is distinct from DNA deletion. MtDNA depletion refers to a state when a cell contains less than normal mtDNA per unit nuDNA. Depletion diseases are
much more severe and earlier in onset compared to deletion diseases[22]. NuDNA encodes for processes within the mitochondria including production and stability of mtDNA. Mutations in nuDNA may result in low levels of or increased destruction of DNA pool essential for mtDNA synthesis[23], thereby reducing the concentration of mtDNA in the cell or tissue as a whole. The end result is suboptimal mitochondrial function. DGUOK mutations lead to predominant neuro-hepatopathy, while TK2 mutations lead to predominant myopathy[23]. As an illustration to above statements, TK2 induced depletions present early in the first few years with myopathy, feeding difficulty, hypotonia, and respiratory failure as a terminal event. However, multiple TK2 deletions present as proximal myopathy and chronic progressive external ophthalmoplegia later in life[24]. Two additional genes, POLG coding for mtDNA polymerase and MPV17, have been described in hepatocerebral form of MDS. POLG mutations in older children have been associated with AHS as already described. It should be understood that MDS and AHS both have mtDNA depletion. AHS got its name earlier and was later found to have its molecular basis as mtDNA depletion, and it characteristically refers to a comparatively delayed onset (> 2 mo age), compared to MDS, which has its onset in the first few weeks of life. The other mutation in nuclear gene MPV17 leads to decreased synthesis of an unknown inner mitochondrial membrane protein that possibly has a role in oxidative phosphorylation, and knockout mice (-/-) have shown impaired oxidative phosphorylation and also mtDNA depletion[25].

Liver failure in infancy is the common presentation of the hepatopathic form. There is notably an overlap between the hepatopathic form of MDS and neonatal liver failure presentation of RCD. The difference exists in the fact that the former has mtDNA quantity that is < 10% of nuDNA, and there is no sequence alteration in mtDNA. Pearson syndrome

Pearson syndrome is one among three mitochondrial diseases (Kearne Sayre syndrome and chronic progressive external ophthalmoplegia being the other two) associated with a single large deletion in mtDNA[26]. This is a multi-systemic fatal disorder with involvement of exocrine pancreas, eyes, skin, hematological system, liver, and kidneys[22]. MtDNA rearrangements form the etiological basis, and it is associated with large 4-5 kbp deletions in a large proportion of cases. All respiratory chain complexes can suffer a decreased synthesis, with complex I most severely affected. Refractory anemia with ring sideroblasts occurs in infancy with vacuolization in bone marrow of myeloid and erythroid precursors[27]. Elevated plasma alanine and
Hepatic steatosis, hepatomegaly, elevated transaminases, and cirrhosis have also been described. Bowel bacterial overgrowth, nutritional deficiencies, and severe weight loss dysphagia, gastroparesis, and pseudo-obstruction leading to consequences like small approximately 60%. GI dysmotility is one of the most important features in the form of begin between the first and fifth decades of life and before 20 years of age in approximately 60%. GI dysmotility is one of the most important features in the form of such as diarrhea, vomiting, and abdominal pain. This disorder was described in 1994 by Cormier-Daire et al.[34] in two unrelated children presenting with chronic diarrhea in infancy with villous atrophy. The defect was identified as mtDNA rearrangements in the form of deletion-duplications. Hepatomegaly and steatosis on biopsy with mildly deranged transaminases was the liver manifestation. Both children survived the diarrheal phase, which subsided by early childhood, including a reversal in histology (Figure 10: Graphical summary). However, the phenotype then changed to neuromuscular and ophthalmic involvement and death by the end of first decade. Complex III defect was detected on muscle biopsy after the advent of neuromuscular symptoms and was normal in lymphocytes.

**Navajo neurohepatopathy**

This is an autosomal recessive disease prevalent in southwestern United States. The genetic defect is a nuclear gene MPV17 (chromosome 2p24)[32], whose product is located on the inner mitochondrial membrane and is responsible for mtDNA maintenance and regulation of oxidative phosphorylation. Hence, there is impaired pool of mtDNA and disrupted oxidative phosphorylation. While earlier only neurological manifestations were known and this entity was called Navajo neuropathy, liver manifestations in form of jaundice, failure to thrive, and liver failure were recognized to be part of the same disease spectrum prompting a change in name to Navajo neurohepatopathy[33]. Clinical features are outlined in Figure 9 graphical summary. All the three subtypes have occurred in same kindred, underscoring the pattern of mitochondrial inheritance.

**Villous atrophy syndrome**

This disorder was described in 1994 by Cormier-Daire et al.[34] in 2 unrelated children presenting as chronic diarrhea in infancy with villous atrophy. The defect was identified as mtDNA rearrangements in the form of deletion-duplications. Hepatomegaly and steatosis on biopsy with mildly deranged transaminases was the liver manifestation. Both children survived the diarrheal phase, which subsided by early childhood, including a reversal in histology (Figure 10: Graphical summary). However, the phenotype then changed to neuromuscular and ophthalmic involvement and death by the end of first decade. Complex III defect was detected on muscle biopsy after the advent of neuromuscular symptoms and was normal in lymphocytes. Intravenous dextrose for resuscitation should not be used in high rates as it may lead to worsening of metabolic acidosis.

**Mitochondrial neurogastrointestinal encephalomyopathy**

This entity is discussed purely as a prototype for GI (non-hepatic) manifestations of mitochondrial disorders, and also since it is a respiratory chain disorder, though not classically a “hepatopathy”, and additionally as it is rewarding to diagnose in view of available therapy[35]. It is to be understood that RCD and non RCD mitochondrial diseases can have some or the other GI manifestation (Table 2). MNGIE was earlier known as polyneuropathy, ophthalmoplegia, leukoencephalopathy and intestinal pseudo-obstruction, oculogastrointestinal encephalopathy syndrome, or oculogastrointestinal muscular distrophy[36]. The current nomenclature was given by Hirano et al.[37].

MNGIE occurs due to mutation in a nuclear gene encoding TYMP, encoding thymidine phosphorylase, deficiency of which leads to toxic accumulation of pyrimidine nucleosides thymidine and deoxyuridine. This impairs mtDNA synthesis thereby leading to a mtDNA depletion state. Clinical symptoms of MNGIE usually begin between the first and fifth decades of life and before 20 years of age in approximately 60%. GI dysmotility is one of the most important features in the form of dysphagia, gastroparesis, and pseudo-obstruction leading to consequences like small bowel bacterial overgrowth, nutritional deficiencies, and severe weight loss[38]. Hepatic steatosis, hepatomegaly, elevated transaminases, and cirrhosis have also been described[38,39].
Table 2 Gastrointestinal manifestations of mitochondrial respiratory chain defects

<table>
<thead>
<tr>
<th>Site</th>
<th>Manifestation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral cavity and esophagus</td>
<td>Sicca syndrome; Dry mouth; Dysphagia</td>
</tr>
<tr>
<td>Stomach</td>
<td>Vomiting; Reflux; Pseudo-obstruction</td>
</tr>
<tr>
<td>Small bowel and large bowel</td>
<td>Pseudo-obstruction; Diarrhea; Megacolon; Constipation</td>
</tr>
<tr>
<td>Extra-luminal/miscellaneous</td>
<td>Poor appetite; Pancreatitis; Pancreatic cysts</td>
</tr>
</tbody>
</table>

Figure 8 Graphical summary of Pearson marrow pancreas syndrome and its natural history. GCSF: Granulocyte colony stimulation factor; insuff: Insufficiency; KSS: Kearns Sayre syndrome; Pancr: Pancreatic; Plt: Platelets; PRBC: Packed red blood cells.

A diagnostic delay of about 5 to 10 years can occur in view of multisystem and complex clinical presentation\[^{40,41}\]. Often there are unnecessary exploratory surgeries for the GI symptoms before being diagnosed as pseudo-obstruction\[^{36}\]. Neurological involvement is mainly in form of peripheral neuropathy (demyelination with or without axonal neuropathy)\[^{38}\], oculoparesis, with subtle central nervous system manifestations due to subcortical white matter involvement, and magnetic resonance imaging changes showing leukencephalopathy. Muscle biopsies may show ragged red fibers due to proliferation of abnormal mitochondria. Current diagnostic methods employ testing for plasma thymidine and deoxyuridine levels (> 3 μmol/L and > 5 μmol/L, respectively)\[^{42}\] or elevated urinary concentrations\[^{43}\] and thymidine phosphorylase activity in leucocytes (< 10% of healthy controls)\[^{43}\]. TYMP gene (nuDNA) mutations and also consequent mtDNA abnormalities can be identified on Sanger sequencing and Southern blot assays\[^{44}\]. A graphical summary is as shown in Figure 11.

Symptomatic management remains the cornerstone. Experimental therapies include hemodialysis and peritoneal dialysis\[^{43}\], platelet transfusions, hematopoietic stem cell transplant, enzyme replacement, and liver transplant\[^{45,46}\]. All above therapies concentrate on 2 aspects: To reduce the toxic load of nucleosides and to replace the enzyme thymidine phosphorylase.

**SETTINGS TO SUSPECT RCD AND DIAGNOSTIC EVALUATION**

The settings of when to suspect a mitochondrial hepatopathy are shown in Figure 12. Individual disorders discussed above and their graphical summaries outlined give specific information. The diagnostic evaluation of mitochondrial disorders follows once a clinical suspicion is raised, and in this section we highlight general steps towards approaching to diagnose a mitochondrial RCD\[^{30}\]. Parallel evaluation of
extra-hepatic and extra-GI symptoms, if present, need to be carried out for “mapping” the disease and for aid in management and improving quality of life. Table 3 elucidates a stepwise evaluation algorithm[30]. Diagnostic steps proceed from non-invasive, easily available, and less expensive investigations to more complex elaborate tests, some of which are available in the research setting only. Level-1 entails workup for basic metabolic causes including checking for hypoglycemia and, if present, whether it is ketotic or non-ketotic. Fatty acid oxidation defects (but not RCDs) are known to have non-ketotic hypoglycemic episodes. Lactate levels more than 2.1 mmol/L (mmol) are significant, and this may often not be observed when not in a metabolic crisis. Notably, lactate may not be elevated much in POLG1 mutations[47]. Normal lactate to pyruvate ratio is less than 20 mol/mol. However, the value often rises above 30 and is typical though not exclusive to RCDs and is discriminatory from pyruvate metabolism defects[48]. Similarly, 3-hydroxy butyrate to acetoacetate ratio is normally less than 4, and values above this should arouse a suspicion of mitochondrial
### Table 3: Stepwise evaluation of mitochondrial hepatopathies (respiratory chain disorder/non-respiratory chain disorders)

<table>
<thead>
<tr>
<th>Steps</th>
<th>Description</th>
<th>Additional action</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Level-1</strong> (body fluids)</td>
<td>Basic: CBC, INR, AFP, CPK, NHD, sugars, phosphorous, urine ketones. Advanced: Lactate: Pyruvate (1 h post feeds); Ketone Body ratio, 3OH-butyrate: Acetoacetate; Serum acylcarnitine profile; Urine organic acidogram; Serum aminoacidogram; 3 Methyl Glutaconic acid in serum/urine; CSF lactate; Pyruvate, CSF alanine, protein; Plasma thymidine (MNGIE); Leucocyte CoQ levels</td>
<td>Parallel level-1: Evaluate other involved systems: CNS: MRI/MR-Spectroscopy, EEG; Eye: Fundus evaluation, clinical evaluation for ophthalmoplegias; Hearing screen; Heart: 2D-Echo, ECG; Renal: urine electrolytes, proteins, amino acids; Muscle: Muscle biopsy (Level-1 in case of primary muscle involvement, level-3 otherwise); Endocrine: HbA1c, 8 AM cortisol; Pancreas: Fecal elastase</td>
</tr>
<tr>
<td><strong>Level-2</strong> (genetics)</td>
<td>Common genes genotyping: POLG-1; DGUOK; MPV-17; SUCLG-1; TRMU; CI10RF2/Twinkle; CPT-1; mtDNA point mutations</td>
<td>Alternative level-2: Next generation sequencing/clinical exome sequencing for simultaneous evaluation of all mitochondrial DNA and nuclear DNA</td>
</tr>
<tr>
<td><strong>Level-3</strong> (invasive)</td>
<td>Tissue diagnosis: (1) Liver biopsy: Light microscopy including oil red O stain for steatosis; Electron microscopy for structural mitochondrial alterations; Frozen tissue analysis for respiratory chain enzymes; DNA quantification. (2) Muscle biopsy: Frozen tissue analysis as above; Blue native page analysis. (3) Skin biopsy: Same as muscle biopsy</td>
<td>Key points to note during level-3 evaluation: Biopsy specimens for electron microscopy need to be preserved in glutaraldehyde and not formalin; It is possible that one invasive test may not give a clue and one has to proceed for an additional invasive test. This is usually because of heteroplasmy. Often liver biopsy molecular analysis provides a final definitive answer; Combination of level-1, level-2 and level-3 studies are sometimes needed to provide comprehensive management and for prognostication</td>
</tr>
</tbody>
</table>

2D Echo: Two-dimensional echocardiography; AFP: Alpha-fetoprotein; CBC: Complete blood count; CNS: Central nervous system; CoQ: Coenzyme Q; CPK: Creatine phosphokinase; CSF: Cerebrospinal fluid; EEG: Electroencephalogram; HbA1c: Glycosylated hemoglobin; INR: International normalized ratio; MRI: Magnetic resonance imaging; NH3: Serum ammonia levels; POLG: DNA polymerase subunit gamma; RCD: Respiratory chain disorders.

### Figure 11: Graphical summary of mitochondrial neurogastrointestinal encephalomyopathy.

BMT: Bone marrow transplantation; dThd: Thymidine; dUrd: Deoxy uridine levels; MRI: Magnetic resonance imaging; TP: Thymidine phosphorylase. Vx: Vomiting.

Urinary organic acids like lactate, succinate, fumarate, malate, and 3-methylglutaconic are seen elevated in Pearson syndrome. Serum alanine elevation is also a clue; however, it is often more elevated in pyruvate dehydrogenase deficiency than in RCDs[48]. Creatine kinase elevation and concomitant low levels of phosphocreatine in brain and muscle tissue are seen in RCDs[49]. Branched chain amino acid to glutamine ratios were highest in RCDs and lowest in pyruvate dehydrogenase deficiency compared to controls, according to one study[48]. Table 4 helps differentiate the common metabolic disorders encountered in the pediatric patient and how to filter out RCD.

### Role of genetic testing

Genetic studies are confirmatory but have a high turnaround time of 4-6 wk. They may not also be available freely at all centers or in resource poor settings. Timely referral to tertiary care centers for management is advisable. A major limitation is selection of the gene panel testing for the phenotypic presentation. In products of consanguineous

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2D Echo: Two-dimensional echocardiography; AFP: Alpha-fetoprotein; CBC: Complete blood count; CNS: Central nervous system; CoQ: Coenzyme Q; CPK: Creatine phosphokinase; CSF: Cerebrospinal fluid; EEG: Electroencephalogram; HbA1c: Glycosylated hemoglobin; INR: International normalized ratio; MRI: Magnetic resonance imaging; NH3: Serum ammonia levels; POLG: DNA polymerase subunit gamma; RCD: Respiratory chain disorders.
Table 4 Biochemical differentiation between various metabolic hepatopathies (respiratory chain disorder vs non respiratory chain disorder comparison)

<table>
<thead>
<tr>
<th></th>
<th>Acidosis</th>
<th>Urine ketones</th>
<th>Blood sugar</th>
<th>Serum lactate</th>
<th>Serum ammonia</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCD</td>
<td>++</td>
<td>++</td>
<td>Normal</td>
<td>+++</td>
<td>±</td>
</tr>
<tr>
<td>FAOD</td>
<td>++</td>
<td>Nil (non-ketotic)</td>
<td>Low (hypoglycemia)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>OA</td>
<td>+++ (persistent)</td>
<td>++/++++</td>
<td>Low/normal/high</td>
<td>Normal</td>
<td>++</td>
</tr>
<tr>
<td>UCD</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>+++</td>
</tr>
</tbody>
</table>

FAOD: Fatty acid oxidation defects; OA: Organic acidemias; RCD: Respiratory chain defects; UCD: Urea cycle defects.

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**Figure 12 Scenarios when to suspect mitochondrial hepatopathy.**

**Core tip:** Multisystem involvement – brain/eye/heart/muscle

**Scenario 1:** Neonate/infant/young child: Acute liver failure
- Suspect mitochondrial if pre-existing/disproportionate CNS symptoms

**Scenario 2:** Infant/child: Chronic liver disease (cirrhosis/cholestasis)
- If steatosis on Bx, hypoglycemia, lactic acidosis/ketonemia

**Scenario 3 (general scenario):** Any age: Liver disease (acute/chronic)
- If chronic neuromuscular disease/eye/heart involvement

Union and multiple affected siblings, genetic evaluation is better guided, and it is possible to identify the index patient’s chromosomal region containing the abnormality by linkage analysis. Targeted gene analysis is performed if the phenotype matches the previous cases in a family and there is an already identified mutation responsible for the clinical features in those particular kindreds. Whole exome analysis for nuDNA and mtDNA is preferred otherwise as an alternative step in case there are no previously affected siblings or if the phenotype does not classically match previously described entities[50]. Targeted analysis can be performed using Sanger method of few genes, while whole exome sequencing refers to a massive parallel sequencing technique of multiple genes or the entire exome using next generation sequencing[51]. Another method to short-list genes for analysis is to study the expression profiles of RNA or specific proteins or polypeptides levels encoded by the gene(s) of interest, especially after a biochemical diagnosis is made. As an example, complex I deficiency can be caused by any of the multiple mtDNA and nuDNA responsible for each of its subunits. To identify a particular gene (of the multiple encoding ones) responsible for causing overall complex I deficiency in an index patient, analyzing the distribution or expression of proteins or RNA and its deviation from healthy controls or known standards can help pinpoint which gene may be defective. Once a specific change is identified, either in RNA expression, which can be detected by microarray assays, or in enzyme levels or stress protein expression, which can be identified by immunoblotting using an antibody panel, indirectly “reverse identification” of the causative gene(s) is facilitated[52]. This of course is only possible when, with time, all genes encoding each subunit of large proteins are identified so
that such indirect, simpler, and time saving methods may be employed. Tables 1 and 3 list genes implicated in mitochondrial RCDs. Figure 13 suggests a two-step strategy of genetic evaluation in mitochondrial RCDs and also a few phenotypes for which specific genes should be tested.

It is pertinent to note that mitochondrial hepatopathies, unlike other metabolic disorders, require analysis of mtDNA in addition to nuDNA defects. Hence, when screening genetics do not yield the diagnosis and the next step of whole exome sequencing is being undertaken, it is essential to specify to the testing lab that mtDNA analysis be included in addition to the wide gamut of nuDNA being tested. While for nuDNA analysis, the tissue of interest may not be specific and whole blood sample may serve the purpose, for mtDNA molecular analysis, specific tissues (liver, muscle) may be required, mainly because of the phenomenon of heteroplasmy.

MtDNA depletions are diagnosed by first isolating the DNA of the tissue biopsied, which is then subjected to electrophoresis and blotting followed by hybridization with probes specific for mtDNA and nuDNA both. The relative levels of autoradiographic signals emitted post hybridization are detected for mtDNA and nuDNA, and this helps in diagnosing mtDNA depletions. MtDNA deletions and point mutations on the other hand can be detected by single strand conformational polymorphisms[33,34].

Next generation sequencing by parallel exome sequencing undertaken with blood or any tissue is limited by its inability to detect mutations in the non-exonic region, like untranslated regions or intronic splice sites. It is also not adept in diagnosing trinucleotide repeat sequences, complex genetic inheritance like synergistic contribution of nuDNA and mtDNA to cause a particular disease, and epigenetic effects[31].

Role of tissue biopsies

Tissue biopsies are important despite having readily evident biochemical abnormalities; only one-third to one-half of mitochondrial disorders have identifiable mutations despite extensive exome sequencing of known genetic defects[31,35]. That is to say, all genes related to mitochondrial disorders have not yet been identified. Biopsies from the most involved site are more likely to yield the diagnosis[36]. Respiratory chain enzymes can be analyzed and activity quantified on tissue biopsy specimens. Quantitative Southern blot analysis or real-time quantitative polymerase chain reaction to detect mtDNA depletion can be done in liver biopsy specimens. Skin biopsy for cultured skin fibroblasts can be stored indefinitely and retrieved for re-culture once newer diagnostic modalities are available. It is simpler to perform and less invasive compared to muscle biopsy. However, the downside is that not all diseases are detectable on skin fibroblast analysis[47].

How to select which tissue to test is an important question that the clinician must be aware. Most mitochondrial disorders involve the muscle, and hence muscle is one of the most useful sites for analysis of enzymes, metabolites, and even molecular DNA studies. While earlier 1-5 g of muscle tissue was required for respiratory chain enzyme assays, now even 100-200 mg of skeletal muscle tissue (usually quadriceps or soleus) is sufficient especially in young children, which then yields a mitochondrial enriched fraction of 400-500 μg of protein, enough to characterize the respiratory chain enzyme deficiencies[57]. Muscle biopsies may be analyzed either as frozen or fresh samples. Samples once collected should be snap frozen immediately bedside or in the procedure room at -80 °C till analysis of mitochondrial enzymes[53]. Fresh muscle samples should not be frozen and transported in cool buffer solution, which offers the advantage of analysis of the entire mitochondrial energy generation system in addition to mitochondrial enzymes being studied in frozen samples[58].

Those diseases that have primary liver involvement and no apparent muscle involvement, especially the ones with liver failure phenotype, liver tissue of up to 10 mg can be more yielding than muscle. Cardiac tissue requirement, when indicated, is even less, about 1-2 mg, obtained by endomyocardial biopsy[53]. These invasive techniques may be gradually substituted by molecular DNA techniques done on whole blood and cater to only research purpose over time as cost and availability of next generation sequencing is eased.

MANAGEMENT OF MITOCHONDRIAL RESPIRATORY CHAIN DEFECTS

There are three aspects to management: Firstly, acute management of crisis, second is general management of children with metabolic liver disease, followed by specific treatment if available including the role of liver transplant. An additional important component relates to parental counseling and to bust myths and avoid patients to
resort to non-scientific therapies and refraining from standard of care.

**Acute crisis management**

Treatment of acute liver failure and progressive liver disease remains unsatisfactory. The aim of therapy is mainly mitigating, postponing, or circumventing damage to the respiratory chain. The basic steps and precautions are outlined as in Table 5. Bicarbonate infusions when used for a longer time may itself worsen cerebral function. An alternative is dichloroacetate, which inhibits pyruvate dehydrogenase kinase, hence favoring persistent levels of active pyruvate dehydrogenase and hence preventing pyruvate accumulation and pyruvate to lactate conversion.

Other supportive therapy is to give packed red cell and platelet transfusions for anemia and thrombocytopenia and pancreatic enzyme replacement in case of insufficiency.

**General considerations for managing a child with mitochondrial hepatopathy**

Once the acute crisis is settled, it is important to improve the nutrition of the child as malnutrition itself can lead to secondary mitochondrial dysfunction[59]. These children often have increased caloric needs and an inability to maintain it owing to either repeated sickness bouts or a general anorexia associated with liver disorders. The issues of swallowing difficulties, impaired gut motility, and gastro-esophageal reflux need to be addressed often to the extent of placement of feeding tubes (orogastric or nasogastric), percutaneous endoscopic feeding gastrostomy, or using parenteral nutrition therapy. Nutritional improvement has led to improved quality of life and an increase in developmental quotients in these children[60]. Ketogenic diet may be useful in some mitochondrial disorders but may worsen fatty acid oxidation defects and should be avoided in them. Exercise helps in reducing the burden of abnormal mitochondria[61]. It is useful to do so regularly, under supervision in a graded manner, and to have a meal prior to exercise[62].

**Pharmacotherapy**

A combination of drugs is often empirically administered to suspected mitochondrial
Table 5 Management during evaluation in acute phase

<table>
<thead>
<tr>
<th>Following thumb rules while attending to a patient with suspected mitochondrial disorder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monitor closely for hypoglycemia and acidosis</td>
</tr>
<tr>
<td>Avoid lactated ringer’s solution for fluid administration: Worsens acidosis</td>
</tr>
<tr>
<td>Bicarbonate infusions as 1st line of defense</td>
</tr>
<tr>
<td>Avoid propofol for sedation/anesthesia</td>
</tr>
<tr>
<td>Avoid fasting &gt; 12 h; avoid high rate glucose only infusions</td>
</tr>
<tr>
<td>Avoid drugs that are toxic to mitochondria: Chloramphenicol, valproate, aminoglycosides, phenytoin, carbamazapine, phenobarbital, statins, linezolid</td>
</tr>
<tr>
<td>Avoid drugs precipitating hepatopathy/liver dysfunction</td>
</tr>
</tbody>
</table>

disorders and comprises: Coenzyme Q, carnitine, thiamine, riboflavin, vitamins C and E, and creatine. Of all, Coenzyme Q shows promise and along with B vitamins remains the most common combination as part of cocktail therapy[63]. The various drugs and their pediatric dosages are outlined in Table 6[13,63,63].

Role of organ transplant
Multisystem involvement in mitochondrial hepatopathies often precludes performing a liver transplant. However, in hepatocerebral form of DGUOK defects when detected in infancy without neurological involvement, liver transplant has shown to be effective. Those with neurological involvement do not benefit from liver transplant [65]. Overall post-transplant survival is less with RCDs than non-RCDs. Sokal et al[66] reported 8 cases with a survival of 50% post transplantation for RCDs. In an elaborate compilation of 40 cases with mitochondrial RCDs across various centers at different time points, it was noted that 22 (55%) patients died within 24 mo post-transplant[67]. Early postoperative multi-organ failure and neuro-degeneration followed by respiratory complications and severe pulmonary hypertension were the cause of death in these patients. The same group recognized that those diagnosed pre-transplant had a higher survival (58%) than those recognized to have RCD after transplant (29%). Thus, the emphasis is on early recognition of the diagnosis and a thorough evaluation for extra-hepatic manifestations, adding investigations like magnetic resonance imaging of the brain and echocardiography.

MNGIE stands out as the single mitochondrial disorder for which replacement of the missing enzyme thymidine phosphorylase by stem cell transplantation can be curative and lead to improvement in long term outcomes. While earlier enzyme levels were artificially increased using repeated platelet transfusions[40], stem cell transplant has come up as a definitive modality[68,69].

Myths in mitochondrial disorders
Immunizations are not contraindicated in children with mitochondrial diseases. This is to be emphasized because of certain misconceptions that immunization may lead to autism in children with mitochondrial diseases for which there is no evidence[64]. The other important aspect that needs to be clarified is that there is no role of hyperbaric therapy in treatment of MH and in fact may lead to oxygen toxicity. Vagus nerve stimulation may not be very helpful in controlling refractory seizures in children with MH[63].

CONCLUSION
In a nutshell: (1) Liver along with other system involvement may not be just sepsis – think of mitochondrial respiratory chain hepatopathy; (2) Lactic acidosis without hypoglycemia is an important clue, avoid ringer lactate and drugs causing hepatopathy; (3) Evaluation should be done in a tiered manner – genetic evaluation and enzyme analysis from tissue of interest; (4) Treatment is largely supportive with transfusions, correction of acidosis, shock, and providing cofactors/salvage therapies; (5) Liver transplantation needs to be considered in only a select group and may worsen disease despite adequate precautions; and (6) Periodic follow-up is mandatory for monitoring evolution of disease including “migration” to other organ systems.
Table 6 Pharmacotherapy used for mitochondrial diseases

<table>
<thead>
<tr>
<th>Drug</th>
<th>Pediatric dose</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coenzyme Q: (1) Ubiquinol form; (2) Ubiquinone form</td>
<td>2-8 mg/kg/d in BD dosing; 10-30 mg/kg/d BD dosing</td>
<td>Preferably had after meals; Most effective and most used therapy; Free radical scavenger; Bypasses complex I</td>
</tr>
<tr>
<td>Idebenone</td>
<td>5 mg/kg/d</td>
<td>Synthetic form of CoQ; Penetrates blood-brain barrier</td>
</tr>
<tr>
<td>L-carnitine</td>
<td>10-100 mg/kg/d IV or oral divided 3 times/d</td>
<td>Avoid in long chain FAO-De: May lead to cardiac arrhythmias</td>
</tr>
<tr>
<td>Creatine</td>
<td>0.1 g/kg PO, OD</td>
<td>Used for repletion of muscle phosphocreatine levels</td>
</tr>
<tr>
<td>L-arginine</td>
<td>500 mg/kg IV per day for 1-3 d followed by 150-300 mg/kg oral daily in BD dosing</td>
<td>Used for acute stroke; Watch for hypotension while infusion; Evidence is anecdotal</td>
</tr>
<tr>
<td>Thiamine</td>
<td>100 mg/d</td>
<td>Cofactor of PDH; useful for thiamine responsive PDH deficiency; Helpful in Leigh disease</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>50-400 mg/d</td>
<td>Give at night time before sleep; Shown to be useful in ACAD9 mutations; Flavin precursor for complex I &amp; II</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>5 mg/kg/d OD</td>
<td>Antioxidant; Artificial electron acceptor</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>Variable dosing, up to 25 IU/kg/d OD (avoid &gt; 400 IU/d)</td>
<td>Absorption better when taken with meals</td>
</tr>
<tr>
<td>Dichloroacetate</td>
<td>25-50 mg/kg/d</td>
<td>Improves lactic acidosis</td>
</tr>
</tbody>
</table>

BD: Twice daily; CoQ: Coenzyme Q; FAO-D: Fatty acid oxidation defects; IV: Intravenous; PDH: Pyruvate dehydrogenase; PO: Per oral; OD: Once daily.

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Cystic fibrosis associated liver disease in children

Joseph J Valamparampil, Girish L Gupte

Abstract

Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations in the CF transmembrane conductance regulator gene. CF liver disease develops in 5%-10% of patients with CF and is the third leading cause of death among patients with CF after pulmonary disease or lung transplant complications. We review the pathogenesis, clinical presentations, complications, diagnostic evaluation, effect of medical therapies especially CF transmembrane conductance regulator modulators and liver transplantation in CF associated liver disease.

Core Tip: Cystic fibrosis(CF) liver disease is caused by abnormal cholangiocyte function, altered biliary secretion and abnormal innate immune response with abnormal response to endotoxins. CF liver disease can present with a wide variety of clinical features from a heterogenous liver on ultrasound, to life threatening gastrointestinal bleeds secondary to portal hypertension. Novel treatment strategies directly targeting the ion channel abnormality-cystic fibrosis transmembrane conductance regulator modulators are available and has significantly improved the clinical status and life expectancy of the cystic fibrosis patients.
INTRODUCTION

Cystic fibrosis (CF) is the most frequent fatal autosomal recessive disorder in Caucasians, and is caused by autosomal recessive disease caused by mutations in the CF transmembrane conductance regulator (CFTR) gene on the long arm of chromosome 7 with more than 2000 variants reported[1]. F508del variant resulting from deletion of three nucleotides that leads to loss of a single phenylalanine residue at codon 508, accounts for approximately 70% mutations[2]. CFTR protein is found in the epithelial cells of lungs, sweat glands, liver pancreas and intestine. Liver disease is one of the classic phenotypes of CF.

CF liver disease (CFLD) usually develops within the first 20 years of life and has a stable non-progressive or mildly progressive course in later life[3,4]. Most children with CF will have some degree of steatosis but clinically significant liver disease develops in < 10% of pediatric CF patients usually by 10 years of age. CF related cirrhosis is a disease of the childhood and adolescence while predominant biliary involvement mimicking sclerosing cholangitis mostly occurs in adulthood[5]. The diagnosis of liver disease has profound implications in short and long term prognosis in CF patients and is the third leading cause of mortality in CF. Analysis of a large cohort of patients from the CF Foundation Patient Registry database showed that in CF patients with liver disease, the estimated 10-year cumulative rate of any adverse liver-related outcomes was approximately 20%[6]. Liver disease with cirrhosis and or portal hypertension has been classified as severe CFLD.

PATHOPHYSIOLOGY

CFLD is a genetic disorder of cholangiocyte transport protein defect, resulting in chronic cholangiopathy caused by reduced ductal bile flow generation and reduction in biliary chloride and bicarbonate secretion caused by the dysfunction of CFTR[6,7]. But this mechanism alone cannot explain CFLD, because CFTR deficiency is present in all patients while CFLD occurs only in a small population of CF patients and has varying clinical manifestations and severity. As described below, a combination of factors including CFTR genotype, non-CFTR genetic variability, abnormal intracellular interactions, abnormal cholangiocyte function, altered biliary secretion, pathologic stimulation of innate immune response with abnormal response to endotoxins lead to CFLD.

Abnormal cholangiocyte function and altered biliary secretion

Abnormal CFTR results in inhibition of cyclic adenosine monophosphate dependent chloride and bicarbonate secretion. This reduces the bile flow and alkalinity resulting in the biliary epithelial damages deriving from the retention of cytotoxic bile acids and xenobiotics and from the reduction in natural defenses against microbiologic pathogens. The response to chronic epithelial damage and the progression in the liver damage depends on the immunogenetic response of the individual and on other modifier genes.

Abnormal protein-protein interactions

CFTR mediated liver injury is also postulated to be caused by ability to regulate the function of other proteins by physically associating in macromolecular complexes at the membrane (protein-protein interaction)[8,9]. CFTR interacting proteins are located not only in the plasma membrane but also in nucleus, endoplasmic reticulum, Golgi apparatus, trafficking vesicles, proteasomes and cytoskeleton[9]. For example, the interaction of CFTR with proteins regulating the function of non-receptor tyrosine kinase Rous sarcoma oncogene cellular homologue can modulate innate immune responses in cholangiocytes[8]. Dysfunction of interactions can have systemic consequences resulting from the perturbation of the interconnected cellular networks accounting for some of the phenotypic variation in CF[8].

Abnormal innate inflammatory response

The conventional theory of CFLD postulates that biliary epithelial CFTR dysfunction causes alterations in the volume and composition of bile, resulting in loss of protective effect of biliary bicarbonate and mucus and an accumulation of toxic bile acids causing damage to the epithelium by initiating an inflammatory response[8]. But it is now postulated that the abnormal inflammatory response is due to lack of tolerance in the innate immune system[7]. CFTR is a now thought as a regulator of cholangiocyte
in innate immune responses and defective CFTR results in aberrant activation of Src tyrosine kinase causing upregulation of innate inflammatory responses via the Toll-like receptor 4/NF-κB axis[7,10]. This results in lack of tolerance of biliary epithelium to endotoxin (e.g. pathogen-associated molecular patterns) from bile and intestine, leading to a para-inflammatory process in the biliary epithelium with the release of cyt/o/chemokines and the infiltration of the portal spaces with inflammatory cells[7,10].

**Gut dysbiosis and role of gut-liver axis**

There is a substantial reduction in the richness and diversity of gut bacteria in patients with CF from early childhood until late adolescence and the changes deviate progressively farther from the path of healthy controls with increasing age[11]. Gut dysbiosis results in reduction in anti-inflammatory short-chain fatty acids, altered ratios of arachidonic acid/Linoleic acid and arachidonic acid/docosahexaenoic acid leading to increased gut inflammation[8,12]. This causes increased permeability of intestinal epithelium, increasing the exposure of biliary epithelial cholangiocytes to endotoxins, perpetuating the inflammatory cascade[8,12]. But it is not certain if intestinal inflammation is caused by the altered microbiota in CF or is the consequence of an altered environment[8,12].

**Genetics**

There is massive heterogenicity in CFTR phenotype among patients with CFLD and CFTR genotype-phenotype correlations are generally weak. The functional consequences of CF-causing variants have been grouped into six classes[1,13] (Figure 1). Mutations in classes I and II are also known as minimal function mutations since they demonstrate no to very little CFTR function, while those in classes IV, V, and VI are known as residual function mutations since they demonstrate some CFTR function, although it is lower compared to the wild type CFTR[14]. CFLD is mostly occurs in pancreatic insufficient patients with biallelic loss-of-function mutations in CFTR (class I, II, or III mutations on both allele)[1,17]. It has been shown that non-CFTR genetic variability also contributes to risk for severe liver disease[15]. This might be one of the reasons in variability of phenotype even between siblings inheriting the same mutations. Though many candidate genes have been postulated, in a large study SERPINA1 (coding for alpha1-antitrypsin) Z allele was significantly associated with CFLD and portal hypertension[16].

**CLINICAL FEATURES**

The prevalence of CFLD varies widely in children and adolescents, based upon the diagnostic criteria used ranging from < 5% to 68%[17,18]. CFLD is more common and the median age of diagnosis is earlier in males[19]. Liver involvement in CF may be subclinical until diffuse liver damage occurs. Liver involvement can vary from mild elevation of aminotransferases to cirrhosis with synthetic failure and portal hypertension. The degree of liver involvement and the rate of progression of liver disease varies significantly among individuals. The awareness of CFLD and its clinical implications has increased as evidenced by an early diagnosis and a drop in the median time at diagnosis from adolescence to < 3 years of age[17,18].

Risk factors for CFLD include male sex, presence of severe mutations, presence of SERPINA1 Z allele, history of meconium ileus, exocrine pancreatic insufficiency and CF-related diabetes[20]. The most common clinical feature is asymptomatic hepatomegaly detected by clinical examination or ultrasonography[18]. Pancreatic insufficiency occurs in 99% of patients with CFLD[19]. Liver involvement in CF can be classified into two broad categories based on the presence of cirrhosis/portal hypertension (Table 1).

**Liver disease without portal hypertension**

**Cholestasis:** Neonatal/infantile cholestasis is the earliest manifestation of liver involvement in CF, but is very rare (< 2%). It is important to exclude other common causes of neonatal cholestasis like biliary atresia and also to consider the diagnosis of CF in infants who present with cholestasis[21].

**Abnormal liver enzymes:** The commonly noticed abnormalities include intermittent rise in serum transaminases (aspartate aminotransferase (AST) and alanine aminotransferase (ALT)) and/or increased serum levels of alkaline phosphatase (ALP)
Table 1 Spectrum of cystic fibrosis liver disease in children

<table>
<thead>
<tr>
<th>Spectrum of cystic fibrosis liver disease in children</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
</tr>
<tr>
<td>Neonatal cholestasis</td>
</tr>
<tr>
<td>Pre-clinical</td>
</tr>
<tr>
<td>Elevated aminotransferases</td>
</tr>
<tr>
<td>Increased GGT</td>
</tr>
<tr>
<td>Steatosis</td>
</tr>
<tr>
<td>Portal hypertension including non-cirrhotic portal hypertension</td>
</tr>
<tr>
<td>Cirrhosis</td>
</tr>
<tr>
<td>Focal biliary</td>
</tr>
<tr>
<td>Multi-lobular</td>
</tr>
<tr>
<td>Gallbladder and biliary system</td>
</tr>
<tr>
<td>Cholelithiasis</td>
</tr>
<tr>
<td>Abnormal size/function</td>
</tr>
<tr>
<td>Intra and extrahepatic biliary strictures (sclerosing cholangitis)</td>
</tr>
</tbody>
</table>

GGT: Gamma glutamyl transferase.

and gamma glutamyl transferase (GGT). Elevated liver enzymes can precede clinical and radiological abnormalities by several years. Bile duct damages can be demonstrated even in asymptomatic cases[22]. About 53%-93% of patients with CF have at least one abnormal value of AST/ALT, while over one-third have abnormal levels of GGT by 21 years of age[23]. CFLD patients with cirrhosis with portal hypertension can have normal liver biochemistry and synthetic function. Fluctuations in liver biochemistry is common and can be due to medications, infection or malnutrition.

Steatosis: Steatosis is common in CF patients, seen in upto 70% children undergoing liver biopsies[24]. The etiology is uncertain, but postulated to be due to malnutrition, deficiencies of essential fatty acid, carnitine and choline[24,25]. Steatosis in CF patients can also be caused by impaired glucose tolerance, diabetes mellitus, hypertriglyceridemia and obesity[23]. Significant steatosis has become uncommon due to earlier diagnosis of CFLD and appropriate nutritional management. Alcohol consumption should be considered in adolescent CF patients with steatosis. Steatosis in CF was previously thought to be a benign condition, but with the emergence of nonalcoholic steatohepatitis as a leading cause of cirrhosis and understanding of the pathology, this might no longer be the case. Other signs of chronic liver disease or portal hypertension are usually not present.

Gallbladder and biliary tract involvement: Abnormalities of gallbladder (GB) can be present in children with CF. Micro-GB has been described in up to 33% of patients and GB might even be absent in CF patients[26]. Abnormal function of gallbladder and gallstones can also present. Black pigmented stones are more commonly found in patients with CF compared to cholesterol gallstones which are common in general population[26]. Symptomatic GB disease (4%) and need for cholecystectomy is common in adults[26].

Intra- or extrahepatic biliary strictures and segmental dilation has been reported in children with CF. Bile duct strictures and associated complications frequently occur even in patients with mild variants of CF. Magnetic resonance (MR) cholangiography data has shown that up to 70% of patients can have abnormalities of biliary tree regardless of biochemical or clinical evidence of liver disease and can mimic primary sclerosing cholangitis[24,26]. There is no correlation between severity of liver disease, abnormal liver tests and the presence of biliary strictures[24,26].
Valampampil JJ et al. Cystic fibrosis liver disease in children

Figure 1 The functional consequences of cystic fibrosis-causing variants have been grouped into six classes. Class I mutations lead to no protein synthesis or translation of shortened, truncated forms. They result from splice site abnormalities, frameshifts due to deletions or insertions, or nonsense mutations, which generate premature termination codons. Class II mutations lead to a misfolding protein that fails to achieve conformational stability in the endoplasmic reticulum and then does not traffic to the plasma membrane (PM), being instead prematurely degraded by proteasomes. Class III mutations lead to a gating channel defect due to impaired response to agonists, although the protein is present at the PM. Class IV mutations lead to a channel conductance defect with a significant reduction in cystic fibrosis transmembrane conductance regulator (CFTR)-dependent chloride transport. Class V mutations lead to a reduction in protein abundance of functional CFTR due to reduced synthesis or inefficient protein maturation. They result from alternative splicing, promoter or missense mutations. Class VI mutations lead to reduced protein stability at the PM, which results in increased endocytosis and degradation by lysosomes, and reduced recycling to the PM. PM: Plasma membrane.

Liver disease with cirrhosis/portal hypertension (severe CFLD)

Portal hypertension: Variceal bleed can occur with or without cirrhosis and frequently occurs in the context of preserved hepatic synthetic function. Varices can be seen in 10-70% with CFLD and may be present at diagnosis of CFLD in 25%[19,27,28]. Isolated gastric varices may be seen in 15%[19]. Variceal bleed can be the sentinel event in CFLD leading to the diagnosis of portal hypertension/cirrhosis in up to 50% and may also be fatal, either from bleed itself or by precipitating liver failure. The age at first bleed can range from 10-30 years and recurrent bleeds can also occur[4]. Variceal bleed is associated with 5 fold risk of liver transplantation (LT)[2]. Thrombocytopenia has been postulated as a marker of severe CFLD with portal hypertension, so decreasing or persistently low platelet counts should prompt evaluation for portal hypertension [19]. Non cirrhotic portal hypertension can also occur in CFLD[28,29]. This has been postulated to be due to perisinusoidal portal venopathy caused by inflammation and fibrosis[24,29].

Focal biliary cirrhosis: Focal biliary cirrhosis is characterized by focal areas of scarring and furrowing in the liver with large areas of normal preserved hepatic architecture in between. Histologically, it is characterized by cholestasis, significant focal fibrosis, plugging of bile ducts with eosinophilic material, bile duct proliferation and expansion of portal tract leading to the postulation that bile duct plugging is the causative factor.

Focal biliary cirrhosis is clinically silent without any abnormalities on physical examination and normal liver biochemistry. Radiological imaging is also frequently noncontributory. Postmortem studies have shown that the incidence of focal biliary cirrhosis increases with advanced age- 11% in infants, 27% at 1 year and 25%–70% of adults[24]. Only a small subset of patients will progress to more severe liver disease and eventually multilobular cirrhosis, but the factors causing this is not known.

Multilobular cirrhosis: Biliary cirrhosis with portal hypertension is the most severe clinical manifestation of CFLD. Clinically, liver is multilobulated and firm- extensive lobulation is characteristic of CF cirrhosis. Signs of chronic liver disease such as clubbing, spider angioma, and palmar erythema may be present but is uncommon and
often occurs late in the disease course. There are no clinical or biochemical abnormalities or radiological features that consistently predict the presence of cirrhosis or risk of development of portal hypertension[25]. Majority of the morbidity due cirrhosis is caused by complications arising from portal hypertension. Hepatic encephalopathy is rare complication of cirrhosis per se in CFLD and mostly has occurred after therapeutic portosystemic shunting for management of portal hypertension[24]. Hepatic decompensation as evidenced by progressive decrease in albumin levels and development of ascites represents poor prognosis and necessitates LT evaluation.

Patients with cirrhosis are at risk of significant malnutrition as compared to CF patients without liver disease. This is due to anorexia, micronutrient deficiency, early satiety due to organomegaly and increased catabolism. In a study comparing CFLD patients with CF patients without liver disease, body fat measurements, including triceps, subscapular, and supra-iliac skinfold measures, were significantly less in the CFLD patients[27]. However, weight, height and mid upper arm circumference were not different between the two groups[27].

**EVALUATION**

Liver enzymes (AST, ALT, GGT) are poor predictors or indicators of cirrhosis or the risk of development of cirrhosis or CFLD and are neither sensitive or specific. There is poor correlation of liver enzymes with histologic findings, with 25% of CFLD patients with biopsy proven severe liver fibrosis having normal ALT levels[28]. But patients presenting with significant or persistently elevated liver biochemistries warrant further investigation for evidence of CFLD and other etiologies (Table 2). Persistently elevated GGT might be a pointer to biliary disease (e.g., sclerosing cholangitis). Thrombocytopenia with splenomegaly is suggestive of development of portal hypertension. The synthetic function of liver (clotting, albumin) should be checked in all patients with suspected CFLD. If deranged after correcting nutritional (poor diet, vitamin deficiency) defects, should be thoroughly investigated.

**Imaging**

Ultrasound (US) of the hepatobiliary system with Doppler measurements of hepatic vasculature is non-invasive and may be a valuable marker of early CFLD[30]. Partial or complete hyper echogenicity liver, suggestive of steatosis is the most common US finding in CF[31]. Another fatty infiltration pattern, pseudomasses, seen as lobulated fatty structures of 1–2cm causing heterogeneity in the liver parenchyma is typical of CF[31]. Focal biliary cirrhosis appears sonographically as regions of increased echogenicity in perportal areas[31,32]. Cirrhotic liver has a nodular appearance with a coarsened echotexture[32]. Right hepatic lobe atrophy and hypertrophy of the caudate and lateral segments of the left lobe may be seen[32]. Splenomegaly, portosystemic shunts, hepatofugal flow in portal vein, and ascites can be seen with portal hypertension.

Abnormal echogenicity frequently precedes biochemical/clinical evidence of liver disease, with one study showing that two thirds of the children with abnormal liver echotexture and 50% with portal hypertension had no biochemical/clinical evidence of CFLD at the time when US changes were first noted[30]. Heterogeneous pattern of liver has been shown to be associated with higher risk of development of advanced liver disease in CF patients[30,33]. However, there is significant intra/ interobserver variability in US imaging and children with normal hepatic US can have advanced fibrosis, so a normal US does not exclude significant liver fibrosis or CFLD[3].

Assessment of the intra and extrahepatic biliary tree is better with MR cholangiography. The typical appearances include strictures, beading, narrowing, or dilatation of the intrahepatic ducts; diffuse narrowing or focal stricture of the common bile duct; and calculi[32].

**Liver biopsy**

Liver biopsy (LB) the gold standard in diagnosing fibrosis and cirrhosis, but is difficult to perform in CF patients because of the invasive nature and presence of associated comorbidities. Also because of the patchy distribution of lesions in CFLD, LB may underestimate the severity of lesions[25]. LB should be reserved for evaluation for other potential causes of fibrosis (autoimmune hepatitis, Wilson’s disease, hepatotropic infections) or drug-induced liver injury.
### Table 2 Causes of acute or chronic liver disease in cystic fibrosis patients showing hepatic abnormalities

<table>
<thead>
<tr>
<th>Condition</th>
<th>Investigation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute/chronic viral hepatitis</td>
<td>Serology for HAV, HBV, HCV, EBV, CMV, adeno virus, HHV 6, parvovirus</td>
</tr>
<tr>
<td>α1 antitrypsin deficiency</td>
<td>Serum α1 antitrypsin level, including phenotype</td>
</tr>
<tr>
<td>Autoimmune hepatitis</td>
<td>Non-organ specific autoantibodies (SMA, anti-LKM1, LC1)</td>
</tr>
<tr>
<td>Celiac disease</td>
<td>Total IgA, IgA anti-tissue transglutaminase</td>
</tr>
<tr>
<td>Wilson disease</td>
<td>Ceruloplasmin, serum copper, 24 h urinary copper</td>
</tr>
<tr>
<td>Drug induced liver injury</td>
<td>Antibiotics (cyclines, macrolides, amoxicillin-based, and cephalexopirins) &amp; antifungals (azoles and polyenes)</td>
</tr>
<tr>
<td>Genetic hemochromatosis (adults)</td>
<td>Iron, Ferritin, Transferrin binding capacity</td>
</tr>
<tr>
<td>Other causes of steatosis</td>
<td>Malnutrition, diabetes, obesity</td>
</tr>
</tbody>
</table>

This table is modified from Debray et al[25]. HAV: Hepatitis A virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; CMV: Cytomegalovirus; EBV: Epstein-Barr virus; HHV6: Herpes hominis virus type 6; SMA: Smooth muscle antibody; LKM1: Liver kidney microsomal type 1; LC: Liver cytosol type 1; IgA: Immunoglobulin A.

**Noninvasive tests of fibrosis and liver disease**

The early detection and monitoring of fibrosis, assessment of stage of fibrosis and progression to CFLD is challenging because routinely available tests to measure liver damage can often be normal even in advanced cirrhosis and liver biopsy is invasive with potential risk of complications. Non-invasive tests are divided into direct and indirect markers of liver fibrosis and imaging modalities as outlined in Table 3.

Direct markers are components of extracellular matrix degradation or fibrogenesis in serum include Matrix Metalloproteinase-9, tissue inhibitor of metalloproteinase-1 and 2, procollagen III peptide, collagen type-IV, hyaluronic acid, laminin, prolyl hydroxylase and YKL-40. These are not readily available in the routine clinical setting, are costly and are not validated in large scale studies. Indirect markers are serum-based tests and consist of readily available biochemical surrogates and clinical risk factors (AST, ALT, platelet count, age) for liver fibrosis. These include aspartate aminotransferase to platelet ratio index (APRI) and Fibrosis-4 (Fib-4). Stonebraker et al [19] demonstrated in a large pediatric cohort (n = 497) with CFLD and portal hypertension that APRI and Fib-4 values could differentiate patients who developed complications of portal hypertension and were significantly different in CFLD patients with and without oesophageal varices.

Advanced imaging modalities which quantify liver stiffness as a marker of fibrosis such as transient elastography (TE, Fibroscan®), acoustic radiation force impulse and MR elastography have been shown to accurately reflect advanced liver disease/end-stage fibrosis in CF. Liver stiffness as measured by TE had high diagnostic accuracy and was increased in CFLD compared to CF patients without liver disease[34]. Serial monitoring using TE is more useful as progressive enhancement of liver stiffness as this might reflect progression of liver disease thereby facilitating early detection[34,35]. MR elastography is currently the most accurate noninvasive method across the spectrum of liver fibrosis and offers promise in the assessment of response to antifibrotic drugs but is not well studied in the context of CF liver disease[36].

Noninvasive methods are valuable for excluding advanced fibrosis or cirrhosis, but are not sufficiently predictive when used in isolation and have not yet been demonstrated to accurately reflect fibrosis change in response to treatment, limiting their role in disease monitoring[36]. Combination of serum markers with liver stiffness analysis might improve the sensitivity and negative predictive value without altering the specificity[34]. The negative predictive value of noninvasive tests is generally very high, allowing the clinician to be confident that advanced fibrosis or cirrhosis has been excluded.

**Differential Diagnosis**

The wide spectrum, variability of presentation at different age groups, presence of confounding factors and the absence of specific markers or tests makes it difficult to diagnose CFLD. The common differential diagnosis to be considered in CFLD are
Table 3 Examples of noninvasive monitoring of liver fibrosis in pediatric cystic fibrosis liver disease

<table>
<thead>
<tr>
<th>Non-invasive marker</th>
<th>Ref.</th>
<th>Outcome measured</th>
<th>AUC</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indirect markers of liver fibrosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APRI</td>
<td>Leung et al[37]</td>
<td>CFLD diagnosis and severe CFLD</td>
<td>0.81</td>
<td>73%</td>
<td>70%</td>
<td>APRI score cut-off &gt; 0.264; Predict CFLD and significant fibrosis in CFLD with a high degree of accuracy</td>
</tr>
<tr>
<td>FIB-4</td>
<td>Leung et al[37]</td>
<td>Portal hypertension</td>
<td>0.91</td>
<td>78%</td>
<td>93%</td>
<td>FIB-4 cutoff 0.358</td>
</tr>
<tr>
<td>Direct markers of liver fibrosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIMP-1</td>
<td>Pereira et al[38]</td>
<td>CFLD diagnosis</td>
<td>0.76</td>
<td>64%</td>
<td>83%</td>
<td>Significantly increased in CFLD vs no-CFLD</td>
</tr>
<tr>
<td>Prolyl hydroxylase</td>
<td>Pereira et al[38]</td>
<td>CFLD diagnosis</td>
<td>0.76</td>
<td>60%</td>
<td>91%</td>
<td>Negative correlation between serum TIMP-1 levels and the stage of histological fibrosis; Prolyl hydroxylase useful in distinguishing CFLD patients with early fibrogenesis vs extensive fibrosis; Not able to differentiate CFLD versus no-CFLD</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>Rath et al [38]</td>
<td>CFLD diagnosis</td>
<td>0.69</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>m-RNA’s</td>
<td>Cook et al [39]</td>
<td>CFLD diagnosis</td>
<td>0.78</td>
<td>47%</td>
<td>94%</td>
<td>Able to differentiate between CFLD versus no-CFLD but quantify not fibrosis stage; Pathological significance not yet certain, more studies needed</td>
</tr>
<tr>
<td>Imaging methods</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transient elastography</td>
<td>Witters et al[40]</td>
<td>Liver stiffness</td>
<td>0.86</td>
<td>63%</td>
<td>87%</td>
<td>Less inter and intra-observer variability; Easy to learn and perform; Regular measurements for serial follow-up feasible</td>
</tr>
<tr>
<td></td>
<td>Rath et al [34]</td>
<td>Liver stiffness</td>
<td>0.68</td>
<td>-</td>
<td>-</td>
<td>Few centres have access to technology</td>
</tr>
<tr>
<td>MR elastography</td>
<td>Palermo et al[41]</td>
<td>Liver stiffness</td>
<td>-</td>
<td>100%</td>
<td>100%</td>
<td>Small study, paucity of data; Shear stiffness significantly elevated in CF patients with cirrhosis; Costly with limited availability</td>
</tr>
</tbody>
</table>

AUC: Area under the curve; APRI: Aspartate aminotransferase to platelet ratio index; CFLD: Cystic fibrosis associated liver disease; Fib-4: Fibrosis-4; TIMP: Tissue inhibitor of metalloproteinase; m-RNA: Messenger ribonucleic acid; MR: Magnetic resonance.

listed in Table 2.

DIAGNOSTIC CRITERIA OF CFLD

The commonly used diagnostic criteria are described in Table 4.

MANAGEMENT

Management of CFLD should be done by a multi-disciplinary team and is mainly supportive since there is no effective therapy to treat or prevent progression of fibrosis, portal hypertension, or cirrhosis in CFLD. The CF foundation guidelines recommends annual screening for CFLD in children with examination of abdomen (hepatosplenomegaly), biochemical evaluation (bilirubin, AST, ALT, GGT, ALP, albumin, prothrombin time, platelet count), abdominal US and pulse oximetry (screening for hepatopulmonary syndrome)[25]. Salicylic acid and non-steroid anti-inflammatory drugs are contraindicated once CFLD is diagnosed and vaccination against hepatitis A and B should be done.

Ursodeoxycholic acid (UDCA) is recommended for all children diagnosed with CFLD at 20 mg/kg/d divided twice daily initially and increased up to 30 mg/kg/d [25]. A Cochrane review[42] had shown that there were only few trials assessing the effectiveness of UDCA with poor quality of evidence and there was no data on the effect of UDCA on long term outcomes including need for LT or mortality. Hence, the long term continuation of UDCA should be individualized.
Table 4 Diagnostic criteria of cystic fibrosis liver disease

<table>
<thead>
<tr>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatomegaly and/or splenomegaly - increased liver span at midclavicular line and spleen size in longitudinal coronal plane for age and sex, confirmed by ultrasonography</td>
<td>CF related liver disease with cirrhosis/portal hypertension (based on clinical exam/imaging, histology, laparoscopy)</td>
</tr>
<tr>
<td>Abnormalities of liver function tests - elevated AST and ALT and GGT levels above the upper limit of normal with at least 3 consecutive determinations over 12 months after excluding other causes of liver diseases</td>
<td>Liver involvement without cirrhosis/portal hypertension consisting of at least one of the following: (1) Persistent AST, ALT, GGT &gt; 2 times upper limit of normal; (2) Intermittent elevations of the above laboratory values; (3) Steatosis (histologic determination); (4) Fibrosis (histologic determination); (5) Cholangiopathy (based on ultrasound, MRI, CT, ERCP); and (6) Ultrasound abnormalities not consistent with cirrhosis</td>
</tr>
<tr>
<td>Ultrasonographic evidence of coarseness, nodularity, increased echogenicity, or portal hypertension</td>
<td>Preclinical: No evidence of liver disease on clinical examination, imaging or laboratory values</td>
</tr>
<tr>
<td>Liver biopsy showing cirrhosis</td>
<td></td>
</tr>
</tbody>
</table>

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma glutamyl transpeptidase; CF: Cystic fibrosis; MRI: Magnetic resonance imaging; CT: Computed tomography; ERCP: Endoscopic retrograde cholangiopancreatography.

**Nutrition**

Optimal nutrition is the cornerstone of CFLD management. Malnutrition in CF is multifactorial including malabsorption due to pancreatic insufficiency, recurrent infections, chronic inflammation, chronic liver disease and anorexia. Nutrition should be managed by experienced CF dietetic team. It is recommended that CFLD patients increase energy intake to 150% of Recommended Daily Allowance preferably by increasing proportion of fat to 40%–50% of the energy content of the feed or diet, with supplementation in medium chain triglycerides and special attention to polyunsaturated fatty acids[25].

About 3 g/kg/d of protein and sufficient pancreatic enzymes to allow optimal absorption of long-chain triglycerides and essential fatty acids is also recommended. High dose oral fat soluble vitamin supplements is recommended- vitamin A (5000–15000 international units daily), vitamin E (alpha tocopherol 100–500 mg daily), vitamin D (alphacalcidiol 50 ng/kg to maximum of 1 μg) and vitamin K (1–10 mg daily)[25]. Plasma levels of vitamins (A, D and E) and prothrombin time needs to be closely monitored to prevent toxicity or deficiencies.

Salt supplementation should be avoided in CF patients with cirrhosis and portal hypertension due to the risk of development of ascites. If adequate caloric intake cannot be achieved orally, nasogastric feeding may be required to ensure adequate caloric intake. CFTR modulator therapy has resulted in less pulmonary exacerbations, decrease in levels of inflammatory makers, better body mass index and pancreatic function resulting in better overall nutritional status[14].

**Management of esophageal varices**

Management of varices in CFLD is complicated by the fact that non-selective beta-blocker (propranolol or carvedilol) might be contraindicated due to the associated lung disease and repeated general anesthesia required for screening of therapeutic endoscopic procedures may also reduce lung function and predispose to infections. Primary variceal prophylaxis in CFLD most commonly involves endoscopic variceal band ligation, but there is lack of quality evidence in children[24].

Variceal bleeding in the absence of decompensated cirrhosis in CFLD is most commonly managed by therapeutic endoscopy (band ligation +/- sclerotherapy)[4]. Sclerotherapy is useful if variceal band ligation is unsuccessful or gastric varices are present. Patients with refractory life threatening bleeds might require transjugular intrahepatic portosystemic shunt (TIPSS) or in rare circumstances surgical portosystemic shunting as an lifesaving procedure. Careful evaluation of liver disease and lung disease is necessary before proceeding with an elective TIPSS procedure. In a study[4] specifically analyzing outcomes of variceal bleeds in CFLD, out of 35 bleeding episodes, 30 were controlled by endoscopic procedures, while 11% (4 episodes) required either TIPSS, surgical shunts procedures.

**Liver transplantation**

LT evaluation should be offered for CFLD patients with intractable complications of portal hypertension and/or end stage liver disease since LT confers significant
Valamparampil JJ *et al*. Cystic fibrosis liver disease in children

survival advantage [43]. The main indications of isolated LT in CFLD is listed in Table 5. Poor growth and nutrition as an indication remains controversial because studies have not shown consistent improvement after LT [43]. LT should be considered when nutritional deficiencies are believed to be sequelae of advanced liver disease and portal hypertensive enteropathy impacting clinical outcomes [43]. Lung function may improve, remain stable or deteriorate after LT and any short term advantage with improvement of lung function is lost within 3 years of LT [44,45]. So, rapidly deteriorating lung function alone should not be an indication for isolated LT in stable CFLD [46].

Long term outcomes after LT are lower in children with CFLD as compared to other etiologies [44]. Table 6 illustrates details of few published series on LT in CFLD in children. For those patients with end-stage liver disease and significant pulmonary complications, combined liver-lung or liver-heart-lung transplantation may be considered, but outcomes are worse compared to isolated LT [45,46].

**Pre-transplant considerations**

Careful assessment of liver disease, pulmonary function, nutritional status and type of transplant to be performed should be done by an experienced multidisciplinary team. Concomitant causes or other etiologies of liver injury as listed in Table 3 should be ruled out before LT is considered. Alpha-1-antitrypsin level and genotype, screening for autoimmune hepatitis and Wilson’s disease should be done as a part of the workup especially if the child is seen for the first time in a LT center. CFLD patients being considered for LT should have endoscopic variceal surveillance and possibly coordinated with bronchoscopy and dental procedures as part of the LT evaluation to minimize the number anaesthetic procedures [43]. Careful evaluation of cardiac function should be done since patients with cardiomyopathy or severe pulmonary hypertension may require combined heart, lung, and liver transplantation.

A thorough evaluation by a pediatric pulmonologist with CF and lung transplantation expertise should be a part of the LT assessment, irrespective of the forced expiratory volume in one second (FEV1). Analysis of United Network for Organ Sharing data from 1987 through 2009 suggested that patients with a predicted forced vital capacity (FVC) > 75% and FEV1 > 60% (possibly even ≥ 40%) may be safely offered isolated LT [50]. The possibility of progressive deterioration in lung function after LT should be communicated to the family. The most difficult group to decide is patients who require LT but present with borderline (FEV1 40%–60% predicted) and/or rapidly declining (10% FEV1 predicted/year) pulmonary function [43].

Microbial considerations, such as multidrug resistant bacterial infections and history of recurrent/invasive fungal infections are critical since post-transplant sepsis is a leading cause of mortality [45,50]. Flexible bronchoscopy with bronchoalveolar lavage with cultures for mycobacteria, fungus, and quantitative bacterial analysis from at least 2 locations within each lung is recommended [43]. The presence of multidrug resistant *Mycobacterium abscessus* in the lungs, even with well-preserved pulmonary function, carries a high risk of mortality in the first year after transplant and needs to be considered carefully before recommendation for LT [43].

Patients should be evaluated for nasal polyps and chronic sinusitis and treated immediately if identified [43]. CF-related diabetes should be evaluated and well controlled prior to LT. Dietetic and nutritional assessment is an integral part of the evaluation.

**Post-transplant considerations**

Immunosuppression after LT in patients with CF will vary from center to center but typically consists of triple drug therapy with tacrolimus, steroids and mycophenolate mofetil/azathioprine. Close collaboration between the CF, transplant and infectious diseases teams is crucial because of the increased risk of mortality from infections. Early mortality (< 6 mo) post-LT is due to disseminated aspergillosis/candidiasis, and sepsis with gram-negative enteric bacteria and staphylococcus aureus while later deaths are a result of progressive pulmonary disease [43]. Post-transplant antibiotic prophylaxis in our unit consists of fluconazole for candida species, acyclovir for herpes simplex virus, valganciclovir for cytomegalovirus and trimethoprim-sulfamethoxazole for *Pneumocystis jiroveci*. Distal intestinal obstructive syndrome (DIOS) causing acute potentially life-threatening intestinal obstruction can develop post-transplant in >20% of pediatric patients [49]. In the pre-transplant period, DIOS occurs typically in older CF patients in adolescence and adulthood, in those with advanced liver disease, severe CFTR mutations, pancreatic insufficiency and diabetes mellitus. In our unit, patients are categorized into low risk (no episodes of DIOS in previous 5 years) and high risk (episodes of DIOS in previous 5 years and previous abdominal surgery) before LT.

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In the lungs, even with well-preserved pulmonary function, carries a high risk of mortality in the first year after transplant and needs to be considered carefully before recommendation for LT [43].
Table 5 Indications and contraindications for liver transplantation in cystic fibrosis liver disease (Modified from Freeman et al[43])

<table>
<thead>
<tr>
<th>Indications</th>
<th>Controversial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strong</td>
<td>(1) Progressive hepatic dysfunction with hypoalbuminemia and coagulopathy (Coagulopathy not corrected by vitamin K, cholestasis not attributed to other causes); (2) Complications of portal hypertension (Intractable/recurrent variceal bleeding which is not controlled by medical or endoscopic management); (3) Hepatorenal and porto-pulmonary syndrome; (4) Overt hepatic encephalopathy; and (5) Hepatorenal syndrome.</td>
</tr>
<tr>
<td>Absolute</td>
<td>(1) Extrahepatic malignancies not amenable to curative therapy; (2) Multiorgan disease for which transplant would not be considered life-sustaining; (3) Uncontrolled systemic or pulmonary infection, active exacerbation, or vaso-arterial extracorporeal membrane oxygenation; and (4) Severe porto-pulmonary hypertension nonresponsive to medical management.</td>
</tr>
<tr>
<td>Relative</td>
<td>(1) Hepatocellular carcinoma; (2) Noncompliance or psychosocial concerns unamenable to transplant; (3) Uncontrollable CF-related diabetes; (4) Substance abuse; (5) Severe cardiopulmonary disease; and (6) Infection/colonization with multi-resistant organism (e.g., Burkholderia cenocepacia and Mycobacterium abscessus).</td>
</tr>
</tbody>
</table>

FEV1: Forced expiratory volume in one second; FVC: Forced vital capacity.

Table 6 Liver transplantation in cystic fibrosis liver disease - data from few published series

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Type</th>
<th>Number of pediatric recipients</th>
<th>Type of transplants</th>
<th>Males</th>
<th>Mean age at isolated liver transplantation (yr)</th>
<th>Lung function after Liver transplantation</th>
<th>5-year survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milkiewicz et al[45], 2002</td>
<td>Single center</td>
<td>9</td>
<td>Liver; Liver-lung -heart</td>
<td>Not available</td>
<td>15</td>
<td>Improved</td>
<td>Not available</td>
</tr>
<tr>
<td>Fridell et al[21], 2003</td>
<td>Single center</td>
<td>12</td>
<td>Liver</td>
<td>83%</td>
<td>10 ± 4.5</td>
<td>Improved or remained unchanged</td>
<td>75%</td>
</tr>
<tr>
<td>Molmenti et al[47], 2003</td>
<td>Single center</td>
<td>10</td>
<td>Liver</td>
<td>90%</td>
<td>9.7 (1.23-19)</td>
<td>Not available</td>
<td>60%</td>
</tr>
<tr>
<td>Mendizabal et al[44], 2011</td>
<td>Analysis of United Network for Organ Sharing database</td>
<td>148</td>
<td>Liver; Liver-lung (3.4%)</td>
<td>62%</td>
<td>11 ± 4.7</td>
<td>Not available</td>
<td>86%</td>
</tr>
<tr>
<td>Miguel et al[40], 2011</td>
<td>Single center</td>
<td>11</td>
<td>Liver</td>
<td>67%</td>
<td>12 (5.4-17)</td>
<td>Worsened or remained unchanged</td>
<td>&gt; 85%</td>
</tr>
<tr>
<td>Dowman et al[49], 2012</td>
<td>Single center</td>
<td>19</td>
<td>Liver</td>
<td>Not available</td>
<td>11.8 (9.5-16.5)</td>
<td>Stable/improved initially, deteriorated &gt; 5 years after transplant</td>
<td>&gt; 60%</td>
</tr>
</tbody>
</table>

Our pre and post-LT protocol for prevention and treatment of DIOS is given in Table 7. High risk patients should be counselled for loop ileostomy formation at transplant assessment.

**CFTR modulators**

CFTR modulator drugs enhance or even restore the expression, function, and stability of a defective CFTR by different mechanisms[14,51] (Table 8). These treatments target the underlying cause of CF and is classified into five main groups depending on their effects on CFTR mutations[14,51] (Table 8). Different CFTR genetic variants can benefit from the same type of modulator and this is the base of a new system recently introduced to classify and group common and rare CFTR variants based on their response to modulators called ‘theratyping’.

The first United States Food and Drug Administration (FDA) approved drug was ivacaftor (Kalydeco, Vertex Pharmaceuticals)[14,51]. Other FDA approved CF modulators combinations are lumacaftor/ivacaftor (Orkambi®, Vertex Pharmaceuticals), tezacaftor/ivacaftor (Symdeko® or Symkevi®, Vertex Pharmaceuticals) for patients aged ≥ 12 years who are F508del-homozygous or F508del-heterozygous with a residual function mutation[14,20]. Lumacaftor/ivacaftor has been approved for F508del homozygous patients aged ≥ 2 years[14]. The triple combination elixacaftor/ivacaftor/tezacaftor (Trikafta™, Vertex Pharmaceuticals) has been by the FDA for the
Table 7 Pre and post-transplant protocol for prevention and treatment of distal intestinal obstructive syndrome

<table>
<thead>
<tr>
<th>Pre and post-transplant protocol</th>
<th>Low risk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(1) 600 mg N-acetyl-cysteine in 120 mL water orally/nasogastric tube twice/day. Senna twice daily; (2) 2 liters of Klean prep per day post-transplant; (3) Consider early nasogastric tube in patients with delayed gastric emptying studies pre-operatively; (4) All patients in intensive care unit should only receive only elemental feed via nasogastric tube as this does not require pancreatic enzyme replacement. Once transferred to ward, can be restarted on regular feeding and pancreatic enzyme supplements; (5) Try and reduce opiates early during hospital stay; and (6) Treat all patients with proton pump inhibitors.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>High risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) As per low risk management; and (2) High risk of developing DIOS and subsequent surgical gut decompression is associated with a high mortality. So these patients should receive a prophylactic loop ileostomy.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment of DIOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Stop feeding, nasogastric tube on free drainage and intravenous fluids; (2) 100 mL gastrografin in 400 mL water enterally and repeat after 6 h; (3) Subsequent management is with Klean prep in 1 L water over 1 h via oral/nasogastric tube and can be repeated up to 4 times every 24 h until bowel movement is achieved; and (4) If no improvement after 48 h, then it is unlikely to resolve without surgery to decompress the gut and also consider total parenteral nutrition.</td>
</tr>
</tbody>
</table>

DIOS: Distal intestinal obstructive syndrome.

Table 8 Cystic fibrosis transmembrane conductance regulator modulators

<table>
<thead>
<tr>
<th>Type of modulator</th>
<th>Mechanism of action</th>
<th>Mutation class in which drug is effective</th>
<th>Example</th>
<th>Clinical effects/present status of modulator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potentiators</td>
<td>Restore or even enhance the channel open probability, thus allowing for CFTR-dependent anion conductance</td>
<td>Classes III and IV</td>
<td>Ivacaftor</td>
<td>Improvement in lung function, pancreatic function and body mass index</td>
</tr>
<tr>
<td>Correctors</td>
<td>Rescue folding, processing and trafficking to the plasma membrane of a CFTR mutant. Enhance protein conformational stability during the endoplasmic reticulum folding process</td>
<td>Class II</td>
<td>Lumacaftor; Tezacaftor; Posenacaftor; Elexacaftor</td>
<td>Significant improvement in lung function when used with Ivacaftor</td>
</tr>
<tr>
<td>Stabilizers</td>
<td>Anchor CFTR at the plasma membrane, thus preventing its removal and degradation by lysosomes</td>
<td>Class VI</td>
<td>Cavosonstat</td>
<td>First CFTR stabilizer studied in clinical trials- studies terminated because of lack of clinical efficacy</td>
</tr>
<tr>
<td>Read-through agents</td>
<td>Induce ribosomal over-reading of premature termination codon, enabling the incorporation of a foreign amino acid in place and continued translation to the normal end of the transcript</td>
<td>Class I</td>
<td>Ataluren (PTC124)</td>
<td>Clinical trials terminated</td>
</tr>
<tr>
<td>Amplifiers</td>
<td>Increase expression of CFTR mRNA and thus biosynthesis of the CFTR protein</td>
<td>Class V</td>
<td>Nesolicaftor (PTI-428)</td>
<td>Clinical trial planned</td>
</tr>
</tbody>
</table>

CFTR: Cystic fibrosis transmembrane conductance regulator; mRNA: Messenger RNA.

treatment of CF patients aged ≥ 12 years with F508del mutation in at least one allele, benefiting 90% of CF population[14,51].

CF MODULATORS AND LIVER

Abnormal elevation aminotransaminases (> 8 times upper limit of normal, more commonly in pediatric patients) and bilirubin (> 3 times upper limit of normal) has been reported 3%-15% of patients on CFTR modulators[52-54]. Lumacaftor/ivacaftor was shown to have less hepatic steatosis as assessed by MR imaging proton density fat fraction in a small cohort[55]. In a study[56] of 117 patients with CFTR gating mutations (partially F508 del heterozygous) treatment with Ivacaftor partially restored disrupted FGF19-regulated bile acid homeostasis. Worsening of liver function and liver failure leading to death has been reported in CF patients with pre-existing cirrhosis and portal hypertension receiving lumacaftor/ivacaftor.

Recommendations for dose adjustment are based on Child Pugh classification: no dose adjustment for Child-Pugh Class A but dose reduction is recommended for Child-Pugh Class B and C. This is applicable to adults and no specific recommendations exist in the literature for children with CFLD. Lumacaftor/ivacaftor should be used with caution in patients with advanced liver disease and only if the benefits are expected to outweigh the risks.
Because an association with liver injury cannot be excluded, assessments of liver function tests (ALT, AST and bilirubin) are recommended before initiation, a month after starting the treatment and every 3 mo during the first year of treatment, and annually thereafter. For patients with a history of ALT, AST, or bilirubin elevations, more frequent monitoring should be considered in collaboration with a pediatric hepatology centre. In the event of significant elevation of ALT or AST, with or without elevated bilirubin [either ALT or AST > 5× the upper limit of normal (ULN), or ALT or AST > 3× ULN with bilirubin > 2× ULN and/or clinical jaundice], dosing with CFTR modulators should be discontinued and closely followed up until the abnormalities resolve. A thorough investigation of potential causes should be conducted and patients should be followed closely for clinical progression. Following resolution of transaminase elevations, the benefits and risks of resuming CFTR modulators should be considered.

Metabolism of CFTR inhibitors is by the CYP450 enzyme pathway. Hence concomitant use of lumacaftor/ivacaftor with these immunosuppressants is not recommended at present as they may reduce efficacy of immunosuppressants by induction of the CYP3A pathway. Given the fact that respiratory function may eventually worsen after LT, CFTR modulators might need to be initiated post-transplant due to significant beneficial effects on lung function, nutritional status and decreased pulmonary exacerbations.[43].

CONCLUSION

CFLD is the most important non-pulmonary cause of death in CF. CFLD has is a wide spectrum from asymptomatic elevation of liver enzymes to severe disease with portal hypertension and cirrhosis with synthetic failure. The degree of liver involvement and the rate of progression of liver disease varies significantly among individuals. There are no specific clinical features or tests for prediction or early detection of CFLD, so regular screening is essential for CF patients. Currently, there is no medical therapy to prevent or treat CFLD. With the advent of CFTR modulators, improvement in medical management has resulted in significantly improved life expectancy in patients with CF and this will have implications in the management of CFLD in future. The long term effects of CFTR modulators on CFLD and liver function is not known, but will hopefully have a beneficial effect. LT is indicated in patients with CFLD with severe portal hypertension or impaired synthetic function of liver either alone or in combination with lung transplantation.

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Valamparampil JJ et al. Cystic fibrosis liver disease in children


Tumor characteristics of hepatocellular carcinoma after direct-acting antiviral treatment for hepatitis C: Comparative analysis with antiviral therapy-naive patients

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Author contributions: Fouad M and El Kassas M conceptualized the idea and design; El Sheemy R drafted, revised and edited the manuscript; all authors participated in and supervised patients’ treatment and follow-up, revised and approved the final version of the manuscript.

Institutional review board statement: The study protocol was approved by the Institutional Research Board at the Faculty of Medicine, Minia University, Egypt. Informed written consent was obtained from all patients of the study. This research was performed in agreement with the guidelines of the 1975 Declaration of Helsinki.

Informed consent statement: Informed consent was obtained from all individual participants included in the study

Conflict-of-interest statement: All authors declare that they do not have any conflict of interest.

Abstract

BACKGROUND
Insufficient and contradictory data are available about the relation between direct-acting antivirals (DAAs) and hepatocellular carcinoma (HCC) development in patients with hepatitis C virus (HCV).

AIM
To analyze differences in basic clinical, radiological, and laboratory characteristics in addition to tumor behavior upon HCC diagnosis between patients with and without a previous history of DAAs exposure.

METHODS
This multicenter case-control study included 497 patients with chronic HCV-related HCC, allocated into one of two groups according to their history of antiviral treatment for their HCV.

RESULTS
Group I included 151 HCC patients with a history of DAAs, while 346 patients who had never been treated with DAAs were assigned to group II. A significant difference was observed between both groups regarding basic assessment scores (Child, MELD, and BCLC), which tended to have more advanced liver disease and HCC stage upon diagnosis in group I. However, serum albumin was
INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third most common cause of cancer-related mortality worldwide[1]. In Egypt, HCC is a significant public health problem responsible for 33.63% and 13.54% of all cancers in males and females, respectively[2]. On the other hand, hepatitis C virus infection (HCV) is considered a leading cause of chronic liver disease in Europe, the United States, and many other countries, including Egypt[3,4]. The risk of HCC development in HCV-related liver cirrhosis is 2% to 8% per year[5]. Multiple studies and meta-analyses demonstrated during the era of interferon (IFN)-based therapy that HCV eradication decreased the risk of hepatocarcinogenesis regardless of fibrosis stage[6,7]. Furthermore, these studies showed that the achievement of sustained virologic response (SVR) after IFN based treatment is directly related to reduced incidence of HCC and increased survival rates[8].

In 2014, the introduction of the more effective direct-acting antivirals (DAAs) for HCV treatment was generally expected to benefit all patients, including those who were not permitted to be treated with IFN-based therapy[7]. However, unexpectedly, the clinical use of DAAs has evoked a significant dilemma about the relationship between DAAs and the development of HCC. Some studies have suggested a direct relation between DAAs and the development of HCC, while others have insisted that DAAs are protective against HCC development[7].

In 2016, the first report in this context showed an unexpectedly high recurrence rate of previously treated HCC after DAAs exposure[6]. This initial report was followed by another retrospective study conducted in Italy which included 344 patients with HCV-related cirrhosis who received different DAA regimens; 91% achieved SVR. The patients were followed for 24 wk. The study revealed a 29% recurrence rate for those with a history of HCC and a 3.16% incidence rate (de novo HCCs) in those without a history of prior HCC irrespective of the used DAA regimen[9].
In addition to HCC recurrence, the different biological behavior of HCC in DAAs exposed patients, and the pattern of recurrence after DAA treatment has also been reported in studies coming from various countries. For example, in 2017, Reig and his colleagues reported more aggressive HCC recurrence after DAA treatment, as defined by an advanced Barcelona Clinic Liver Cancer (BCLC) stage[6]. Moreover, Renzulli et al[10] found a more aggressive HCC recurrence pattern with vascular invasion evidence after DAA therapy.

This study aimed to analyze differences in basic clinical, radiological, and laboratory characteristics and tumor behavior upon HCC diagnosis between patients with and without a previous history of DAAs exposure.

**MATERIALS AND METHODS**

**Study design**

The current study is a multicenter retrospective case-control study designed to compare the basic demographic, laboratory, and radiological criteria of HCC in patients with a history of DAAs treatment for their chronic HCV infection compared to HCC patients with no previous history of HCV antiviral treatment. Patients were recruited from December 2016 to April 2019 from Minia university hospital and Minia fever hospital, Minia, Egypt. Study patients were assigned to one of 2 groups according to previous DAAs exposure. The first group included 151 HCC patients who were previously treated with DAAs (Group I). According to a standardized treatment protocol, all patients were treated in one of the specialized viral hepatitis treatment centers affiliated to the Egyptian National Committee for Control of Viral Hepatitis. Group II included 346 HCC patients with the first presentation as HCC and no history of antiviral treatment for their HCV infection. Patients with combined HBV or HIV infections and patients with extrahepatic malignancies were excluded from the study.

**Methods**

All patients were recruited and diagnosed according to EASL guidelines and updated AASLD practice guidelines for managing HCC and BCLC guidelines[11-13]. In addition, baseline demographic, clinical, laboratory, and radiological criteria were studied. The Child-Turcotte Pugh score (CTP), Model for End-stage Liver Disease (MELD) score, BCLC score, and FIB 4 as a non-invasive marker for fibrosis were calculated and presented.

Lines of treatment for HCV have been verified as well as the viral response. In addition, all baseline characteristics, laboratory, radiological and medical scores were compared between the two groups.

The study was performed according to the ethical guidelines of the 1975 Declaration of Helsinki after approval from the Research Ethics Committee for human subject research at the Faculty of Medicine, Minia University (Serial: 165: 2/2019) on Feb 25, 2019. In addition, written informed consent was obtained from all participants before enrolment in the study.

**Statistical analysis**

Statistical analyses were performed using IBM SPSS advanced statistics, version 26 (SPSS Inc., Chicago, Illinois, USA. Numerical data were presented as mean ± SD and median (range), whereas categorical data were presented as number (percent). The Mann-Whitney U-test and the χ²-test are used when appropriate. Statistical significance is considered if P value is less than or equal to 0.05.

**RESULTS**

This study included 497 patients with chronic HCV-related HCC, allocated into one of two groups according to their history of antiviral treatment for their HCV. Group I included 151 patients with chronic HCV and HCC who were previously treated with DAAs. Group II included 346 patients representing all participants recruited in the same period with HCV-related HCC and age and sex-matched with group I. Most of the studied patients in both groups were males: (76.2%) and (72.0%) (P value 0.33), with a mean age of 60.2 years and 59.8 years in groups I and II, respectively (P value 0.70) (Table 1). Regarding the received DAAs regimen in group I patients, 44.4% of patients
Table 1 Basic demographic data and underlying liver status in both groups

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HCC with previous DAAs (n = 151)</td>
<td>HCC without previous DAAS (n = 346)</td>
<td></td>
</tr>
<tr>
<td>Age (mean ± SD)</td>
<td>60.17 ± 7.75</td>
<td>59.84 ± 9.12</td>
<td>0.70</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td>0.33</td>
</tr>
<tr>
<td>Female</td>
<td>36 (23.8)</td>
<td>97 (28.0)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>115 (76.2)</td>
<td>249 (72.0)</td>
<td></td>
</tr>
<tr>
<td>Residence</td>
<td></td>
<td></td>
<td>0.28</td>
</tr>
<tr>
<td>Rural</td>
<td>131 (86.8)</td>
<td>287 (82.9)</td>
<td></td>
</tr>
<tr>
<td>Urban</td>
<td>20 (13.2)</td>
<td>59 (17.1)</td>
<td></td>
</tr>
<tr>
<td>BCLC</td>
<td></td>
<td></td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>0</td>
<td>5 (3.3)</td>
<td>15 (4.3)</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>47 (31.1)</td>
<td>134 (38.7)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>17 (11.3)</td>
<td>68 (19.7)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>49 (32.5)</td>
<td>50 (14.5)</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>33 (21.9)</td>
<td>79 (22.8)</td>
<td></td>
</tr>
<tr>
<td>MELD (mean ± SD)</td>
<td>14.35 ± 5.041</td>
<td>36.10 ± 30.22</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>CTP score</td>
<td></td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td>A</td>
<td>50 (33.1)</td>
<td>88 (25.4)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>65(43.0)</td>
<td>138 (39.9)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>36 (23.8)</td>
<td>120 (34.7)</td>
<td></td>
</tr>
<tr>
<td>FIB4</td>
<td></td>
<td></td>
<td>&lt;0.001c</td>
</tr>
<tr>
<td>mean ± SD</td>
<td>3.25 ± 9.87</td>
<td>7.11 ± 7.68</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>0.023</td>
<td>4.49</td>
<td></td>
</tr>
<tr>
<td>IQR</td>
<td>4.51</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>HCC detection time</td>
<td>Range: 1-72 moMedian: 8 mo</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*aP < 0.05.
*bP < 0.001.

HCC: Hepatocellular carcinoma; MELD: Model for end stage liver disease; CTP: Child Turcotte-pough; BCLC: Barcelona cancer liver clinic.

received sofosbuvir/daclatasvir (SOF/DAC), 40.1% received SOF/DAC/RBV, 13.2% received SOF/RBV, and only 2% received SOF/RBV/PEG IFN. Figure 1 shows patients’ distribution among various treatment regimens and treatment duration, in addition to treatment viral response.

Notably, significant differences were observed between the two groups regarding the case assessment scores that reflect the severity of the underlying liver condition upon HCC discovery. A total of 34.7% of patients in group II were CTP class C, and only 23.8% of group I patients were class C. Mean MELD score in group I was 14, while the mean MELD in group II was 36 (P value < 0.001). Moreover, a significant difference was observed in the BCLC score (P value < 0.001). A significant difference was encountered in FIB4 as a method for non-invasive fibrosis assessment with a mean FIB4 of 3.25 in group I, compared to 7.11 in group II (P value < 0.001). Basic demographic data and underlying liver status in both groups are detailed in Table 1. The time between stopping DAAs and the development of HCC ranged from 1 to 72 mo with a median of 8 mo.

When comparing both groups’ clinical data, no significant differences were observed except in the current smoking status, which was significantly increased in group I compared to the other group (P value 0.005). On the other hand, a significant history of blood transfusion was observed in patients with no previous history of DAAs (P value 0.01); cellular decompensation in the form of hepatic encephalopathy is
significantly observed in patients with no previous history of DAAs (P value 0.01). Detailed clinical data of the two studied groups are well presented in Table 2.

Comparing laboratory data in both groups, hemoglobin level and total leukocytic count were significantly different (P values are 0.02 and 0.004, respectively). Median ALT in group I was 77 IU in comparison to 54 IU in group II (P value 0.001). Mean albumin in groups I and II was (3.3 and 2.9 respectively) (P value 0.004), and mean urea in groups I and II was (40 and 54 respectively) (P value 0.04). Median AFP in group I was 184 in comparison to 60 in group II (P value < 0.001). An illustrated comparison of all laboratory data is presented in Table 3.

Regarding the radiological characters of HCC in both groups, HCC in group I patients was more multifocal (53%) in comparison to (25%) in group II (P value < 0.001). Moreover, HCCs in group I patients tended to present with a bigger tumor size at the initial presentation than group II patients. More precisely, less than 1% of group I patients were presented with tumors less than 2 cm, while more than 15% of group II patients presented with tumors less than 2 cm (P value < 0.001), indicating more aggressive tumor behavior associated with the previous history of DAAs. The right lobe was the dominant victim in both groups. Early vascular invasion was significantly higher in group I compared to group II as evidenced radiologically by portal vein thrombosis (PVT), which present in 45% of group I patients and only 21% of group II patients (P value < 0.001), all radiological data for HCCs in the studied patients are detailed in Table 4.

DISCUSSION

Chronic HCV infection is a significant risk factor for developing liver cirrhosis in approximately 20%-30% of patients with subsequent increased risk for HCC development in those patients with an estimated annual incidence of 3.5%[14]. This risk is shown to be lower in patients with chronic HCV infection without cirrhosis and in patients who succeeded in achieving eradication, as proved by their SVR[15]. Despite the notable decrease in the overall incidence of HCV infection, its prevalence in HCC patients is still high[16]. Surprisingly, HCC development’s risk is continuous in HCV-induced liver cirrhosis even after viral eradication and SVR achievement[16]. During the interferon-based treatment era, successful viral eradication decreases the risk for HCC and improvement in the fibrosis stage[9].

The emergence of DAAs with their extended patient spectrum, improved efficacy, and safety profile increased our expectations regarding a decrease in HCC occurrence and recurrence. However, unpleasant data from new studies showed that DAAs might encourage tumor occurrence in patients with cirrhosis or recurrence in patients with previously treated HCC[9,17]. The same was reported in some studies regarding HCC
Table 2 Clinical presentation in both groups

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HCC with previous DAAs (n = 151)</td>
<td>HCC without previous DAAs (n = 346)</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>56 (37.1)</td>
<td>135 (39.0)</td>
<td>0.68</td>
</tr>
<tr>
<td>DM</td>
<td>56 (37.1)</td>
<td>113 (32.7)</td>
<td>0.33</td>
</tr>
<tr>
<td>Smoking</td>
<td>73 (48.3)</td>
<td>121 (35.0)</td>
<td>0.005*</td>
</tr>
<tr>
<td>Surgical operations</td>
<td>32 (21.2)</td>
<td>101 (29.2)</td>
<td>0.06</td>
</tr>
<tr>
<td>Blood transfusion</td>
<td>23 (15.2)</td>
<td>87 (25.1)</td>
<td>0.01*</td>
</tr>
<tr>
<td>Jaundice</td>
<td>60 (39.7)</td>
<td>154 (44.5)</td>
<td>0.32</td>
</tr>
<tr>
<td>Ascites</td>
<td>90 (59.6)</td>
<td>197 (56.9)</td>
<td>0.58</td>
</tr>
<tr>
<td>LL edema</td>
<td>48 (31.8)</td>
<td>143 (41.3)</td>
<td>0.07</td>
</tr>
<tr>
<td>Hepatic encephalopathy</td>
<td>14 (9.3)</td>
<td>61 (17.6)</td>
<td>0.01*</td>
</tr>
</tbody>
</table>

*p < 0.05.
HCC: Hepatocellular carcinoma; DM: Diabetes mellitus; LL: Lower limb.

Table 3 Comparison of laboratory data in both groups

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HCC with previous DAAs (n = 151)</td>
<td>HCC without previous DAAs (n = 346)</td>
<td></td>
</tr>
<tr>
<td>HB (mean ± SD)</td>
<td>10.41 ± 1.88</td>
<td>10.78 ± 1.99</td>
<td>0.02*</td>
</tr>
<tr>
<td>TLC (mean ± SD)</td>
<td>6.55 ± 6.20</td>
<td>7.74 ± 8.60</td>
<td>0.004*</td>
</tr>
<tr>
<td>PLATELETS (mean ± SD)</td>
<td>147.28 ± 79.76</td>
<td>135.95 ± 61.17</td>
<td>0.22</td>
</tr>
<tr>
<td>TBIL (median)</td>
<td>3.07</td>
<td>2.5</td>
<td>0.93</td>
</tr>
<tr>
<td>DBIL (median)</td>
<td>0.7</td>
<td>0.9</td>
<td>0.84</td>
</tr>
<tr>
<td>ALB (mean ± SD)</td>
<td>3.32 ± 1.47</td>
<td>2.98 ± 0.85</td>
<td>0.004*</td>
</tr>
<tr>
<td>INR</td>
<td>1.31 ± 0.35</td>
<td>1.44 ± 0.47</td>
<td>0.4</td>
</tr>
<tr>
<td>ALT (median)</td>
<td>77</td>
<td>54</td>
<td>0.001*</td>
</tr>
<tr>
<td>AST (median)</td>
<td>76</td>
<td>70</td>
<td>0.62</td>
</tr>
<tr>
<td>CREAT (mean ± SD)</td>
<td>1.21 ± 0.45</td>
<td>1.43 ± 3.67</td>
<td>0.15</td>
</tr>
<tr>
<td>UREA (mean ± SD)</td>
<td>40.81 ± 16.01</td>
<td>54.93 ± 46.17</td>
<td>0.04*</td>
</tr>
<tr>
<td>AFP (median)</td>
<td>184.0</td>
<td>60.0</td>
<td>&lt; 0.001*</td>
</tr>
</tbody>
</table>

*p < 0.05.
*P < 0.01.
**P < 0.001.
HCC: Hepatocellular carcinoma; CBC: Complete blood picture; TLC: Total leucocytic count; TBIL: Total bilirubin; DBIL: Direct bilirubin; ALB: Albumin, ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AFP: Alfa feto protein.

recurrence after initial management upon treatment with DAAs[18].

This study stands at the current dilemma between DAAs’ benefits and drawbacks; studying the basic characteristics of HCC patients previously treated with DAAs and comparing them with HCC patients never treated with DAAs provides the central part of this controversy.

In our study, significant differences were found in the CTP, MELD, and BCLC scores in HCC patients without DAAs and those who received DAAs; these findings are contrary to what proved by Abdelaziz et al.[19], who found matching between patients with HCC and previous DAAs and HCC without DAAs regarding CTP score. In accordance with our results, a large study from Pakistan reported a raised neutrophil to lymphocyte ratio and younger patient age with more aggressive tumor
Table 4 Radiological characters of hepatocellular carcinoma in both groups

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>P value&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HCC with previous DAAs (n = 151)</td>
<td>HCC without previous DAAS (n = 346)</td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td></td>
<td></td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Single</td>
<td>71 (47.0)</td>
<td>259 (74.9)</td>
<td></td>
</tr>
<tr>
<td>Multiple</td>
<td>80 (53.0)</td>
<td>87 (25.1)</td>
<td></td>
</tr>
<tr>
<td>Size</td>
<td></td>
<td></td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Less than 2 cm</td>
<td>1 (0.7)</td>
<td>54 (15.6)</td>
<td></td>
</tr>
<tr>
<td>2.5 cm</td>
<td>91 (60.3)</td>
<td>177 (51.2)</td>
<td></td>
</tr>
<tr>
<td>Greater than 5 cm</td>
<td>59 (39.1)</td>
<td>115 (33.2)</td>
<td></td>
</tr>
<tr>
<td>Site</td>
<td></td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>Bilobar</td>
<td>21 (13.9)</td>
<td>29 (8.4)</td>
<td></td>
</tr>
<tr>
<td>Lt lobe</td>
<td>24 (15.9)</td>
<td>23 (6.6)</td>
<td></td>
</tr>
<tr>
<td>RT lobe</td>
<td>106 (70.2)</td>
<td>294 (84.9)</td>
<td></td>
</tr>
<tr>
<td>PVT</td>
<td>68 (45.0)</td>
<td>75 (21.7)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td></td>
<td></td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Average</td>
<td>38 (25.2)</td>
<td>98 (28.3)</td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>113 (74.8)</td>
<td>214 (61.8)</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>0 (0.0)</td>
<td>34 (9.8)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>P < 0.001.

HCC: Hepatocellular carcinoma; DAAs: Directly acting antiviral agents; PVT: Portal vein thrombosis.

Behavior in HCV-treated HCC patients[20].

The pattern of HCC invasion either locally inside the liver manifested by multiplicity and larger size or vascularly manifested by PVT is significantly increased with the previous history of DAAs, suggesting a possible DAAs role in such aggressive behavior. In accordance with the current study, Reig et al[6] stated the increased aggressiveness of HCC, but in recurrent cases, he omitted de novo HCC in his study. Also, Renzulli et al[10] noticed a faster rate of development of HCC after DAA therapy with an aggressive course of microvascular invasion. Similarly, Faillaci et al[21] proved that DAAs are associated with increased aggressiveness and tumor recurrence growth. Another study done by Romano et al[21] demonstrated an aggressive behavior of tumors after DAA in the form of a higher number of nodules and extrahepatic metastases, suggesting that such patients’ tumor growth is faster than usual. Many theories have been proposed to explain this unexpected event; some researchers have related the development of HCC to baseline risk factors such as advanced fibrosis grade, HBV co-infection, or age[7]. Another theory proposes that DAAs cause immune surveillance mechanisms to become dysregulated due to the rapid viral clearance, and this behavior has been confirmed by several investigations [16,19]. With the downregulation of type II and III IFNs, their receptors, and IFN-stimulated genes, this dysregulation may result in the re-establishment of innate immunity. Due to the anti-angiogenic and anti-proliferative capabilities of IFN, which DAAs lack, a reduction in IFN activation may promote the proliferation of malignant cells. Furthermore, after HCV eradication, one of the immune system alterations observed is a decrease in the number of cytotoxic activity of natural killer cells in the liver, favoring a faster progression of HCC foci[7,22].

A significant difference was observed in AFP levels between the two groups, explained mainly by the invasive pattern and prominent vascular invasion in group I, and this is in agreement with Abdelaziz et al[20].

The strengths of our study include its design and the large number of included subjects. Limitations include the exclusive existence of genotype four patients because of its prevalence in Egypt and lack of tight evaluation of other risk factors for HCC, like non-alcoholic fatty liver disease and aflatoxin effect, and the lack of further longitudinal follow up of the studied cohort.
CONCLUSION
In conclusion, despite the introduction of newer DAAs, HCV-related HCC will continue to be a significant public health concern in the coming period. Post-treatment HCV-related HCC has been discovered to be an emerging issue due to unmet needs for early HCC identification and intervention. In this study, more aggressive tumor behavior was encountered in DAAs exposed patients. Such finding needs to be investigated further in prospective studies with larger cohorts and more longitudinal follow-up for comparing survival and necessitates proactive screening for HCC in HCV-treated patients via public or private pharmacovigilance programs. Furthermore, anti-HCV therapy in HCC patients should be postponed until a consistent risk-benefit ratio is established through further research.

ARTICLE HIGHLIGHTS
Research background
The evidence on the link between direct-acting antivirals (DAAs) and the development of hepatocellular carcinoma (HCC) in hepatitis C virus (HCV) patients is insufficient and conflicting.

Research motivation
Due to unmet needs for early HCC detection and care, post-treatment HCV-related HCC is an increasing concern.

Research objectives
To compare fundamental clinical, radiographic, and laboratory features and tumor behavior in individuals with and without a history of DAAs exposure after HCC diagnosis.

Research methods
A multicenter case-control study including 497 patients with chronic HCV-related HCC, allocated into one of two groups according to their history of antiviral treatment for their HCV.

Research results
Group I consisted of 151 HCC patients who had previously been treated with DAAs, while group II included 346 patients who had never been treated with DAAs. Regarding basic assessment scores (Child, MELD, and BCLC), there was a substantial difference between the two groups, with group I showing a tendency for more advanced liver disease and HCC stage at diagnosis. However, serum albumin levels were considerably lower in group II, and serum-fetoprotein levels were significantly greater ($P = 0.001$). In addition, HCC mult centricity was substantially higher in group I than in group II, and the rate of portal vein thrombosis was significantly higher in group I ($P = 0.001$).

Research conclusions
HCC patients who are naïve to DAAs have more advanced clinical scores and laboratory features than those who have never been treated with DAAs; yet, HCC behavior is more aggressive in DAA-treated patients.

Research perspectives
The findings of this study warrant additional investigation in prospective trials with larger cohorts and longer follow-up for comparing survival and proactive screening for HCC in HCV-treated patients through public or private pharmacovigilance programs.

ACKNOWLEDGEMENTS
The authors acknowledge the physicians of Minia University who helped in this research.
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Case Control Study

Circulating microRNA 9-3p and serum endocan as potential biomarkers for hepatitis C virus-related hepatocellular carcinoma

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ORCID number: Amany Mohamed Salah Eldin Wahb 0000-0001-7063-4285; Mohamed El Kassas 0000-0002-3396-6894; Ahmed Kamal Khamis 0000-0002-9326-662X; Mostafa Elhelbawy 0000-0002-6945-6205; Nesreen Elhelbawy 0000-0002-9231-0342; Mona Salah Eldin Habieb 0000-0002-4433-0584.

Author contributions: Wahb A designed the research; Wahb A and Habieb M performed the laboratory work, performed the RT-qPCR work and drafted the manuscript; El Kassas M, Khamis A and Elhelbawy M performed the case selection and the clinical and radiological evaluations; Habieb M supplied reagents; Elhelbawy N performed the data analysis, biological parameter assessment and performed the RT-qPCR work; all authors contributed equally to the manuscript drafting and revision; All authors approved the final version of the manuscript.

Institutional review board statement: The study was approved by the Menoufia University Faculty of Medicine Research Ethics Committee.

Informed consent statement: Written informed consent was obtained from the patient or his/her guardians prior to the

Abstract

BACKGROUND

The high mortality rate of hepatocellular carcinoma (HCC) in Egypt is due mainly to the increasing prevalence of hepatitis C virus infection (HCV) and late diagnosis of the carcinoma. MicroRNAs (miRNA), which regulate tumor proliferation and metastasis in HCC, may serve as a useful diagnostic approach for the early detection of HCC, thus decreasing its mortality. Meanwhile, endocan is a protein with angiogenic and inflammatory properties that are associated with tumor progression and poor outcomes.

AIM

To analyze the levels of miRNA 9-3p and endocan in HCV-infected HCC patients and correlate them with clinicopathological parameters.

METHODS

We compared levels of endocan and circulating miRNA 9-3p from 35 HCV-related HCC patients to 33 patients with HCV-induced chronic liver disease and 32 age and gender matched healthy controls recruited from inpatient and outpatient clinics of the National Liver Institute, Menoufia University, Egypt in the period from January to March 2021 in a case-control study. Serum samples from all groups were analyzed for HCV. Endocan was measured by enzyme-
study.

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INTRODUCTION

Worldwide, hepatocellular carcinoma (HCC) is the sixth most prevalent cancer and the third leading cause of cancer deaths[1]. In Egypt, HCC is a significant health problem, as it is the most prevalent and second most prevalent cancer in males and females, respectively. It is the most prevalent malignancy in general, accounting for 32.35% of the total cancer deaths[2,3]. One reason for these high prevalence rates is the high prevalence of hepatitis C virus in Egypt[4]. Moreover, HCC has been attributed to molecular aberrations, such as errors in gene expression, which may result in translational repression and/or degradation[5,6]. To improve the overall survival from HCC, extensive research is needed, focusing particularly on more accurate and monitored management of the disease[7].

MicroRNAs (miRNAs) are small (approximately 22 nucleotides long), endogenous, non-protein coding RNAs that are key post-transcriptional regulators of gene expression[8]. miRNAs regulate different cellular pathways, including the cell cycle, cell proliferation and apoptosis. Dysregulation of miRNAs can therefore impact cellular processes involved in tumorigenesis and cancer. Thus, serum miRNAs may serve as non-invasive biomarkers for the diagnosis of cancer[9].

Linked immunosorbent assays, and the expression levels of circulating miRNA 9-3p were measured by real-time quantitative reverse transcriptase PCR.

RESULTS

The levels of circulating miRNA 9-3p were significantly lower in the HCC group compared to the chronic liver disease (P < 0.001) and control (P < 0.001) groups, while levels in the chronic liver disease were significantly lower than those in the control group (P < 0.001). The levels of serum endocan were significantly higher in the HCC group compared to the chronic liver disease (P < 0.001) and control (P < 0.001) groups. Moreover miRNA 9-3p and endocan performed better than α-fetoprotein in discriminating HCC patients from cirrhosis and healthy patients. The levels of miRNA 9-3p were significantly inversely correlated to vascular invasion (P = 0.002), stage of advancement of Barcelona Clinical Liver Cancer (P < 0.001) and the metastatic site (P < 0.001) of the HCC group.

CONCLUSION

Circulating miRNA 9-3p and endocan can be used as novel biomarkers for the early diagnosis of HCV-related HCC.

Key Words: MicroRNA 9-3p; Hepatocellular carcinoma; Endocan; Diagnostic; Biomarker; Egypt

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Three genes encode miRNA 9-3p: MIR9-1, MIR9-2 and MIR9-3, located on chromosomes 1 (1q22), 5 (5q14.3) and 15 (15q26.1), respectively[10]. miRNA 9-3p is expressed abnormally in various types of human cancer, suggesting that it is functionally versatile[11]. miRNA 9-3p has been identified to have a tumor suppressive role by targeting an oncogenic tafazzin expression in HCC cells[12].

Endocan is a 50 kDa soluble proteoglycan that circulates freely in the bloodstream of healthy individuals and is expressed by the vascular endothelium. It has angiogenic and inflammatory properties that may affect vascular permeability, thus it plays crucial roles in regulating major physiological and pathophysiological processes, such as cell adhesion, inflammation and tumor progression[13]. Endocan expression is upregulated in cancer cells derived from the lung, kidney, brain, astrocytes and liver [14].

A single study has reported that miRNA 9-3p expression decreases in bladder cancer patients, resulting in reduced inhibition of endocan, thus increasing its expression, which promotes cell proliferation[15]. These results indicate that the gene encoding endocan is a target of miRNA 9-3p. To the best of our knowledge, simultaneous assessment of the roles of both miRNA 9-3p and inflammatory role of endocan that may induce tumorigenesis and tumor progression have not been evaluated in HCV-related HCC, which is triggered by the viral inflammation. Thus, the current work aimed to study the diagnostic value of circulating levels of miRNA 9-3p and endocan in HCV-related HCC patients and to correlate them with clinicopathological parameters.

MATERIALS AND METHODS

Study subjects
This case-control study included a total of 100 subjects recruited from inpatient and outpatient clinics of the National Liver Institute, Menoufia University, Egypt in the period from January to March 2021. Participants were categorized into three groups: Group I: 35 patients with HCV-related HCC; Group II: 33 patients with chronic liver disease due to chronic HCV; and Group III: 32 healthy and free of viral infection volunteers of matched age and gender. Patients were selected based on restrictive inclusion criteria including patients whose age was more than 18 years with confirmed HCV infection by both HCV antibody (anti-HCV) detection and positive HCV RNA. In Group I, HCC was diagnosed (trifasic spiral computed tomography or dynamic magnetic resonance imaging together with elevated α-fetoprotein and/or liver biopsy), and its stage was identified according to the Barcelona Clinical Liver Cancer (BCLC) system[16]. In Group II, chronic liver disease was diagnosed based on history, clinical examination, laboratory results and imaging that included abdominal ultrasonography and computed tomography. Liver disease severity was assessed by the Child-Pugh score. Patients with positive hepatitis B surface antigen and/or hepatitis B c antibody, secondary liver cancer, other malignancies, chronic hepatitis or cirrhosis due to any cause other than HCV infection, significant associated comorbidities (such as renal failure or heart failure) and those receiving chemotherapy, radiotherapy or an immunosuppression medication were excluded. Detailed histories of all participants were taken, and they all underwent physical examination, liver imaging (abdominal ultrasound) and routine laboratory tests that included complete blood counts, kidney and liver function tests [albumin, total bilirubin, direct bilirubin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST)], serum levels of α-fetoprotein, and serological tests for hepatitis B virus and HCV. Serum samples from Groups I and II were analyzed for HCV by reverse transcriptase PCR (RT-PCR).

Laboratory procedures
Seven milliliters of venous blood were withdrawn by venipuncture; of this, 5 mL were transferred into a plain tube, left to clot and centrifuged for 10 min at 4000 rpm. The serum obtained was stored at -80 °C until subsequent analyses for serum α-fetoprotein levels, liver function tests, hepatitis viral markers and endocan levels. The remaining 2 mL of blood were placed into an EDTA containing tube for HCV RT-PCR and miRNA 9-3p expression analysis. Anti-HCV levels were determined by electrochemiluminescence immunoassay using the Cobas immunoassay analyzer.

The hepatitis B surface antigen in serum was determined using (Sorin Biomedica Co. kits, Italy). Serum levels of ALT and AST were determined by the kinetic UV optimized method of the IFCC (ELTEC Kit, England). Serum levels of total bilirubin were measured using the DIAMOND diagnostics Kit, Germany. Serum albumin levels
were quantified using a colorimetric method of enhanced specificity of bromocresol green (DIAMOND diagnostics Kit, Germany). Prothrombin time was determined by the STA-Stago Compact computed tomography autoanalyzer. Serum α-fetoprotein levels were measured by enzyme-linked immunosorbent assays using the IMMULITE 1000 system (Siemens Medical Solutions Diagnostics, United States).

**Endocan detection**

Serum endocan levels were measured by enzyme-linked immunosorbent assay using the PicokineTM ELISA Kit for human ESMI/Endocan (Boster Biological Technology Co., Ltd., CA, United States, cat# EK0752).

**RT-PCR for HCV**

Nucleic acids were extracted using the Qiagen viral RNA Mini Extraction Kit.

**Expression assay for miRNA 9-3p**

miRNA was isolated from plasma using the Qiagen™ RNA extraction Kit MiRNeasy Kit (QIAGEN). miRNA was purified and then its concentration and purity were quantified using a NanoDrop® N50 nanophotometer (Implant GmbH and Implen, Inc. Schatzbogen 52 81829 München, Germany). Purified miRNA was stored at -80 °C until reverse transcription, which was accomplished using the Qiagen®miScript II RT Kit (QIAGEN) following the manufacturer’s instructions. Each 20-μl reaction tube contained 4 μL 5 × miScript HiSpec Buffer, 2 μL 10 × miScript Nuclease Mix, 2 μL RNase-free water, 2 μL miScript Reverse Transcriptase Mix, and 10 μL template RNA. Reverse transcription was carried out at 37 °C for 60 min and 95 °C for 5 min on an Applied Biosystems 2720 thermal cycler (Bioline, Singapore, United States). The cDNA product was diluted to 5 ng/ul before determining the transcript levels by real-time quantitative PCR. Real-time quantitative PCR was performed using the miScript SYBR Green PCR Kit (QIAGEN) according to the manufacturer’s instructions. The reaction mixture contained 12.5 μL 2x QuantiTect SYBR Green PCR Master Mix, 2.5 μL 10x miScript Universal Primer based on mRNA sequences obtained from the miRBase database for miRNA 9-3p, 2.5 μL template cDNA and 3.5 μL RNase-free water. The Applied Biosystems®7500 real-time thermal cycler (Applied Biosystems, Foster City, CA, United States) was programmed to run 40 cycles of the following steps: 95 °C for 15 min (initial denaturation step), denaturation at 94 °C for 15 s, annealing for 30 s at 55 °C and extension for 30 s at 70 °C. U6 snRNA was used as an endogenous control. Relative quantification expression levels were calculated using the comparative 2−ΔΔCt method with Applied Biosystems 7500 software version 2.0.1. Each run was completed using melting curve analysis to confirm the specificity of the amplification and absence of primer dimers.

All procedures involving human participants were performed according to the ethical standards of the institutional and/or national research committee and the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the Menoufia University Faculty of Medicine Research Ethics Committee. Every patient in this research provided their written consent to participate in the research, provided that they were not identified in the paper.

**Statistical analysis**

Data were analyzed using IBM SPSS statistics software version 20 (SPSS Inc. Released 2011. IBM SPSS statistics for windows, version 20.0, Armonk, NY, United States: IBM Corp). Quantitative data are presented as means, standard deviations, medians and interquartile ranges, while qualitative data are presented as frequencies and percentages. The relationship between qualitative variables was evaluated by the χ² test. Pairs of groups of non-normally distributed quantitative data were compared by the Mann–Whitney test, while three groups were compared by the Kruskal–Wallis test (non-parametric analysis of variance). Based on Kruskal–Wallis distribution, a post-hoc test was performed for pairwise comparisons. Correlations were assessed using the Spearman correlation test. The diagnostic values of serum miRNA 9-3p and endocan in HCC patients were evaluated by the receiver operating characteristic (ROC) curve analysis. The most independent factor associated with metastasis was identified by logistic regression analysis. P values of < 0.05 were considered statistically significant.
RESULTS

The patients in all three groups did not differ statistically in terms of age and gender. Biochemical analyses results (Table 1) showed that the HCC group and the chronic liver disease group differed in the following: ALT, direct bilirubin, international normalized ratio, endocan and miRNA 9-3p ($P = 0.005$, $P = 0.003$, $P = 0.002$, $P < 0.001$ and $P < 0.001$, respectively).

The HCC group differed significantly from the control group in terms of ALT, AST, platelet count, serum albumin, direct bilirubin, international normalized ratio, α-fetoprotein level, endocan and miRNA 9-3p ($P < 0.001$). The chronic liver disease group differed significantly from the control group in terms of ALT, AST, hemoglobin level, platelet count, international normalized ratio, α-fetoprotein level, endocan and miRNA 9-3p ($P = 0.003$, $P < 0.001$, $P = 0.019$, $P = 0.022$, $P = 0.001$, $P < 0.001$ and $P < 0.001$ respectively).

Serum endocan levels in the HCC group were significantly higher than those in the chronic liver disease and control groups ($P < 0.001$). Furthermore, serum miRNA 9-3p expression levels in the HCC group were significantly lower than those in the chronic liver disease and control groups ($P < 0.001$), while levels in the chronic liver disease group were significantly lower than those in the control group ($P < 0.001$).

In the HCC group, 62.9% of patients (22 patients) were classified as grade A and (37.1%) of patients (13 patients) were classified as grade B according to Child-Pugh classifications; 4 (11.4%) patients were classified as grade A in BCLC stage, 18 (51.4 %) patients in stage B and 13 (37.1%) patients were in stage C. Detailed tumor characteristics of the HCC group are shown in Table 2.

The correlations between serum miRNA 9-3p levels and clinical data in HCC patients are shown in Table 3. miRNA 9-3p expression levels were significantly inversely correlated to vascular invasion, BCLC classification and metastatic site. Moreover, miRNA 9-3p expression levels were also significantly inversely correlated to serum endocan levels (Figure 1).

Univariate and multivariate logistic regression analyses on the HCC group indicated that miRNA 9-3p is an independent predictor factor of metastasis ($P = 0.041$; 95% confidence interval: 0.089-0.951) (Table 4).

ROC analysis of miRNA 9-3p and endocan levels indicated that at a cutoff point of 0.26, miRNA 9-3p can discriminate between patients with HCC and those with chronic liver disease with a sensitivity of 91.43%, a specificity of 87.88%, a positive predictive value of 88.90% and a negative predictive value of 90.60%. Meanwhile, at a cutoff point of 2370 pg/mL, endocan can discriminate between HCC and chronic liver disease patients with a sensitivity of 82.86%, a specificity of 84.85%, a positive predictive value of 85.30% and a negative predictive value of 82.40%. In comparison, α-fetoprotein was less sensitive and specific (60.00% and 33.30%, respectively).

At a cutoff point of 1.01, miRNA 9-3p can discriminate between HCC and control group patients with a sensitivity of 91.43%, a specificity of 87.50%, a positive predictive value of 88.90% and a negative predictive value of 90.30%. Meanwhile, at a cutoff point of 1510 pg/mL, endocan can discriminate between HCC and chronic liver disease patients with a sensitivity of 85.71%, a specificity of 87.50%, a positive predictive value of 88.20% and a negative predictive value of 84.80%. In comparison, α-fetoprotein was less sensitive and specific (80.00% and 71.87%, respectively). Diagnostically, both miRNA 9-3p and endocan performed better than α-fetoprotein at discriminating HCC patients from both chronic liver disease and healthy patients (Figures 2 and 3).

ROC analysis of miRNA 9-3p levels in the HCC group indicated that at a cutoff point of 0.02, miRNA 9-3p can discriminate between metastatic and non-metastatic HCC patients with a sensitivity of 91.67%, a specificity of 82.61%, a positive predictive value of 73.30% and a negative predictive value of 95.00%.

DISCUSSION

The increasing prevalence of HCC worldwide and its associated poor prognosis make it a global health problem. Studies in Egypt shows the increasing role of HCV infection in liver cancer etiology, and among all cancer deaths in Egypt, HCC is the primary cause[17,18].

HCC is often detected late, when it is no longer operable, which limits curative surgical treatment to only a few cases involving small HCC malignancies. Moreover, as a diagnostic tool, α-fetoprotein is limited in its accuracy[19]. In contrast, circulating
miRNAs may serve as biomarkers and a useful diagnostic approach for the early detection of HCC[20].

In the present study, clinical and laboratory data from the three different groups of patients revealed that serum α-fetoprotein levels of HCC and chronic liver disease patients was significantly different from those of control patients. α-fetoprotein is known to be overexpressed in HCC[21-23], and the severity of cirrhosis is a significant predictor of elevated serum α-fetoprotein levels; higher serum α-fetoprotein levels are significantly correlated with advanced cirrhosis in patients with chronic HCV[24].

We found that serum α-fetoprotein levels in the HCC group did not differ significantly from those of the chronic liver disease group. This agrees with the results of Massironi et al[25], who reported similar findings in HCC and liver cirrhosis subjects. In our study, at a cutoff value of 23 ng/dL, α-fetoprotein discriminates between HCC and control patients at a sensitivity of 80.00% and a specificity of 71.87%. These results are similar to those of Massironi et al[25] and Metwaly et al[26], who reported a sensitivity of 75% and a specificity of 80% at a cutoff value of 16.9 ng/dL.

Our findings show higher serum endocan levels in HCC patients than in chronic liver disease patients, which agrees with previous studies by Nault et al[27] and Ozaki et al[28].

Recent studies on HCC show that elevated serum endocan levels and endocan expression by stromal endothelial cells in HCC tissues are correlated with poor survival[29]. Endocan expression in tumors undergoing angiogenesis reflects the
Table 2 Clinical characteristics of tumors in the hepatocellular carcinoma group, n = 35

<table>
<thead>
<tr>
<th>Number of the focal lesions</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single</td>
<td>16 (45.7)</td>
</tr>
<tr>
<td>Multiple</td>
<td>19 (54.3)</td>
</tr>
<tr>
<td>Tumor size in cm</td>
<td></td>
</tr>
<tr>
<td>Small &lt; 3</td>
<td>7 (20.0)</td>
</tr>
<tr>
<td>Medium 3-5</td>
<td>15 (42.9)</td>
</tr>
<tr>
<td>Large &gt; 5</td>
<td>13 (37.1)</td>
</tr>
<tr>
<td>Location of the focal lesions</td>
<td></td>
</tr>
<tr>
<td>Rt. Lobe</td>
<td>19 (54.3)</td>
</tr>
<tr>
<td>Lt. Lobe</td>
<td>8 (22.9)</td>
</tr>
<tr>
<td>Both</td>
<td>7 (20.0)</td>
</tr>
<tr>
<td>Caudate lobe</td>
<td>1 (2.9)</td>
</tr>
<tr>
<td>BCLC stage</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>4 (11.4)</td>
</tr>
<tr>
<td>B</td>
<td>18 (51.4)</td>
</tr>
<tr>
<td>C</td>
<td>13 (37.1)</td>
</tr>
<tr>
<td>Vascular invasion</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>25 (71.4)</td>
</tr>
<tr>
<td>Positive</td>
<td>10 (28.6)</td>
</tr>
<tr>
<td>LN metastasis</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>28 (80.0)</td>
</tr>
<tr>
<td>Positive</td>
<td>7 (20.0)</td>
</tr>
<tr>
<td>Ascites</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>25 (73.5)</td>
</tr>
<tr>
<td>Mild</td>
<td>8 (23.5)</td>
</tr>
<tr>
<td>Moderate</td>
<td>1 (2.9)</td>
</tr>
<tr>
<td>Child Pugh class A</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>22 (62.9)</td>
</tr>
<tr>
<td>B</td>
<td>13 (37.1)</td>
</tr>
<tr>
<td>C</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>23 (65.7)</td>
</tr>
<tr>
<td>Yes</td>
<td>12 (34.3)</td>
</tr>
</tbody>
</table>

Rt.: Right; Lt.: Left; BCLC: Barcelona Clinic Liver Cancer; LN: Lymph node.

processes of angiogenesis and tumor invasion. Structurally, the glycan form and phenylalanine-rich region of endocan are its key effective sections through the nuclear factor-κB/IκB pathway\[30\]. However, the involvement of endocan in HCC development remains unclear.

We found that plasma miRNA 9-3p levels are significantly lower in HCC patients compared to chronic liver disease and control patients. Overall, the order of miRNA 9-3p expression among the different groups is as follows: HCC < chronic liver disease < control.

This supports the concept of the antitumor function of miRNA 9-3p as reported by Higashi et al\[12\], Yang et al\[31\] and Tang et al\[32\]. In contrast, Sun et al\[33\] showed that miR-9 increases the levels of migration and invasion of HCC cell lines. It is possible that miR-9 (i.e. miR-9-5p) and miR-9* (miR-9-3p) are two different miRNAs that...
Table 3 Correlations between microRNA 9-3p levels and clinical data in hepatocellular carcinoma group

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>microRNA 9-3p Median (IQR)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vascular invasion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>25</td>
<td>0.04 (0.02-0.26)</td>
<td>0.002</td>
</tr>
<tr>
<td>Positive</td>
<td>10</td>
<td>0.02 (0.02-0.02)</td>
<td></td>
</tr>
<tr>
<td>LN metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>28</td>
<td>0.04 (0.02-0.17)</td>
<td>0.072</td>
</tr>
<tr>
<td>Positive</td>
<td>7</td>
<td>0.02 (0.02-0.03)</td>
<td></td>
</tr>
<tr>
<td>Distant metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>23</td>
<td>0.04 (0.03-0.26)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Yes</td>
<td>12</td>
<td>0.02 (0.02-0.02)</td>
<td></td>
</tr>
<tr>
<td>Child Pugh class</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>22</td>
<td>0.03 (0.02-0.04)</td>
<td>0.389</td>
</tr>
<tr>
<td>B</td>
<td>13</td>
<td>0.04 (0.02-0.26)</td>
<td></td>
</tr>
<tr>
<td>Tumor number</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>16</td>
<td>0.03 (0.02-0.17)</td>
<td>0.935</td>
</tr>
<tr>
<td>Multiple</td>
<td>19</td>
<td>0.03 (0.02-0.05)</td>
<td></td>
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<tr>
<td>Tumor size in cm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small &lt; 3</td>
<td>7</td>
<td>0.03 (0.02-0.15)</td>
<td>0.852</td>
</tr>
<tr>
<td>Medium 3-5</td>
<td>15</td>
<td>0.03 (0.02-0.06)</td>
<td></td>
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<tr>
<td>Large &gt; 5</td>
<td>13</td>
<td>0.04 (0.02-0.04)</td>
<td></td>
</tr>
<tr>
<td>Tumor site</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rt lobe</td>
<td>19</td>
<td>0.04 (0.02-0.06)</td>
<td>0.432</td>
</tr>
<tr>
<td>Lt lobe</td>
<td>8</td>
<td>0.04 (0.03-0.15)</td>
<td></td>
</tr>
<tr>
<td>Both</td>
<td>7</td>
<td>0.02 (0.02-0.04)</td>
<td></td>
</tr>
<tr>
<td>Caudate lobe</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCLC stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>4</td>
<td>0.26 (0.17-0.26)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>B</td>
<td>18</td>
<td>0.04 (0.03-0.05)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>13</td>
<td>0.02 (0.02-0.02)</td>
<td></td>
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</tbody>
</table>

Rt.: Right; Lt.: Left; BCLC: Barcelona Clinic Liver Cancer; IQR: Interquartile range; LN: Lymph node.

originates from the same precursor, and they can play either synergistic or opposite roles within one malignancy [34].

Interestingly, we observed significantly lower levels of miRNA 9-3p expression and vascular invasion at the advanced stage of BCLC and at the metastatic site of the HCC group.

In cervical adenocarcinoma, miRNA 9-3p is downregulated and acts as a tumor suppressor. Ectopic expression of miR-9-3p inhibits the JAK/STAT3 pathway by targeting interleukin 6, leading to the upregulation of vascular endothelial growth factor and increased angiogenesis. This results in decreased proliferation and migration and reduced tumor growth in vivo [35]. Moreover, Tang et al [32] reported that exosomal miRNA 9-3p suppresses the development and progression of HCC.

Cai et al [15] reported that increased exosomal miR-9-3p counteracts bladder cancer growth and metastasis and decreases endocan protein expression in nude mice. We similarly observed that miR-9-3p expression is inversely correlated to serum endocan levels in the HCC group.
Table 4 Univariate and multivariate regression analyses for the parameters affecting metastasis in hepatocellular carcinoma group

<table>
<thead>
<tr>
<th></th>
<th>Univariate</th>
<th></th>
<th>Multivariate</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P value</td>
<td>OR (95%CI)</td>
<td>P value</td>
<td>OR (95%CI)</td>
</tr>
<tr>
<td>microRNA 9-3p</td>
<td>0.008</td>
<td>0.193 (0.057-0.653)</td>
<td>0.041</td>
<td>0.291 (0.089-0.951)</td>
</tr>
<tr>
<td>Endocan</td>
<td>0.023</td>
<td>1.002 (1.000-1.003)</td>
<td>0.358</td>
<td>1.001 (0.999-1.002)</td>
</tr>
</tbody>
</table>

Statistically significant at $P \leq 0.05$. OR: Odds ratio; CI: Confidence interval.

Figure 1 Correlation between microRNA 9-3p and endocan levels in the hepatocellular carcinoma group.

We performed ROC analysis to compare the diagnostic accuracies of miRNA 9-3p, endocan and the traditional HCC tumor marker, α-fetoprotein. Diagnostically, both miRNA 9-3p and endocan perform better than α-fetoprotein in discriminating patients with HCC from those with or without (i.e. healthy) chronic liver disease. Furthermore, ROC analysis revealed that miRNA 9-3p performed well at discriminating between metastatic and non-metastatic patients in the HCC group. Statistically, miRNA 9-3p is an independent predictor factor of metastasis. This study could be the nucleus of a larger study working on a larger number of patients that may include those with other causes of chronic liver disease like alcoholism as our study was limited to HCV-induced chronic liver disease as it is highly prevalent in Egypt.

CONCLUSION

Endocan and miRNA 9-3p could be biomarkers with potential use for the early diagnosis of HCV-related HCC. In this regard, they are more valuable than α-fetoprotein. Moreover, miRNA 9-3p is an independent predictor of metastasis in HCC patients.
Figure 2 Receiver operating characteristic curve analysis of microRNA 9-3p, endocan and α-fetoprotein for discriminating between hepatocellular carcinoma and chronic liver disease.

Figure 3 Receiver operating characteristic curve analysis of microRNA 9-3p, endocan and α-fetoprotein for discriminating between hepatocellular carcinoma and control.

ARTICLE HIGHLIGHTS

Research background
The high mortality rate of hepatocellular carcinoma (HCC) in Egypt is due mainly to the increasing prevalence of hepatitis C virus infection (HCV) and late diagnosis of the carcinoma.

Research motivation
MicroRNAs (miRNA), which regulate tumor proliferation and metastasis in HCC, may serve as a useful diagnostic approach for the early detection of HCC, thus decreasing its mortality. Meanwhile, endocan is a protein with angiogenic and inflammatory properties that are associated with tumor progression and poor outcomes.
**Research objectives**
To analyze the levels of miRNA 9-3p and endocan in HCV-infected HCC patients and correlate them with clinicopathological parameters.

**Research methods**
We compared levels of endocan and circulating miRNA 9-3p from 35 HCV-related HCC patients to 33 patients with HCV-induced chronic liver disease and 32 age and gender matched healthy controls.

**Research results**
The levels of circulating miRNA 9-3p were significantly lower in the HCC group compared to the chronic liver disease \((P < 0.001)\) and control \((P < 0.001)\) groups, while levels in the chronic liver disease were significantly lower than those in the control group \((P < 0.001)\). While the levels of serum endocan were significantly higher in the HCC group compared to the chronic liver disease \((P < 0.001)\) and control \((P < 0.001)\) groups. Moreover, miRNA 9-3p and endocan performed better than α-fetoprotein in discriminating HCC patients from cirrhosis and healthy patients. The levels of miRNA 9-3p are significantly inversely correlated to vascular invasion \((P = 0.002)\), stage of advancement of Barcelona Clinical Liver Cancer \((P < 0.001)\) and the metastatic site \((P < 0.001)\) of the HCC group.

**Research conclusions**
Endocan and miRNA 9-3p could be biomarkers with potential use for the early diagnosis of HCV-related HCC. In this regard, they are more valuable than α-fetoprotein. Moreover, miRNA 9-3p is an independent predictor of metastasis in HCC patients.

**Research perspectives**
The findings of this study warrant additional investigation in prospective trials with larger cohorts and longer follow-up for confirming our results and validating the potential clinical use of these markers in early HCC detection.

**ACKNOWLEDGEMENTS**
We thank all medical staff and technicians of dialysis centers who agreed to participate in this study.

**REFERENCES**

Wahb A et al. microRNA 9-3p and endocan in HCC


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Retrospective Cohort Study

Do peripartum and postmenopausal women with primary liver cancer have a worse prognosis? A nationwide cohort in Taiwan


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Author contributions: Tseng GW did the study conception and design, and initial draft of the manuscript; Lin MC did the data analysis and interpretation, and initial draft of the manuscript; Lai SW, C Peng CY, Chuang PH, Su WP, Kao JT participated in the study conception; Lai HC did the data analysis and interpretation, manuscript drafting and revision. All authors have read and approved the final manuscript.

Institutional review board statement: The study was reviewed and approved for publication by our Institutional Reviewers.


Conflict-of-interest statement: All

Abstract

BACKGROUND
While primary liver cancer (PLC) is one of the most common cancers around the world, few large-scale population-based studies have been reported that evaluated the clinical survival outcomes among peripartum and postmenopausal women with PLC.

AIM
To investigate whether peripartum and postmenopausal women with PLC have lower overall survival rates compared with women who were not peripartum and postmenopausal.

METHODS
The Taiwan National Health Insurance claims data from 2000 to 2012 was used for this propensity-score-matched study. A cohort of 40 peripartum women with PLC and a reference cohort of 160 women without peripartum were enrolled. In the women with PLC with/without menopause study, a study cohort of 10752
INTRODUCTION

Primary liver cancer (PLC), the sixth most common cancer, and the fourth leading cause of cancer-related death around the world in 2018, put a heavy burden on global health [1]. Hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma, account for 70%-75% and 15% of cases, respectively, and comprise most primary liver malignancies [2]. The common risk factors of PLC are male gender, excess body fat, type II diabetes mellitus, chronic infection with hepatitis B virus (HBV) and/or hepatitis C virus (HCV), cigarette smoking, aflatoxin, and heavy alcohol consumption [3, 4]. Men appear to have a higher occurrence and worse outcomes, with two to three times higher incidence and mortality compared with women [5, 6]. Thus, most studies have included too few women to draw accurate conclusions.

Animal studies indicated that the primary etiology behind the protective effect of the female sex hormone might involve the anti-inflammatory modulation of estrogen, as chronic inflammation was a major contributor to carcinogenic processes [7-9]. Nevertheless, controversial results were obtained in research targeting women of reproductive age. Despite the rarity, PLC diagnosed during pregnancy generally caused a shorter survival compared with non-pregnant patients with inoperable PLC.
Several early reports suggested that the adverse influence of pregnancy for the development of PLC was probably due to an alteration of the hormonal milieu\cite{10-12}. In contrast, other recent papers attributed the consequence to delayed diagnosis\cite{11,15}. However, the latest cohort analysis needs further interpretation, as most of the published articles were case reports, with the largest including 48 cases published in 2011\cite{12,16}. In addition, evidence implied that the downturn in ovarian function in menopause is related to the spontaneous elevation in pro-inflammatory cytokines\cite{17-19}, which may have an undesirable effect on PLC development and progression. While there were limited epidemiologic statistics with the survival outcome among females, the research indicated that there was a reduced risk. It increased overall survival times of PLCs in postmenopausal patients receiving hormone replacement therapy (HRT)\cite{20}. It is estimated that 1.2 billion women worldwide will be menopausal or postmenopausal by the year 2030\cite{21}. Therefore, there is a growing necessity to make a thorough exploration of the morbidity and mortality of PLCs among this sector of the population.

To date, few large-scale population-based studies have been conducted to elucidate the relationship between pregnancy, menopause, and survival outcomes among women with PLCs. Our primary aim was to determine if pregnant and postmenopausal female patients with PLCs have a lower survival rate relative to population-based controls using a nationwide database in Taiwan.

MATERIALS AND METHODS

Data source
Taiwan government built a nationwide health record-related database named the National Health Insurance Research Database (NHIRD) in 1995. The database contains comprehensive health information, representative study subjects, and long-term follow-up periods. This study was conducted using the population-based hospitalization file, including all hospitalization records of Taiwan citizens. The identification was encrypted before the database released the records for medical research to protect the privacy of each patient.

All previous diagnoses in the database were coded according to the International Classification of Disease, Ninth Revision, Clinical Modification (ICD-9-CM). The Research Ethics Committee of China Medical University and Hospital in Taiwan approved the study (CMUH-104-REC2-115-R3).

Study population
According to the study objective, we would like to confirm the association between peripartum PLC and survival. We selected patients with peripartum PLC (ICD-9-CM: 155) who were diagnosed between 10 mo before and six months after delivery, during 2000-2012, as the exposed cohort. We defined the date of newly diagnosed PLC as the index date. The unexposed group was defined as patients with PLC who were diagnosed outside of the pregnancy period and selected by 4:1 propensity score matching with the exposed cohort. The matching variables included age, index year, and comorbidities, such as HBV, unspecified chronic hepatitis, alcoholic liver disease, cirrhosis, biliary stones, cholecystitis, and cholangitis. To further realize the correlation between menopause and PLC prognosis, we defined women aged 50 and beyond as the postmenopausal period. While natural menopause may occur from 45 to 55 of age\cite{22}, a recent cohort analysis including 36931 postmenopausal women indicated that the mean age at menopause is 50.2 years in Taiwan\cite{23}. Propensity score matching and matching variables mentioned above were applied. Patients with PLC before the index date were excluded from the study. The study population was followed up until death, withdrawn from NHIRD, or until December 31, 2013.

The comorbidities of concern in this study were HBV (ICD-9-CM: 070.2, 070.3, and V02.61), unspecified chronic hepatitis (ICD-9-CM: 070.9, 571.4, 571.8, 571.9), alcoholic liver disease (ICD-9-CM: 571.0, 571.1, 571.2, 571.3), cirrhosis (ICD-9-CM: 571.5, 571.6), biliary stones (ICD-9-CM: 574), cholecystitis (ICD-9-CM: 575), and cholangitis (ICD-9-CM: 576). The comorbidities above were defined as at least one hospitalization before the index date.

Statistical analysis
This study included demographic and comorbidities variables. The continuous and the categorical variables were shown by mean ± SD and number (%), and to compare the difference of each variable in two groups, a t-test and chi-square test were used,
respectively. To calculate the risk of death in the exposed and the unexposed cohorts, Cox proportional hazard models were used and presented using hazard ratios, adjusted hazard ratios (aHR) and 95% confidence intervals (CIs). The survival rate of death in the two cohorts was presented by the Kaplan-Meier method. The log-rank test was used to compare the difference between two survival curves. All statistical analyses were performed with SAS statistical software version 9.4 (SAS Institute Inc., Cary, NC). The Figure of the cumulative incidence curve was plotted by R software. The significance criteria were set up as a two-sided test with a P value of less than 0.05.

RESULTS

Of 200 eligible subjects in this study (Table 1), 40 were diagnosed with peripartum PLC, and the other 160 were selected as the unexposed cohort. Among patients with peripartum PLC, the dominant age group was younger than 30 years old (47.5%), 11 (27.5%) with HBV, one (2.5%) with unspecified chronic hepatitis, one with alcoholic liver disease, three (7.5%) with cirrhosis, two (5%) with biliary stone, four (10%) with cholecystitis, and three (7.5%) with cholangitis. The mean age of the exposed and unexposed cohort was 30.9 and 31.3 years, respectively. The characteristics and comorbidities showed a non-significant difference between the two cohorts after propensity matching (P > 0.05).

Table 2 presents the risk factors of death associated with and without peripartum PLC. Patients with peripartum PLC had a non-significant risk of death compared with the unexposed cohort (aHR = 1.40, 95% CI: 0.89-2.20, P = 0.149). Considering their older age and comorbidities, patients with HBV (aHR = 0.48, 95% CI: 0.30-0.77, P = 0.002) and cholecystitis (aHR = 0.30, 95% CI: 0.12-0.75) showed a decreased risk of death; patients with cholangitis showed a significantly higher risk of death (aHR = 3.34, 95% CI: 1.49-7.47, P = 0.003). Figure 1 illustrates the non-significant difference in the survival curves between the two cohorts (P = 0.1649).

The survival rate at different follow-up durations between patients with peripartum PLC and the unexposed cohort (Table 3) revealed a non-significant difference. When followed for less than 0.5 years, 1 year, 3 years, or 5 years, the survival rate in patients with peripartum PLC was lower than that in the unexposed cohort (71.79% vs 78.94%; 60.84 vs 63.61%; 30.42 vs 44.85%; 27.38 vs 39.59%), but without a significant difference between the two cohorts (P > 0.05).

We enrolled 13440 study subjects to learn more about the influence of age and menopause on survival outcomes. Of these women, 2688 were diagnosed with PLC, younger than 50 years, and without menopause (Table 4). The other group comprised 10752 women who were PLC patients, aged 50 years and older, and with menopause (postmenopausal). The mean ages were 39.7 and 69.1 years, respectively. The percentage of comorbidities had no significant difference between the two cohorts after propensity score matching by age and comorbidities (P > 0.05), except alcoholic liver disease (P = 0.041).

Table 5 shows the risk factors for developing death. Patients who were diagnosed with PLC at less than 50 years old had a substantially lower risk of death compared with patients diagnosed with PLC at 50 years or older (aHR = 0.64, 95% CI: 0.61-0.68, P < 0.001). Patients with HBV (aHR = 0.76, 95% CI: 0.72-0.80, P < 0.001), HCV (aHR = 0.72, 95% CI: 0.67-0.78, P < 0.001) and cholecystitis (aHR = 0.71, 95% CI: 0.64-0.78, P < 0.001) showed a significantly lower risk of developing death. Patients with comorbidities such as cirrhosis (aHR = 1.18, 95% CI: 1.13-1.24, P < 0.001), and cholangitis (aHR = 1.77, 95% CI: 1.63-1.92, P < 0.001) had a notably higher risk of death. Figure 2 shows that the survival rate was significantly higher in women younger than 50 years old with PLC than in the older cohort (P < 0.001).

Table 6 presents the survival rates at different follow-up durations. The survival rate in women < 50 years with PLC was significantly higher than in older women with PLC when followed for 0.5 year (72.44% vs 64.16%), 1 year (60.37% vs 51.66%), 3 years (42.92% vs 31.28%), and 5 years (37.02% vs 21.83%), respectively (P < 0.001).

DISCUSSION

To our knowledge, this large-scale, population-based, cohort study is one of the pioneering research investigations that focused on women under different conditions to determine the relationship between peripartum and postmenopause and the risk of death from liver cancer. Based on our results, despite no significant difference, overall
Table 1 Demographic characteristics and comorbidities of patients with newly diagnosed peripartum primary liver cancer in Taiwan during 1996-2012

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total, N</th>
<th>Peripartum primary liver cancer</th>
<th></th>
<th></th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No, n = 160</td>
<td>Yes, n = 40</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>n (%) / mean ± SD</td>
<td>n (%) / mean ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td>0.788</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 30</td>
<td>88</td>
<td>69 (43.1)</td>
<td>19 (47.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30-34</td>
<td>64</td>
<td>53 (33.1)</td>
<td>11 (27.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>35-49</td>
<td>48</td>
<td>38 (23.8)</td>
<td>10 (25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean ± SD¹</td>
<td></td>
<td>31.3 ± 5.1</td>
<td>30.9 ± 4.8</td>
<td>0.673</td>
<td></td>
</tr>
<tr>
<td>Baseline comorbidity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>HBV</td>
<td>58</td>
<td>47 (29.4)</td>
<td>11 (27.5)</td>
<td>0.815</td>
<td></td>
</tr>
<tr>
<td>Unspecified chronic hepatitis</td>
<td>2</td>
<td>1 (0.6)</td>
<td>1 (2.5)</td>
<td>0.286</td>
<td></td>
</tr>
<tr>
<td>Alcoholic liver disease</td>
<td>6</td>
<td>5 (3.1)</td>
<td>1 (2.5)</td>
<td>0.836</td>
<td></td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>9</td>
<td>6 (3.8)</td>
<td>3 (7.5)</td>
<td>0.306</td>
<td></td>
</tr>
<tr>
<td>Biliary stone</td>
<td>6</td>
<td>4 (2.5)</td>
<td>2 (5)</td>
<td>0.407</td>
<td></td>
</tr>
<tr>
<td>Cholecystitis</td>
<td>12</td>
<td>8 (5)</td>
<td>4 (10)</td>
<td>0.234</td>
<td></td>
</tr>
<tr>
<td>Cholangitis</td>
<td>10</td>
<td>7 (4.4)</td>
<td>3 (7.5)</td>
<td>0.417</td>
<td></td>
</tr>
</tbody>
</table>

¹ t-test, Chi-square test.  
HBV: Hepatitis B virus; SD: Standard deviation.

low survival was found in PLCs diagnosed either within or outside of the peripartum period among women of reproductive age (15-49 years old). Our data revealed that five-year survival rates in non-peripartum and peripartum PLCs were 39.59% and 27.38% (aHR = 1.40, 95%CI: 0.89-2.20, \( P = 0.149 \)), respectively. However, postmenopausal women (> 50 years old) with PLCs have a considerable decrease in survival rates (five-year survival rates in fertile and postmenopausal women were 37.02% and 21.83%, respectively), compared with a significantly higher risk of death in premenopausal female patients (aHR = 0.64; 95%CI: 0.61-0.68). Although the molecular mechanisms underlying this protective effect are complicated, previous research suggested that the inhibitory role of estrogen was responsible for the gender disparity of PLCs partly via micro RNA, DNA repair, and obesity-associated pathways[7]. Moreover, the number of estrogen receptors (ERs) correlated with the risk of tumor occurrence and invasion. Some research proposed that ERs suppressed the proliferation and progression of liver cancer by decreasing the peroxisome proliferator-activated receptor γ and transcription of metastatic tumor antigen 1[24,25]. In the time of limited estrogen supply (e.g., Postmenopause), sex hormone binding globulin (SHBG), a plasma protein that involved in the maintenance of a reservoir of sex steroid hormones, played a crucial role in potentiating estrogenic action[26].

We focused on women of childbearing age to gain a deeper understanding of the influence of reproductive hormones. Because of the elevation of estrogen and progesterone during pregnancy, the diagnosis of PLCs within this period is rare. Nevertheless, among the 62 cases reported to date worldwide, all ended with poor outcomes when compared with non-pregnant women with PLCs[10]. As early as in 1995, Lau and his colleague[27] concluded that pregnancy has an adverse effect on the prognosis of patients with HCC, and therefore measurement of AFP level is recommended for screening HCC in pregnant women at high risk. The largest retrospective review published by Choi et al[12] demonstrated poor yet improving survival rates over time (median survivals of the groups before and during/after 1995 were 18 and 25.5 mo, respectively) among all 48 HCC cases in pregnancy. Contrary to prior research, our analysis of the nationwide database revealed an overall unpleasant prognosis among women of childbearing age. There was no significant difference in survival rates between parous and non-parous women with PLCs. This could probably be explained by the limited number of cases and the nationwide coverage of health insurance. Since almost all women received check-ups during the prenatal and
Table 2 Cox model measured hazard ratios and 95% confidence intervals of death associated non-peripartum primary liver cancer and peripartum primary liver cancer patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Event, n = 124</th>
<th>Person, yr</th>
<th>IR</th>
<th>Crude HR (95%CI)</th>
<th>adjusted HR (95%CI)</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>Peripartum primary liver cancer</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>97</td>
<td>587</td>
<td>16.53</td>
<td>Ref.</td>
<td>Ref.</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>27</td>
<td>99</td>
<td>27.17</td>
<td>1.35 (0.88-2.08)</td>
<td>1.40 (0.89-2.20)</td>
<td>0.166</td>
</tr>
<tr>
<td>Age at baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 30</td>
<td>54</td>
<td>350</td>
<td>15.44</td>
<td>Ref.</td>
<td>Ref.</td>
<td></td>
</tr>
<tr>
<td>30-34</td>
<td>39</td>
<td>169</td>
<td>23.08</td>
<td>1.21 (0.80-1.84)</td>
<td>1.47 (0.95-2.28)</td>
<td>0.083</td>
</tr>
<tr>
<td>35-49</td>
<td>31</td>
<td>167</td>
<td>18.52</td>
<td>1.29 (0.83-2.00)</td>
<td>1.13 (0.69-1.85)</td>
<td>0.617</td>
</tr>
<tr>
<td>Baseline comorbidity</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>HBV</td>
<td>27</td>
<td>230</td>
<td>11.72</td>
<td>0.52 (0.34-0.80)</td>
<td>0.48 (0.30-0.77)</td>
<td>0.002</td>
</tr>
<tr>
<td>Unspecified chronic hepatitis</td>
<td>1</td>
<td>11</td>
<td>9.04</td>
<td>0.86 (0.12-6.17)</td>
<td>0.56 (0.08-4.10)</td>
<td>0.565</td>
</tr>
<tr>
<td>Alcoholic liver disease</td>
<td>6</td>
<td>8</td>
<td>73.37</td>
<td>2.85 (1.25-6.49)</td>
<td>2.15 (0.73-6.36)</td>
<td>0.165</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>7</td>
<td>40</td>
<td>17.55</td>
<td>1.12 (0.52-2.40)</td>
<td>1.49 (0.57-3.90)</td>
<td>0.411</td>
</tr>
<tr>
<td>Biliary stone</td>
<td>4</td>
<td>11</td>
<td>35.47</td>
<td>1.23 (0.45-3.33)</td>
<td>0.64 (0.17-2.35)</td>
<td>0.499</td>
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<tr>
<td>Cholecystitis</td>
<td>5</td>
<td>80</td>
<td>6.26</td>
<td>0.43 (0.18-1.06)</td>
<td>0.30 (0.12-0.75)</td>
<td>0.010</td>
</tr>
<tr>
<td>Cholangitis</td>
<td>7</td>
<td>4</td>
<td>179.30</td>
<td>3.76 (1.71-8.26)</td>
<td>&lt; 0.001</td>
<td>3.34 (1.49-7.47)</td>
</tr>
</tbody>
</table>

Adjusted HR: Adjusted for gender, age, and all comorbidities in Cox proportional hazards regression; CI: Confidence interval; HR: Hazard ratios; HBV: Hepatitis B virus; IR: Incidence rate.

Table 3 Survival rates of different follow-up durations between non-peripartum primary liver cancer and peripartum primary liver cancer patients

<table>
<thead>
<tr>
<th>Follow-up duration</th>
<th>Survival rate (%)</th>
<th>Non-peripartum primary liver cancer</th>
<th>Peripartum primary liver cancer</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 0.5</td>
<td>78.94</td>
<td>71.79</td>
<td>0.254</td>
<td></td>
</tr>
<tr>
<td>≤ 1</td>
<td>63.61</td>
<td>60.84</td>
<td>0.611</td>
<td></td>
</tr>
<tr>
<td>≤ 3</td>
<td>44.85</td>
<td>30.42</td>
<td>0.111</td>
<td></td>
</tr>
<tr>
<td>≤ 5</td>
<td>39.59</td>
<td>27.38</td>
<td>0.117</td>
<td></td>
</tr>
</tbody>
</table>

postnatal period under the national health insurance program, proper management could be provided in time to improve outcomes.

Because menopause represents a state of gradual estrogen deficiency in the setting of physiologic aging, we also divided the study population into two groups by age, either younger or older than 50 years. According to Yang’s research[28] investigating patients with HCC, women of 18 years old to 64 years old were noted as having longer survival than men of the same age, with the largest difference in survival among women aged 18 years to 44 years. Furthermore, Shimizu et al[29] reported that hepatic ER levels, which were inversely related to the progression of HCC, were significantly higher in premenopausal women compared with postmenopausal women. While El Mahdy Korah et al[30] stated that there was no clear relationship between sex hormone and HCC development or progression by analyzing total testosterone, estrogen, progesterone and prolactin levels among 40 selected HCC patients, Petrick’s cohort study in 2019[31] indicated that higher levels of SHBG and circulating estradiol were associated with an increased risk of HCC and ICC, respectively, among women after menopause. These data suggest that climacteric status may adversely mediate the outcomes of PLCs. Our results are consistent with those of previous studies, that
Table 4: Demographic characteristics and comorbidities of female patients newly diagnosed with and without menopause primary liver cancer patients in Taiwan during 1996-2012

| Characteristics                  | Liver cancer |          |          |          |
|----------------------------------|--------------|----------|----------|
|                                  |              | ≥ 50 yr, n = 10752 | < 50 yr, n = 2688 |          |
|                                  |              | n (%)/mean ± SD | n (%)/mean ± SD | P value |
| Age                              |              | 69.1 ± 9.6 | 39.7 ± 10.5 | < 0.001 |
| Baseline comorbidity             |              |          |          |          |
| HBV                              | 2971         | 2358 (21.9) | 613 (22.8) | 0.329   |
| HCV                              | 1168         | 931 (8.7)  | 237 (8.8)  | 0.795   |
| Unspecified chronic hepatitis    | 780          | 619 (5.8)  | 161 (6)    | 0.645   |
| Alcoholic liver disease          | 211          | 157 (1.5)  | 54 (2)     | 0.041   |
| Cirrhosis                        | 4142         | 3321 (30.9) | 821 (30.5) | 0.730   |
| Biliary stone                    | 1269         | 1012 (9.4) | 257 (9.6)  | 0.813   |
| Cholecystitis                    | 626          | 489 (4.5)  | 137 (5.1)  | 0.227   |
| Cholangitis                      | 818          | 649 (6)    | 169 (6.3)  | 0.626   |

Table 5: Cox model measured hazard ratios and 95% confidence intervals of death associated with and without menopause primary liver cancer patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Event, N = 9982 Person, yr IR</th>
<th>Crude</th>
<th></th>
<th>Adjusted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>HR (95%CI)</td>
<td>P value</td>
</tr>
<tr>
<td>Liver cancer</td>
<td></td>
<td></td>
<td>Ref.</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>≥ 50 yr</td>
<td>8279</td>
<td>23410</td>
<td>35.37</td>
<td></td>
</tr>
<tr>
<td>&lt; 50 yr</td>
<td>1703</td>
<td>9149</td>
<td>18.61</td>
<td></td>
</tr>
<tr>
<td>Baseline comorbidity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBV</td>
<td>2049</td>
<td>7552</td>
<td>27.13</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>HCV</td>
<td>831</td>
<td>3513</td>
<td>23.65</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Unspecified chronic hepatitis</td>
<td>584</td>
<td>2224</td>
<td>26.25</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Alcoholic liver disease</td>
<td>165</td>
<td>449</td>
<td>36.72</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>3186</td>
<td>9924</td>
<td>32.10</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Biliary stone</td>
<td>955</td>
<td>2730</td>
<td>34.98</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Cholecystitis</td>
<td>416</td>
<td>2162</td>
<td>19.25</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Cholangitis</td>
<td>687</td>
<td>989</td>
<td>69.45</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Adjusted HR: Adjusted for comorbidities in Cox proportional hazards regression; CI: Confidence interval; HR: Hazard ratios; HBV: Hepatitis B virus; HCV: Hepatitis C virus; IR: Incidence rate.

implied a negative interplay between age and hormonal factors in the disease course since women beyond reproductive age (> 50 years old) with PLCs were found to have lower half-year, one-year, three-year, and five-year survival rates. Although it is difficult to distinguish how the two factors account for the consequence individually, it is certain that they interact with each other. This interaction results in diminishing immunologic responses to injury, and the imbalance between antioxidant formation and oxidative stress.
Table 6 Survival rates of different follow-up durations between primary liver cancer patients with and without menopause

<table>
<thead>
<tr>
<th>Follow-up duration</th>
<th>Survival rate (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≥ 50 yr</td>
<td>&lt; 50 yr</td>
</tr>
<tr>
<td>≤ 0.5</td>
<td>64.16</td>
<td>72.44</td>
</tr>
<tr>
<td>≤ 1</td>
<td>51.66</td>
<td>60.57</td>
</tr>
<tr>
<td>≤ 3</td>
<td>31.28</td>
<td>42.92</td>
</tr>
<tr>
<td>≤ 5</td>
<td>21.83</td>
<td>37.02</td>
</tr>
</tbody>
</table>

Figure 1 The estimated survival rates between non-peripartum primary liver cancer and peripartum primary liver cancer patients by Kaplan-Meier analysis.

Figure 2 The estimated survival rates between patients younger than 50 years old with primary liver cancer (without menopause) and those older with primary liver cancer (with menopause) by Kaplan-Meier analysis.

The use of a broad, representative, nationwide, population-based sample to observe the survival outcome of PLC in reproductive and postmenopausal female patients increased the validity of the results. Nevertheless, these results should be interpreted with caution because of several limitations in this study. First, detailed information related to the risk of PLC is not available. This information includes data on body mass index, smoking and alcohol use, high-fat diet, lower physical activity lifestyle, history of receiving HRT, and family history of PLC. Second, tumor burden, staging, and management strategies of PLC are not accessible from the NHIRD and therefore cannot be analyzed. Third, defining menopause by age alone may not be comprehensive enough since it is hard to make an optimal covariate adjustment. Fourth, the
generalization of the findings to Western or non-Taiwanese populations is a concern. For instance, the high incidence of PLC warrants further follow-up in other populations. Fifth, the small number of cases during the peripartum period may lead to biased findings. Hence, future studies with an improved design, larger sample sizes, and better control of confounding factors are required to enable a more thorough understanding.

CONCLUSION
In summary, among female patients with PLC, we found a trend for older age to be associated with increased risk for both incidence and mortality of PLC. In contrast, no apparent relationship was noted between pregnancy and prognosis. Even though subsequent clinical studies are necessary for further validation, the present research demonstrates that age and hormonal factors have a protective influence on the occurrence and deterioration of PLCs. Moreover, patients with more risk factors are recommended to follow up regularly to achieve a better prognosis.

ARTICLE HIGHLIGHTS

Research background
Primary liver cancer (PLC), the sixth most common cancer, accounts for the fourth leading cause of cancer-related death worldwide. Given the continuous rise of the global burden, there are increasing concerns about PLC outcomes in different populations.

Research motivation
For a long time, most studies about PLC put their focus on men due to higher incidence and riskier morbidities compared to women. Even with growing evidence on the protective effects of female sex hormones in animal research, few clinical cohorts pay attention to women with PLCs. Therefore, we are interested in the issue of how female reproductive status is related to the prognosis of PLCs.

Research objectives
This study aimed to assess whether peripartum and postmenopausal women with PLC have lower overall survival rates in a large cohort of subjects in Taiwan.

Research methods
This is a retrospective cohort of the PLC prognosis among peripartum, non-peripartum, premenopausal, and postmenopausal women using the Taiwan National Health Insurance Research Database from 2000-2012. There were 200 eligible subjects enrolled in the study of peripartum PLC, whereas 13440 subjects enrolled in the research of menopausal PLC. 4:1 Propensity score matching was applied to adjust the covariates.

Research results
While the survival rate was overall lower in patients with peripartum PLC, there was no significant difference in the risk of death and the survival rate at different follow-up durations among patients with/without peripartum PLC. In the menopausal PLC cohort, significantly lower risk of death (aHR = 0.64, 95%CI: 0.61-0.68, \( P < 0.001 \)) and higher survival rate when followed for 0.5 year (72.44% vs 64.16%), 1 year (60.57% vs 51.66%), 3 years (42.92% vs 31.28%), and 5 years were seen in patients diagnosed with PLC younger than 50 years old (without menopause) compared with patients diagnosed with PLC at or older than 50 years (with menopause).

Research conclusions
According to our dataset, it is concluded that younger age and female hormonal factors may reduce the occurrence and deterioration of PLCs. Females with paripartum PLC have no difference in survival rates compared with those patients without peripartum. Menopausal females with PLC have worse survival rates compared with those patients without menopause.
To further clarify the association between sexual hormone and PLC outcome, future studies with more detailed information and better-controlled confounders are required.

REFERENCES


Tseng GW et al. Prognosis of women with PLC


Nonalcoholic fatty liver disease is associated with worse intestinal complications in patients hospitalized for *Clostridioides difficile* infection

Yi Jiang, Salil Chowdhury, Bing-Hong Xu, Mohamad Aghaie Meybodi, Konstantinos Damiris, Samanthika Devalaraju, Nikolaos Pyrsopoulos

**Abstract**

**BACKGROUND**
Nonalcoholic fatty liver disease (NAFLD) has become the leading cause of chronic liver disease with increasing prevalence worldwide. *Clostridioides difficile* infection (CDI) remains the most common cause of nosocomial diarrhea in developed countries.

**AIM**
To assess the impact of NAFLD on the outcomes of hospitalized patients with CDI.

**METHODS**
This study was a retrospective cohort study. The Nationwide Inpatient Sample database was used to identify a total of 7239 adults admitted as inpatients with a primary diagnosis of CDI and coexisting NAFLD diagnosis from 2010 to 2014 using ICD-9 codes. Patients with CDI and coexisting NAFLD were compared to those with CDI and coexisting alcoholic liver disease (ALD) and viral liver disease (VLD), individually. Primary outcomes included mortality, length of stay, and total hospitalization charges. Secondary outcomes were in-hospital complications. Multivariate regression was used for outcome analysis after adjusting for possible...
Nonalcoholic fatty liver disease (NAFLD) is a heterogeneous disease with a spectrum from simple steatosis to nonalcoholic steatohepatitis (NASH), cirrhosis, and hepato-cellular carcinoma[1,2]. With a prevalence of 10 to 46 percent in the United States and 6% to 35% worldwide[3,4], NAFLD has become the leading cause of chronic liver disease, and its prevalence continues to increase, paralleled by the increase of obesity and type 2 diabetes[5].

Clostridioides difficile (C. difficile) is a gram-positive, spore-forming bacterium, known as the most common pathogen causing nosocomial diarrhea in developed countries [6]. Symptoms of C. difficile infection (CDI) range from mild to severe diarrhea, which can progress to sepsis, fulminant colitis, and bowel perforation[7]. Severe colitis may also present as ileus and megacolon, which are characterized by symptoms of intestinal obstruction[8,9]. Gut microbiota dysbiosis due to the administration of antibiotics is the most prominent risk factor for the development of CDI. Advanced age, prolonged hospitalization and gastric acid suppression are some common additional risk factors for CDI[10,11].
Recently, a number of animal and human studies have revealed the role of the gut microbiota in the pathophysiology of NAFLD. It is proposed that dysbiosis-induced dysregulation of the gut barrier function and translocation of the bacteria link the gut microbiome to NAFLD\cite{12,13}. In addition, it has been well documented that patients with chronic liver disease are more susceptible to CDI due to frequent hospitalization and antibiotics use. Specifically, recent studies have observed that NAFLD is an independent risk factor for CDI by single-centered retrospective design\cite{14,15}.

Although a strong association between NAFLD and CDI has been observed, gut microbiota dysbiosis likely plays a vital role in the pathogenesis of both aforementioned diseases. However, the inpatient outcomes of CDI in the NAFLD population, have not been well studied in large populations. The aim of this nationwide study was to assess the impact of NAFLD on the outcomes of hospitalized patients with CDI.

**MATERIALS AND METHODS**

**Data source and study population**

The largest all-payer inpatient care database in the United States, the Nationwide Inpatient Sample (NIS) database was accessed. The NIS database represents approximately 20\% of all inpatient hospitalizations. Weighted, it estimates more than 35 million hospitalizations nationally\cite{16}. It includes demographic information (age, sex, race, income), hospital characteristics (e.g., bed size, type), insurance status, discharge status, diagnoses and procedures (identified by The International Classification of Diseases-Ninth Edition Revision Clinical Modification (ICD-9 CM) codes), total hospitalization charges, length of stay (LOS), severity and other comorbidity measures. Yearly sampling weights are applied to generate national estimates.

This retrospective cohort study examined all adult (18-90 years old) patients hospitalized with CDI as the primary diagnosis from 2010 to 2014. Within this CDI population, patients with NAFLD were selected to compare to those with viral liver disease (VLD) (including hepatitis B infection and hepatitis C infection) and those with alcoholic liver disease (ALD). Notably, CDI was identified by ICD-9 CM code 008.45. NAFLD was identified by ICD-9 CM code 571.80 with the exclusion of all diagnostic codes for previous organ recipients and donors as well as other causes of chronic liver disease including hepatitis B and hepatitis C infection, ALD, hemochromatosis, primary biliary cholangitis, autoimmune hepatitis, and other unspecified liver diseases. The diagnosis of VLD was identified by the ICD-9 CM codes for hepatitis B and C caused liver diseases with the exclusion of previous organ recipients and donors, as well as other causes of chronic liver disease including NAFLD, ALD, hemochromatosis, primary biliary cholangitis, autoimmune hepatitis, and other unspecified liver diseases. Similarly, ALD was identified by the ICD-9 CM codes for ALD with the exclusion of previous organ recipients and donors as well as other causes of chronic liver disease including NAFLD, VLD, hemochromatosis, primary biliary cholangitis, autoimmune hepatitis, and other unspecified liver diseases (see Supplementary Table 1, supplemental digital content 1, which demonstrates ICD-9 diagnostic and procedure codes). VLD and ALD were assessed as separate groups which excluded patients with concomitant diagnoses of VLD and ALD. Information such as patients’ demographics, comorbidities, disposition, selected outcomes and surgical interventions were extracted from the NIS database. Elixhauser Comorbidity Index (ECI)\cite{17}, which measures 29 general medical conditions, then assigns different weights to compile a longitudinal score, allowing for further description of comorbidity burden.

Primary outcomes included mortality, length of stay, and total hospitalization charges. Secondary outcomes were CDI related complications and interventions.

**Statistical analysis**

SAS Survey Procedures (SAS 9.4, SAS Institute Inc, Cary, NC, United States) was utilized for all statistical analyses. The national estimates were calculated after accounting for sample design elements (clusters, strata, and trend weights) provided by the NIS. Continuous variables were reported as weighted mean ± SE; categorical variables were reported as weighted numbers (n) and percentages (%). The SEs of weighted means were estimated using the Taylor linearization method that incorporated the sample design. Weighted Student’s t-tests were used to analyze the normally distributed continuous variables, while Rao-Scott modified chi-square tests were used to test the difference of distribution for categorical variables. Wilcoxon Rank-Sum Tests were used to test the variables that are not normally distributed.
Multivariate linear regression was used to estimate the average change in LOS and total hospitalization charges after adjusting for patient demographics, hospital characteristics, insurance type, median household income, ECI score, obesity, diabetes, tobacco use disorder, hypertension, dyslipidemia, cirrhosis and its complications, numbers of cirrhosis complications, and hepatocellular carcinoma. Multivariate logistic regression was used to estimate the odds ratio (OR) of mortality, CDI complications and interventions after adjusting for the same confounding variables as noted above.

The statistical methods of this study were reviewed by Dr. Chunyi Wu, PhD of Epidemiology from University of Michigan Medical School.

RESULTS

Patient demographics and baseline characteristics
From 2010 to 2014, the numbers of patients hospitalized for CDI with coexisting NAFLD, VLD and ALD were 7239, 11857 and 5938, respectively. The CDI with NAFLD cohort in this study was predominantly Caucasian with an average age 56.3 years old. In the aforementioned cohort, 69.4% of the patients were female, 41.6% were admitted to southern hospitals, and 58.6% were admitted to large hospitals (Table 1).

Compared to CDI with VLD or ALD individually, the CDI with NAFLD group had significantly more patients in the 18-39 and greater than 70-year-old age groups (P < 0.0001), were more likely to be female (P < 0.0001), from the southern hospital region (P < 0.0001), and less likely to be Medicaid insured (P < 0.0001). Additionally, the CDI with coexisting VLD group was associated with a higher percentage of African American patients and had less patients with a high household income (Q3 and Q4, median household income for ZIP code between 51th and 100th percentile) compared to the CDI with NAFLD group.

In regard to comorbidities (Table 2), when compared to the CDI with VLD or ALD groups individually, CDI patients with NAFLD had a greater prevalence of obesity (P < 0.0001, P < 0.0001), diabetes (P < 0.0001, P < 0.0001), hypertension (P = 0.0006, P < 0.0001) and dyslipidemia (P < 0.0001, P < 0.0001). CDI with NAFLD was also associated with a significantly lower rate of cirrhosis (P < 0.0001, P < 0.0001) when compared to the other two groups. None of the patients in the CDI with NAFLD group had cirrhosis-related ascites, esophageal varices bleeding, spontaneous bacterial peritonitis or hepatorenal syndrome. Moreover, a lower rate of hepatocellular carcinoma (P < 0.0001, P = 0.0217) was observed in the CDI with NAFLD group compared to the CDI with VLD or ALD groups individually.

Outcomes and regression analysis of CDI patients with NAFLD vs VLD
When compared to the CDI with NAFLD group, the CDI with VLD group was associated with higher rates of acute kidney injury (AKI) [adjusted OR (aOR) = 1.35, 95%CI: 1.10-1.67, P = 0.0041], respiratory failure (RF) (aOR = 1.83, 95%CI: 1.22-2.76, P = 0.0036), longer LOS (adjusted LOS ratio = 1.12, 95%CI: 1.06-1.18, P < 0.0001) and higher hospitalization charges (adjusted cost ratio = 1.13, 95%CI :1.06-1.2, P < 0.0001). However, a lower rate of intestinal perforation rate was observed in the CDI with VLD group (aOR = 0.12, 95%CI: 0.03-0.57, P = 0.0075). CDI with VLD was initially associated with higher rates of mortality, colectomy and ileostomy, however this difference no longer existed after adjusting for confounding factors (Table 3).

Outcomes and regression analysis of CDI patients with NAFLD vs ALD
When compared to CDI patients with NAFLD, CDI patients with ALD had higher rates of RF (aOR = 1.72, 95%CI: 1.09-2.72, P = 0.0201), mortality (aOR = 2.63, 95%CI: 1.25-5.51, P = 0.0107), longer LOS (adjusted LOS ratio = 1.18, 95%CI: 1.10-1.25, P < 0.0001) and higher hospitalization charges (adjusted cost ratio = 1.17, 95%CI: 1.09-1.26, P < 0.0001). However, a lower rate of intestinal obstruction (aOR = 0.45, 95%CI: 0.28-0.72, P = 0.0010) was found in the CDI with ALD group when compared to the CDI with NAFLD group. Higher rates of AKI and septic shock, and a lower rate of colectomy were initially observed in CDI with ALD group, but the difference no longer existed after adjusting for the aforementioned confounders (Table 4).
### Table 1 Comparison of demographic data for patients hospitalized with *Clostridioides difficile* infection with coexisting nonalcoholic fatty liver disease, viral liver disease and alcoholic liver disease

<table>
<thead>
<tr>
<th>Variables</th>
<th>CDI with NAFLD</th>
<th>CDI with VLD</th>
<th>CDI with ALD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n (weighted)</strong></td>
<td>7239</td>
<td>11857</td>
<td>5938</td>
<td>P value</td>
</tr>
<tr>
<td><strong>Age (yr)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18-39</td>
<td>56.32 ± 0.42</td>
<td>57 ± 0.26</td>
<td>56.13 ± 0.37</td>
<td>0.15</td>
</tr>
<tr>
<td>40-49</td>
<td>56.32 ± 0.42</td>
<td>57 ± 0.26</td>
<td>56.13 ± 0.37</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>50-59</td>
<td>56.32 ± 0.42</td>
<td>57 ± 0.26</td>
<td>56.13 ± 0.37</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>60-69</td>
<td>56.32 ± 0.42</td>
<td>57 ± 0.26</td>
<td>56.13 ± 0.37</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>≥ 70</td>
<td>56.32 ± 0.42</td>
<td>57 ± 0.26</td>
<td>56.13 ± 0.37</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>5023 (69.4%)</td>
<td>5795 (48.9%)</td>
<td>2300 (38.7%)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><strong>Race</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>5427 (75%)</td>
<td>6920 (58.4%)</td>
<td>4358 (73.4%)</td>
<td>0.17</td>
</tr>
<tr>
<td>African American</td>
<td>482 (6.5%)</td>
<td>2773 (23.4%)</td>
<td>525 (8.8%)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Hispanic</td>
<td>648 (9%)</td>
<td>1144 (9.6%)</td>
<td>515 (8.7%)</td>
<td>0.17</td>
</tr>
<tr>
<td><strong>Hospital bed size</strong></td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>Large</td>
<td>4241 (58.6%)</td>
<td>7414 (62.6%)</td>
<td>3461 (58.3%)</td>
<td>0.033</td>
</tr>
<tr>
<td><strong>Hospital region</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Northeast</td>
<td>1091 (15.1%)</td>
<td>2618 (22.1%)</td>
<td>1243 (20.9%)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Midwest</td>
<td>1618 (22.3%)</td>
<td>2514 (21.1%)</td>
<td>1584 (26.7%)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>South</td>
<td>3008 (41.6%)</td>
<td>4208 (35.5%)</td>
<td>1671 (28.1%)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>West</td>
<td>1522 (21%)</td>
<td>2517 (21.2%)</td>
<td>1440 (24.3%)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><strong>Hospital type</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urban teaching</td>
<td>3401 (47%)</td>
<td>7207 (60.8%)</td>
<td>3065 (51.6%)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><strong>Insurance</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medicare</td>
<td>3086 (42.6%)</td>
<td>5493 (46.3%)</td>
<td>2239 (37.7%)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Medicaid</td>
<td>914 (12.6%)</td>
<td>3329 (28.1%)</td>
<td>1261 (21.2%)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Private</td>
<td>2526 (34.9%)</td>
<td>1835 (15.5%)</td>
<td>1391 (23.4%)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><strong>Median household income for ZIP Code, %</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q1</td>
<td>1790 (24.7%)</td>
<td>4205 (35.5%)</td>
<td>1592 (26.8%)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Q2</td>
<td>1824 (25.2%)</td>
<td>3128 (26.4%)</td>
<td>1407 (23.7%)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Q3</td>
<td>1926 (26.6%)</td>
<td>2353 (19.8%)</td>
<td>1503 (25.3%)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Q4</td>
<td>1511 (20.9%)</td>
<td>1657 (14%)</td>
<td>1252 (21.1%)</td>
<td>0.61</td>
</tr>
</tbody>
</table>

Values reported as weighted mean ± SE and weighted number [n (%)]. CDI: *Clostridioides difficile* infection; NAFLD: Nonalcoholic fatty liver disease; VLD: Viral liver disease; ALD: Alcoholic liver disease; Q1: Quartile 1, 0-25th percentile; Q2: Quartile 2, 26th-50th percentile; Q3: Quartile 3, 51th-75th percentile; Q4: Quartile 4, 76th-100th percentile.

**DISCUSSION**

This nationwide retrospective cohort study investigated the inpatient clinical characteristics and outcomes of CDI in hospitalized patients with coexisting liver diseases, with comparisons between NAFLD, VLD and ALD. We demonstrated that patients hospitalized with CDI and coexisting NAFLD had overall more favorable outcomes including a lower rate of RF, lower hospitalization charges and a shorter LOS when
Table 2 Comparison of comorbid conditions and complications for patients hospitalized with \textit{Clostridioides difficile} infection with coexisting nonalcoholic fatty liver disease, viral liver disease and alcoholic liver disease

<table>
<thead>
<tr>
<th>Variables</th>
<th>CDI with NAFLD</th>
<th>CDI with VLD</th>
<th>CDI with ALD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( n ) (weighted)</td>
<td>7239</td>
<td>11857</td>
<td>5938</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Number of Elixhauser comorbidities</td>
<td></td>
<td></td>
<td></td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>0</td>
<td>0 (0%)</td>
<td>114 (1%)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>244 (3.4%)</td>
<td>574 (4.8%)</td>
<td>116 (2%)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>656 (9.1%)</td>
<td>1409 (11.9%)</td>
<td>354 (6%)</td>
<td></td>
</tr>
<tr>
<td>( \geq 3 )</td>
<td>6338 (87.6%)</td>
<td>9760 (82.3%)</td>
<td>5463 (92%)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Obesity</td>
<td>2012 (27.8%)</td>
<td>850 (7.2%)</td>
<td>372 (6.3%)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Diabetes</td>
<td>2750 (38%)</td>
<td>3451 (29.1%)</td>
<td>1170 (19.7%)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Hypertension</td>
<td>4300 (59.4%)</td>
<td>6347 (53.5%)</td>
<td>2980 (50.2%)</td>
<td>0.00058</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>2619 (36.2%)</td>
<td>1868 (15.8%)</td>
<td>905 (15.2%)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>-</td>
<td>253 (2.1%)</td>
<td>45 (0.8%)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Cirrhosis related comorbidities(^1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>401 (5.5%)</td>
<td>2508 (21.2%)</td>
<td>3407 (57.4%)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Number of cirrhosis complications</td>
<td>401 (5.5%)</td>
<td>2508 (21.2%)</td>
<td>3407 (57.4%)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>0</td>
<td>137 (34.2%)</td>
<td>1773 (70.7%)</td>
<td>2105 (61.8%)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>244 (60.8%)</td>
<td>688 (27.4%)</td>
<td>1104 (32.4%)</td>
<td></td>
</tr>
<tr>
<td>( \geq 2 )</td>
<td>20 (5.0%)</td>
<td>47 (1.9%)</td>
<td>198 (5.8%)</td>
<td></td>
</tr>
<tr>
<td>Ascites</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>NA</td>
</tr>
<tr>
<td>Esophageal varices bleeding</td>
<td>0 (0%)</td>
<td>-</td>
<td>20 (0.6%)</td>
<td>NA</td>
</tr>
<tr>
<td>Hepatic encephalopathy</td>
<td>110 (27.4%)</td>
<td>60 (2.4%)</td>
<td>569 (16.7%)</td>
<td>0.003338</td>
</tr>
<tr>
<td>Hepatorenal syndrome</td>
<td>0 (0%)</td>
<td>15 (0.6%)</td>
<td>33 (1.0%)</td>
<td>NA</td>
</tr>
<tr>
<td>Portal hypertension</td>
<td>175 (43.6%)</td>
<td>661 (26.4%)</td>
<td>843 (24.7%)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Spontaneous bacterial peritonitis</td>
<td>0 (0%)</td>
<td>38 (1.5%)</td>
<td>40 (1.2%)</td>
<td>NA</td>
</tr>
</tbody>
</table>

\(^1\)Value reported as percentage of all cirrhotic patients.

Values reported as weighted number \((n [\%])\). Numbers were not displayed due to extremely small numbers were associated with increased risk for identification of persons; CDI: \textit{Clostridioides difficile} infection; NAFLD: Nonalcoholic fatty liver disease; VLD: Viral liver disease; ALD: Alcoholic liver disease; NA: Not available.

compared to those with ALD and VLD individually. Interestingly, higher rates of intestinal complications were observed in the CDI with NAFLD group when compared to the CDI with ALD or VLD groups. Specifically, a significantly higher rate of intestinal obstruction was seen in the CDI with NAFLD group when compared to the CDI with ALD group, and a higher rate of intestinal perforation was seen when compared to CDI patients with concomitant VLD.

Our findings of worse intestinal complications in patients hospitalized with CDI and coexisting NAFLD compared to CDI patients with VLD and ALD, linked the gut pathology to the liver. The crosstalk between the gut and liver is increasingly recognized as the gut-liver axis\(^{18}\). Receiving more than 70% of the blood supply from the intestinal venous outflow, the liver represents the first line of defense against gut derived antigens with a broad array of immune cells\(^{19}\). The liver also releases many bioactive mediators into the systemic circulation, allowing for communication with the intestine. In the intestine, the endogenous and exogenous products from host and microbial metabolism translocate to the liver through the portal venous system, ultimately influencing liver function\(^{20}\).
Table 3 Multivariate regression analysis of outcomes for patients hospitalized for *Clostridioides difficile* infection with coexisting nonalcoholic fatty liver disease vs viral liver disease

<table>
<thead>
<tr>
<th>Outcomes</th>
<th>CDI with NAFLD</th>
<th>CDI with VLD</th>
<th>Unadjusted ratio (95%CI)</th>
<th>P value</th>
<th>Adjusted ratio (95%CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7239</td>
<td>11857</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hospital mortality</td>
<td>59 (0.8%)</td>
<td>186 (1.6%)</td>
<td>1.94 (1.44, 2.6)</td>
<td>&lt; 0.0001</td>
<td>1.87 (0.95, 3.7)</td>
<td>0.071</td>
</tr>
<tr>
<td>Acute kidney injury</td>
<td>933 (13%)</td>
<td>2035 (17.2%)</td>
<td>1.39 (1.28, 1.51)</td>
<td>&lt; 0.0001</td>
<td>1.35 (1.1, 1.67)</td>
<td>0.0041</td>
</tr>
<tr>
<td>Respiratory failure</td>
<td>192 (2.7%)</td>
<td>504 (4.2%)</td>
<td>1.63 (1.37, 1.92)</td>
<td>&lt; 0.0001</td>
<td>1.83 (1.22, 2.76)</td>
<td>0.0036</td>
</tr>
<tr>
<td>Septic shock</td>
<td>39 (0.5%)</td>
<td>115 (1%)</td>
<td>1.8 (1.25, 2.59)</td>
<td>0.0015</td>
<td>1.64 (0.67, 4.02)</td>
<td>0.27</td>
</tr>
<tr>
<td>Intestinal perforation</td>
<td>- (0.0%)</td>
<td>0.3 (0.1, 0.89)</td>
<td>0.03</td>
<td>0.12 (0.03, 0.57)</td>
<td>0.0075</td>
<td></td>
</tr>
<tr>
<td>Intestinal obstruction</td>
<td>331 (4.6%)</td>
<td>527 (4.4%)</td>
<td>0.97 (0.84, 1.12)</td>
<td>0.67</td>
<td>0.94 (0.66, 1.33)</td>
<td>0.725</td>
</tr>
<tr>
<td>Peritonitis</td>
<td>61 (0.8%)</td>
<td>106 (0.9%)</td>
<td>1.06 (0.77, 1.45)</td>
<td>0.71</td>
<td>0.72 (0.35, 1.52)</td>
<td>0.39</td>
</tr>
<tr>
<td>Colectomy</td>
<td>45 (0.6%)</td>
<td>105 (0.9%)</td>
<td>1.43 (1.01, 2.03)</td>
<td>0.044</td>
<td>1.38 (0.6, 3.15)</td>
<td>0.44</td>
</tr>
<tr>
<td>Ileostomy</td>
<td>- (0.0%)</td>
<td>41 (0.3%)</td>
<td>2.47 (1.24, 4.92)</td>
<td>0.01</td>
<td>2.62 (0.66, 10.41)</td>
<td>0.17</td>
</tr>
<tr>
<td>LOS (days)</td>
<td>5.75 ± 0.16</td>
<td>6.77 ± 0.16</td>
<td>1.11 (1.06, 1.16)</td>
<td>&lt; 0.0001</td>
<td>1.12 (1.06, 1.18)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Total hospitalization charges</td>
<td>38150.34 ± 1757.01</td>
<td>46326.72 ± 1809.82</td>
<td>1.14 (1.07, 1.2)</td>
<td>&lt; 0.0001</td>
<td>1.13 (1.06, 1.2)</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

1 Adjusted for age, sex, race, primary insurance payer, hospital type, hospital bed size, hospital region, income quartile, Elixhauser Comorbidity Index score, obesity, diabetes, tobacco use disorder, hypertension, dyslipidemia, cirrhosis and its complications, numbers of cirrhosis complications, and hepatocellular carcinoma.

< Numbers were not displayed due to extremely small numbers were associated with increased risk for identification of persons. Values reported as weighted mean ± SE and weighted numbers (%); CDI: *Clostridioides difficile* infection; NAFLD: Nonalcoholic fatty liver disease; VLD: Viral liver disease; CI: Confidence interval; LOS: Length of stay.

How does NAFLD influence the intestinal complications of CDI through the gut-liver axis? Convincing evidence has shown that NAFLD is associated with significantly increased gut permeability and inflammation in both animal[21] and human models. Miele et al.[22] found that NAFLD patients had significantly increased gut permeability measured by urine radiolabeled markers and immunohistochemical analysis of zona occludens-1 expression in intestinal biopsy specimens, compared with healthy volunteers. They also discovered that both gut permeability and the prevalence of small intestinal bacterial overgrowth are correlated with the severity of steatosis. Verdam et al.[23] found that plasma immunoglobulin G levels against endotoxin were increased in NASH patients, which positively correlated with the severity of inflammation. Furthermore, transmission electron microscopy observed irregular microvilli and widened tight junctions in the gut mucosa of the NAFLD patients[24]. In addition, decreased numbers of CD4+ and CD8+ T lymphocytes and increased levels of inflammation at both the tissue and cellular levels in NAFLD compared to healthy control. All of these results suggested impaired gut permeability and increased levels of inflammation at both the tissue and cellular levels in NAFLD disease models.

The gut microbiota-mediated inflammation, the related disturbance of the intestinal integrity and the impairment in mucosal immune function have been reported to play important roles, not only in the pathophysiology of CDI[25] but also in the pathogenesis of NAFLD[13,24,26]. The gut microbiota normally exerts significant influence on intestinal epithelial cell health, nutrient metabolism and mucosal defense[19,27]. Early evidence in animal studies demonstrated that altered gut microbiota composition[28] independently contributed to the development of NAFLD in mice. In addition, altered interaction between the gut and the host (produced by defective inflammasome sensing in inflammasome-deficient mouse models) may govern the rate of progression of multiple metabolic syndrome-associated abnormalities[29]. With the recent developments in genome sequencing technologies, bioinformatics, and culturomics; it has been recognized that NAFLD and NASH are associated with decreased richness of the gut flora and increased risk of pathogenic flora in pediatric and adult patients[30-34], which are both well known risk factors for CDI. Although it is still unclear which specific microorganisms are harmful given conflicting results in
Table 4 Multivariate regression analysis of outcomes for patients hospitalized for *Clostridioides difficile* infection with coexisting nonalcoholic fatty liver disease vs alcoholic liver disease

<table>
<thead>
<tr>
<th>Outcomes</th>
<th>CDI with NAFLD (n = 7239)</th>
<th>CDI with ALD (n = 5938)</th>
<th>Unadjusted ratio (95% CI)</th>
<th>P value</th>
<th>Adjusted ratio* (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hospital mortality</td>
<td></td>
<td>59 (0.8%)</td>
<td>159 (2.7%)</td>
<td>3.34 (2.48, 4.52)</td>
<td>&lt; 0.0001</td>
<td>2.63 (1.25, 5.51)</td>
</tr>
<tr>
<td>Acute kidney injury</td>
<td></td>
<td>938 (13%)</td>
<td>935 (15.8%)</td>
<td>1.26 (1.14, 1.39)</td>
<td>&lt; 0.0001</td>
<td>1.2 (0.93, 1.54)</td>
</tr>
<tr>
<td>Respiratory failure</td>
<td></td>
<td>192 (2.7%)</td>
<td>249 (4.2%)</td>
<td>1.61 (1.33, 1.94)</td>
<td>&lt; 0.0001</td>
<td>1.72 (1.09, 2.72)</td>
</tr>
<tr>
<td>Septic shock</td>
<td></td>
<td>39 (0.5%)</td>
<td>79 (1.3%)</td>
<td>2.48 (1.69, 3.64)</td>
<td>&lt; 0.0001</td>
<td>2.14 (0.84, 5.46)</td>
</tr>
<tr>
<td>Intestinal perforation</td>
<td>-</td>
<td>0 (0%)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Intestinal obstruction</td>
<td></td>
<td>331 (4.6%)</td>
<td>133 (2.2%)</td>
<td>0.48 (0.39, 0.59)</td>
<td>&lt; 0.0001</td>
<td>0.45 (0.28, 0.72)</td>
</tr>
<tr>
<td>Peritonitis</td>
<td></td>
<td>61 (0.8%)</td>
<td>69 (1.2%)</td>
<td>1.38 (0.97, 1.95)</td>
<td>0.071</td>
<td>0.54 (0.25, 1.18)</td>
</tr>
<tr>
<td>Colectomy</td>
<td></td>
<td>45 (0.6%)</td>
<td>15 (0.3%)</td>
<td>0.42 (0.23, 0.74)</td>
<td>0.003</td>
<td>0.44 (0.14, 1.39)</td>
</tr>
<tr>
<td>Ileostomy</td>
<td>-</td>
<td>-</td>
<td>0.65 (0.23, 1.85)</td>
<td>0.42</td>
<td>0.99 (0.15, 6.61)</td>
<td>0.98</td>
</tr>
<tr>
<td>LOS (days)</td>
<td></td>
<td>5.75 ± 0.16</td>
<td>6.84 ± 0.23</td>
<td>1.14 (1.08, 1.21)</td>
<td>&lt; 0.0001</td>
<td>1.18 (1.1, 1.25)</td>
</tr>
<tr>
<td>Total hospitalization charges</td>
<td></td>
<td>38150.34 ± 1757.01</td>
<td>44641.74 ± 1660.66</td>
<td>1.14 (1.07, 1.22)</td>
<td>&lt; 0.0001</td>
<td>1.17 (1.09, 1.26)</td>
</tr>
</tbody>
</table>

*Adjusted for age, sex, race, primary insurance payer, hospital type, hospital bed size, hospital region, income quartile, Elixhauser Comorbidity Index score, obesity, diabetes, tobacco use disorder, hypertension, dyslipidemia, cirrhosis and its complications, numbers of cirrhosis complications, and hepatocellular carcinoma.*

Values reported as weighted mean ± SE and weighted numbers [n (%)]. Numbers were not displayed due to extremely small numbers were associated with increased risk for identification of persons. CDI: *Clostridioides difficile* infection; NAFLD: Nonalcoholic fatty liver disease; ALD: Alcoholic liver disease; LOS: Length of stay.

Human and animal studies[35], it is believed that gut microbiota-derived signatures extracted by whole-genome shotgun sequencing of DNA can be used for diagnosis of advanced fibrosis in NAFLD[36], and modification of gut microbiota analyzed by 16S ribosomal RNA pyrosequencing can be used for therapeutic purposes in NASH patients[37]. Additionally, increased pathogenic flora in NAFLD and NASH further disturb the immune balance and cause worsened dysbiosis through various mechanisms involving short-chain fatty acids[38], lipopolysaccharide[21], choline metabolism[39], bile acid metabolism[40] and bacteria-derived ethanol[41]. Collectively, NAFLD and NASH related alterations of gut microbiota and its downstream dysbiosis pathways may contribute to CDI risk and worsen intestinal complications.

On the other end, we sought to identify the characteristics of gut microbiota changes in ALD and VLD. Compared to NAFLD, ALD is remarkably similar histologically[42] and initiated directly from the gut by alcohol intake or binges. It has been well documented that alcohol intake can lead to changes in gut microbiota composition[43] and gut permeability[44] early on, even before the development of liver disease. These alterations involve multiple physical and biochemical layers of defense in the intestinal barrier[19]. In VLD, the gut microbiome works as an effective tool early on for immunity against the hepatitis virus, and helps with viral clearance[45]. In chronic VLD, large translocations of intestinal microbiota were observed and thought to contribute to not only dysregulation of immune cells and dysfunction of the intestinal barrier, but also viral replication[27]. Comparison analysis revealed that, compared to other cirrhosis etiologies, alcoholic cirrhosis is associated with worse gut dysbiosis after adjusting for Model For End-Stage Liver Disease score and body mass index[46]. In two other studies[47,48], which primarily compared the gut microbiota composition in HBV/HCV related and alcoholic cirrhosis, no difference was observed at the phylum and class level.

Intriguingly, in our study, the majority (94.5%) of patients in CDI with NAFLD group were non-cirrhotic; the percentage of cirrhotic patients in CDI with NAFLD group was significantly less than those in CDI with ALD or VLD group. CDI with NAFLD group was associated with a higher rate of intestinal complications after adjusting for cirrhosis and its complications. These results suggested that NAFLD is...
associated with altered gut microbiota that is predisposed to CDI and its complications, likely independent from the liver disease severity. In fact, NAFLD has been reported as an independent risk factor for CDI[14]. Although ALD and VLD cirrhosis was previously found to be associated with worse gut dysbiosis than NAFLD cirrhosis, this finding should be treated cautiously for non-cirrhotic patients, because the alteration of the gut microbiome is associated with the severity of liver disease, as significant differences in gut microbiota have been found between non-cirrhotic, compensated and decompensated cirrhotic patients[49,50]. Importantly, the standard of care therapies in cirrhotic patients such as lactulose, rifaximin, antibiotics and acid-suppressants that can affect the gut microbiota, may be playing a critical role[51]. In summary, our study suggested that NAFLD may be associated with worse dysbiosis in early liver disease stages and therefore a higher risk for CDI and its complications compared to ALD and VLD.

Aside from aforementioned gut microbiota changes that directly link NAFLD to CDI and intestinal complications, NAFLD related metabolic syndrome and systemic inflammation also play crucial roles in intestinal pathology. Recently, metabolic dysfunction-associated fatty liver disease has been proposed as a more appropriate name to replace NAFLD by an international panel of experts, with emphasis on the underlying metabolic dysfunction[52,53]. Clinical evidence has demonstrated that NAFLD, along with other components of metabolic syndrome, such as diabetes and obesity, are associated with an increased prevalence of small intestinal bacterial overgrowth (SIBO)[54,55] by insulin resistance, oxidative stress and chronic low grade inflammation[56]. Subsequently, the dysmotility induced by SIBO can further promote SIBO in NAFLD patients, causing a vicious cycle[57]. In fact, dysmotility itself is associated with NAFLD and may be a potential therapeutic target for NAFLD from a Japanese study[58,59]. Moreover, diabetes, a component of metabolic syndrome which may cause vasculopathies and neuropathies in the intestines, also contributes to dysmotility[60]. Additionally, diverticular disease, irritable bowel disease[61] and inflammatory bowel disease[62], together with SIBO and dysmotility have all been shown to have increased prevalence in NAFLD patients. Not surprisingly, the structural and functional abnormalities in the gut associated with NAFLD and metabolic dysfunction further increase the risk of CDI and its complications.

The strengths of this study include the utilization of the NIS database to provide a unique opportunity to investigate a nationwide population hospitalized for CDI. To the best of our knowledge, this study is a leading clinical research analysis that provided a comprehensive nationwide comparison of outcomes between NAFLD and other common chronic liver diseases, ALD and VLD, in hospitalized CDI patients. There are also limitations in this study. Particularly, NIS data acquisition relies on the accuracy ICD-9-CM codes for medical diagnoses and no lab results, biopsy or image studies were available for NAFLD diagnosis and severity stratification. It is also difficult to determine which cases of CDI were hospital acquired or community acquired because ICD-9 codes are assigned at discharge. To strengthen the validity of ICD-9 codes for NAFLD, VLD and ALD, we used not only diagnostic codes but also excluded the codes for all other chronic liver diseases (Supplementary Table 1)[63].

CONCLUSION

In conclusion, this study found more favorable overall outcomes but higher rates of intestinal complications in patients hospitalized with CDI and coexisting NAFLD, compared to CDI with coexisting ALD and VLD, individually. These results suggested that NAFLD may be associated with a higher risk of CDI associated intestinal complications through alteration of gut microbiota. Our study also suggested that NAFLD associated metabolic syndrome may contribute significantly to the gut dysbiosis even in the early liver disease stages and cause increased risk for CDI and its complications. During the last few years, the novel and rapidly evolving research technologies for the gut microbiome have been opening up an exciting era in the microbiota therapeutics for different disease models[66]. Tremendous progress has been observed in the treatment of NAFLD and CDI through gut microbiome manipulation. Our study may help increase awareness and diagnose intestinal complications in patients with two common diseases: CDI and NAFLD. Unraveling the significance of interactions between gut microbiota, gut immunity and systemic metabolic impact of NAFLD with prospective studies will provide more insights into the future microbiota therapeutics.
Research background
The ongoing exploration of liver-gut axis has discovered strong association between gut dysbiosis and nonalcoholic fatty liver disease (NAFLD) in both basic science and clinical research. Small-scaled studies have observed that NAFLD is an independent risk factor for *Clostridioides difficile* infection (CDI).

Research motivation
CDI, as the most common cause of nosocomial diarrhea in developed countries, carries high hospitalization burden. NAFLD, as the leading cause of chronic liver disease, is commonly seen in hospitalized patients with CDI. So far the inpatient outcomes of CDI in the NAFLD population have not been well studied.

Research objectives
The authors aimed to examine the impact of NAFLD on the inpatient outcomes of hospitalized patients with CDI, by comparing the effect of NAFLD with alcoholic liver disease (ALD) and viral liver disease (VLD) individually.

Research methods
This nationwide retrospective cohort study was conducted according to STROBE statement using the National Inpatient Sample database. Inpatient CDI with coexisting NAFLD cases were selected using ICD-9 codes. Multivariate regression analysis was used with adjustment for a large group of possible confounders. Elixhauser Comorbidity Index (ECI) was used for a full description of comorbidity burden.

Research results
CDI with NAFLD was independently associated with lower rates of acute respiratory failure, shorter length of stay and lower hospitalization charges when compared to CDI with VLD and CDI with ALD. However, CDI with NAFLD was associated with a higher rate of intestinal perforation when compared to VLD, and a higher rate of intestinal obstruction when compared to ALD.

Research conclusions
CDI and coexisting NAFLD is associated with favorable overall outcomes, but higher rates of intestinal complications compared to CDI with coexisting ALD and VLD, individually.

Research perspectives
This finding suggests that alteration of gut microbiota may play an important role in the pathogenesis of both CDI and NAFLD. NAFLD associated metabolic syndrome may contribute significantly to the gut dysbiosis and cause increased risk for CDI and its complications. This study provides potential directions for future prospective clinical research to identify the clinical meaningfulness of interactions between gut microbiota, gut immunity and systemic inflammation. The study may open the door for potential microbiota therapeutic targets and manipulation as future treatment options for chronic liver diseases.

ACKNOWLEDGEMENTS
The authors thank Dr. Chunyi Wu, PhD of Epidemiology from University of Michigan Medical School for her assistance with the statistical analysis.

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associated with inflammation and impaired mucosal immune function in intestine of humans with non-alcoholic fatty liver disease. *Sci Rep* 2015; 5: 8096 [PMID: 25644696 DOI: 10.1038/srep08096]


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Six-minute walking test performance is associated with survival in cirrhotic patients

Carolina Frade M G Pimentel, Ana Cristina de Castro Amaral, Adriano Miziara Gonzalez, Michelle Lai, Daniel de Oliveira Mota, Maria Lucia Gomes Ferraz, Wilson Mathias Junior, Mario Kondo

Abstract

BACKGROUND
Patients with cirrhosis are at risk of cirrhotic cardiomyopathy, with resulting cardiac dysfunction and exercise limitations. Six min walk test (6MWT) assesses functional status and predicts morbidity and mortality in cardiopulmonary diseases.

AIM
To determine if it associates with mortality by analyzing 6MWT performance in patients with liver cirrhosis.

METHODS
A cohort of 106 cirrhotic patients was evaluated in the outpatient setting with echocardiogram and 6MWT and follow up for one year to document hepatic decompensation and mortality. The distance in meters was recorded at the end of six minutes.

Institutional review board statement: The study has been performed in accordance with the Declaration of Helsinki (2000) and approved by the Ethics Committee of our institution, Federal University of Sao Paulo, Brazil (CAAE: 30942714.8.0000.5505; May 28, 2014).
INTRODUCTION

Liver cirrhosis is related to functional impairment leading to reduction in physical fitness[1,2]. Some possible factors implicated in this process are profound muscle wasting (or cirrhotic myopathy)[3], cardiac dysfunction (cirrhotic cardiomyopathy)[4], autonomic dysfunction (chronotropic incompetence) and concurrent pulmonary disease (portopulmonary hypertension and hepatopulmonary syndrome). Recently studies reinforce the importance of frailty scores as a prediction of mortality in liver transplantation list[5,6], giving emphasis in sarcopenia and physical fitness as important factors associated with mortality[7].

The six-minute walk test (6MWT) is a practical simple inexpensive test that provides a global assessment of all systems involved during exercise[8]. Although it does not give information about specific organ impairment, it evaluates overall exercise capacity and has been shown, in patients with cardiac disease, to correlate with the maximal oxygen consumption (VO2) and survival[9].

Some studies demonstrated that short distance during 6MWT (6MWD) predicted poorer prognosis and disease outcome in patients with heart failure[10] and chronic obstructive pulmonary disease[11]. In addition, this test can be used to assess the overall functional status and quantify response to a certain intervention[8] in a variety of other chronic diseases and in the elderly population[9-12].

Previous studies highlight the importance of 6MWD in predicting survival in cirrhotic and non-cirrhotic patients[13-16]. There are also evidences suggesting an association between exercise performance and increase risk of death on the waiting liver transplantation list[15-18]. Despite its role in long term survival in different chronic diseases, the impact in mortality prediction in cirrhotic patients is underes-
The aim of this study was to analyze the association between 6MWT and long-term mortality in a cohort of cirrhotic patients.

**MATERIALS AND METHODS**

A total of 106 outpatients with liver cirrhosis (57 male, mean age 51.2 ± 12.9 years) was included in the present study. Cirrhosis was defined by clinical history, physical examination, laboratory analysis and at least one imaging data. Disease prognosis and severity were established based on Child and MELD scores, according to original scores definitions\[19,20]. Exclusion criteria were any previous or current cardiovascular or pulmonary disease, heart failure or diagnosis of hemochromatosis (when cardiac involvement was documented). Patients who had a history of alcohol abuse (more than 20 g and 60 g of ethanol per day for women and men, respectively) \[21\] were included if they had abstained from alcohol use for at least 6 mo prior to enrollment. Patients with non-sinus rhythm, decompensated arterial hypertension, low peripheral oxygen saturation (\(\text{SpO}_2 < 90\%\)), recent history (less than 3 mo) of new liver related decompensation or hospitalizations were also excluded (patients with previous ascites or encephalopathy were included, those characterized with chronic decompensated patients). Patients with neuromuscular diseases, myopathy, balance deficits or orthopedic disorders were also excluded. Patients who have previously received a liver transplant were not included. No paracentesis was performed within at least one week prior to exercise, avoiding volume depletion or electrolyte imbalances.

One hundred and sixty-four patients were consecutively screened from two liver transplantation centers between October 2014 and December 2014, 58 out of 164 were excluded according to previous criteria, most of the due to cardiovascular disorders (26%) or active alcohol consumption (19%). On the day of enrollment, patients provided written informed consent and had blood samples collected and 6MWT done. Electrocardiogram and transthoracic bubble echocardiogram were performed within 1 mo of enrollment.

Patients were followed-up by clinical visits, hospital records or telephone calls to patients to capture deaths and their causes. Patients were stratified according to their ability to complete 6MWT, whether they achieved or not predicted distance according to gender and age, and pattern of symptom secondary to physical effort due to the test. Patients included were follow-up to one year, main outcomes were defined as death or liver transplantation.

The study has been performed in accordance with the Declaration of Helsinki (2000) and approved by the Ethics Committee of our institution.

**6MWT**

The 6MWT was conducted according to American Thoracic Society guidelines[8] and supervised by a qualified physician. The test was performed indoors, along a 30 m flat, straight corridor with a hard surface and free of any type of obstacles. Before starting the test, all patients were provided instructions by the evaluator, encouraged to walk as far as possible within 6 min, and instructed to stop if pain, dyspnea, or other symptoms. The distance in meters was recorded at the end of the six minutes (6MWD). Predicted distances were computed according to specific equations for gender, weight, height and age[22]. Predicted distance achieved percentage (%6MWD) is then derived by dividing the actual 6MWD divided by the predicted distance.

**Statistical analysis**

Data were analyzed using a statistical software program (IBM® SPSS® Statistics, version 22.0). Logistic regressions were performed to evaluate the independent association between 6MWD and death. Receiver operating curves (ROC) and the area under ROC (AUROC) were computed to estimate sensitivity, specificity and cut-off points for 6MWD used in regression models, selected by Youden’s index. COX regression analysis and Kaplan-Meier curves were performed and significant differences between the later were assessed by means of the log-rank test. We performed subgroup analysis according achievement of liver transplantation in order to evaluated 6MWT distance as a predictor of death.
Patient characteristics

The main demographic, clinical, and laboratory characteristics of the patients are presented in Table 1. One hundred and six patients were selected from two liver transplantation centers in Sao Paulo, Brazil. The majority was male (56%), and non-alcoholic etiology of the liver disease was the most common (69.8%). The mean MELD was 11.1, Child B more common (66%), and 74% of patients presented a history of at least one liver related decompensation. Ascites was identified in 32.1% and hepatic encephalopathy in 10.4% of patients on the day of the test.

All patients were followed until death, time of transplantation or end of study follow-up (12 mo). During the study period, 11 patients died and 3 underwent liver transplantation. All deaths were related to hepatic decompensation.

The majority of this cohort (71.7%) did not achieve the predicted distance adjusted for age and gender according to standardized equations[22] (678 ± 131 m, 402-890 m) (see Figure 1). 6MWT performance is demonstrated in Table 2. The mean 6MWD of this cohort was 515 ± 138 m, 180-960 m. Not surprisingly, older patients with higher Child score, worse hepatic synthetic function (lower albumin) and anemia performed worse. It was found to be inversely correlated with age (r = -0.391, P < 0.001) and Child score (r = -0.228, P = 0.019), and positively correlated with albumin (r = 0.242, P = 0.012), creatinine (r = 0.242, P = 0.018) and hemoglobin (r = 0.192, P = 0.048). Patients with a history of at least one hepatic decompensation in the past (74.5%) presented with significant shorter 6MWD (496 ± 141 m vs 571 ± 115 m, P = 0.015).

The mean 6MWD was progressively shorter among Child classes (A = 570 ± 144 m, B = 504 ± 137 m and C = 471 ± 115 m) and statistical significance was demonstrated between Child A and C (P = 0.04) and when Child A was compared with more advanced stages (B and C), P = 0.02. 6MWD was different among compensated (Child Pugh A) and decompensated (Child Pugh B and C) patients (P = 0.031) (see Figure 2). Patients decompensated with ascites or hepatic encephalopathy on the day of the test achieved shorter distances than those who did not have ascites or hepatic encephalopathy (472 vs 534 m, P = 0.03; 440 vs 525 m, P = 0.04, respectively). All patients previously included were submitted to 6MWT, even those with hepatic decompensation at the moment of evaluation, ascites or encephalopathy. 6MWD did not differ according to the etiologies of cirrhosis (P = 0.08), past history of alcohol abuse (P = 0.58), use of beta-blocker (P = 0.19), tobacco (P = 0.97) and presence of anemia (P = 0.84).

None of the patient presented with liver related decompensation within 2 wk following the exercise, meaning no detectable clinically significant portal hypertension increase induced by exercise. All patients were able to perform exercise adequately, without help, interruptions, or any significantly adverse effect.

To emphasize the role of 6MWD and %6MWD in the prediction mortality, as an additional factor besides liver disease severity, logistic regression models were designed to evaluate if the inclusion of 6MWT parameters improves the model performance and increases the AUROC computed using regression models. MELD and Child score were used to quantify the severity of liver disease. When 6MWT parameters were added to the models designed to predicted mortality using MELD or Child score, we observed an improvement in model performance, defined as a significant difference according to Omnibus Chi-square test (P = 0.01) and higher AUROCs in combining models (see Figure 3).

Cutoff points associated with mortality was 387 m for 6MWD (sensibility 90.9 and specificity 88.4) and 0.82 for %6MWD (sensibility 100 and specificity 83.2). After exclusion of patients who were submitted to liver transplantation, patients who died (11, 10.4%) had a shorter mean 6MWD (423 m vs 526 m, P = 0.006) and lower %6MWD (0.72 vs 0.92, P = 0.004). Just one of them achieved the predicted distance during 6MWT, 6MWD and %6MWD were independent predictors of mortality, after adjusted for Child scores, according to multivariate regression model analysis (Table 3). Patients who achieved distances shorter than 387 m or %6MWD < 0.82 presented higher mortality, and statistical difference according to Kaplan-Meier and log-rank analysis (P = 0.004 and P = 0.006, respectively) (Figure 4).

DISCUSSION

6MWT is a safe, easy-to-administer, and inexpensive test to determine the functional capacity of cirrhotic patients and also has prognostic value. We found that a decreased
## Table 1 Patients’ characteristics (n = 106), n (%)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n (%) or means ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender M/F</td>
<td>59/47 (56/44)</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>51 ± 13</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.7 ± 4.7</td>
</tr>
<tr>
<td>PASP (mmHg)</td>
<td>25.4 ± 8.0</td>
</tr>
<tr>
<td>Cirrhosis etiology</td>
<td></td>
</tr>
<tr>
<td>Virus</td>
<td>36 (33.9)</td>
</tr>
<tr>
<td>Alcohol</td>
<td>32 (30.2)</td>
</tr>
<tr>
<td>NASH</td>
<td>8 (7.5)</td>
</tr>
<tr>
<td>Others</td>
<td>30 (28.4)</td>
</tr>
<tr>
<td>Child-Pugh class n</td>
<td>7.1 ± 1.8</td>
</tr>
<tr>
<td>A</td>
<td>23 (21.7)</td>
</tr>
<tr>
<td>B</td>
<td>70 (66)</td>
</tr>
<tr>
<td>C</td>
<td>13 (12.3)</td>
</tr>
<tr>
<td>MELD</td>
<td>11.1 ± 3.1</td>
</tr>
<tr>
<td>Previous history of liver related decompensation</td>
<td>76 (73.8)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>19 (17.9)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>26 (24.5)</td>
</tr>
<tr>
<td>Tobacco smoking</td>
<td>12 (11.4)</td>
</tr>
<tr>
<td>Beta-blocker use</td>
<td>32 (30.2)</td>
</tr>
<tr>
<td>Hepatic decompensation on the day of the test</td>
<td></td>
</tr>
<tr>
<td>Ascites</td>
<td>34 (32.1)</td>
</tr>
<tr>
<td>(Grade 1, 2, and 3)</td>
<td>(11.3, 17, 5)</td>
</tr>
<tr>
<td>Peripheral edema</td>
<td>13 (12.3)</td>
</tr>
<tr>
<td>Hepatic encephalopathy</td>
<td>13 (12.3)</td>
</tr>
<tr>
<td>(Grade 1, 2, 3, and 4)</td>
<td>(10.4, 1.9, 0, 0)</td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>5 (4.7)</td>
</tr>
<tr>
<td>Patient on the liver transplantation waiting list</td>
<td>35 (33)</td>
</tr>
<tr>
<td>Baseline laboratory</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin (mg/dL)</td>
<td>13.1 ± 1.9</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>39.3 ± 5.4</td>
</tr>
<tr>
<td>Albumin (g/dL)Bilirubin (mg/dL)INR</td>
<td>3.5 ± 0.62.0 ± 1.51.2 ± 0.2</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>Na (mmol/L)</td>
<td>137.8 ± 2.1</td>
</tr>
<tr>
<td>K (mmol/L)</td>
<td>4.1 ± 0.5</td>
</tr>
<tr>
<td>Mg (mg/dL)</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>Ca (mmol/L)</td>
<td>1.2 ± 0.1</td>
</tr>
</tbody>
</table>

1 Continuous variables are shown as means ± SD.

Reference range values: Na (136-145); K (3.5-5.0); Mg (1.6-2.6) and Ca (1.15-1.29).

M: Male; F: Female; PASP: Pulmonary arterial systolic pressure.
Pimentel CFMG et al. Six-minute walking test in cirrhotic patients

Table 2 Six minute walking test performance in 106 patients with liver cirrhosis

<table>
<thead>
<tr>
<th>Variable</th>
<th>6MWD (m)</th>
<th>P (t-test when applicable)</th>
<th>6MWD (%)</th>
<th>P (t-test when applicable)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean 6MWD (m)</td>
<td>515 ± 138</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean 6MWD (%)</td>
<td></td>
<td></td>
<td>0.91 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>6MWD according to Child classes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>570 ± 144</td>
<td></td>
<td>0.97 ± 0.22</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>504 ± 137</td>
<td></td>
<td>0.88 ± 0.21</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>471 ± 115</td>
<td></td>
<td>0.82 ± 0.25</td>
<td></td>
</tr>
<tr>
<td>6MWD according to Liver decompensation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascites (w vs wo)</td>
<td>473 ± 20 vs 535 ± 17</td>
<td>0.03</td>
<td>0.86 ± 0.22 vs 0.95 ± 0.21</td>
<td>0.028</td>
</tr>
<tr>
<td>Hepatic encephalopathy (w vs wo)</td>
<td>435 ± 34 vs 525 ± 14</td>
<td>0.04</td>
<td>0.87 ± 0.25 vs 0.91 ± 0.21</td>
<td>0.87</td>
</tr>
<tr>
<td>History of previous hepatic decompensation (w vs wo)</td>
<td>496 ± 141 vs 571 ± 115</td>
<td>0.02</td>
<td>0.86 ± 0.22 vs 1.02 ± 0.17</td>
<td>0.004</td>
</tr>
<tr>
<td>Hospital admission during follow-up (w vs wo)</td>
<td>444 ± 172 vs 531 ± 125</td>
<td>0.01</td>
<td>0.77 ± 0.25 vs 0.92 ± 0.20</td>
<td>0.004</td>
</tr>
<tr>
<td>Survival (died vs survived)</td>
<td>423 ± 122 vs 526 ± 137</td>
<td>0.02</td>
<td>0.72 ± 0.21 vs 0.93 ± 0.21</td>
<td>0.21</td>
</tr>
</tbody>
</table>

6MWT: Six-minute walking test; 6MWD: Six-minute walking distance; 6MW (%): Predicted distance achieved percentage; w: With; wo: Without.

Figure 1 Relationship between predicted (line) and performed (bars) walking distance during six minute walking test. 6MWD: Six minute walking distance.

6MWD, as a marker of impaired exercise capacity, is associated with hepatic dysfunction. In addition, 6MWD and %6MWD performed as independent predictors of mortality, becoming an important tool during risk evaluation of severe complications and death in liver cirrhosis. Also, this study reinforces the key importance of physical evaluation during cirrhotic patients, especially those referred to liver transplantation team.

Basal exercise capacity was significantly impaired in our patients, as only 28.3% achieved the pre-test predicted distance. The 6MWD results in our cohort of patients was similar to previous studies in patients with cirrhosis which found a significantly lower 6MWD values than expected for healthy population[22]. Our cohort had a mean 89.7% (34.8%-149%) of predicted 6MWD (vs 63% found by Román et al[18]), and a mean
WJH

November 27, 2021 | Volume 13 | Issue 11

Table 3 Association between six-minute walking test parameters and unfavorable clinical outcomes (hospital admissions and mortality) using logistic regression models

<table>
<thead>
<tr>
<th>Predictors</th>
<th>Hospital Admission</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Univariate</td>
<td>Multivariate</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>p</td>
</tr>
<tr>
<td>Child score</td>
<td>0.74</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>6MWD</td>
<td>-0.005</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>%6MWD</td>
<td>-0.04</td>
<td>0.01</td>
</tr>
<tr>
<td>6MWD ≤ 444 m</td>
<td>-1.395</td>
<td>0.007</td>
</tr>
<tr>
<td>6MWD ≤ 387 m</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1Confidential intervals for odds ratio are not represented but consider adequate for all analysis except for odds ratio.

OR: Odds ratio.

6MWD of 515 m (180-960 m), compared to 306 m in Alameri et al[14]'s cohort of 98 patients with cirrhosis. The poor performance during 6MWT meets with the current knowledge about the abnormal exercise capacity in cirrhotic patients. Future studies should verify those findings and evaluate if 6MWD can be used as a more general tool able to evaluate outcomes and quality of life in this group[15].

We reported a weak inverse correlation between 6MWD and Child scores ($r = -0.228, P = 0.019$), although it was clear the tendency in walk distance reduction along Child classes. Carey et al[15], studying 121 cirrhotic patients, showed a strong correlation with MELD. In this particular study, all patients were listed for liver transplant, denoting a population with more advanced disease, making us understand that this stronger correlation reflects a major prevalence of their patient's overall disability when comparing to our study group. In the same way, by comparing subjects with advanced disease (Child B and C) and those without it (Child A), we detected a significant difference between these groups ($P = 0.02$), supporting the previous interpretation. Furthermore, patients with a history of at least one hepatic decompensation in the past, presented shorter 6MWD ($P = 0.015$) and subjects presenting with ascites or encephalopathy at the moment of evaluation performed worse, these facts highlight the relationship between shorter distances and severity of liver disease in our study. Similarly, Wong et al[23] reported that patients with decompensated cirrhosis with ascites performed worse during cycle ergometer evaluation when compared to well compensated patients, however, no specific data is available regarding 6MWT.

Although the gold standard measurement of exercise capacity is maximal VO$_2$[24] measurement during treadmill or cycle ergometer tests, 6MWT is a cheap and simple test found to correlate with oxygen consumption that can be administered without
Figure 2 Distance in meters was recorded at the end of the six minutes among compensated (Child Pugh A) and decompensated (Child Pugh B and C) patients.

Figure 3 Progressive improvement in prediction of mortality using models combing six minute walking test parameters and Child scores. 6MWD: Six minute walking distance.

Figure 4 Kaplan Meier analysis for overall survival.

special equipment or skilled staff that you can perform in clinic to give an immediate result. Noticeable that all patients in our study completed the full test, independently of the presence of ascites or encephalopathy, demonstrating one great advantage above other exercise tests, that sometimes require a more complex adaptation and comprehension about the technique. Cahalin et al[9] performed 6MWT and symptom-limited cardiopulmonary exercise testing in patients with heart failure during cardiac
transplant evaluation. The authors described a significant correlation between 6MWD and peak VO$_2$ ($r = 0.64$, $P < 0.001$), concluding that 6MWT is a valuable tool to predict VO$_2$ and short-term survival. These results should be validated in cirrhotic population, but represent a good evidence that 6MWT could be introduced in routine practice without loss of diagnostic accuracy in exercise capacity estimation. While our study did not evaluate the association between VO$_2$ and 6MWD, it did show the safety and practicality of this procedure. García-Pagán et al.[25] reported that moderate exercise (30% of the maximum) significantly increases portal pressure in patients with portal hypertension, and, therefore, could increase the risk of variceal bleeding, ascites and encephalopathy. Although 6MWT is a submaximal exercise, we did not identify any clinical event directly associated with it during the period following the test. Recent studies do not mention the prevalence of adverse events induced by exercise, and more studies designed to respond this issue should be carried out.

Previously studies who reported the relationship between 6MWT and mortality were conducted with small populations and during a short period of follow-up[11,12]. Poor performance during 6MWT may warrant that the at-risk patients should be followed more closely due to the risk of adverse events. Notwithstanding, 6MWT has been proposed as a tool during frailty status evaluation, giving emphasis in this role as a practical and cheap method for this proposal. This study reinforces this importance, adding more powerful results due to our long period of follow-up, demonstrating how physical exercise evaluation may be an interesting long predictor of prognosis in cirrhotic patients.

In our study, 6MWD was an independent predictor of death, consistent with findings from previous studies by Alameri et al.[14], and Carey et al.[15]. In the first study, mortality was evaluated in the whole group, including patients with non-cirrhotic chronic hepatitis, which may bias the interpretation about causality between 6MWD and cirrhosis. Also, Carey et al.[15] studied a population with more advanced disease, all of them on the liver transplant waiting list with a high frequency of liver transplantation (50.4%) performed in a short period of time (5-6 mo). The statistical power of 6MWT in predicting mortality could be affected by pulling out so many patients after transplant from this cohort.

The role of 6MWD and %6MWD in the prediction of mortality were independently of Child scores as demonstrated by multivariate logistic regression analysis. These facts highlight the association of 6MWT parameters with disease progression and adverse outcomes, despite the severity of liver disease.

There are several limitations to our study. First, we did not proceed an external validation of 6MWD cutoffs used in our study, although our main objectives were focused in the transversal and descriptive characterization of study population. Second, we did neither evaluated nutritional status nor calculate the Frailty score of our patients. When study was designed there were no clear parameters specific settle for this diagnosis and a retrospective evaluation was not possible due to lack of complete data. Although recent studies suggest a close relationship between malnourished patients and physical capacity, in order to better evaluate this relationship, another specific protocol must be designed, which was not in accordance with our main objectives. Finally, we did not submit this cohort to a second phase 6MWT to evaluate the relationship between test performance and disease progression. Maybe this analysis could enhance the comprehension about the association of shorter 6MWD and severity of liver disease and its role as a marker of liver decompensation episodes. As we proposed a sectional evaluation of cirrhotic patients with 6MWT, future prospective studies should be able to better answer the previous questions.

**CONCLUSION**

In summary, 6MWT is a very simple, inexpensive, well tolerated, noninvasive test to assess exercise capacity and the result of which is related to MELD and Child scores. The present study showed that 6MWD is an independent predictor of mortality in this population. 6MWT is a promising prognostic marker in patients with liver cirrhosis and should be considered as part of liver transplantation evaluation especially in those referred for the liver transplantation team.
Pimentel CFMG et al. Six-minute walking test in cirrhotic patients

ARTICLE HIGHLIGHTS

Research background
Patients with cirrhosis are at risk of exercise limitations due to progressive limitations related to liver dysfunction. Sarcopenia and cirrhotic cardiomyopathy may be possible related factors. The six-minute walking test (6MWT) is a known simple and practical tool used to evaluate patients with cardiopulmonary disease.

Research motivation
In face of limited diagnosis tools focused on exercise capacity, we purposed to evaluate the role of 6MWT in this population.

Research objectives
The aim of our study was to analyzed 6MWT performance in patients with liver cirrhosis to determine if it associates with mortality.

Research methods
We analyzed 6MWT performance in 106 cirrhotic patients. They were evaluated in the outpatient setting with 6MWT and follow up for one year. Hepatic decompensation and mortality were documented.

Research results
This cohort had a mean age of 51 years and 56% male; patients were staged as Child A in 21.7%, B 66%, and C 12.3%. Walk distance inversely correlated with Child scores, and was significantly reduced as Child stages progress. Patients who died (10.4%) showed a shorter mean 6MWD ($P = 0.006$). Low 6MWD was an independent predictor of mortality ($P = 0.01$).

Research conclusions
6MWT is a noninvasive inexpensive test whose result is related to Child scores and mortality.

Research perspectives
It is a useful, simple, practical test that can be incorporated into cirrhotic evaluation due to its relation with mortality for closer monitoring and potential early intervention.

ACKNOWLEDGEMENTS
This study was supported by Sao Luis Hospital, D’Or Institute of Research and Education (IDOR), and Fleury Institution. The content is solely the responsibility of the authors and does not necessarily represent the official views of these institutions.

REFERENCES
Physical exercise increases portal pressure in patients with cirrhosis and portal hypertension.


Incidence of umbilical vein catheter-associated thrombosis of the portal system: A systematic review and meta-analysis

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Author contributions: Bersani I, Piersigilli F, Iacona G, Di Stasio E and Garcovich M contributed to conceptualization, systematic review, investigation, supervision, writing first draft, review and editing; Savarese I, Campi F, Dotta A and Auriti C contributed to conceptualization, investigation, review and editing; all authors revised the manuscript critically for intellectual content and have approved the final version.

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

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

Country/Territory of origin: Italy

Abstract

BACKGROUND
The use of umbilical venous catheters (UVCs) in the perinatal period may be associated with severe complications, including the occurrence of portal vein thrombosis (PVT).

AIM
To assess the incidence of UVC-related PVT in infants with postnatal age up to three months.

METHODS
A systematic and comprehensive database searching (PubMed, Cochrane Library, Scopus, Web of Science) was performed for studies from 1980 to 2020 (the search was last updated on November 28, 2020). We included in the final analyses all
INTRODUCTION

The placement of an umbilical venous catheter (UVC) is a common procedure in neonatology and has multiple clinical indications driven by the need for quick and secure access for medication administration[1]. During placement, the UVC should run through the umbilical vein, pass the medial portion of the left portal vein at the umbilico-portal confluence, join the direct communication existing between the umbilical vein and the ductus venosus and, through it, bypass the liver and join the inferior vena cava[2,3]. The UVC has to be placed in a central position, ideally at the junction between the inferior vena cava and the right atrium. If a central position is not achieved, then the tip of the catheter can be left below the liver, i.e., below the level of umbilical-portal confluence (peripheral position). The UVC in peripheral position can be used as an emergency access, but it has to be replaced as soon as possible by a peer-reviewed prospective cohort studies, retrospective cohort studies and case-control studies. The reference lists of included articles were hand-searched to identify additional studies of interest. Studies were considered eligible when they included infants with postnatal age up to three months with UVC-associated PVT. Incidence estimates were pooled by using random effects meta-analyses. The quality of included studies was assessed using the Newcastle-Ottawa scale. The systematic review was performed according to the Preferred Reporting Items for Systematic reviews and Meta-Analysis (PRISMA) guidelines.

RESULTS

Overall, 16 studies were considered eligible and included in the final analyses. The data confirmed the relevant risk of UVC-related thrombosis. The mean pooled incidence of such condition was 12%, although it varied across studies (0%-49%). In 15/16 studies (94%), diagnosis of thrombosis was made accidentally during routine screening controls, whilst in 1/16 study (6%) targeted imaging assessments were carried out in neonates with clinical concerns for a thrombus. Tip position was investigated by abdominal ultrasound (US) alone in 1/16 (6%) studies, by a combination of radiography and abdominal US in 14/16 (88%) studies and by a combination of radiography, abdominal US and echocardiography in 1/16 (6%) studies.

CONCLUSION

To the best of our knowledge, this is the first systematic review specifically investigating the incidence of UVC-related PVT. The use of UVCs requires a high index of suspicion, because its use is significantly associated with PVT. Well-designed prospective studies are required to assess the optimal approach to prevent UVC-related thrombosis of the portal system.

Key Words: Portal vein thrombosis; Umbilical venous catheter; Portal system thrombosis; Hepatic thrombosis; Neonate; Incidence
central venous catheter. To prevent UVC-related complications, a proper assessment of catheter tip position is mandatory before its use. In fact, if the tip of the catheter is too deep, it can cause complications such as thrombo-embolic disorders, arrhythmias, and pericardial effusion. On the other hand, if the tip of the UVC is too low, it can be associated with necrotizing enterocolitis, colon perforation, hepatic abscess, and portal vein thrombosis (PVT)[1,4-9]. Furthermore, if the ductus venosus is not perfectly aligned to the umbilical vein, the UVC may unintentionally enter the portal system through the left portal vein during placement and possibly lead to severe complications involving both the hepatic vasculature and parenchyma[1,2,5-8,10-16]. Such liver complications may arise from multiple mechanisms including thrombosis of the portal system vasculature, infusion of irritating drugs and/or hypertonic solutions within the UVC leading to hepatic necrotizing direct mechanical injury[3,17-19]. Besides individual hereditary or acquired predisposing factors (such as prematurity, hereditary prothrombotic disorders, sepsis, the need of transfusions, hyper-viscosity syndrome, dehydration, asphyxia, congenital malformations etc.), whose actual role is still debated[3,10,19-26], umbilical venous catheterization itself represents a risk factor for the development of PVT[18]. In fact, multiple factors may explain the association between UVC and PVT: The introduction of a foreign surface with thrombogenic properties in a small diameter vessel, endothelial damage, and the well-known pro-thrombotic predisposition typical of the neonatal period[27-29]. Symptoms/signs suggestive of PVT may include unexplained thrombocytopenia, catheter-obstructed fluid delivery, increased UVC in-line pressure, impaired lower body/extremity perfusion, although PVT may remain completely asymptomatic[30,31]. When persisting, PVT may inflict substantial damage to the liver leading to portal hypertension, mainly related to the increased vascular resistance in the portal venous system, and to liver atrophy[11,19,32].

In the present systematic review, we specifically focused our search attention on the risk of UVC-related PVT. Although multiple observational studies have provided an overview about the risk of PVT after UVC positioning, to the best of our knowledge no reviews explored systematically this issue. Our aim was to investigate the most accurate information about the actual incidence of UVC-related PVT in the neonatal setting, and to assess if any particular risk factor was systematically associated with the development of such complication.

MATERIALS AND METHODS

The review followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) reporting guidelines[33].

The PICOS strategy was used, which comprised the following (PRISMA): Population: Infants with less than three months of postnatal age; Intervention (or exposure): Umbilical venous catheter; Comparison: No catheter; Outcome (primary): Incidence of PVT; Outcome (secondary): Association with a specific risk factor; Study type: Peer-reviewed observational, cohort and case-control studies.

There was no funding agency for this study. The systematic review did not require ethical approval/informed consent since there was no direct contact with individual patients, and only previously published data were included in the analyses.

Outcomes

The primary outcome was the incidence of PVT related to the use of UVCs (UVC only/attempted UVC/UVC + umbilical artery catheters) in infants with postnatal age up to three months. The secondary outcome was the identification of any risk factor associated with the development of UVC-related PVT.

Search strategy and selection criteria

The following search strategy was used: (portal OR vein OR system OR hepatic) AND (thrombosis) AND (neonat* OR newborn OR pediatric*) AND (catheter* OR umbilical). For reliability, three review authors (Bersani I, Iacona G and Piersigilli F) independently analyzed the currently available literature through systematic and comprehensive database searching (PubMed, Cochrane Library, Scopus, Web of Science) from 1980 to 2020 (the search was last updated on November 28, 2020). Reviews, in vitro studies, animal studies, autopsy studies and conference abstracts were excluded. The reference lists of the included articles were hand-searched to identify additional studies of interest. We obtained the full texts of all the potentially eligible studies.
Eligibility criteria
Three review authors independently undertook eligibility assessment (Bersani I, Iacona G and Piersigilli F). Any disagreement about study eligibility was resolved by discussion with a fourth review author (Garcovich M) until consensus. We considered the studies eligible if they investigated the incidence of UVC-related PVT in infants with postnatal age up to three months. For articles resulting eligible based on the title or abstract, the full paper was retrieved. Case reports were considered not eligible for the final analyses being the calculation of an incidence not possible for such study design. Non-English studies were considered not eligible for the final analyses. We finally included all peer-reviewed, English-language, prospective/retrospective cohort studies and case-control studies.

Study quality assessment
To assess the risk of bias, two authors (Bersani I and Garcovich M) independently used the Newcastle-Ottawa Scale for comparative nonrandomized studies corresponding to each study’s design (cohort/cross-sectional)[34]. Such scale is a validated quality assessment instrument for non-randomized trials which evaluates three parameters of study quality: selection, comparability and exposure assessment. The scale assigns a maximum score of 4 for selection, 2 for comparability, and 3 for exposure, for a maximum total score of 9. Studies with a total score of ≥ 5 or ≥ 7 were considered to be of moderate or high quality, whereas those with a score of less than 5 were considered low-quality studies with high risk of bias. The scale results were tabulated in Table 1.

Data extraction
Three review authors independently performed data extraction (Bersani I, Iacona G and Piersigilli F). Disagreements about data extraction were resolved by discussion with a fourth review author (Garcovich M) until consensus. Pertinent findings from the included studies were tabulated in Table 2 and assessed according to pre-specified subgroups analyses: (1) Year of publication: 1980-2000 or 2001-2020; (2) Indication for thrombosis assessment: Abdominal US as systematic screening or abdominal ultrasound (US) only in case of a clinical concern for thrombosis; (3) Type of diagnostic technique to detect tip position: Radiography or/and (US) evaluation; (4) UVC model: UVC material, size (French), single or double lumen; (5) Thrombosis localization and type: Exact localization within the portal system, complete or partial; (6) Dwell time: Mean UVC in situ persistence (in days); and (7) Prophylaxis: None or heparin infusion or other.

Statistical analysis
Because of high heterogeneity, pooled data on the incidence of UVC-related PVT were analyzed using a random effects (DerSimonian and Laird method) model approach. Statistical heterogeneity among studies was assessed with Cochran’s Q and quantified with Higgins P statistic[35,36]. We considered an P of < 25% as low heterogeneity, P of 25% to 75% as moderate heterogeneity and P > 75% as high heterogeneity. Publication bias was assessed graphically using funnel plots and qualitatively using Egger’s regression and Begg rank correlation method. Statistical analysis was performed by using the Statistical Package for Social Science (SPSS 22.0; SPSS Inc, Chicago, IL, United States) and Microsoft Excel (Version 16.45).

RESULTS
The searches identified 2460 potentially relevant papers, 1835 after duplicates were removed. After title and abstract screening, 53 full-text studies were considered potentially eligible for inclusion and 37 studies were then excluded for the following reasons: (1) Not relevant comparators (n = 23); (2) Non-English language (n = 3); and (3) Wrong study design (n = 11) (Figure 1). Since the design/methodologies varied among different studies, information was not uniformly available for all analyses. For example, some studies could not be considered eligible, although pertinent, since the exact incidence UVC-associated PVT and/or the exact site of a catheter-related thrombosis and/or the exact age of patients with PVT could not be clearly extrapolated from the results.

According to the Newcastle-Ottawa Scale assessing the risk of bias, all the included studies were of moderate-high quality (Table 1). The characteristics and most relevant findings of the included studies are summarized in Table 2[5,21,30-32,37-45]. Of the 16
Table 1 Risk of bias assessment (Newcastle-Ottawa scale for non-randomized studies)

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Selection</th>
<th>Comparability</th>
<th>Outcome</th>
<th>Total score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levit et al[42], 2020</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Dubbink-Verheij et al[31], 2020</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Chen et al[15], 2020</td>
<td>4</td>
<td>0</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Hwang et al[46], 2020</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Çakir et al[38], 2020</td>
<td>4</td>
<td>0</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Cabannes et al[32], 2018</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Derinkuyu et al[5], 2018</td>
<td>4</td>
<td>0</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Chandrashekhar et al[43], 2015</td>
<td>4</td>
<td>0</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Michel et al[37], 2012</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Gharehbaghi et al[39], 2011</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>9</td>
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<td>Sakha et al[41], 2007</td>
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<td>9</td>
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<tr>
<td>Turebylu et al[21], 2007</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>9</td>
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<tr>
<td>Kim et al[30], 2001</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>9</td>
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<tr>
<td>Boo et al[44], 1999</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Schwartz et al[40], 1997</td>
<td>4</td>
<td>0</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Yadav et al[43], 1993</td>
<td>4</td>
<td>0</td>
<td>2</td>
<td>6</td>
</tr>
</tbody>
</table>

included studies, 14 were prospective and 2 were retrospective[15,46]. In some cases, the information about the clinical features of the included population was generically related to the overall cohort rather than specifically to neonates with UVC-related PVT and could not be extrapolated.

In the present review a total pooled sample of 4509 of neonates aged less than three months with UVC was included, 195 of whom experienced UVC-related PVT. The sample sizes ranged widely across studies (median, 83 patients; range, 22-2017). Mean gestational age and birth weight were 30.9 wk and 1738 g respectively, but it was not possible to extrapolate these data from each study, since neonates with PVT sometimes only represented a subgroup, whilst the available data mostly referred to the overall cohort. Figure 2 presents the results of overall meta-analysis with a random effects overall pooled-estimated incidence of UVC-related PVT of 12% [95% confidence interval (CI): 5.91-20.16], with high heterogeneity [I² = 97.5% (95% CI: 97.1%-97.9%)]. Figure 3 shows evidence of publication bias, as indicated by visual inspection of the funnel plot and by the Egger test for small study effects for the primary outcome [bias coefficient for the main analysis, 3.5309 (95% CI: 1.983176-5.078624); P = 0.0002].

When investigating the pre-specified subgroups analyses, we found the following data (Table 2): (1) Year of publication: Overall, 3/16 (19%) studies were published between 1980 and 2000, whereas 13/16 (81%) between 2001 and 2020; (2) Indication for thrombosis assessment: In 15/16 studies (94%), the diagnosis of thrombosis was made accidentally during routine screening controls, whilst in 1/16 study (6%) targeted imaging assessments were carried out in neonates with clinical concerns for a thrombus. In most studies it was not possible to extrapolate mean age at the time of PVT diagnosis (Table 2); (3) Type of diagnostic technique used to assess tip position: Tip position was never assessed exclusively by radiography or echocardiography alone, while it was investigated by abdominal US alone in 1/16 (6%) studies, by a combination of radiography and abdominal US in 14/16 (88%) studies and by a combination of radiography, abdominal US and echocardiography in 1/16 (6%) studies. Only a minority of studies (3/16 studies, with a total number of 39/195 neonates) explicitly specified wrong tip position at the first imaging assessment, in UVC-related PVT cases[32,37,39]. However, most of the studies did not provide such information specifically for neonates who developed PVT, but rather for the overall population. Follow-up imaging controls were scheduled differently across studies; (4) UVC model: Information about UVC material, size and lumen number was only specified by a minority of studies. When the information was available, the studies reported the use of polyvinyl UVCs (n = 3/16) or polyurethane (n = 3/16) UVCs. When described, UVC size varied from 2.5 French to 5 French; (5) Thrombosis...
Table 2 Characteristics of included studies

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Study design</th>
<th>UVC with PVT</th>
<th>UVC without PVT</th>
<th>Dwell time UVC with PVT</th>
<th>Dwell time UVC without PVT</th>
<th>Indication to UVC control</th>
<th>Type of imaging</th>
<th>Country/territory</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levit et al[42], 2020</td>
<td>Prospective</td>
<td>1</td>
<td>2016</td>
<td>N/A</td>
<td>N/A</td>
<td>Clinical Suspicion</td>
<td>X-ray + US</td>
<td>United States</td>
</tr>
<tr>
<td>Dubbink-Verheij et al[41], 2020</td>
<td>Prospective</td>
<td>13</td>
<td>27</td>
<td>N/A</td>
<td>N/A</td>
<td>Screening</td>
<td>X-ray + US</td>
<td>The Netherlands</td>
</tr>
<tr>
<td>Chen et al[13], 2020</td>
<td>Retrospective</td>
<td>7</td>
<td>1320</td>
<td>N/A</td>
<td>N/A</td>
<td>Screening</td>
<td>X-ray + US</td>
<td>Taiwan</td>
</tr>
<tr>
<td>Hwang et al[46], 2020</td>
<td>Retrospective</td>
<td>15</td>
<td>54</td>
<td>N/A</td>
<td>N/A</td>
<td>Screening</td>
<td>X-ray + US</td>
<td>South Korea</td>
</tr>
<tr>
<td>Çakır et al[38], 2020</td>
<td>Prospective</td>
<td>13</td>
<td>83</td>
<td>10.5 ± 4.3(^1)</td>
<td>12.2 ± 4.1(^1)</td>
<td>Screening</td>
<td>X-ray + US</td>
<td>Turkey</td>
</tr>
<tr>
<td>Cabannes et al[32], 2018</td>
<td>Prospective</td>
<td>51</td>
<td>53</td>
<td>N/A</td>
<td>N/A</td>
<td>Screening</td>
<td>X-ray + US</td>
<td>France</td>
</tr>
<tr>
<td>Derinkuyu et al[3], 2018</td>
<td>Prospective</td>
<td>15</td>
<td>229</td>
<td>N/A</td>
<td>N/A</td>
<td>Screening</td>
<td>X-ray + US</td>
<td>Turkey</td>
</tr>
<tr>
<td>Chandrashekhar et al[5], 2015</td>
<td>Prospective</td>
<td>3</td>
<td>27</td>
<td>N/A</td>
<td>N/A</td>
<td>Screening</td>
<td>X-ray + US</td>
<td>India</td>
</tr>
<tr>
<td>Michel et al[37], 2012</td>
<td>Prospective</td>
<td>2</td>
<td>59</td>
<td>N/A</td>
<td>N/A</td>
<td>Screening</td>
<td>X-ray + US + Echocardiography</td>
<td>France</td>
</tr>
<tr>
<td>Gharehbaghi et al[59], 2011</td>
<td>Prospective</td>
<td>5</td>
<td>159</td>
<td>N/A</td>
<td>N/A</td>
<td>Screening</td>
<td>X-ray + US</td>
<td>Iran</td>
</tr>
<tr>
<td>Sakha et al[41], 2007</td>
<td>Prospective</td>
<td>17</td>
<td>33</td>
<td>2 ± 1.12(^1)</td>
<td>N/A</td>
<td>Screening</td>
<td>US</td>
<td>Iran</td>
</tr>
<tr>
<td>Turebylu et al[21], 2007</td>
<td>Prospective</td>
<td>2</td>
<td>26</td>
<td>N/A</td>
<td>6</td>
<td>Screening</td>
<td>X-ray + US</td>
<td>United States</td>
</tr>
<tr>
<td>Kim et al[30], 2001</td>
<td>Prospective</td>
<td>43</td>
<td>57</td>
<td>&gt; 6 d in 23/43</td>
<td>&gt; 6 d in 6/57</td>
<td>Screening</td>
<td>X-ray + US</td>
<td>South Korea</td>
</tr>
<tr>
<td>Boo et al[44], 1999</td>
<td>Prospective</td>
<td>0</td>
<td>57</td>
<td>N/A</td>
<td>N/A</td>
<td>Screening</td>
<td>X-ray + US</td>
<td>Malaysia</td>
</tr>
<tr>
<td>Schwartz et al[40], 1997</td>
<td>Prospective</td>
<td>1</td>
<td>99</td>
<td>3</td>
<td>4 (0-12)(^2)</td>
<td>Screening</td>
<td>X-ray + US</td>
<td>United States</td>
</tr>
<tr>
<td>Yadav et al[45], 1993</td>
<td>Prospective</td>
<td>7</td>
<td>15</td>
<td>N/A</td>
<td>N/A</td>
<td>Screening</td>
<td>X-ray + US</td>
<td>India</td>
</tr>
</tbody>
</table>

\(^1\)Results are expressed as mean ± SD, if reported.  
\(^2\)Results are expressed as median (range), if reported.  
UVC: umbilical venous catheter; PVT: portal vein thrombosis; N/A: Not applicable; US: Ultrasound (abdominal).

localization and type: Only a minority of studies specified PVT exact localization within the portal system. When reported, the left portal vein was the most frequently involved. Similarly, only a minority of studies (in a total number of 84/195 neonates) specified if PVT was complete or partial[5,30,38-41]. According to the available data, PVT was complete in 27/84 (32%) cases and partial in 57/84 (68%) cases; (6) Dwell time: Only a minority of studies reported explicitly the mean UVC dwelling time in neonates with PVT (since most of the studies provided mean dwelling time for the overall population); and (7) Prophylaxis: Only 6/16 (37%) studies reported a prophylactic administration of heparin[21,38,39,42,44,46].

DISCUSSION

To the best of our knowledge, this is the first systematic review specifically investigating the issue of UVC-related PVT. One of the most important limitations that emerged when reviewing the scientific literature was the extreme heterogeneity of study designs across the investigated studies (Table 2 and Figure 3). As a whole, the data achieved by our systematic review confirmed the relevant risk of PVT associated with umbilical catheterization. The mean reported pooled incidence of neonatal UVC-related PVT among studies was 12%, with a range which varied from 0% to 49% from study to study (Figure 2). Such large difference might be attributed to...
multiple factors, including the different indication to imaging diagnostics, the different imaging time schedules, the heterogeneous UVC size/position/duration, and the proportion of preterm/term neonates\cite{30,40,43}. Moreover, the time frame of research and publication may have influenced the incidence of UVC-related PVT as well. In fact, across literature, PVT was more frequently reported in the most recent studies. For example, a large multicenter registry assessing all thrombotic events occurring between 1989 and 1992 in 22 Canadian and 42 international centers from Europe, Australia and United States, recorded only 97 thrombotic events but did not explicitly report any case of PVT at all\cite{47}. In contrast, a more recent large multicenter survey which included 187 children with a diagnosis of PVT (mean age at diagnosis: 4 years) reported a history of neonatal UVC placement in 65\% of cases\cite{19}. The higher incidence of PVT in recent years might be explained by the fact that clinicians are more aware of the thrombotic risk associated with the use of UVC and are more attentive to its detection. Furthermore, advances in US techniques make the detection of PVT easier.

The scientific literature emphasizes that UVC-related PVT is mostly related to improper tip position. Considering the small distance required for an UVC to become dislodged, UVC may migrate into the portal vein even following an initial proper positioning\cite{2,15,16,42,48-52}. Therefore, tip location must be verified with accuracy not only soon after placement but also at regular intervals throughout time\cite{30,31}. For this purpose, US is the ideal tool to check the position of the tip, since it is easy to perform for clinicians, it can be done at bedside and is not invasive for the patient.

When reviewing the literature, we found differences regarding the indication for US assessment, i.e., systematic surveillance in asymptomatic neonates with history of UVCs vs targeted diagnostic test in neonates with clinical concerns for a thrombus. However, in the studies which were finally included in the analyses, UVC-related PVT was mostly asymptomatic and only detected thanks to systematic imaging surveillance. Levit et al\cite{42} found that in their neonatal unit, where routine US screening for PVT was not conducted, the rate of clinically identified thrombi was only 0.15\% of all UVCs placed and 1.1\% of all UVC-associated complications. On the other hand, Kim et al\cite{30} found clinically silent PVT after UVC placement in 43\% of critically ill neonates undergoing systematic US assessment. This indicates that UVC-related PVT might be largely underestimated if not properly investigated\cite{42}, once more confirming the need for routine imaging screenings in all neonates with UVC to exactly determine the incidence of UVC related PVT. Notably, PVT might also be associated with short- and long-term severe complications, deserving meticulous clinical evaluation\cite{5,15}. 

Figure 1 Flow-chart of study selection process.
According to the results of our systematic review, UVC-related PVT was reliably investigated by US assessment. Nevertheless, we found large discrepancies across studies concerning data presentation. As described above in the text, only a minority of studies reported the exact thrombus position/extension within the portal system and if the occlusion was partial/complete. After PVT detection, imaging follow-up controls were performed with heterogeneous time schedules across studies. As a whole, however, the data confirmed that US is a valid, non-invasive, bedside diagnostic technique for PVT detection. But whereas assessment of tip position is easy, requires a minimal training, and can be performed by the neonatologist bedside, detection of PVT at an early stage usually warrants a higher degree of US expertise. Besides the skill level of the radiologist/neonatologist, correct US examination might also depend on further technical factors (neonatal cooperation, abdominal gas distension, clinical instability, small-sized anatomical structures etc.) which may...
influence the assessment.

A meticulous assessment of UVC tip position is needed to decrease catheter-related complications. Radiography is the most widely used technique to assess and follow-up UVC tip location[53, 54]. However, most of the studies used only the anteroposterior view to assess tip location, although such view alone is not able to safely define the correct UVC tip position[54]. In case of wrong tip position within the portal system, radiography may show: (1) The tip below the diaphragm (below the vertebral body T10), overlying the liver; (2) Portal venous gas; and (3) Hypoechoic lesions in the liver if fluid extravasation into liver parenchyma occurred[2, 9, 10, 12, 13]. However, radiographic assessments expose neonates to repeated ionizing radiations. US evaluation can be used in daily practice to check UVC tip position as well as the possible occurrence of UVC-associated hepatic complications. In fact, point-of-care US is able to assess in real-time UVC navigation and tip position during catheter placement[55]. Once UVC is correctly in place, US is the technique of choice to detect the development of UVC-related liver complications[5, 30, 31, 35, 56, 57]. US and Doppler findings demonstrating hepatic complications include: (1) Detection of air in the portal venous system; (2) Portal venous thrombosis with impaired vascular patency; and (3) Liver parenchymal lesions presenting as nodular echogenic lesions/branched echogenic lesions/wide irregular heterogeneous lesions with laceration and the presence of peri-hepatic fluid[2, 5, 9, 10, 52]. Data exist comparing the ability of radiography and sonography to assess UVC positioning. A recent study found that US testing of UVC placement was able to identify catheter location in 100% of cases when compared to radiographic assessment[57]. Moreover, US is more accurate in the assessment of tip position compared to an estimation of catheter position achieved by its relationship to external structures on a radiograph[2, 5, 54, 58]. Echocardiographic evaluation of UVC tip position was also assessed with success in recent years, although most studies focused on its ability to detect intra-cardiac abnormal tip position or atrial/inferior vena cava thrombosis, considering its limited ability to detect thrombi outside of the thoracic great vessels[24, 59-62].

To date, the latest guidelines recommend the removal of UVCs after 7-10 d, although some authors reported an UVC in situ duration up to 28 d, once more proving how the management of UVCs is highly heterogeneous[4, 22, 24, 38, 42, 61, 63, 64]. Unfortunately, the mean UVC dwell time in neonates with PVT was explicitly reported only by a minority of the included studies. Some authors found comparable UVC duration both in neonates with or without PVT[38-40], whilst in a large prospective study Kim et al[30] found an increased risk of PVT with a dwell time longer than 6 d. Noteworthy, PVT occurrence may develop soon after UVC position, as demonstrated by studies describing its detection already 12 h after placement[37]. It could be put forward that the presence of an UVC may itself represent a trigger for PVT development, presumably by raising vascular pressure in the ductus venosus and slowing down blood flow[18], and that such risk may eventually increase if catheterization persists. Such hypothesis deserves proper validation and large randomized controlled trials are warranted to achieve conclusive data about the benefits of early UVC removal.

Only a minority of studies described the occurrence of difficult or failed umbilical catheterization[30, 65]. Considering that traumatic catheterization and/or failed insertion may induce vasculature injury and predispose to PVT by damaging the endothelial wall and decreasing portal flow[8], also the occurrence and number of failed attempts to UVC placement may play a role in PVT development and should be therefore considered either when programming diagnostic/follow-up controls for PVT or in the design of future studies.

The studies included in the final analyses reported the use of different models of UVCs, but unfortunately several studies did not specify the UVC model at all. Today, the most used UVC are dedicated catheters in polyurethane or in polyvinyl chloride but in the past several units used nasogastric tubes for venous umbilical catheterization. Furthermore, most of the studies did not specify the size and the number of lumens of the catheters that have been used. The use of different UVC models/materials may have influenced the incidence of UVC-related PVT in each study.

Concerning the presence of hereditary risk factors, the literature is, once more, quite vague and inconclusive. Turebylu et al[21] evaluated prospectively the prevalence of hereditary prothrombotic mutations in neonates with umbilical catheterization developing thrombotic lesions (including two cases of PVT). Interestingly, the authors found no increase in the risk of catheter-related thrombosis in patients carrying such prothrombotic mutations. In contrast, Heller et al[25] found that among 65 neonates, 24 of whom had PVT, the rate of genetic prothrombotic risk factors was higher than
healthy, age-/sex-matched controls.

Sepsis was suggested as possible risk factor for pediatric PVT development[3,66,67]. However, only a minority of patients affected by PVT presented with infection[3]. Furthermore, as for the studies included in the present review, only a minority of authors explicitly reported the presence of sepsis in case of PVT.

Recently, Hwang et al[46] reported for the first time significantly higher serum calcium concentrations in infants with umbilical catheter-related thrombosis. The authors assessed that such finding may reflect a possible role of calcium as a clotting factor leading to a hypercoagulable state. Further evidence is however required to confirm these results.

Only a minority of the studies included in our review reported a prophylactic treatment with heparin which, moreover, varied in terms of dosage[21,38,39,42,46]. After UVC-related PVT development, spontaneous resolution may often occur in UVC-related PVT, but this warrants close monitoring to determine either progression or resolution of the thrombus[21,30,32,40,46,64,68-70]. However, in case of thrombus extension with occlusion of the portal venous tract or clinical deterioration, antithrombotic therapy with unfractionated or low molecular weight heparin can be considered[64,68,70,71]. Kim et al[30] investigated prospectively the occurrence of UVC-related PVT in 100 neonates by subsequent US assessment. The authors found that 43% of neonates had a clinically silent PVT and reported complete resolution in 56% of neonates at follow-up controls, with recanalization being more frequent in neonates with partial rather than occlusive thrombi. Cabannes et al[32] investigated prospectively the occurrence of PVT in a cohort of patients including preterm neonates. PVT occurred in 53/123 of which 51 had an UVC. In these cases, the authors reported a spontaneous favorable evolution of left PVT in 95% of cases. In a prospective observational study, Dubbink-Verheij et al[31] investigated by serial US evaluations the incidence of catheter-related thrombosis in neonates with UVCs compared to a control group of neonates without UVC. The authors found the presence of thrombotic lesions in the UVC route in 30/40 cases (75%), of which 13 in the portal vein system. Most of the thrombotic lesions were asymptomatic and regressed spontaneously, whilst a minority required treatment with heparin. In contrast, Derinkuyu et al[5] treated with low-molecular-weight heparin all neonates with a diagnosis of UVC-related PVT (all described as asymptomatic). This heterogeneous approach may reflect the absence of solid evidence about safety/efficacy of antithrombotic therapy specifically addressing the neonatal period.

Our systematic review has multiple limitations, mostly attributable to the heterogeneity across studies. First, the intrinsic limitation of having included either retrospective studies or “old” studies (from 1980 onwards), i.e., performed at time-points during which clinical approach to patients and awareness about PVT was presumably different compared to more recent studies. Second, the lack of correlation between PVT and UVC tip position in most studies. Third, the different study designs regarding the indication and time schedule for imaging assessment. Fourth, the different approach of clinicians about the use of prophylactic/therapeutic treatment in neonates with indwelling UVCs.

CONCLUSION

In conclusion, the use of umbilical lines requires a high index of suspicion for PVT development, especially if considering that the need for an UVC obviously preselects ill newborns in whom multiple risk factors for the development of thrombotic disorders may coexist. To avoid or minimize the risk of PVT, some crucial key-points have to be followed, as checking the correct position before infusing in the catheter, checking again the correct tip position every 48 h, and removing the UVC after a maximum of 7 d.

As a whole, this systematic review revealed relevant gaps also in knowledge about the optimal diagnostic approach and treatment for UVC-related PVT, maybe related to the lack of updated, evidence-based guidelines addressing step-by-step all the aspects of what the best approach to the management of this complication should be. According to our opinion, this represents a call to action addressed to researchers and clinicians to design large prospective randomized studies and to draft specific, concrete and updated guidelines.
ARTICLE HIGHLIGHTS

Research background
The use of umbilical venous catheters (UVCs) in the perinatal period may be associated with severe complications, including the occurrence of portal vein thrombosis (PVT).

Research motivation
Although multiple observational studies have provided an overview about the risk of PVT after UVC positioning, no studies/reviews explored systematically this issue.

Research objectives
The main goal was to investigate the most accurate information about the actual incidence of UVC-related PVT in the neonatal setting, and to assess if any particular risk factor was systematically associated with the development of such complication.

Research methods
A systematic and comprehensive database searching (PubMed, Cochrane Library, Scopus, Web of Science) was performed for prospective cohort studies, retrospective cohort studies and case-control studies from 1980 to 2020. Incidence estimates were pooled by using random effects meta-analyses. The quality of included studies was assessed using the Newcastle-Ottawa scale.

Research results
Sixteen studies were considered eligible and included in the final analyses. The data confirmed the relevant risk of UVC-related thrombosis with a mean pooled incidence of 12%, although it varied across studies (0%-49%).

Research conclusions
This is the first systematic review specifically investigating the incidence of UVC-related PVT. The use of UVCs requires a high index of suspicion, because its use is significantly associated with PVT.

Research perspectives
Large prospective randomized studies and updated guidelines are warranted in order to define the best management of this dreaded complication.

REFERENCES


