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The *WJH* covers topics concerning liver biology/pathology, cirrhosis and its complications, liver fibrosis, liver failure, portal hypertension, hepatitis B and C and inflammatory disorders, steatohepatitis and metabolic liver disease, hepatocellular carcinoma, *etc*. Priority publication will be given to articles concerning diagnosis and treatment of hepatology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, *etc*.

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MINIREVIEWS

Endoscopic ultrasound guided liver biopsy for parenchymal liver disease

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Abstract

Liver biopsy plays an essential role in the diagnosis, evaluation and management of a vast proportion of liver diseases. Conventionally, percutaneous and trans-jugular approaches have been used to obtain liver biopsies. Endoscopic ultrasound guided liver biopsy (EUS-LB) has emerged as a safe and effective alternate in the past two decades. EUS-LB carries a role in evaluation of both benign and malignant diseases of the liver. It can offer higher resolution imaging of the liver and can detect smaller lesions than computed tomography scan of the abdomen or ultrasound scans with the option for doppler assistance to reduce complications. Current evidence demonstrates the superiority of EUS-LB for a targeted approach of focal lesion and there is also evidence of less sampling variability in heterogeneous parenchymal pathologies. These advantages combined with an improved safety profile had led to the rapid progress in the development of new techniques, equipment and procedures for EUS-LB. We provide a comprehensive review of EUS-LB for parenchymal liver disease.

Key words: Liver biopsy; Endoscopic ultrasound; Endoscopic ultrasound guided liver biopsy; Liver disease

Core tip: In this review, we provide a comprehensive discussion on the role of Endoscopic ultrasound guided liver biopsy (EUS-LB) in parenchymal liver disease. This article summarized the technical aspects of EUS-LB; as well as debated its advantages and disadvantages. We also highlight new advancements and recently reported research

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INTRODUCTION

Liver biopsy plays an essential role in the diagnosis, evaluation and management of a vast proportion of liver diseases. History, physical exam, laboratory testing and radiological exams continue to be the mainstay in diagnosis of disease, however, the use of these modalities without liver biopsy can miss up to one-third cases of cirrhosis[1]. Histological examination of biopsy samples can not only help identify the underlying pathophysiology of liver disorder but also quantify its severity. This has a significant impact on the management of as many as one-fifth of all patients with liver disease[2]. In the past, liver biopsy was performed *via* the percutaneous route without image guidance[3]. However this has now been largely replaced by ultrasound-guided or computed tomography (CT) guided biopsy to limit potential complications[4,5]. When a percutaneous biopsy is not feasible a trans-jugular approach is used. Open or laparoscopic surgical biopsy is usually last resort. Endoscopic ultrasound guided liver biopsy (EUS-LB) is a technique that has recently gained popularity since it offers certain advantages over the traditional methods of obtaining tissue samples. Endoscopic ultrasound is an established imaging modality that is essential in the assessment of a broad array of luminal, hepatobiliary, and pancreatic disorders. It provides high resolution images of both lobes of the liver, hence allowing a potentially easier and safer biopsy technique whilst providing the opportunity to target focal hepatic lesions[6]. The choice of technique is eventually based upon expertise of the operator, anatomical barriers and risk profile of the patient (coagulopathy, hepatic vascularity and presence of ascites). In this review, we provide a detailed comparison between EUS-LB and traditional modalities of liver biopsy.

APPROACHES TO LIVER BIOPSY

The advent of liver biopsies is dated back to the 19th century, with Paul Ehrlich reporting the first successful liver aspiration in 1883[4,7]. However, it wasn't until four decades later that the first percutaneous liver biopsy was successfully performed in Germany in 1923[4,5]. Even a century later this remains the preferred approach to obtaining hepatic parenchymal tissue[3]. Over time, advances in imaging modalities have led to the addition of ultrasound and CT scan guided liver biopsy[5,8].

Previously percutaneous liver biopsy (PC-LB) was performed "blindly" using percussion to identify the liver anatomy[3]. This has now largely been replaced by image-guidance, either by ultrasonography or CT scan[8]. Most commonly large gauge needles (16-18) are used, although depending on expertise smaller needles are also utilized at some centers[9]. Percutaneous biopsy can be classified as transthoracic (transpleural) or subcostal depending on the site of entry. This requires an enlarged liver extending below the diaphragm for a safe approach. Pain, bleeding, infection, peritonitis, pleural injury resulting in pneumo- or hemo-thorax continue to be common complications of percutaneous biopsy[9,10]. Image guidance partly mitigates these risks, but studies have reported adverse event rates up to 1%[3,10].

Trans-jugular biopsy of the liver emerged as a viable technique in the 1960s based on the works of an interventional radiologist – Charles Dotter[11]. Over time it has become an accepted and safe alternative to percutaneous biopsy in select settings[12]. The internal jugular vein is cannulated to gain access to the hepatic vein, allowing tissue acquisition without the need to traverse the liver capsule[13]. This approach is preferred in patients who have coagulopathy, hepatic peliosis, large volume ascites or morbid obesity[12]. Rates of success have been reported as high as 97% and the complication rates as low as 1.3%[14]. Complications include hepatic capsule perforation, major hemorrhage, pain from hematoma, hemobilia, arterial aneurysms and arrhythmias[12]. Fortunately however, major complications are rare and minor bleeding from the access site and transient abdominal pain from hematomas are the

most commonly reported adverse events[14,15].

EUS was developed in the 1980s and has been gaining popularity. This was further revolutionized in the early 1990s with the advent of Fine Needle Aspiration (FNA)[16]. Significant advances have been made since then and now EUS is widely used as both a diagnostic and therapeutic modality. EUS-LB has recently emerged as another technique for obtaining liver tissue. Although as described earlier; several approaches and techniques are there for obtaining a liver biopsy, EUS-LB offers several advantages over conventional approaches. EUS offers a more precise localization and characterization of target tissue which helps to improve diagnostic yield[17,18]. More so, it is arguably a less invasive and better tolerated approach than conventional methods[15,19]. It offers the advantage of good access to both the lobes of liver and the presence of doppler assistance decreases the chances of complications[20]. Other important considerations for choosing EUS-LB over conventional methods of liver biopsy include contraindications to percutaneous biopsy.

EUS GUIDED SAMPLING TECHNIQUES FOR PARENCHYMAL LIVER DISEASE

Since the onset of EUS-LB, multiple needle types and techniques have been reported for its use. In the next few segments, we review the historic timeline for various needle types and discuss the different techniques used to help in increasing the diagnostic yields of EUS-LB. Core sample obtained *via* EUS-LB is shown in Figure 1.

How is a good quality liver biopsy defined?

The criteria for an adequate liver biopsy had been well defined. Per the American Association for the Study of Liver Disease (AASLD), adequacy of samples are defined as number of complete portal triads (CPTs) to be 11 and total sample length (TSL) of 30 mm, and with no or minimum fragmentation of the sample[21]. However, the definition of adequacy for liver biopsy sample remains controversial in the literature[22-26].

Needle types

Tru-cut biopsy: In 2002, the initial experience with EUS-LB was in swine models using Tru-Cut biopsy needles. In a study by Wiersema *et al*[27], they performed EUS-LB of multiple peri-gastric organs using a 19 gauge Tru-Cut needle. The median TSL was 4 mm for liver samples and 78% had fragmentation, however the number of CPT was not reported. They reported difficulty in the procedure due to the use of the stiff needle since problems were encountered in making the needle bend to traverse through the flexible endoscope. They concluded that the method is safe and feasible, however did not meet criteria for adequate liver biopsy samples and were technically difficult. Due to the aforementioned reasons this method was not widely used.

However, in 2009, Gleeson *et al*[28] also reported outcomes using the Tru-Cut needle. They reported that results of EUS Tru-Cut needle biopsy are comparable to those of trans-jugular liver biopsy. In their small study with the use of Tru-Cut needle on 9 patients, they reported the TSL of 16.9 mm and a median of 7 CPTs. Although the results do not suffice the criteria of having at least 11 CPT per AASLD[21], they were able to reach a histopathologic diagnosis in all 9 patients. However, the study was retrospective and only included 9 patients.

Overall, the EUS Tru-Cut biopsy did not gain wide spread popularity for diagnosing parenchymal liver disease and more novel needles and techniques emerged which made Tru-Cut biopsies fall out of favor.

19 Gauge FNA "non tru-cut" needle: Several studies reported using 19 gauge FNA needle, with the first one published in 2012[24]. In this study, Stavropoulos *et al*[24] performed EUS-LB on patient undergoing EUS to rule out biliary obstruction when the exam was unrevealing. The median length of obtained specimens was 36.9 mm ranging from 2 to 184.6 mm, nine complete portal tracts (range: 1-73), diagnostic adequacy of 91%, and no post-procedure complications. The outcomes showed comparable biopsy quality results to percutaneous and trans-jugular liver biopsies. They concluded that for patients being investigated by endoscopic ultrasound for biliary obstruction; EUS-LB was a safe, reliable and cost-effective option to diagnose parenchymal disease.

In one large study published in 2015 by Diehl *et al*[29], liver biopsy specimens obtained *via* EUS were sufficient for pathological diagnosis in 98% of the cases. The aggregate length of tissue acquired ranged from 0 to 203 mm with a median of 38 mm. A total of 0 to 68 CPTs were obtained and the median was 14. This led the authors to conclude that EUS-LB using a 19 gauge FNA needle, is a safe technique yielding

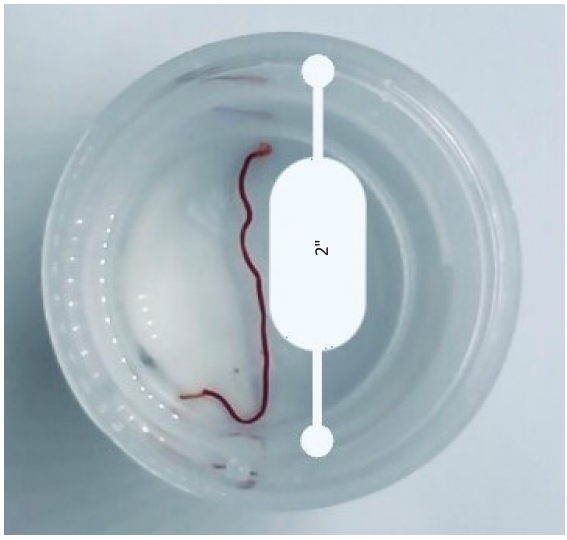

Figure 1 Core biopsy sample obtained *via* endoscopic ultrasound guided liver biopsy.

adequate tissue for pathological diagnosis in 98% of the patients.

This needle provides a higher diagnostic yield and is less technically challenging to use in comparison with the Tru-Cut biopsy needle. However, the ideal technique has not been yet defined and larger studies are needed. Different biopsy techniques are discussed in the next section.

19 Gauge Fine Needle Biopsy needle: A new 19 gauge fine needle biopsy (FNB) needle has shown promising results and a higher diagnostic yield. Schulman *et al*[30] compared the different needle types and biopsy techniques. They studied 6 different needle types (four EUS-LB needles and two percutaneous needles) on human cadaveric tissue and had a total of 288 liver samples. They concluded that a novel 19 gauge needle (SharkCore 19 gauge FNB needle) had significantly better diagnostic yield compared to all other needle types in the study including the second FNB needle used in the study, the ProCore FNB (Echo Tip HD ProCore).

In this study four discrete suction techniques were used in addition to one fanning pass *vs* three fanning pass patterns of needle excursion. Analysis of the subgroups showed that three fanning pass needle excursion pattern was an independent prognosticator of CPT, however the suction techniques had no effect on the adequacy of the tissue sample when other variables were controlled. This type of needle also had overall less tissue fragmentation rates compared to the other needles with an 84% mean of core samples from tissue obtained using this needle. This study was double blinded and randomized, however was done on ex-vivo on cadaveric tissue. This needle type seems to have a promising future, however more studies need to be done on non-cadaveric diseased liver with comparison to other biopsy modalities to confirm its superiority and overall cost-effectiveness.

Biopsy techniques

Several techniques have been used to increase the diagnostic yield of EUS-LB. The technique for performing EUS-LB is demonstrated in Video 1. Diehl *et al*[29] reported performing multiple movements with the needle "fanning technique" in which the needle is advanced to-and-fro at different areas to obtain tissue samples[31].

Most endoscopists use full suction with needle aspiration as well. Suction techniques include ten-/twenty-/thirty-mL method. Alternatively, a slow-pull technique may be used where the stylet is drawn out from the needle once it is in the desired location[7].

More recently, newer techniques have emerged in attempts to increase the diagnostic yields of EUS-FNB including the use of "wet suction". In this technique a heparinized needle is used to reduce chances of coagulation and hence, improve tissue retrieval whilst minimizing comminution of tissues samples. A recent study by Mok *et al*[32] compared this method to both "dry heparin" and "dry needle" technique. This was a prospective study on 40 patients, were they all had 3 EUS-FNA liver biopsies using the 3 different methods. In this method, needles are heparinized till drops are seen at the needle-point while ensuring no air is pushed through. Two milliliters of water are then drawn into the syringe and a twenty-milliliter vacuum-syringe is connected. They concluded that "wet suction" technique had better tissue

yield in comparison to "dry needle" method. However, the question remains if this technique is necessary or even useful when using the more novel FNB needles as these needles provided superior results regardless if wet suction was used or not.

Another recently described technique is the "modified 1-pass 1 actuation wet suction technique [EUS-modified liver biopsy sampling (EUS-MLB)]". This was described in a study by Nieto *et al*[33] They used the FNB-needle (SharkCore) which was prepped normal saline. Suction was applied to the FNB-needle at a depth of seven cm into the hepatic parenchyma. Large vessels were avoided using Doppler assistance. Both right and left lobes were sampled *via* a "rapid-puncture" and one actuation for each lobe. The authors concluded that "EUS-MLB" was effective and safe in evaluation of inexplicable liver disease. The median TSL in their study was 6 cm, and the median number of CPTs per TSL was 18.

ADVANTAGES OF EUS-GUIDED BIOPSY

Historically percutaneous liver biopsy was done "blind", however in present times this has largely been replaced by image guided biopsy. Image guidance is postulated to help increase sampling adequacy and more importantly reduce complications[4]. There is some controversy regarding this and conflicting data have been reported[4,34]. In 1991, Vautier *et al*[34] reported that ultrasound guidance dose not reduce bleeding complications from PC-LB and concluded that image guided liver biopsy may not be safer than blind biopsy. A retrospective analysis looked at the complications and safety profile of liver biopsy in patients enrolled in the HALT-C trial[35]. All the patients included in this trial had advanced chronic liver disease. A total of 2740 liver biopsies were performed and 90% were ultrasound guided. 16 of the total 29 cases of significant adverse events were from bleeding complications. EUS guidance aims to mitigate these complications further by better anatomical definition and doppler assistance.

Linear echoendoscopes were first introduced in the 1990s[36]. Linear echoendoscopes allowed the use of doppler ultrasound and the ability to track needles in real-time. Combined with high resolution imaging, the intrahepatic vessels and major bile ducts can be easily identified and avoided during biopsy, hence reducing potential complications[37].

Commonly a sixteen-gauge needle is used for percutaneous liver biopsy. On the contrary using nineteen-gauge needles for EUS-LB reduces possible complications. A large trial comprising over a hundred patients studied the diagnostic yield and safety of EUS-FNA using a 19-gauge needle[29]. Reported diagnostic yield was 98% as measured by the tissue sample length and presence of complete portal tracts. Serious adverse event was reported in one patient who developed a sub-capsular hematoma that required only conservative management. The authors concluded that EUS-LB was a safe technique with comparable diagnostic accuracy to PC-LB. Adler *et al*[38] performed a multicenter retrospective review of 200 patients, specifically looking at safety and performance when sampling solid lesions. They reported excellent diagnostic yield at 98.5%, however 6.5% of the patients needed a repeat procedure at some point. No adverse event was identified in the population. Table 1 summarizes the diagnostic accuracy and adverse event rates of EUS-LB.

EUS guidance has the benefit to sample and evaluate both lobes of the liver, hence achieving more accurate representation of liver histology, potentially addressing concerns about sampling error[24]. PC-LB and transjugular biopsies are both subject to sampling variability due to heterogeneity of parenchymal diseases[39]. This variability can be reduced by sampling both the liver lobes. EUS-LB allows easier access to the right and left lobe of liver; thus minimizing this variability[39].

For Pancreatic lesions EUS guided biopsy has proven superiority as an imaging modality for as it allows greater anatomical definition and higher resolution with the ability to sample ascites, local lymphatic structures and small liver nodules[40]. There is some evidence suggesting that for smaller liver lesions EUS-LB is indeed also superior and safer than PC-LB with CT or ultrasound guidance[41,42].

Other advantages of EUS-LB include a much shorter recovery time (about 4 h) than that of PC-LB (commonly at least 10 h)[13,43,44]. Another potential benefit is that patients are sedated for the EUS procedure, thus EUS-LB is better tolerated in most instances as compared with PC-LB[44,45]. However, it is important to remember that the benefits of sedation and anesthesia must be balanced against risk of respiratory depression. As described for trans-jugular biopsy, EUS-LB also has the potential advantage in patients with morbid obesity, large ascites, peliosis hepatis, and coagulopathy[14,15].

Table 1 Current evidence of diagnostic accuracy and adverse event rates of endoscopic ultrasound guided liver biopsy

	Sample size	Diagnostic yield	Serious adverse events
Gleeson *et al*[28], 2009	9	0%	0%
Schulman *et al*[30], 2017	288	84%	NA (cadaveric tissue)
Stavropoulos *et al*[24], 2012	22	91%	0%
Diehl *et al*[29], 2015	110	98%	0.9%
Mok *et al*[32], 2018	40	98% (wet suction)	0%
Nieto *et al*[33], 2018	165	> 90%	1.8%
Adler *et al*[38], 2018	200	98.5%	0%

DISADVANTAGES

Despite its advantages over the other liver biopsy techniques, the application of EUS-LB in everyday practice has yet to reach its full potential. One of the barriers to this is the relative novelty of the technique. PC-LB and trans-jugular approaches have been used in clinical practice much longer than EUS-LB and hence operators have more experience with these. The conventional techniques are also easier and require less technical expertise. This is especially true with the use of Tru-Cut needle for biopsy which is more technically demanding and may have variable sample yield[46]. Tru-Cut needles have been largely replaced with more flexible needles that can be navigated with more ease[27,47].

Left lobe of the liver can be approached through the gastric wall, whilst the right lobe is accessed *via* the duodenum. Right Hepatic Lobe sampling can be difficult in some cases due to difficulty in navigating the biopsy needle at sharp angles across the duodenum[15,48]. This again is more of an issue with Tru-Cut needles which have limited flexibility[47].

Another important consideration is the cost of procedure. EUS-LB has a much higher cost when compared to PC-LB and this can be prohibitive to the widespread use[15,39]. However, this drawback is offset in patients who are undergoing an endoscopy for another indication (such as esophageal varices screening). EUS-LB may be done during the same session with little additional time and risk. Patients intolerant or non-compliant of pre-procedure preparation can also prove to be a challenge to successful EUS-LB. Table 2 summarizes the comparison between PC-LB and EUS-LB.

CONCLUSION

Liver biopsy remains essential in diagnosis, evaluation and management of numerous liver diseases. Whilst percutaneous biopsy remains the test of choice, it has its drawbacks; and hence EUS guided liver biopsy has emerged over the resent past as a safe and effective alternative. Advantages of EUS-LB include easier access to both lobes of liver and improved diagnostic accuracy in heterogeneous parenchymal diseases as well as detecting multiple focal lesions. Procedure and recovery times are shorter with less reliance on patient cooperation. Additionally, the use of doppler assistance helps avoid blood vessels reducing risk of hemorrhage; which is the most common complication of liver biopsy.

Techniques of obtaining liver samples with EUS guidance and the equipment used, such as types of needles; is constantly changing. However, current evidence on EUS-LB techniques is conflicting and there is no consensus on the best technique and type of needle used. Nevertheless, most centers have published positive results from their individual experiences. Despite these advances cost barriers and sparsity of technical expertise continue to remain limiting factors for the wide spread use of EUS-LB.

Table 2 Comparison of percutaneous liver biopsy with endoscopic ultrasound guided liver biopsy

	PC-LB	EUS-LB
Anatomical definition	Likely improved with ultrasound guidance	Better than PC-LB and the addition of Doppler reduces complications
Imaging resolution	Lower	Higher
Diagnostic yield/accuracy	Comparable	
Sampling error	Higher	Lower
Access to liver lobes	Predominantly right lobe sampling	Can easily sample both right and left lobes
Average recovery time	10 h	4 h
Technical difficulty	Lower	High
Cost	Low	High

PC-LB: Percutaneous liver biopsy; EUS-LB: Endoscopic ultrasound guided liver biopsy.

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MINIREVIEWS

MicroRNAs contribute to ATP-binding cassette transporter- and autophagy-mediated chemoresistance in hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) has an elevated mortality rate, largely because of high recurrence and metastasis. Additionally, the main obstacle during treatment of HCC is that patients usually develop resistance to chemotherapy. Cancer drug resistance involves many different mechanisms, including alterations in drug metabolism and processing, impairment of the apoptotic machine, activation of cell survival signaling, decreased drug sensitivity and autophagy, among others. Nowadays, miRNAs are emerging as master regulators of normal physiology- and tumor-related gene expression. In HCC, aberrant expression of many miRNAs leads to chemoresistance. Herein, we particularly analyzed miRNA impact on HCC resistance to drug therapy. Certain miRNAs target ABC (ATP-binding cassette) transporter genes. As most of these miRNAs are downregulated in HCC, transporter levels increase and intracellular drug accumulation decrease, turning cells less sensitive to death. Others miRNAs target autophagy-related gene expression, inhibiting autophagy and acting as tumor suppressors. Nevertheless, due to its downregulation in HCC, these miRNAs do not inhibit autophagy or tumor growth and, resistance is favored. Concluding, modulation of ABC transporter and/or autophagy-related gene expression or function by miRNAs could be determinant for HCC cell survival under chemotherapeutic drug treatment. Undoubtedly, more insights on the biological processes, signaling pathways and/or molecular mechanisms regulated by miRNAs are needed. Anyway, miRNA-based therapy together with conventional chemotherapeutic drugs has a great future in cancer therapy.

Key words: Hepatocellular carcinoma; Chemoresistance; ABC transporter; Autophagy; miRNA

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Core tip: The main obstacle during treatment of hepatocellular carcinoma (HCC) is resistance to chemotherapy. We analyzed microRNAs (miRNAs) impact on HCC chemoresistance. Certain miRNAs target ABC transporter genes. As most of these miRNAs are downregulated in HCC, transporter levels increase and intracellular drug accumulation decrease, turning cells less sensitive to death. Others miRNAs target autophagy-related gene expression, inhibiting autophagy and acting as tumor suppressors. Nevertheless, due to its downregulation in HCC, these miRNAs do not inhibit autophagy or tumor growth and, resistance is favored. ABC transporter and/or autophagy-related gene expression modulated by miRNAs affect HCC cell survival under chemotherapy.

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INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for around 80%-90% of liver cancer. It's the fifth most common cancer worldwide and the third cancer-related death cause. In general, HCC develops in a background of cirrhosis. The main factors that contribute to this tumor are infections with hepatitis B or C virus, alcohol abuse and non-alcoholic fatty liver disease[1]. Despite the development of early detection methods, 80% of HCC patients are diagnosed at a stage of the disease so advanced that the patient's survival is just of about few months[2]. When HCC is diagnosed at early stages the most effective approaches are partial hepatectomy or liver transplantation, however in most patients the tumor is not detected promptly or it is hard to find a compatible donor. In intermediate stages, transarterial chemoembolization is the treatment of choice[3]. For patients with unresectable HCC, sorafenib oral administration slightly improves survival[4]. Even the ones who qualify for surgery have a modest improvement in the survival because of its high rate of recurrence, occurring in more than 90% of patients[2].

Treatment with anti-cancer drugs (chemotherapy) can destroy tumor cells helping patients to control cancer growth. Some of these drugs to treat HCC are 5-fluorouracil (5-FU), cisplatin, doxorubicin[5], paclitaxel and the multitarget tyrosine kinase inhibitor sorafenib. But unfortunately, liver cancer patients usually develop drug resistance to chemotherapy[6]. Cancer drug resistance is a multifactorial phenomenon involving many different mechanisms such as gene mutation, DNA repair pathway aberrations, impairment of the apoptotic machine, alterations in drug metabolism and processing, activation of cell survival signaling and escape of drug sensitivity, autophagy, epigenetic regulation, altered lipid metabolism and tumor microenvironment participation[2]. Among these mechanisms, modifications in drug uptake or efflux produce a diminished intracellular drug concentration, leading to tumor cell survival and resistance to death. Until some years ago, the reduction of intracellular drug concentration was thought to be the only important mechanism for resistance. Nevertheless, many other processes have been appeared to be involved more lately. Autophagy is one of these novel processes that also contribute to tumor chemotherapy resistance; moreover, autophagy inhibitors are used to sensitize different cancers to chemotherapy. Under this scenario, microRNAs (miRNAs) are nowadays emerging as master regulators of normal physiology- and tumor-related gene expression. Thus, it is not surprising that these molecules can also regulate chemoresistance.

In this review we summarize the findings from the last decade regarding the molecular mechanisms of chemoresistance in HCC, focusing on the involvement of miRNAs. We especially highlight the role of these molecules in drug efflux through ABC (ATP-binding cassette) transporters and autophagy.

miRNAs IN THE DEVELOPMENT OF HCC

CHEMORESISTANCE

miRNAs are short RNA molecules of 18-25 nucleotides in length. They are non-coding molecules that regulate the expression of many genes. By binding to the 3′-untranslated region (UTR) of target genes through base complementarity, miRNAs lead to mRNA degradation or translational repression, acting as negative regulators of gene expression[6]. Many reports have described a role of these molecules in the control of diverse biological processes such as development, differentiation, cell proliferation and apoptosis[7]. They can also act as oncogenes or tumor suppressors in different human cancers.

Up to date more than 2500 miRNAs have been described in humans and each of them has been reported to silence more than one gene. miRNAs take a crucial part in different processes in normal and abnormal liver development. They are involved in lipid, cholesterol and glucose metabolism, and liver differentiation (see Ref.[8] review). They take part in apoptosis, necrosis, cell cycle, proliferation, epithelial-mesenchymal transition and inflammation[9]. Further, studies derived from the comparison between normal liver and human liver cancer showed the aberrant expression of many miRNAs in cancer cells. It was interesting to find that some of them such as, miR-21, miR-221 and miR-222 are upregulated in HCC whereas miR-122, miR-199 and the let7 family members are downregulated[7], showing the complex pattern of miRNA expression in this type of cancer. Regarding the molecular mechanisms, protein kinase B (AKT), mammalian target of rapamycin (mTOR), Wingless-type MMTV integration site family (Wnt), Janus kinase/signal transducers and activators of transcription (JAK/STAT), and Mitogen-activated protein kinases (MAPK) are the main miRNA-regulated pathways during HCC tumorigenesis[10].

Thus, many miRNAs are deregulated in HCC and some of them participate in the progression of chemoresistance[11]. Nevertheless, their precise roles in the development of drug resistance in liver cancer are not fully understood. In the following sections we particularly analyze the relationship between miRNAs and ABC transporter-mediated drug resistance in HCC. Besides, we summarize the novel evidence that relates miRNAs, autophagy and chemoresistance in HCC.

miRNAs AND ABC TRANSPORTER-MEDIATED DRUG RESISTANCE IN HCC

The ABC transporters are cell membrane proteins that couple the hydrolysis of ATP to extrude different types of xenobiotics and metabolites against concentration gradient. Efflux transporters such as P-glycoprotein (P-gp/*ABCB1*), breast cancer resistance protein (BCRP/*ABCG2*) and multidrug resistance-associated protein 1 (MRP1/*ABCC1*), among others, limit the exposure to chemotherapeutic drugs by extruding them from cells. Consequently, these transporters are main actors in the drug resistance phenomenon. Furthermore, their overexpression in HCC cells represents a big obstacle for chemotherapy treatment and the central mechanism that contributes to drug resistance[12]. Here we summarize the recent studies describing the role of major miRNAs on ABC transporter family proteins and their impact on HCC chemoresistance.

miR122

This is a liver specific miRNA[13] and it participates in lipid and cholesterol metabolism and its decreased expression has detrimental effects on the liver[8]. Frequently, miR122 is downregulated in HCC tumors denoting poor prognosis and metastatic properties[14]. In human HCC cell lines (HepG2, HuH-7, Hep3B), miR122 levels were also found reduced. Interestingly, when these cells were adenovirus-transduced to overexpress miR122 they became more sensitive to DOX- and vincristine-induced death. Remarkably, miR122-overexpressing HCC cells showed reduced levels of *ABCB1* and *ABCC1* mRNA expression (Table 1). The latter was also found downregulated at the protein level[12]. These results indicate that miR122 controls sensitivity to drug-induced HCC cell death by downregulating P-gp and MRP1 expression.

miR27a

miR27a is involved in tumorigenesis in different types of cancers. In leukemia cells it was shown that resistance to DOX correlates with high expression of P-gp and low levels of miR27a. The upregulation of this miRNA produced greater mortality in the presence of DOX than the observed in control cells, demonstrating the role of miR27a in chemoresistance in leukemia cells[15]. On the contrary, miR27a upregulation lead to

Table 1 miRNAs and ABC transporter-mediated drug resistance in hepatocellular carcinoma cells

miRNA	Expression Level in HCC	Role in ABC transporter expression and/or function	Involvement in cell viability and/or drug resistance	Ref.
miR122	Downregulated in tumors (reduced levels correlate with patient poor prognosis and metastasis) and in human HepG2, HuH-7 and Hep3B HCC cell lines	miR122-overexpressing HCC cells treated with DOX and vincristine showed reduced levels of P-gp mRNA expression, and MRP1 mRNA and protein levels	Adenovirus-transduced cells to overexpress miR122 became more sensitive to DOX- and vincristine-induced death	[12,14]
miR27a	Low in drug-resistant Bel-7402 cells	Negatively correlated with P-gp levels. Upregulation of miR27a reduced P-gp mRNA and protein expression	Cells transfected to overexpress miR27a sensitized resistant cells to 5-FU, mitomycin and DOX	[17]
miR503	Downregulated in HCC tissues (reduced levels correlate with malignant tumor progression), in HCC cell lines (SMMC-7721, Hep3B, HepG2, MHCC97H and LM3) and HepG2 resistant to drugs	Cells transfected to overexpress miR503 showed downregulation of both P-gp and MRP1, at mRNA and protein levels, and accumulated more intracellular rhodamine-123 (extruded through P-gp)	miR503 overexpression restored sensitivity to DOX in HepG2 resistant cells	[19,20]
miR375	Downregulated in patient tumor tissues and cells lines (HepG2, HuH-7, Hep3B)	Delivered within nanoparticles decreased P-gp protein expression	Delivered within nanoparticles improved DOX antitumor effect, prevented tumor cell growth *in vitro* and *in vivo*	[21,22,24]
miR133a	Downregulated in patient tumor tissues (its low expression correlated with poor differentiated tumors) and in HepG2, SMMC-77231, Hep3B, HuH-7 HCC cells	Through its binding to the 3'UTR of *ABCC1* gene specifically downregulated MRP1 expression	miR133a-overexpressing HepG2 cells were more sensitive to DOX-induced death	[27,28]
miR326	Downregulated in human tissues (its low expression correlated with tumor progression and lymph node metastasis) and in HepG2, SMMC-77231, Hep3B, HuH-7 HCC cells	Specifically targeted MRP1 expression through its binding to the 3'UTR of *ABCC1* gene	miR326-overexpressing HepG2 cells were more sensitive to DOX than control cells	[28,31]
miR223	Downregulated in HCC patient sera and liver biopsies	Through its binding to the 3'UTR of *ABCB1* gene, it specifically downregulated P-gp expression	miR223 overexpression increased sensitivity to DOX and paclitaxel in SMMC-7721 and HepG2 cells	[34,35]
miR491-3p	Downregulated in HCC tissues and in human cell lines (Hep3B, Bel-7402 and SMMC-7721 cells)	Negatively correlated with P-gp expression. Through its binding to the 3'UTR of *ABCB1* gene, it specifically downregulated P-gp expression and increased DOX intracellular concentration. Also, miR491-3p downregulated SP3 expression (transcription factor suggested to induce P-gp expression).	Conferred sensitivity to DOX and vinblastine in Hep3B and SMMC-7721 HCC cells	[38]
miR183	Overexpressed in liver tissues and in drug-resistant Bel-7402 cells	Positively correlated with P-gp and MRP2 protein expression	Conferred resistance to 5-FU in Bel-7402 cells	[41,42]

miRNA: MicroRNA; ABC: ATP-binding cassette; HCC: Hepatocellular carcinoma; DOX: Doxorubicin; 5-FU: 5-Fluoroacil; P-gp/*ABCB1*: P-glycoprotein; MRP1/*ABCC1*: Multidrug resistance-associated protein 1; 3'-UTR: 3'-untranslated region; MRP2: Multidrug resistance-associated protein 2.

the overexpression of P-gp in ovarian cancer and cervical carcinoma cells[16].

There was no information about the relationship between the miR27a and ABC transporters in HCC until 2013. Chen *et al*[17] developed Bel-7402 HCC cells resistant to 5-Fluoroacil (5-Fu). They found that these cells overexpressed P-gp while miR27a was almost undetectable. But when cells were transfected with miR27a, the authors observed more sensitivity to 5-Fu, DOX and mitomycin, and diminished expression of P-gp[17] (Table 1). Thus, levels of miRNA27a in HCC cells are low and seem to

negatively correlate with P-gp expression.

miR503

This molecule is downregulated in some types of cancers such as gastric and endometrial tumors, while it is overexpressed in others, like retinoblastoma or parathyroid carcinoma[18]. In HCC samples, this miRNA is downregulated with respect to non-tumor liver tissues, and this low level is related to malignant progression[19]. Furthermore, the expression of miR503 in different HCC cell lines is also decreased. Wang *et al*[20] found that miR503 was reduced in DOX-resistant HepG2 HCC cells. Besides, these cells overexpressed both P-gp and MRP1 transporters[20]. Further, HepG2 cells transfected to overexpress miR503 showed a reduction in the expression of both transporters at protein level, and at mRNA level as well (Table 1). Interestingly, cells overexpressing miR503 also showed an increased sensitivity to DOX and accumulated more intracellular rhodamine-123[20]. This fluorophore is known to be extruded from the cells through P-gp, therefore these results indicate that miR503 controls sensitivity to DOX-induced death in HepG2 cells by reducing P-gp and MRP1 expression and increasing intracellular drug concentration.

miR375

Initially, this molecule was identified in pancreatic cells. It regulates insulin secretion so it takes part of glucose homeostasis. In many cancers, it is downregulated and acts as a tumor suppressor[21]. In HCC, miR375 is one of the most downregulated miRNAs in tumor tissues and cell lines. Its ectopic overexpression was demonstrated to decrease liver cancer cell growth and invasion, and to promote apoptosis[21,22]. Moreover, miR-375 suppressed astrocyte elevated gene-1 (*AEG-1*) expression by binding directly to its 3'-UTR[22], and AEG-1 overexpression increased P-gp protein levels in HCC cells[23].

In a recent study, DOX-resistant HepG2 cells were established and they overexpressed P-gp. However, when miR375-containing lipid-coated nanoparticles were delivered to these cells, P-gp expression decreased under control cell levels. Also, in cell viability assays, the half inhibitory concentration (IC_{50}) of different HCC cell lines (HepG2, HuH-7, Hep3B and HepG2 resistant to DOX) incubated with nanoparticles containing both DOX and miR375 was lower than the IC_{50} of cells cultured with DOX or nanoparticles containing DOX alone (Table 1). Importantly, nanoparticles loaded with miR375 suppressed liver tumor growth, enhanced the therapeutic effect of DOX and reduced drug toxicity in mice[24].

miR133a

This miRNA is downregulated in breast cancer cell lines and tissues, and this decrease is related to advanced clinical stages[25]. Besides, it's downregulation has been associated with metastasis and recurrence of prostate cancer[26]. In HCC cell lines and tissues, miR133a was also found downregulated and its low expression was related with poor differentiated tumors[27]. Interestingly, predictions performed with computational programs revealed that the 3'UTR of MRP1/*ABCC1* transporter gene includes a binding site for miR133a (and also for miR326). By luciferase reporter assay it was confirmed that miR133a binds to the 3'UTR of *ABCC1* gene and controls MRP1 expression. Remarkably, miR133a overexpression in HepG2 cells induced a significant decrease in MRP1 mRNA and protein levels (Table 1). Besides, in the presence of DOX, cells overexpressing miR133a were more sensitive to drug-induced death than control cells[28].

miR326

This miRNA was found to be downregulated in gastric cancer[29]. In breast cancer cells and tissues its expression negatively correlated with MRP1 transporter levels. Furthermore, elevated miR326 levels sensitized cells to cytotoxic drugs[30]. In human HCC tissues, this miRNA was also found downregulated and its reduced expression correlated with tumor malignancy and patient lymph node metastasis. *In vitro*, miR326 inhibited tumor cell proliferation and invasion, and promoted apoptosis *in vivo*[31]. Regarding its role as modulator of ABC transporter expression, computational programs predicted a binding site for miR326 at the 3'UTR of *ABCC1* gene. This binding site was also confirmed by luciferase reporter assay (Table 1). In addition, it was demonstrated that HCC cells overexpressing miR326 had reduced levels of MRP1, both at mRNA and protein levels. Further, miR326-overexpressing HepG2 cells showed more sensitivity to DOX than control cells[28].

miR223

In colon cancer, miR223 is upregulated and acts as an oncogene. On the contrary, it is frequently downregulated in leukemia and lymphomas[32]. In HCC, this miRNA is

also reduced and it was shown to induce HepG2 and Bel-7402 cell growth suppression and to promote apoptosis[33]. Moreover, lower miR223 levels were found in HCC patient sera respect to healthy volunteers, and a reduced expression was also determined in liver biopsies compared with normal liver. Thus, this miRNA has been proposed as a novel potential biomarker for HCC[34].

Yang *et al*[35] showed that P-gp protein expression was higher in some HCC cell lines such as Hep3B, HCC3, LM-6, SMMC7721, HuH-7 and HepG2, than in others like Hep3B and BEL-7402 cells. Moreover P-gp levels positively correlated with DOX IC_{50}. Interestingly, using bioinformatics techniques miR223 was first predicted to bind to the 3'UTR of *ABCB1* gene. This was later confirmed by performing an EGFP reporter assay where miR223 was proved to specifically interact with the 3'UTR of *ABCB1* gene. Further, miR223 expression was found to be differentially expressed and inversely correlated with P-gp levels when comparison was made among the eight cell lines. Besides, inhibition of miR223 expression was verified to induce P-gp expression in most of the HCC cell lines analyzed[35]. In addition, transfection of HCC cells with miR223 mimics showed the inhibition of P-gp expression (Table 1), both at mRNA and protein levels (Table 1), and an increase in cell mortality under paclitaxel and DOX treatment. These results were confirmed by upregulating P-gp expression and through a rescue experiment by overexpressing *ABCB1* lacking the 3'UTR. Therefore, this study demonstrates that miR223 has a relevant role in HCC cell chemoresistance by controlling P-gp expression[35].

miR491-3p

In multidrug-resistant cancer tongue cells, this miRNA was found to be decreased and the induction of its expression sensitized cells to chemotherapy[36]. Also in osteosarcoma cells and tissues, miR491-3p was observed downregulated and when its expression was restored, the suppression of tumor growth and invasion was demonstrated[37].

Recently, it was demonstrated that this miRNA is also downregulated in HCC cell lines. Furthermore, in liver tumors miR491-3p levels were reduced compared with matched non-tumor tissues. Besides, P-gp levels were higher in HCC Hep3B, BEL-7402 and SMMC-7721 cells than in normal human liver cell lines (THLE-2 and THLE-3). Notably, P-gp expression inversely correlated with miR491-3p in both tumor and normal liver cells (Table 1). This negative correlation was also found in clinical samples[38]. Moreover, using bioinformatic algorithms, different miRNAs -including miR491-3p- were predicted as candidates to bind to the 3'UTR of *ABCB1* gene. miR491-3p specific interaction with the 3'UTRs of *ABCB1* was confirmed performing luciferase reporter gene assays. Interestingly, specificity protein 3 (SP3, a transcription factor suggested to induce *ABCB1* expression) was demonstrated to be another target of miR491-3p. miR491-3p levels inversely correlated with SP3 expression in both HCC cells and liver tumors. Remarkably, Hep3B cells transfected with miR491-3p mimics were more sensitive to DOX and vinblastine. On the contrary, overexpression of P-gp or SP3 restored chemoresistance reverting the sensitivity conferred by miR491-3p. Therefore, a loop including miR491-3p, SP3 and MDR1 in HCC cell chemoresistance was proposed[38].

miR183

The expression of this miRNA is deregulated in some malignancies such as leukemia, breast cancer and liver tumors[39]. miR183 downregulation correlated with metastasis in lung cancer[40]; however, this mRNA was upregulated in HCC tissues as compared to the adjacent non-tumor liver zone[41]. Further, the programmed cell death 4 (*PDCD4*) tumor suppressor gene was downregulated in HCC cells transfected with miR183[41]. Wang *et al*[42] showed that miR183 was overexpressed in HCC Bel-7402/5-Fu (resistant to 5-FU) cells. P-gp and MRP2 protein expression was also increased in Bel-7402/5-Fu cells (Table 1), and the suppression of miR183 in these cells diminished P-gp and MRP2 protein levels. In addition, the authors established that high levels of miR183 induced the expression of both transporters in control cells. So, these results indicate that miR183 modulates P-gp and MRP2 expression.

Thus, a large amount of evidence points that many miRNAs are deregulated in HCC and some of them are involved in chemoresistance. Nevertheless, until the recent years the precise mechanisms underlying miRNA-induced drug resistance in this malignancy have not been fully understood. Many efforts have been targeted to decipher how aberrantly expressed miRNAs affect tumor cell proliferation or apoptosis pathways. Here, we investigated thoroughly the recent findings that reveal that certain miRNAs regulate ABC transporter expression by specifically targeting, for instance, *ABCB1* and/or *ABCC1* genes. Most of these miRNAs are found downregulated in HCC tissues and cells. As a consequence, P-gp and/or MRP1 expression levels increase and intracellular therapeutic drug accumulation decrease,

making HCC cells less sensitive to death (Table 1). Altogether, these findings indicate that miRNAs have a key role in ABC transporter-mediated drug resistance in HCC.

AUTOPHAGY IN THE DEVELOPMENT OF HCC CHEMORESISTANCE

Autophagy is an evolutionary conserved mechanism that involves proteolytic degradation of cytosolic components at the lysosome through lysosomal enzyme action that facilitate degradation of sequestered products[43]. It occurs mainly as a response to cellular stress (infection, hypoxia, *etc.*) and its leading function is to grant nutrients for cellular functions and to remove unwanted material from the cytosol such as damaged organelles, acting as a cytoprotective system[44].

Autophagy includes five phases: initiation, elongation and autophagosome formation, fusion, and autolysosome formation. This process begins with the development of an isolated membrane, called phagophore, which origin is controversial. It expands and engulfs intracellular organelles or protein aggregates (for example) in a double-membrane vesicle called autophagosome. Then, maturation occurs when autophagosome fuses with a lysosome to form an autolysosome, promoting the degradation of the inside contents. Finally, aminoacids, fatty acids, and nucleotides are transported to the cytoplasm, so they can be re-used by the cell[43]. Thus, autophagy works as a recycling machinery that removes non-functional proteins and organelles.

The molecular pathway of autophagy has been thoroughly reviewed elsewhere[45,46]. Briefly, the initial phase is driven by the unc-51-like autophagy activating kinase (ULK) complex and the class III phosphatidylinositol 3-kinase (PtdIns3K) complex. The last complex produces phosphatidylinositol 3-phosphate for recruitment of other factors to the phagophore, and contains the key autophagy regulators vacuolar protein sorting 34 (VPS34), VPS15, Beclin 1 and activating molecule in Beclin 1-regulated autophagy (AMBRA1). Downstream, the autophagy-related gene 8 (ATG8) and ATG12 systems (two ubiquitin-like conjugation systems) mediate vesicle expansion. The E1-like protein ATG7 is required for activation of ATG8 [light chain 3 (LC3) in mammals] and ATG12. ATG8/LC3 is subjected to proteolysis and then is covalently attached to the lipid phosphatidylethanolamine (in mammalian cells, the precursor form is LC3-I and LC3-II is the lipidated one), by which it associates with the phagophore membrane. Thus, autophagy can be detected biochemically (by assessing LC3-II generation) or microscopically (by observing LC3 puncta formation, indicative of LC3 redistribution to the autophagosomes under development). The pathway includes other proteins such as ATG9, factors required for autophagosome–lysosome fusion (*e.g.*, lysosomal-associated membrane protein 2), vacuolar permeases that mediate amino acid efflux from the lysosome, and lysosomal enzymes involved in cargo degradation[45,46].

In normal liver, damaged mitochondria and mutated cells are removed through autophagy, and this mechanism suppresses tumor initiation. But once the tumor is established, autophagy acts as a pro-oncogenic factor, as it promotes tumor growth metastasis and resistance to therapeutic drugs (revised in[47,48]). Actually, inhibition of autophagy in HCC cells incubated with sorafenib and bevacizumab enhanced cell lethality[49,50]. Moreover, autophagy modulation participated in oncolytic virotherapy in a liver cancer stem cell model[51]. Thus, appropriate autophagy regulation could effectively suppress HCC growth and metastasis. In this section, we describe the recent findings concerning the effect of miRNAs in autophagy and chemoresistance in HCC.

miR26a/b

This molecule is upregulated in some malignant cancers such as glioma and glioblastoma. On the other hand, it is downregulated in some bladder tumors, breast cancer cell lines and tissues, and anaplastic thyroid carcinoma, among others[52]. Particularly in HCC, miR26a/b is downregulated and its overexpression was shown to reduce HepG2 and MHCC97-H HCC cell proliferation, migration and invasion[53]. Thus, miR26a/b might play a suppressive role in liver tumor progression.

Jin *et al*[54] found that DOX treatment induced autophagy and reduced miR26a/b levels in HepG2 cells. Further, in DOX-resistant HepG2 cells this miRNA was also downregulated. In both cell lines, incubation with autophagy inhibitors resulted in the upregulation of miR26a/b, whereas the opposite effect was observed when an autophagy inducer was used. This implies that autophagy can modulate the expression of this miRNA[54]. Remarkably, miR26a/b overexpression in resistant cells sensitized them to apoptosis induced by DOX. Moreover, when miR-26a/b was

combined with DOX treatment, miR-26a/b further improved the therapeutic effect of this drug on tumor growth *in vivo*. Besides, the authors performed bioinformatics analysis and reporter gene assays and found that ULK1 (a protein that participates in the initial stage of autophagy) is a target of miR26a/b in HCC cells (Table 2). The overexpression of miR26a/b induced the reduction not only of ULK1, but also of another proteins involved in autophagy (Beclin-1, ATG7 and LC3-II), on the contrary, downregulation of miR26a/b induced the increase of the formers. Thus, miR26 modulates autophagy (suppressing ULK1 expression) in order to promote apoptosis and sensitize HCC to chemotherapy. The authors propose that the combination of miR26a/b with chemotherapy could introduce a new strategy to overcome cancer[54].

miR199a-5p

In triple negative breast cancer cells this miRNA is downregulated, and suppresses cell migration and invasion[55]. On the other hand, it is upregulated in osteosarcoma cells and patient tissues and its knock-down brings about a reduction in cell proliferation and tumor growth[56]. In HCC cells and tissues, miR199a-5p is frequently downregulated and its overexpression inhibits cell proliferation, migration and invasion *in vitro* and *in vivo*[57]. Moreover, low miR199a-5p expression in HCC patients was associated with poor prognosis. Interestingly, cisplatin treatment decreased miR199a-5p levels and induced autophagy in two HCC cell lines, HuH-7 and HepG2. However, when the expression of miR199a-5p was forced, cisplatin-induced autophagy was inhibited and cisplatin-induced decrease of cell proliferation was increased. Accordingly, it was found that miR199a-5p interacts with the 3'UTR region of *ATG7* transcript (Table 2). Thus, cisplatin, *via* miR199a-5p downregulation, increases drug resistance by inducing autophagy in HCC cells[58].

miR101

This miRNA is involved in the development and progression of oral squamous-cell cancer. In these tissues, it is downregulated and related to patient poor prognosis[59]. In non-small cell lung cancer, it is almost undetectable and this absence is related to lymph node metastasis and poor prognosis of patients[60]. Further, in HCC it was observed that patients with distant metastasis had lower levels of miR101, and the downregulation of this miRNA correlated with adverse prognosis. Besides, lentivirus-delivered miR101 avoided tumor growth and metastasis in a HCC murine model[61].

In a study performed with HepG2 HCC cells, it was shown that miR101 induced apoptosis upon cisplatin treatment. By using cells transfected with a sequence of RNA that mimics miR101 it was observed that proteins involved in the phagosome formation stathmin 1 (STMN1), RAS related protein (RAB5A), autophagy-related 4D cysteine peptidase (ATG4D) and mTOR were miR101 targets (Table 2). On the other hand, the use of a sequence of RNA that specifically binds and inhibits miR101 increased the mRNA levels of the four genes. Moreover, miR101 inhibitor induced much more phagosome formation than miR101 mimics, showing that the absence of the miRNA induces autophagy. Besides, it was also demonstrated that in the presence of cisplatin, cells with lower levels of miR101 showed less apoptosis than the ones transfected with miR101 mimics. In conclusion, miR101 inhibits autophagy and increases apoptosis induced by cisplatin in HCC cells[62].

miR216b

miR216b is downregulated in colorectal cancer compared with normal tissues. High expression of this miRNA inhibited cell proliferation, migration and invasion in this type of cancer through targeting the expression of the oncogene serine-arginine protein kinase 1 (SRPK1)[63]. In cervical cancer tissues and cells it is also downregulated. Its overexpression inhibited cell proliferation in cultured HeLa cells, suggesting a possible tumor suppressor activity in this type of cancer[64]. In HCC patient plasma and tissues it was also found downregulated compared with healthy volunteers and non-tumor adjacent liver tissue, respectively. Further, low expression of miR216b correlated with patient poor prognosis[65]. In HepG2 and SMMC-7721 cells, low levels of miR216b stimulated cell proliferation, migration and invasion, while its overexpression produced the opposite effect[65]. Besides, miR216b levels in BEL-7402 HCC cells inversely correlated with the expression levels of metastasis associated lung adenocarcinoma transcript 1 (*MALAT1*), an oncogenic long non-coding RNA (LncRNA) that is generally upregulated in this tumor and can modulate chemosensitivity[66]. By performing gene reporter assays, it was shown that miR216b has two binding sites in *MALAT1*. Both *MALAT1*-siRNA and miR216b mimics inhibited autophagy in BEL-7402/5-FU (BEL-7402 cells 5-FU resistant) cells, and autophagy inhibition significantly increased 5-FU-induced cell apoptosis (Table 2). Moreover, these treatments decreased the IC_{50} of 5-FU, DOX, and mitomycin in BEL-7402/5-FU cells[66]. These results revealed the relationship between autophagy,

Table 2 miRNAs and autophagy in hepatocellular carcinoma cell drug resistance

miRNA	Expression Level in HCC	Effect on autophagy	Involvement in drug resistance	Ref.
miR26a/b	Downregulated in tissues and cell lines (HepG2, Hep3B, MHCC97-H and SMCC-7721), also by chemotherapeutic drugs and autophagy inhibitors	Inhibited autophagy, by targeting the expression of ULK1, the key initiator of autophagy	Sensitized HepG2 cells and tumors to apoptosis induced by DOX through targeting autophagy	[53,54]
miR199a-5p	Downregulated in HepG2 and HuH-7 cells and tissues, also by chemotherapeutic drugs	Inhibited cisplatin-induced autophagy, by interacting with the 3'UTR region of ATG7 transcript (an autophagy related gene)	Protected HepG2 and HuH-7 cells from cisplatin-induced resistance	[56,58]
miR101	Downregulated in cell lines (SMMC-7721, HepG2, Bel-4404, and 97L) and tissues, associated with distant metastasis and related to poor prognosis	Inhibited autophagy, by reducing STMN1, RAB5A, ATG4D and mTOR expression (involved in the phagosome formation)	Sensitized HepG2 cells to apoptosis induced by cisplatin	[61,62]
miR216b	Downregulated in patient plasma and tissues, related to poor prognosis	Inhibited autophagy, by targeting . *MALAT1* (an oncogenic long non-coding RNA generally upregulated in HCC that modulates chemosensitivity)	Sensitized BEL-7402/5-FU resistant cells to 5-FU-, DOX- and mitomycin-induced death	[65,66]
miR142-3p	Downregulated in tissues, also by sorafenib treatment	Inhibited sorafenib-induced autophagy by binding to the 3'-UTR of ATG5 and ATG16L1	Sensitized SMCC-7721 and HepG2 cells to sorafenib-induced death	[69,70]
miR21	Overexpressed in patient tissues and in drug-resistant cells	Inhibited autophagy, and downregulated PTEN pathway	miR21-dependent autophagy inhibition contributed to sorafenib resistance in Huh7 and HepG2 cells and HCC tumors developed in mice	[72-74]
miR423-5p	Overexpressed in tissues, also elevated in serum of sorafenib-treated patients, and secreted in high levels from sorafenib-treated cells	Induced autophagy	Proposed as a helpful tool to predict patient response to sorafenib treatment	[77,78]

miRNA: MicroRNA; HCC: Hepatocellular carcinoma; ULK1: Unc-51 like autophagy activating kinase; DOX: Doxorubicin; 3'-UTR: 3'-untranslated region; ATG: autophagy-related protein; STMN1: Stathmin 1; RAB5A: RAS related protein; ATG4D: autophagy-related 4D cysteine peptidase; mTOR: The mammalian target of rapamycin; *MALAT1*: Metastasis associated lung adenocarcinoma transcript 1; 5-FU: 5-Fluoroacil; PTEN: Phosphatase and tensin homologue.

MALAT1 and miR216b, contributing to HCC chemosensitivity.

miR142-3p

This miRNA is downregulated in non-small cell lung cancer (NSCLC) tissues and cell lines. At high levels, it prevents tumorigenesis inhibiting the expression of one of the high mobility group protein superfamily, HMGB1, involved in cancer cell migration and invasion[67]. Besides, this miRNA is diminished in ovarian cancer tissues and cell lines, and its downregulation is related to poor differentiation. Further, it was demonstrated that overexpression of miR142-3p inhibited proliferation and chemoresistance in ovarian cancer cells[68]. In HCC tissues it is also downregulated, and the expression levels decreased as the disease progressed. miR142-p3 overexpression inhibited BEL-7402 and SMMC-7721 HCC cell migration and invasion, thus, it was suggested to suppress metastasis[69].

Zhang *et al*[70] studied the role of miR142-3p in sorafenib resistance in SMMC-7721 and HepG2 HCC cells. They showed that cells incubated with this drug presented a higher autophagic flux than control cells in a dose- and time-dependent manner. When autophagy was inhibited, cells become sensible to the drug. They also showed that miR142-3p was downregulated in HCC cells under sorafenib treatment. Furthermore, it was shown that high levels of the miRNA negatively regulated sorafenib-induced autophagy in HCC cells by binding to the 3'-UTR of ATG5 and ATG16L1 mRNAs (both part of the autophagy machinery) (Table 2). Therefore, these data indicate that miR142-3p downregulation induces autophagy by increasing the

level of ATG5 and ATG16L1, and thus promoting sorafenib resistance in HCC[70].

miR21

This miRNA is frequently upregulated in cancer cell lines and human tumors. It is very important in oncogenic processes as it is associated with high proliferation, reduced apoptosis, metastasis potential and invasion[71]. In HCC it is also overexpressed and it was suggested to be related to tumor progression in patients[72]. Treatment with an oligonucleotide anti-miR21 led to the loss of viability, induction of apoptosis and necrosis in different HCC cell lines. Further, anti-miR21 also diminished cell migration and suppressed clonogenic growth[73].

He *et al*[74] developed sorafenib-resistant HuH-7 and HepG2 HCC cells and found that miR21 was upregulated. Interestingly, drug-sensitive parental cells transfected with miRNA21 mimics also became resistant. It was also demonstrated that sorafenib upregulated the expression of two key autophagic proteins (LC3-II and Beclin-1), but the expression level of both proteins in parental cells was higher than in drug-resistant ones. Besides, parental cells showed more acidic vesicular organelles than resistant cells. These results indicated that autophagy is more activated in parental cells than in the resistant ones. Interestingly, autophagy induction with rapamycin inhibited sorafenib-resistant cell growth, indicating that this process increases sensitivity to sorafenib in resistant cells[74]. Further, inhibition of miR21 by using an antisense miRNA oligonucleotide, re-sensitized resistant cells to sorafenib by promoting autophagy and stimulating apoptosis. On the other hand, miR21 mimics reduced autophagy, apoptosis, and the expression of phosphatase and tensin homologue (PTEN) protein (a known target of miR21) in parental cell lines (Table 2). Finally experiments performed with resistant cell-derived tumors established in mice showed that sorafenib administration plus intratumoral injection of anti-miR21 oligonucleotides induced a reduction in tumor size. Moreover, sorafenib administration downregulated PTEN, upregulated LC3-II and Beclin-1 expression in tumor lysates, and increased the activation of p-AKT. While anti-miR21 induced the upregulation of PTEN, LC-II and Beclin-1, and reduced expression of p-AKT[74]. Therefore, acquired sorafenib resistance might be mediated by the overexpression of miR21 and the inhibition of autophagy, probably by regulating the PTEN/AKT pathway.

miR423-5p

This miRNA, overexpressed in glioblastoma, was shown to enhance angiogenesis, tumor cell growth and invasion[75]. In gastric cancer, miR423-5p was also upregulated, and this overexpression favored cell cycle progression and decreased cell migration and invasion[76]. Particularly in HCC tissues it is upregulated, and high levels of this miRNA constitute a factor that increases cell invasiveness in HCC cells[77].

Interestingly, sorafenib treatment increased miR423-5p levels in conditioned media of HepG2 and HuH-7 HCC cells and in serum of patients, suggesting a potential role of miR423-5p as a marker of sorafenib response in HCC patients. Transfection of HCC cells with an anti-miRNA diminished LC-II and ATG7 protein levels (an autophagic vacuole formation protein). Accordingly, transfection with miR423-5p mimic induced vacuole and autophagosome formation (Table 2), and the opposite was observed with the miRNA inhibitor. In conclusion, it was suggested that this miRNA has a biological role in autophagy in HCC and it was proposed as a helpful tool to predict patient response to sorafenib treatment[78].

Thus, most miRNAs described here target the expression of autophagy-related genes, inhibiting autophagy and acting as tumor suppressors. However, in HCC tissues and cells many of these miRNAs are downregulated, or their levels are reduced after therapeutic drug treatment. As a consequence of this downregulation, miRNAs do not inhibit autophagy and tumor growth and resistance is favored (Table 2).

CONCLUSION

miRNAs are deregulated in HCC cells and tissues, and they play crucial roles in the development of chemoresistance. Emerging studies point that the modulation of ABC transporter and/or autophagy-related gene expression could be determinant for HCC cell survival under chemotherapeutic drug treatment. Our knowledge about the precise mechanisms regarding miRNA involvement in resistance will lead us to find new ways of making HCC treatment more effective. The development of miRNAs, miRNAs mimics or anti-miRNA with long half lives and their use in combination with chemotherapeutic drugs could be a powerful option. Even so, there are still difficulties to overcome prior the possibility of being able to use miRNAs in clinical

trials, for instance, the right delivery system. Nowadays, much research is being conducted on the use of nanoparticles to get over this trouble. Undoubtedly, more insights on the biological processes, signaling pathways and/or molecular mechanisms regulated by miRNAs are needed. Anyway, miRNA-based therapy together with conventional chemotherapeutic drugs has a great future in cancer therapy.

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ORIGINAL ARTICLE

Basic Study

Beneficial effects of losartan or telmisartan on the local hepatic renin-angiotensin system to counter obesity in an experimental model

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Author contributions: Graus-Nunes F performed the majority of experiments and analyzed the data; Santos FO, Marinho TS and Miranda CS performed the molecular investigations; Graus-Nunes F, Oliveira FO, Marinho TS and Miranda CS participated equally in the handling of animals; Graus-Nunes F, Barbosa-da-Silva S and Souza-Mello V designed and coordinated the research; Graus-Nunes F, Oliveira FO and Souza-Mello V wrote the paper.

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Abstract

BACKGROUND

Obesity has been associated with hepatic overexpression of the renin-angiotensin system (RAS).

AIM

To evaluate the action of two angiotensin II (ANGII) receptor blockers (losartan or telmisartan) on the modulation of local hepatic RAS and the resulting metabolic effects in a diet-induced obesity murine model.

METHODS

Twenty C57BL/6 mice were randomly divided into two nutritional groups for 10 wk: control group (C, n = 5, 10% of energy as fat) or high-fat group (HF, n = 15, 50% of energy as fat). After treatment started, the HF group was randomly divided into three groups: untreated HF group (n = 5), HF treated with losartan (HFL, n = 5) and HF treated with telmisartan (HFT, n = 5). The treatments lasted for 5 wk, and the dose was 10 mg/kg body mass.

RESULTS

HF diet induced body mass gain (+28%, $P < 0.0001$), insulin resistance (+69%, $P = 0.0079$), high hepatic triacylglycerol (+127%, $P = 0.0004$), and overexpression of intrahepatic angiotensin-converting enzyme (ACE) 1/ ANGII type 1 receptor (AT1r) (+569.02% and +141.40%, respectively, $P < 0.0001$). The HFL and HFT groups showed higher ACE2/rMAS gene expression compared to the HF group (ACE2: +465.57%, $P = 0.0002$ for HFL and +345.17%, $P = 0.0049$ for HFT; rMAS:

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+711.39%, $P < 0.0001$ for HFL and +539.75%, $P < 0.0001$ for HFT), followed by reduced insulin/glucose ratio (-30% for HFL and -33% for HFT, $P = 0.0181$), hepatic triacylglycerol levels (-28%, $P = 0.0381$ for HFL; and -45%, $P = 0.0010$ for HFT, and Plin2 expression.

CONCLUSION
Modulation of the intrahepatic RAS, with favored involvement of the ACE2/rMAS axis over the ACE1/AT1r axis after losartan or telmisartan treatments, caused hepatic and metabolic beneficial effects as demonstrated by reduced hepatic triacylglycerol levels coupled with reduced PLIN 2 expression and improved glycemic control.

Key words: Liver; Telmisartan; Losartan; Obesity; Angiotensin II type 1 receptor; C57BL/6 mouse

Core tip: Studies that address regulation of the local renin-angiotensin system (RAS) have accumulated since it was established that obese subjects overexpress components of the classical RAS in organs like the liver. Herein, we show evidence that two different angiotensin II receptor blockers modulate the intrahepatic RAS, favoring the angiotensin-converting enzyme (ACE) 2/rMAS axis over the ACE1/angiotensin II type 1 receptor in mice fed a high-fat diet. These drugs mitigated hepatic steatosis by reducing hepatic triacylglycerol levels and decreasing body mass and improving glycemic control. These drugs could be a viable option to treat non-alcoholic fatty liver disease in obese and/or hypertensive patients.

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INTRODUCTION

The most prevalent liver disease in the world, non-alcoholic fatty liver disease (NAFLD), affects 20 to 30% of the adult population and has a significant association with obesity, insulin resistance, dyslipidemia and hypertension[1]. Considered to be a hepatic manifestation of metabolic syndrome, NAFLD is comprised of histological changes ranging from isolated steatosis to non-alcoholic steatohepatitis (NASH) characterized by steatosis, ballooning, and inflammation of hepatocytes with or without fibrosis[2].

The renin-angiotensin system (RAS) is an important physiological regulator of blood pressure, electrolyte balance, and fluid homeostasis. angiotensin II (ANGII) is the major effector of the cascade and is related to organ dysfunction and chronic tissue damage through its profibrotic effects[3]. Almost all of the standard RAS components are expressed in the liver, and local RAS activation has been associated with liver lesion pathophysiology[4]. Based on elevated levels of several RAS components during the progression of hepatic fibrosis, the role of RAS in hepatic fibrosis has been attributed to the classical RAS axis [angiotensin-converting enzyme (ACE) 1/ANGII/ ANGII type 1 receptor (AT1r)][5,6].

The RAS has been a frequent target for pharmacological intervention regarding systemic arterial hypertension, but the use of these drugs may also contribute to minimizing organ damage such as in the pancreas and the liver[7,8]. Therefore, attenuating the effects of ANGII by ANGII receptor blockers (ARBs, losartan or telmisartan) could be beneficial for NASH and other components of metabolic syndrome. The present study aimed to evaluate the role of two ARBs (losartan or telmisartan) in modulating the local hepatic RAS and the resulting metabolic effects in a diet-induced obesity murine model.

MATERIALS AND METHODS

Ethical approval

The experimental protocol and all procedures were carried out in accordance with the guide for the care and use of laboratory animals from the National Institute of Health (NIH, publication number 85–23, revised in 1996) and approved by the Ethical Committee in animal experimentation from the State University of Rio de Janeiro (CEUA/013/2015).

Animals, diets and treatment

Male C57BL/6 mice (n = 20) were group housed in pathogen-free cages under controlled conditions (temperature 20°C ± 2°C, humidity 60% ± 10%, 12/12 h light/dark cycle) and with free access to food and water.

At 3-mo-old, mice were randomly divided into two nutritional groups to be fed for 10 wk: control group (C, n = 5), who received a purified control diet (76% of total energy as carbohydrate, 14% of total energy as protein and 10% of total energy as lipids, total energy 15 kJ/g), and high-fat group (HF, n = 15), who received a purified diet rich in saturated fatty acids from lard (36% of total energy as carbohydrate, 14% of total energy as protein and 50% of total energy as lipids, total energy 21 kJ/g). Then, the animals fed HF chow were randomly allocated into three groups, and each group received one of the following treatments over 5 wk (the drugs were mixed into the diets, as follows): (1) HF group (n= 5), untreated; (2) HFL group (n= 5), HF diet treated with losartan (L, 10 mg/kg/d, Cozaar, Merck); (3) HFT group (n= 5), HF diet treated with telmisartan (T, 10 mg/kg·d, Micardis; Boehringer, Ingelheim). Sample size was established according to a minimum that could be conclusive with a $P < 0.05$, as previously reported[9]. The diets were produced by the PragSolucoes company (Jau, São Paulo, Brazil) based on the recommendations of the American Institute of Nutrition (AIN-93M)[10]. The drugs were mixed with the diet in order to minimize differences in the daily dose intake by each animal.

Energy intake was measured as the product of food consumption by the energy content of the diet (in kJ/g). The body mass (BM) of the animals was measured weekly.

Systolic blood pressure

Before the official evaluations, animals were trained for 4 wk in constraint conditions to minimize their stress. Systolic blood pressure (SBP) was monitored weekly by tail-cuff plethysmography in conscious mice (Letica LE 5100, Harvard/Panlab, Barcelona, Spain).

Sacrifice and tissue extraction

At the conclusion of the experiment, the animals were deprived of food for six hours and were deeply anesthetized (sodium pentobarbital 150 mg/kg). Blood was collected by cardiac puncture. Plasma was separated by centrifugation (400 G for 20 min) at room temperature and stored at -20°C or –80°C until analysis. The liver was weighed and sliced into several fragments and frozen at -80°C for molecular studies.

Biochemical analysis

Blood glucose was measured using a glucometer (Accu-Chek, Roche, Sao Paulo, SP, Brazil) after the six-hour fast period before sacrifice. The concentration of insulin was analyzed using a kit (Rat/Mouse Insulin ELISA kit Cat # EZRMI-13 K, Millipore, Missouri, United States) and a TP-READER Thermoplate (Bio Tek Instruments, Inc Highland Park, United States). The ratio of insulin and glucose was calculated to evaluate insulin resistance.

The hepatic cholesterol and triacylglycerol levels were measured in hepatic tissue by routine protocols described previously[11]. Briefly, frozen samples (50 mg) were placed in an ultrasonic processor with 1 ml isopropanol, and the homogenate was centrifuged at 2000 G. The supernatant (5 μL) was analyzed using a kit for measuring triacylglycerol or cholesterol (automatic analyzer K55, Bioclin System II; Quibasa, Belo Horizonte, MG, Brazil). Alanine aminotransferase (ALT) was measured in the plasma using a kinetic colorimetric method (Bioclin System II; Quibasa, Belo Horizonte, MG, Brazil).

Immunofluorescence

Fixed tissue fragments were embedded in Paraplast Plus (Sigma-Aldrich Co., St. Louis, MO, United States) and sectioned at a thickness of 5 micrometers.

Liver sections (deparaffinized and hydrated) underwent antigen retrieval using citrate buffer at pH 6.0, and were blocked in 2% glycine and 5% BSA in PBS. Sections were incubated with anti-AT1R (anti-rabbit, AB15552, 1:100, Millipore) or anti-PLIN 2

(anti-rabbit, CSB-PA920084, 1:100, Millipore), in 1% BSA in PBS for 2 h.

Subsequently, samples were treated with a secondary antibody conjugated to Alexa 488 fluorophore (anti-rabbit IgG-Alexa 488, for AT1R and PLIN 2, 1:100), and slides were mounted with SlowFade (Invitrogen, Molecular Probes, Carlsbad, CA, United States). Digital images were captured using a confocal laser scanning microscope (Nikon C2; Nikon Instruments Inc., Tokyo, Japan).

Quantitative real-time PCR

Quantitative real-time PCR (RT-qPCR) was performed to examine RAS-related mRNA levels in the livers of mice. Total liver RNA was extracted using Trizol (Invitrogen, CA, United States). The RNA concentration was determined by spectroscopy using the Nanovue (GE Life Sciences) equipment using 1 μg RNA and DNAse I (Invitrogen). The cDNA was synthesized using Oligo (dT) primers and Superscript III transcriptase-reverse (Invitrogen, CA, United States). Real-time PCR was performed using the Biorad CFX96 thermal cycler and SYBR Green mix (Invitrogen, CA, United States). The primers were designed using Primer 3web online software version 4.0. The beta-actin gene was used as an endogenous control to normalize target gene expression. The primer efficiency of the target genes and the control gene were approximately equal, as calculated by using serial dilutions of cDNA. PCR reactions were performed following a program of denaturation and activation of the polymerase (4 min at 95°C), with 44 cycles, each consisting of 95°C for 10 s and 60°C for 15 s, followed by a melting curve (60–95°C, with a heating rate of 0.1 °C/s). Negative controls consisted of wells in which cDNA was replaced by deionized water. The relative expression ratio of mRNA was calculated by the $2^{\Delta\Delta}$Ct method. Sense and antisense primers sequences are described in Table 1.

Statistical analysis

After confirming normality and homoscedasticity of variances, data were displayed as the mean and standard deviation. Comparisons among groups were tested by a *t*-test or one-way ANOVA followed by Holm-Sidak post-hoc test when indicated. In all cases, $P < 0.05$ was considered statistically significant (GraphPad Prism version 7.04 for Windows, GraphPad Software, La Jolla, CA, United States).

RESULTS

Food intake and body mass

All animals tolerated the diet well and did not show any sign of disease during the experiment. All animals were included in the analysis ($n = 5$ per group).

Food intake was evaluated and showed no difference between the HF group and the C group, or between the HFL and the HF group. The HFL group did not demonstrate any differences in dietary energy intake compared to the HF group. However, the HFT group had lower energy intake compared to the HF group (-21%, $P = 0.0010$) and the HFL group (-22%, $P = 0.0008$). These results are detailed in Table 2.

At the 10th week, after the induction of obesity, the HF group was heavier than the C group (+28%, $P < 0.0001$). At the end of the treatment, at the 15th week, the HFT group had a significant reduction in BM compared to the HF group (-23%, $P < 0.0001$) and HFL group (-17%, $P < 0.0001$). Losartan treatment reduced BM compared to the HF group (-9%, $P < 0.0001$). Figure 1A depicts these findings.

Systolic blood pressure

The HF group had an 18% increase in SBP values compared to the C group (+15%, $P < 0.0001$). Both treated groups showed a marked reduction in SBP values compared to the untreated HF group (-14%, $P < 0.0001$ for both HFL and HFT, Figure 1B). There was no difference between the HFL and the HFT groups regarding SBP values.

Fasting glucose levels and I/G ratio

We did not observe a difference in fasting glucose between the HF-L and HF groups. Nevertheless, the HF-T group showed significantly reduced fasting glucose levels compared to the HF group (-9%, $P = 0.0210$, Table 2).

Insulin resistance was detected in the HF group due to a higher I/G than the C group (+69%, $P = 0.0079$, Table 2). Both treatments significantly decreased the I/G ratio compared to the HF group (-30%, $P = 0.0181$, HFL; -33%, $P = 0.0181$, HFT, Table 2), with levels similar to the C group.

Hepatic parameters

The HF group showed higher hepatic cholesterol (+9%, $P < 0.0001$) and triacylglycerol levels (+127%, $P = 0.0004$) than the C group. Only telmisartan treatment significantly

Table 1 Forward and reverse sequences of RT-qPCR primers

Primers		
Gene	5′- 3′	3′-5′
Renin	ACCTTGCTTGTGGGATTCAC	CCTGATCCGTAGTGGATGGT
ACE1	GTGGCTGGAAGAGCAGAATC	GCCTTGGCTTCATCAGTCTC
ACE2	CAACAGAAGCCAGACAACA	GCCTTGGCTTCATCAGTCTC
AT1r	CCCTGGCTGACTTATGCTTT	ACATAGGTGATTGCCGAAGG
AT2r	GAAGCTCCGCAGTGTGTTTA	TGGCTAGGCTGATTACATGC
rMAS	TTCTCCACCATCAACAGCAG	CCTGGGTTGCATTTCATCTT
B-actin	TGTTACCAACTGGGACGACA	GGGGTGTTGAAGGTCTCAAA

ACE1: Angiotensin-converting enzyme; ACE2: Angiotensin-converting enzyme 2; AT1r: Angiotensin type-1 receptor; AT2r: Angiotensin type-2 receptor; rMAS: MAS receptor.

reduced hepatic cholesterol levels compared to the HF group (-3%, P = 0.0271). Conversely, hepatic triglyceride levels were decreased by both treatments compared to the HF group (-28%, P = 0.0381 for HFL; and -45%, P = 0.0010 for HFT).

ALT enzyme concentrations were markedly enhanced by the HF diet compared to the C group (+56%, P < 0.0001). The treatments were effective in reducing ALT levels compared to the HF group (-14%, P = 0.0082 for HFL and – 15%, P = 0.0064 for HFT). The effects of losartan or telmisartan treatment on ALT concentrations were not statistically different. These results are detailed in Table 2.

Immunofluorescence

Immunofluorescence for AT1r revealed a negative reaction for both treatment groups (losartan and telmisartan), thus confirming the blockade of AT1r by both drugs and validating our study design. In contrast, the HF group showed widespread positive immunostaining for AT1r, consistent with over-activation of the classic local RAS axis in the liver of obese animals (Figure 2, upper panel).

The beneficial effects of both treatments on weight management and insulin sensitivity might be, at least in part, mediated by decreased expression of PLIN 2 on lipid droplet surface membranes. Both HFL and HFT groups showed weaker PLIN 2 expression than the HF group, which showed more intense immunostaining. Figure 2 (lower panel) depicts these findings.

RT-qPCR

Renin gene expression was reduced in the HF group compared to the C group (-84%, P < 0.0001). Neither treatment significantly changed renin gene expression, which remained lower than the C group (-82.30%, P < 0.0001 for HFL and -79.93%, P < 0.0001 for HFT). These results are detailed in Figure 3A.

ACE1 gene expression was markedly increased by the HF diet, as the HF group showed higher ACE1 gene levels than the C group (+569.02%, P < 0.0001). On the contrary, both treatments restored ACE1 gene expression to levels lower than the HF group (-94.97%, P < 0.0001 for HFL and -81.65%, P < 0.0001 for HFT) and similar to the C group. These results are depicted in Figure 3B.

The HF group also showed higher AT1r gene expression than the C group (+141.40%, P < 0.0001). Conversely, both treatments restored AT1r gene expression to values lower than the HF group (-78.95%, P < 0.0001 for HFL and -82.54%, P < 0.0001 for HFT). These results are found in Figure 3C.

In contrast to the AT1r results, the losartan and telmisartan treatments enhanced AT2r gene expression compared to the HF group (+320.70%, P<0.0001 for HFL and +354.11% for HFT). These results are detailed in Figure 3D.

ACE2 gene expression was reduced in the HF group in comparison to the C group (-75.69%, P = 0.004). Both losartan and telmisartan significantly enhanced ACE2 gene expression (+465.57%, P = 0.0002 for HFL and +345.17%, P = 0.0049 for HFT). These results are depicted in Figure 4A.

In agreement with ACE2 gene expression, the HF group showed lower rMAS gene expression than the C group (-51.37%, P = 0.041). Notably, both treatments enhanced rMAS gene expression compared to the HF group (+711.39%, P < 0.0001 for HFL and +539.75%, P < 0.0001 for HFT). These results are shown in Figure 4B.

Table 2 Food behavior, carbohydrate metabolism and hepatic parameters

Data	C	HF	HFL	HFT
Food behavior				
Energy intake (kJ/ mouse)	36.8 ± 0.8	53.3 ± 4.7[a]	53.4 ± 3.5[a]	41.9 ± 4.3[c,e]
Carbohydrate metabolism				
Fasting glycemia (mg/dL)	120.4 ± 3.9	152.4 ± 8.5[a]	151.8 ± 6.3[a]	139.4 ± 6.8[a,c,e]
I/G	0.16 ± 0.03	0.27 ± 0.02[a]	0.19 ± 0.01[c]	0.18 ± 0.01[c]
Hepatic parameters				
Hepatic Cholesterol (mg/g liver)	8.8 ± 0.2	9.6 ± 0.1[a]	9.5 ± 0.1[a]	9.3 ± 0.1[a,c]
Hepatic Triglycerides (mg/g liver)	10.4 ± 4.1	23.6 ± 4.7[a]	17.1 ± 3.9[a,c]	12.9 ± 2.2[c]
ALT (IU/L)	18.3 ± 2.8	28.6 ± 3.1[a]	22.8 ± 3.2[a,c]	22.5 ± 2.0[ac]

Data are expressed as the mean ± SD, (n = 5). $P < 0.05$ compared with the C group (a), HF group (c) and HFL group (e) (one-way ANOVA and post-hoc Holm-Sidak test). C: Control group; HF: High-fat diet; HFL: HF diet treated with losartan; HFT: HF diet treated with telmisartan; I/G: Insulin/glucose ratio; ALT: Alanine aminotransferase.

DISCUSSION

The present results confirm that diet-induced obesity induces overexpression of ACE1-AT1r genes in the liver of mice, whereas both losartan and telmisartan restored local ACE1-AT1r gene expression to values similar to the lean animals. Moreover, the beneficial effects from both ARBs on insulin resistance, body mass, SBP, hepatic cholesterol, and triacylglycerol levels might stem from the stimulation of the ACE2-rMAS axis, whose components showed increased hepatic gene expression after selective AT1r blockade.

Telmisartan treatment has been linked to decreased energy intake and reduced body mass gain despite the maintenance of a HF diet during the experimental protocol. Some mechanistic explanations rely on the activation of PPARdelta-dependent lipolytic pathways and activation of the local RAS in the brain, reducing energy intake and increasing energy expenditure[12,13].

The reduced energy intake might raise some doubts regarding the real contribution of telmisartan to the observed body mass loss. However, a previous study revealed that the reduced energy intake in the absence of treatment (pair-feeding group) did not produce a significant reduction in body mass. Hence, the noticeable body mass loss can be attributed to telmisartan action once the pair-feeding group remained overweight[7]. On the other hand, recent reports stated that losartan treatment does not influence food intake[12,14]. In agreement with these observations, losartan treatment did not change energy intake herein, though it elicited a slight reduction in body mass.

Both losartan and telmisartan were effective in reducing SBP values, even with a chronic HF diet intake, as previously shown[14,15]. A recent human study reported equal efficacy of both losartan and telmisartan on blood pressure control. Nevertheless, telmisartan shows more favorable effects on lipid profiles, implying an additive beneficial effect in patients[16].

It is worth mentioning that the reduction of fasting glycemia was only observed following telmisartan treatment, but both treatments affected insulin resistance, suggesting that the normalization of insulin sensitivity is independent of the effects of PPAR-gamma activation and relies more on AT1r blockade[17]. This is consistent with our previous findings in the pancreas, where relief in hyperinsulinemia stemmed from adequate glucose-stimulated insulin secretion by pancreatic islets as a result of the selective AT1r blockade[7].

Favorable effects of telmisartan on metabolism have been related to its dual property as an ARB and partial PPAR-gamma agonist[18]. In the liver, combined partial PPAR-gamma and PPAR-alpha activation play a pivotal role in regulating carbohydrate and lipid metabolism through the transcription of their target genes, with marked effects on lipotoxicity and insulin resistance control[8,19,20]. Hence, the normalization of hepatic triacylglycerol levels due to telmisartan is consistent with the augmented insulin sensitivity and better glycemic control, implying a favored hepatic beta-oxidation over lipogenesis[21].

On the contrary, losartan treatment did not minimize the harmful effects of the HF diet upon fasting glycemia and hepatic cholesterol levels, besides the less noticeable impact on hepatic triacylglycerol levels than telmisartan. In agreement with our

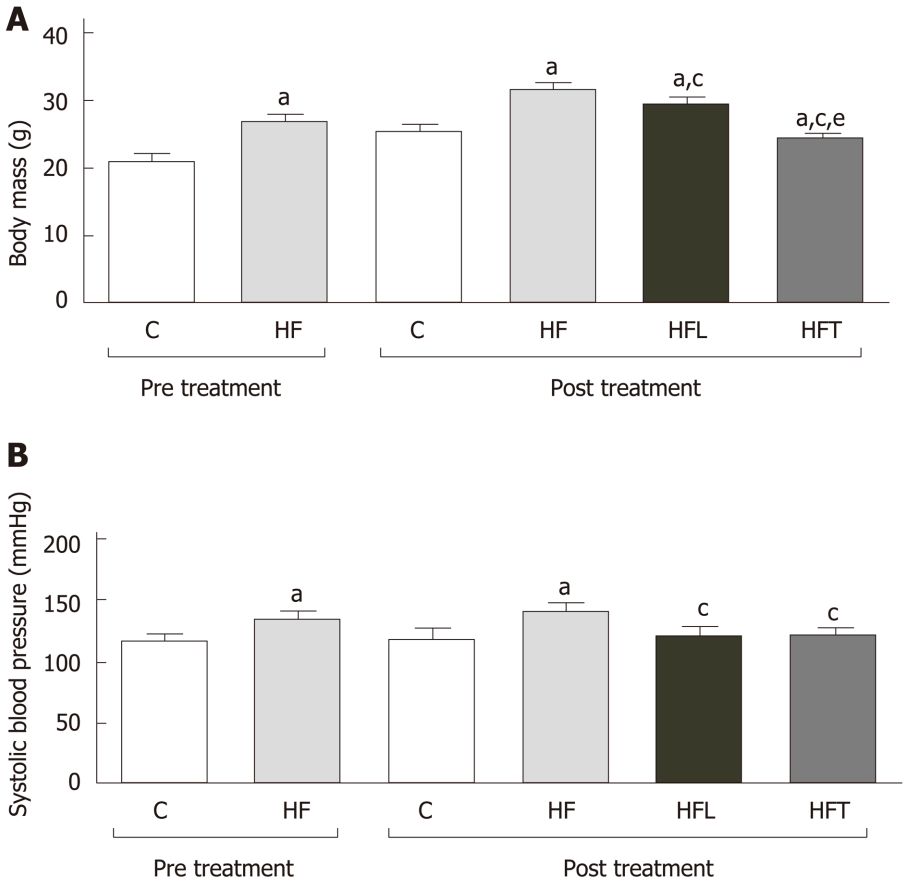

Figure 1 Body mass (A) and systolic blood pressure (B) pre-treatment (10th week) and end of treatment (15th week). Data are the mean ± SD, $n = 5$ for each group. Differences between the groups were tested with one-way ANOVA and Holm–Sidak post hoc test ($P < 0.05$) compared to the C group (a), HF group (c) and HFL group (e). C: Control group; HF: High-fat diet; HFL: HF diet treated with losartan; HFT: HF diet treated with telmisartan.

observations, a recent study using a high fat-high sucrose diet showed that losartan did not significantly change the glucose levels nor influence total cholesterol levels[22]. Increased hepatic cholesterol levels imply that the liver is more susceptible to harmful forms of liver disease because it correlates with hepatic inflammation and impaired transcriptional response[23].

In obesity, there is an interaction between hepatic cholesterol metabolism and intrahepatic RAS activation[24]. HF animals showed hepatic overexpression of ACE1/AT1r and reduced expression of ACE2/MASr parallel to enhanced hepatic cholesterol levels. Intrahepatic RAS overexpression triggers extracellular matrix synthesis and impairs LDL receptor function, leading to adverse liver remodeling[25]. These events are mediated by AT1r activation by ANGII and predispose the subject to adverse hepatic remodeling and hepatic steatosis[26].

Conversely, HF animals treated with losartan or telmisartan benefited from AT1r blockade, as they favored the ACE2/MASr axis over the ACE1/AT1r axis, which has been previously associated with the amelioration of glucose intolerance, hepatic inflammation, and prevention of steatosis and fibrosis[8,15,20]. The RAS modulation by telmisartan can explain previous beneficial effects on the livers of an animal model of hepatic fibrosis[27] and in human with steatohepatitis[28], without side effects. Although the HF diet did not induce fibrosis, our results show that the AT1R blockade reduced PLIN 2 expression and hepatic triacylglycerol levels, both of which are strongly correlated with reduced hepatic steatosis[11,29].

Some tissues can store excessive lipids in cytoplasmic lipid droplets, which are dynamic organelle-like structures[29]. PLIN 2 is a cytoplasmic lipid droplet-associated protein, and mice lacking PLIN 2 are resistant to obesity-associated fatty liver disease, as its deficiency is linked to increased PPAR-alpha activation and subsequent increases in beta-oxidation[30]; suppressed SREBP-1 and SREBP-2 and the resulting suppression of *de novo* lipogenesis and hepatic cholesterol biosynthesis[30]; and enhanced hepatic FGF21 expression and the browning of white adipose tissue with metabolic improvements[31].

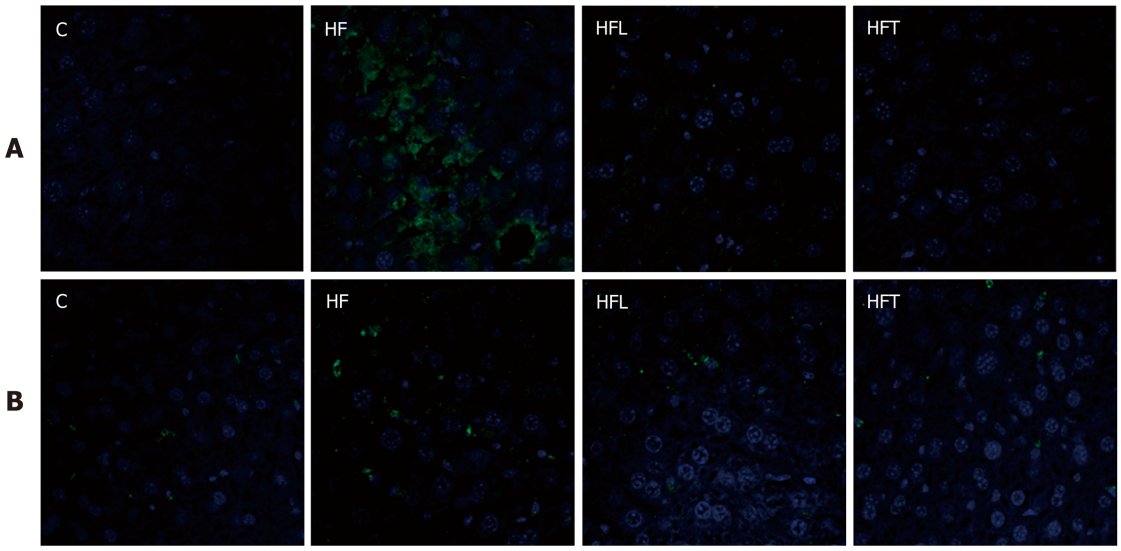

Figure 2 Angiotensin type-1 receptor (A) and PLIN 2 (B) assessed by immunofluorescence (green) in the liver. Scale bars correspond to 50 μm, same magnification for all immunoreactions (40 x). C: Control group; HF: High-fat diet; HFL: HF diet treated with losartan; HFT: HF diet treated with telmisartan.

In conclusion, we propose that the modulation of intrahepatic RAS, with a preference for the ACE2/rMAS axis over the ACE1/AT1 axis after losartan or telmisartan treatments, cause beneficial hepatic and metabolic effects, as demonstrated by reduced hepatic triacylglycerol levels coupled with reduced PLIN 2 expression and improved glycemic control. Additional beneficial effects of telmisartan were perceived in hepatic cholesterol levels and normalization of fasting glycemia. Both drugs, which are frequently used as anti-hypertensive agents, can be a useful option for obese patients to control IR and NAFLD through AT1r blockade.

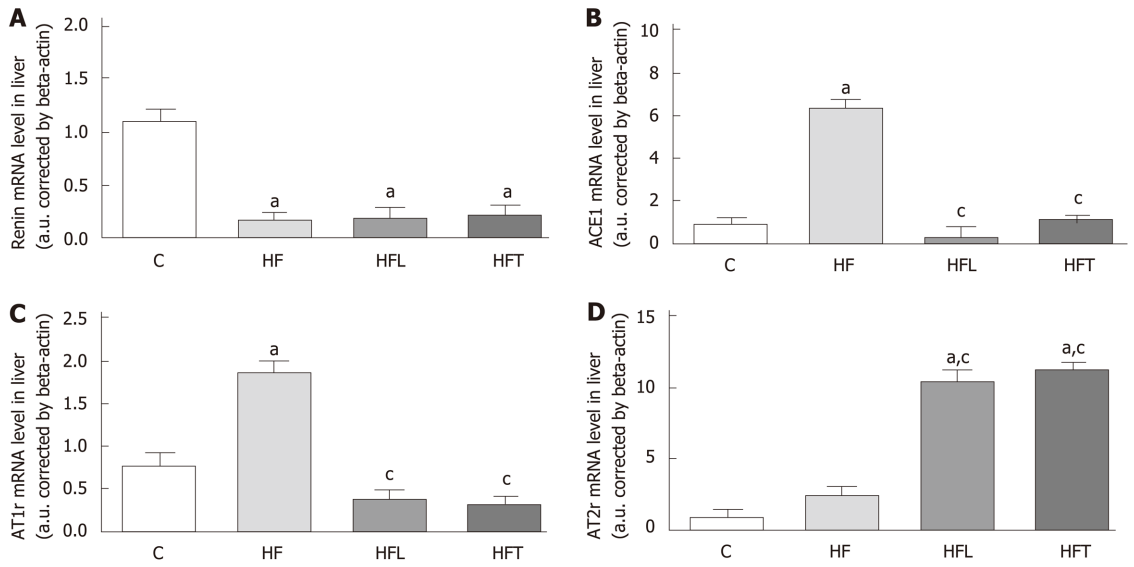

Figure 3 Local gene expression of components of the classical ACE1/AT1r axis in the liver: Renin (A), ACE1 (B), AT1r (C) and AT2r (B). B-Actin was used as an endogenous control to normalize the expression of the selected genes. Data are the means ± SD, n = 5 for each group. Differences between the groups were tested with one-way ANOVA and post hoc test of Holm–Sidak (P < 0.05) when compared with the C group (a) and HF group (c). ACE1: Angiotensin-converting enzyme 1; AT1r: Angiotensin type-1 receptor; AT2r: Angiotensin type-2 receptor; C: Control group; HF: High-fat diet; HFL: HF diet treated with losartan; HFT: HF diet treated with telmisartan.

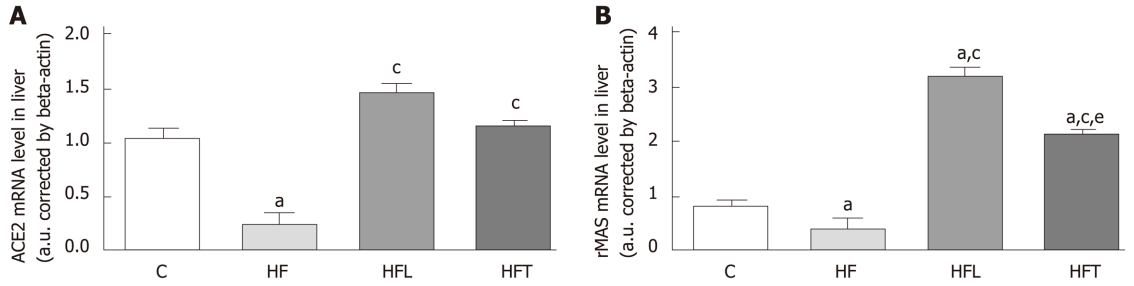

Figure 4 Local gene expression of the ACE2/MASr axis in the liver: ACE2 (A) and rMAS (B). B-Actin was used as an endogenous control to normalize the expression of the selected genes. Data are the means ± SD, n = 5 for each group. Differences between the groups were tested with one-way ANOVA and post hoc test of Holm–Sidak (P < 0.05) compared to the C group (a), HF group (c) and HFL group (e). ACE2: Angiotensin-converting enzyme 2; rMAS: MAS receptor; C: Control group; HF: High-fat diet; HFL: HF diet treated with losartan; HFT: HF diet treated with telmisartan.

ARTICLE HIGHLIGHTS

Research background

Drugs that target the renin-angiotensin system (RAS) could benefit the adverse hepatic remodeling and glycemic control in the diet-induced obese mouse.

Research motivation

Most obese subjects show insulin resistance and non-alcoholic fatty liver disease (often referred to as NAFLD), whose consequences lead to high healthcare costs. Currently, there is no established drug treatment for NAFLD.

Research objectives

To evaluate the action of losartan or telmisartan on the modulation of intrahepatic RAS and the resulting metabolic effects in a diet-induced obesity murine model.

Research methods

Twenty C57BL/6 mice were randomly divided into two nutritional groups for 10 wk and, then, into four groups for a 5-wk treatment: control group (C, n = 5), high-fat group (HF, n = 15), HF treated with losartan (HFL, n = 5, 10 mg/kg body mass) and HF treated with telmisartan (HFT, n = 5, 10 mg/kg body mass).

Research results

The HF group showed increased weight, glucose intolerance, high hepatic triacylglycerol, and overexpression of intrahepatic angiotensin-converting enzyme (ACE) 1/ angiotensin II (ANGII) type 1 receptor (AT1r). Losartan and telmisartan modulated the intrahepatic RAS, with preference for the ACE2/rMAS axis, resulting in ameliorated glucose intolerance and reduced hepatic triacylglycerol levels, both of which are related to diminished Plin2 expression in the liver. Only telmisartan could restore body mass, fasting glucose levels, and hepatic cholesterol levels, implying additional benefits.

Research conclusions

The modulation of intrahepatic RAS, with preference for the ACE2/rMAS axis over the ACE1/AT1r axis after losartan or telmisartan treatments, caused hepatic and metabolic beneficial effects, as demonstrated by reduced hepatic triacylglycerol levels coupled with reduced PLIN 2 expression and improved glycemic control.

Research perspectives

The pharmacological modulation of intrahepatic RAS showed beneficial effects in terms of hepatic steatosis, evaluated by reduced hepatic triacylglycerol levels, in addition to its effects on decreased body mass and better glycemic control. These drugs could be a viable option to treat NAFLD in obese and/or hypertensive patients.

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ORIGINAL ARTICLE

Retrospective Study

Being accompanied to liver discharge clinic: An easy measure to identify potential liver transplant candidates among those previously considered ineligible

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Abstract

BACKGROUND

Patients with cirrhosis deemed ineligible for liver transplantation are usually followed in general hepatology or gastroenterology clinics, with the hope of re-evaluation once they meet the appropriate criteria. Specific strategies to achieve liver transplant eligibility for these patients have not been studied.

AIM

To assess clinical and sociodemographic factors associated with future liver transplant eligibility among patients initially considered ineligible.

METHODS

This is a retrospective study of patients with cirrhosis considered non-transplant eligible, but without absolute contraindications, who were scheduled in our transitional care liver clinic (TCLC) after discharge from an inpatient liver service. Transplant candidacy was assessed 1 year after the first scheduled TCLC visit. Data on clinical and sociodemographic factors were collected.

RESULTS

Sixty-nine patients were identified and the vast majority were Caucasian men with alcoholic cirrhosis. 46 patients (67%) presented to the first TCLC visit. Seven of 46 patients that showed to the first TCLC visit became transplant candidates, while 0 of 23 patients that no-showed did (15.2% *vs* 0%, $P = 0.08$). Six of 7 patients who showed and became transplant eligible were accompanied by family or

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friends at the first TCLC appointment, compared to 13 of 39 patients who showed and did not become transplant eligible (85.7% *vs* 33.3%, $P = 0.01$).

CONCLUSION
Patients who attended the first post-discharge TCLC appointment had a trend for higher liver transplant eligibility at 1 year. Being accompanied by family or friends during the first TCLC visit correlated with higher liver transplant eligibility at 1 year (attendance by family or friends was not requested). Patient and family engagement in the immediate post-hospitalization period may predict future liver transplant eligibility for patients previously declined.

Key words: Cirrhosis; Transitional clinic; Transplant listing; Support; Family

Core tip: Being declined as a liver transplant candidate is not always an irreversible decision, but there is limited information about predictors for eventually achieving liver transplant eligibility. This study shows that among patients who were found not to be transplant candidates, those who presented to their post hospital discharge liver clinic appointment with family and friends had a higher chance of liver transplant eligibility within one year. This finding suggests the importance of engaging family and friends in the complex care of patients with cirrhosis.

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INTRODUCTION

The limited supply of donor livers necessitates careful selection of potential liver transplant candidates by institutional transplant recipient review committees. Each institution has different criteria for listing patients with cirrhosis based on medical, social, and economic factors, some of which remain controversial[1-3]. Patients considered to be unsuitable candidates for liver transplantation are often followed by their providers with the hope of candidacy reassessment once they fulfill the appropriate medical and psychosocial criteria. There is limited literature on specific strategies to achieve liver transplant eligibility for patients with cirrhosis who are initially declined.

Few studies have looked at causes of liver transplant ineligibility among patients with cirrhosis. A single center study found that after initial transplant referral, patients were often declined because they were too well, had co-existing medical contraindications, or needed addiction rehabilitation[4]. Another study found that patients who were declined for non-medical reasons often did not meet the minimum alcohol abstinence requirements and lacked social support[5]. These barriers to liver transplantation are often challenging to overcome, and it can be difficult to determine which patients would be able to achieve the changes needed to become suitable candidates.

Identifying and addressing psychosocial issues early among potential liver transplant candidates with cirrhosis is important. Alcohol relapse after liver transplantation has been associated with mental health issues, lack of a stable life partner, or less than six months of sobriety[6,7]. Additionally, a survey assessing the role of psychosocial evaluations on liver transplant candidacy found that transplant psychosocial evaluators assigned greater importance to coping skills and the ability to adapt to stress, and were less likely to recommend transplant listing for those with poor social support[8]. Furthermore, it has been suggested that psychological characteristics could be used to identify patients less likely to be suitable liver transplant candidates, allowing for targeted support and engagement to improve chances for transplant eligibility[9].

These observations underscore the importance of identifying patients' support

networks and psychosocial barriers early. As these issues are major reasons for liver transplant ineligibility among those who might otherwise be suitable candidates, an early intervention could potentially improve liver transplant candidacy. Unfortunately, there are no specific strategies for achieving liver transplant eligibility among those considered unsuitable. We aimed to identify clinical and sociodemographic factors associated with future liver transplant eligibility among patients seen at a transitional care liver clinic (TCLC) who were considered transplant ineligible but without absolute contraindications.

MATERIALS AND METHODS

This is a retrospective study of discharged patients whose first TCLC visits were scheduled between March 2015 and December 2015, with follow up through December 2016 at a single tertiary academic center. Patients were included if they had cirrhosis, were not considered liver transplant candidates, were discharged from the liver inpatient service, were alive but not hospitalized at the first scheduled TCLC appointment, and were not seen by an outpatient hepatologist or gastroenterologist at another institution. Patients were excluded if they had received a liver transplant previously or within 90 days of the hospital discharge prior to the first TCLC visit, if they were on hospice within 90 days of that discharge, if they were older than 70 years, or if they had an irreversible contraindication to liver transplantation. Transplant listing was assessed 1 year after the first scheduled TCLC encounter. Charts were reviewed for demographics, clinical data, previous liver care, show rate at first TCLC visit, whether they were accompanied by family or friends during the first TCLC visit (being accompanied was not asked or required), and liver transplant listing at 1 year. Yale University Institutional Review Board approval was obtained.

Statistical analysis

Statistical analyses were performed using student's *t*-test for numerical data and chi-square test for categorical data. The statistical software package SPSS for Windows (SPSS Inc, version 25) was used to analyze the data, and $P < 0.05$ was considered a significant difference.

RESULTS

Eighty-six patients met the inclusion criteria and were scheduled in TCLC. Seventeen patients were excluded given the very low probability of transplant candidacy at age 70 or older, 6 patients were excluded for untreatable malignancies, and 4 patients were excluded for extensive comorbidities that indefinitely precluded transplantation, leaving a total of 69 eligible patients, Figure 1.

The majority of patients were unmarried Caucasian men with decompensated alcoholic cirrhosis, and with mean Model of End-stage Liver Disease (MELD) score of 15, Table 1. Forty-six patients (66.7%) showed to the first scheduled post-discharge TCLC appointment. Mean time from hospital discharge to first TCLC appointment was 9.7 days (range 3-29 d) as compared to 8.2 days (range 4-24 d) for those that did not show. The patients who showed were not transplant candidates because of active alcohol use (63.0%), lack of social support (17.4%), active substance use (10.9%), low MELD (4.3%), and poor medical optimization (4.3%). The patients who did not show to the scheduled TCLC visit were alive and not hospitalized at the time of the scheduled appointment, and were not transplant candidates because of active alcohol use (56.5%), lack of social support (21.7%), low MELD (13.0%), and active substance use (8.7%). There was no statistical difference between those that showed and those that did not show based on demographics, recent alcohol or substance use, cirrhosis etiology, cirrhosis decompensations, Child-Pugh score (CPS), MELD, or prior hepatology care, Table 2.

Seven of the 46 patients that showed to the first scheduled TCLC appointment became liver transplant candidates at 1 year while none of the 23 patients that no-showed did. (15.2% *vs* 0.0%, $P = 0.08$). These 7 patients were initially not transplant eligible because of active alcohol use (57.1 %), active substance use (14.3%), lack of social support (14.3%), and need for medical optimization (14.3%).

Among the patients who showed to the first TCLC visit, 7 patients became transplant eligible at 1 year and 39 did not. The only statistically significant finding between these two groups was the presence of a family member or friend to TCLC visit (this was not asked or required). Six of the 7 patients who became transplant eligible and 13 of the 39 patients who did not become transplant eligible had been

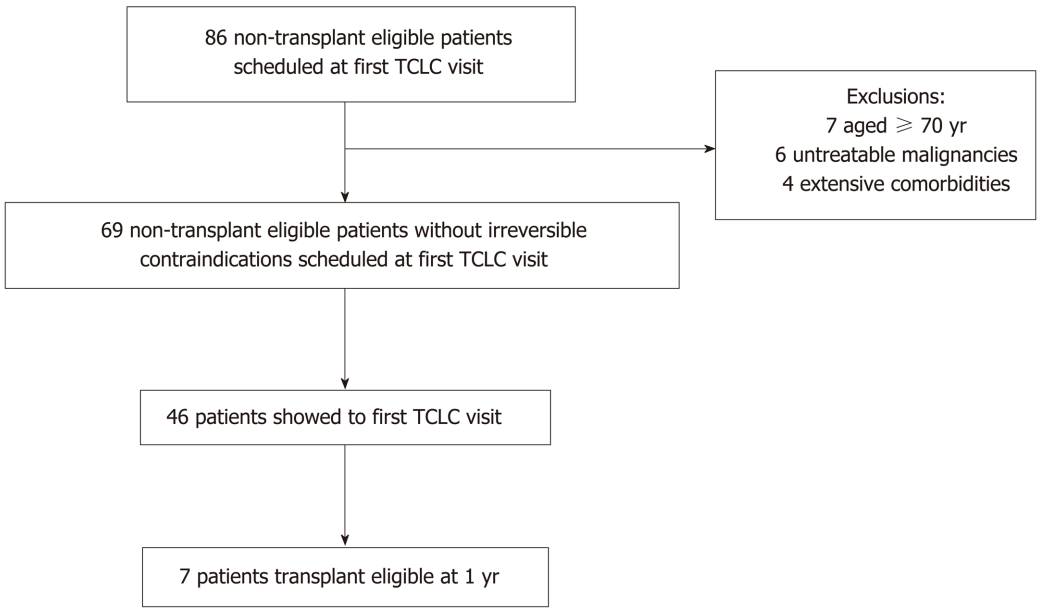

Figure 1 Number of patients who met study criteria, who showed to the first transitional care liver clinic (TCLC) appointment, and who became transplant eligible at 1 year.

accompanied at the first TCLC appointment (85.7% *vs* 33.3%, $P = 0.01$). Though not statistically significant, those that became transplant eligible at 1 year had a trend for having alcoholic cirrhosis and for being Caucasian. Those who did not become transplant eligible at 1 year had a trend for recent active alcohol use and for having Medicaid insurance. There were no statistical differences based on demographics, cirrhosis etiology, CPS, MELD, or prior hepatology care, Table 3.

DISCUSSION

There is limited literature on strategies to optimize liver transplant candidacy for patients considered ineligible. At our institution, about 30% of referred patients are ultimately accepted as liver transplant candidates. Transplant eligibility requires an adequate support system, among other medical and psychosocial criteria.

This study followed non-transplant eligible patients seen at their post discharge TCLC visit to identify which patients would become transplant candidates at 1 year. We found that patients who showed to the first TCLC visit had a trend for increased liver transplant eligibility at 1 year. Being accompanied by family or friends during the first TCLC visit was correlated with an even higher rate of liver transplant candidacy at 1 year. Of note, patients were not required or asked to bring family or friends to the TCLC encounter.

These observations suggest that patient and family involvement in the immediate post-hospitalization period may predict future liver transplant eligibility for patients previously considered unsuitable but did not have absolute contraindications. All 7 patients who became liver transplant candidates at 1 year had shown to the initial TCLC appointment. 6 of those 7 had been accompanied by a family member or friend. After review of demographics and clinical history, being accompanied at the first TCLC visit was the only statistically significant difference between the 7 patients that showed to TCLC and became transplant candidates at 1 year, and the 39 patients that showed to TCLC but did not become transplant candidates at 1 year. Though there was a trend for more Medicaid insurance and recent active alcohol use for those that showed and did not became transplant candidates at 1 year, both patient populations consisted mostly of unmarried men with decompensated alcoholic cirrhosis and active alcohol use. Moreover, both patient groups had been previously declined liver transplant candidacy because of active alcohol use and poor social support. These findings reinforce the importance of continued liver care for transplant ineligible patients because the window for transplant candidacy can re-open, even among patients with challenging psychosocial situations.

The observed correlation of transplant eligibility at 1 year with being accompanied by family or friends at the first transitional liver care clinic is significant and should be

Table 1 Characteristics of overall patient population

Variables	Included patients[*n* = 69]
Age	
Mean (range)	51.4 (26-69)
Sex	
Male	45 (65.2%)
Race/Ethnicity	
Caucasian	44 (63.8%)
Hispanic	12 (17.4%)
African American	11 (15.9%)
Other	2 (2.9%)
Insurance	
Medicaid	38 (55.1%)
Medicare	13 (18.8%)
Private	14 (20.3%)
Uninsured	4 (5.8%)
Homeless	2 (2.9%)
English primary language	63 (91.3%)
Marital Status	
Married	27 (39.1%)
Single	42 (60.9%)
Cirrhosis etiology	
EtOH	39 (56.5%)
EtOH/HCV	15 (21.7%)
HCV	7 (10.1%)
NASH	4 (5.8%)
PBC	1 (1.4%)
NASH/EtOH	1 (1.4%)
AIH	1 (1.4%)
HBV	1 (1.4%)
Other	0 (0%)
Decompensation	63 (91.3%)
Ascites	54 (78.3%)
Hepatic encephalopathy	38 (55.1%)
Variceal hemorrhage	20 (29.0%)
Child Pugh Score	
A	9 (13.0%)
B	34 (49.3%)
C	26 (37.7%)
MELD mean (range)	15.0 (6-30)
Patient reported active alcohol use on last admission	40 (58.0%)
Patient reported active substance use on last admission	9 (13.0%)
Previous 1 yr hospitalizations	44 (63.8%)
Previous 1 yr hepatology visit	25 (36.2%)
Accompanied at first TCLC	19 (27.5%)
Deceased at 1 yr	20 (29.0%)

EtOH: Alcohol; HCV: Hepatitis C virus; PBC: Primary biliary cholangitis; NASH: Non-alcoholic steatohepatitis; AIH: Autoimmune hepatitis; PSC: Primary sclerosing cholangitis; HBV: Hepatitis B virus; TCLC: Transitional care liver clinic.

explored further in future studies. We do not suggest that having someone come with a patient to clinic is sufficient for transplant eligibility, but rather consider it a potential marker of the support available at home, which is especially important for transplant centers such as ours that require a strong support system. Involvement of family and caregivers at a visit may help them understand the patient's liver disease

Table 2 Characteristics of patients by show and no-show to the first transitional care liver clinic visit

Variables	Show patients[*n* = 46]	No Show patients[*n* = 23]	*P* value
Age			
Mean (range)	51.8 (26-69)	50.6 (30-68)	0.63
Sex			
Male	31 (67.4%)	14 (60.9%)	0.59
Race/Ethnicity			
Caucasian	32 (69.6%)	12 (52.2%)	0.16
Hispanic	6 (13.0%)	6 (26.1%)	0.18
African American	6 (13.0%)	5 (21.7%)	0.35
Other	2 (4.3%)	0 (0%)	0.55
Insurance			
Medicaid	22 (47.8%)	16 (69.6%)	0.09
Medicare	10 (21.7%)	3 (13.0%)	0.38
Private	11 (23.9%)	3 (13.0%)	0.29
Uninsured	3 (6.5%)	1 (4.3%)	0.72
Homeless	1 (2.2%)	1 (4.3%)	0.61
English primary language	43 (93.5%)	20 (87.0%)	0.36
Marital status			
Married	19 (41.3%)	8 (34.8%)	0.60
Single	27 (58.7%)	15 (65.2%)	0.60
Cirrhosis etiology			
EtOH	28 (60.9%)	11 (47.8%)	0.30
EtOH/HCV	11 (23.9%)	4 (17.4%)	0.54
HCV	3 (6.5%)	4 (17.4%)	0.16
NASH	2 (4.3%)	2 (8.9%)	0.47
PBC	1 (2.2%)	0 (0%)	1
NASH/EtOH	1 (2.2%)	0 (0%)	1
AIH	0 (0%)	1 (4.3%)	0.33
HBV	0 (0%)	1 (4.3%)	0.33
Other	0 (0%)	0 (0%)	1
Decompensation	43 (93.5%)	20 (87.0%)	0.36
Ascites	36 (78.2%)	18 (78.3%)	1
Hepatic Encephalopathy	27 (58.7%)	11 (47.8%)	0.39
Variceal hemorrhage	13 (28.3%)	7 (30.4%)	0.85
Child Pugh Score			
A	5 (10.9%)	4 (17.4%)	0.45
B	24 (52.2%)	10 (43.5%)	0.50
C	17 (37.0%)	9 (39.1%)	0.86
MELD mean (range)	15.3 (6–30)	14.4 (7-26)	0.54
Patient reported active alcohol use on last admission	27 (58.7%)	13 (56.5%)	0.86
Patient reported active substance use on last admission	5 (10.9%)	4 (17.4%)	0.45
Previous 1 yr hospitalizations	29 (63.0%)	15 (65.2%)	0.86
Previous 1 yr hepatology visit	16 (34.8%)	9 (39.1%)	0.72
Accompanied at first TCLC	19 (41.3%)	0 (0.0%)	0.0001
Deceased at 1 yr	13 (28.3%)	7 (30.4%)	0.85

EtOH: Alcohol; HCV: Hepatitis C virus; PBC: Primary biliary cholangitis; NASH: Non-alcoholic steatohepatitis; AIH: Autoimmune hepatitis; PSC: Primary sclerosing cholangitis; HBV: Hepatitis B virus; TCLC: Transitional care liver clinic.

as well as the barriers that preclude transplantation, allowing for better care of their loved ones at home. Many of these patients with decompensated cirrhosis and psychosocial issues likely face challenges caring for themselves and fully understanding all content discussed at a liver clinic visit - over half of our study patients had hepatic encephalopathy and were single. It is also plausible that follow

Table 3 Characteristics of patients that showed to the first transitional care liver clinic visit by transplant eligibility at 1 yr

Variables	Show patients, transplant eligible 1 yr [*n* = 7]	Show patients, non-transplant eligible 1 yr [*n* = 39]	*P* value
Age			
Mean (range)	51.9 (26-69)	51.8 (34-69)	0.98
Sex			
Male	5 (71.4%)	26 (66.7%)	0.80
Race/Ethnicity			
Caucasian	7 (100.0%)	25 (64.1%)	0.08
Hispanic	0 (0.0%)	6 (15.4%)	0.57
African American	0 (0.0%)	6 (15.4%)	0.57
Other	0 (0.0%)	2 (5.1%)	1
Insurance			
Medicaid	1 (14.3%)	18 (46.2%)	0.11
Medicare	2 (28.6%)	9 (23.1%)	0.75
Private	3 (42.9%)	9 (23.1%)	0.27
Uninsured	1 (14.3%)	3 (7.7%)	0.57
Homeless	0 (0.0%)	1 (2.6%)	1
English Primary Language	7 (100.0%)	36 (92.3%)	1
Marital Status			
Married	3 (42.9%)	16 (41.0%)	0.93
Single	4 (57.1%)	23 (59.0%)	0.93
Cirrhosis Etiology			
EtOH	6 (85.7%)	22 (56.4%)	0.14
EtOH/HCV	0 (0.0%)	11 (28.2%)	0.17
HCV	1 (14.3%)	2 (5.1%)	0.37
NASH	0 (0.0%)	2 (5.1%)	1
PBC	0 (0.0%)	1 (2.6%)	1
NASH/EtOH	0 (0.0%)	1 (2.6%)	1
Other	0 (0%)	0 (0%)	1
Decompensation	7 (100.0%)	36 (92.3%)	1
Ascites	6 (85.7%)	30 (76.9%)	0.60
Hepatic Encephalopathy	4 (57.1%)	23 (59.0%)	0.93
Variceal hemorrhage	1 (14.3%)	12 (27.3%)	0.79
Child Pugh Score			
A	1 (14.1%)	4 (10.3%)	0.75
B	5 (71.4%)	19 (48.7%)	0.27
C	1 (14.1%)	16 (41.0%)	0.18
MELD mean (range)	13.7 (10-20)	15.6 (6-30)	0.44
Patient reported active alcohol use on last admission	2 (28.6%)	25 (64.1%)	0.08
Patient reported active substance use on last admission	0 (0.0%)	5 (12.8%)	1
Previous 1 yr hospitalizations	5 (71.4%)	24 (61.5%)	0.62
Previous 1 yr hepatology visit	3 (42.9%)	13 (33.3%)	0.63
Accompanied at first TCLC	6 (85.7%)	13 (33.3%)	0.01
Deceased at 1 yr	0 (0.0%)	13 (33.3%)	0.17

EtOH: Alcohol; HCV: Hepatitis C virus; PBC: Primary biliary cholangitis; NASH: Non-alcoholic steatohepatitis; AIH: Autoimmune hepatitis; PSC: Primary sclerosing cholangitis; HBV: Hepatitis B virus; TCLC: Transitional care liver clinic.

up in TCLC with family members who know the patient enabled physicians to identify and provide appropriate resources for addressing specific psychosocial issues. More research is needed to identify and to support patients deemed ineligible for liver transplantation because of psychosocial reasons, with the hope of achieving

transplant eligibility.

The limitations of this study are that it is retrospective with a small sample size at a single tertiary academic center. However, the patients in all groups had similar characteristics and were evaluated at a single liver transplant center which allowed for consistency in the assessment of transplant eligibility. An advantage of our study was that it included a high-risk population consisting of predominantly unmarried men with decompensated alcoholic cirrhosis who had been mostly declined for active alcohol and substance use as well poor social support. Six of the 7 patients who became transplant eligible at 1 year had one of these significant barriers.

While these findings suggest that patient and family engagement after hospital discharge may predict future liver transplant eligibility for those initially considered unsuitable, we should continue to advocate for liver transplant evaluations for all patients provided that there are no absolute contraindications.

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ARTICLE HIGHLIGHTS

Research background

There is minimal data on the long-term outcomes of patients with cirrhosis who are declined for liver transplantation. Many of these ineligible patients are followed by general hepatology and gastroenterology providers with the hope of re-eligibility for transplantation. Specific strategies to achieve liver transplant eligibility for these patients have not been studied.

Research motivation

We were motivated to pursue this project so that the field may have a better understanding of the clinical and sociodemographic factors that may predict future liver transplant eligibility for those initially considered ineligible.

Research objectives

The objective of our study was to assess clinical and sociodemographic factors associated with one-year liver transplant eligibility among patients with cirrhosis seen in a transitional care liver clinic who were considered unsuitable transplant candidates but did not have absolute contraindications.

Research methods

Retrospective, single-center study.

Research results

69 patients were identified, predominantly Caucasian men with alcoholic cirrhosis. 46 patients (67%) presented to the first TCLC visit. Seven of 46 patients that presented to the first TCLC visit became transplant candidates at one year, while 0 of 23 patients that no-showed did (15.2% *vs* 0%, $P = 0.08$). Six of 7 patients who showed and became transplant eligible were accompanied by family or friends at the first TCLC appointment, compared to 13 of 39 patients who showed and did not become transplant eligible (85.7% *vs* 33.3%, $P = 0.01$).

Research conclusions

Patients ineligible for liver transplantation, but without absolute contraindications, who presented to our TCLC were more likely to be listed for liver transplantation at one year if they were joined by family or friends at the first clinic visit. While more research is needed, patient and family participation in clinical care may serve as a surrogate marker of social support for patients previously declined for liver transplant.

Research perspectives

This study reinforced the importance of investigating the long-term outcomes of patients with cirrhosis who are declined for liver transplantation. Given our small study population and known variations in transplant listing policies at each institution, larger multi-centered prospective studies are needed.

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ORIGINAL ARTICLE

Retrospective Study

Effectiveness of venous thromboembolism prophylaxis in patients with liver disease

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Author contributions: Yerke J, Welch S, Bauer S, Bass S, Torbic H and Militello M conceived the research study design; Yerke J collected data; Yerke J and Welch S interpreted data; Yerke J drafted the manuscript; Yerke J, Welch S, Bauer S, Bass S, Torbic H, Militello M, Hanouneh I and Roach E critically revised the manuscript.

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Abstract

BACKGROUND

Patients with liver disease are concomitantly at increased risk of venous thromboembolism (VTE) and bleeding events due to changes in the balance of pro- and anti-hemostatic substances. As such, recommendations for the use of pharmacological VTE prophylaxis are lacking. Recent studies have found no difference in rates of VTE in those receiving and not receiving pharmacological VTE prophylaxis, though most studies have been small. Thus, our study sought to establish if pharmacological VTE prophylaxis is effective and safe in patients with liver disease.

AIM

To determine if there is net clinical benefit to providing pharmacological VTE prophylaxis to cirrhotic patients.

METHODS

In this retrospective study, 1806 patients were propensity matched to assess if pharmacological VTE prophylaxis is effective and safe in patients with cirrhosis. Patients were divided and evaluated based on receipt of pharmacological VTE prophylaxis.

RESULTS

The composite primary outcome of VTE or major bleeding was more common in the no prophylaxis group than the prophylaxis group (8.7% *vs* 5.1%, $P = 0.002$), though this outcome was driven by higher rates of major bleeding (6.9% *vs* 2.9%, $P < 0.001$) rather than VTE (1.9% *vs* 2.2%, $P = 0.62$). There was no difference in

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length of stay or in-hospital mortality between groups. Pharmacological VTE prophylaxis was independently associated with lower rates of major bleeding (OR = 0.42, 95%CI: 0.25-0.68, P = 0.0005), but was not protective against VTE on multivariable analysis.

CONCLUSION
Pharmacological VTE prophylaxis was not associated with a significant reduction in the rate of VTE in patients with liver disease, though no increase in major bleeding events was observed.

Key words: Fibrosis; Venous thromboembolism; Venous thrombosis; Liver; Embolism

Core tip: While patients with cirrhosis have historically been considered to be coagulopathic, recent data suggests that these patients may be both hypo- and hypercoagulable. Recommendation for provision of chemoprophylaxis to prevent venous thromboembolism (VTE) in this group of patients is lacking. In our study, pharmacological VTE prophylaxis decreased composite rates of major bleeding and VTE but was not protective against VTE, further demonstrating the uncertain utility of chemoprophylaxis in this population.

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INTRODUCTION

It is widely recognized that patients with liver disease, particularly end stage, have acquired bleeding disorders resulting from a reduction of procoagulant factors, thrombocytopenia, and abnormalities in fibrinolysis[1]. More recently, however, the risk of venous thromboembolism (VTE) is being recognized and is likely due to elevations in factor VIII and von Willebrand factor along with a reduction of the endogenous anticoagulant protein C[1]. Several studies have evaluated the risk of VTE in patients with end-stage liver disease with varying results ranging from an incidence of 0.5% to 6.3%[2-9]. Factors that have been implicated in a higher rate of VTE occurrence include albumin levels < 3 g/dL and concomitant comorbidities, particularly chronic kidney disease, heart failure, and malignancy[3-5].

These variable findings are likely due to several factors including differences in severity of liver disease, etiology of liver disease, concomitant comorbidities, and potentially the receipt of pharmacological VTE prophylaxis. The use of pharmacological VTE prophylaxis is commonly omitted in patients with end stage liver disease due to the widely held belief that the risk of bleeding outweighs the benefit of prophylaxis[6,7,10,11]. Additionally, it is unclear if pharmacological prophylaxis is effective in preventing thrombosis as it has been shown to be in other patient populations.

Few studies have evaluated the effectiveness and risk of VTE prophylaxis in patients with liver disease. Those that have suggest no reduction in rate of VTE events, but suggest potential increases in the rate of major bleeding[6,7,9,12]. However, these studies have significant limitations, most notably the lack of defined prophylactic therapy, defined VTE and bleeding events, heterogeneity among patients, and small sample size. The net benefit of pharmacological VTE prophylaxis is of particular interest because VTE events have been shown to confer a higher 30-d mortality when they occur in a patient with cirrhosis compared to the general population[13]. Therefore, our study seeks to compare differences in the rate of VTE and major bleeding between patients with liver disease receiving and not receiving pharmacological VTE prophylaxis.

MATERIALS AND METHODS

Study site

This retrospective cohort study was performed at a large, tertiary-care academic medical center and approved by the Cleveland Clinic Institutional Review Board (Cleveland, OH, United States). Adult patients admitted for 48 h or more from November 2008 through July 2015 with discharge International Classification of Diseases, 9th edition (ICD-9) diagnosis codes corresponding to cirrhotic liver disease were included in the study. Patients were excluded if they developed an incident VTE within 48 h of admission, if they had a diagnosis of congenital or acquired thrombophilia (defined as factor V Leiden, anti-phospholipid syndrome, prothrombin G20210A, protein C or S deficiency, prothrombin mutation, or anti-thrombin deficiency) or hemophilia, or if they received treatment dose anticoagulation for any indication other than an incident VTE. For patients with multiple admissions, the most recent admission was included for analysis. Patients admitted for liver transplantation were included up until their transplant.

Outcomes

The primary outcome was the composite rate of incident VTE and major bleeding. Secondary outcomes included the rate of incident VTE, rate of major bleeding, length of hospital stay, and rate of in-hospital mortality. Incident VTE was defined as a new thrombosis occurring 48 h or more after admission, extension of a VTE in a patient with an untreated prevalent VTE, or additional VTE formation in a patient with an untreated prevalent VTE. An incident VTE was required to be demonstrated by unequivocal radiographic imaging by compression ultrasonography, venography, computed tomography angiography, or ventilation-perfusion scanning[12]. Prevalent VTE was defined as a documented VTE at admission that was not being treated with anticoagulation. An incident bleeding event was considered any new-onset major bleeding event or any major bleeding event that occurred 24 h or more following hemostasis of a previous bleeding event[12]. For example, if a patient was admitted for variceal hemorrhage and did not have further bleeding the patient was not regarded as having incident bleeding, but if the patient developed bleeding more than 24 h after initial hemostasis the patient was regarded as having incident bleeding. Major bleeding was defined as bleeding that was symptomatic and at a critical site (intracranial, intraspinal, intraocular, retroperitoneal, intraarticular, pericardial, or intramuscular with compartment syndrome), or that required transfusion of at least 2 units of whole red blood or red cells within 24 h[14].

Included patients

Patients were allocated to the pharmacological VTE prophylaxis group if they received pharmacological prophylaxis for at least 50% of their hospital stay. Patients receiving prophylaxis less than 50% of their stay were allocated to the no prophylaxis group. Those experiencing a VTE event were grouped according to receipt or no receipt of prophylaxis within 48 h prior to the event and those experiencing a bleeding event were grouped according to receipt or no receipt of prophylaxis in the 24 h prior to the event. All major bleeding and VTE events were identified by the use of ICD-9 codes and manually verified in the electronic medical record.

Appropriate dosing of prophylactic anticoagulants was considered to be two or three doses per day of subcutaneous unfractionated heparin 5000 units, one or two doses per day of subcutaneous enoxaparin 40mg or two doses per day of enoxaparin 30 mg (or renally adjusted equivalent), one dose per day of subcutaneous fondaparinux 2.5 mg (or renally adjusted equivalent), or aspirin 160mg or more per day in orthopedic surgery patients.

Statistical analysis

The statistical methods of this study were reviewed by R. Samuel Butler from the Cleveland Clinic Department of Biostatistics. Assuming a rate of 7.7% for occurrence of the primary outcome in the no pharmacological VTE prophylaxis group and a rate of 4.4% in the pharmacological VTE prophylaxis group, a sample of 513 patients would provide 80% power to detect a 3.3% difference with a two-sided $\alpha = 0.05$. This estimate assumes an incidence of VTE of 6.3% in the no prophylaxis group as found by Dabbagh *et al*[7] as it was thought that this patient population most closely mirrored our study population. No study with a similar patient population to our own that compared incidence of major bleeding in patients with liver disease receiving or not receiving pharmacological prophylaxis was found, so a rate of 1.4% was chosen for this portion of the composite primary outcome. Patients were matched in a 1:1 fashion based on propensity score. Variables included in the propensity score were history of VTE, baseline platelet count, use of mechanical VTE prophylaxis, baseline model for

end-stage liver disease (MELD) score, age, the presence of heart failure, the presence of chronic kidney disease, the presence of lung disease (chronic obstructive pulmonary disease, asthma, or idiopathic pulmonary fibrosis), and the etiology of liver disease. Missing data required to calculate the MELD score was considered to be normal. Univariate analyses were completed using Pearson's Chi-square test for categorical variables and analysis of variance for continuous variables. Multivariable logistic regression was performed to identify independent risk factors for VTE, major bleeding, and in-hospital mortality.

RESULTS

A total of 9547 patients were identified with ICD-9 codes for liver disease, of which 3114 patients met inclusion criteria. The most common reasons for exclusion were those already receiving full dose anticoagulation (n = 1090), hospital length of stay less than 48 h (n = 1504), and liver disease without cirrhotic morphology (n = 3742). Of the 3114 patients, 903 patients in the prophylaxis group were matched by propensity score to 903 patients in the no prophylaxis group (Figure 1) and were included in the analyses. Baseline characteristics according to group are summarized in Table 1. Patients in the no pharmacological prophylaxis group were less likely to require renal replacement therapy (24.5% *vs* 29.6%, P = 0.015) and had a different distribution of MELD scores (fewer patients with MELD 10-39, more patients with MELD < 10 or ≥ 40). Differences in VTE risk score were noted; however, patients in both groups were predominately categorized as medium (65.4% *vs* 75.3%) and high risk (20.3% *vs* 14.2%) in the no prophylaxis and prophylaxis groups respectively. No difference was noted in the primary etiology of hepatic disease. Statistically significant differences were also noted in baseline INR and hemoglobin, but these were considered to be of negligible clinical significance. All other baseline characteristics were similar between groups.

Patients in the no prophylaxis group were more likely to experience the composite endpoint of VTE or major bleeding compared to those in the prophylaxis group (8.7% *vs* 5.1%, P = 0.002), although this was driven by an increased rate of major bleeding events (6.9% *vs* 2.9%, P < 0.0001) with no difference observed in the rate of VTE events (1.9% *vs* 2.2%, P = 0.61). There was no difference in in-hospital mortality (12.1% *vs* 11.5%, P = 0.72) or hospital length of stay (10.5 ± 12.6 d *vs* 10.8 ± 14.8 d, P = 0.67) between groups (Table 2).

Multivariable logistic regression for VTE events (Table 3), bleeding events (Table 4), and in-hospital mortality (Table 5) was performed. Lower baseline serum albumin (OR = 0.23, 95%CI: 0.13-0.42, P < 0.0001) was independently associated with development of VTE events, while decreasing baseline hemoglobin (OR = 0.76, 95%CI: 0.68-0.87, P < 0.0001) and albumin (OR = 0.61, 95%CI: 0.42-0.90) were independently associated with development of a major bleed. Pharmacological VTE prophylaxis was independently associated with lower rates of major bleeding (OR = 0.42, 95%CI: 0.25-0.68, P = 0.0005), but was not significantly associated with a difference in rate of incident VTE (OR = 0.99, 95%CI: 0.48-2.06, P = 0.97).

Risk factors independently associated with in-hospital mortality included occurrence of the primary endpoint (OR = 2.30, 95%CI: 1.44-3.70, P = 0.0005), decreasing baseline albumin (OR = 0.68, 95%CI: 0.52-0.88, P = 0.004), and increasing MELD category (OR = 0.31, 95%CI: 0.13-0.70, P = 0.0005 for comparison of MELD 20-29 with MELD > 40).

DISCUSSION

While antihemostatic changes of cirrhosis have been well characterized, prohemostatic changes have also been more recently recognized[1,15-17]. Although liver disease associated coagulopathy results in elevated laboratory tests for coagulation, thrombin generation is not proportionately reduced, leaving some subsets of patients with a hypercoagulable thrombin generation profile[18-21]. However, the propensity of a patient to be hypo- or hypercoagulable is challenging to predict, particularly when using standard laboratory tests of coagulation, such as INR or activated partial thromboplastin time, that have not been validated in this patient population[7,18,22].

While the incidence of VTE has been well established in patients with cirrhosis, whether pharmacological VTE prophylaxis should be provided in an attempt to decrease this incidence is not well known. Major VTE prophylaxis guidelines are silent on this topic[23]. Therefore, this study was conducted to evaluate whether patients with cirrhosis experienced net benefit or harm when prophylactic

Table 1 Baseline characteristics

	No prophylaxis(*n* = 903)	Prophylaxis(*n* = 903)
Mean age (yr)	60.1 ± 11.9	60.2 ± 11.8
Male	572 (63.3)	535 (59.2)
RRT[a]	221 (24.5)	267 (29.6)
CKD	226 (25.0)	238 (26.4)
Lung disease	218 (24.1)	218 (24.1)
Heart failure	192 (21.3)	196 (21.7)
MVP	291 (32.2)	286 (31.7)
VTE risk score[c]		
Unknown risk score	103 (11.4)	78 (8.6)
Low risk	26 (2.9)	17 (1.9)
Medium risk	591 (65.4)	680 (75.3)
High risk	183 (20.3)	128 (14.2)
BMI (kg/m^2)	24.4 ± 0.20	24.4 ± 0.20
INR[c]	1.4 ± 0.57	1.3 ± 0.32
Platelet count (k/μL)	144.2 ± 92.5	151.0 ± 97.0
aPTT (s)	34.8 ± 12.0	34.4 ± 10.2
Albumin (g/dL)	2.9 ± 0.70	2.9 ± 0.67
Tbili (mg/dL)	4.0 ± 7.1	3.8 ± 6.6
SCr (mg/dL)	1.5 ± 1.3 [a]	1.6 ± 1.5
Hemoglobin (g/dL)	10.4 ± 2.3 [b]	10.7 ± 2.1
Liver disease etiology		
Alcoholic	278 (30.8)	286 (31.7)
NASH	27 (3.0)	22 (2.4)
Acute Hepatitis	35 (3.9)	38 (4.2)
Other[1]	563 (62.3)	557 (61.7)
Mean MELD score	17.0 ± 9.1	17.4 ± 8.3
MELD category[c]		
MELD > 40	20 (2.2)	8 (0.89)
MELD 30-39.9	71 (7.9)	74 (8.2)
MELD 20-29.9	205 (22.7)	233 (25.8)
MELD 10-19.9	338 (37.4)	386 (42.7)
MELD < 10	269 (29.8)	202 (22.4)

Data presented as *n* (%) or mean ± SD.
[1]Other liver disease includes primary sclerosing cholangitis, biliary cirrhosis, cirrhosis due to alpha-1 antitrypsin deficiency, and any other liver disease not included above. This categorization is for the primary hepatic International Classification of Diseases, 9th edition (ICD-9) code for an admission; all patients had an ICD-9 code for cirrhosis.
[a]$P < 0.05$ *vs* prophylaxis group;
[b]$P < 0.01$ *vs* prophylaxis group;
[c]$P < 0.001$ *vs* prophylaxis group. RRT: Renal replacement therapy; CKD: Chronic kidney disease; MVP: Mechanical venous thromboembolism prophylaxis; BMI: Body mass index; INR: International normalized ratio; aPTT: Activated partial thromboplastin time; Tbili: Total bilirubin; SCr: Serum creatinine; NASH: Non-alcoholic steatohepatitis; MELD: Model for end-stage liver disease.

anticoagulation was provided. We found that those who received pharmacological prophylaxis experienced the primary outcome of incident VTE or incident major bleeding less frequently, although this difference was driven by decreased rates of major bleeding (Table 2).

The overall rate of VTE in our study (2.0%) closely correlates with the incidence seen in several other studies of patients with liver disease, but was significantly lower than the VTE rate used in our power analysis[3,5,7,12,24]. Dabbagh *et al*[7] was chosen to inform the power analysis as this study contained a large proportion of patients with Child-Pugh Class C liver disease, which more closely mirrors the liver disease population seen at our institution. The differences in incidence of VTE may partially be explained by higher rates of mechanical prophylaxis (31.9% *vs* 16.3%) in the current study. Similar to previous data, there was no difference in the incidence of VTE

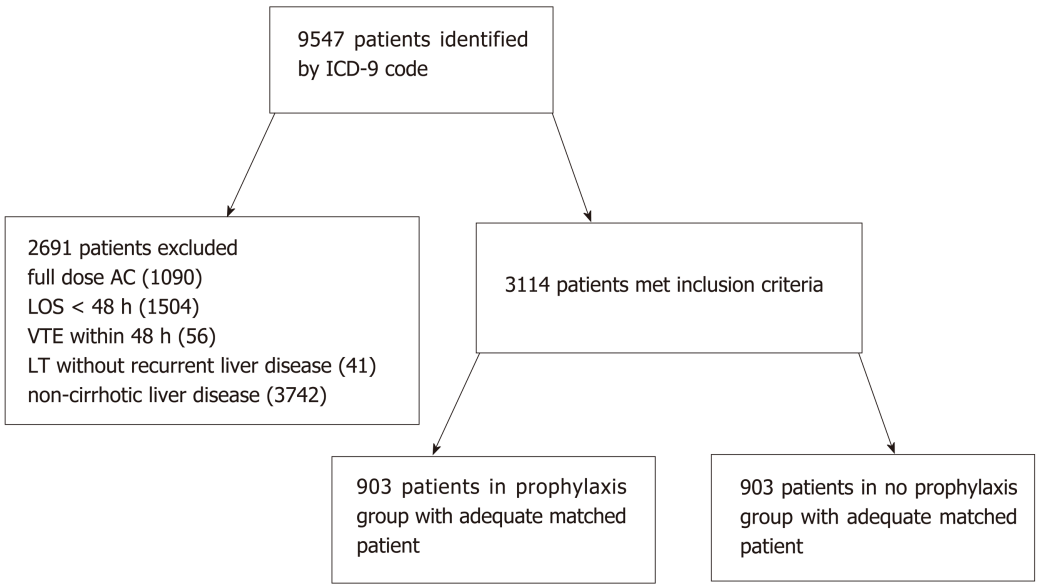

Figure 1 Inclusion/exclusion schema. AC: Anticoagulation; LOS: Hospital length of stay; LT: Liver transplantation; ICD-9: International Classification of Diseases, 9th edition.

between the no prophylaxis group and the prophylaxis group (1.9% *vs* 2.2%, P = 0.62)[6,9,12,25]. While the matched analysis was likely still susceptible to bias due to an imbalance in baseline characteristics, further correction for between-group differences by multivariable logistic regression also revealed no difference (OR for VTE in the prophylaxis group 0.99, 95%CI: 0.48-2.06). This lack of difference when correcting for other factors may indicate that only minimal, if any, protection from VTE is provided by pharmacological prophylaxis in patients with cirrhosis.

As noted in previous literature, low baseline serum albumin was independently associated with VTE development in this population[4,5]. Low baseline serum albumin was also independently associated with increased odds of major bleeding and in-hospital mortality, as has been observed widely throughout the literature[26-30]. These findings complicate the use of serum albumin as an indicator of appropriateness of pharmacological prophylaxis. These data may also suggest that patients with severe liver disease could concomitantly be at elevated risk of bleeding and thrombosis, with the type of event experienced influenced by acute physiologic insults.

While several different types of liver disease have been associated with increased risk for thrombosis, little is known about how different types of liver disease compare to each other in regards to thrombotic risk[31-37]. In addition, very few studies have evaluated how risk factors for thrombosis have translated to actual thrombotic events. One large study evaluating nearly 5 million patients with liver disease found an increased rate of VTE in patients with non-alcoholic liver disease compared to those with alcoholic liver disease (0.9% *vs* 0.6%, P < 0.0001)[34]. However, significant differences in baseline characteristics between the non-alcoholic and alcoholic groups were present, including age (60 *vs* 52 years, P < 0.0001), which is a known risk factor for VTE[34]. Patients with cholestatic cirrhosis have also been shown to be more hypercoagulable on evaluation by thromboelastography than a cohort of mainly patients with alcoholic cirrhosis[33]. However, no difference in etiology of cirrhosis was noted in our study.

Our study found an overall rate of major bleeding of 4.9%, with significantly more major bleeding events occurring in the no prophylaxis group than in the prophylaxis group (6.9% *vs* 2.9%). Overall bleeding rates in a previous study found no significant difference in rates of any bleeding between those who did not receive prophylaxis versus those that received prophylaxis (8.1% *vs* 5.5%, P = 0.258) as well as in rates of gastrointestinal hemorrhage (3.2% *vs* 3.0%, P = 0.52)[12]. However, on multivariable analysis the use of pharmacological prophylaxis was significantly associated with in-hospital bleeding (OR = 2.355, 95%CI: 1.116-4.971)[12]. This result contrasts sharply with our own multivariable analysis, which found that prophylaxis was associated with a decreased incidence of major bleeding (OR = 0.42, 95%CI: 0.25-0.68). Importantly, the bleeding definitions used in the respective studies differed, with Shatzel *et al*[12] evaluating all bleeding events compared to major bleeding in the current evaluation. However, these discrepant findings are unlikely explained by

Table 2 Matched univariate results (*n* = 903)

	No prophylaxis	Prophylaxis
VTE or major bleeding	79 (8.7)[a]	46 (5.1)
VTE[1]	17 (1.9)	20 (2.2)
Major Bleeding[1]	62 (6.9) [b]	26 (2.9)
In-hospital mortality	109 (12.1)	104 (11.5)
LOS (d)	10.5 ± 12.6	10.8 ± 14.8

Data presented as *n* (%) or mean ± SD.
[1]One patient in each group experienced both a venous thromboembolism and major bleeding event.
[a]$P < 0.05$ *vs* prophylaxis group;
[b]$P < 0.001$ *vs* prophylaxis group. LOS: Hospital length of stay; VTE: Venous thromboembolism.

bleeding definitions[12]. Our study found that lower baseline serum albumin (OR = 0.676, 95%CI: 0.484-0.943) was independently associated with major bleeding, a result that likely highlights the increased bleeding risk that occurs as cirrhosis severity progresses. However, this does not explain the difference in major bleeding observed in the propensity score matched analysis as the mean albumin was not different between groups. Notably, it does not seem that prophylactic anticoagulation imparted any additional bleeding risk within our patient population.

There was no difference in in-hospital mortality between those who did not receive prophylaxis versus those that did (12.1% *vs* 11.5%, $P = 0.72$). Factors found to increase the risk of in-hospital mortality include occurrence of the primary endpoint (OR = 2.30, 95%CI: 1.44-3.70, $P = 0.0005$), decreasing baseline albumin (OR = 0.68, 95%CI: 0.52-0.88, $P = 0.004$), and increasing MELD category. A higher incidence of mortality in patients with hypoalbuminemia has been consistently observed throughout the literature, a finding that is corroborated by our study[26-30]. Overall, these findings seem to suggest that progression of cirrhosis leads to worsened outcomes in regards to VTE and bleeding events as well as in-hospital mortality.

The results of our study can be applied clinically in many ways. First, decreased serum albumin has consistently been shown to be an independent risk factor for VTE within this population, and was also associated with increased odds of major bleeding and in-hospital mortality. While these results may not be useful in stratifying patients that should receive pharmacological prophylaxis from those that should not, they can help provide insight into patients that require mechanical prophylaxis, as well as heighten the clinician's suspicion of VTE if signs and symptoms meet this clinical picture. Second, these data suggest that pharmacological VTE prophylaxis is safe in patients with cirrhosis, as patients that received prophylaxis did not experience increased risk of major bleeding. However, efficacy of pharmacological prophylaxis within this population was not established by this study. Finally, our findings suggest that a validated risk tool, such as the Padua Predictive Score, may be more useful in stratifying liver disease patients at risk for VTE[9,12,38].

Our study has several limitations. First, this retrospective review is subject to inherent flaws of the study design; we were reliant on the accuracy of the medication administration record for group allocation. Second, although selection bias was minimized through propensity matching, baseline differences between groups remained. Despite efforts to collect a comprehensive list of baseline characteristics, there may be additional unaccounted differences that influenced the clinician's decision to administer prophylaxis. Additionally, while absolute standardized differences of baseline characteristics between groups decreased following propensity score matching, some differences remained (Table 6). While we made every effort to make the prophylaxis and no prophylaxis groups as similar as possible, the chance remains that there is a fundamental difference in the patient populations for which we could not account. Thirdly, few patients in this study received low molecular weight heparin. A previous study, primarily evaluating bleeding risk associated with pharmacological prophylaxis, found that patients receiving unfractionated heparin, but not low molecular weight heparin, were at an increased risk of in-hospital bleeding events[12]. This finding may in part be explained by a greater effect on thrombin generation with unfractionated heparin when compared to low molecular weight heparin, suggesting a more potent anticoagulant effect for unfractionated heparin in cirrhotic patients[20]. Therefore, our results should only be applied to patient's receiving unfractionated heparin. A VTE risk scoring tool that includes risk factors similar to those included in the Caprini score and Padua predictive score was used to evaluate patients within our study[38,39]. This tool was developed,

Table 3 Multivariable analysis of risk factors for development of venous thromboembolism in patients with hepatic cirrhosis

	OR	95%CI
Prophylaxis group	0.99	0.48-2.06
BMI (kg/m^2)	2.33	0.38-14.13
Baseline albumin (g/dL)	0.23	0.13-0.42
Baseline hemoglobin (g/dL)	1.18	0.99-1.41
Male sex	1.57	0.68-3.61

MELD: Model for end-stage liver disease; BMI: Body mass index.

implemented, and validated at the study site, but has not been evaluated within a population of patients with liver disease. Because a validated VTE risk score for this population was not collected and analyzed, it is possible that there was a difference in baseline VTE risk for which we could not account. However, baseline characteristics that were collected that are risk factors for VTE (such as hospital length of stay and comorbidities) were balanced between groups. Finally, our study relied on *ICD-9* codes to identify all patient diagnoses, including liver disease, VTE and bleeding events, and comorbid conditions. Although this is consistent with other studies on this topic, confirmation of clinical conditions aside from VTE and bleeding events was not manually performed. Additionally, validation of these events was only completed if events had appropriate ICD-9 codes, leaving us unable to account for events that were not documented appropriately.

Our study does have some notable strengths. Incident VTE and major bleeding were clearly defined and confirmed by manual chart review. Second, our study had clear definitions for what constituted prophylaxis and no prophylaxis. Third, baseline albumin and comorbid conditions, factors known to increase the risk of VTE in liver disease, were well balanced between groups, decreasing the risk that these variables could have confounded the results. Finally, our study included patients with varying degrees and etiologies of liver disease making these results more generalizable.

In conclusion, patients receiving pharmacological VTE prophylaxis experienced a lower rate of the composite endpoint of VTE and major bleeding, though this was driven by a reduction in the rate of major bleeding. Pharmacological VTE prophylaxis was not associated with a significant reduction in the rate of VTE in patients with liver disease, but was also not associated with an increase in rates of major bleeding.

Table 4 Multivariable analysis of risk factors for development of a major bleed in patients with hepatic cirrhosis

	OR	95%CI
Prophylaxis group	0.42	0.25-0.68
Baseline albumin (g/dL)	0.61	0.42-0.90
Baseline hemoglobin (g/dL)	0.77	0.68-0.87
BMI (kg/m^2)	0.49	0.16-1.53
Male sex	0.87	0.54-1.39
MELD < 10	0.07	0.02-0.21
MELD 10-19	0.07	0.03-0.18
MELD 20-29	0.19	0.07-0.49
MELD 30-39	0.43	0.16-1.15
All MELD categories compared to MELD score > 40		

MELD: Model for end-stage liver disease; BMI: Body mass index.

Table 5 Multivariable analysis of risk factors for in-hospital mortality in patients with hepatic cirrhosis

	OR	95%CI
Prophylaxis group	1.03	0.73-1.43
Baseline albumin (g/dL)	0.68	0.52-0.88
Baseline hemoglobin (g/dL)	1.00	0.92-1.08
Male sex	1.00	0.71-1.42
Occurrence of primary endpoint	2.30	1.44-3.68
MELD < 10	0.05	0.02-0.14
MELD 10-19	0.09	0.04-0.21
MELD 20-29	0.31	0.13-0.70
MELD 30-39	0.93	0.39-2.20
All MELD categories compared to MELD score > 40		

MELD: Model for end-stage liver disease.

Table 6 Absolute standardized differences in baseline characteristics before and after propensity score matching

	Unmatched			Matched		
	No prophylaxis (*n* = 2166)	Prophylaxis (*n* = 948)	ASD	No prophylaxis (*n* = 903)	Prophylaxis (*n* = 903)	ASD
Age (yr)	58.1 ± 11.6	60.3 ± 11.8	0.153	60.1 ± 11.9	60.2 ± 11.8	0.00844
Male	1349 (62.3)	561(59.2)	0.0639	572 (63.3)	535 (59.2)	0.0842
RRT	819 (37.8)	270 (28.5)	0.199	221 (24.5)	267 (29.6)	0.115
CKD	577 (26.6)	247 (26.1)	0.0133	226 (25.0)	238 (26.4)	0.0304
Lung disease	397 (18.3)	236 (24.9)	0.160	218 (24.1)	218 (24.1)	0.000
Heart failure	360 (16.6)	210 (22.2)	0.140	192 (21.3)	196 (21.7)	0.0108
MVP	755 (34.9)	300 (31.6)	0.0682	291 (32.2)	286 (31.7)	0.0119
VTE risk score			0.390			0.218
Unknown	447 (20.6)	80 (8.4)		103 (11.4)	78 (8.6)	
Low	86 (4.0)	17 (1.8)		26 (2.9)	17 (1.9)	
Medium	1341 (61.9)	716 (75.5)		591 (65.4)	680 (75.3)	
High	292 (13.5)	135 (14.2)		183 (20.3)	128 (14.2)	
BMI (kg/m^2)	24.2 ± 0.19	24.4 ± 0.20	0.0180	24.4 ± 0.20	24.4 ± 0.20	0.00
INR	1.51 ± 0.67	1.27 ± 0.31	0.478	1.4 ± 0.57	1.3 ± 0.32	0.216
Platelet count (k/μL)	108.3 ± 76.2	159.8 ± 108.0	0.551	144.2 ± 92.5	151.0 ± 97.0	0.0718
aPTT (s)	37.68 ± 13.6	34.3 ± 10.1	0.282	34.8 ± 12.0	34.4 ± 10.2	0.0359

Albumin (g/dL)	2.8 ± 0.70	2.9 ± 0.68	0.118	2.9 ± 0.70	2.9 ± 0.67	0.00
Tbili (mg/dL)	5.88 ± 8.57	3.68 ± 6.49	0.288	4.0 ± 7.1	3.8 ± 6.6	0.0292
SCr (mg/dL)	1.65 ± 1.41	1.59 ± 1.50	0.0414	1.5 ± 1.3	1.6 ± 1.5	0.0713
Hemoglobin (g/dL)	10.0 ± 2.3	10.7 ± 2.1	0.269	10.4 ± 2.3	10.7 ± 2.1	0.136
Liver disease etiology			0.0964			0.0416
Alcoholic	745 (34.4)	296 (31.2)		278 (30.8)	286 (31.7)	
NASH	75 (3.5)	25 (2.6)		27 (3.0)	22 (2.4)	
Other[1]	1239 (57.2)	585 (61.7)		563 (62.3)	557 (61.7)	
Acute hepatitis	107 (4.9)	42 (4.4)		35 (3.9)	38 (4.2)	
MELD score	21.1 ± 10.3	17.2 ± 8.3	0.422	17.0 ± 9.1	17.4 ± 8.3	0.0459
MELD category			0.431			0.210
>40	102 (4.7)	8 (0.8)		20 (2.2)	8 (0.89)	
30-39	364 (16.8)	74 (7.8)		71 (7.9)	74 (8.2)	
20-29	637 (29.4)	238 (25.1)		205 (22.7)	233 (25.8)	
10-19	692 (31.9)	402 (42.4)		338 (37.4)	386 (42.7)	
< 10	371 (17.1)	226 (23.8)		269 (29.8)	202 (22.4)	

Data presented as *n* (%) or mean ± SD.
[1]Other liver disease includes primary sclerosing cholangitis, biliary cirrhosis, cirrhosis due to alpha-1 antitrypsin deficiency, and any other liver disease not included above. RRT: Renal replacement therapy; CKD: Chronic kidney disease; MVP: Mechanical venous thromboembolism prophylaxis; BMI: Body mass index; INR: International normalized ratio; aPTT: Activated partial thromboplastin time; Tbili: Total bilirubin; SCr: Serum creatinine; NASH: Non-alcoholic steatohepatitis; MELD: Model for end-stage liver disease; ASD: Absolute standardized difference.

ARTICLE HIGHLIGHTS

Research background

Hepatic cirrhosis has historically been considered a coagulopathic disease, as traditional measurements of coagulation are often deranged. However, more recent literature suggests an altered coagulation cascade that may be tipped toward thrombosis or bleeding based on acute insults. Major venous thromboembolism (VTE) prophylaxis guidelines currently make no recommendation on whether to provide pharmacological prophylaxis to hospitalized cirrhotic patients. This study sought to improve on previously published retrospective data that has studied this topic, and attempted to provide data about whether pharmacological prophylaxis provides net clinical benefit or causes harm in a cirrhotic population.

Research motivation

The main problem that this study attempted to solve is whether pharmacological VTE prophylaxis prevents thrombotic events in patients with cirrhosis without causing a significant additional bleeding burden. Solving this problem could provide clarity in regards to the optimal strategy to prevent thrombotic events in cirrhotic patients.

Research objectives

The main objective of this study was to determine whether pharmacological VTE prophylaxis was beneficial overall to cirrhotic patients. This was assessed using a composite outcome of incident VTE and incident major bleeding, as the authors considered either of the events included in the composite outcome to be similarly detrimental to a patient. We feel that this study evaluated the benefits of pharmacological prophylaxis to the best of the capabilities of a retrospective study, and showed no harm to patients receiving prophylactic anticoagulation. Our findings could be used to demonstrate that pharmacological prophylaxis is likely safe in a population such as ours, which could allow for a future prospective, randomized controlled trial to be completed in an ethical manner.

Research methods

This study was a retrospective, cohort trial of patients with cirrhosis that received or did not receive pharmacological VTE prophylaxis during a hospitalization for any indication. Cirrhosis and other baseline past medical history that may have contributed to bleeding or thrombosis were identified using *ICD-9* codes. Incident major bleeding and incident VTE were identified using *ICD-9* codes and verified in the patient's medical record by reviewing relevant imaging reports and lab values. We attempted to balance the patient groups by performing propensity score matching, and to account for any additional imbalance through multivariable logistic regression.

Research results

Baseline characteristics were largely balanced when comparing groups. Our primary outcome (the composite of incident major bleeding or incident VTE) was found to occur significantly less frequently in the prophylaxis group than in the no prophylaxis group (5.1 *vs* 8.7%, $P < 0.05$), though this result was driven largely by a higher rate of major bleeding in the no prophylaxis

group. This result was confirmed on multivariable analysis, as receipt of pharmacological prophylaxis was significantly associated with a lower odds of major bleeding (though no significant association with pharmacological prophylaxis was noted on multivariable analysis of VTE).

Research conclusions

The major finding of this study was that pharmacological VTE prophylaxis did not increase the incidence of major bleeding in a large cohort of hospitalized cirrhotic patients. This challenges the historical idea that pharmacological prophylaxis should be withheld from cirrhotic patients due to an increased bleeding risk, and is more in line with recent findings that while cirrhotic patients have an altered coagulation cascade, they are at risk for both thrombotic and bleeding complications depending on acute insults. This finding could be the impetus for a large, randomized controlled trial in this patient population that could better answer the question of whether prophylactic anticoagulation truly prevents incident thrombotic events in a cirrhotic population.

Research perspectives

We feel that the only way to definitively answer the question of whether pharmacological prophylaxis is effective in preventing incident thrombotic events in a cirrhotic population is through a randomized, controlled trial. However, we feel that the lack of an increase in bleeding complications observed in this study is significant, and should allow for the pursuit of such a study without significant concern for harming a cirrhotic population similar to ours by providing pharmacologic VTE prophylaxis.

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ORIGINAL ARTICLE

Observational Study

Nonalcoholic fatty liver disease prevalence in an Italian cohort of patients with hidradenitis suppurativa: A multi-center retrospective analysis

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Abstract

BACKGROUND

Nonalcoholic fatty liver disease (NAFLD) includes two distinct conditions, with different histologic features and prognosis: non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH). Furthermore, NASH is the more aggressive necro-inflammatory form, which may accumulate fibrosis and result in End stage liver disease (ESLD). NAFLD is also linked to systemic inflammatory conditions such as psoriasis. NAFLD is currently the most common cause of ESLD in Western countries, becoming a serious public health concern. Hidradenitis suppurativa (HS) is a systemic inflammatory/autoinflammatory disease of the terminal follicular epithelium of the apocrine gland with a prevalence of 0.05% to 4.10%. Due to its systemic inflammatory behavior several comorbidities were recently associated, however liver ones were scarcely assessed.

AIM

To evaluate the prevalence and characteristics of NASH/NAFL in HS patients.

METHODS

This retrospective study is a sub-analysis of a larger study carried out in 4 Italian dermatological centers. In this cohort, there were 83 patients: 51 patients with HS only, 20 patients with HS/NAFL and 12 with HS/NASH.

RESULTS

Inflammatory comorbidities were present in 3.9% of HS only patients, 25% of HS/NAFL patients and 58.3% of HS/NASH patients ($P < 0.001$). Similarly, mean Autoinflammatory Disease Damage Index (ADDI) was significantly higher among patients with HS/NASH (5.3 ± 2.2, $P < 0.001$) compared to patients with HS/NAFL or HS only (2.8 ± 1.6 and 2.6 ± 1.4 respectively). Furthermore, ADDI correlates with IHS4 in HS, HS/NAFL and HS/NASH. Diabetic patients have higher Hurley score than not diabetic ones. Ultrasound examination was significantly different in the three groups.

CONCLUSION

HS patients displayed a high prevalence of NASH/NAFLD and ultrasound examination should be particularly addressed to patients that display high ADDI scores.

Key words: Non-alcoholic steatohepatitis; Non-alcoholic fatty liver; Nonalcoholic fatty liver disease; End stage liver disease; Hidradenitis suppurativa

Core tip: Nonalcoholic fatty liver disease (NAFLD), in its two variants non-alcoholic fatty liver and non-alcoholic steatohepatitis, is often linked to systemic inflammatory conditions, such as psoriasis. Remarkably, hidradenitis suppurativa (HS) is a new affirming systemic inflammatory disorder of the follicular epithelium of skin apocrine glands with a prevalence in normal population ranging from 0.05% to 4.10%. Furthermore, HS patients display a significant comorbidities burden (*e.g.*, cardiovascular risk, metabolic syndrome, diabetes, and spondyloarthritis) but the association with NAFLD was not previously investigated. This is the first study which evaluated NAFLD prevalence and its characteristics in HS patients.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) includes two distinct entities, with different histologic clues and prognosis: non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH), the more aggressive necro-inflammatory form, which may accumulate fibrosis and result in End stage liver disease (ESLD) and its complications, including hepatocellular carcinoma (HCC)[1].

Nowadays, NAFLD represents the main cause of chronic liver disease in Europe and North America, where is found in 17%-30% of the population, worldwide the prevalence is 2%-4% of the population becoming a serious public health concern[2]. Evidences suggest that NAFLD is the hepatic sign of metabolic syndrome; therefore, is linked not only with an increase of liver-related mortality, but also of the overall mortality. Noninvasive techniques, such as biological tests and elastography can be used for the evaluation of NAFLD patients. Today, liver biopsy (diagnostic gold standard) should be recommended in selected cases.

Patients with NAFLD would benefit from their lifestyle changes by progressive weight loss through exercise and low sugar and fat intake. Pharmacotherapy should be reserved for patients with significant fibrosis. Unfortunately, there are no Food and Drug Administration (FDA) approved therapies[3].

Hidradenitis suppurativa (HS) is a systemic, chronic, inflammatory/autoinflammatory disease with a relapsing remitting behavior and a deep impact on patient's quality of life. Despite its elusive pathogenesis, clinical manifestations are clear and space from painful nodules to fistula, mainly involving areas rich in apocrine gland-bearing, such as armpits, inguinal and anogenital regions (Dessau definition)[4-6]. HS is an affirming systemic inflammatory disease and this idea was sustained by the recent acquisitions in the pathogenesis[7], epidemiology[8] and therapy[9]. Until recently, it was considered to be a rare disease with a prevalence cited as approximately 1%[10]. Actually, the prevalence of HS seems to be greater varying from 0.05% to 4.10%; this variability is intrinsically affected by study type, being lower in retrospectively designed studies and the higher in prospective or self-reported ones[11].

European guidelines for the management of HS have been published[12]: No therapy is actually able to guaranty a high rate of complete disease remission. As for patients with NAFLD also patients with HS would benefit from their lifestyle changes by losing weight. Furthermore, topical and systemic antibiotics, injected corticosteroids, or biologics and other systemic treatments may be used. Oral antibiotics may be used to help prevent new lesions. Moderate stages may be treated with oral antibiotics, oral retinoids such as isotretinoin, hormonal therapy, and/or surgery[11,12]. For moderate to severe disease, target therapy directed against TNF-alpha proteins which are involved in the inflammation process are used: adalimumab has been approved by the FDA as orphan product for HS treatment. Adalimumab, a TNF blocker, is actually the only biologic drug approved in Italy for HS patients and notable in October 2018 it received an extension also for children over 12 years old[13]. Due to the increased body of comorbidities currently associated with HS[14,15], the liver metabolic comorbidities were neglected.

NAFLD is considered a multisystem pathology increasing the risk of diabetes mellitus, cardiovascular and chronic renal disorders, diseases with an increased incidence in HS patients[16].

Over the last decade, it has been growing the evidence that NAFLD is associated with psoriasis, another systemic chronic inflammatory disease[17-19]. Despite the high incidence of NAFLD and the current evidence that HS is not an uncommon disease, there are currently no studies in the literature investigating the association between NAFLD and chronic skin diseases other than psoriasis.

MATERIALS AND METHODS

Study population

This retrospective study is a sub-analysis of a larger one carried out in the Department of Dermatology of Ospedale Maggiore Policlinico at the beginning and after extended to other 3 primary dermatological Italian centers, namely San Donato Hospital, San Gallicano Hospital and Galeazzi Hospital. The study started in January 2018 and

ended in December 2018. Patients were recruited by filling the recently proposed visual-aided questionnaire for the self- assessment of HS[20]. The positive patients were after assessed in a dedicated HS-Lab. The diagnosis of HS was performed by two independent board-certified dermatologists following the Dessau criteria[21]. The inclusion criteria comprehended HS diagnosis, Alcohol Use Disorders Identification Test (AUDIT) < 8[22], last 3 complete blood count (CBC) available with transaminases. The exclusion criteria comprehended AUDIT score > 7, pre-existent hepatic cirrhosis, viral hepatitis (B, C and E) and recent drug-related hepatitis (< 5 years), congenital hepatic malformations, hepatic or cholangitic autoimmune conditions.

All patients underwent a hepatologic visit and ultrasonographic (US) evaluation of the liver. Patients with raised liver enzymes underwent liver biopsy to evaluate the presence of NAFLD according the European Association for the Study of the Liver (EASL), European Association for the Study of Diabetes (EASD) and European Association for the Study of Obesity (EASO) Clinical Practice Guidelines for the management of NAFLD[23]. Patients were also screened for diabetes, a predisposing factor for NASH and NAFLD. Diabetes diagnosis was performed following these criteria: a random blood sugar level of equal or greater than 200 mg/dL or 11.1 mmol/L or fasting blood sugar test of 126 mg/dL (7 mmol/L) or higher on two separate tests or oral sugar test of equal of higher than 200 mg/dL (11.1 mmol/L) after two hours.

Outcomes of the study

During dermatological assessment, besides demographics, drug-history and comorbidities, were collected HS clinical phenotypes[24], static score as Hurley[25], dynamic score as international HS 4 (iHS4)[26], the Autoinflammatory Disease Damage Index (ADDI)[27,28] and Dermatology Life Quality Index (DLQI)[29].

Statistical analysis

Variables were described as number and/or percentages. All variables were preliminarily assessed with Shapiro-Wilk test to establish the parametric behavior. The Wilcoxon-Mann-Whitney test was employed to deal with quantitative variables, whilst Fisher's exact test was applied with qualitative variables comparison. A P value < 0.05 was considered significant. The analysis was performed with the statistical software SPSS ver. 20.0 (Armonk, NY: IBM Corp.).

RESULTS

Demographics and clinical characteristics were summarized in Table 1. Interestingly from the pool of 86 patients that had a positive visual-aided questionnaire for the self-assessment of HS, after clinical assessment we enrolled 83 HS patients with the above HS clinical phenotypes: 54 regular type, 6 frictional type, 10 scarring folliculitis type, 5 conglobata type, 5 syndromic type, 3 ectopic type.

In this cohort, there were 51 patients with HS only, 20 patients with HS and NAFL (HS/NAFL) and 12 with HS and NASH (HS/NASH)(Table 1). The groups were predominantly composed by females, in fact males were 33.3% of HS only, 43.8% HS/NAFLD, 41.7% HS/NAFL and 45% HS/NASH patients being female and did not display significant difference (P = 0.62, P = 0.52, P = 84). The average age between groups was similar (HS only 43 ± 8.9; HS/NAFLD 41.3 ± 9.0; HS/NAFL 40.6 ± 10.3; HS/NASH 41.6 ± 7.4, P = 0.56). Patients also had similar Body Mass Index (BMI) with HS only having an average BMI of 28.3 ± 2.5 kg/m^2, HS/NAFLD patients being 27.6 ± 1.9 kg/m^2 (P = 0.38), HS/NAFL 27.6 ± 1.7 kg/m^2 (P = 0.22) and HS/NASH having 27.6 ± 2.7 kg/m^2 (P = 0.38). Diabetes was present in 24% of HS only patients, 30% of HS/NAFL and 25% of HS/NASH patients. Inflammatory comorbidities (Table 1) were present in 3.9% of HS only patients, 37.5% of HS/NAFLD, 25% of HS/NAFL patients and 58.3% of HS/NASH patients with a statistically different prevalence (P < 0.001). Specifically, in HS only patients one had acne conglobata and 1 patient had lichen sclerosus; while in HS/NAFL there was one patient with Crohn's disease, 1 with Pyoderma gangrenosum, Acne, and Hidradenitis Suppurativa (PASH), 1 with psoriasis, 1 with spondyloarthritis and 1 with uveitis. Finally, of the HS/NASH patients, 1 had Crohn's disease, 4 had PASH, 1 had psoriasis and 1 had spondyloarthritis.

The average IHS4 score among HS/NASH patients (12.7 ± 3.6, P = 0.03) was the highest, while it was similar among those with HS only and HS/NAFL patients (9.6 ± 3.6 and 9.4 ± 3.9 respectively, P = 0.86). Likewise, mean ADDI was significantly higher among HS/NASH patients (5.3 ± 2.2, P < 0.001) compared to HS only and HS/NAFL patients (2.8 ± 1.6 and 2.6 ± 1.4 respectively) (Table 1). There were no significant differences in Hurley score, however 83% of HS/NASH patients had a Hurley score

Table 1 Characteristics of 83 patients with hidradenitis suppurative, Nonalcoholic fatty liver and Nonalcoholic steatohepatitis and intercalsses charactersitics

	HS only	NAFLD	*P* value	NASH	*P* value	NAFL	*P* value
n	51	32		12		20	
Age, mean(SD)	43.04 (8.9)	41.32 (9.0)	0.564	41.58 (7.4)	0.602	40.55 (10.3)	0.315
Age cat (%)			0.646		0.463		0.629
< 30	3 (5.9)	4 (12.5)		1 (8.3)		3 (15.0)	
30-39	16 (31.4)	8 (25.0)		2 (16.7)		6 (30)	
40-49	18 (35.3)	14 (43.8)		7 (58.3)		7 (35.0)	
> 50	14 (27.5)	6 (18.8)		2 (16.7)		4 (20.0)	
Male, *n* (%)	17 (33.3)	14 (43.8)	0.623	5 (41.7)	0.835	9 (45.0)	0.52
Diabetes, *n* (%)	12 (23.5)	9 (28.1)	0.853	3 (25.0)	0.764	6 (30.0)	0.794
BMI, mean(SD)	28.31 (2.5)	27.56 (1.9)	0.381	27.58 (2.7)	0.376	27.55 (1.7)	0.218
bmi_cat (%)			0.559		0.515		0.384
Normal Weight	4 (7.8)	4 (12.5)		2 (16.7)		2 (10.0)	
Overweight	38 (74.5)	26 (81.3)		9 (75.0)		17 (85.0)	
Obese	9 (17.6)	2 (6.3)		1 (8.3)		1 (5.0)	
IHS4, mean(SD)	9.57 (3.6)	11.32 (2.8)	0.025	12.67 (3.6)	0.009	9.40 (3.9).	0.861
IHS4 cat (%)			0.028		0.007		0.97
Mild	5 (9.8)	3 (9.4)		1 (8.3)		2 (10.0)	
Moderate	24 (47.1)	10 (31.3)		0 (0)		10 (50.0)	
Severe	22 (43.1)	19 (59.4)		11 (91.7)		8 (40)	
Hurley (%)			0.494		0.197		0.785
1	5 (9.8)	3 (9.4)		1 (8.3)		2 (10.0)	
2	24 (47.1)	6 (18.8)		1 (8.3)		5 (25.0)	
3	22 (43.1)	23 (71.9)		10 (83.3)		13 (65.0)	
Elevated_liver_enzymes, *n* (%)	19 (37.3)	9 (28.1)	0.617	4 (33.3)	0.998	5 (25.0)	0.482
ADDI_score, mean(SD)	2.55 (1.4)	3.72 (1.8)	< 0.001	5.33 (2.2)	< 0.001	2.75 (1.6)	0.603
Inflammatory comorbidities, *n* (%)	2 (3.9)	12 (37.5)	< 0.001	7 (58.3)	< 0.001	5 (25.0)	0.025
In detail (%)			0.001		< 0.001		0.047
Acne conglobata	1 (2.0)	0 (0)		0 (0)		0 (0)	
Crohn disease	0 (0)	2 (6.3)		1 (8.3)		1 (5.0)	
Lichen sclerosus	1 (2.0)	0 (0)		0 (0)		0 (0)	
PASH	0 (0)	5 (15.6)		4 (33.3)		1 (5.0)	
Psoriasis	0 (0)	2 (6.3)		1 (8.3)		1 (5.0)	
Spondyloarthritis	0 (0)	2 (6.3)		1 (8.3)		1 (5.0)	
Uveitis	0 (0)	1 (3.1)		0 (0)		1 (5.0)	
Positive ultrasound, *n* (%)	11 (21.6)	32 (100.0)	< 0.001	12 (100.0)	< 0.001	20 (100.0)	< 0.001
NASH	0 (0)	12 (37.5)	< 0.001	12 (100.0)		0 (0)	
NAFL	0 (0)	20 (62.5)	< 0.001	0 (0)		20 (100.0)	

ADDI: Autoinflammatory disease damage index; BMI: Body mass index; HS: Hidradenitis suppurativa; IHS4: International Hidradenitis Suppurativa Severity Scoring System, NASH: NonAlcoholic SteatoHepatitis, NAFL: NonAlcoholic Fatty Liver, PASH: Pyoderma gangrenosum, Acne, and hidradenitis suppurativa; SD: Standard deviation. Normal weight: 18.5–24.9 kg/m^2, Overweight: 25–29.9 kg/m^2 Obese: >29. 9 kg/m^2.

of 3, whereas only 65% of HS/NAFL and 57% of HS only patients had a Hurley score of 3 (P = 0.49). Presence of elevated liver enzymes was similar among the three groups (HS only 37.3%; HS/NAFL 25%; HS/NASH 33.3%, P = 0.62). Finally, ultrasound revealed a bright liver in 22% of HS only patients and all HS/NAFL and HS/NASH patients (P < 0.001).

HS only and HS/NAFL patients displayed a significant difference in inflammatory comorbidities (P = 0.025) and positivity of ultrasound (P < 0.001) (Table 1).

HS/NASH compared with patients with HS only displayed a significant difference in IHS4 (P = 0.009), ADDI (P < 0.001), inflammatory comorbidities rate (P < 0.001) and ultrasound positivity (P < 0.001) (Table 1).

HS patients with and without diabetes had a significant difference only in Hurley stage (P = 0.022) (Table 2).

Table 2 Differences among hidradenitis suppurativa patients with and without diabetes

	Non diabetes	Diabetes	*P* value
n	62	21	
Age, mean(SD)	42.44 (9.4)	41.62 (8.2)	0.722
Age cat (%)			0.203
< 30	4 (6.5)	3 (14.3)	
> 50	17 (27.4)	3 (14.3)	
30-39	20 (32.3)	4 (19.0)	
40-49	21 (33.9)	11 (52.4)	
Male, *n* (%)	21 (33.9)	10 (47.6)	0.387
Diabetes, *n* (%)	0 (0.0)	21 (100.0)	<0.001
BMI, mean(SD)	28.10 (2.6)	27.81 (1.4)	0.636
bmi_cat (%)			0.161
Normal Weight	8 (12.9)	0 (0.0)	
Obese	9 (14.5)	2 (9.5)	
Overweight	45 (72.6)	19 (90.5)	
IHS4, mean(SD)	10.37 (3.6)	8.81 (4.1)	0.101
IHS4 cat, *n* (%)			0.051
Mild	4 (6.5)	4 (19.0)	
Moderate	23 (37.1)	11 (52.4)	
Severe	35 (56.5)	6 (28.6)	
Hurley, *n* (%)			0.022
1	4 (6.5)	4 (19.0)	
2	14 (22.6)	9 (42.9)	
3	44 (71.0)	8 (38.1)	
Elevated_liver_enzymes, *n* (%)	21 (33.9)	7 (33.3)	1
ADDI_score, mean(SD)	3.13 (1.8)	2.62 (2.0)	0.275
Inflammatory comorbidities, *n* (%)	9 (14.5)	5 (23.8)	0.518
In.detail, *n* (%)			0.563
	53 (85.5)	16 (76.2)	
Acne conglobata	1 (1.6)	0 (0.0)	
Crohn	1 (1.6)	1 (4.8)	
Lichen sclerosus	0 (0.0)	1 (4.8)	
PASH	4 (6.5)	1 (4.8)	
Psoriasis	1 (1.6)	1 (4.8)	
Spondyloarthritis	1 (1.6)	1 (4.8)	
Uveitis	1 (1.6)	0 (0.0)	
Positive_ultrasound, *n* (%)	31 (50.0)	12 (57.1)	0.754
NASH, *n* (%)	9 (14.5)	3 (14.3)	1
NAFL, *n* (%)	14 (22.6)	6 (28.6)	0.795
Disease, *n* (%)			0.853
HS only	39 (62.9)	12 (57.1)	
NAFL	14 (22.6)	6 (28.6)	
NASH	9 (14.5)	3 (14.3)	

ADDI: Autoinflammatory Disease Damage Index; BMI: Body mass index; HS: Hidradenitis suppurativa; IHS4: International Hidradenitis Suppurativa Severity Scoring System; NASH: Non-alcoholic steatohepatitis; NAFL: Nonalcoholic fatty liver; PASH: Pyoderma gangrenosum, Acne, and hidradenitis suppurativa; SD: Standard deviation. Normal weight: 18.5–24.9 kg/m^2; Overweight: 25-29.9 kg/m^2; Obese: > 29.9 kg/m^2.

Age had a significant moderately positive correlation with ADDI among HS/NAFL patients (r = 0.57, P = 0.05). Next, BMI and ADDI were moderately negatively correlated in HS patients with inflammatory comorbidities (R^2 = 0.43, Figure 1).

BMI and ADDI were weakly negatively correlated in patients with HS only (r = -0.25, P = 0.05) and in those who had HS and diabetes (r = -0.46, P = 0.04). Correlation between BMI and IHS4, age and IHS4, BMI and ADDI, among the three groups was

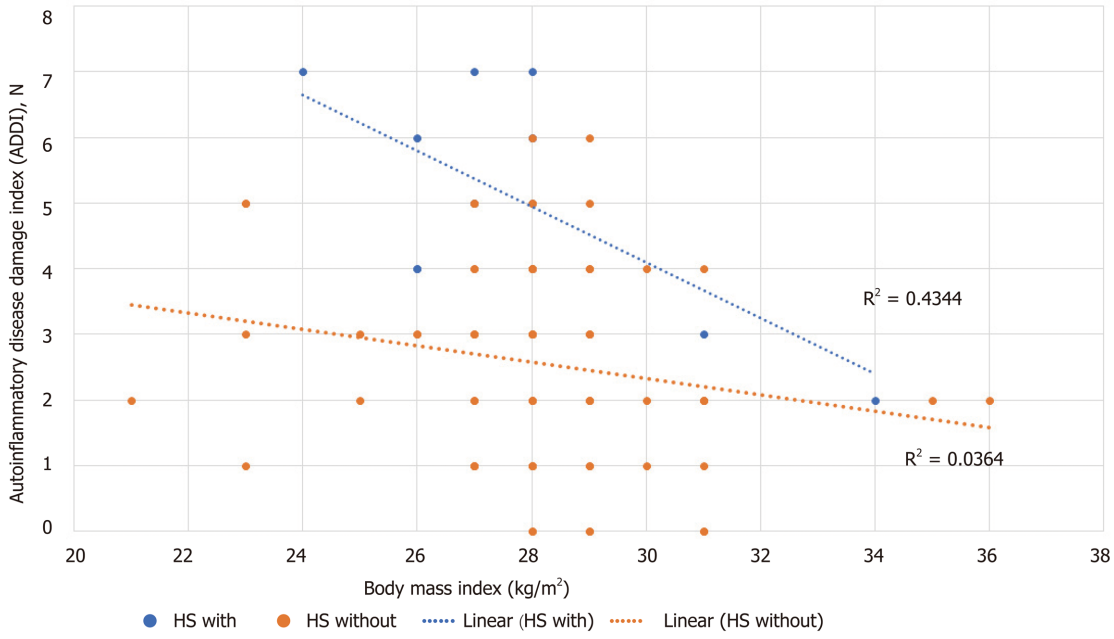

Figure 1 Correlation between body mass index and Autoinflammatory Disease Damage Index among patients with hidradenitis suppurative (HS) only, or HS with other inflammatory comorbidity.

not significant. In addition, correlation between BMI and IHS4, age and IHS4, age and ADDI based on presence of other inflammatory comorbidity was not significant. Finally, BMI and IHS4, age and IHS4, age and ADDI based on diabetes status was not significant.

Hurley score and categorical IHS4 score had good overlap in Hurley 1 and 2 scores, with 8 Hurley 1 patients also having mild IHS4 categorical score, 22 (96%) Hurley 2 patients having a moderate IHS4 categorical score, and 1 (4%) Hurley 2 patient having a severe IHS4 categorical score. However, among 52 patients with Hurley score 3, 12 (23%) were considered moderate IHS4, and 40 (77%) were considered severe (P < 0.001). Average ADDI score among Hurley 1 patients was 0.75 ± 1.2, 1.9 ± 1.4 among Hurley 2 patients and 3.8 ± 1.6 among Hurley 3 patients (P < 0.001).

There was a moderate correlation between IHS4 and ADDI scores among all 3 groups [R^2 = 0.48 (P < 0.001) for HS only; R^2 = 0.51(P < 0.001), for HS/NAFL; R^2 = 0.57 (P < 0.001), for HS/NASH, Figure 2].

DISCUSSION

In our cohort of HS patients, for the first time, was described a 38,5% NAFLD prevalence: 24% of NAFL and 14,5% of NASH. Likewise, in psoriasis, HS patients with NAFLD displayed the higher severity scores, namely IHS4 and ADDI. These findings, together with pathogenetic[7], epidemiologic[8] and therapeutic[9] evidences, further confirm the recent idea that HS is a systemic inflammatory disease. NAFLD is the main entity to cause ESLD in Europe and North America, this is easy to predict that it will become the most frequent liver transplantation indication by 2030[16]. Although the weight of the disease is so overwhelming, there are no really effective drugs in treatment[3]. Therefore, it is essential to investigate all co-morbidities that can worsen the prognosis, among these in our study emerges the role of HS, whose treatment is a controversial issue[30-32]. Microbiological data show that HS is associated with polymicrobial flora, including anaerobic microorganisms[33,34]. On this point, Guet-Revillet *et al*[33], in a French prospective microbiological study on 102 HS lesions from 82 patients, found that Staphylococcus lugdunensis was cultured in 58% of HS lesions and anaerobic microorganisms, including actinomycetes, were observed in 24% of abscesses or nodules and in 87% of chronic lesions. More recently, in a prospective metagenomic study, the same Author, using high-throughput sequencing, confirmed the high prevalence of polymicrobial anaerobic flora in HS[35]. Overall, topical or oral antibiotics (monotherapy or combination therapy) is commonly suggested for the management of HS flares[12,36]. The most common antibiotic regimens used for the treatment of HS included topical clindamycin, oral

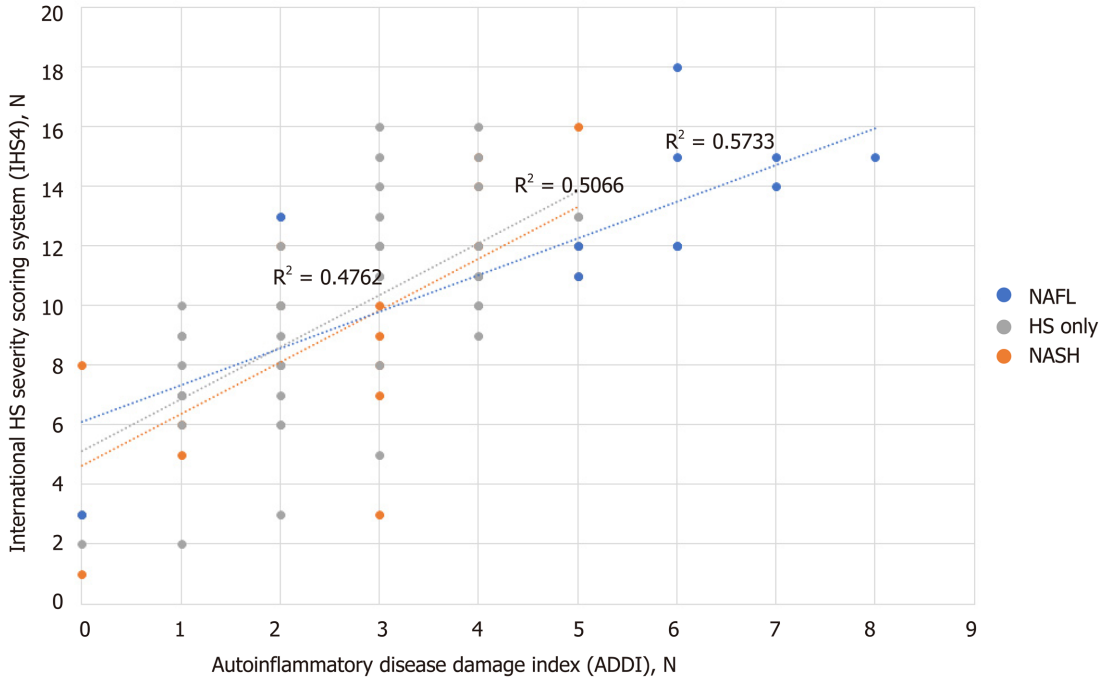

Figure 2 Correlation between international hidradenitis suppurativa severity scoring system and autoinflammatory Disease Damage Index among patients with hidradenitis suppurative only, or non-alcoholic staetohepatitis or non-alcoholic fatty liver.

tetracyclines, oral clindamycin-rifampicin combination and parenteral ertapenem followed by oral rifampicin-moxifloxacin-metronidazole combination[12,37]. No data are available for the antibiotic management of HS with the newer drugs, including dalbavancin, daptomycin and tigecycline[38-40]. Moreover, there are insufficient data to support intravenous antibiotics[41-43]. A major concern of the antibiotic use in HS is the increasing of antimicrobial resistance[44,45]. Finally, a clinical monitoring and a dose adjustment in patients with liver disease can be required[46,47] in view of the fact that NAFLD remains the main source of ESLD in Western countries[1]. It is clear, with these premises, that the available therapeutic armamentarium for the treatment of both diseases is very inadequate. Of our findings, the most obvious appears the US finding of bright liver in 22% of HS only and all HS/NAFL or HS/NASH patients ($P < 0.001$). However liver biopsy (histology) remains the gold standard in the diagnosis of NAFLD, as recently suggested in a meta-analysis that compared US and histology quantifying diagnostic sensitivity to 84.8% (79.5-88.9), specificity to 93.6% (87.2-97.0), positive likelihood ratio to 13.3 (6.4-27.6) and negative likelihood ratio to 0.16 (0.12-0.22)[48].

Interestingly the newly proposed ADDI score displayed a clinically meaning in addressing ultrasound examination in patients with NASH. PASH syndrome patients all displayed NAFLD and this confirm that higher levels of inflammations trigger the development of liver disease. Adipose tissue is not inert but metabolically active and release pro-inflammatory cytokines, furthermore the metabolic syndrome is a recognized comorbidity of both NASH and HS. Thus, the finding is that ADDI correlates with BMI in patients with inflammatory comorbidities. To further enforce its clinical capability, ADDI and IHS4, the dynamic severity index, correlated in the examined groups. Therefore ADDI, a composite index derived from the global examination of monogenic autoinflammatory diseases and applied to HS[27], is related to the dynamic index that monitor HS skin inflammation. This assumption empowers the thesis that HS should be considered an autoinflammatory polygenic disease and treated by physicians as a systemic condition. From this point of view, is it really correct talk about comorbidities or it is more proper define them as different manifestations of a common spectrum of disease, namely HS as a systemic disease. As for psoriasis, the real goal for the newly introduced biological therapy will be to act on both cutaneous and systemic manifestation of HS.

In conclusion high prevalence of NAFLD was found in HS patients and an US screening to exclude liver abnormalities should be performed especially in HS patients with active disease and inflammatory comorbidities.

ARTICLE HIGHLIGHTS

Research background

Nonalcoholic fatty liver disease (NAFLD), in its two variants non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH), is the main cause of End stage liver disease (ESLD) and its complications, including hepatocellular carcinoma (HCC) in North America and Europe. Due to its impact on morbility and mortality, the identification of population with high risk of NAFLD is mandatory and in literature some systemic inflammatory diseases are described to be linked with NAFLD. Hidradenitis suppurativa (HS) is a new affirming systemic inflammatory disorder of the follicular epithelium of skin apocrine glands with a prevalence in normal population ranging from 0.05% to 4.10%. No data are present in literature towards the prevalence of NAFLD in HS.

Research motivation

The estimation of NAFLD in HS patients may lead to an early and optimized treatment.

Research objectives

This study aimed first to evaluate the overall prevalence of NAFLD and specifically of NAFL and NASH. Secondary aims were the clinical characterization of these patients. Depict a profile of HS patients with NAFLD will be crucial in optimizing clinical and therapeutic management.

Research methods

This retrospective multicenter carried out 4 primary dermatological Italian centers started in January 2018 and ended in December 2018. Patients were recruited by filling the recently proposed visual-aided questionnaire for the self- assessment of HS and after underwent a dermatologic visit that evaluate HS with static (Hurley score) and dynamic indexes (ADDI: Autoinflammatory Disease Damage Index, IHS4: International Hidradenitis Suppurativa Severity Scoring System). Transaminases were assessed and all patients underwent liver sonography (US). NASH suspected cases were biopsied.

Research results

We included 83 HS patients, in detail 51 patients with HS only and 32 with NAFLD (20 with NAFL, 12 NASH). Inflammatory comorbidities were present in 3.9% of HS only patients, 37.5% of HS/NAFLD, 25% of HS/NAFL patients and 58.3% of HS/NASH patients ($P < 0.001$). The average IHS4 score among HS/NASH patients (12.7 ± 3.6, $P = 0.03$) was the highest, while it was similar among those with HS only and HS/NAFL patients (9.6 ± 3.6 and 9.4 ± 3.9 respectively, $P = 0.86$). Likewise, mean ADDI was significantly higher among HS/NASH patients (5.3 ± 2.2, $P < 0.001$) compared to HS only and HS/NAFL patients (2.8 ± 1.6 and 2.6 ± 1.4 respectively). While no significant differences were found in Hurley score. There was a significant positive correlation between IHS4 and ADDI scores among all 3 groups ($r = 0.7$, $P < 0.001$ for HS only; $r = 0.71$, $P = 0.0004$ for HS/NAFL; $r = 0.76$, $P = 0.004$ for HS/NASH). Finally, BMI and ADDI were weakly negatively correlated in patients with HS only ($r = -0.25$, $P = 0.05$) and in those who had HS and diabetes ($r = -0.46$, $P = 0.04$).

Research conclusions

HS patients have a high prevalence of NAFLD. In particular clinicians should sonographically assess HS patients with more active disease (high IHS4 score) and with other inflammatory comorbidities (high ADDI).

Research perspectives

The present study highlighted the association between HS and NAFLD. However other issues remain still open to future investigations. In particular related issues,that should be addressed to optimize patient management are the prevalence of NAFLD HS-related in different ethnicity and the impact of systemic therapies on NAFLD development in HS patients.

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CASE REPORT

Leukocytoclastic vasculitis caused by hepatitis C virus in a liver transplant recipient: A case report

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Author contributions: Trevizoli NC and Watanabe ALC designed the report; Ferreira GSA and Araujo MCCL collected the patient's clinical data; Ferreira GSA, Araujo GC and Machado AC analyzed the data and wrote the paper; Jorge FMF and Diaz LGG reviewed the paper.

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Abstract

BACKGROUND

Infection by the hepatitis C virus (HCV) is currently considered to be a global health issue, with a high worldwide prevalence and causing chronic disease in afflicted individuals. The disease largely involves the liver but it can affect other organs, including the skin. While leukocytoclastic vasculitis has been reported as one of the dermatologic manifestations of HCV infection, there are no reports of this condition as the first symptom of HCV recurrence after liver transplantation.

CASE SUMMARY

We report here a case of leukocytoclastic vasculitis in a liver transplant recipient on maintenance immunosuppression. The condition presented as a palpable purpura in both lower extremities. Blood and urine cultures were negative and all biochemical tests were normal, excepting evidence of anemia and hypocomplementemia. Imaging examination by computed tomography showed a small volume of ascites, diffuse thickening of bowel walls, and a small bilateral pleural effusion. Skin biopsy showed leukocytoclasia and fibrinoid necrosis. Liver biopsy was suggestive of HCV recurrence in the graft, and HCV polymerase chain reaction yielded 11460 copies/mL and identified the genotype as 1A. Treatment of the virus with a 12-wk direct-acting antiviral regimen of ribavirin, sofosbuvir and daclatasvir led to regression of the symptoms within the first 10 d and subsequent complete resolution of the symptoms.

CONCLUSION

This case highlights the difficulties of diagnosing skin lesions caused by HCV

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infection in immunosuppressed patients.

Key words: Hepatitis C; Liver transplantation; Leukocytoclastic vasculitis; Immunosuppression; Direct-acting antivirals; Case report

Core tip: Leukocytoclastic vasculitis is an uncommon extrahepatic manifestation of infection by the hepatitis C virus (HCV). We report the case of a patient who underwent liver transplantation for the treatment of cirrhosis and hepatocellular carcinoma associated with HCV infection, and who developed skin lesions and systemic symptoms, such as fever, post-transplantation. A short time after HCV antiviral treatment was started, the patient showed complete regression of all symptoms. While there are previous reports of leukocytoclastic vasculitis in solid organ transplant recipients, we have found no previous case in the literature of this being a symptom of HCV recurrence in this context.

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INTRODUCTION

Hepatitis C is a chronic liver disease caused by infection with the hepatitis C virus (HCV). According to the Global Hepatitis Report 2017, around 71 million people worldwide carry the HCV infection[1]. As this disease leads to liver fibrosis, cirrhosis and/or hepatocellular carcinoma (HCC), it is currently one of the most common causes for liver transplant worldwide, being the main cause of liver transplant in the United States and Europe[1,2].

Recurrence of HCV after liver transplantation is a common event, occurring in practically all patients who were not successfully treated prior to the surgical procedure[3,4]. Diagnosis is made by either liver biopsy or detection of HCV in the blood *via* polymerase chain reaction (PCR). While the timing to recurrence varies among individuals, the manifestations of the HCV infection after the transplant are typically more aggressive than with the original infection due to the post-transplantation immunosuppressive drug regimen[3-5].

Infection by HCV is not limited to the liver and extrahepatic manifestations can include mixed cryoglobulinemia, insulin resistance, and depression, as well as cardiovascular, renal and dermatological diseases[6]. Among the dermatological manifestations in particular, the more common are late cutaneous porphyria, cryoglobulinemic vasculitis, lichen planus, psoriasis, erythema multiforme, erythema nodosum, and necrolytic acral erythema[7].

Leukocytoclastic vasculitis is an inflammatory syndrome that affects small-sized vessels, and the most prominent histological features are leukocytoclasia (neutrophil fragmentation) and fibrinoid necrosis[8]. It can develop secondary to drugs, infection, collagen disease, or cancer. The most common clinical presentation is a palpable purpura in the lower extremities[8,9], although cutaneous manifestations can also include petechiae, nodules or ulcers. Ultimately, skin biopsy is necessary for diagnostic confirmation[10,11].

Cases of leukocytoclastic vasculitis in organ transplant patients have been reported, but none of those previous cases have cited HCV recurrence as the underlying cause.

CASE PRESENTATION

A 61-year-old female with cirrhosis due to chronic HCV infection, cirrhosis and HCC, underwent liver transplant.

At the time of transplantation, the patient's model for end-stage liver disease (MELD) score was 6 and Child-Pugh classification was grade A (5 points). Three years

prior to the procedure, the patient had undergone treatment with pegylated-interferon and ribavirin for 1 year, achieving no sustained virologic response. The interferon had been discontinued, but the ribavirin had been continued as monotherapy for another 6 mo. At the end of treatment, the patient's HCV RNA load was undetectable by PCR and the treatment had been considered effective. A screening ultrasound of the patient's abdomen, however, showed a suspicious nodule in the liver. Computerized tomography (CT) scan revealed a hypervascular lesion with rapid washout, suggestive of HCC, measuring 38 mm × 36 mm in the liver segment IVA. This lesion had close contact with the middle hepatic vein and retrohepatic inferior vena cava, and was deemed to be unresectable.

The patient was submitted to orthotopic liver transplantation with a graft from a cadaveric donor (37-year-old male). Total ischemia time was 4 h and 41 min, with no intraoperative complications and no need for blood product transfusions. She was discharged from the intensive care unit 2 d after the procedure and from the hospital 8 d after the transplant.

Chief complaints

At 5 mo after the surgery, the patient returned to the emergency room complaining of pain, swelling and redness on both lower extremities, associated with fever, diarrhea and vomiting.

History of present illness

The patient reported that the skin lesions appeared 17 d before she presented to the emergency room, and they had gradually increased in extension during that period. She had already presented to another institution and given treatment for a presumptive diagnosis of cellulitis, consisting of cephalexin and then amoxicillin–clavulanate for 1 wk. The treatment induced no improvement and her clinical condition worsened, as she developed diarrhea, vomiting and loss of appetite.

History of past illness

The patient had no previous history of skin disease and no significant comorbidities, other than HCV infection and the recent transplant. She reported contact with ticks 1 wk before the lesions appeared.

Physical examination

A painful palpable purpura was observed on both lower extremities, affecting the lateral and posterior aspect of the lower legs (Figure 1). The patient was admitted to the hospital for investigation, and intravenous cefepime was started. In addition, a short course of doxycyclin was administered, to address the history of contact with ticks.

Laboratory testing

Blood and urine cultures were obtained, yielding negative results. Biochemical tests showed normal leukocyte and platelet counts, and levels of blood urea nitrogen, creatinine, aspartate aminotransferase and alanine aminotransferase within the normal range (NR). Remarkable findings were anemia (hemoglobin concentration of 8.6 g/dL) and hypocomplementemia [C3 52 mg/dL (NR: 90-170), C4 < 2 mg/dL (NR: 12-36), total complement CH50 < 60 U/CAE (NR: 60-265)]. Tests for anti-RO, anti-LA, anti-DNA, anti-RNP, FAN, anti-SM, ANCA and lupic anticoagulant antibodies and cryoglobulins, as well as cytomegalovirus and Zika virus (by PCR), were negative.

Imaging examination

During investigation, CT scans of chest and abdomen were carried out, as well as an echocardiogram. The CT showed a small volume of ascites, diffuse thickening of bowel walls, and a small bilateral pleural effusion (Figure 2). The echocardiogram was normal, with an ejection fraction of 67.45% and no pericardial effusion.

MULTIDISCIPLINARY EXPERT CONSULTATION

A skin biopsy was obtained from the patient's left leg and investigated by histological analysis carried out by Dr. Silvia Conde Watanabe, an expert in skin pathologies.

FINAL DIAGNOSIS

Histology of the skin biopsy showed discrete acanthosis, and neutrophil and erythrocyte exocytosis in the epidermis (Figures 3 and 4). The superficial and medium

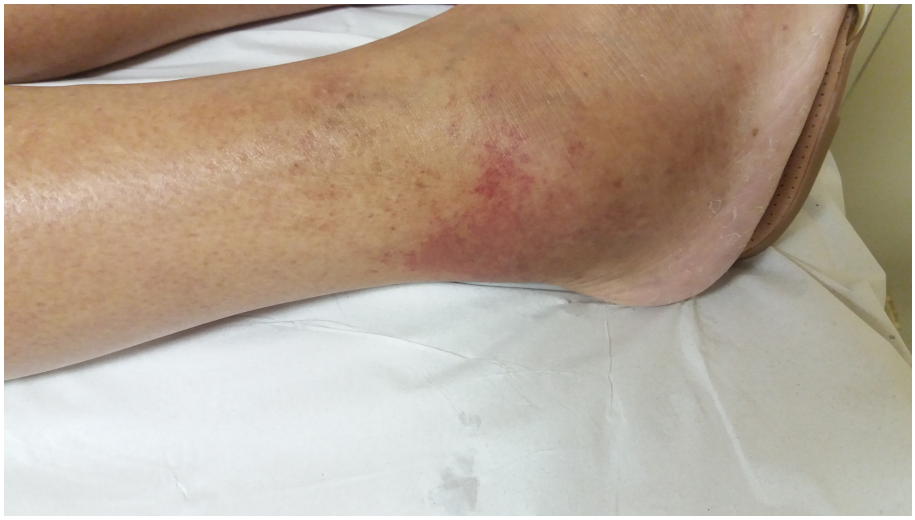

Figure 1 Aspect of the lesion on the patient´s right foot. The image was taken on the day she presented to the emergency room.

dermis showed a moderate perivascular and interstitial inflammatory infiltrate, with a predominance of neutrophils. Focal vasculitis was also found, characterized by small-size vessels surrounded by polymorphonuclear leukocytes and fibrinoid necrosis of their walls, along with eosinophil leukocytoclasia. A liver biopsy was also performed, and showed chronic hepatitis with moderate activity, suggesting HCV recurrence in the graft; METAVIR score of A2F1 was assigned. HCV PCR yielded 11460 copies/mL and identified the genotype as 1A.

TREATMENT

A 12-wk antiviral regimen was initiated, consisting of sofosbuvir (400 mg/d), daclatasvir (60 mg/d) and ribavirin (1000 mg/d). The patient experienced no adverse effects associated with the treatment. After 10 d, all skin lesions showed appreciable regression, and the patient remained afebrile.

OUTCOME AND FOLLOW-UP

At the 6-mo follow-up after finishing the 12-wk treatment, the patient remained well and with normal graft function. Blood tests detected no HCV, and there was no fever or any other sign of vasculitis.

DISCUSSION

Reportedly, half of the patients infected with HCV develop chronic liver disease[12] and about 30% progress to hepatic cirrhosis, with many eventually needing a liver transplant[4]. After the transplant, there is a 50% recurrence rate of the virus within the first year, and recurrence within 5 years is an almost a universal phenomenon[4].

Besides the characteristic effects on the liver, HCV can affect other organs and systems as well. Indeed, 10%-15% of patients infected with HCV develop symptomatic extrahepatic disease[13]. A small portion (2%-4%) present to clinic with leukocytoclastic vasculitis, accounting for 8%-19% of all leukocytoclastic vasculitis cases[13,14].

The most frequent HCV subtype found in cases of human HCV infection is genotype 1, corresponding to roughly 70%-75% of the cases in the United States alone[12]. Unfortunately, this is also the subtype with the greatest resistance to interferon, requiring longer treatment duration and greater doses[7]. No studies in the current literature, however, have reported on the association of leukocytoclastic vasculitis with any particular HCV genotype.

The treatment of HCV-related leukocytoclastic vasculitis focuses on the virus itself, aiming to reduce or eradicate it[10,11]. Prednisone has been used successfully in some cases of vasculitis, but it has no curative effect and can increase the HCV viral load

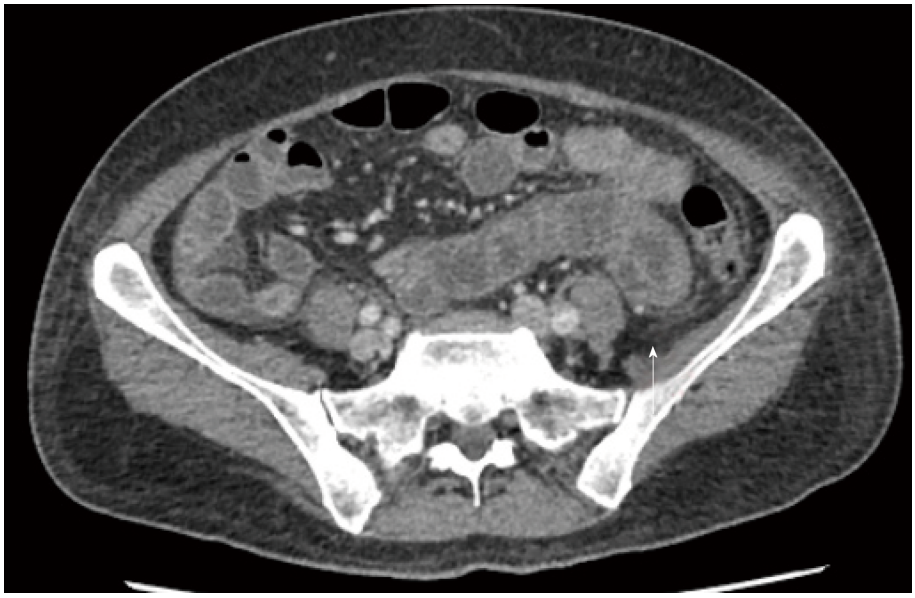

Figure 2 Computerized tomography scan showing a small volume of ascites and diffuse thickening of bowel walls (white arrow).

and associated damage to the liver[15]. One case of successful treatment with tiaprofenic acid and topical clobetazole has been reported from India, with the outcome being complete resolution after 3 wk of treatment[12].

The most important differential diagnoses of leukocytoclastic vasculitis are other vasculitis conditions, pigmented purpuric dermatosis, acute meningococcemia, disseminated gonococcal infection, disseminated intravascular coagulation, monoclonal paraproteinemia, thrombotic thrombocytopenic purpura, Gardner-Diamond syndrome, atrial myxoma, cholesterol embolism and infectious emboli[16].

CONCLUSION

The challenge of correctly diagnosing HCV-associated leukocytoclastic vasculitis after liver transplantation lies in the great number of immunological and infectious agents that can cause similar skin lesions in immunosuppressed patients. Skin biopsy is of great importance in investigating these cases. Once specific treatment is initiated against HCV, the patient usually experiences a rapid recovery and has a favorable prognosis.

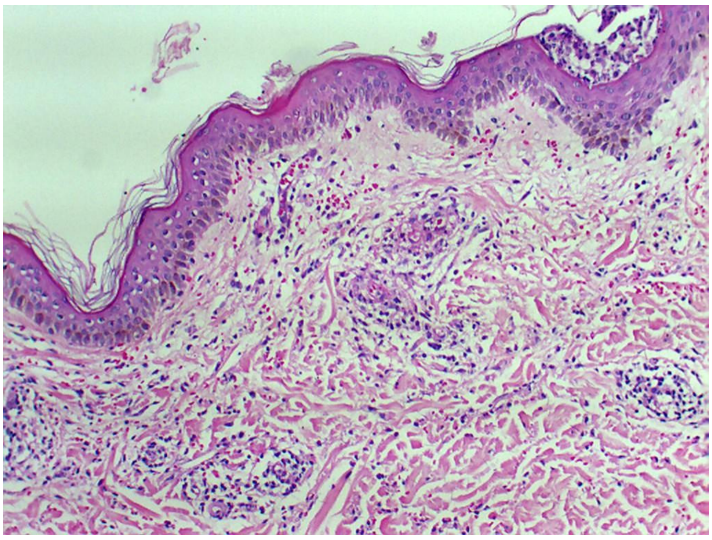

Figure 3 Focal vascular damage with mild perivascular neutrophilic infiltrate and fragmentation of the neutrophils resulting in nuclear dust (leukocytoclasis), suggestive of Urticarial vasculitis (HE 100 x).

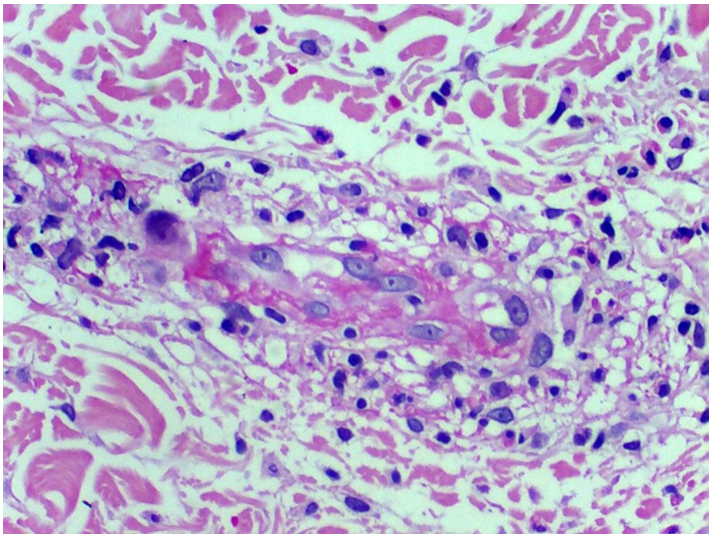

Figure 4 Perivascular neutrophilic infiltrate (HE 40 x).

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LETTER TO THE EDITOR

Yes-associated protein at the intersection of liver cell fate determination

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Abstract

A recent publication highlights the importance of high yes-associated protein (YAP) expressing cells in liver regeneration following partial hepatectomy. Although the names of the cell populations described in these articles [hybrid periportal hepatocytes (HybHP) or epithelial-mesenchymal transition (EMT)-reprogrammed hepatocytes] are not identical, they all express high levels of YAP. We hypothesize that the HybHP and EMT-reprogrammed hepatocytes might be a similar cell population. Hippo signaling is the primary pathway that regulates YAP activity. According to the contribution of these two types of cells to liver regeneration and the high YAP expression, Hippo-YAP signaling activation may be a common regulatory pathway experienced by cells undergoing dedifferentiation and reactivating proliferative activity during liver regeneration. Although no evidence has shown that HybHP cells contribute to hepatocellular carcinoma in mouse models, we can not rule out the possibility that these highly regenerative cells can further develop into tumor cells when they acquire mutations caused by viral infection or other risk factors like alcohol. The detailed mechanistic insight of the regulation of YAP expression and activity in HybHP (or other types of cells contributing to liver regeneration) is unknown. We hypothesize that liver regeneration under various conditions will eventually lead to divergent consequences, likely due to the duration of YAP activation regulated by Hippo-large tumor suppressor 1 and 2 pathway in a context- and cell type-dependent manner.

Key words: Hybrid periportal hepatocytes; Yes-associated protein; SOX9; Epithelial-mesenchymal transition; Hepatocellular carcinoma

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Core tip: Hybrid periportal hepatocytes (HybHP) and epithelial-mesenchymal transition-reprogrammed hepatocytes might be a similar cell population. The contribution of HybHPs to liver regeneration is associated with high expression of yes-associated protein (YAP). The detailed mechanistic insight of the regulation of YAP activity in HybHPs is undetermined. The context- and cell type-dependent regulation of Hippo-large tumor suppressor 1 and 2-YAP axes might lead to divergent consequences in liver regeneration.

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TO THE EDITOR

The recent publication in *Hepatology* by Jiang *et al* described that nuclear pregnane X receptor (PXR) activates hybrid periportal hepatocyte (HybHP) cell proliferation through YAP and played a key role in liver regeneration following partial hepatectomy[1]. The authors found that HybHPs comprise a SOX9-positive, YAP-high cell population and that YAP is a key protein that regulates HybHP cell proliferation. Inhibition of YAP abolished PXR-induced liver enlargement in mice. HybHP was first characterized in 2015 as a pre-existing group of periportal hepatocytes in healthy livers[2]. These cells have low Sox9 (a progenitor cell marker) expression and hepatic gene features. Another study published several months ago examined the role of epithelial-mesenchymal transition (EMT) in liver regeneration[3]. They found that some hepatocytes overexpressed YAP during the repair process after liver damage and underwent an EMT-like process. YAP interacted with Smad2 in the TGF-β pathway to promote cell proliferation.

Although the names of the cell populations described in these articles (HybHP or EMT-reprogrammed hepatocytes) are different, they both express high levels of YAP. Even though whether such EMT-reprogrammed cells express SOX9 is unknown, EMT is an important pathway to generate progenitor cells. We hypothesize that the HybHP and EMT-reprogrammed hepatocytes described in these studies are a similar cell population. According to the contribution of these two cell types to liver regeneration and the high YAP expression, Hippo-YAP pathway activation may be a common regulatory pathway experienced by cells undergoing dedifferentiation and reactivating proliferative activity during liver regeneration, regardless of whether these highly proliferative cells are derived from hepatocytes or HybHP cells. YAP may be an effective target for promoting liver regeneration in liver failure patients.

Although no evidence showed that HybHP cells contribute to hepatocellular carcinoma (HCC) in three different mouse models[2], we cannot rule out the possibility that these highly regenerative cells can further develop into tumor cells or cancer stem cells when they gain mutations caused by viral infection or other risk factors like alcohol. YAP is activated in 50% of human HCCs, and its activation level correlates with decreased survival after resection. Endogenous YAP activation perturbs hepatocyte differentiation and maintains this state in advanced tumors, and YAP silencing restores hepatocyte differentiation and leads to tumor regression[4]. It is interesting to question whether YAP is activated during controlled liver regeneration and excessive cell proliferation is prevented by inactivating YAP. However, under pathological conditions, the control of YAP activity is disrupted, resulting in continuous YAP activation and the generation of HCC.

Hippo signaling is the primary signaling pathway that regulates YAP activity, and MST1/2 phosphorylation of large tumor suppressor 1 and 2 (LATS1/2) can inhibit YAP entry into the nucleus. However, LATS1/2 phosphorylation by MST1/2 is context- and cell type-dependent. Loss of MST1/2 in mouse embryonic fibroblasts does not significant affect LATS1/2 phosphorylation or activation[5]. Therefore, we hypothesize that liver regeneration under various conditions will eventually lead to divergent consequences, probably due to the duration of YAP activation regulated by Hippo-LATS1/2 pathway in a context- and cell type-dependent manner. A deeper understanding of this aspect may uncover the key to target YAP to promote liver regeneration in pathological conditions and to control its tumorigenicity at the same time.

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