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Redox therapeutics in hepatic ischemia reperfusion injury

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Abstract

Ischemia-reperfusion plays a major role in the injury experienced by the liver during transplantation. Much work has been done recently investigating the role of redox species in hepatic ischemia-reperfusion. As animal models are better characterized and developed, and more insights are gained into the pathophysiology of hepatic ischemia reperfusion injury in humans the questions into exactly how oxidants participate in this injury are becoming more refined. These questions include effects of cellular location, timing of injury, and ability of therapeutics to access this site are increasing our appreciation of the complexity of ischemia reperfusion and improving attempts to ameliorate its effects. In this review, we aim to discuss the various methods to alter redox chemistry during ischemia reperfusion injury and future prospects for preventing organ injury
during hepatic ischemia reperfusion.

Key words: Ischemia; Reperfusion; Pre-conditioning; Nitrite

Core tip: Reactive oxygen and nitrogen species play a central role in the pathology of ischemia-reperfusion injury. In this review we discuss the efforts of many groups to trial therapeutics to ameliorate this damage in animal models of disease as well as clinical trials in humans. The failure of some trials has served to highlight the complexity of timing and compartmentalization of Ischemia Reperfusion injury. Finally, we discuss the emerging potential of replenishing nitric oxide by nitrite therapy and the uniquely broad therapeutic profile of nitrite.

INTRODUCTION

Ischemia reperfusion injury (IRI) identified in animal models in the mid-70's was not a widely used clinical term until the mid-80's[1-3]. Ischemia reperfusion is a process whereby the initial damage caused to tissue by compromised blood flow (and associated deficit in oxygen and nutrient delivery) is then compounded by additional and more severe injury caused by restoration of blood flow (*i.e.*, reperfusion). Multiple participants play a role in the post ischemic stress that is caused by restoration of blood flow and oxidant formation, the interplay of these participants form the initial layer of complexity to the problem of IRI. An excellent review by Jaeschke and

Woolbright discusses in greater detail the cellular and molecular participants in post ischemic stress of IRI[4]. Adding to this complexity is the multiple and diverse situations in which IRI occurs in the clinic which we posit likely leads to unique signatures of IRI in different diseases. In this review we discuss IRI that occurs in liver transplantation and how our understanding of these redox mechanisms may be critical as we attempt to define and implement strategies aimed at expanding donation pools by utilizing marginal donors[5]. The term marginal donor refers to grafts from donors of old age, steatosis, viral infection or other known insults to the liver graft. The added complexity resulting from each distinct clinical scenario has required further development of our scientific models to more closely mimic the problem[6]. These models have revealed a more prominent role for IRI when marginal grafts are used. The goal of this review is to provide the reader a brief review of the pathophysiology of IRI and focus the discussion on attempts to ameliorate IRI and how these therapeutics are suggesting a direction forward for clinical strategies.

One emerging candidate for the inhibition of mitochondrial dysfunction and restoration of nitric oxide bioactivity during ischemia reperfusion injury is the Nitrite anion. Emphasis on nitrite within this review will discuss the unique profile of *in vivo* nitrite to address the multiple insults of IRI injury.

IRI

During the ischemic phase of injury liver tissue is left with anaerobic metabolism to keep up with the demand of various cellular processes for high energy phosphates. Eventually the supply of high energy phosphates becomes inadequate resulting in disruption of cellular homeostasis. Primarily disruption of Na^+/K^+ ATPase function results in loss of membrane gradients which then allows for Ca^{2+} influx to the cell. Further production of reactive oxygen and nitrogen species (ROS, RNS), H^+ and toxic metabolites amplify injury and attract water into the cell and the resultant edema further impairs cellular function. As cell death begins within the hepatocytes, nearby endothelial cells and Kupffer cells begin to express adhesion molecules and chemokines, that recruit neutrophils to the site of injury and amplify tissue injury[7]. Below we discuss individual cell types implicated in liver IRI during transplantation and their respective roles in controlling the redox milieu (Figure 1).

Kupffer cells

Kupffer cells are perhaps the most important producer of ROS during the ischemic stress that occurs during the cold preservation of transplantation. A general lack of appreciation of the details of the hepatic IRI led to studies providing more detail into the timing and compartmentalization of IRI. The observation that glutathione was oxidized in the extracellular space but not within the hepatocyte drew attention to leukocytes. Given that very few neutrophils had infiltrated the liver during the early phase of ischemia focus was directed at the resident macrophages of the liver, the Kupffer cells, as possible mediators of glutathione oxidation[8,9]. Given that circulating leukocytes have not begun to infiltrate the liver in the early phase of ischemia and that donor blood is largely flushed from the liver during the initiation of cold preservation it is likely that the role of Kupffer cells in ROS production is relatively more important during the IRI experienced by the transplanted organ. The main ROS generated by Kupffer cells has been demonstrated to be the superoxide anion radical, and selective inhibition of Kupffer cells has been shown to ameliorate IRI further emphasizing the role of ROS derived from these cells[8,10,11]. Targeting the Kupffer cells clinically is challenging given their multiple roles in normal liver function and defense. However, this work reveals the clinical relevance of inhibiting ROS within the more accessible extracellular or vascular space.

Neutrophils

As Kupffer cells contribute to the early ischemic phase, neutrophils infiltrate and cause much of the damage after reperfusion. Cytokines and chemokines produced by the activated Kupffer cells initiate expression of the cellular adhesion molecules such as ICAM-1 and VCAM-1. Neutrophils become activated, adhere, and begin to infiltrate the hepatic tissue. Once activated neutrophils are capable of producing large amounts of superoxide, hydrogen peroxide, and hypochlorous acid[2,12]. These ROS are then capable of injuring the hepatocyte with the mitochondria being principal targets[13,14].

Mitochondria

Mitochondria play a central and complex role in IRI as both sources and targets of reactive oxygen species (ROS). Mitochondria produce ROS such as the superoxide anion as a result of electron leak during normal respiration. This process is enhanced during ischemia leading to decreased ATP production and organelle dysfunction[15]. Ultimately, mitochondria become central to the fate of the cell as opening of the mitochondrial permeability transition pore (MPT) instantly short circuits the membrane potential resulting in the cessation of ATP production and necrosis ensues[16]. Interestingly, whilst opening of the MPT may occur if the ischemic phase is long enough, it has also been shown that the MPT opening can be triggered by oxidant stress that ensues with the restoration of oxygen supply thereby supporting the model of IRI in that the tissue injury is amplified during the post ischemic stress phase[14].

ANTI-OXIDANT BASED THERAPIES TO INHIBIT IRI

As described above, elucidation of the mediators, timing, and location of IRI has proven complex and presented many potential targets for therapy. Indeed further experiments to evaluate the role of many therapeutics within

Figure 1 Ischemia initiates injury leading to reactive oxygen species formation from mitochondria and Kupffer cells. Restoration of blood flow introduces neutrophils and substrate that further amplify injury. Nitrite reduction in the setting of hypoxia and acidosis restores nitric oxide bioavailability and augments mitochondrial tolerance to ischemia if administered prior to injury. ROS: Reactive oxygen species; ATP: Adenosine triphosphate; TNF: Tumor necrosis factor; IL: Interleukin; ICAM: Intercellular adhesion molecule; O_2^-: Superoxide anion; H_2O_2: Hydrogen peroxide; HOCL: Hypochlorous acid.

animal models of IRI have displayed promise[6]. Further appreciation for the complexities of IRI within humans will develop as we attempt to intervene with therapies in clinical trials. Further, attempts to expand organ donation pools to decrease mortality within the waiting list for organ transplantation will result in a greater need to optimize graft function by inhibiting IRI. The following will discuss several potential therapies of relevance to ROS and RNS during IRI and their translational potential.

Ischemic preconditioning
Since initial observations that exposure of organs to brief periods of non-lethal ischemia provides protection from injury against a subsequent lethal ischemic event[17], many studies have established the concept of ischemic preconditioning (IP) in multiple organs[18-20] and across various species, including humans[18,21,22]. Unlike many pathologies characterized by a period of ischemia, liver transplantation presents an ideal model for therapies such as ischemic preconditioning as liver is exposed to a defined, scheduled, and relatively controlled period of cold ischemia.

Animal models of IP have shown that IRI can be inhibited by a defined therapy period of multiple[3-5], brief (5-10 min) bouts of ischemia followed by a reperfusion phase of equal time. As alluded to above although organ transplantation may represent an ideal model for IP it also emphasizes that the greatest limitation to IP in other human diseases, such as myocardial ischemia, is the inability to predict when the injury may occur and provide the therapeutic IP. This limitation has led to the search for the mechanism and mediators of IP in hopes of duplicating its effects pharmacologically. Interestingly, this research has led to the finding that the effects of IP are not completely limited to the timing or location of the ischemic therapy in order to provide a protective effect and have led to the advent of ischemic post conditioning[23] and remote ischemic preconditioning[24].

The hypotheses to explain the phenomena of ischemic conditioning generally fall into the general categories of IP causing changes directly on the organ subjected to the repeated IP therapy or that a humoral factor is produced by the tissue undergoing IP that then provides a systemic effect of protection. Given the advent of remote IP the evidence would suggest the latter mechanism predominates. Further, blood taken from rabbits that have undergone preconditioning confer their protective effects during myocardial infarction in rabbits that have not undergone a preconditioning phase[25]. Mechanistic studies have revealed that adenosine, bradykinin, or perhaps other circulating factors signal through G-protein coupled receptors[26]. This initiates a signaling cascade involving activation of protein kinase C[27], heat shock factor 1[28], and mitogen-activated protein kinase[29]. Activation of these receptors during preconditioning result in up regulation of multiple systems capable of attenuating IRI including superoxide dismutase, heat shock proteins, and eNOS[30-32]. Interestingly, the mild oxidation associated with the sub-lethal insult of IP as opposed to the hypoxia itself has been shown to confer protection against a subsequent lethal insult. Specifically, inducing a mild oxidative insult of infusing a peroxide analog into the portal vein of a mouse protected the liver from a subsequent ischemic insult enforcing the concept that timing and amount of exposure to oxidants results in either protection by inducing anti-oxidant de-

fenses, or overwhelm the cell and initiate cell death[33].

Attempts to translate preconditioning whether pre-, post-, or remote into human disease has had mixed results[34]. In part this is due to variance in the research and clinical models such as the timing of preconditioning. Mechanistic studies into IP have led to many potential candidates to confer the protective effects of IP pharmacologically. Some of these molecules will be discussed further below and include redox active effectors. Ongoing work in humans will further assess the clinical relevance of IP on liver transplantation outcomes[35].

Volatile anesthetics

Further evidence to support the principle of providing a pharmacologic agent that imparts the benefits of ischemic preconditioning is the finding that volatile anesthetics impart a similar profile of protection against ischemia reperfusion injury in the heart[36]. Termed anesthetic preconditioning, this phenomenon has been reproduced in multiple species and by multiple volatile anesthetics. Mechanistically, it appears that anesthetic preconditioning does closely parallel ischemic preconditioning and interestingly *via* ROS species induces a minute stress that activates similar pathways of ischemic preconditioning within target cells. The net effect is the up regulation of anti-oxidant systems such as heme oxygenase, and eNOS that help protect the cell from an ensuing IRI[37,38]. Human studies have revealed that patients preconditioned with sevoflurane experienced a reduction in peak transaminase levels, an improvement in clinical outcomes, and enhanced benefit in those with steatotic livers. Interestingly, iNOS mRNA was significantly increased in the preconditioned group suggesting a role for NO although further investigation into mechanism or eNOS expression was not performed[39]. As the role of nitric oxide in IRI and protection is more complex a detailed discussion is saved for a later sub-section.

Glutathione/NAC

Glutathione (GSH) is an important intracellular anti-oxidant and reductant found in high concentrations within hepatocytes. The protective role of glutathione and its importance in intracellular detoxification is emphasized by the model of glutathione oxidation and depletion found in acetaminophen overdose. Glutathione may react directly with oxidants such as peroxynitrite and hydrogen peroxide, but also provides reducing equivalents to maintain catalytic antioxidant systems (*e.g.*, glutathione peroxidase) that provide protection against these ROS and RNS in intracellular compartments[40,41]. Additionally, hepatocytes export glutathione and thereby detoxify the important ROS produced by Kupffer cells within the vascular space as described above during the early phases of IRI. GSH can either be administered itself or reduced GSH levels can be replenished by N-acetylcysteine (NAC) administration. Both GSH and NAC have been shown to reduce ROS production and oxidant stress after IRI[42,43]. Additionally, clinical trials of NAC and GSH

have shown a reduction in biochemical markers of liver injury. However, few have reported differences in clinical outcomes associated with GSH or NAC infusion[44]. It should be noted that GSH or NAC therapy is limited in that non-specific effects are likely, and neither target oxidants in specific compartments. Emerging and exciting recent findings indicate that targeted expression of antioxidants for example in the mitochondria may result in more effective and safer strategies[45].

α-Tocopherol

α-Tocopherol is an orally administered analogue of vitamin E that limits lipid peroxidation. In general, α-tocopherol therapy studies have led to mixed results. In IRI of the liver, pre-treatment of α-Tocopherol in mice was shown to be protective. In humans, α-Tocopherol had no effect on biomarkers of hepatic damage after hepatic ischemia; however, patients in the α-Tocopherol treatment group had a reduction in ICU stay. Clearly, the implications on ICU length of stay are complex but suggest the potential for α-Tocopherol in human liver transplantation remains and require further testing.

Allopurinol

Xanthine oxidase has long been considered one of the major producers of ROS during IRI largely due to evidence of the protective effect of the xanthine oxidase inhibitor, allopurinol[46]. However, multiple studies have demonstrated the limited contribution of xanthine oxidase mediated generation of ROS to the post ischemic stress of IRI. These findings are further supported by the dose and length of pretreatment required to convey the protective effects of allopurinol relative to the much smaller dose and time demonstrated to effectively inhibit xanthine oxidase[47,48]. Additionally, the length of time required (d) for pretreatment with allopurinol to be effective will limit its potential clinically due to the limited lead time a patient has from notification until transplantation due to the nature of organ donation.

Superoxide dismutase

One of the problems with targeting ROS in IRI is the separation of intracellular and extracellular sources of the oxidant stress during IRI as well of the timing of ROS generation within these locations. Generally, Kupffer cells produce ROS early within the ischemic phase that injures intracellular targets within the hepatocytes. Later, during the reperfusion phase previously damaged and dysfunctional mitochondria in addition to infiltrating neutrophils contribute to the oxidant stress within the intra and extracellular spaces. This delineation of timing and location was emphasized by the failure of one of the early attempts of scavenging free radicals as a means to ameliorate IRI. In these studies catalase and superoxide dismutase were administered intravenously either in combination or alone[49]. This study found only partial protection from IRI implicating a significant role for other ROS or highlighting the weakness that this therapy

cannot target the intracellular effects of ROS. Subsequent studies have utilized carbohydrate modifications of these enzymes or gene delivery to up regulate the intracellular activity of these enzymes and have shown potential benefit and cause for further investigation[50,51].

Augmentation of endogenous nitric oxide and application of exogenous nitric oxide and Nitrite

Nitric oxide (NO) produced at low levels by endothelial nitric oxide synthase (eNOS) is associated with protection against IRI *via* multiple possible mechanisms including preventing leukocyte adhesion and limiting reactive oxygen species production by mitochondria. Moreover, deficits in eNOS-derived NO have been documented in numerous inflammatory disorders and IRI, although how eNOS activity is altered in human liver transplantation remains to be clearly defined[52,53]. Consistent with protective effects of eNOS are gene therapy studies that show overexpression of this enzyme in the liver protects against IRI in mice[54].

An alternate strategy to gene therapy is to augment NO using NO-repletion strategies. Many NO-donors exist but suffer from a lack of compartmentalized release that can result in unwanted effects (*e.g.*, hypotension)[55]. Recent studies are beginning to address this limitation. As alluded to above, targeting drugs to the mitochondria is now a possibility. This has also been demonstrated with NO, with a mitochondrial targeted S-nitrosothiol showing protection against IRI in the heart[56,57] in part by limiting ROS production in this organelle. The potential for this strategy in liver transplantation remains to be tested. In addition, recent studies have shown that nitrite administration can replete NO-signaling in areas of ischemia[58]. The underlying concept is that nitrite will only be reduced to NO by ischemia sensitive mechanisms and thus only produce NO in the environments where needed and avoid unwanted systemic effects (Figure 1). This concept has been validated in numerous experimental models, including hepatic IRI and lung and liver transplantation providing a rationale for testing in humans[59-64]. Supporting this rationale is the protective effects of inhaled NO in preventing IRI and improving graft function in liver transplant patients[52]; the protective effects of inhaled NO was posited to be mediated by increased circulating nitrite levels. In addition to a therapy that can be administered during the ischemic phase, nitrite may also be a candidate therapeutic to mimic IP exemplified by studies showing nitrite administration to normal rats, resulting in protection against myocardial and hepatic IRI 24#h thereafter *via* mechanisms that involved limiting mitochondrial dysfunction during the IRI period[61].

CONCLUSION

Further appreciation of the time course and mediators of IRI has led to the discovery of many potential

therapeutics. Each of these faces the hurdle of increased complexity and other unknowns when trying to translate to the pathology seen in human disease. Take for instance the IRI of liver transplantation and the question of when best to administer a proposed therapeutic to: the donor, the graft after harvest, the graft during flushing prior to reperfusion, or after reperfusion. Now, clinical trials must utilize the best data available to choose timing of administration and the most valuable targets to investigate whether the therapy is working by the proposed mechanism. These studies will provide the mechanistic insights currently needed into the IRI of human pathology.

Additionally, the demand for a better understanding of IRI is increasing as we try to reduce the significant wait list mortality caused by demand outpacing supply. This push is causing a closer look at marginal grafts deemed as such because they are steatotic or come from donors of advanced age. These conditions are important to the field of IRI as many of the therapeutics outlined above show an enhanced benefit in steatotic livers. In order to optimize these grafts system changes are occurring within the organ donation network primarily to reduce cold ischemic times associated with transportation. These proposed changes will result in donors being transported to specialized centers more experienced with organ harvest which should minimize organ harvest time and as the recipient will be located at the same medical center this will significantly limit cold ischemic time. Importantly, the advent of donation centers will also create an opportunity to further study these processes. As our clinical trials are often controlled for safety by regulatory boards it has been logistically difficult to administer a therapeutic to a donor that will conceivably affect multiple recipients at multiple institutions. Although donation centers do not remove all logistical barriers of such studies they will create more opportunities to address IRI prior to the ischemic phase and certainly allow for tissue collection to improve our understanding of the human disease.

Given the outlined complexity of IRI it seems that the ideal candidate therapeutic will function by multiple pathways. By preventing ROS production, scavenging ROS in the vascular space and preventing the intracellular damage and mitochondrial dysfunction caused by ROS in the reperfusion phase we may envision an ideal therapeutic against IRI. It is unlikely a single candidate will fulfill the multitude of needs of the ideal therapeutic but as we gain further information on the mechanisms of human IRI it will be possible to identify a combination of therapeutics to best ameliorate the effects of IRI.

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REVIEW

Complementary and alternative medications in hepatitis C infection

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Abstract

Chronic hepatitis C (CHC) infection affects almost 3% of the global population and can lead to cirrhosis, liver failure, and hepatocellular carcinoma in a significant number of those infected. Until recently, the only treatments available were pegylated interferon and ribavirin, which traditionally were not very effective and have considerable side effects. For this reason, interest in complementary and alternative medications (CAM) in the management of hepatitis C has been investigated. Some CAM has demonstrated therapeutic potential in chronic hepatitis C treatment. Unfortunately, some CAM has been shown to have the potential to cause drug-induced liver injury. This article will review and evaluate many of the natural molecules that interact with the hepatitis C virus (HCV) life cycle and discuss their potential use and safety in HCV therapy, as well as highlight some important interactions between medical and complementary treatments.

Core tip: Over the last 10 years there has been a substantial increase in reports of natural compounds displaying anti-viral activity against hepatitis C. At this time, there is no firm evidence supporting complementary and alternative medications for hepatitis C virus infection. Due to a limited number of trials and small numbers of subjects included in them, it is not possible to fully evaluate the risk of adverse events connected with the use of these products.

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INTRODUCTION

Hepatitis C virus (HCV) infection affects an estimated 180 million people globally and is a leading cause of chronic hepatitis, cirrhosis, and liver cancer[1,2]. To prevent the complications of chronic hepatitis C (CHC), the goal of therapy is complete viral eradication. For the past decade, a combination of pegylated interferon-α (peg-IFN) and ribavirin was used to treat CHC with disappointing viral eradication rates. These rates were particularly suboptimal in patients with genotype 1 HCV, which is responsible for approximately 60% of worldwide infections[3]. Sustained virological response (SVR) rates for genotype 1 HCV are approximately 40% following 48 wk of peg-IFN/ribavirin and are even lower in patients with HIV co-infection, high baseline viral load, advanced fibrosis, or those of African descent[4-7].

The life cycle of HCV can be divided into three major steps: (1) entry of the virus into its target cells by receptor-mediated endocytosis; (2) cytoplasmic and mem-

brane-associated replication of the RNA genome; and (3) assembly and release of the progeny virions[8]. In recent years, there has been improvement in SVR rates with the development and approval of the first HCV-specific direct-acting antiviral agents (DAAs), namely boceprevir and telaprevir[9,10]. In contrast to the non-specific antiviral activity of peg-IFN and ribavirin, DAA are designed to inhibit viral proteins involved in the HCV life cycle. Still, the first DAAs require coadministration with peg-IFN and ribavirin, and many patients remain intolerant to treatment-associated side effects, including fevers, influenza-like symptoms, headache, cytopenias, fatigue, anorexia, rash, and depressive symptoms.

CAM is being used increasingly across the globe for many chronic diseases[11,12]. The Cochrane Library included nearly 50 systematic reviews of complementary medicine interventions as of 2003[13]. Many people turn to CAM when conventional medicine fails, or they believe strongly in its effectiveness. During the last few years, a substantial increase of reports on natural compounds displaying an anti-HCV activity has been published. There is data that some of these medicinal herbs might have therapeutic potential in CHC, or may alleviate side effects of conventional therapy[13]. CAM use is common among people with CHC. A survey of 1145 participants in the National Institutes of Health (NIH)-supported HALT-C (Hepatitis C Antiviral Long-Term Treatment Against Cirrhosis) trial found that 23% of the participants used herbal products[14]. Although sometimes thought by the public to be safer then conventional therapy, there are many reports about liver toxicity and other adverse events from some herbal products[11,15]. The aim of this review is to evaluate the efficacy and safety of treating HCV infection using complementary and alternative medicine.

MEDICINAL HERBAL AND DIETARY SUPPLEMENTS WITH ANTI-HCV ACTIVITY

Silymarin
An extract of the milk thistle plant, silymarin *(Silybum marianum)*, has been used to treat chronic liver disease since the time of the ancient Greeks[16]. Owing to its purported hepatoprotective properties, it is the most commonly used herbal product by individuals with chronic liver disease in the United States[16,17]. A recent publication from the HALT-C study group indicated that 33% of patients with CHC and cirrhosis reported current or past use of silymarin[14]. A follow-up study found silymarin use among CHC patients was associated with reduced progression from fibrosis to cirrhosis, but had no impact on clinical outcomes[16].

The major active component of silymarin, silibinin (a mixture of the two diastereoisomers silybin A and silybin B), is thought to be responsible for silymarin's hepatoprotective properties[18]. Silymarin appears to inhibit HCV infection at two or more different levels: (1)

it inhibits HCV replication in cell culture; and (2) it displays anti-inflammatory and immunomodulatory actions that may contribute to its hepatoprotective effect[19,20]. The inhibition of HCV replication has been attributed to inhibitory action on the NS5B RNA-dependent RNA polymerase.

Clinical studies that have evaluated milk thistle for a variety of liver diseases have yielded inconsistent results and low bioavailability of oral silymarin components[21]. Studies with IV silibinin have shown substantial antiviral effect against HCV in liver transplant recipients, and even in nonresponders with good safety outcomes[22-24]. Although oral administration of silymarin is not effective for the treatment of HCV, intravenous silibinin formulation may represent a future potential therapeutic option.

Green tea extract
Green tea, made from the unfermented leaves of *Camellia sinensis*, is comprised of several polyphenolic compounds called catechins, and can be concentrated into a green tea extract (GTE). Epigallocatechin-3-gallate (EGCG) is the most abundant and potent catechin contained within GTE, comprising typically approximately 40% of the total polyphenol content[25]. EGCG is a potent inhibitor of HCV entry in primary human hepatocytes independent of the genotype, by blocking virus attachment. This novel inhibitor may provide a new approach to prevent HCV infection, especially in the setting of liver transplantation of chronically infected HCV patients[26,27]. Beyond its antiviral effect on HCV, EGCG may have potential use as a chemopreventative agent for hepatocellular cancer as EGCG may inhibit cancer cell growth. This mechanism of action is thought to be due to tyrosine kinase inhibition and modulation of target gene expression associated with induction of apoptosis and cell cycle arrest in cancer cells[28-34].

GTE is a common ingredient in several dietary supplements, some of which have been withdrawn from the market due to safety concerns. An example of this is *Exolise* (Arkopharma, France), a weight loss supplement containing high EGCG levels that was withdrawn from the market in April 2003 due to 13 cases of attributable liver injury[35]. Between 1966 and 2008, 216 case reports of toxicity with green tea extracts were identified by the United States Pharmacopeia, of which 34 were concerning for liver toxicity[36]. Recent animal studies with high doses of GTE and EGCG have described dose-dependent hepatotoxicity resulting in severe morbidity and mortality[37]. However, chronic moderate to high dose daily GTE and EGCG use in healthy human volunteers, and selected patients with cirrhosis, was safe and did not impair liver function[38-40]. Although GTE may be very useful in further treatment of CHC and prevention of HCC, its hepatotoxic potential must be acknowledged and monitored carefully in future studies.

Naringenin
HCV associates with β-lipoproteins [very low density lipoprotein (vLDL) and low-density lipoprotein (LDL)]

circulating in blood[41]. In addition, HCV replication can be up-regulated by fatty acids and inhibited by statins; this suggests an interaction between HCV, cholesterol, and lipid metabolism[42]. Recent research has found that of HCV secretion is dependent on both apolipoprotein B (ApoB) expression and vLDL assembly in a chromosomally integrated complementary DNA (cDNA) model of HCV secretion[43].

Naringenin is the predominant flavanone present in the grapefruit and is responsible for its bitter taste. Naringenin has been shown to reduce cholesterol levels both *in vitro* and *in vivo*[44,45]. Furthermore, naringenin inhibits ApoB secretion by reducing the activity and the expression of the microsomal triglyceride transfer protein (MTP) and the acyl-coenzyme A cholesterol acyltransferase 2 (ACAT)[44,46]. Due to the close link between HCV assembly/secretion and lipoprotein metabolism, there has been extensive study on the impact of naringenin on the secretion of HCV particles[43]. A dose-dependent decrease of core protein, HCV-positive strand RNA, infectious particles, and ApoB has been observed in the supernatant of infected primary hepatocytes in culture after naringenin treatment[43]. Overall, naringenin blocked the assembly of intracellular infectious viral particles without affecting intracellular levels of the viral RNA or protein. Although still at the cell culture phase, naringenin may offer new insight into a promising and novel HCV therapeutic target.

Glycyrrhizin

Glycyrrhizin, a natural compound extracted from the roots of *Glycyrrhiza glabra*, has been used for more than 20 years as a treatment for chronic hepatitis[47]. It has been used for many centuries in traditional Chinese medicine as an anti-allergic agent. Because of its sweet taste it is also used as a food additive, for example in beverages and licorice[48]. In an attempt to use glycyrrhizin as a treatment for "allergic" hepatitis it was found to lower the transaminases. In a study by Suzuki *et al*[49] in 1977, plasma transaminases activity improved significantly with glycyrrhizin in patients with chronic liver disease compared to a placebo group.

The mechanism by which glycyrrhizin improves the biochemistry and histology in liver disease is unknown. It is thought to have anti-inflammatory, antioxidant and immunomodulatory activities. Due to this there has been much interest in use of glycyrrhizin in CHC. In the only randomized clinical trial of glycyrrhizin, ALT levels declined modestly during treatment, compared with placebo, but this was not sustained after cessation of treatment and there was no significant effect on HCV RNA levels[50]. In the another trial, statistically significant differences in liver enzyme levels, but not viral loads, between treatment groups were identified during treatment, however, again no sustained response occurred at follow-up[51]. Use of glycyrrhizin is not without side effects. It has been found to cause pseudo-aldosteronism, manifested by sodium retention, hypokalemia and hyper-

tension[52]. Cardiac arrhythmia and acute rhabdomyolysis due to severe hypokalemia caused by excess licorice consumption have also been reported[52-54].

Oxymatrine

Oxymatrine is the major alkaloid extract from the root of *sophora flavescens*, a deciduous shrub native to China, Japan, South Korea and Russia. It is reported to have antiviral activity against HCV in cell cultures and in animal studies[55-57]. Clinical studies have shown that oxymatrine has some hepatoprotective activity in alcohol toxicity and hepatitis B infection, but not carbon tetrachloride, acetaminophen or cadmium chloride-induced acute hepatitis[58,59]. Oxymatrine is considered to be an antifibrotic, likely through inhibition of lipid peroxidation[60-62]. In a study of HCV-infected subjects randomized subjects to receive either an intramuscular injection of oxymatrine 600 mg/d or other support products such as oral vitamins 47% of the treated cases had complete HCV viral suppression after 3 mo, compared with only 5% in the control group[61]. No serious adverse events were reported. The treated group had significantly more ALT normalizations than the control group in the first 2 mo, but this improvement waned by the end of the third month of treatment. While treatment with oxymatrine holds promise, it is difficult to draw conclusions from the small studies currently available.

Traditional chinese herbal medications

The primary goal of Chinese traditional medicine is to create wholeness and harmony within a person, allowing the mind/body/spirit to heal itself. There have been several randomized clinical trials of traditional Chinese medicine in the treatment of hepatitis C, however, the methodological quality of these studies is generally considered poor[63-70]. In two trials of herbal formulations in combination with interferon-alfa, there was a trend toward greater clearance of HCV RNA and ALT normalization with the combination treatment compared with patients receiving monotherapy[63,64]. In the only placebo-controlled trial of solo therapy with traditional Chinese medicine, a significant reduction in ALT levels during treatment occurred, though no virologic effect was identified[69]. Detailed descriptions of adverse events were not provided for most of these trials. The safety of these medicines is unclear due to the individualized nature of many of the herbal compounds involved, the large number of different herbs in each formulation, and the relatively small number of subjects within each clinical trial.

Vitamin D

The traditional role of Vitamin D (Vit D) was thought to be based upon its interaction in calcium homeostasis, *via* regulation of intestinal calcium absorption and of bone health. However, over the last several years Vit D has been shown to have a much more complex role in many other host functions, including its interaction with

chronic hepatitis C. 25-OH Vit D is made in the liver *via* cytochrome P450 (CYP27A1) activated hydroxylation of Vit D, brought into the body either by intestinal absorption or endogenous synthesis through sun-exposed skin. It is then converted to 1.25 OH Vit D (calcitriol) in the kidneys, the most active form, where it becomes available to bind to Vit D receptors throughout the body[71,72].

A growing body of clinical evidence has demonstrated an increased prevalence of Vit D deficiency in patients with CHC. As such, Vit D supplementation has been proposed as an adjunct to current standard regimens for treatment of hepatitis C[72]. One study found that mean 25-OH Vit D serum levels were significantly lower in CHC (25 μg/L) than in the controls (43 μg/L)[73]. Importantly, low Vit D has been linked to increased fibrosis and impaired sustained virologic response (SVR) in IFN-based therapy[71]. One clinical trial demonstrated that the addition of Vit D to the standard IFN plus ribavirin treatment significantly increased SVR in patients with genotype 1 CHC[74]. Regarding the underlying molecular mechanisms, an *in vitro* study showed that Vit D remarkably inhibits HCV production in Huh7.5 hepatoma cells[75]. These cells express Vit D hydroxylases and can eventually generate calcitriol. Notably, treatment with calcitriol resulted in HCV inhibition through induction of IFN-beta. Overall, 25-OH Vit D levels appear to be an important prognostic marker in helping determine the likelihood of SVR. 25-OH Vit D levels should be checked routinely before HCV treatment and supplementation provided to deficient patients, in an effort to enhance treatment response.

Antioxidants

Antioxidants are one of the most common dietary supplements taken by patients with CHC[14]. The use of these supplements is based on the fact that oxidative stress has been attributed to both host inflammatory processes and induction by viral proteins. By increasing antioxidants, one may be able to decrease oxidative stress and therefore decrease liver injury[76]. Existence of oxidative stress in CHC is well documented, as oxidized protein and nucleic acid markers are increased and antioxidant levels are decreased[77-80]. Studies have shown levels of oxidative stress markers to correlate with disease severity, HCV RNA, iron overload, and insulin sensitivity[78,79]. Oxidative stress has also been shown to be an early event in carcinogenesis and is a risk factor for development of HCC in patients with chronic HCV[81].

Multiple trials have shown antioxidants, such as Vitamin E and N-acetyl cysteine, only lead to small reductions in ALT after chronic administration in some instances[82-93]. Further, the decrease in ALT levels in most studies is marginal and is not sustained after stopping the treatment, raising the question of their clinical significance. No study has shown an improvement in outcome. In addition, no study has shown clear benefit of antioxidants as adjuvant to interferon based therapy of HCV. At the doses studied, these antioxidants appear to be well-tolerated, with no specific adverse events reported in any of the trials. However, very large oral doses of *N*-acetyl cysteine are commonly associated with nausea and vomiting and intravenous administration of *N*-acetyl cysteine can result in anaphylactoid reactions, which may be more common in patients with chronic liver disease[94]. Therefore, evidence supporting use of antioxidants as useful therapeutic agents in CHC is lacking.

HERBAL SUPPLEMENTS AND DRUG INDUCED LIVER INJURY IN CHRONIC HCV

Drug-related hepatotoxicity is a serious health problem, with broad implications for patients, healthcare providers, the pharmaceutical industry and governmental regulatory agencies. The Drug Induced Liver Injury Network (DILIN), a federally funded consortium of 12 centers in the United States, recently reported the preliminary results of its prospective study[94]. Dietary supplements were implicated in 9% of reported DILI cases. This may be potentially related to increasing use of herbal or dietary supplements in the US population. The importance of these supplements as a cause of DILI is further underscored by a retrospective Japanese study, in which 10% of 879 cases of single agent DILI from 1997 to 2006 were attributed to dietary supplements and 7% to Chinese herbal drugs[95].

In general, chronic liver diseases such as HCV infection are thought to be associated with an increased incidence of hepatotoxicity induced by several specific drugs. Furthermore, patients with underlying liver disease potentially have worse outcomes than healthy individuals if they do develop DILI[96]. For example, the presence of underlying CHC has been shown to increase the risk of DILI caused by the antituberculosis drugs isoniazid and rifampin, as well as ibuprofen and methimazole[15,97,98]. Due to this, patients with chronic hepatitis C should be counseled and screened by physicians on potential risks associated with herbal medications.

DRUG-CAM INTERACTIONS

Another major area of awareness when patients are considering using CAM is whether or not drug-CAM interactions may exist that could impact the medical therapy. This issue is becoming even more complicated with the addition of new medications for the treatment of CHC infection such as simeprevir and sofosbuvir approved for use in the U.S. in December 2013. St. John's wort (*Hypericum perforatum*), a common CAM used for the treatment of depression, is an inducer of cytochrome P450 3A4[99]. This cytochrome is also the primary metabolizer of many medications, including the HCV protease inhibitors: telaprevir, boceprevir, and simeprevir. Additionally, St. John's wort is a potent intestinal P-gp inducer and may lead to a reduced therapeutic effect of

Table 1 Herbal supplements to discontinue and/or avoid while taking hepatitis C virus treatment

Herbal Product	Effect
Milk thistle (*Silybum marianum*)	Concomitant use of milk thistle may result in increased plasma concentrations of simeprevir
St. John's wort (*Hypericum perforatum*)	Concomitant use of St. John's wort may result in decreased plasma concentrations of telaprevir, boceprevir, simeprevir and sofosbuvir

the HCV nucleotide polymerase inhibitor sofosbuvir[100]. Concomitant use of St. John's wort and these HCV treatments is contraindicated and can lead to treatment failure by reducing blood concentrations. Additionally, concomitant use of milk thistle use is contraindicated with simeprevir. This combination may increase levels of simeprevir by milk thistle CYP3A inhibition leading to possible toxicity[101] (Table 1). Garlic extracts, grapefruit juice, and germander also have cytochrome P450 3A4 interactions[102].

CONCLUSION

Many human studies have shown improvements in subjective symptoms and liver biochemistries in HCV patients with CAM, but there is no convincing data to suggest a definite histological and/or virologic improvement with any of the herbal agents currently available. Vit D seems to have the best available data as adjunctive therapy to antiviral medications in patients with Vit D deficiency. Poorly designed studies, heterogeneous patient populations, lack of standardized preparations, and poorly defined nonobjective end points may partly explain the conflicting reports in the literature.

The safety profiles of the interventions discussed within this review are encouraging at the doses studied. However, the long-term safety for use in the treatment of hepatitis C, either alone or in combination with conventional medicines, has not been established. Comparative and placebo-controlled trials suggest that patients experience no more adverse events with these interventions than with placebo or comparative medications, although short-term clinical trials are not designed to detect rare or delayed adverse events. Physicians need to be cognizant of known or occult use of CAM by their patients because hepatotoxicity and drug interactions may occur with many herbal medications, and may occur more frequently in patients with chronic liver disease.

There is an undoubted need for further research into the treatment of hepatitis C, and this review has identified several promising compounds, including Vit D, silymarin, oxymatrine, naringenin, and GTE. Some or all of these may be integral components of future HCV management.

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REVIEW

Drug and herb induced liver injury: Council for International Organizations of Medical Sciences scale for causality assessment

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Abstract

Causality assessment of suspected drug induced liver injury (DILI) and herb induced liver injury (HILI) is hampered by the lack of a standardized approach to be used by attending physicians and at various subsequent evaluating levels. The aim of this review was to analyze the suitability of the liver specific Council for International Organizations of Medical Sciences (CIOMS) scale as a standard tool for causality assessment in DILI and HILI cases. PubMed database was searched for the following terms: drug induced liver injury; herb induced liver injury; DILI causality assessment; and HILI causality assessment. The strength of the CIOMS lies in its potential as a standardized scale for DILI and HILI causality assessment. Other advantages include its liver specificity and its validation for hepatotoxicity with excellent sensitivity, specificity and predictive validity, based on cases with a positive reexposure test. This scale allows prospective collection of all relevant data required for a valid causality assessment. It does not require expert knowledge in hepatotoxicity and its results may subsequently be refined. Weaknesses of the CIOMS scale include the limited exclusion of alternative causes and qualitatively graded risk factors. In conclusion, CIOMS appears to be suitable as a standard scale for attending physicians, regulatory agencies, expert panels and other scientists to provide a standardized, reproducible causality assessment in suspected DILI and HILI cases, applicable primarily at all assessing levels involved.

Core tip: We propose that the attending physicians caring for patients with assumed drug induced liver injury and herb induced liver injury should use the Council for International Organizations of Medical Sciences (CIOMS) scale for causality assessment. This approach includes the option of subsequent refinement of the CIOMS based results by expert panels and regulatory agencies. The use of the CIOMS scale as an identical

tool for all involved parties will allow early and prospective collection of all relevant data required for a valid causality assessment in clinical hepatology.

INTRODUCTION

Drug induced liver injury (DILI) and herb induced liver injury (HILI) are complex diseases and often overdiagnosed[1-5]. An expert review of suspected DILI reports from primary and secondary care physicians to the UK Committee on the Safety of Medicine revealed that 47.1% of the cases were not DILI and that the misdiagnoses delayed arriving at the correct diagnoses, possibly worsening patient outcome[1]. Misdiagnosis was a common phenomenon in other DILI studies[2-4], including publications in which DILI was initially assumed, but hepatitis E virus infection later on evolved as the correct diagnosis[2,3]. Similarly, in a recent assessment of initially suspected HILI, correct diagnoses were missed in 278/573 cases, corresponding to 48.5%[5]. Given these frequencies because of insufficient case assessment, DILI and HILI represent major issues for physicians who care for patients with these diseases.

Physicians commonly are confronted with a wealth of published data about hepatic adverse drug and herb reactions and may use this information for evaluating the cases of their patients. Reviews addressed general aspects of DILI[6,7] or HILI[4,8-10], whereas other reports focused on various basic features like clinical course, prognosis, alternative causes, case definition and phenotype standardization[5,11-17]. They suggest a similar or identical clinical presentation of DILI and HILI, raising the question of whether HILI needs a separate term. However, major differences exist between DILI and HILI; DILI is caused by a single chemically characterized drug, whereas HILI is triggered by a chemical mixture constituted of the herbal extract, which often lacks the benefit of regulatory surveillance. Herbal product quality varies and is a major issue in HILI, adding to the complexity in evaluating causality for herbs. This may explain why HILI is considered as a poorly defined entity, is a neglected disease, and requires special attention.

Potential genetic risk factors and biomarkers, including micro-RNA, are presently being investigated to explain DILI and HILI disease[18-20]. These data provide promising clinical and scientific results but currently contribute little to diagnose DILI or HILI correctly and in time, or to exclude alternative causes. Recognizing that the best approach is still not available in clinical practice, the physician needs a pragmatic guideline to quickly evaluate suspicious cases and reach a conclusive diagnosis. This is at present best achieved by the combination of clinical judgement and a liver specific causality assessment algorithm like the CIOMS (Council for International Organizations of Medical Sciences) scale[21,22], as has been summarized recently[10,14,23-25]. For DILI and HILI case reports, the CIOMS scale based on international consensus meetings[21,22,26,27] is the commonly applied method to assess causality[4,5,10,14,23,24,28,29]. In clinical practice, causality assessment of suspected hepatotoxicity is hampered by the lack of a standardized approach, which is applicable to all levels of causality assessment[4,14,24] and requires simplicity of the assessment method rather than complexity to evaluate DILI and HILI cases.

This review analyzes the suitability of the liver specific CIOMS scale for causality assessment in DILI and HILI cases as a standard for attending physicians, regulatory agencies, expert panels and the scientific community. It focuses on the characteristic features of the CIOMS scale, discusses strengths and weaknesses, and suggests approaches for the clinician who lacks a stand-by panel of DILI or HILI experts.

The PubMed database was searched for the following terms: drug induced liver injury; herb induced liver injury; DILI causality assessment; and HILI causality assessment. The literature search was done on June 4, 2013. Several hundreds of records were initially obtained, depending of the term used. The first 50 publications of each search were analyzed in depth for suitability in the analysis of the CIOMS scale quality, with numerous duplicated reports found in each category. The final compilation of evaluated publications consists of original papers, case series, case reports, consensus reports and review articles. All relevant reports were included in the reference list to be presented in this review. Analyzed reports were published between 1977 and 2013, preferentially within the last decade.

GENERAL ASPECTS

The liver specific and quantitative CIOMS scale was conceptualized and developed in consensus meetings organized at the request of the Council for International Organizations of Medical Sciences (CIOMS), with details published in 1993[21,22]. This CIOMS scale represented a breakthrough in DILI causality assessment methods and extended, specified and quantified the preceding qualitative RUCAM (Roussel Uclaf Causality Assessment Method) of 1988[26] and qualitative CIOMS method of 1990[27]. The basis for the CIOMS scale was provided by eight experts in hepatology from 6 countries and included J P Benhamou (France), J Bircher (Germany), G Danan (France), W C Maddrey (United States), J Neuberger (United Kingdom), F Orlandi (Italy), N Tygstrup (Denmark) and H J Zimmerman (United States)[21]. This expert panel evaluated DILI cases for case characteris-

tics, hepatotoxicity criteria, liver injury pattern and reexposure criteria, standardized DILI case assessment with specific, quantitative items[21], and the experts validated their method with established positive reexposure DILI case results[22]. The CIOMS scale was developed for assessment of a single drug containing a synthetic product and may be used for a single herb containing multiple chemical constituents, but does not allow causality attribution to a specific constituent. The scale is a learning system and not immutable; room for improvement and refinement of the CIOMS scale has been outlined[29], with modifications of the CIOMS scale based on improved diagnostic instruments[14].

STRENGTHS

Prospective use

Its prospective application enables the CIOMS scale to provide an early causality grading for patients with suspected DILI or HILI; its results can be adapted further to diagnostic and therapeutic measures. This scale is easily used as a bedside tool at a time the disease is developing (Tables 1 and 2). Results do not depend on expert opinion and are quickly available for trained physicians to decide whether DILI and/or HILI should be considered as relevant differential diagnoses due to their clinical experience. Assessment is best started on the day of suspecting DILI or HILI, with a continuous update of the required data and a change in the diagnostic and therapeutic concept if needed. Finally, a complete data set for presentation to regulatory agencies, expert panels and eventually for publication is obtained[24,30-33], including a checklist with additional data helpful in overall case evaluation and causality assessment (Table 3). Therefore, the CIOMS scale should be considered as a standard for causality assessment of DILI and HILI, both for the attending physician and later evaluation stages. Using one single assessment method at all evaluating levels allows comparison of different assessment outcomes.

Liver specificity

Liver specificity is a hallmark of the CIOMS scale, in contrast to liver unspecific causality assessment methods or ad hoc approaches[4,24]. The CIOMS items are specially tailored to liver injury and not applicable to liver unrelated adverse drug reactions[24]. All current core elements of hepatotoxicity are considered in the CIOMS scale (Tables 1 and 2): time to onset of increased liver values or symptoms from the beginning and cessation of the drug/herb; course of liver enzymes after cessation; risk factors such as alcohol, age and pregnancy; comedication with other drugs/herbs; search for alternative causes, previously known drug/herb hepatotoxicity; and response to unintentional reexposure[21-25] based on specific criteria (Table 4). The individual items are transparent and facilitate quick and precise answers.

The CIOMS scale is structured and all its items undergo quantitative rather than qualitative assessment

and scoring (Tables 1 and 2)[4,5,10,14,21,23,24,29]. Each item is weighted with specific scores based on the answer. The sum of the individual scores gives a final score that may range from -9 to +14 points, allowing for sufficient discrimination. The final score provides causality levels for the individual synthetic drug or herb as highly probable, probable, possible, unlikely or excluded (Tables 1 and 2)[12,24,30-32].

Hepatotoxicity definition

The international CIOMS expert panel defined liver injury in its consensus report as increased alanine aminotransferase (ALT) and/or alkaline phosphatase (ALP) activities of at least 2N, with N as the upper limit of normal[21]. Conversely, the consensus of the international DILI Expert Working Group with participants from Europe, the United States and Japan raised the ALT cut off point to 5N or 3N if total bilirubin values exceeded 2N and considered the 2N of ALP as an appropriate definition criterion[14]. Whereas the DILI Expert Working Group recommendations were based on expert opinion alone[14], those of the CIOMS expert panel were derived from both expert opinion and assessment of reference reexposure DILI cases[21,22].

Raising the ALT cut off to 5N increases the specificity of the hepatotoxicity causality assessment[24], eliminates false positive cases and substantiates hepatotoxicity causality at a higher level of probability[16,24]. The lower threshold of ALT > 2N will include multiple cases with nonspecific enzyme increases and requires more stringent exclusion of causes unrelated to drug(s) and herb(s)[24]. Also for low threshold N values, the inclusion rate of alternative diagnoses must be higher; false positive fulfilment of a hepatotoxicity definition results in high numbers of misattributed cases due to overdiagnosing and overreporting[8,12,17,24,34-48]. This phenomenon is illustrated in a recent HILI study where initial ALT values were available in only 8/22 cases (36%), including 3 cases with a range of 50-69 U/L serum activity[36]. None withstanding, regulatory assessment attributed a possible causality for the incriminated herb to all 22 cases[36,42]. In other spontaneous case collections, initial ALT values were available in 5/24 cases (21%)[35], 19/22 cases (86%)[37], 12/15 cases (80%)[38], and 7/13 cases (54%)[39]. The corresponding figures for ALT in published case reports of HILI were 16/16 cases (100%)[35], 21/21 cases (100%)[32], and 5/8 cases (63%)[33]. ALT values were included in DILI reports for amoxicillin/clavulanic acid, troglitazone, pioglitazone and montelukast in 11% to 88% of the cases, which were not further scored for causality by the Drug Induced Liver Injury Network (DILIN)[6]. ALT underreporting is therefore an issue for both DILI and HILI.

Other arguments merit further considerations. An ALT cut off point of 5N may not be applicable to some types of chronic liver injury like methotrexate liver fibrosis or nodular regenerative hyperplasia; misinterpretation is also possible in some forms of acute liver injury by mitochondrial toxicity in cases of valproate or

Table 1 Council for International Organizations of Medical Sciences scale for the hepatocellular type of injury and cholestatic or mixed type of injury in drug induced liver injury and herb induced liver injury cases

Items for hepatocellular injury	Score	Result
1 Time to onset from the beginning of the drug/herb		
5-90 d (rechallenge: 1-15 d)	2	-
< 5 or > 90 d (rechallenge: > 15 d)	1	-
Alternative: Time to onset from cessation of the drug/herb		
⩽ 15 d (except for slowly metabolized chemicals: > 15 d)	1	-
2 Course of ALT after cessation of the drug/herb		
Percentage difference between ALT peak and N		
Decrease ⩾ 50 % within 8 d	3	-
Decrease ⩾ 50 % within 30 d	2	-
No information or continued drug/herb use	0	-
Decrease ⩾ 50 % after the 30th day	0	-
Decrease < 50 % after the 30th day or recurrent increase	-2	-
3 Risk factors		
Alcohol use (drinks/d: > 2 for women, > 3 for men)	1	-
Alcohol use (drinks/d: ⩽ 2 for women, ⩽ 3 for men)	0	-
Age ⩾ 55 yr	1	-
Age < 55 yr	0	-
4 Concomitant drug(s) or herbs(s)		
None or no information	0	-
Concomitant drug or herb with incompatible time to onset	0	-
Concomitant drug or herb with compatible or suggestive time to onset	-1	-
Concomitant drug or herb known as hepatotoxin and with compatible or suggestive time to onset	-2	-
Concomitant drug or herb with evidence for its role in this case (positive rechallenge or validated test)	-3	-
5 Search for non drug/herb causes	Tick if negative	-
Group Ⅰ (6 causes)		
Anti-HAV-IgM	□	-
HBsAg, anti-HBc-IgM, HBV-DNA	□	-
Anti-HCV, HCV-RNA	□	-
Hepatobiliary sonography/colour doppler sonography of liver vessels/endosonography/CT/MRC	□	-
Alcoholism (AST/ALT ⩾ 2)	□	-
Acute recent hypotension history (particularly if underlying heart disease)	□	-
Group Ⅱ (6 causes)		
Complications of underlying disease(s) such as sepsis, autoimmune hepatitis, chronic hepatitis B or C, primary biliary cirrhosis or sclerosing cholangitis, genetic liver diseases	□	-
Infection suggested by PCR and titer change for CMV (anti-CMV-IgM, anti-CMV-IgG)	□	-
EBV (anti-EBV-IgM, anti-EBV-IgG)	□	-
HEV (anti-HEV-IgM, anti-HEV-IgG)	□	-
HSV (anti-HSV-IgM, anti-HSV-IgG)	□	-
VZV (anti-VZV-IgM, anti-VZV-IgG)	□	-
Evaluation of group Ⅰ and Ⅱ		
All causes-groups Ⅰ and Ⅱ - reasonably ruled out	2	-
The 6 causes of group Ⅰ ruled out	1	-
5 or 4 causes of group Ⅰ ruled out	0	-
Less than 4 causes of group Ⅰ ruled out	-2	-
Non drug or herb cause highly probable	-3	-
6 Previous information on hepatotoxicity of the drug/herb		
Reaction labelled in the product characteristics	2	-
Reaction published but unlabelled	1	-
Reaction unknown	0	-
7 Response to unintentional readministration		
Doubling of ALT with the drug/herb alone, provided ALT below 5N before reexposure	3	-
Doubling of ALT with the drug(s) and herb(s) already given at the time of first reaction	1	-
Increase of ALT but less than N in the same conditions as for the first administration	-2	-
Other situations	0	-
Total score for patient		

The CIOMS scale is based on the original CIOMS scale[21] and was adapted from previous modifications[4,14,23,24,44,45]. The above items specifically refer to the hepatocellular type of injury rather than to the cholestatic or mixed type (shown in Table 2). Regarding risk factor of alcohol use, 1 drink commonly contains about 10 g ethanol and details were discussed recently[14,44,45]. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CIOMS: Council for International Organizations of Medical Sciences; CMV: Cytomegalovirus; CT: Computer tomography; DILI: Drug induced liver injury; EBV: Epstein Barr virus; HAV: Hepatitis A virus; HBc: Hepatitis B core; HBsAg: Hepatitis B antigen; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HEV: Hepatitis E virus; HILI: Herb induced liver injury; HSV: Herpes simplex virus; MRC: Magnetic resonance cholangiography; N: Upper limit of the normal range; VZV: Varicella zoster virus. Total score and resulting causality grading: ⩽ 0: Excluded; 1-2: Unlikely; 3-5: Possible; 6-8: Probable; ⩾ 9: Highly probable.

fialuridine hepatotoxicity[14]. Aspartate aminotransferase (AST) activities may be used instead if ALT activities are unavailable[14,44,45] and other pathologies for AST increases are excluded[14]. ALP increases should be paral-

Table 2 Council for International Organizations of Medical Sciences scale for the cholestatic or mixed type of injury and cholestatic or mixed type of injury in drug induced liver injury and herb induced liver injury cases

Items for cholestatic or mixed injury	Score	Result
1 Time to onset from the beginning of the drug/herb		
5-90 d (rechallenge: 1-90 d)	2	-
< 5 or > 90 d (rechallenge: > 90 d)	1	-
Alternative: Time to onset from cessation of the drug/herb		
⩽ 30 d (except for slowly metabolized chemicals: > 30 d)	1	-
2 Course of ALP after cessation of the drug/herb		
Percentage difference between ALP peak and N		
Decrease ⩾ 50 % within 180 d	2	-
Decrease < 50 % within 180 d	1	-
No information, persistence, increase, or continued drug/herb use	0	-
3 Risk factors		
Alcohol use (drinks/d: > 2 for women, > 3 for men) or pregnancy	1	-
Alcohol use (drinks/d: ⩽ 2 for women, ⩽ 3 for men)	0	-
Age ⩾ 55 yr	1	-
Age < 55 yr	0	-
4 Concomitant drug(s) or herbs(s)		
None or no information	0	-
Concomitant drug or herb with incompatible time to onset	0	-
Concomitant drug or herb with compatible or suggestive time to onset	-1	-
Concomitant drug or herb known as hepatotoxin and with compatible or suggestive time to onset	-2	-
Concomitant drug or herb with evidence for its role in this case (positive rechallenge or validated test)	-3	-
5 Search for non drug/herb causes	Tick if negative	-
Group Ⅰ (6 causes)		
Anti-HAV-IgM	☐	-
HBsAg, anti-HBc-IgM, HBV-DNA	☐	-
Anti-HCV, HCV-RNA	☐	-
Hepatobiliary sonography/colour doppler sonography of liver vessels/endosonography/CT/MRC	☐	-
Alcoholism (AST/ALT ⩾ 2)	☐	-
Acute recent hypotension history (particularly if underlying heart disease)	☐	-
Group Ⅱ (6 causes)		
Complications of underlying disease(s) such as sepsis, autoimmune hepatitis, chronic hepatitis B or C, primary biliary cirrhosis or sclerosing cholangitis, genetic liver diseases	☐	-
Infection suggested by PCR and titer change for CMV (anti-CMV-IgM, anti-CMV-IgG)	☐	-
EBV (anti-EBV-IgM, anti-EBV-IgG)	☐	-
HEV (anti-HEV-IgM, anti-HEV-IgG)	☐	-
HSV (anti-HSV-IgM, anti-HSV-IgG)	☐	-
VZV (anti-VZV-IgM, anti-VZV-IgG)	☐	-
Evaluation of group Ⅰ and Ⅱ		
All causes-groups Ⅰ and Ⅱ - reasonably ruled out	2	-
The 6 causes of group Ⅰ ruled out	1	-
5 or 4 causes of group Ⅰ ruled out	0	-
Less than 4 causes of group Ⅰ ruled out	-2	-
Non drug or herb cause highly probable	-3	-
6 Previous information on hepatotoxicity of the drug/herb		
Reaction labelled in the product characteristics	2	-
Reaction published but unlabelled	1	-
Reaction unknown	0	-
7 Response to unintentional readministration		
Doubling of ALP with the drug/herb alone, provided ALP below 5N before reexposure	3	-
Doubling of ALP with the drug(s) and herb(s) already given at the time of first reaction	1	-
Increase of ALP but less than N in the same conditions as for the first administration	-2	-
Other situations	0	-
Total score for patient		

The CIOMS scale presented in this table is designed specifically for the cholestatic or mixed type of liver injury rather than for the hepatocellular type, which differs in a few items and is presented separately in Table 1. Additional details and abbreviations are provided in the legend of Table 1. Abbreviation: ALP, Alkaline phosphatase. Total score with resulting causality grading: ⩽ 0, excluded; 1-2, unlikely; 3-5, possible; 6-8, probable; ⩾ 9, highly probable.

leled by γ-glutamyltranspeptidase (γGT) to rule out isolated increases of ALP activities due to bone rather than hepatobiliary disease. However, γGT alone is not an appropriate parameter for liver cell injury[14,36], contrary to published claims[42]. In addition, isolated hyperbilirubinemia is not DILI or HILI specific and may be caused by Gilbert's syndrome[1,14].

Liver injury pattern

The CIOMS scale takes into account divergent laboratory constellations of the liver injury pattern in the hepatocellular and the cholestatic type of liver injury and

Table 3 Data checklist for drug induced liver injury and herb induced liver injury diagnosis assessment				
Items to be assessed	**Information obtained**			**Individual result**
	Yes	**No**	**Partial**	
Brand name with batch number and expiration date	□	□	□	-
Indication of drug/herb use	□	□	□	-
Begin of symptoms leading to drug/herb treatment	□	□	□	-
Daily dose	□	□	□	-
Application form of drug/herb product	□	□	□	-
Exact date of drug/herb start	□	□	□	-
Exact date of drug/herb end	□	□	□	-
Exact dates of emerging new symptoms after drug/herb start in chronological order	□	□	□	-
Exact date of initially increased liver values	□	□	□	-
Time frame of challenge	□	□	□	-
Time frame of latency period	□	□	□	-
Time frame of dechallenge	□	□	□	-
Verification of temporal association	□	□	□	-
Exclusion of temporal association	□	□	□	-
Gender, age, body weight, height, BMI	□	□	□	-
Ethnicity, profession	□	□	□	-
Preexisting general diseases with past medical history and actual assessment	□	□	□	-
Preexisting liver diseases with past medical history and actual assessment regarding	□	□	□	-
Risk factors such as age and alcohol	□	□	□	-
Alcohol use with quantification	□	□	□	-
Comedication by synthetic drugs, herbal drugs, herbal and other dietary supplements with all details of product, daily dose, exact dates of start and end of use, indication	□	□	□	-
ALT value initially including exact date and normal range	□	□	□	-
ALT values during dechallenge at least on days 8 and 30, and later on, with exact dates	□	□	□	-
ALT values during dechallenge to exclude a second peak, with exact dates	□	□	□	-
ALT normalization with exact date and actual value	□	□	□	
ALP value initially including exact date and normal range	□	□	□	-
ALP values during dechallenge at least on days 8 and 30, and later on, with exact dates	□	□	□	-
ALP values during dechallenge to exclude a second peak, with exact dates	□	□	□	-
ALP normalization with exact date and actual value	□	□	□	-
AST value initially including normal range	□	□	□	-
Laboratory criteria for hepatotoxicity	□	□	□	-
Laboratory criteria for injury pattern	□	□	□	-
Liver and biliary tract imaging including hepatobiliary sonography, CT, MRT, MRC	□	□	□	-
Color Doppler sonography of liver vessels	□	□	□	-
Unintended reexposure	□	□	□	-
Known hepatotoxicity caused by the drug/herb	□	□	□	-
Other possible causes, consideration and exclusion	□	□	□	-
Hepatitis A - Anti-HAV-IgM	□	□	□	-
Hepatitis B - HBsAg, anti-HBc-IgM, HBV-DNA	□	□	□	-
Hepatitis C - Anti-HCV, HCV-RNA	□	□	□	-
Hepatitis E - Anti-HEV-IgM, anti-HEV-IgG, HEV-RNA	□	□	□	-
Cytomegalovirus (CMV) - CMV-PCR, titer change for anti-CMV-IgM and anti-CMV-IgG	□	□	□	-
Epstein Barr virus (EBV) - EBV-PCR, titer change for anti-EBV-IgM and anti-EBV-IgG	□	□	□	-
Herpes simplex virus (HSV) - HSV-PCR, titer change for anti-HSV-IgM and anti-HSV-IgG	□	□	□	-
Varicella zoster virus (VZV) - VZV-PCR, titer change for anti-VZV-IgM and anti-VZV-IgG	□	□	□	-
Other virus infections - specific serology of Adenovirus, coxsackie-B-Virus, echovirus, measles virus, rubella virus, flavivirus, arenavirus, filovirus, parvovirus, HIV, and others	□	□	□	-
Other infectious diseases - specific assessment of bacteria (such as campylobacter, coxiella, leptospirosis, listeria, salmonella, treponema pallidum), fungi, parasites, worms, tropical diseases, and others	□	□	□	-
Autoimmune hepatitis (AIH) type Ⅰ - Gamma globulins, ANA, SMA, AAA, SLA/LP	□	□	□	-
Autoimmune hepatitis (AIH) type Ⅱ - Gamma globulins, anti-LKM-1 (CYP 2D6), anti-LKM-2 (CYP 2C9), anti-LKM-3	□	□	□	-
Primary biliary cirrhosis (PBC) - AMA, anti PDH-E2	□	□	□	-
Primary sclerosing cholangitis (PSC) - p-ANCA, MRC	□	□	□	-
Autoimmune cholangitis (AIC) - ANA, SMA	□	□	□	-
Overlap syndromes - see AIH, PBC, PSC, and AIC	□	□	□	-
Non alcoholic steatohepatitis (NASH) - BMI, insulin resistance, hepatomegaly, echogenicity of the liver	□	□	□	-
Alcoholic liver disease (ALD) - patient's history, clinical and laboratory assessment, sonography	□	□	□	-
Drug/herb induced liver injury - patient's history, clinical and laboratory assessment, sonography, use of the CIOMS scale	□	□	□	-
Toxin Screening - cocaine, ecstasy and other amphetamines	□	□	□	-
Rare intoxications - toxin screening for household and occupational toxins	□	□	□	-
Hereditary hemochromatosis - serum ferritin, total iron-binding capacity, genotyping for C2824 and H63D mutation, hepatic iron content	□	□	□	-

Wilson's disease - copper excretion (24 h urine), ceruloplasmin in serum, free copper in serum, coombs-negative hemolytic anemia, hepatic copper content, Kayser-Fleischer-Ring, neurologic-psychiatric work-up, genotyping	□	□	□	-
Porphyria - corphobilinogen in urine, total porphyrines in urine	□	□	□	-
α1 - antitrypsin deficiency - α1- Antitrypsin in serum	□	□	□	-
Biliary diseases - clinical and laboratory assessment, hepatobiliary sonography, endosonography, CT, MRT, MRC	□	□	□	-
Pancreatic diseases - clinical and laboratory assessment, sonography, CT, MRT	□	□	□	-
Celiac disease - TTG antibodies, endomysium antibodies, duodenal biopsy	□	□	□	-
Anorexia nervosa - clinical context	□	□	□	-
Parenteral nutrition - clinical context	□	□	□	-
Cardiopulmonary diseases with shock liver (cardiac hepatopathy, ischemic hepatitis) - cardiopulmonary assessment of congestive heart disease, myocardial infarction, cardiomyopathy, cardiac valvular dysfunction, pulmonary embolism, pericardial diseases, arrhythmia, hemorrhagic shock, and various other conditions	□	□	□	-
Addison's disease - plasma cortisol	□	□	□	-
Thyroid diseases - TSH basal, T4, T3	□	□	□	-
Grand mal seizures - clinical context of epileptic seizure (duration > 30 min)	□	□	□	-
Heat stroke - shock, hyperthermia	□	□	□	-
Polytrauma - shock, liver injury	□	□	□	-
Systemic diseases - specific assessment of M. Boeck, amyloidosis, lymphoma, other malignant tumors, sepsis, and others	□	□	□	-
Graft *vs* host disease - clinical context	□	□	□	-
Other diseases - clinical context	□	□	□	-

This checklist is far from complete and considered as a reminder for the physician. Some listed liver diseases like AIH require a liver biopsy to establish the diagnosis. Few elements are not directed to causality assessment but are important for overall case evaluation. AAA: Anti-actin antibodies; AMA: Antimitochondrial antibodies; ANA: Antinuclear antibodies; BMI: Body mass index; CT: Computed tomography; CYP: Cytochrome P450; HAV: Hepatitis A virus; HBc: Hepatitis B core; HBsAg: Hepatitis B antigen; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HEV: Hepatitis E virus; HILI: Herb induced liver injury; HIV: Human immunodeficiency virus; LKM: Liver kidney microsomes; LP: Liver-pancreas antigen; MRC: Magnetic resonance cholangiography; MRT: Magnetic resonance tomography; p-ANCA: Perinuclear antineutrophil cytoplasmatic antibodies; PDH: Pyruvate dehydrogenase; PCR: Polymerase chain reaction; SLA: Soluble liver antigen; SMA: Smooth muscle antibodies; TSH: Thyroid stimulating hormone; TTG: Tissue transglutaminase.

Table 4 Conditions of unintentional reexposure tests in drug induced liver injury and herb induced liver injury cases

Reexposure test result	Hepatocellular type of liver injury		Cholestatic or mixed type of liver injury	
	ALTb	ALTr	ALPb	ALPr
Positive	< 5N	≥ 2ALTb	< 5N	≥ 2ALPb
Negative	< 5N	< 2ALTb	< 5N	< 2ALPb
Negative	≥ 5N	≥ 2ALTb	≥ 5N	≥ 2ALPb
Negative	≥ 5N	< 2ALTb	≥ 5N	< 2ALPb
Negative	≥ 5N	NA	≥ 5N	NA
Uninterpretable	< 5N	NA	< 5N	NA
Uninterpretable	NA	NA	NA	NA

Conditions and criteria for an unintentional reexposure test are described in previous reports[4,21,22,24,26,27]. **Accordingly, required data for the hepatocellular type of liver injury are the ALT levels just before reexposure, designed as baseline ALT or ALTb, and the ALT levels during reexposure, designed as ALTr.** Response to reexposure is positive, if both criteria are met: first, ALTb is below 5N with N as the upper limit of the normal value, and second ALTr ≥ 2ALTb. Other variations lead to negative or uninterpretable results. **For the cholestatic or mixed type of liver injury, corresponding values of ALP are to be used rather than of ALT. ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; NA: Not available.**

therefore provides two different subscales[21,23,24] for the hepatocellular type of injury (Table 1) and for the cholestatic or mixed type (Table 2). These types are differentiated by the ratio R, calculated as the ALT/ALP activity measured at the time liver injury is suspected, with both activities expressed as multiples of N[21,24]. Injury is hepatocellular, if only ALT > 2N, alternatively if R ≥ 5; cholestatic injury is assumed, if only ALP > 2N or R ≤ 2; mixed damage is prevalent, if ALT > 2N and ALP is increased, with R > 2 and R < 5[21,23,24]. Of note, R may vary during the later course of the liver injury independent from the initial attribution of damage type.

Time to onset from the beginning of the drug/herb

Clear challenge criteria are defined with a time frame between beginning of the drug/herb use as the first day of intake and the onset of increased liver enzymes or symptoms at the time of ongoing use, with a high score for 5-90 d and a lower one for < 5 d or > 90 d (Tables 1 and 2). If drug/herb use has been terminated prior to the onset of challenge criteria, then this specific condition must be considered and scored exclusively. Scoring is only possible when the onset occurs within 15 d after cessation for the hepatocellular injury (Table 1) or 30 d for the cholestatic or mixed type (Table 2), a longer interval commonly excludes causality (Tables 1 and 2). An exemption is provided for slowly metabolized chemicals like amiodarone, leflunomide and clavulanate[44,45]; no definitive time frame can be provided in these cases due to varying half lives. The time frame of challenge and latency period were neither specified nor individually scored by other causality assessment methods[4,24], including the DILIN method[49,50].

Course of liver enzymes after cessation of the drug/herb

Precise dechallenge criteria are cornerstones of the CIOMS scale and facilitate causality assessment (Tables 1 and 2). In analogy to the periods mentioned, the physician can easily determine relevant future time points for repeated liver enzyme tests. When dechallenge data are missing in retrospective analyses, the CIOMS scale considers this and provides 0, but not negative points, so

Table 5 Frequency of risk factors of alcohol and age among herb induced liver injury patients

HILI study cohort	Total study cases (n)	Cases scored with risk factors (n)			Total cases scored with risk factors n (%)	Cases with references
		Alcohol	Age	Alcohol + Age		
Kava	26	0	6	1	7 (26.9)	Cases 2, 4, 10, 17, 20, 24, 26[12]
Kava	5	0	3	0	3 (60.0)	Cases 1-3[46]
Ayurvedic herbs	1	0	1	0	1 (100.0)	Case 1[30]
Black cohosh	4	2	1	0	3 (75.0)	Cases 2, 3, 4[47]
Black cohosh	9	1	1	0	2 (22.2)	Cases 4, 9[48]
Black cohosh	22	4	2	0	6 (27.3)	Cases 2, 8, 10, 16, 17, 21[36]
Greater Celandine	22	1	9	0	10 (45.5)	Cases 3, 5, 8, 11, 14-18, 21[37]
Greater Celandine	21	0	7	0	7 (33.5)	Cases 4,9, 11, 16-18, 20[32]
Pelargonium sidoides	15	0	3	0	3 (20.0)	Cases 1, 7, 14[38]
Pelargonium sidoides	13	0	7	0	7 (53.9)	Cases 2, 3, 5, 8, 9, 11, 13[39]
Herbalife	8	0	3	0	3 (37.5)	Cases 1, 2, 4[33]
Total	146	8	43	1	52 (35.6)	

The study cohort consisted of 146 herb induced liver injury patients patients assessed for the frequency of the risk factors alcohol and age ≥ 55 years. In 52/146 cases (35.6%), risk factors were evident.

that the overall score may still present a probable causality level. Of note, the dechallenge time frame was not specifically considered or scored by the DILIN method[49,50] or by virtually any of the other methods[4,24].

Risk factors

The consensus report of the international CIOMS expert panel considered alcohol and age ≥ 55 years as risk factors each scoring +1 point (Tables 1 and 2)[21], as suggested by DILI cases with positive reexposure[21,22]. The international DILI Expert Working Group specified alcohol intake of > 2 drinks per day (> 14 units/week) in women and > 3 drinks per day (21 units/week) in men as the lower threshold for alcohol intake as a risk factor (Tables 1 and 2)[14]. This limit is in line with the recommendations of NIH LiverTox equalling 1 drink to 10 g ethanol[44,45].

The impact of including alcohol as a risk factor on the overall CIOMS scoring was negligible as only 9/146 patients (6%) of a HILI study cohort were allotted an alcohol related scoring point (Table 5)[12,30,32,36-39,46-48]. In these 9 patients, CIOMS causality grading was changed in only one case (patient 16) and unchanged in the other 8 cases (patients 2, 13, 14, 17, 20, 21, 22, 32) (Table 6). In the single case (patient 16), alcohol as risk factor raised the overall CIOMS scoring from 0 to +01 point, *i.e.*, from excluded to unlikely causality (Table 6). Therefore, alcohol per se as risk factor upgrades the CIOMS causality level in virtually none of the cases in this study cohort.

Age ≥ 55 years was as a risk factor in 44/146 cases (30%) of the analyzed HILI study cohort (Table 5)[12,30,32,36-39,46-48]. In 35/44 patients, the overall CIOMS causality grading remained unchanged whether or not age as a risk factor was included in the CIOMS scale scoring (Table 6). Deletion of age as a risk factor reduced the overall CIOMS grading by one causality level in 9/44 patients, *i.e.*, from unlikely to excluded in 5 cases (patients 1, 3, 15, 42, 43), from highly probable to probable in 1 case (patient 23), and from probable to possible in 3 cases (patients 9,

25, 34). Therefore, within this cohort the risk factor age upgraded the causality levels only marginally, which appears to have no clinical relevance. Overall, age as a risk factor has a limited impact on the final causality gradings by the CIOMS scale.

Risk factors are not considered and/or not scored by various other methods[4,24,49,50], including the DILIN method[49,50]. Conversely, the international DILI Expert Group also accepts the risk factors defined in the CIOMS scale, with modified specifications and limitations, if risk factors for hepatotoxicity are present in addition to those listed in the CIOMS algorithm[14].

Concomitant drug(s) and herbs(s)

Concomitant drugs and herbs are individually assessed for temporal association and hepatotoxic potency (Tables 1 and 2). For reasons of comparison and transparency, each comedicated drug or herb requires a separate analysis by the complete CIOMS scale. This is feasible and easily tabulated (Table 7)[30,47,48]. In patients with multiple drug or herb intakes, causality should be attributed primarily to the product with the highest score.

Search for non drug/herb causes

In this section, the CIOMS scale considers the clinically most relevant alternative causes (Tables 1 and 2). There is no difference in alternative causes made between the two types of liver injury, avoiding the need of subsequent reassessment, if the laboratory based typology changes during the clinical course[45]. Complications of underlying disease(s) are exemplified, such as sepsis, autoimmune hepatitis, chronic hepatitis B or C, primary biliary cirrhosis and sclerosing cholangitis and genetic liver diseases (Tables 1 and 2), in accordance with recent suggestions[45]. Other rare alternative causes are included in a checklist of differential diagnoses as a reminder for the clinician in case of unclear clinical diagnosis (Table 3)[24].

To improve its performance when used as an investigational tool, criteria for competing liver injury causes have been proposed for the CIOMS scale[14,29,44,45,49,50]

Table 6 Changes of the Council for International Organizations of Medical Sciences gradings with considering the risk factors

HILI study	Scored risk		CIOMS assessment with RF	CIOMS assessment without RF	Grading change	Cases with references
	Alcohol	Age	Score/grading	Score/grading		
Kava	0	+	+1/Unlikely	0/Excluded	↓	Case 2[12]
Kava	+	+	-1/Excluded	-3/Excluded	0	Case 4[12]
Kava	0	+	+1/Unlikely	0/Excluded	↓	Case 10[12]
Kava	0	+	-1/Excluded	-2/Excluded	0	Case 17[12]
Kava	0	+	+8/Probable	+7/Probable	0	Case 20[12]
Kava	0	+	-1/Excluded	-2/Excluded	0	Case 24[12]
Kava	0	+	-1/Excluded	-2/Excluded	0	Case 26[12]
Kava	0	+	+5/Possible	+4/Possible	0	Case 1[46]
Kava	0	+	+6/Probable	+5/Possible	↓	Case 2[46]
Kava	0	+	+8/Probable	+7/Probable	0	Case 3[46]
Ayurvedic herbs	0	+	+8/Probable	+7/Probable	0	Case 1[30]
Black cohosh	0	+	-2/Excluded	-3/Excluded	0	Case 1[47]
Black cohosh	+	0	-2/Excluded	-3/Excluded	0	Case 2[47]
Black cohosh	+	0	-3/Excluded	-4/Excluded	0	Case 3[47]
Black cohosh	0	+	+1/Unlikely	0/Excluded	↓	Cases 4[48]
Black cohosh	+	0	+1/Unlikely	0/Excluded	↓	Case 9[48]
Black cohosh	+	0	-1/Excluded	2/Excluded	0	Case 2[36]
Black cohosh	0	+	-1/Excluded	-2/Excluded	0	Case 8[36]
Black cohosh	0	+	-1/Excluded	-2/Excluded	0	Case 10[36]
Black cohosh	+	0	0/Excluded	-1/Excluded	0	Case 16[36]
Black cohosh	+	0	0/Excluded	-1/Excluded	0	Case 17[36]
Black cohosh	+	0	-2/Excluded	-3/Excluded	0	Case 21[36]
Greater Celandine	0	+	+9/Highly probable	+8/Probable	↓	Case 2[37]
Greater Celandine	0	+	+10/Highly probable	+9/Highly probable	0	Case 5[37]
Greater Celandine	0	+	+6/Probable	+5/Possible	↓	Case 8[37]
Greater Celandine	0	+	+5/Possible	+4/Possible	0	Case 11[37]
Greater Celandine	0	+	+8/Probable	+7/Probable	0	Case 14[37]
Greater Celandine	0	+	+5/Possible	+4/Possible	0	Case 15[37]
Greater Celandine	0	+	-1/Excluded	-2/Excluded	0	Case 16[37]
Greater Celandine	0	+	+8/Probable	+7/Probable	0	Case 17[37]
Greater Celandine	0	+	0/Excluded	-1/Excluded	0	Case 18[37]
Greater Celandine	+	0	+4/Possible	+3/Possible	0	Case 21[37]
Greater Celandine	0	+	+5/Possible	+4/Possible	0	Case 4[32]
Greater Celandine	0	+	+6/Probable	+5/Possible	↓	Case 9[32]
Greater Celandine	0	+	+3/Possible	+2/Possible	0	Case 11[32]
Greater Celandine	0	+	+7/Probable	+6/Probable	0	Case 16[32]
Greater Celandine	0	+	+7/Probable	+6/Probable	0	Case 17[32]
Greater Celandine	0	+	+5/Possible	+4/Possible	0	Case 18[32]
Greater Celandine	0	+	+7/Probable	+6/Probable	0	Case 20[32]
Pelargonium sidoides	0	+	0/Excluded	-1/Excluded	0	Case 1[38]
Pelargonium sidoides	0	+	+2/Unlikely	+1/Unlikely	0	Case 7[38]
Pelargonium sidoides	0	+	+1/Unlikely	0/Excluded	↓	Case 14[38]
Pelargonium sidoides	0	+	+1/Unlikely	0/Excluded	↓	Case 2[39]
Pelargonium sidoides	0	+	+4/Possible	+3/Possible	0	Case 3[39]
Pelargonium sidoides	0	+	0/Excluded	-1/Excluded	0	Case 5[39]
Pelargonium sidoides	0	+	0/Excluded	-1/Excluded	0	Case 8[39]
Pelargonium sidoides	0	+	+2/Unlikely	+1/Unlikely	0	Case 9[39]
Pelargonium sidoides	0	+	+2/Unlikely	+1/Unlikely	0	Case 11[39]
Pelargonium sidoides	0	+	0/Excluded	-1/Excluded	0	Case 13[39]
Herbalife	0	+	+7/Probable	+6/Probable	0	Case 1[33]
Herbalife	0	+	+2/Unlikely	+1/Unlikely	0	Case 2[33]
Herbalife	0	+	+2/Unlikely	+1/Unlikely	0	Case 4[33]

Based on details described in Table 5, in all 52 patients with evident risks factors of alcohol, age ≥ 55 years, or both, scores and Council for International Organizations of Medical Sciences (CIOMS) gradings with risk factors were compared with conditions without risk factor consideration. In 9 patients, there was a CIOMS downgrading when risk factors would not have been considered. RF: Risk factor.

and were included in the updated CIOMS scale (Tables 1 and 2)[24]. This update ensures correct diagnosis of alternative causes but was limited to details of hepatitis serology and hepatobiliary sonography, as specified by the current knowledge in the field and adapted to actual diagnostic methods[23,24]. The update of the original CIOMS scale substantially improved specificity, *i.e.*, exclusion of alternative causes by hepatitis serology. HBsAg and HBV-DNA quantification were added to distinguish HBV infection from immunization, as was hepatitis C virus (HCV)-RNA to correctly assess HCV infections. Also, clinical and/or biological parameters for cytomegalovirus (CMV), Epstein Barr virus (EBV) or herpes simplex virus (HSV) infection were vague or

Table 7 Council for International Organizations of Medical Sciences scale as an example with items required for causality assessment in a patient with herb induced liver injury by four different Indian Ayurvedic herbs

Items for hepatocellular injury	Possible score	Psoralea corylifolia	Acacia catechu	Eclipta alba	Vetivexia zizaniodis
1 Time to onset from the beginning of the herb 5-90 d (rechallenge: 1-15 d)	2				
< 5 or > 90 d (rechallenge: > 15 d)	1	1	1	1	1
Alternative: Time to onset from cessation of the herb					
⩽ 15 d (except for slowly metabolized herbal chemicals: > 15 d)	1				
2 Course of ALT after cessation of the herb					
Percentage difference between ALT peak and N					
Decrease ⩾ 50% within 8 d	3	3	3	3	3
Decrease ⩾ 50% within 30 d	2				
No information or continued herbal use	0				
Decrease ⩾ 50% after the 30th day	0				
Decrease < 50% after the 30th day or recurrent increase	-2				
3 Risk factors					
Alcohol use (drinks/d: > 2 for women, > 3 for men)	1				
Alcohol use (drinks/d: ⩽ 2 for women, ⩽ 3 for men)	0	0	0	0	0
Age ⩾ 55 yr	1	1	1	1	1
Age < 55 yr	0				
4 Concomitant herbs(s) and drug(s)					
None or no information	0				
Concomitant herb or drug with incompatible time to onset	0				
Concomitant herb or drug with compatible or suggestive time to onset	-1	-1			
Concomitant herb or drug known as hepatotoxin and with compatible or suggestive time to onset	-2		-2	-2	-2
Concomitant herb or drug with evidence for its role in this case (positive rechallenge or validated test)	-3				
5 Search for non herb causes					
Group Ⅰ (6 causes)					
Anti-HAV-IgM		-	-	-	-
HBsAg, anti-HBc-IgM, HBV-DNA		-	-	-	-
Anti-HCV, HCV-RNA		-	-	-	-
Hepatobiliary sonography/colour Doppler sonography of liver vessels/ endosonography/CT/MRC		-	-	-	-
Alcoholism (AST/ ALT ⩾ 2)		-	-	-	-
Acute recent hypotension history (particularly if underlying heart disease)		-	-	-	-
Group Ⅱ (6 causes)					
Complications of underlying disease(s) such as sepsis, autoimmune hepatitis, chronic hepatitis B or C, primary biliary cirrhosis or sclerosing cholangitis, genetic liver diseases		-	-	-	-
Infection suggested by PCR and titre change for		-	-	-	-
CMV (anti-CMV-IgM, anti-CMV-IgG)		-	-	-	-
EBV (anti-EBV-IgM, anti-EBV-IgG)		-	-	-	-
HEV (anti-HEV-IgM, anti-HEV-IgG)		-	-	-	-
HSV (anti-HSV-IgM, anti-HSV-IgG)		-	-	-	-
VZV (anti-VZV-IgM, anti-VZV-IgG)		-	-	-	-
Evaluation of group Ⅰ and Ⅱ					
All causes-groups Ⅰ and Ⅱ - reasonably ruled out	2	2	2	2	2
The 6 causes of group Ⅰ ruled out	1				
5 or 4 causes of group Ⅰ ruled out	0				
Less than 4 causes of group Ⅰ ruled out	-2				
Non herb cause highly probable	-3				
6 Previous information on hepatotoxicity of the herb					
Reaction labelled in the product characteristics	2				
Reaction published but unlabelled	1	1			
Reaction unknown	0		0	0	0
7 Response to unintentional readministration					
Doubling of ALT with the herb alone, provided ALT below 5N before reexposure	3				
Doubling of ALT with the herb(s) and drug(s) already given at the time of first reaction	1				
Increase of ALT but less than N in the same conditions as for the first administration	-2				
Other situations	0				
Total score for each individual herb used by the patient		7	5	5	5

The data of the patient with severe hepatotoxicity by four different Indian Ayurvedic herbs are derived from a published report[30], using the CIOMS scale for the hepatocellular type of liver injury (Table 1). The symbol of - signifies that this particular item has been evaluated and no abnormality was found. For the four herbs, the total score was either +7 (probable causality) or +5 (possible causality). Abbreviations see legend to Table 1.

unknown at the time of compilation[21] but specified in the updated CIOMS scale; also included and specified were infections by hepatitis E virus (HEV) and varicella zoster virus (VZV) (Tables 1 and 2)[24]. Specific diagnostic criteria include polymerase chain reaction detection and titer changes of the respective antibodies (IgM, IgG) for CMV, EBV, HEV, HSV and VZV infections. Hepatobiliary sonography was supplemented by color Doppler sonography, including assessments of the liver vessels, endosonography, computed tomography (CT) and magnetic resonance cholangiography (MRC), if these tests were indicated clinically (Tables 1 and 2). For comparison and method validation, causality has been evaluated in 101 hepatotoxicity cases by both the original and updated CIOMS scales, with identical causality results published in 6 studies[32,33,36-39]. Therefore, the updated CIOMS scale was validated and there is no need for further validation of the updated CIOMS scale versus the original CIOMS scale.

Previous information on hepatotoxicity of the drug/herb
Hepatotoxicity listed in the product information sheet must be checked; in addition, a quick literature search in PubMed will be sufficient to determine whether the observed reaction has been published before. Appropriate information may also be obtained from the NIH LiverTox database[44,45].

Response to unintentional readministration
To classify an unintentional reexposure test as positive, few criteria are required (Tables 1 and 2), as specified (Table 4)[21,22,24,26,27]. Although reexposure is an important domain, probable causality gradings with the CIOMS scale are achievable even in the absence of a reexposure (Table 7)[12,17,30-32,37].

Scoring system
Each item of the CIOMS scale receives an individual score and the sum of the individual scores provides the final score for the patient (Tables 1 and 2). With +14 down to -9 points, there is a wide range of the final scores, leading to the following causality levels: ≤ 0 points, excluded causality; 1-2, unlikely; 3-5, possible; 6-8, probable; and ≥ 9, highly probable (Tables 1 and 2)[21].

Sensitivity, specificity and predictive value
Cases with positive reexposure tests were proposed for validation of the CIOMS scale and used as gold standard[22]. Articles from two databanks were compiled with liver injury confirmed by a positive rechallenge. The mandatory information for inclusion in this series contained the type of liver injury, time interval between administration of the drug and occurrence of the reaction, and results of the positive response to readministration of the drug, in accordance with the conclusions of the International Consensus Meeting on drug induced liver injuries. For the final validation, 49 cases and 28 controls were assessed, as described in detail[22]. Most importantly, the discriminative power of the score was quantified

in terms of sensitivity, specificity and predictive values. The cut off point was offset to maximize the combined sensitivity and specificity. Using +5 points as the cut off, sensitivity was 86%, specificity 89%, positive predictive value 93%, and negative predictive value 78% for the CIOMS causality assessment. In another study with 81 cases and 46 controls, sensitivity was 78% and specificity 100% for the CIOMS scale[51], confirming the validation of the CIOMS scale.

The interrater reliability of CIOMS assessment was good by one group[52] but mediocre by the DILIN group[49]. In the latter report, however, 40 cases going back to 1994 were studied. Uncertainties arose from numerous missing, incomplete or outdated medical reports and charts, especially for older cases. In particular, there were high rates (28%) of preexisting liver diseases like chronic hepatitis C virus infection, hemochromatosis and unspecified cirrhosis. Liver sonography was reported in 26/40 cases and found abnormal in 15/26 (58%). These data were nevertheless described as "best-case scenario"[49]. Considering these limitations and numerous confounding variables, poor case data quality likely results in mediocre assessment quality, including low interrater reliability[49]. Moreover, problematic data presentation by the principal assessor to external reviewers may have influenced the results as the external reviewers received only a subset of the case report forms and had no access to the original data of the cases[49]. Of interest, no proof has been provided that an expert group opinion improves the CIOMS assessment evaluation, at least according to recent comments and studies[11,49]. In another study comparing the CIOMS scale with the DILIN method, there was considerable interobserver variability in both methods[50].

Usage frequency
The CIOMS scale for hepatotoxicity assessment in its original or updated form[4,5,10,14,23,24,28,29] has been extensively used in epidemiological studies, clinical trials, case reports, case series, regulatory analyses and genotyping studies[4]. Additional efforts are still needed to reevaluate causality in most HILI reports for 60 different herbs and herbal products[53]. CIOMS based results were published by the DILIN group[49,50] and by the European Medicines Agency (EMA)[54]. Individual studies[10,16,55,56], the NIH LiverTox[44,45], the international DILI Expert Working Group[14], the Spanish Group for the Study of Drug-Induced Liver Disease[29], and the Hong Kong Herb-Induced Liver Injury Network (HK-HILIN)[57] provided further support for the CIOMS scale.

Among various causality assessment methods, the original and updated CIOMS scales were the preferred tools in cases of DILI[28] and HILI (Table 8)[5], seen for 573 cases from 23 HILI reports evaluating alternative causes[12,32,34,36-39,42,43,47,48,54,57-67].

Transparency
CIOMS based assessments should be reported or published as an original data set suitable for subsequent and independent assessments, rather than as final scores and

Table 8 Compilation of causality assessment methods used in suspected herb induced liver injury cases

Herbs/Herbal products	Ad hoc (*n*)	WHO (*n*)	CIOMS (*n*)	Naranjo (*n*)	DILIN (*n*)	KL (*n*)	Ref.
Kava	20						BfArM[58]
Kava		30					Denham *et al*[59]
Kava	20						Teschke *et al*[60]
Kava			36				Stickel *et al*[61]
Kava		80					Schmidt *et al*[62]
Greater Celandine	23						BfArM[63]
Black cohosh			31				EMA[54]
Herbalife products		12					Elinav *et al*[64]
Herbalife products		12					Schoepfer *et al*[65]
Kava			26				Teschke *et al*[12]
Black cohosh				30			Mahady *et al*[42]
Green tea				34			Sarma *et al*[43]
Black cohosh			4				Teschke *et al*[47]
Black cohosh			9				Teschke *et al*[48]
Kava			31				Teschke[34]
Hydroxycut					17		Fong *et al*[66]
Black cohosh			22				Teschke *et al*[36]
Greater Celandine			22				Teschke *et al*[37]
Herbalife products						20	Manso *et al*[67]
Various herbs			45				Chau *et al*[57]
Greater Celandine			21				Teschke *et al*[32]
Pelargonium sidoides			15				Teschke *et al*[38]
Pelargonium sidoides			13				Teschke *et al*[39]
Sum (*n*)	63	134	275	64	17	20	
Sum (percent)	11.00%	23.40%	48.00%	11.20%	3.00%	3.40%	

The data are derived from a study evaluating alternative causes in suspected HILI cases (*n* = 573) comprising the study cohort[5]. For the 275 CIOMS cases, causality assessment was performed with the updated CIOMS scale the original CIOMS scale, or both. Ad hoc: ad hoc approach; CIOMS: Council for International Organizations of Medical Sciences scale; DILIN: Drug Induced Liver Injury Network method; KL: Karch and Lasagna method; Naranjo: Naranjo scale; WHO: World Health Organization method.

corresponding causality levels, to improve data transparency. Scientists, editors and reviewers should strive to obtain appropriate CIOMS based details for all DILI and HILI case reports. This can easily be achieved since the CIOMS scale provides all items in tabulated form for each individual case (Tables 1, 2 and 7). These forms may be communicated as a spontaneous report to regulatory agencies and expert panels or presented for publication to scientific journals as a case report[24,30,31] or case series[12,24,32]. This tabulation is a good basis for further regulatory or scientific assessments and discussions. For regulatory and expert based assessments, there is no need for other causality assessment algorithms to be used subsequently since CIOMS based data are also amenable to regulatory and expert panel evaluations.

Comparison to precursor scales
The CIOMS scale resulted from intensive expert discussions[21], integrating medical progress and improving the initial qualitative RUCAM[26] and the qualitative CIOMS method[27]. The qualitative RUCAM represented the first objective attempt to assess causality in drug induced liver injury and considered some characteristic features of liver injury, but it had a qualitative rather than a quantitative approach[26]. As an improved version of the qualitative RUCAM[26], the qualitative CIOMS method differentiated the hepatocellular, the cholestatic and the mixed type of liver injury[27]. However, both the qualitative

RUCAM[26] and the qualitative CIOMS method[27] were not quantitative, as opposed to the current quantitative CIOMS scale[21] that is now the preferred tool[24].

Other liver specific methods
The scale of Maria and Victorino (MV)[68] was developed to improve upon the CIOMS scale by deleting laboratory items and adding clinical elements, along with simplifying and changing the relative weight of elements in their algorithm[23,44,45]. No data are available for specificity, sensitivity, positive and negative predictive values for the MV scale[68]. Compared to the original CIOMS scale[21], the MV scale[68] showed shortcomings and the results are not equivalent, causing major concern[10,14,23,24,29,44,45,69-71]. This may explain why the MV scale was used in a few DILI studies[1,72,73], but not in 38 other publications of DILI cases[28] or in 23 publications of HILI cases[5]. The MV scale is not commonly recommended for assessing causality in assumed DILI and HILI cases and is certainly no substitute for the CIOMS scale[24].

The TTK scale[25], named for the first three authors Takikawa, Takamori, Kumagi *et al*[74], is a modification of the CIOMS scale[21] with different evaluations of the chronology, exclusion of comedication, inclusion of the drug lymphocyte stimulation test (DLST) and of eosinophilia in their assessment system[74,75]. The TTK scale is widely used in Japan[74], as recently reviewed[75]. In other countries, this scale is not or rarely

considered[5,10,14,24,28,29,44,45,76]. Limited access and lack of standardization have prevented general clinical use of the DLST and consequently TTK scale applications outside Japan[29]; this may be due to methodological difficulties with false positive and false negative cases in the DLST[25,75]. For clinicians, the TTK scale cannot replace the CIOMS scale[25].

The DILIN method provided by the DILIN group requires an expert panel[3,6,11,24,44,45,49,50,77,78], in contrast to the CIOMS scale[21,24]. Consequently, the DILIN method is of limited availability to physicians in need of early results for therapeutic decisions[24]. In particular, the DILIN method is not an appropriate substitute for the CIOMS scale, nor are other expert panel based approaches[24]. This includes the **novel Causality Assessment Tool (CAT)** specifically designed for herbs and dietary supplements (HDS), which was presented as an abstract[15]. As opposed to CIOMS based results with transparent data presentation (Table 7)[12,30,32,36-39,47,48,71], publications based on the DILIN method lack transparency for individual cases regarding assessed and scored items since only final causality levels are published without details and thereby open for discussions, not allowing valid conclusions[3,6,11,49,50,77,78]. The DILIN method also lacks data on specificity, sensitivity and predictive values, as an expert opinion based method no items can be validated. Individual weighing and scoring of items remain undisclosed and undiscussed, hampering thorough analysis of assessment results by the DILIN method.

Liver unspecific methods

In contrast to the liver specific core elements of the original and updated CIOMS scale (Tables 1 and 2)[4,21-24], numerous causality algorithms are liver unspecific[4,24,76,79,80], including the Naranjo scale[81], the World Health Organization (WHO) global introspection method as the WHO method in short[82], and the KL method of Karg and Lasagna[83]. Particularly intensive discussions focused on the Naranjo scale[4,24,25,84-87], the WHO method[4,24,84,87], and the KL method[24,25]. All these methods are obsolete for causality assessment of assumed hepatotoxicity as they lack liver specificity and do not consider hepatotoxicity characteristics.

WEAKNESSES

Retrospective use

Retrospective analysis of case data is problematic and may require some assistance evaluating the CIOMS items[14,44,45]; unselected and sometimes undefined, low quality data have to be adapted into a structured algorithm like the CIOMS scale. Therefore, physicians should prospectively use the CIOMS scale, which then may provide complete case data (Table 7)[30].

Dechallenge criteria

Missing ALT dechallenge data are factored as 0 points given (Table 1); this condition has been interpreted as a

limitation of the CIOMS scale[14]. Retrospective studies commonly lack dechallenge results[6,12,32,34-39] which are included in prospective evaluations (Table 7)[30]. CIOMS performs inaccurately in acute liver failure and liver transplantation if liver values are not available within 30 d after cessation of the incriminated drug or herb. Under these circumstances, 0 but not negative points are credited due to lacking ALT data (Tables 1 and 2).

Risk factors

The CIOMS scale includes only the risk factors of alcohol and age ≥ 55 years[21]. Diabetes, metabolic syndrome, sex, ethnicity and body mass index[14], as well as genetic predisposition[29], are also proposed as potential risk factors; the lack of inclusion in the CIOMS scale has been considered as a limitation[14,29]. However, these factors have not been validated as risk factors; their inclusion into the CIOMS scale requires evidence as independent contributors and a subsequent new validation.

Alternative causes

It may be argued that rare alternative causes were not listed in the CIOMS scale (Tables 1 and 2) but this shortcoming was compensated for by the checklist for numerous rare liver diseases as a reminder for the clinician (Table 3)[24].

Previous information on hepatotoxicity of drug/herb

Safety labels are available for both synthetic and herbal drugs but rarely for other herbal products. This shortcoming of the CIOMS scale may be compensated by a thorough search for prior publications of hepatotoxicity by herbal products; published reports provide an even higher scoring than information obtained only from safety labels.

CONCLUSION

The major strength of the CIOMS is its potential as a standard scale for DILI and HILI causality assessment by attending physicians, regulatory agencies, expert panels and the scientific community. Other advantages include its liver specificity and its validation for hepatotoxicity cases, with excellent sensitivity, specificity and predictive validity based on results obtained from cases with a positive reexposure test. This scale will allow the physician treating patients with suspected DILI and HILI an early preliminary result of the likelihood, facilitates timely and prospective collection of all relevant data required for a subsequent valid causality assessment, does not require an expert panel, and has the option of subsequent refinement by regulatory agencies, expert panels and the scientific community. With the CIOMS scale, an identical causality assessment algorithm can used by all evaluating parties, which facilitates the overall procedure of causality association. Minor weaknesses of the CIOMS scale include the limited exclusion of alternative causes and the handling of poor case data in

retrospectively rather than prospectively assessed cases.

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Fatty liver in childhood

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Abstract

Fatty liver is a growing health problem worldwide. It might evolve to nonalcoholic steatohepatitis, cirrhosis and cause hepatocellular carcinoma. This disease, which has increased because of eating habits, changes in food content and lifestyle, affects people from childhood. The most important risk factors are obesity and insulin resistance. Besides these factors, gender, ethnicity, genetic predisposition and some medical problems are also important. Cirrhosis in children is rare but is reported. Nonalcoholic fatty liver disease (NAFLD) has no specific symptoms or signs but should be considered in obese children. NAFLD does not have a proven treatment. Weight loss with family based treatments is the most acceptable management. Exercise and an applicable diet with low glycemic index and appropriate calorie intake are preferred. Drugs are promising but not sufficient in children for today.

Core tip: Nonalcoholic fatty liver disease (NAFLD) consists of steatosis in liver, steatohepatitis and cirrhosis. Histological type 2 pattern (macrovesicular steatosis with portal inflammation and/or fibrosis, generally without evidence of cellular injury or lobular inflammation) is seen differently in children than in adults. The most important risk factors are obesity and insulin resistance, as well as gender, ethnicity, genetic predisposition and some medical problems. Progression to cirrhosis in children is rare but possible. NAFLD does not have a proven treatment. Losing weight and increasing physical activity provide improvement in histological and biochemical findings in fatty liver. Drugs are used in specific situations. More research is needed for drug therapy.

INTRODUCTION

Fat is stored as triglyceride (TG) in human liver. Steatosis is defined as fat accumulation in hepatocytes and is seen in many liver diseases[1-3].

Nonalcoholic fatty liver disease (NAFLD) defines the spectrum of histological changes in liver in which macrovesicular steatosis is outstanding[3]. NAFLD includes simple hepatic steatosis due to obesity and/or insulin resistance, nonalcoholic steatohepatitis (NASH) and cirrhosis. Hepatosteatosis usually limits itself but it may advance to NASH. NASH differs from simple steatosis by hepatocyte damage, inflammatory infiltrate and collagen deposition[4-6].

In many ways, the NASH pattern and characteristics differ between children and adults[7]. In adults, common features are the combination of macrovesicular steatosis with ballooning degeneration and lobular inflammation

with or without pericellular fibrosis localized primarily in acinar zone 3 (type 1). Pediatric NASH is characterized by macrovesicular steatosis with portal inflammation and/or fibrosis, generally without evidence of cellular injury or lobular inflammation (Type 2). Type 1 and type 2 NASH are distinct subtypes of pediatric NAFLD associated with different clinical demographic and possible pathophysiological features. In children with NAFLD aged 2-18 years, 51% is type 2 and 17% is type 1. In most of the children with extensive fibrosis, type 2 pattern is demonstrated[8]. These children are younger and more obese compared to children displaying type 1 pattern. Type 2 NASH is more common in boys than girls. Asian, Native American race and those of Hispanic ethnicity predominantly demonstrate type 2. Among children with type 2 NASH, it is not known whether the pattern evolves into a more characteristically adult type 1 pattern as the children grow older[9].

There has been an increase in NAFLD frequency in the last 30 years[1,8-11]. Nowadays, NAFLD is the most common form of liver disease in children[7]. A chronic obesity associated condition, NAFLD can lead to cirrhosis and liver failure over time[8]. It is also an independent risk factor for cardiovascular disease and liver cancer[9]. Studies have demonstrated differences in NAFLD prevalence rates across race/ethnicity, gender and weight status[12-14].

EPIDEMIOLOGY

In developed countries, hepatosteatosis is seen in 20%-30% of an unselected population[15]. The prevalence of NAFLD in Hong Kong Chinese is 27.3%. Around 4% of patients with fatty liver in the community had advanced fibrosis, as estimated by transient elastography[16].

The frequency of NASH is considered to be 2%-3%. It is reported that 10%-29% of NASH cases develop cirrhosis in 10 years[17]. Cirrhosis may progress to liver cancer. Hepatocellular carcinoma may occur in 4%-27% of the individuals with NASH-induced cirrhosis[18-20].

Since childhood obesity became epidemic in developed countries, NAFLD became the most common cause of chronic liver disease in pediatrics[7].

In fact, the true NAFLD prevalence in children is unknown. A population based autopsy study reported that 13% of children and adolescents are affected with NAFLD, 23% of the subjects with NAFLD had evidence for steatohepatitis, whereas bridging fibrosis or cirrhosis was observed in 9% of the children with NASH. Overweight and obese children accounted for 81% of all of the cases of NAFLD. A male-to-female ratio was 2: 1[7,20-23].

It is suggested that NAFLD prevalence increases with age, with a mean age at diagnosis between 11 and 13 years[24]. This tendency is explained by adolescent hormonal changes which result in an increase in serum insulin levels and fat accumulation in the liver[25,26].

Obesity and insulin resistance are the most common

risk factors for NAFLD. However, differing amounts of fat accumulation in individuals with similar adipose tissue suggests that other factors are also responsible. Gender, ethnicity and genetic predisposition are emphasized[27-35].

The prognosis of children with NAFLD is still unknown. Patients with simple steatosis may still develop NASH and fibrosis progression. It is reported that weight reduction is associated with non-progressive disease in adult patients[28]. It is suggested that long term survival of NAFLD pediatric patients is shorter than non-affected patients[29].

PATHOGENESIS

Triglycerides are preferred as storage nutrients in cells to regulate the changes between intake and usage. Triglycerides supply high calories. Additionally, because they are not dissolved in water, they might be stored intracellularly in high amounts without causing any colloidal or osmotic problems. Triglycerides are basic material stocks of adipocytes and are not accumulated in other cells, except in unusual situations. Steatosis in liver is not an adaptive process; indeed it may cause severe chronic problems[1].

Fat droplets should be seen in at least 5% of hepatocytes in order to be named as steatosis. Another definition is TG deposition in the liver above 95th percentile or more than 55 mg per gram of liver tissue in a healthy lean person[2].

Hepatosteatosis might be seen in two different types; macrovesicular and microvesicular. In macrovesicular steatosis, one or a few lipid droplets are present, filling the total hepatocyte. These lipid droplets propel the nucleus to the edge. In microvesicular type, multiple small lipid droplets are seen, giving a foamy appearance[3].

Microvesicular steatosis might be seen in Reye syndrome, salicylate, sodium valproate or ethanol intake, fulminant hepatitis D, mitochondrial fatty acid beta oxidation defects and urea cycle disorders. In these disorders, liver function tests are usually affected and the patient is comatose. If the patient survives, permanent damage will not occur in the liver. Macrovesicular type occurs in alcoholic liver disease, obesity, diabetes, kwashiorkor, AIDS, total parenteral nutrition therapy, phosphorus intoxication and steroid treatment[3].

High concentration of serum saturated free fatty acids is important in the pathogenesis of steatosis. This high concentration of saturated free fatty acids creates hepatotoxic impulse. Besides, esterification of these free fatty acids into TGs is a process of detoxification. The balance between TG deposition and removal is disrupted. There are three sources of fatty acids causing TG deposition in liver: from diet, 15%; *de-novo* synthesis (carbohydrates from diet), 26%; and adipose tissue circulating, 59%[1,5].

Twenty percent of the fat present in the systemic circulation (100 g/d) is taken by the liver. Daily intake of

TGs from diet (approximately 20g/d) and free fatty acids from adipose tissue (approximately 20 g/d) enter the liver as TG[1,36]. There has been an increase in NAFLD frequency in the last 30 years. It is considered that this is due to changes in the amount and content of food. Changes in food composition cause steatosis in liver. Generally, carbohydrates and fructose play the most important role in this issue. Fructose influences the dietary carbons to move to liver and participate in lipogenesis. Despite glucose, fructose is almost totally taken from the systemic circulation. Fructose is phosphorylated at C1 instead of C6 and because of this it cannot be used in glycogen synthesis. Instead, fructose is changed to glyceraldehyde-3-phosphate, which provides substrate for *de-novo* lipogenesis. Yearly fructose intake of the population is increasing day by day and consequently NAFLD incidence is rising[1,37]. As the adipose tissue increases in obesity, death receptors in adipose tissue and apoptosis pathway are activated. Increase in adipocyte death causes more macrophage migration. Insulin resistance and hepatosteatosis occur as a result. Approaches blocking apoptosis of adipose cells are considered to improve complications related to obesity, including NAFLD. Lipoapoptosis is related to AST/ALT > 1 and liver fibrosis[38-40].

Insulin stimulates fatty acid production while preventing glucose production in the liver. As insulin resistance develops in the liver, the effect of insulin on preventing glucose production diminishes. However, the effect of insulin on stimulating fat synthesis in the liver is preserved. When the insulin level decreases with therapy, steatosis in the liver also decreases. Additionally, high insulin levels increase hepatotoxicity by preventing FFA oxidation[41].

It is suggested that NAFLD pathogenesis is multifactorial with many factors affecting disease development and progression. The "multiple-hit" hypothesis is currently the established pathogenetic model[42]. At the onset, NAFLD is characterized by fat accumulation in the liver and insulin resistance, influenced by genetic susceptibility, epigenetic mechanisms, a sedentary lifestyle and hypercaloric diets[43]. Hepatic fat accumulation leads to exacerbating insulin resistance by interfering with phosphorylation of insulin receptor substrates[44]. Free fatty acid accumulation and insulin resistance predispose the fatty liver, including oxidative stress, inflammatory cytokines, stellate cells activation and mitochondrial disturbance, which lead to inflammation, necrosis and fibrosis[45]. A changing of gut microbiota and excess gut permeability increase liver exposure to gut-derived bacterial products in NAFLD. These products stimulate innate immune receptors and trigger liver inflammation and fibrogenesis[46].

Hepatic progenitor cell activation is correlated with fibrosis and NASH progression[47]. Adiponectin, leptin, resistin and tumor necrosis factor-alpha are also thought to be involved in the progression of steatosis to NASH. Adipocytes or inflammatory cells infiltrating the adipose tissue in insulin resistance are responsible for adipocytokine secretion. Leptin may activate hepatic stellate cells. The expansion of adipose tissue, especially visceral fat, is associated with a decrease in the release of insulin-sensitizing and anti-inflammatory cytokines and an increase in the release of pro-inflammatory molecules[48]. Tumor necrosis factor-α and interleukine-6 levels are elevated in the liver and blood of NASH patients. These cytokines are involved in Kupffer and hepatic stellate cell activation in myofibroblasts[49]. NAFLD results from the relationship between multiple organs, including adipose tissue, liver, gut and the pancreas[50,51].

CLINICAL FINDINGS

Most of the cases are asymptomatic but nonspecific symptoms like abdominal pain may be present[30]. The most common admission reason is slightly elevated transaminases or coincidentally noticed hepatomegaly. Multiple diseases like Wilson's disease, drug-induced liver injury and autoimmune hepatitis should be excluded before a diagnosis of NAFLD[30,31].

Obesity is distinctive[7-15,31]. In adults, 10%-75% of fatty liver occurs with insulin resistant type 2 diabetes. Fatty liver is defined in poorly regulated type 1 diabetes (Mauriac syndrome) in children. Children with typical NAFLD have insulin resistance with hyperinsulinemia but they are euglycemic. Type 2 diabetes mellitus is present in 5.5% of NASH cases[15,32]. Acanthosis nigricans, defined as hyperplasia of pigmented skin cells, is an important physical examination seen with insulin resistance. This can be found in more than 50% of children with NASH. Family story is important in NAFLD because familial clustering is common[33-35].

Obesity is reported, especially after ALL chemotherapy, hypothalamic dysfunction or hypothalamic surgery. Even NAFLD progressing to cirrhosis is defined in these children. NAFLD is also seen in Prader-Willi syndrome. Besides these, fatty liver may be seen concurrently with some inborn errors of metabolism and genetic diseases. Insulin resistance, obesity, type 2 DM and NAFLD progressing to cirrhosis may be seen in Alström syndrome. Liver fibrosis is reported in Turner's syndrome. Also, in lipodystrophy, cases are present with cirrhosis with liver transplantation[35,52].

LABORATORY FINDINGS

In NAFLD, serum aminotransferases are moderately high with ALT being higher than AST. Increase in AST and reversing of the AST/ALT ratio in NAFLD predicts a bad prognosis. Raised ALT and GGT levels, especially if they are within normal ranges, are found to be related to hepatic steatosis evaluated by USG or magnetic resonance imaging. Therefore, changing the normal ranges is being discussed. Serum GGT > 96.5 U/L is a marker of advanced fibrosis. Serum bilirubin levels are normal or near normal. Biochemical findings of cholestasis are

not present[32-57]. Serum IgG and nonspecific tissue auto-antibodies imply autoimmunity. Mostly, the anti-smooth muscle antibody is positive at low titer[52-54].

The other markers synthesized in liver, like sex hormone binding globulin, ferritin and plasminogen activating inhibitor-1, may be used in the diagnosis of NAFLD[54]. Homocysteine levels may increase in steatohepatitis. High hyaluronic acid levels are the most powerful independent marker of severe fibrosis and distinguishes steatosis and NASH[15,55-57]. Laminin and ELF (enhanced liver fibrosis) scores may also be used. Low adiponectin with low adipokines are important in NASH diagnosis. A combination of serum adiponectin, homoeostasis model assessment of insulin resistance (HOMA-IR) and type IV collagen 7S, at cut-off limits of $\leq$ 4.0 μg/mL, $\geq$ 3.0 and $\geq$ 5.0 ng/mL respectively, was shown to have a sensitivity of 94% and specificity of 74% for identifying early NASH[58]. Cut-off values of HOMA-IR for insulin resistance are higher than in adults. When an obese patient loses weight, normal ALT decreases more and a decrease in HOMA-IR also occurs with insulin resistance.

Hypoadiponectinemia and high tumor necrosis factor-alpha levels were found to be related to NAFLD[59-71]. However, adiponectin and tumor necrosis factor-alpha gene polymorphism were not shown to be associated with NAFLD or significant fibrosis in Chinese people[72].

Urea, electrolytes, thyroid function tests, glucose, HbA1c and serum lipids should be controlled. The most common lipid disorder is hypertriglyceridemia. Autoantibodies, immunoglobulins, viral markers for hepatitis B, hepatitis C, cytomegalovirus and Ebstein-Barr virus are important in excluding chronic liver diseases. Chronic hepatitis C, Wilson's disease, cystic fibrosis and drug intoxication (*e.g.*, methotrexate) should especially be excluded[72].

Steatosis may be diagnosed by ultrasound, computed tomography or MRI scanning. Ultrasound, the cheapest option, has been reported to have a sensitivity of 89% and specificity of 93% for the identification of fatty liver[73]. Abdominal USG does not reflect changes in liver histology and it is not useful in distinguishing steatosis and NASH. Microvesicular steatosis is due to hereditary inborn errors of metabolism, urea cycle disorders and valproic acid toxicity, and it is more severe. USG with a good history taking and metabolic tests may be sufficient in diagnosis of microvesicular steatosis[15]. ALT and AST levels are not always in parallel with the histological state and therefore, in children with risk factors, USG should be performed even if ALT and AST are normal[74,75].

New non-invasive tests such as proton-magnetic resonance spectroscopy and transient elastography allow relatively accurate estimation of hepatic steatosis and fibrosis in the community[74-81].

Liver biopsy may be essential in the diagnosis of NAFLD and distinguishing NASH from other disorders. In obese patients, biopsy may be needed to differentiate NAFLD from hepatitis. Optimal timing for this is not certain. Some physicians delay biopsy for 3-6 mo, make the patient lose weight and perform biopsy if ALT is still high. In younger children and cases with acanthosis nigricans, biopsy may be performed but there is insufficient data for this[30,82].

TREATMENT

In childhood, fatty liver does not have a proven treatment[83]. In a meta-analysis evaluating studies on adults, losing weight is reported to improve histological activity in NASH but > 50% of the patients could not reach the estimated weight[84,85]. In the literature, results of the studies about antioxidants in NASH therapy are conflicting and heterogeneous. In studies with pentoxifylline, telmisartan, L-carnitine and polyunsaturated fatty acids, it is stated that these agents may improve different parameters (radiology, biochemistry, histology) of NASH[86-88]. Vitamin E or metformin is not efficient in fatty liver in children[89].

As apoptosis is the key pathogenic mechanism in NAFLD, antiapoptotic agents are considered to be efficient in treatment. Studies are proceeding on chemical chaperones (glycerol, 4-phenyl butyric acid, TUDCA), PUFA (decreases ER stress and cell death in liver caused by saturated FFA), protease inhibitors (pan-caspase inhibitor Z-VAD-fmk, VX-166) and kinase inhibitors[11,90-94].

Drugs increasing insulin sensitivity are also studied in NASH. Indeed, the best management of insulin resistance is losing weight but drugs are also used. In pediatric NASH, 1000 mg/d metformin decreased ALT, in 40% ALT became normal, and in 90% steatosis in liver detected by MR spectroscopy decreased 23%. Metformin is effective on SREBP-1c and it is used in adulthood NASH. If evidence of childhood obesity and insulin resistance is present, it is useful and advised to be used. It is used in childhood type 2 diabetes, PCOS and Prader-Willi syndrome[92]. Thiazolidinedione is reported to improve steatosis and inflammation but causes severe weight gain[85].

Exercise, diet and bariatric surgery improve liver histology. Standard obesity surgery is not studied in children and the effect on NAFLD is not known. None of the drug therapies in children is efficient in NAFLD[9,66,71,95,96].

Multi-disciplinary management is needed in obesity treatment. Decrease in weight normalizes transaminases and liver histology. The most acceptable strategy is lowering weight gain and regular medium level exercise. For losing weight, diets with a low glycemic index and realistic portions are helpful. Special diets bringing hyperinsulinism to a minimal level instead of standard low calorie diets are more effective in childhood obesity. Diets with low postglycemic index may be carried out longer than calorie restriction[96-98]. In the management of obesity, family based behavior therapies increase success. The other valuable factor is exercise because it decreases hyperinsulinemia[99-101].

CONCLUSION

Fatty liver is a growing health problem worldwide. It might evolve to nonalcoholic steatohepatitis, cirrhosis and cause hepatocellular carcinoma. There are two distinct subtypes of pediatric NAFLD associated with different clinical, demographic and possible pathophysiological features. In children with NAFLD aged 2-18 years, 51% is type 2 and 17% is type 1. The most important risk factors for NAFLD are obesity and insulin resistance. In general, NAFLD has no specific symptoms or signs but should be considered in obese children. The most common admission reason is slightly elevated transaminases or coincidentally noticed hepatomegaly. In NAFLD, serum aminotransferases are moderately high, ALT being higher than AST. Increase in AST and reversing of the AST/ALT ratio in NAFLD predicts a bad prognosis. Progression to cirrhosis in children is rare but possible. The treatment of this disease is not certain. It is demonstrated that decrease in weight normalizes transaminases and liver histology. Therefore, weight loss with regular medium level exercise and an applicable diet with low glycemic index and appropriate calorie intake are preferred. Drugs are promising but not sufficient in children for today.

In conclusion, since childhood obesity became epidemic in developed countries, NAFLD has become the most common cause of chronic liver disease in pediatrics. Therefore, it should be taken into consideration in obese children. After excluding other diseases, multidisciplinary management should be started for weight loss.

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Hepato-cardiac disorders

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Abstract

Understanding the mutual relationship between the liver and the heart is important for both hepatologists and cardiologists. Hepato-cardiac diseases can be classified into heart diseases affecting the liver, liver diseases affecting the heart, and conditions affecting the heart and the liver at the same time. Differential diagnoses of liver injury are extremely important in a cardiologist's clinical practice calling for collaboration between cardiologists and hepatologists due to the many other diseases that can affect the liver and mimic haemodynamic injury. Acute and chronic heart failure may lead to acute ischemic hepatitis or chronic congestive hepatopathy. Treatment in these cases should be directed to the primary heart disease. In patients with advanced liver disease, cirrhotic cardiomyopathy may develop including hemodynamic changes, diastolic and systolic dysfunctions, reduced cardiac performance and electrophysiological abnormalities. Cardiac evaluation is important for patients with liver diseases especially before and after liver transplantation. Liver transplantation may lead to the improvement of all cardiac changes and the reversal of cirrhotic cardiomyopathy. There are systemic diseases that may affect both the liver and the heart concomitantly including congenital, metabolic and inflammatory diseases as well as alcoholism. This

review highlights these hepatocardiac diseases

Core tip: Acute and chronic heart failure may lead to acute ischemic hepatitis or chronic congestive hepatopathy. Treatment in these cases should be directed to the primary heart disease. In patients with advanced liver disease, cirrhotic cardiomyopathy may develop including hemodynamic changes, diastolic and systolic dysfunctions, reduced cardiac performance and electrophysiological abnormalities. Cardiac evaluation is important for patients with liver diseases especially before and after liver transplantation. Liver transplantation may lead to improvement of all cardiac changes and reversal of cirrhotic cardiomyopathy. There are systemic diseases that may affect both liver and heart concomitantly including congenital, metabolic, inflammatory diseases and alcoholism.

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INTRODUCTION

The heart and liver are organs that are closely related both in health and disease. According to traditional medicine, each body has its organ own specific temperament composed of four qualities (elements): "warmth", "coldness", "wetness" and "dryness". "Wetness" and "dryness" are considered on a spectrum of "tissue moistures" and "warmth" and "coldness" may be regarded as the basic metabolism of the organ. In his famous book ,"Canon" (The Law), Avicenna pointed to some of the interactive

effects occurring in the heart and the liver. Some of the most important include: (1) dominance of the "heart warmth" over "liver coldness" and (2) the dominance of "liver dryness" over "heart wetness". The impact and position of "heart temperament" as well as its effect on "liver intemperaments" may be definitive in diagnosis and assessment of the general prognosis of liver disease and in the treatment process[1,2].

Chronic liver diseases may affect cardiac functions in the absence of other heart disease. These effects are called cirrhotic cardiomyopathy and may aggravate the course during orthotropic liver transplantation (OLT). Most of these effects are reversed after OLT[3,4]. In case of ischemic hepatitis, patients with severe heart failure usually remain asymptomatic, while for patients with congestive hepatopathy, signs of right-sided heart failure could mask hepatic injury. However, changes in hepatic function, that are proven by laboratory tests are significant in predicting the survival of patients with severe heart failure. Therefore, the evaluation of cardiac and hepatic function is very important in patients with severe heart failure and hepatic injury. Their treatment options should be revised in order to ensure stable hemodynamics, as well as optimal liver function, and so in this way their survival and prognosis could be improved[5]. This review highlight the liver diseases affecting the heart, heart diseases affecting the liver and some systemic diseases affecting both heart and liver.

LIVER DISEASES AFFECTING THE HEART

Chronic hepatitis C virus

In hepatitis C virus (HCV) heart disease, most patients develop chronic inflammation of the myocardium and, later, dilated cardiomyopathy attributable to necrosis and loss of myocytes. However, because myocytes do not replicate, the proliferative stimuli induced by HCV infection may promote myocyte hypertrophy and hypertrophic cardiomyopathy[6]. A role of direct effect of HCV core proteins was suggested in the pathogenesis of cardiomyopathy[7]. Cardiac damage is a rare manifestation of HCV-related mixed cryoglobulinemia vasculitis. Despite favourable early outcomes, patients with cardiac damage had poorer survival than those without[8]. Chronic hepatitis C viral infection is independently associated with presence of metabolic conditions (insulin resistance, type 2 diabetes mellitus and hypertension) and congestive heart failure[9].

The connection between hyperlipidemia and atherosclerosis is not linear in people with hepatitis C. In a population-based study, although chronic HCV infection was associated with severe insulin resistance, the patients only had mild atherosclerosis, suggesting a unique characteristic of HCV-related metabolic abnormality. Chronic HCV-associated steatosis was suggested as a leading cause of coronary artery diseases through the modulation of atherogenic factors, such as inflammation and dysmetabolic milieu. Interestingly, interferon-based therapies

in patients with chronic HCV were found to reduce the long-term risk of stroke. Thus, atherosclerosis in patients with hepatitis C is likely due to an inflammatory process rather than to a lipid related source[10-12]. Thus, even patients having healthy cholesterol and triglyceride levels in the presence of chronic hepatitis C infections should not engage in activities that could further increase the disease risk of their cardiovascular vessels.

Liver cirrhosis

Patients with liver cirrhosis (LC) frequently experience autonomic cardiovascular dysfunction, such as increased activity of the sympathetic nervous system and reduced vagal cardiac function, which has important implications for liver dysfunction and poor survival[13-15]. Baroreflex has been shown to be an important determinant of electrical stability in the heart and can predict increased mortality and end-organ damage[16-19]. Patients with liver cirrhosis have an enhanced activity of the sympathetic nervous system and hyperdynamic circulation showing increased cardiac output and reduced systemic vascular resistance. These changes may induce myocardial remodelling and LV hypertrophy (LVH), resulting in systolic and diastolic functional abnormalities and cardiomyopathy[20-22]. Cirrhotic cardiomyopathy was defined by a working group as a cardiac dysfunction in patients with cirrhosis characterized by impaired contractile responsiveness to stress and or altered diastolic relaxation with electrophysiological abnormalities in the absence of known cardiac disease[23]. The criteria for the diagnosis of cirrhotic cardiomyopathy are shown in Table 1[24].

Systolic dysfunction is related to the inability of the heart to meet its demands with respect to the generation of an adequate arterial blood pressure and cardiac output. This dysfunction can be unveiled by physical exercise that increases left ventricular pressure, volume, and left ventricular ejection fraction and heart rate in some cirrhotic patients. Similarly, the administration of vasoconstrictors, such as angiotensin II and terlipressin, increases the SVR and thereby the left ventricular afterload unmasking a latent left ventricular dysfunction in cirrhosis. In contrast, vasodilators, such as angiotensin-converting enzyme inhibitors and other afterload-reducing agents, should be used with caution due to the risk of further aggravation of the vasodilatory state[24]. Systolic dysfunction may have an impact on the development of complications, such as sodium and water-retention and ascites formation, as well as development and prognosis of renal dysfunction[25,26].

Diastolic dysfunction in cirrhosis is due to an increased stiffness of the myocardial wall owing to myocardial hypertrophy, fibrosis, and subendothelial edema. The prevalence of diastolic dysfunction has been reported to range from 45% to 56%. Diastolic dysfunction is most prominent in patients with severe decompensation, in whom, the combination of myocardial hypertrophy, contractile dysfunction, changes in heart volumes, and diastolic dysfunction may represent an essential element

Table 1 Proposal for diagnostic and supportive criteria for cirrhotic cardiomyopathy agreed upon at a working party held at the 2005 World Congress of Gastroenterology

A working definition of cirrhotic cardiomyopathy
A cardiac dysfunction in patients with cirrhosis characterised by impaired contractile responsiveness to stress and/or altered diastolic relaxation with electrophysiological abnormalities in the absence of other known cardiac disease
Diagnostic criteria
Systolic dysfunction
Blunted increase in cardiac output with exercise, volume challenge or pharmacological stimuli
Resting EF < 55%
Diastolic dysfunction
E/A ratio < 1.0 (age-corrected)
Prolonged deceleration time (> 200 ms)
Prolonged isovolumetric relaxation time (> 80 ms)
Supportive criteria
Electrophysiological abnormalities
Abnormal chronotropic response
Electromechanical uncoupling/dyssynchrony
Prolonged QTc interval
Enlarged left atrium
Increased myocardial mass
Increased BNP and pro-BNP
Increased troponin I

BNP: Brain natriuretic peptide; E/A: Early diastolic/atrial filling ratio; EF: Left-ventricular ejection fraction.

in cirrhotic cardiomyopathy[26-28]. The diastolic dysfunction may adversely affect the prognosis of patients with cirrhosis, by favouring the occurrence of complications and impairing the outcomes of manoeuvres that lead to rapid increases in preload, such as transjugular intrahepatic porto-systemic shunt (TIPS) insertion[24].

Patients with advanced cirrhosis usually exhibit tachycardia. The inability to increase the heart rate further contributes to an impaired ability to keep the cardiac output at a level adequate to meeting the needs of systemic circulation. At this point, the effective volemia suddenly worsens, similar to the events of post-paracentesis circulatory dysfunction and hepatorenal syndrome[29-31]. The prolongation of the electrocardiographic QT interval is common in cirrhosis, with a prevalence that exceeds 60% in patients with an advanced disease. In this case, drugs affecting QT should be avoided or used with caution and under close ECG monitoring[32]. Systemic and cardiac changes in patients with liver cirrhosis are shown in Figure 1.

Almost all cardiovascular abnormalities reverse a few months after liver transplantation[4,24,33].

Nonalcoholic fatty liver disease

It has been shown that the leading cause of death in patients with nonalcoholic fatty liver disease (NAFLD) is coronary events. In patients with diabetes mellitus, NAFLD is associated with cardiovascular disease (CVD) independent of the classical risk factors, glycaemic control, medications, and metabolic syndrome features. When diabetic patients with and without NAFLD were compared, those with NAFLD had a higher prevalence of coronary vascular disease, hypertension, central obesity, poor glycaemic control, and dyslipidaemia and greater carotid intimal thickness. Furthermore, with the development of steatohepatitis, the degree and severity of CVD became directly proportional to the severity of inflam-

mation on liver biopsy. Cardiovascular mortality is also increased at least two-fold in non-alcoholic steatohepatitis (NASH). The presence of liver fat is associated with lower adiponectin levels and increased levels of fibrinogen, C-reactive protein (CRP), and plasminogen activator inhibitor 1 (PAI-1), which are markers of inflammation and risk factors of coronary vascular disease independent of BMI and intra-abdominal obesity. Patients with NAFLD also have significantly higher mean values of intima-media thickness and prevalence of plaques resulting in an increased risk of atherosclerosis in subjects with metabolic syndrome. It has also been shown that NASH predicts plasma inflammatory biomarkers independent of visceral adiposity and other potential confounders. These findings suggest that NASH is not simply a marker of CVD but may also be involved in its pathogenesis. Steatosis has been found to be the strongest independent risk predictor of vascular damage, followed by age and blood pressure. Patients with NAFLD and systolic BP ≥ 130 mmHg are 4.7 times more likely to have a positive treadmill test[34-36].

In a recent study, asymptomatic obese children with NAFLD exhibited features of early LV diastolic and systolic dysfunction. These abnormalities were more severe in those with NASH[37].

Primary biliary cirrhosis

Circulating cholesterol levels are elevated in most with primary biliary cirrhosis. Hypercholesterolemia in patients with primary biliary cirrhosis should be considered a cardiovascular risk factor only when other factors are present. Ursodeoxycholic acid, the standard treatment for primary biliary cirrhosis, improves cholestasis, thereby lowering the circulating levels of cholesterol. Thus, hypercholesterolemia in the absence of other cardiovascular risk factors does not require specific therapeutic interventions in patients with primary biliary cirrhosis.

Figure 1 **Proposal of changes in cardiac output during the course of the liver disease.** DT: Deceleration time; LAV: Left atrial volume; LVEDV: Left end-diastolic volume; LVEF: Left ventricular ejection time.

Epidemiological studies have shown significantly increased all-cause mortality rates in comprehensive PBC patient groups, with a significant component of this increased mortality coming from non-liver-related causes[38,39]. These studies were not designed to address the cause of this increase in non-liver-related mortality. There is, however, convincing evidence from the same populations to suggest that malignant disease makes little or no contribution to this excess non liver mortality[38,40]. Given the importance of cardiovascular mortality in the general population, the possibility must be considered that cardiac mechanisms contribute to the excess non liver mortality rates seen in these populations. Autonomic dysfunction has been seen in PBC and was associated with an increased cardiac mortality risk in non-liver chronic disease states[41,42]. Furthermore, a significant peripheral muscle bioenergetics abnormality has also been reported in PBC[43]. Raising the possibility that similar bioenergetic abnormalities may also be present in the cardiac muscle. The effects of autonomic dysfunction may alter the perfusion patterns in tissues, potentially reducing muscle perfusion and contributing to peripheral mechanisms of fatigue. A generic tendency towards altered myocardial function was shown in PBC and did not typically appear to be symptomatic in terms of "classical" myocardial dysfunction symptoms[44].

Primary sclerosing cholangitis

Primary sclerosing cholangitis (PSC) is a chronic inflammatory disease of affecting the large bile ducts and is characterized by periductal fibrosis and stricture formation. Arteriosclerosis involves the accumulation of altered lipids and lipoproteins in large arteries; this drives inflammation and fibrosis and ultimately leads to the narrowing of the arteries and hypoperfusion of dependent organs and tissues. Knowledge of the causative factors is crucial to the understanding of disease mechanisms and the development of specific treatment. Based on the pathogenic similarities and common molecular, cellular, and morphological features that provide the conceptual framework for a deeper understanding of their pathogenesis between PSC and arteriosclerosis, it has been hypothesized that PSC represents "arteriosclerosis of the bile duct" initiated by toxic biliary lipids[45]. This hypothesis should stimulate translational research to facilitate the search for novel treatment strategies for both diseases.

Hepatocellular carcinoma

Cardiac complications of hepatocellular carcinoma hepatocellular carcinoma are rare. Cases of right atrial invasion of HCC had been reported[46], which led in some

cases to right ventricular outflow obstruction and Budd Chiari syndrome[47,48]. Hepatocellular carcinoma patients with cardiac metastases are usually found in advanced stages. These patients have limited survival from the diagnosis of cardiac metastases. The most common causes of death are related to HCC itself or to the underlying liver disease. Only a few patients will die due to cardiac metastases[49]. The palliative treatments for tumor thrombi may include transcatheter chemotherapy, transarterial chemoembolization and radiation therapy with a partial improvement of patient symptoms[50].

Budd-chiari syndrome

Primary Budd-chiari syndrome (BCS) is a rare clinical entity characterized by hepatic venous outflow obstruction at various levels from the small hepatic veins to the inferior vena cava. There are three main types of BCS: Type Ⅰ, occlusion of the IVC; type Ⅱ, occlusion of the hepatic veins; and type Ⅲ, occlusion of the IVC and the hepatic veins. The incidence of HCC combined with BCS varies among the types of BCS[50,51]. Type Ⅰ BCS is more prone to inducing HCC and the incidence ranges between 10.7% and 43.5%. The mechanisms of HCC induction unknown. The therapeutic treatments of BCS combined with HCC includes TACE, surgery and more recently angioplasty followed by percutaneous microwave ablation[52-55].

Portal hypertension

Three important complications are associated with portal hypertension hepatopulmonary syndrome, portopulmonary hypertension, and hepatic hydrothorax.

The hepatopulmonary syndrome: This entity is defined by an oxygenation defect caused by the development of intrapulmonary vascular dilation in patients with either advanced liver disease and/or portal hypertension[56]. Angiogenesis was shown to be induced by an increased level of nitric oxide and vascular endothelial growth factor in patients with advanced liver disease or portal hypertension[57,58]. Patients with the hepatopulmonary syndrome (HPS) may present with the insidious onset of dyspnea or remain completely asymptomatic during the early stages. Dyspnea upon standing (platypnea) and hypoxemia exacerbated in the upright position (orthodeoxia) are present in almost 25% of HPS patients[59]. Patients with severe HPS may display digital clubbing and cyanosis.

Chest radiographs may be normal or show bibasilar nodular or reticulonodular opacities, reflecting diffuse vascular pulmonary dilation[60,61]. Pulmonary function tests typically demonstrate a reduced diffusion capacity for carbon monoxide[61]. There is no established medical therapy currently available for HPS. In patients with $PaO_2 < 60$ mmHg at rest or with exertion, the administration of supplemental oxygen is appropriate, because chronic hypoxemia itself may contribute to the mortality in HPS[62,63]. The administration of garlic resulted in im-

provements in the PaO2, in two uncontrolled trials and a small randomized study[64].

Portopulmonary hypertension: Portopulmonary hypertension (POPH) is characterized by pulmonary arterial hypertension (PAH) that occurs in the setting of portal hypertension, with or without advanced liver disease[65]. The severity of POPH does not correlate with the degree of liver dysfunction or the severity of portal hypertension[66,67]. The pathophysiology of POPH is not fully understood. The histopathology of POPH is similar to that of idiopathic PAH, and is triggered by vascular injury as reflected by the development of plexiform arteriopathy, concentric intimal fibrosis, and proliferation and muscularization of the pulmonary arterioles[68]. Dyspnea on exertion is the most common initial symptom of POPH and fatigue, orthopnea, chest pain, peripheral edema, syncope, and dyspnea at rest may develop as the disease progresses[69,70]. Medical treatment includes the following: prostacyclin analogs (prostanoids), Endothelin receptor antagonist and Phosphodiesterase-5 inhibitors[71-73]. A single short-term study in patients with moderate to severe POPH, found that the use of β-blockers was associated with worsening exercise capacity[72].

Hepatic hydrothorax: This entity is characterized by a transudative pleural effusion in the absence of underlying cardiac or pulmonary disease. Its prevalence has been estimated to be 5%-10% in cirrhotics, based on retrospective observational data[73]. The most important mechanism leading to the passage of ascitic fluid from the peritoneal into the pleural cavity is the presence of diaphragmatic defects. These defects were corroborated by showing passage of 99mTc-human albumin from the abdominal into the pleural cavity, even in the absence of underlying ascites[74]. Symptoms include cough, dyspnea, chest discomfort, hypoxia, and in the most severe cases respiratory failure with or without ascites[74,75]. Spontaneous bacterial pleuritis (SBPL) results when hepatic hydrothorax (HH) becomes infected in the absence of pneumonia. Symptoms in SBPL vary from fever and pleuritic chest pain to subtle worsening of encephalopathy or renal function, necessitating a high index of suspicion. A PMN > 500 cells/mm^3 is diagnostic for SBPL in a pleural effusion, although SBPL with PMN between 250-500 cells/mm^3 is documented by positive pleural fluid culture[76]. Chest tube placement is contraindicated in SBPL, in the absence of empyema, due to the risk of protein loss, prolonged drainage, secondary infection and hepatorenal syndrome[77]. Treatment of HH includes the restriction of sodium intake with the administration of diuretics. This approach is effective in controlling HH, although fluid mobilization from the pleural cavity may be slower than from the peritoneal cavity and approximately 20% of patients develop refractory HH[77]. Percutaneous drainage, and chest tube placement can be used in some cases[77,78]. The standard of care treatment for refractory HH is TIPS placement with response rates

of 70% to 80%[79,80]. Video assisted thoracoscopy (VATS) with pleurodesis is a potential treatment alternative for patients with refractory HH, who are not eligible for or who have failed TIPS[81-83].

Liver transplantation

Patients with cirrhosis requiring liver transplantation (LT) usually demonstrate increased cardiac output. Low systemic vascular resistance and bradycardia are also commonly seen in cirrhosis and can be aggravated by beta-blocker use. These physiologic changes increase the risk of cardiovascularn complications, in addition to altered hemodynamic stresses that LT patients face in the immediate post-operative period. Post-transplant reperfusion may result in cardiac death due to a multitude of causes, including arrhythmia, acute heart failure (HF), and myocardial infarction[84].

The unusually high perioperative mortality in transplant patients with CAD warrants a systematic evaluation in every patient that thought to have a greater risk of atherosclerotic coronary disease. No single test has a predictive value of 100%. Therefore, diagnostic protocols must account for the variation in prevalence that occurs in subsets of transplant candidates and the limitation of each type of test[85]. In contrast to ischemic heart disease, most patients with advanced liver disease have myocardial defects that cause systolic and diastolic impairments not always evident at rest. There are also underlying electrophysiological defects that cause an uncoupling of the mechanical and electrical activity. Diagnosis of "cirrhotic cardiomyopathy" is difficult because the findings can be subtle as some patients develop frank heart failure when exposed to pharmacological or physiological stressors such as during liver transplantation[85].

Almost all cardiovascular abnormalities can be reversed 6 to 12 mo after liver transplantation. Namely, indices of both systolic and diastolic function, cardiac workload, and exercise capacity can be substantially improved or normalized. QT interval prolongation can also revert after OLT, even though this occurs in about half of cases suggesting that liver disease may not be the only pathogenic factor[4,24,33].

CARDIAC CAUSES OF HEPATIC DISORDERS

Heart failure

The cardiac causes of hepatic dysfunction include constrictive pericarditis, severe pulmonary arterial hypertension (PAH), mitral stenosis, tricuspid regurgitation (TR), corpulmonale, ischemic cardiomyopathy, and postoperative consequences of the Fontan procedure for pulmonary atresia and hypoplastic left heart syndrome. All of these causes can lead to passive congestion due to the elevated right ventricular (RV) pressure and right sided heart failure. The outcomes of heart failure have dramatically improved, due to the increased efficiency of medical treatment, as a result, cardiac cirrhosis prevalence is declining[24,86].

Pathophysiology: In chronic heart failure (backward failure), the increase in venous pressure caused by RV dysfunction leads to the atrophy of hepatocytes and causes perisinusoidal edema which can impair the diffusion of oxygen and nutrients to the hepatocytes[87,88]. This backward failure is also responsible for the enhanced hepatic lymph formation, leading to ascites when its production rate exceeds the draining capacity of the lymphatic system. Moreover, increased pressure within the hepatic sinusoid favours bile duct damage by disrupting endothelial cells and the interhepatocyctic tight junctions that separate the extravascular space from the bile canaliculus. Finally, stagnant flow favors thrombosis within sinusoids, hepatic venules, and portal tracts; thereby contributing to liver fibrosis[24,89,90].

On gross examination, the congestive liver is enlarged, with a purple or reddish hue and prominent hepatic veins. The cut surface shows a classic nutmeg appearance, reflecting an alternating pattern of haemorrhage and necrosis of zone 3 with normal or slightly steatotic areas in zones 1 and 2. Microscopically, the hallmark features of hepatic venous hypertension are the prominence of the central veins, central vein haemorrhage, and sinusoidal engorgement[87,91,92]. Untreated, longstanding congestion can lead to cardiac fibrosis and,ultimately cardiac cirrhosis[93]. Acute HF (forward failure): most commonly arises in the context of profound systemic hypotension from acute cardiopulmonary collapse after myocardial infarction, exacerbation of HF, or pulmonary embolism. In the absence of established hypotension, ischemic hepatitis has been shown in instances of severe hypoxemia, such as obstructive sleep apnea, respiratory failure, and in conditions of increased metabolic demand, such as those seen in toxic/septic shock[94-96]. Ischemic liver injury is characterized by centrolobular necrosis of zone 3 hepatocytes in the absence of histological evidence of inflammation characteristic of viral hepatitis[97-101].

Oxygen consumption can be easily increased when the hepatic blood flow is decreased. The mechanism by which the liver protects itself from damage in hypoxia is increasing oxygen extraction by the hepatocytes up to 95% as the blood passes through the liver. When inadequate end-organ perfusion and tissue hypoxia is persistent or when acute shock develops this protecting mechanism against hypoxic liver damage is overwhelmed. Hepatocellular injury ensues, accompanied by a sharp elevation of the serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactic dehydrogenase (LDH), prolongation of the prothrombin time, and occasionally functional renal impairment. These abnormalities reach their peak 1 to 3 d after the onset of cardiogenic ischemic hepatitis and return to normal within 5 to 10 d from onset of the disorder[102].

These forward and backward factors often coexist and potentiate each other. Additionally, the presence of hepatic steatosis due to diabetes, obesity, or other causes may increase liver susceptibility to the ischaemic reperfu-

sion injury[24,103].

Clinical presentations: As for the acute ischemic hepatitis, no specific symptoms but patients may present with symptoms of nausea, vomiting, anorexia, malaise, right-upper quadrant pain, jaundice, oliguria, and flapping tremors representing cerebral hypoperfusion rather than hepatic encephalopathy. Ischemic hepatitis is usually benign and self-limited. The clinical diagnosis of liver injury is almost always incidental when liver enzymes are found to be massively elevated 1 to 3 d after an episode of systemic hypotension. This condition may be associated with increased serum creatinine level from acute tubular necrosis[102].

Congestive hepatopathy: The term congestive hepatopathy replaced cardiac cirrhosis. Patients experience mild, dull right upper quadrant pain caused by the stretching of the liver capsule. Hepatomegaly with a firm, tender liver edge and peripheral edema are typically the most prominent findings in patients with chronic right-sided HF, but these may also occur rapidly in acute HF. Ascites may be present in up to 25% of these patients and splenomegaly is characteristically absent[86]. Jaundice is not commonly reported. In patients with considerable TR, a prominent systolic pulsation of the liver, attributable to an enlarged right atrial V wave, is often noted. A presystolic pulsation of the liver, attributable to an enlarged right atrial A wave, can occur in tricuspid stenosis, constrictive pericarditis, restrictive cardiomyopathy involving the RV, and pulmonary hypertension[5].

Laboratory data: As for the acute ischemic hepatitis, severe jaundice is common, with a bilirubin level as high as 15 to 20 mg/dL, elevation of AST to more than 10 times the upper reference range limit, a marked increase in serum LDH, an elevated ALP level, and prolongation of the prothrombin time. Increases in LDH tend to be massive and an ALT/LDH ratio of less than 1.5 helps distinguishing ischemic injury from other forms of acute hepatitis[104,105].

As for the congestive hepatopathy, the usual findings are moderate elevations of the biochemical parameters of liver function 2 to 3 times the upper normal reference level. These parameters include AST, ALT, LDH, gamma-glutamyl transpeptidase (GGT), and alkaline phosphatase (ALP). Hyperbilirubinemia, secondary to an increase in both the direct and indirect bilirubin, is also common. The total bilirubin level is rarely greater than 3 mg/dL. In patients with long-standing HR, albumin synthesis may be impaired, leading to hypoalbuminemia and intensifying the accumulation of fluid[102].

Treatment: Treatment of the cardiac problem is the key to improvement in hepatic dysfunction.

As for the AHF and ischemic hepatitis, correcting underlying circulatory or respiratory disturbances is the main treatment. It is recommended that doctors identify and remove any precipitating cause, such as medications with negative inotropic or hypotensive effects (certain antiarrhythmic drugs, calcium-channel blockers, and vasodilators), medications likely to cause impairment of renal function (high doses of angiotensin-converting enzyme (ACE) inhibitors, angiotensin Ⅱ receptor blockers), or medications likely to accumulate with evolving renal failure (like digoxin)[106]. Oxygen should be administered as early as possible in hypoxemic patients to achieve an arterial oxygen saturation > 95%. Administration of intravenous diuretics is recommended with caution in acute HF patients in the presence of symptoms secondary to congestion and volume overload. Inotropic agents should be considered in patients with low output states and low systolic blood pressure. When needed, inotropic agents should be administered as early as possible and withdrawn as soon as adequate organ perfusion is restored and/or the congestion is reduced. Vasopressors are only indicated in cases of cardiogenic shock when the combination of an inotropic agent and fluid challenge fails to restore systolic blood pressure > 90 mmHg, with inadequate organ perfusion, despite an improvement in cardiac output[5,106].

As for the chronic HF and congestive hepatopathy, the main lines of treatment are angiotensin-converting enzyme (ACE) inhibitors and beta blockers. The addition of a low-dose aldosterone antagonist should be considered in all patients with an LV systolic dysfunction. ACE Inhibitors increase cardiac output and decrease LV filling pressure due to their vasodilatory effect. Some ACE inhibitors are prodrugs, which require transformation by the liver into active metabolites. These drugs include enalapril, ramipril, fosinopril, trandolapril, quinapril, benazepril, and moexipril. With liver dysfunction, decreases in the prodrug transformation and inactivation of the active drug may occur[107]. For those patients who cannot tolerate ACE inhibitors due to cough, angiotensin receptor blockers (ARBs) are recommended instead. ARBs reduce morbidity and mortality in patients with systolic HF. Losartan is metabolized to the active metabolite *via* hepatic carboxylation. In patients with hepatic impairment, the bioavailability is doubled and the total plasma clearance is halved. Therefore, lower initial doses are recommended. Valsartan undergoes little metabolic conversion. Caution is recommended in patients with mild to moderate liver dysfunction but dosage adjustments are generally not needed. Similar to valsartan, irbesartan does not require biotransformation, thus dosage modification is not necessary[108-111].

The use of *b*-blockers is associated with a 30% reduction in total mortality in HF. Propranolol should be administered cautiously in patients with hepatic impairment. No dose adjustments are necessary for atenolol, nadolol, esmolol, sotalol, or acebutolol[112-116].

Diuretics: Loop diuretics, such as furosemide, bumetanide, and torsemide, are used for volume management in HF because of their superior natriuretic effects compared with other classes of diuretics. For unknown

Table 2 Comparison between acute and chronic hepatic complications of cardiac failure

	Chronic congestive hepatopathy	Acute ischemic hepatitis
Aetiology	Chronic heart failure	Acute heart failure
Pathophysiology	Perisinusoidal edema	Tissue hypoxia
	Increased lymph flow	Zone 3 necrosis
	Zone 3: alternating necrosis and hemorrhage	
	Sinusoidal thrombosis	
Manifestations	Right hypochondrial pain	Asymptomatic or nonspecific
	Edema, ascites, jaundice	(nausea, vomiting, jaundice, right hypochondrial pain)
Laboratory data		
Bilirubin	Mild increase	Marked elevation
ALT and AST	Normal mild elevation	Marked elevation
LDH	Normal or mild elevation	Marked elevation
Prothrombin time	Prolonged	Normal or prolonged
ALP	Normal or mild elevation	Increased
Albumin	Hypoalbuminemia	Normal
Traetment	ACE inhibitors	Oxygen therapy
	b-blockers	Avoid precipitating factors
	Diuretic	Inotropic agents with caution
	Amiodarone	Vasopressor with caution
	Statins with caution	Diuretics in hypervolemia
Prognosis	Slowly progressive course	Benign and usually self limited

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; LDH: Lactic dehydrogenase; ALP: Alkaline phosphatase; ACE: Angiotensin-converting enzyme.

reasons, the pharmacologic response in patients with liver dysfunction and HF is diminished, and there is a net decreased in sodium excretion when compared with healthy individuals taking the same dose. No adjustments are necessary if renal function is normal[115].

In patients with severe HF, amiodarone has proven to be effective for suppressing ventricular arrhythmias, reducing sudden death and cardiac mortality, and improving exercise tolerance and ejection fraction. This drug undergoes an extensive hepatic metabolism to active metabolite, but no dosage reduction is indicated in hepatic impairment[116].

Statins undergo extensive hepatic metabolism. In patients with active liver disease or persistent unexplained elevations in serum transaminases to above 3 times the upper limit of normal, the use of statins is contraindicated as they may worsen liver function[117].

For patients, who are refractory to medical therapy and who may be candidates for cardiac surgery, CH due to chronic HF can improve and reverse after temporary LV assistive device support or for selected patients or cardiac transplantation[118,119]. The differences between acute and chronic hepatic impairment are summarized in Table 2.

Ventricular assist devices

Ventricular assist devices (LVADs) lead to volume shifts from the intrathoracic area to the systemic circulation, thus improving liver blood flow, as assessed by indocyanine green clearance. Studies have shown improvement in the liver function in patients with mild abnormalities in pre-implant liver tests, and no deterioration in those with normal baseline values, up to 6 mo[118,120]. However, pre-existing or post-LVAD severe liver dysfunction strikingly influences patients' prognosis and endangers their sur-

vival[121]. Liver dysfunction can also occur or worsen after LVAD implantation. Pre-, peri-, and post-operative factors, such as large doses of vasopressors, prolonged cardiopulmonary bypass time, arterial hypotension, systemic inflammatory responses and, mainly, right ventricular failure predispose a patient to liver damage, often presenting with intrahepatic cholestasis[121]. The model for end-stage liver disease (MELD), a scoring system assessing the severity of chronic liver disease based on serum bilirubin, creatinine and INR for prothrombin time that is widely used to determine prognosis and prioritize the receipt of a liver transplant, is able to predict mortality and morbidity following LVAD. The severity and course of post-ventricular assist devices liver damage can be monitored by sequential assessment of MELD-XI, a modified MELD score excluding INR to overcome the problem posed by concomitant anticoagulation[24,122].

Heart transplantation

Chronic cardiac hepatopathy is common in patients evaluated for heart transplantation-x (HTx), and liver dysfunction predicts an adverse outcome following transplantation. At the same time, altered pre-HTx liver tests can significantly improve after surgery, suggesting that chronic cardiac hepatopathy is a potentially reversible disease. In a large cohort study over 10 years among patients who had received LTx, all cholestatic parameters, transaminases and LDH improved after the procedure. Interestingly, a complete reversal of cardiac cirrhosis 10 years after HTx has even been reported[123,124].

A careful assessment of liver function and detection of liver cirrhosis is required in all candidates for HTx. An ultrasound of the abdomen with an echo-Doppler study of portal and tributary veins should be performed. Endoscopy may be necessary to assess the presence of

Table 3 Diseases affecting both the liver and the heart concomitantly		
	Hepatic manifestations	**Cardiac manifestations**
Congenital		
Allagile syndrome	Cholestasis	Congenital heart defects
Situs Inversus totalis	Concerns with liver or heart transplantation	
Infections		
Sepsis	Acute liver failure	Acute heart failure
Hepatitis C	Hepatitis	Myocarditis, cardiomyopathy
Cytomegalovirus	Hepatiitis	Myopericarditis
HIV	Hepatitis, granuloma	Myocarditis , cardiomyopathy
Malaria	Hepatic necrosis	Cariac failure
Dengue fever	Hepatic necrosis	Myocarditis
Amebiasis	Hepatitis, hepatic abscess	Pericarditis, effusion
Metabolic		
Wilson disease	Cirrhosis, hepatitis	Left ventricular remodeling
Hemochromatosis	Cirrhosis, hepatitis	Cardiomyopathy
Systemic		
SLE	Steatosis, hepatomegaly	Endocarditis, pericarditis
Amyloidosis	Hepatomegaly, cholestasis	Cardiomyopathy
Sarcoidosis	Granuloma, cholestasis	Conduction defects, HF
Chronic alcoholism	Cirrhosis	Cariomyopathy
Autoimmune		
Grave's disease	Hepatitis, cholestasis	HF
Autoimmune hepatitis	Hepatitis, cirrhosis	Carditis

HF: Heart failure.

gastro-esophageal varices and congestive gastropathy in patients with portal hypertension. Liver biopsy may be needed in some cases. MELD or modified MELD scores should be calculated. Higher MELD scores predict higher postoperative complication rates, including reoperation for bleeding, bacterial infections, and in-hospital death[124].

Patients with chronic hepatitis C or chronic hepatitis B should be treated before HTx to avoid antiviral drug intake after HTx which may be associated with graft rejection[125]. Finally, patients with heart failure and irreversible cirrhosis could be offered combined heart and liver transplantation[126].

DISEASES AFFECTING BOTH THE HEART AND THE LIVER

There are many systemic diseases in addition to chronic alcoholism, that affect both the liver and the heart. This fact may have important implications, because the heart and the liver also interact particularly during surgical procedures, OLT, and TIPS insertion, thereby influencing the outcomes. The spectrum of these diseases include congenital , autoimmune, metabolic and infectious causes (shown in Table 3). We highlight some examples of these diseases.

Congenital causes
The famous example is Alagile syndrome (AS), which is a multisystemic disease that is autosomal dominant, with variable expression. The major clinical manifestations are as follows: chronic cholestasis, congenital heart disease, posterior embryotoxon in the eye, characteristic facial phenotype, and butterfly vertebrae. AS is caused by mutations in JAGGED1 (more than 90%) and in NOTCH2. Cholestasis, pruritus and xanthomas have been successfully treated with choleretic agents (ursodeoxycholic acid) and other medications (cholestyramine, rifampin, naltrexone). In certain cases, partial external biliary diversion has also proven successful. Liver transplantation is indicated in children with cirrhosis and liver failure[127].

Infections
Cytomegalovirus (CMV) infection in inmunocompetent hosts generally is asymptomatic; whoever, it rarely can lead to severe organ complications. A rare, but serious complication of cytomegalovirus infection is the presence of myopericarditis concomitant with hepatitis with a possible role of oral valganciclovir in these patients[128].

Metabolic causes
Wilson disease: Wilson disease is an inherited autosomal recessive disorder of the copper metabolism resulting in the pathological accumulation of copper in the liver, brain and other tissues. One of the reported manifestations is cardiac involvement. Cardiac involvement in Wilson disease patients is characterized by LV parietal thickening with an increased prevalence of concentric LV remodelling. Children with Wilson diseases were asymptomatic upon cardiological examination, but had significantly lower mitral E velocities, mitral E/A ratios as estimated by pulsed wave Doppler echocardiography[129,130].

Hemochromatosis: Hemochromatosis is an autosomal recessive disorder affecting the white population. In this disorder, the inappropriate absorption and deposi-

tion of dietary iron may result in the development of hepatic and non-hepatic end-organ injury, leading to liver cirrhosis, hepatocellular carcinoma, diabetes, arthritis, skin pigmentation and cardiac diseases[131]. Cardiac involvement in hemochromatosis affects mainly the myocardium: iron overload of the myocytes reduces left ventricular distensibility. Heart failure is the most frequent manifestation of cardiac involvement. Diagnosis of cardiac involvement depends essentially on Doppler echocardiography showing abnormal left ventricular filling and, later, ventricular dilatation with left ventricular systolic dysfunction. Magnetic resonance imaging can quantify intrahepatic and intramyocardial iron levels. The two principal means of treatment by iron depletion are phlebotomy in primary hemochromatosis and excretion of iron by chemical chelation in secondary hemochromatosis. Early diagnosis and iron depletion improve survival by reducing the organ iron overload, especially in the liver and myocardium. The recent guidelines issued by Anaes (national agency for health evaluation) make it possible to identify risk factors for complications early, to determine the disease stage, and to provide appropriate management as a function of disease severity. Combined liver transplantation and cardiac surgery may be needed in cases of hemochromatosis with end stage liver disease and heart failure[132].

Autoimmune diseases: The atypical clinical presentations of Graves' disease (GD) include anemia, vomiting, jaundice, and right heart failure. Hyperthyroidism may present with jaundice, and on the other hand, deep jaundice may develop with the onset of overt hyperthyroidism in previously compensated chronic liver disease patients. Jaundice may be caused by hepatitis or intrahepatic cholestasis. Pulmonary hypertension is reported to be associated with GD and to respond to its treatment. GD-related pulmonary hypertension may be so severe that it produce isolated right-sided heart failure, which is occasionally identified as the presenting manifestation of GD[133].

Chronic alcoholism: Patients with chronic alcoholism can be presented with both hepatic and cardiac complications. Actively drinking alcoholics with cirrhosis have significantly lower mean ejection fraction and shortening fraction, as well as a greater mean end-diastolic diameter and left ventricular mass than abstaining alcoholics with cirrhosis. Alcoholics admitted solely for cardiomyopathy have a higher prevalence of cirrhosis than unselected alcoholics without heart disease. Actively drinking alcoholics admitted only for cirrhosis show impaired cardiac performance, whereas abstaining alcoholics with liver disease tend to manifest normal cardiac function[134]. Patients with alcoholic cirrhosis should be screened for cardiomyopathy for at least three reasons: (1) asymptomatic systolic and diastolic dysfunctions can precede the overt manifestation of cardiomyopathy; (2) hyperdynamic circulatory syndrome may disguise the clinical expression of initial heart failure; and (3) prevention or treatment

of some complications of cirrhosis, such as hepatorenal syndrome, is based on plasma expansion with albumin and the administration of vasoconstrictors. This would lead to deleterious effects if latent heart failure goes unrecognized[135].

CONCLUSION

Chronic liver diseases may induce systolic and diastolic dysfunctions in addition to electrophysiological changes, and the prolongation of QT interval in conditions of cirrhotic cardiomyopathy; all of these may improve completely after liver transplantations. Recent studies have found cardiac changes in patients with NAFLD, hepatitis C and primary biliary cirrhosis. On the contrary, acute and chronic heart failure have been shown to lead to acute hepatic injury and chronic congestive hepatopathy with manifestations of liver failure and laboratory data specific to ischemic hepatitis or congestive hepatopathy. There are systemic diseases that affect both the heart and the liver, thus necessitating good cardiac and hepatic evaluation. Collaboration between hepatologists and cardiologists is needed in these categories of patients for better diagnosis, treatment and prognosis. Liver and cardiac transplantation may solve this problem in some patients with heart and liver failure.

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SYSTEMATIC REVIEW

Human immunodeficiency virus and nodular regenerative hyperplasia of liver: A systematic review

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Abstract

AIM: To investigate the diagnosis, pathogenesis, natural history, and management of nodular regenerative hyperplasia (NRH) in patients with human immunodeficiency virus (HIV).

METHODS: We performed a systematic review of the medical literature regarding NRH in patients with HIV. Inclusion criteria include reports with biopsy proven NRH. We studied the clinical features of NRH, in particular, related to its presenting manifestation and laboratory values. Combinations of the following keywords were implemented: "nodular regenerative hyperplasia", "human immunodeficiency virus", "noncirrhotic portal hypertension", "idiopathic portal hypertension", "cryptogenic liver disease", "highly active antiretroviral therapy" and "didanosine". The bibliographies of these studies were subsequently searched for any additional relevant publications.

RESULTS: The clinical presentation of patients with NRH varies from patients being completely asymptomatic to the development of portal hypertension – namely esophageal variceal bleeding and ascites. Liver associated enzymes are generally normal and synthetic function well preserved. There is a strong association between the occurrence of NRH and the use of antiviral therapies such as didanosine. The management of NRH revolves around treating the manifestations of portal hypertension. The prognosis of NRH is generally good since liver function is preserved. A high index of suspicion is required to make a identify NRH.

CONCLUSION: The appropriate management of HIV-infected persons with suspected NRH is yet to be outlined. However, NRH is a clinically subtle condition that is difficult to diagnose, and it is important to be able to manage it according to the best available evidence.

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Key words: Human immunodeficiency virus; Nodular regenerative hyperplasia; Ascites; Systematic review; Liver complications

Core tip: Liver complications in patients with human immunodeficiency virus (HIV) is emerging as a public health concern. The appropriate management of HIV-infected persons with suspected nodular regenerative hyperplasia (NRH) is yet to be outlined. However, NRH is a clinically subtle condition that is difficult to diagnose, and it is important to be able to manage it according to the best available evidence. We believe the implications of our manuscript will have immediate clinical implications.

Sood A, Castrejón M, Saab S. Human immunodeficiency virus and nodular regenerative hyperplasia of liver: A systematic

INTRODUCTION

Nodular regenerative hyperplasia (NRH) is the diffuse transformation of the liver parenchyma into micronodules without intervening fibrosis[1]. NRH is associated with a number systemic diseases, including human immunodeficiency virus (HIV) infection, and over the past several years, it has become an increasingly recognized entity that causes noncirrhotic portal hypertension (NCPH)[2].

The pathogenesis of NRH is thought to be vascular in origin, initiated by endothelial damage and causing an uneven distribution of obliteration of the small portal venules throughout the liver parenchyma[3]. In addition to endothelial damage, recurrent micro-thrombosis of the portal vasculature is also thought to contribute to the obliterative phenomenon. These micronodules tend to form in areas of preserved blood flow and are thought to represent a compensatory hypertrophic response to the neighboring acini with impaired blood flow[2,4]. The diagnosis of NRH is definitively made with the histological presence of micronodules not greater than 3 mm thick and without intervening fibrosis[1].

The pathogenesis of NRH in HIV infection remains unclear. Several theories have been proposed, including a "two-hit" model in which recurrent gut bacterial translocation to the portal tract in combination with vascular endothelial damage ultimately results in portal hypertension[5]. Other theories suggest that direct viral or immune-mediated damage contribute to the obliterative venopathy[6]. However drug-induced hepatotoxicity has become the prevailing theory. The prolonged exposure to highly active antiretroviral therapy (HAART), namely didanosine (DDI) has been strongly correlated, and in all cases of proposed HAART-associated NRH, DDI has been present in each[5-15].

The significance of the association between NRH and HIV is underscored by the evolving patterns of disease in HIV-infected persons. Approximately 34 million people are currently infected with the virus worldwide. In developed nations, where individuals have ready access to HAART the mortality of HIV is decreasing[5]. The causes of death are shifting and studies have shown that liver disease accounts for as high as 18% of deaths in the post-HAART era[6]. These findings place liver disease in the top three causes of mortality in HIV-infected persons, out ranking opportunistic infections and acquired immune deficiency syndrome (AIDS)-defining illnesses. While hepatic viral co-infection accounts for a majority of the liver disease in HIV patients, both alcoholic and non-alcoholic liver diseases are also important. Of emerging relevance, is the burden of NCPH, namely NRH.

The purpose of this paper is to perform a systematic review of the clinical syndrome and outcomes in patients with NRH specifically related to HIV. The manifestations of NRH in HIV patients are similar to those that have previously been well defined in patients with NRH of other etiologies. We will focus on the clinical manifestations reported and suggest a methodical approach to the HIV patient with known or suspected NRH.

MATERIALS AND METHODS

We performed a search of the MEDLINE database for all published studies in all available languages on NRH in HIV-infected patients. Combinations of the following keywords were implemented: "nodular regenerative hyperplasia", "human immunodeficiency virus", "noncirrhotic portal hypertension", "idiopathic portal hypertension", "cryptogenic liver disease", "highly active antiretroviral therapy" and "didanosine". The bibliographies of these studies were subsequently searched for any additional relevant publications.

Our search yielded 68 unique hits (Figure 1). These publications were reviewed for relevance to the topic of interest. A total of 49 publications pertaining to the phenomenon of NCPH in the context of HIV-infection and its treatment were found. The papers were then reviewed in further detail to identify cases where the specific diagnosis of NRH was made, and cases were only included if diagnosis was based on histologic identification. A total of 95 cases that met our inclusion criteria were found. We excluded any cases where the diagnosis of NCPH was attributed to conditions other than NRH, therefore cases of hepatoportal sclerosis (HPS) and other obliterative portal venopathies not meeting diagnostic criteria for NRH were excluded. Of note, we did include cases where both HPS and NRH were found in the same liver. We excluded any cases where liver biopsy was not done and therefore no definitive diagnosis of NRH could be made.

RESULTS

Epidemiology
NRH is a rare condition, and although it has become increasingly recognized, its epidemiology remains poorly understood. The incidence in the general population is approximately 2.6%, an estimate that comes from a large autopsy study done in 1990[1]. The majority of the existing data is based from case reports and case series, and the incidence appears to be increasing as NRH is becoming more widely recognized[7]. The diagnosis is frequently missed as liver biopsy is usually only undertaken in symptomatic patients and NRH often exists subclinically. Moreover, diagnosis requires adequate specimen, appropriate use of reticulin staining and evaluation by a skilled pathologist, making histologic confirmation difficult[8].

The first published case of a patient with HIV found

Figure 1 Flow diagram outlining methods of search. NRH: Nodular regenerative hyperplasia; HIV: Human immunodeficiency virus.

Table 1 Literature review of nodular regenerative hyperplasia in human immunodeficiency virus positive patients (biopsy confirmed)			
Ref.	Study type	Number of patients	Clinical presentation
Arey *et al*[21]	Case report	1	Abdominal distention, abdominal, pain, EV
Bihl *et al*[31]	Case report	1	Abdominal pain, ALP, ascites, EV, GIB, splenomegaly
Bissonnette *et al*[11]	Case report	2	Ascites, EV, GIB
Cachay *et al*[15]	Case series	1	ALP
Saifee *et al*[52]	Case series	11	Ascites, EV, GIB
Cesari *et al*[53]	Case control	5	Ascites, EV, GIB, splenomegaly
Cotte *et al*[12]	Case control	13	Abdominal pain, ALP, ascites, EV, GIB, splenomegaly
Alvarez Díaz *et al*[33]	Case report	2	ALP, ascites, EV, GIB
Ding *et al*[54]	Case report	1	ALP, EV, GIB, ascites
Dinh *et al*[55]	Case control	3	Ascites, encephalopathy, EV
Fernandez-Miranda *et al*[9]	Case report	1	Unknown
Garvey *et al*[19]	Case report	2	EV, splenomegaly
Hofmaenner *et al*[27]	Case report	1	Epigastric pain, ALP
Kochin *et al*[56]	Case report	1	EV, GIB
Kovari *et al*[23]	Case control	1	EV
Maida *et al*[8]	Case series	2	Splenomegaly
Mallet *et al*[4]	Case control	13	ALP, EV, GIB
Mallet *et al*[57]	Case report	1	Portal hypertension
Mallet *et al*[30]	Case series	8	ALP, ascites, EV, splenomegaly
Mendizabal *et al*[58]	Case control	5	EV, GIB
Podevin *et al*[59]	Case report	1	Ascites, EV, splenomegaly
Sandrine *et al*[20]	Case report	1	ALP, ascites, EV, splenomegaly
Santiago *et al*[28]	Case report	1	Abdominal distention, splenomegaly
Schiano *et al*[22]	Case report	1	ALP, EV, splenomegaly
Scourfield *et al*[60]	Retrospective cohort	4	Unknown
Schouten *et al*[61]	Case report	3	EV, GIB
Stebbing *et al*[62]	Retrospective cohort	2	ALP
Tateo *et al*[13]	Case report	3	ALP, ascites, EV, GIB
Vispo *et al*[29]	Case report	3	ALP, ascites, EV, GIB

ALP: Abnormal liver panel; EV: Esophageal varices; GIB: Gastrointestinal bleed.

to have NRH was reported from Spain in 1993 in a patient with HIV infection and visceral leishmaniasis[9]. We identified a total of 94 additional cases of biopsy-confirmed NRH in HIV-positive patients, most of which were reported within the last decade (Table 1). Given that the same limitations in diagnosis apply to HIV-infected persons, the incidence is likely grossly underestimated in this patient population as well. Epidemiological studies geared toward select patient populations are needed and

may shed more light on the actual incidence of NRH in HIV patients as well as in the general population.

Clinical presentation

The clinical presentation of the cases found was highly variable. The diagnosis was prompted by incidental lab abnormalities in asymptomatic patients, and in other cases, patients presented with manifestations of portal hypertension such as esophageal varices, ascites, and

Table 2 Manifestations of portal hypertension in patients with nodular regenerative hyperplasia and human immunodeficiency virus *n* (%)		
Manifestation	**Patients identified**	**References**
Ascites	30 (32)	[4,12,19,20,28,33,52,53,58,60]
Esophageal varices	61 (66)	[4,12,19,20,28,33,52,53,58,60]
Hepatic encephalopathy	1 (1.1)	[54]
Splenomegaly	25 (27)	[4,12,19,20,31,52,53,54,58,60]
Portal thrombosis	11 (12)	[4,12,19,20,31,52,53,54,58]

hypersplenism (Table 1). Jaundice was universally not reported. According to our findings, the most common manifestation of NRH in HIV-positive patients was esophageal varices, which were identified in at least 66 of 95 cases. Only 28 patients developed gastrointestinal bleeding in the setting of esophageal varices (Table 2). In many cases, once the clinical syndrome of portal hypertension was identified, patients underwent screening esophagogastroduodenoscopy and were placed on prophylactic non-selective beta-blockers (NSBB) if varices were found. Patients were reported to be on NSBB in almost all cases.

Liver synthetic function as indicated by INR and albumin is well preserved across all cases in which it was reported. Liver associated enzymes may be only mildly elevated (Table 3). Patients were also not uncommonly found to have thrombophilias including protein C and protein S deficiencies, which may be associated with the pathogenesis of NRH and the development of portal vein thrombosis in these patients (Table 3).

When compared to the clinical presentation of patients with NRH without HIV, similar findings have been reported. In one recent series including 42 patients, the most common presenting abnormality was an abnormal liver profile, existing in 76% of cases. Varices were detected in 26% of patients. None of these patients had synthetic liver dysfunction as implicated by normal INR[10]. In another series of 24 patients, similar rates of various clinical features of NRH were reported[11]. These findings mimic those of other similar case reports and are also similar to the findings presented in patient's specifically with NRH and HIV.

Diagnosis
Tthe diagnosis of NRH is a histologic one, requiring liver biopsy. Histologic features are shown in Figure 2. The use of a reticulin stain is usually necessary to make the diagnosis. Important features on the reticulin stain include: nodular apperance, characterized by alternating hypertrophic and atrophic hepatocytes. Highlighting the frequent delay in diagnosis, in one report of 13 patients on HAART who developed NRH, the mean time from presentation to diagnosis of NRH was approximately 38 mo[12]. This point highlights the sub-optimal diagnosis of NRH, leading to its under-appreciation as an important clinical entity in the HIV population. Diagnosis is further limited by the presence of a clear workup bias in that it

is usually either the symptomatic patient, or the patient with long-term DDI exposure who undergoes diagnostic testing for NRH. Furthermore, consideration of NRH is certainly more common in the academic setting[13].

Radiologically, the diagnosis of NRH is also difficult. Findings are variable and range from none to diffuse hypoechoic nodules. On ultrasound, findings may include widespread nodularity of the liver can mimic cirrhosis[14]. On computed tomography, the nodules are usually hypodense and typically do not enhance with contrast. Finally on magnetic resonance imaging, suface nodularity and nodules of similar signal intensity to the liver may be noted[14]. Because the findings on imaging are non-specific and non-diagnostic, clinical correlation is key in determining the next best step in diagnosis.

Natural history
The natural history of NRH is poorly understood. There is likely an inherent bias to diagnose and report symptomatic cases, and NRH is likely more indolent than appreciated. This notion is supported by the large autopsy study by Wanless in which only one of 64 patients had been diagnosed with NRH prior to death. Few of these patients had developed manifestations of portal hypertension prior to death[1]. These findings are likely explained by the preservation of hepatic synthetic function observed in patients with NRH. From the available data, the presentation of NRH is variable and ranges from subtle findings on serum liver tests, to vague abdominal symptoms, to overt gastrointestinal hemorrhage and other severe manifestations of portal hypertension. To date, the overall prognosis has not been well defined, as there have not been substantial outcomes data reported in the literature. In one long-term follow up study of a cohort of eight HIV-infected patients with NCPH, there was only one death, which was attributed to non-NCPH related causes. The remainder of the patients underwent regular screening EGD and was placed on NSBB therapy. If the patients were found to have NCPH prior to the development of variceal bleeding, they tended to remain minimally symptomatic with supportive and preventative care. On the other hand patients who were not treated in a timely manner tended to develop refractory bleeding. One patient with severe NRH required repeated TIPS and was placed on the liver transplant waiting list while another underwent surgical hepatorenal shunt[15].

Associated conditions and medications
Nodular regenerative hyperplasia has long been associated with rheumatologic, autoimmune, hematologic and myeloproliferative disorders[7]. Of particular clinical consequence is the link between NRH and thrombophilias. The association is theoretically attributed to the increased chronic micro-thrombosis of the small portal venules, leading to their constriction, obliteration and eventually to intrahepatic portal hypertension[16]. Many of these patients are also predisposed to the development of portal vein thrombosis, which can lead to end-

Table 3 Select laboratory tests associated with nodular regenerative hyperplasia		
Laboratory test	Range	References
Albumin (g/dL)	1.9-4.5	[22,28,52,54,55]
Aspartate transaminase (IU/L)	15-139	[4,8,20-22,30,31,38,54,55,59,60]
Alanine transaminase (IU/L)	13-196	[3,7,19-21,30,31,52,54,55,59,60]
Alkaline Phosphatase (IU/L)	92-541	[4,20-23,28,31,52,53,55,59,60]
Total bilirubin (mg/dL)	0.6-3.6	[21,22,31,52,54,55,59,60]
Gamma glutamyl transpeptidase (IU/L)	12-771	[4,20,22,28,31,53,55,59,60]
White blood cell count (cells/mm^3)	1090-4800	[4,21,22,28,30,31,33,53-55,60]
Hemoglobin (g/dL)	6.6-13	[22,55,60]
Platelet (1000/mm3)	61-273	[4,22,23,52,54,55,59,60]

Figure 2 Needle liver biopsy of male infected with human immunodeficiency virus. A: His risk factor for the development of Nodular Regenerative Hyperplasia was long term use of didanosine. Hepatocytes size varies zonally, occasional areas of small hepatocytes with increased nuclear cytoplasmic ratio alternate with areas of a more normal appearing morphology in the hematoxylin-eosin stain (original magnification × 100); B: The reticulin stain highlights and confirms nodular regeneration throughout the specimen with nodular areas of regenerative hepatocytes as characterized by widened hepatocellular plates alternating with areas of normal appearing lobular architecture and then areas of narrowed, attenuated hepatocytes (original magnification × 100).

stage disease. In one study, 12 out of 2600 persons with HIV infection were found to have NCPH, and NRH was a common histologic diagnosis. Of the 12 patients with NCPH, half were found to have portal thrombosis[17]. Among all cases, the percentage of patients with portal thrombosis was close to 15% (Table 2).

The association between NRH and antiretroviral therapy has been identified mostly through case reports and small case-control studies. In particular, specific associations have been made with DDI. One of the earliest associations with DDI and NCPH and NRH was from a case-control analysis[18]. Their findings were supported in a number of succeeding publications[14,15,17,19-23]. More recently, the results of another analysis suggested that patients with NRH had a longer exposure not only to DDI, but stavudine, tenofovir, and a combination of DDI and stavudine and DDI and tenofovir as well[12].

Aside from HAART, other medications have also been linked to NRH. Its association with azathioprine (AZA), 6-mercaptopurine, 6-thioguanine, busulphan, cyclophosphamide and most recently, oxaliplatin-based therapies have also been reported[24,25]. A presumed mechanism is damage of the endothelium in the small hepatic veins leading to the phenomenon of obliterative portal venopathy. Interestingly, in one report of AZA-induced NRH, some patients demonstrated normalization of liver enzyme markers and histological regression of NRH after

withdrawal of AZA[26]. Such histological regression has never been reported in the case of HAART and HIV-associated NRH.

Treatment

There is no definitive treatment for NRH. The mainstay of management is removal of the offending agent and prevention and supportive care of disease manifestations. It is important to note that in many of the cases in which NRH was linked to DDI, the exposure had been long-since stopped prior to the time of NRH diagnosis. Cessation of DDI or other implicated HAART agents may improve outcomes, however it has not been shown to result in reversal of the disease process[27]. In part because of its associated hepatotoxicity, the use of DDI has declined substantially and it is now regarded as a lower-tier anti-retroviral medication[12]. Although it is clear that DDI-induced NRH does persist for years after cessation of the medication; interruption has still been associated with an overall better prognosis[27-29].

Treatment of the clinical manifestations of NRH revolves around the primary prophylaxis of esophageal variceal bleeding in asymptomatic patients. For cases in which details were provided, we observed that in almost all asymptomatic cases of esophageal varices, primary prophylaxis with NSBB was started, and propranolol was the usual drug of choice. Many patients also under-

went endoscopic band ligation (EBL), either as primary prophylaxis or after manifestation with bleeding. There were several cases of failure of NSBB or EBL, for which patients underwent transjugular intrahepatic portosystemic shunt (TIPS). At least one of these cases was documented as refractory and TIPS was done as bridge to transplant[15]. In contrast to non-HIV related NRH, ascites was not uncommonly reported in our review of HIV patients with NRH. At least 12 cases of ascites were reported, however the need for paracentesis was not reported. Ascites is infrequently reported in NRH with patients without HIV[10].

The utility of anticoagulation in patients with NRH is a burgeoning concept. Mallet *et al*[30] described a series of 21 patients with clinical evidence of prothrombotic state in the setting of NCPH. The median protein S level in this group was nearly half of normal. The coagulation abnormalities in patients with NRH have particular significance as suggested by Bihl *et al*[31], who reported a case in which the patient's clinical course improved dramatically after initiation of anticoagulation. It appears that the benefit of anticoagulation in these patients may be multifactorial. One theory is that it slows disease progression by preventing the micro-thrombosis thought to play a pathogenic role in NRH. In addition, given the noted thrombophilias in these patients, anticoagulation prevents portal vein thrombosis, which can lead to refractory manifestations of portal hypertension and end-stage liver disease[30-32]. These findings suggest potential benefit of anticoagulation therapy and warrant further studies specifically in the context of NRH.

Patients with severe liver disease and intractable symptoms of portal hypertension should be considered for liver transplant, which has been successfully described in 7 cases of patients with HIV and NRH[13,30]. In most of these cases, the indication for liver transplant was severe and intractable symptoms portal hypertension, with or without complete portal vein thrombosis. In one case, the indication to consider transplant was hepatic encephalopathy secondary to TIPS placement[33]. All 7 patients who underwent liver transplant reportedly had excellent survival. However, it is important to note that there have been cases in which NRH reappears in the transplanted liver[34], although this has yet to be described in a patient with NRH in the setting of HIV. Regardless, for this reason, continued anticoagulant therapy even after transplant may be warranted and necessitates further investigation.

DISCUSSION

The burden of liver disease in HIV-infected persons is substantial and a comprehensive and interdisciplinary approach to management is crucial. All HIV-positive individuals should undergo regular liver function testing at least biannually as they are routinely exposed to hepatotoxic medications and are generally at increased risk for both viral and non-viral hepatitides[35]. NRH should be considered in all HIV-positive patients with unexplained signs and symptoms of portal hypertension. A thorough history of past and current antiretroviral drug regimens should be obtained as it has been shown that the effects of NRH persist long after cessation of the offending agent.

Diagnosis of NRH requires a high index of suspicion given its uncommon incidence and varied presentation of portal hypertension. Imaging techniques, such as abdominal ultrasound or computed tomography (CT) are frequently used to further evaluate patients with liver disease. Hepatic nodularity may be appreciated on liver ultrasound, and when combined with a clinical context of relatively preserved hepatic function, NRH should remain high on the differential. The hepatic nodularity on ultrasound is often followed up with CT imaging, however findings of diffuse micronodularity can also mimic cirrhosis[14,56].

NRH is one of the potential causes of non-cirrhotic portal hypertension in patients with HIV. Other potential causes of non-cirrhotic intrahepatic portal hypertension include schistosomiasis, sinusoidal obstruction syndrome, and idiopathic portal hypertension (hepatoportal sclerosis)[7,22,37]. In particular, antiretroviral therapy has been associated with both NRH and idiopathic portal hypertension. It has been hypothesized that idiopathic portal hypertension can lead to both NRH and portal vein thrombosis[2].

Accurate diagnosis of NRH requires the histological assessment[7]. Since the nodularity is often heterogeneously distributed, liver biopsy requires an ample sized tissue. The transjugular route is often used to minimize bleeding complications[38]. However, there have been cases where diagnosis was initially missed and later found only *via* wedge biopsy[39]. Classification of non-neoplastic, diffuse parenchymal liver disease necessitates a sample of at least 2-3 cm in length and at least a 16-gauge caliber needle[40]. The hepatocyte atrophy, a key histologic feature is best identified with reticulin staining which must be employed to make the diagnosis[41].

The pathogenesis of NRH is believe to be related to the differential of blood supply to the liver that leaves some areas ischemic, and other with compensatory hypertrophy which leads to the formation of hepatic nodules. There are multiple diseases, conditions, and medications associated with its development[7]. In contrast to non-HIV patients, patients with HIV and NRH tend to have a higher incidence of ascites. It is unclear why the incidence differs but the variance may be potentially related to the underlying cause of NRH in HIV patients being largely pharmacologic from antiretroviral drugs.

DDI which has been most strongly linked to the development of NRH, belongs to a class of medications known as nucleoside reverse transcriptase inhibitors (NRTI). It functions as a purine analogue and interferes with the transcription of DNA and RNA[27]. Azathioprine, 6-mercaptopurine 6-thioguanine and other chemotherapeutic agents that have been previously linked to NRH have a similar mechanism of action[42]. Although

current knowledge suggests that NRH is not a reversible condition, several studies have shown improved outcomes with cessation of causative medications.

Treatment of NRH generally revolves around manifestations of portal hypertension. Once the diagnosis is confirmed, patients should undergo screening for esophageal varices with esophagogastroduodenoscopy (EGD)[43]. Patients with NRH should be considered for screening for HCC. Although this remains a relatively controversial topic, a possible pathogenetic relationship between NRH and HCC has been described[1,25,44-50]. The inability to conclusively establish a propensity of NRH to develop into HCC may be a result of its underestimated incidence, rather than a lack of association between the two conditions. Liver transplantation is an uncommon treatment for NRH since liver function is usually preserved. In a recently published systematic review, the authors found severe portal hypertension as the most common indication for liver transplantation[51].

The importance of NRH in HIV-infected individuals is growing, especially as patients are experiencing increased longevity and longer exposure to medications. High index of suspicion is required to make a diagnosis since there are patients with HIV who develop manifestations of portal hypertension such as ascites and variceal bleeding from cirrhotic and non-cirrhotic causes. Treatment generally revolves around treating the manifestation of portal hypertension.

COMMENTS

Background
Nodular regenerative hyperplasia (NRH) causes portal hypertension in patients with human immunodeficiency virus (HIV). Unlike cirrhosis, NRH is not associated with liver synthetic function. On imaging, NRH and cirrhosis appear similar with nodular liver contours. Patients with NRH can present with ascites and variceal bleeding.

Research frontiers
Further research in needed in the utility of non-invasive methods of making the diagnosis of NRH. Studies are essential to determine the long term consequences of NRH. More research is needed to understand the pathogenesis of NRH.

Innovations and breakthroughs
There is increasing appreciation of the epidemiology and association of NRH with HIV. There is also enhanced awareness of the association of certain antiretroviral medications such as didanosine and NRH.

Applications
The article adds to the literature the current understanding of the diagnosis, risk factors, natural history and, management of NRH.

Terminology
The diagnosis of NRH requires a liver biopsy. Liver parenchymal cells are clustered in nodules. Unlike cirrhosis, there are fibrotic bands encompassing the nodule.

Peer review
One of the most important issues raised during peer review was that there may be differences in the presentation in patients affected by NRH with and without HIV. Patients with NRH and HIV are more likely to have ascites than those patients with NRH without HIV.

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Books
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15   Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

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16   **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Portal vein thrombosis in liver cirrhosis

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Abstract

Portal vein thrombosis (PVT) is considered to be a frequent complication of liver cirrhosis. However, unlike PVT in patients without cirrhosis, very few data are available on the natural history and management of PVT in cirrhosis, despite its association with potentially life-threatening conditions, such as gastroesophageal bleeding and acute intestinal ischemia. Moreover, no consensus regarding PVT in cirrhosis exists. Suggested causes of PVT in cirrhosis include reduced portal blood flow velocity, multiple congenital or acquired thrombophilic factors, inherited or acquired conditions, and derangement of liver architecture. However, the understanding of PVT in cirrhosis is incomplete. In addition, information on the management of PVT in cirrhosis is inadequate. The aims of this review are to: (1) assemble data on the physiopathological mechanism, clinical findings, diagnosis and management of PVT in cirrho-sis; (2) describe the principal factors most frequently involved in PVT development; and (3) summarize the recent knowledge concerning diagnostic and therapeutic procedures.

Core tip: Portal vein thrombosis (PVT) is considered to be a frequent complication of liver cirrhosis; however, very few data are available on the natural history and management of PVT in cirrhosis, despite its association with potentially life-threatening conditions. The understanding and information on the management of PVT in cirrhosis are incomplete. The aims of this review are to: (1) assemble data on the physiopathological mechanism, clinical findings, diagnosis and management of PVT in cirrhosis; (2) describe the principal factors most frequently involved in PVT development; and (3) summarize the recent knowledge concerning diagnostic and therapeutic procedures.

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INTRODUCTION

Portal vein thrombosis (PVT), an obstruction of the portal vein or its branches by a blood clot, is encountered in a variety of clinical settings, such as myeloproliferative disease, cirrhosis, cancer and infection. More patients with cirrhosis are being diagnosed with PVT be-

cause current imaging techniques allow for the detection of asymptomatic PVT during routine ultrasonographic examination. PVT has a variety of clinical presentations, from asymptomatic to life-threatening conditions such as gastroesophageal bleeding and acute intestinal ischemia[1-3]. Although liver transplantation has altered the prognosis of patients with cirrhosis, the presence of PVT can exclude a patient from a transplant listing or negatively impact post-transplantation survival[4].

It remains unclear whether PVT is a consequence of severe liver disease, a factor aggravating underlying liver disease, or both. PVT is considered to be a frequent complication of liver cirrhosis; however, unlike PVT in patients without cirrhosis, very few data are available on its natural course and management despite its association with potentially life-threatening conditions. In addition, no consensus regarding PVT with cirrhosis exists. There is a growing need for optimal, evidence-based management of PVT in cirrhosis.

The aims of this review are to: (1) assemble evidence regarding the physiopathological mechanism, clinical findings, diagnosis and management of PVT in cirrhosis; (2) describe the principal factors most frequently involved in PVT development; and (3) summarize the most recent knowledge concerning diagnostic and therapeutic procedures.

DEFINITION OF ACUTE AND CHRONIC PVT

From a clinical point of view, PVT comprises two different entities: acute PVT and chronic PVT. Each represents a successive stage of the same disease. Although they share similar causes, they differ with respect to their management[5].

Acute PVT is characterized by the sudden formation of a thrombus within the portal vein[6]. The thrombus can involve variable portions of the mesenteric veins and/or splenic vein. Occlusion can be complete or partial, leaving a peripheral circulating channel.

In patients with chronic PVT, also known as portal cavernoma, the obstructed portal vein is replaced by a network of hepatopetal collateral veins connecting the patent portion of the vein upstream of the thrombus to the patent portion downstream. The number, size and location of these collaterals are extremely variable from patient to patient[6].

However, in patients with cirrhosis, it may be difficult to establish the "age" of the thrombosis because the criteria commonly used in patients without cirrhosis to define acute or chronic PVT (presence of collateral circulation and signs of portal hypertension) are already features of liver disease[7,8].

PREVALENCE

The prevalence of PVT in patients with cirrhosis has been reported more frequently in recent years. The reported prevalence of PVT is in the range of 0.6%-15.8% in patients with liver cirrhosis or portal hypertension[9-15]. The presence of PVT is reportedly 0.6% when evaluated by angiographic studies[9], 4.4% when evaluated by ultrasound[10], and 10%-12% when evaluated by computed tomography and magnetic resonance imaging[1,11]. Moreover, the prevalence of PVT increases with patient age and liver disease severity, reaching 15% in patients awaiting liver transplantation[12-15].

The etiology of liver disease influenced the prevalence of PVT in a study of 885 patients who underwent liver transplantation. The prevalence of PVT was 3.6% in primary sclerosing cholangitis, 8% in primary biliary cirrhosis, 16% in alcoholic and hepatitis B virus-related cirrhosis, and 35% in hepatocellular carcinoma (HCC)[12].

ETIOLOGY OF PVT IN CIRRHOSIS

Inherited and acquired thrombophilic disorders, bacterial infection[16,17] and sluggish portal flow[2,18,19] may all play a role in the high prevalence of PVT in patients with cirrhosis.

Cirrhosis was recently considered to be a hypercoagulable state, not a hypocoagulable state. The levels of both pro- and anti-coagulation proteins are reduced under conditions of hepatic synthetic impairment in patients with liver cirrhosis. Coagulation and anticoagulation mechanisms remain balanced but are carried out at a lower level[20-22]. The net result is a hemostatic balance that is compensated under normal circumstances, with no tendency for bleeding or thrombosis[22]. In cirrhosis, however, this equilibrium can easily tilt towards either bleeding or thrombosis[19,23-26]. Some authors have demonstrated that elevated levels of factor VIII (a procoagulant driver) in combination with decreased levels of protein C (an anticoagulant driver), both of which are typically found in patients with cirrhosis (*i.e.*, procoagulant imbalance), are probably related to partial resistance to the *in vitro* anticoagulant action of thrombomodulin[27-30]. However, in patients with impaired synthetic function and low plasma levels of natural coagulation inhibitors, there is currently no simple way to ascribe such a low level to a pre-existing deficiency[31].

A thrombophilic genotype, including factor V Leiden G1691A mutation[32,33], methylenetetrahydrofolate reductase (TT677) mutation[34,35] and prothrombin (G20210A)[11,36], is associated with the formation of PVT. However, they may play a minor pathogenic role in the formation of PVT.

Reduced portal flow velocity seems to be the most important predictive variable for PVT development in patients with cirrhosis[37-39]. Amitrano *et al*[38] suggested that portal blood stasis in patients with cirrhosis is the main change favoring thrombosis, even in the presence of other local, systemic, congenital and acquired factors. Kinjo *et al*[39] performed Doppler ultrasonographic examinations after splenectomy in patients with cirrhosis and showed that portal venous flow was dramatically decreased by 49.2% in the PVT group but only by 6.6% in

the non-PVT group.

Splenectomy has recently been reported to play a role in the surgical strategy for HCC and interferon-based therapy for hepatitis C[40-45]. In addition, it can improve the prognosis for patients with cirrhosis by allowing them to receive interferon therapy or undergo treatment for HCC[44,45]. Despite the good results demonstrated in these studies, the high prevalence of PVT after splenectomy in patients with cirrhosis remains problematic[39,46]. It has been suggested that blood turbulence or stasis in the stump of the splenic vein after splenectomy might result in increased coagulability, leading to the propagation of splenic venous thrombus formation in the portal system after splenectomy[47]. Splenomegaly and a large splenic vein diameter are independent risk factors for PVT after splenectomy in patients with concomitant cirrhosis and portal hypertension[39,46,47].

The role of sclerotherapy as a potential trigger for PVT is controversial[48,49]. Some recent reports showed that thrombopoietin receptor agonists might be associated with an increased incidence of PVT in patients with cirrhosis[50,51].

CLINICAL FINDINGS

Clinical findings of PVT in cirrhosis vary from asymptomatic to life-threatening conditions. Partial PVT, which is now often detected by routine ultrasonography or computed tomography, might be associated with few symptoms. However, complete PVT may present as abdominal or lumbar pain with sudden onset or progression over a few days. Rapid, complete obstruction of the portal vein or mesenteric veins without involvement of the mesenteric venous arches induces intestinal congestion, which manifests as severe, continuous, colicky abdominal pain and occasionally as nonbloody diarrhea[1-3]. The bleeding risk appears to be higher in patients with PVT and cirrhosis than in patients with cirrhosis alone (39% *vs* 27%, respectively)[52]. In many patients, however, the thrombus is partial and its aspects and location change in follow-up images. Laboratory findings, including the levels of aminotransaminase, fibrin and fibrinogen degradation products, and d-dimers, are often normal in many cases of developing PVT.

Chronic PVT is commonly diagnosed after a fortuitous finding of hypersplenism or portal hypertension. In the majority of patients it is asymptomatic. Gastrointestinal bleeding is better tolerated by patients with chronic PVT than in those with other forms of portal hypertension, probably because patients with PVT are usually younger and have no liver dysfunction. The occurrence of ascites or encephalopathy in patients with chronic PVT is uncommon and is usually encountered only transiently following gastrointestinal bleeding or when unrelated renal failure or marked sepsis is present in older patients[3]. Liver test results are typically normal in patients with portal cavernoma in the absence of underlying liver disease. Biliary symptoms related to portal cholangiopathy (jaundice, biliary pain, cholangitis, cholecystitis or pancreatitis) rarely reveal the presence of a cavernoma[53,54]. Hepatopulmonary syndrome is present in about 10% of patients.

DIAGNOSIS

More of these patients are being diagnosed with PVT because current imaging techniques allow for the detection of asymptomatic PVT during routine ultrasonography in patients with cirrhosis.

Ultrasound and Doppler ultrasound are almost always sufficient for a diagnosis of PVT[55,56]. In most patients, the diagnosis of acute PVT can be rapidly established using noninvasive imaging. Ultrasound sonography can show hyperechoic material in the vessel lumen with distension of the portal vein and its tributaries. Doppler imaging shows the absence of flow in part or all of the lumen.

Enhanced computed tomography (CT) can show a lack of luminal enhancement in the portal vein, increased hepatic enhancement in the arterial phase, and decreased hepatic enhancement in the portal phase[57]. CT and magnetic resonance (MR) angiography are more sensitive techniques than Doppler imaging with respect to assessment of the extent of the thrombus within the portal venous system[56-58]. Definitive diagnosis of PVT can be obtained by MR imaging (MRI) and CT; the former provides a better evaluation of the extent of the thrombosis, particularly in the mesenteric vein, reaching a sensitivity and specificity of 98%-100%. CT provides information not only about the extent of the thrombosis and the development of collateral circulation, but also about the state of the abdominal organs. It is the procedure of choice when intestinal ischemia or hepatocellular carcinoma is suspected[59,60]. A diagnosis of cavernoma is readily achieved by abdominal imaging with ultrasound, CT or MRI, which shows serpiginous structures while the main portal vein and/or its main branches are not visible.

A recent study showed that positive intrathrombus enhancement on contrast-enhanced sonograms is an accurate predictor of recanalization in patients with recent portal thrombosis[61].

TREATMENT

Optimal management of PVT in cirrhosis is not addressed in any current consensus publication. There are a few reports about the factors that influence recanalization or the extent of thrombosis; however, the actual impact of PVT treatment on the natural course of cirrhosis has not been investigated. No randomized controlled trials have been performed and most existing evidence concerning PVT treatment is based on case series and is of low quality.

PVT increases the risk of variceal bleeding and is reportedly an independent risk factor for the inability to

control variceal bleeding[62]. In addition, PVT can be a life-threatening emergency when it extends to the superior mesenteric vein, leading to intestinal infarction. Anticoagulated patients with cirrhosis have better recanalization rates and PVT extension than non-anticoagulated patients[63]. Therefore, in patients with concomitant cirrhosis and PVT, a treatment algorithm that includes anticoagulation and transjugular intrahepatic portosystemic shunting (TIPS) provides a good chance of complete repermeation, reduces portal hypertensive complications and decreases the rate of thrombosis progression[63]. Francoz *et al*[4] evaluated patients with cirrhosis awaiting liver transplantation and found that survival was significantly lower in those with complete PVT at the time of surgery ($P = 0.04$). Furthermore, the rate of partial or complete recanalization was significantly higher among patients receiving anticoagulation therapy than among those not receiving anticoagulation therapy ($P = 0.002$).

Conversely, some reports have shown that PVT has little influence on prognosis in patients with cirrhosis. Maruyama *et al*[64] evaluated 150 patients with virus-related cirrhosis but without PVT at baseline; PVT developed in 28% of patients (42/150), with a cumulative incidence of 12.8%, 20% and 38.7% at 1, 5 and 8-10 years, respectively. The natural course of thrombosis was improvement in 47.6% of patients, unchanged in 45.2%, and worsened in 7.2%. Spontaneous resolution or an unchanged appearance was the most common outcome of PVT; therefore, cirrhotic PVT had little influence on prognosis. In their multivariate analysis, Luca *et al*[65] noted that there was no clear association between progression or regression of partial PVT and clinical outcome and that the Child-Pugh score at the time of diagnosis was the only independent predictor of survival.

In the field of liver transplantation, there is accumulating evidence that PVT, especially thrombus extension to the superior mesenteric vein, may adversely affect the outcome of transplantation. Thus, patients with concomitant cirrhosis and PVT who are on the waiting list for liver transplantation should be treated with anticoagulation therapy[66,67]. PVT prior to liver transplantation is an independent prognostic factor for post-transplant survival[68,69] and complete or partial PV recanalization has been associated with a better survival rate after liver transplantation[4]. It has also been shown that individuals with PVT at the time of liver transplantation are at higher risk of recurrent PVT after transplantation and of requiring retransplantation[30,70]. The increased mortality and morbidity rates associated with PVT are mostly restricted to the first year after liver transplantation[4,62] and actuarial survival after 1 year is good. Therefore, PVT cannot be considered to be a contraindication to liver transplantation[71].

Anticoagulation therapy is of proven benefit in patients with acute deep vein thrombosis[72]. The optimal anticoagulation regimen for the treatment and monitoring of PVT has not yet been fully explored and no clear recommendations exist regarding this issue in recent guidelines or consensus publications[6,28]. Treatment strategies most often include the use of anticoagulation, while thrombectomy and TIPS are considered second-line options.

The goal of anticoagulation therapy for acute PVT is to recanalize the obstructed veins, which will prevent intestinal infarction and portal hypertension. Correction of the causal factors should be achieved as soon as possible.

Vitamin K antagonists (VKA) have been used in some studies to treat PVT in patients with cirrhosis. The rate of PV recanalization in patients with cirrhosis treated with VKA is about 40%[4,73]. Orally administered VKA is more acceptable to patients; however, treatment with VKA is particularly difficult in patients with cirrhosis, mostly because anticoagulation monitoring is complex in this particular situation. Notably, international normalized ratio (INR) monitoring in patients with liver disease probably overestimates the bleeding risk because this international sensitivity index is determined using plasma from patients taking VKA[74]. The INR has only been validated in individuals with normal liver function on stable anticoagulation. A 29% variation in the mean INR was reported in patients with cirrhosis in a study in which three different thromboplastin reagents were used[75]. It is also unclear whether a target INR between 2 and 3 is adequate in individuals with an abnormal INR before anticoagulation therapy[30].

No consensus exists regarding the optimal duration of anticoagulation therapy in these settings. Complete recanalization can be delayed until the sixth month of anticoagulation therapy[5,76]. However, whether this is also true for patients with cirrhosis who develop acute PVT remains to be determined.

Randomized controlled trials of anticoagulation therapy for the prevention of recurrent thrombosis are lacking in cirrhotic PVT. In patients with deep vein thrombosis, a lack of complete recanalization indicates a high risk of recurrence after cessation of anticoagulation therapy[77]. The frequent association with permanent prothrombotic disorders and the risk of intestinal infarction support the use of anticoagulation. However, an increased risk of bleeding secondary to portal hypertension raises concerns. Delgado *et al*[73] reported that re-thrombosis after complete recanalization occurred in 38.5% of patients with cirrhosis after anticoagulation therapy was stopped. Thatipelli *et al*[78] stated that prolonged anticoagulant therapy does not appear to be justified based on the low rate of recurrence and substantial rate of major hemorrhage. To avoid the extension of thrombosis to the splanchnic vessels, prophylactic anticoagulation should be continued in patients with underling thrombophilic conditions or in patients who are likely candidates for future liver transplantation[4,63].

The choice of the anticoagulation regimen must also account for the potential need to reverse the effect of anticoagulation. There is no current consensus or guideline on whether nonselective beta blockers, endoscopic

variceal ligation or combination therapy is better for variceal bleeding prophylaxis[7,79].

An attractive alternative to oral anticoagulants could be the use of low-molecular-weight heparin (LMWH), the dosing of which is weight based and thus does not necessitate screening. A 50%-80% portal vein recanalization rate was recently reported with the use of LMWH in 38 patients with cirrhosis, with only a few episodes of non-severe variceal bleeding[62,80]. Villa *et al*[81] performed a randomized controlled trial to evaluate the safety and efficacy of enoxaparin, a LMWH, in preventing PVT in patients with advanced cirrhosis. They demonstrated that the actuarial probability of PVT was lower in the enoxaparin group ($P = 0.006$). The actuarial probability of survival was higher in the enoxaparin group ($P = 0.020$). No relevant side effects or hemorrhagic events were observed in their study. Enoxaparin appeared to delay the occurrence of hepatic decompensation and improve survival. However, an increased volume of distribution, such as that produced by ascites and edema, in patients with cirrhosis makes it difficult to determine the optimal dose of LMWH[82].

The administration of antithrombin Ⅲ (AT-Ⅲ) could be an attractive alternative to PVT in cirrhosis. Kawanaka *et al*[83] demonstrated that the low AT-Ⅲ activity and further decreases in this activity are associated with PVT after splenectomy in patients with cirrhosis and that treatment with AT-Ⅲ concentrates is likely to prevent the development of PVT in these patients.

TIPS and anticoagulation therapy are considered to be optimal treatment choices for PVT in cirrhosis[84]. TIPS completely recanalized the portal venous system in 57% of patients with cirrhosis and resulted in a marked decrease in 30% without major procedure-related complications. Despite problems associated with patency (bare stents, 38% in 12 mo and 85% in 24 mo; covered stents, 21% in 12 mo and 29% in 24 mo) and encephalopathy (27% at 12 mo, 32% at 24 mo), the long-term outcome of TIPS placement for cirrhotic PVT is excellent[85]. In addition, PVT prior to liver transplantation is an independent prognostic factor for post-transplant survival and TIPS prevents total portal vein occlusion in liver transplantation candidates with partial PVT[86].

In patients with both cirrhosis and chronic PVT, there is no consensus on the indication for anticoagulant therapy. As described in the consensus for PVT in patients without cirrhosis, therapy for chronic PVT can be separated into prevention and treatment of gastrointestinal bleeding, prevention of recurrent thrombosis and treatment of portal cholangiopathy. When PVT is longstanding and cavernous transformation has occurred in the portal vein, prophylactic anticoagulation is reversed only in patients with thrombophilic conditions and/or a high risk of thrombus extension into the superior mesenteric vein. There is still sufficient evidence in favor of interventional therapy such as TIPS[87]. Data on endoscopic ligation are lacking in adult patients with chronic PVT.

CONCLUSION

PVT is a common problem in patients with cirrhosis, mostly in individuals with advanced liver disease. However, many unknown pathophysiological aspects of PVT and unresolved issues encountered in everyday practice remain to be addressed. The most optimal, most efficient and safest modalities for treatment, screening and monitoring must be established in future controlled trials.

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ORIGINAL ARTICLE

Oxidative stress and extracellular matrices after hepatectomy and liver transplantation in rats

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Abstract

AIM: To investigate oxidative stress (OS)-mediated damage and the behavior of extracellular matrices in various rat models because shear stress with portal hypertension and cold ischemia/warm reperfusion injury trigger the liver regeneration cascade after surgery. These injuries also cause fatal liver damage.

METHODS: Rats were divided into four groups according to the surgery performed: control; hepatectomy with 40% liver remnant (60% hepatectomy); orthotopic liver transplantation (OLT) with whole liver graft (100% OLT); and split OLT (SOLT) with 40% graft (40% SOLT). Survival was evaluated. Blood and liver samples were collected at 6 h after surgery. Biochemical and histopathological examinations were performed. OS-induced damage, 4-hydroxynonenal, ataxia-telangiectasia mutated kinase, histone H2AX, phosphatidylinositol 3-kinase (PI3K) and Akt were evaluated by western blotting. Behavior of extracellular matrices, matrix metalloproteinase (MMP)-9, MMP-2, tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 were also evaluated by western blotting and zymography.

RESULTS: Although 100% OLT survived, 60% hepatectomy and 40% SOLT showed poor survival. Histopathological, immunohistological, biochemical and protein assays revealed that 60% hepatectomy, 100% OLT and 40% SOLT showed liver damage. PI3K and Akt were decreased in 60% hepatectomy and 40% SOLT. For protein expression, 40% SOLT showed differences in MMP-9, MMP-2 and TIMP-2. TIMP-1 showed differences in 60% hepatectomy and 40% SOLT. For protein activity, MMP-9 demonstrated significant differences in 60% hepatectomy, 100% OLT and 40% SOLT.

CONCLUSION: Under conditions with an insufficient liver remnant, prevention of OS-induced damage *via* the Akt/PI3K pathway may be key to improve the postoperative course. MMP-9 may be also a therapeutic target after surgery.

Key words: Free radicals; Akt; Phosphatidylinositol 3-kinase; Matrix metalloproteinase; Tissue inhibitors of metalloproteinase

Core tip: Although shear stress with portal hypertension and cold ischemia/warm reperfusion injury trigger the liver regeneration cascade after surgery, these injuries also cause fatal liver damage. Postoperative liver damage is still a critical matter in the field of liver surgery. Oxidative stress and extracellular matrices are important for liver regeneration after surgery and these may be important keys to overcome current problems in the field of liver surgery. Here, we investigated oxidative stress-mediated damage and the behavior of extracellular matrices in various rat models with liver surgery.

INTRODUCTION

Liver resection is considered the standard treatment for primary malignant tumors and liver metastases. Advanced surgical techniques for hepatectomy, development of preoperative evaluation, and improvements in intensive postoperative care have resulted in a decline in perioperative morbidity and mortality. However, postoperative liver failure still occurs despite these developments. Extended hepatectomy has the advantage of high curability but increases morbidity and mortality[1]. Insufficient volume of the remnant liver is correlated with perioperative morbidity and mortality[1]. Prognosis of postoperative liver failure due to insufficient liver remnant is poor[1,2].

Orthotopic liver transplantation (OLT) is an accepted therapy for end-stage liver disease and currently provides long-term survival and good quality of life. However, cold ischemia/warm reperfusion (CIWR) injury is still a major cause of morbidity and mortality after OLT[3]. Currently, strategic procedures are needed to improve the liver tolerance against CIWR injury. A small-for-size graft (SFSG) is used for deceased donor liver transplantation (DDLT) and living donor liver transplantation (LDLT)[4,5]. The SFSG is defined as a ratio of graft weight against standard liver volume < 40%[6,7]. An inevitable insufficiency of graft size cannot be avoided in the LDLT or split orthotopic liver transplantation (SOLT) for DDLT. The SFSG in LDLT or SOLT is accompanied with CIWR injury and shear stress with portal hypertension. Hence, the SFSG results in high mortality and morbidity. The choice of a left-side graft is preferred from the viewpoint of greater donor safety and expanded donor candidates in LDLT[7,8]. Guaranteed SOLT with successful outcomes resolves a donor shortage in DDLT[4,5]. Currently, the 40% SFSG is a critical matter to overcome the donor shortage in DDLT and ensure donor safety in LDLT[4].

Oxygen is required for cell survival. However, it also poses a potential hazard *via* reactive oxygen species (ROS) and reactive nitrogen species (RNS), with biological and functional alterations of lipids, proteins and DNA[9-11]. Control of ROS/RNS production plays physiological roles, especially in regulating cell signaling, cell proliferation, differentiation and apoptosis[9-11]. Oxidative stress (OS) mediated by free radicals is defined as an imbalance between the production of ROS/RNS and the antioxidant capacity of the cell[9-11].

The extracellular matrix has important effects on inflammation, carcinogenesis and regeneration[12-14]. There are diverse types of proteases that control remodeling of the extracellular matrix, trigger liver regeneration and drive tumor progression[12-14]. Matrix metalloproteinases (MMPs) are a family of enzymes that degrade constituents of extracellular matrices and basement membranes. Currently, a total of 28 MMPs have been identified[14]. MMPs have been intensively studied and shown to play key roles in inflammation, carcinogenesis and regeneration[12-15]. MMP-2 and MMP-9 are implicated in liver injury and remodeling. In particular, previous researchers reported that MMP-9 and MMP-2 contribute to liver failure after liver surgery[12-21]. Tissue inhibitors of metalloproteinases (TIMPs) are a family of endogenous inhibitors of MMPs. Alteration in the MMP-TIMP balance is linked to pathophysiological conditions[22,23]. Currently, four members have been identified in the TIMP family which can inhibit various MMPs[24]. In particular, many researchers have focused on TIMP-1 and TIMP-2 during liver regeneration[25-28].

Although shear stress with portal hypertension and CIWR injury trigger the liver regeneration cascade after liver surgery, these injuries also cause fatal liver damage[29-31]. Initial damage is confirmed at the early postoperative period after liver surgery[3,12,13,18,29-31]. Therapeutic strategies to reduce this damage have the advantage of improving clinical results after liver surgery and overcoming the current issue of insufficient liver volume in the field of liver surgery. In the present preliminary study, we investigated OS-mediated damage and the behavior of extracellular matrices in various rat models with shear stress and portal hypertension and/or CIWR injury.

MATERIALS AND METHODS

Animals

Lewis rats (RT-1^l) were purchased from Harlan Laboratories (Indianapolis, IN, United States). Male rats were 8-12 wk old and weighed 250 g. The experimental protocols were approved by the Ethical Committee of our institution (Mayo Clinic, Institutional Animal Care and Use Committee, No. A19609). Rats were cared for in accordance with the Institutional Guidelines for Animal Welfare based on The National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Surgical procedures and postoperative care

Comprehensive details of the surgical procedures for rat

Table 1 Study design			
Group	Hepatic remnant volume	Cold ischemia warm reperfusion	Shear stress portal hypertension
Control	100%, native liver	-	-
60%-hepatectomy	40%, native liver	-	+
100%-OLT	100%, syngeneic graft	+	-
40%-SOLT	40%, syngeneic graft	+	+

OLT: Orthotopic liver transplantation; SOLT: Split orthotopic liver transplantation.

and postoperative care in our institution have been previously described[32-34]. In the hepatectomy model, 40% of liver remnant consisted of the left median and lateral segments[32,33]. In the transplantation model, the syngeneic graft had a cold ischemic time of 3-4 h at 4 ℃ in normal Ringer's solution[33]. The 40% SFSG was also formed by the left median and lateral segments at the back table[34]. To avoid any irrelevant signaling, the hepatic artery was reconstructed by ultramicrosurgery[33]. Each rat was kept separately after surgery and body temperature was maintained by a heating pad. Postoperative observation was performed every 30 min until 6 h after surgery and 1.0 mL of warm lactate Ringer's solution was routinely administered every 1 h until 6 h after surgery. In the transplantation model, we previously demonstrated the importance of a shortened anhepatic phase and exclusion of unreliable samples based on autopsy findings[33,34]. In this study, the anhepatic phase was kept within 20 min in the transplantation model. No surgical complications were observed in each case at sampling autopsy.

Study design

Rats were divided into four groups according to the surgery performed: (1) laparotomy only (control); (2) hepatectomy with 40% liver remnant (60% hepatectomy); (3) OLT with whole liver graft (100% OLT); and (4) SOLT with 40% SFSG (40% SOLT) (Table 1). The survival study was performed on 10 rats in each group. Cell signaling involved in proliferation, differentiation and apoptosis was confirmed at the early postoperative period after liver surgery and subsequently progressive necrosis was observed, as described previously[3,12,13,18,29-31]. Serum and plasma were collected at 6 h after surgery (*n* = 5, in each group). Liver samples were also collected at 6 h after surgery for histopathological/immunohistological assessments, western blotting and gelatin zymography (*n* = 5, in each group).

Biochemical assays and coagulation profile

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (T-Bil), the international normalized ratio of prothrombin time (PT-INR) and hyaluronic acid (HA) were measured. Serum AST, ALT and T-Bil were assessed by commercial kits (SGOT, SGPT and total bilirubin reagent, respectively; Biotron, Hemet, CA, United States). The PT-INR in the plasma was measured by the i-STAT System (Abbott, Princeton, NJ, United States). Serum HA was measured using a commercial kit (Quantikine Hyaluronan ELISA Kit; R

and D Systems, Minneapolis, MN, United States).

Histopathological and immunohistological assessments

Liver tissue was fixed in 10% neutral-buffered formalin, embedded in paraffin, and sliced into 4-μm sections. The morphological characteristics and graft injury scores were assessed after hematoxylin-eosin (HE) staining. The graft damage score has been described previously[34]. Scores were counted in 10 fields (× 100 magnification) in each slide and these scores were averaged in each HE slide.

Induction of apoptosis was assessed by immunostaining of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) (ApopTag Peroxidase *In Situ* Apoptosis Detection Kit, S7100; Chemicon International, Billerica, MA, United States) and cysteine aspartic acid protease (caspase) 3 [Cleaved Caspase-3 (Asp175) Antibody, 9661S; Cell Signaling Technology, Danvers, MA, United States]. A TUNEL-positive nucleus was stained brown and a negative nucleus was counterstained light blue. A caspase-3-positive nucleus was stained brown and a negative nucleus was counterstained blue. Slides were scanned with an automated high-throughput scanning system (Scanscope XT, Aperio Technologies, Vista, CA, United States). To quantify the immunohistological findings, positive-stained nuclei were counted by Aperio Imagescope software (Aperio Technologies). All nuclei were classified into four color intensity levels and the higher two levels were considered as positive. The ratio of positive-stained nuclei to all nuclei was calculated and the mean ratio/mm^2 was determined.

Western blotting and gelatin zymography

The primary antibodies for malondialdehyde (MDA) (Anti-Malondialdehyde antibody, ab6463; Abcam, Cambridge, MA, United States); 4-hydroxynonenal (4-HNE) (4 Hydroxynonenal antibody, ab46545; Abcam); ataxia telangiectasia mutated kinase (ATM) (Phospho-ATM/ATR Substrate Rabbit mAb, 2909; Cell Signaling Technology); phosphorylated histone H2AX (γH2AX) (Phospho-Histone H2A.X Antibody, 2577; Cell Signaling Technology); phosphatidylinositol 3-kinase (PI3K) (Phospho-PI3K p85/p55 Antibody, 4228; Cell Signaling Technology); Akt (Phospho-Akt Rabbit mAb, 4058; Cell Signaling Technology); superoxide dismutase (SOD) (Cu/Zn Superoxide Dismutase, LS-B2907; LifeSpan Biosciences, Seattle, WA, United States); catalase (Catalase, LS-B2554; LifeSpan BioSciences); MMP-9 (Anti-MMP-9, Catalytic domain, AB19016; Millipore, Temecula, CA,

Figure 1 Survival curves. OLT: Orthotopic liver transplantation; SOLT: Split orthotopic liver transplantation.

United States); MMP-2 [MMP-2 antibody (MMP2/8B4), ab7032; Abcam]; TIMP-1 [Anti-TIMP-1 Mouse mAb (102D1), IM63; Calbiochem, San Diego, CA, United States]; and TIMP-2 [Anti-TIMP2 antibody (3A4), ab1828; Abcam] were used. Glyceraldehyde-3-phosphate dehydrogenase served as a control. Signals were quantified using ImageQuant 5.0 software (Molecular Dynamics, Sunnyvale, CA, United States). Gelatinase activity was visualized by fluorescence microscopy (Olympus BX50; Olympus Optical, Tokyo, Japan).

Statistical analysis

The results were presented as mean $\pm$ SD. Student's t test was used for the comparison of unpaired continuous variables between groups. Survival curves were constructed by the Kaplan-Meier method (Log-rank test). Statistical calculations were performed using SPSS version 16.0 (SPSS Inc., Chicago, IL, United States). $P < 0.05$ was considered statistically significant.

RESULTS

Survival curves

Survival curves for each group are shown in Figure 1. All rats that underwent a laparotomy or 100% OLT survived. The 60% hepatectomy and 40% SOLT groups clearly showed poorer survival than the controls ($P < 0.0001$). Insufficient liver remnant resulted in poor survivals after 60% hepatectomy. Especially, 40% SOLT showed very poor survivals.

Liver parenchymal damage

In comparison with the controls (0.1 $\pm$ 0.1 points), there were significant differences in the graft damage score for 60% hepatectomy (3.7 $\pm$ 0.7 points, $P < 0.0001$), 100% OLT (4.0 $\pm$ 0.6 points, $P < 0.0001$) and 40% SOLT (5.8 $\pm$ 1.1 points, $P < 0.0001$) (Figure 2A).

Immunohistological assessment of apoptosis induction

In comparison with the controls (0.003 $\pm$ 0.004), the rates of TUNEL-positive nuclei showed significant dif-

ferences in 60% hepatectomy (0.017 $\pm$ 0.009, $P = 0.0278$), 100% OLT (0.107 $\pm$ 0.012, $P = 0.0001$) and 40% SOLT (0.166 $\pm$ 0.052, $P < 0.0001$) (Figure 2B). In comparison with the controls (0.002 $\pm$ 0.002), the rates of caspase-3-positive nuclei revealed significant differences in 60% hepatectomy (0.044 $\pm$ 0.023, $P = 0.0033$), 100% OLT (0.063 $\pm$ 0.014, $P < 0.0001$) and 40% SOLT (0.115 $\pm$ 0.019, $P < 0.0001$) (Figure 2C).

Conventional liver function tests, coagulation profile and endothelial damage

In comparison with the controls (42.5 $\pm$ 8.6 U/L), AST levels showed significant differences in 60% hepatectomy (202.4 $\pm$ 41.9 U/L, $P < 0.0001$), 100% OLT (290.5 $\pm$ 31.9 U/L, $P < 0.0001$) and 40% SOLT (387.4 $\pm$ 36.8 U/L, $P < 0.0001$) (Figure 3A). In comparison with the controls (59.8 $\pm$ 9.6 U/L), ALT levels showed significant differences in 60% hepatectomy (213.8 $\pm$ 57.0 U/L, $P < 0.0001$), 100% OLT (309.4 $\pm$ 38.3 U/L, $P < 0.0001$) and 40% SOLT (392.2 $\pm$ 76.7 U/L, $P < 0.0001$) (Figure 3B). In comparison with the controls (0.41 $\pm$ 0.13 mg/dL), there were no significant differences in T-Bil levels in 60% hepatectomy (0.50 $\pm$ 0.26 mg/dL, $P = 0.4798$) and 100% OLT (0.58 $\pm$ 0.15 mg/dL, $P = 0.0801$), but there was in 40% SOLT (1.37 $\pm$ 0.29 mg/dL, $P = 0.0001$) (Figure 3C).

In comparison with the controls (0.99 $\pm$ 0.04), PT-INR values revealed significant differences in 60% hepatectomy (1.16 $\pm$ 0.09, $P = 0.0052$), 100% OLT (1.12 $\pm$ 0.04, $P = 0.0008$) and 40% SOLT (1.22 $\pm$ 0.06, $P < 0.0001$) (Figure 3D).

In comparison with the controls (76.6 $\pm$ 14.9 ng/mL), HA levels demonstrated significant differences in 60% hepatectomy (264.0 $\pm$ 58.8 mg/dL, $P = 0.0001$), 100% OLT (188.0 $\pm$ 29.0 mg/dL, $P < 0.0001$) and 40% SOLT (350.2 $\pm$ 136.6 mg/dL, $P = 0.0021$) (Figure 3E).

Oxidative stress

The western blotting intensities of MDA in each group are shown in Figure 4A. In comparison with the controls (1.00 $\pm$ 0.10), normalized MDA showed significant differences in 60% hepatectomy (1.64 $\pm$ 0.39, $P = 0.0074$), 100% OLT (2.12 $\pm$ 0.78, $P = 0.0133$) and 40% SOLT (2.30 $\pm$ 0.26, $P < 0.0001$) (Figure 4B).

Lipid peroxidation

In comparison with the controls (1.00 $\pm$ 0.09), normalized 4-HNE showed significant differences in 60% hepatectomy (1.30 $\pm$ 0.20, $P = 0.0152$), 100% OLT (1.41 $\pm$ 0.20, $P = 0.0028$) and 40% SOLT (1.40 $\pm$ 0.19, $P = 0.0032$) (Figure 4C).

Responses and repairs to DNA damage

In comparison with the controls (1.00 $\pm$ 0.098), normalized ATM showed significant differences in 60% hepatectomy (1.15 $\pm$ 0.09, $P = 0.0336$), 100% OLT (1.28 $\pm$ 0.10, $P = 0.0015$) and 40% SOLT (1.21 $\pm$ 0.09, $P = 0.0053$) (Figure 4D). In comparison with the controls (1.00 $\pm$ 0.17), normalized γH2AX showed significant differences

A

B

C

Figure 2 Histopathological and immunohistological assessments. A: Liver damage score in HE staining; B: TUNEL-positive rate; C: Caspase-3-positive rate. [a]P < 0.05 *vs* control. OLT: Orthotopic liver transplantation; SOLT: Split orthotopic liver transplantation.

in 60% hepatectomy (1.39 ± 0.29, P = 0.0071), 100% OLT (1.67 ± 0.38, P = 0.0303) and 40% SOLT (2.59 ± 0.66, P = 0.0008) (Figure 4E).

Promotion of cell survival
The western blotting intensities of PI3K and Akt in each group are shown in Figure 4F.

In comparison with the controls (1.00 ± 0.08), there was no significant difference in normalized PI3K in 100% OLT (0.92 ± 0.09, P = 0.1726), but there were significant differences in 60% hepatectomy (00.36 ± 0.11, P < 0.0001) and 40% SOLT (0.42 ± 0.19, P = 0.0002) (Figure 4G). In comparison with the controls (1.00 ± 0.12), there was no significant difference in normalized Akt in 100% OLT (0.92 ± 0.37, P = 0.6486), but there were significant differences in 60% hepatectomy (0.37 ± 0.23, P = 0.0007) and 40% SOLT (0.34 ± 0.24, P = 0.0006) (Figure 4H).

Activities of antioxidant enzymes
In comparison with the controls (1.00 ± 0.09), normalized SOD did not show significant differences in 60%

hepatectomy (0.97 ± 0.09, P = 0.6503), 100% OLT (0.96 ± 0.11, P = 0.5461) and 40% SOLT (0.87 ± 0.09, P = 0.0595) (Figure 4I). In comparison with the controls (1.00 ± 0.17), normalized catalase also revealed no significant differences in 60% hepatectomy (0.91 ± 0.11, P = 0.3665), 100% OLT (0.90 ± 0.15, P = 0.3365) and 40% SOLT (0.95 ± 0.14, P = 0.6454) (Figure 4J).

Behavior of MMP-9, MMP-2, TIMP-1 and TIMP-2
Protein expression and activity of MMP-9 are shown in Figure 5A. Protein expression was evaluated by western blot densitometry (Figure 5B-D). In comparison with the controls (1.00 ± 0.34), there were no significant differences in normalized MMP-9 in 60% hepatectomy (1.14 ± 0.43, P = 0.5811) and 100% OLT (1.18 ± 0.35, P = 0.4254), but there was a significant difference in 40% SOLT (2.16 ± 0.26, P = 0.0003) (Figure 5B). In comparison with the controls (1.00 ± 0.16), there were no significant differences in normalized MMP-2 in 60% hepatectomy (0.78 ± 0.17, P = 0.0716) and 100% OLT (0.80 ± 0.23, P = 0.1437), but there was a significant difference in 40% SOLT (0.78 ± 0.12, P = 0.0385) (Figure

Figure 3 Biochemical and coagulation profiles. A: Serum aspartate aminotransferase (AST); B: Serum alanine aminotransferase (ALT); C: Serum total bilirubin (T-Bil); D: Plasma international normalized ratio of prothrombin time (PT-INR); E: Serum hyaluronic acid (HA). [a]$P < 0.05$ *vs* control. NS: Not significant ($P \geqslant 0.05$); OLT: Orthotopic liver transplantation; SOLT: Split orthotopic liver transplantation.

5C). In comparison with the controls (1.00 ± 0.30), there was no significant difference in normalized TIMP-1 in 100% OLT (0.82 ± 0.43, $P = 0.4654$), but there were significant differences in 60% hepatectomy (1.41 ± 0.26, $P = 0.0491$) and 40% SOLT (1.46 ± 0.32, $P = 0.0486$) (Figure 5D). In comparison with the controls ($1.00 \pm$ 0.24), there were no significant differences in normalized TIMP-2 in 60% hepatectomy (1.23 ± 0.24, $P = 0.1605$) and 100% OLT (0.95 ± 0.17, $P = 0.6846$), but there was a significant difference in 40% SOLT (1.28 ± 0.12, $P = 0.0471$) (Figure 5E).

Protein activities were evaluated by intensity in zy-

Figure 4 Protein expression of malondialdehyde, 4-hydroxynonenal, ataxia-telangiectasia mutated kinas/H2AX, phosphatidylinositol 3-kinase/Akt and antioxidant enzymes. A: Actual intensities of malondialdehyde (MDA) in western blotting; B: Normalized MDA; C: Normalized 4-hydroxynonenal (4-HNE); D: Normalized ataxia-telangiectasia mutated kinase (ATM); E: Normalized γH2AX; F: Actual intensities of phosphatidylinositol 3-kinase (PI3K) and Akt in western blotting; G: Normalized PI3K; H: Normalized Akt; I: Normalized superoxide dismutase (SOD); J: Normalized catalase. [a]P < 0.05 *vs* control. NS: Not significant ($P \geqslant 0.05$); OLT: Orthotopic liver transplantation; SOLT: Split orthotopic liver transplantation.

mography (Figure 5F-I). In comparison with the controls (1.00 ± 0.15), relative MMP-9 clearly demonstrated significant differences in 60% hepatectomy (1.37 ± 0.23, P = 0.0156), 100% OLT (1.47 ± 0.33, P = 0.0211) and 40% SOLT (2.10 ± 0.75, P = 0.0125) (Figure 5F). In comparison with the controls (1.00 ± 0.17), relative MMP-2 did not reveal significant differences in 60% hepatectomy (1.03 ± 0.12, P = 0.7444), 100% OLT (0.98 ± 0.15, P = 0.8821) and 40% SOLT (1.04 ± 0.13, P = 0.6847) (Figure 5G). In comparison with the controls (1.00 ± 0.15), relative TIMP-1 did not reveal significant differences in 60% hepatectomy (0.96 ± 0.29, P = 0.7926), 100% OLT (0.98 ± 0.09, P = 0.8217) and 40% SOLT (0.91 ± 0.26, P = 0.5347) (Figure 5H). In comparison with the controls (1.00 ± 0.12), relative TIMP-2 did not show significant differences in 60% hepatectomy (1.04 ± 0.09, P = 0.5974), 100% OLT (1.03 ± 0.11, P = 0.6845) and 40% SOLT (1.03 ± 0.16, P = 0.7495) (Figure 5I).

Statistical differences between groups

As described above, the data in comparisons with the controls are shown. Statistical differences between groups are summarized in Table 2.

DISCUSSION

In survival and histopathological studies, 40% SOLT involved dual damage (*i.e.*, shear stress with portal hypertension and CIWR injury) and showed the poorest survival and most severe liver damage. Although 100% OLT showed good survival, CIWR injury was observed by histopathological and biochemical findings. Here, we used plasma PT-INR and serum HA levels as markers of sinusoidal endothelial damage and all groups showed significant differences. Survival in the 60% hepatectomy and 40% SOLT groups seemed to be higher than in

the 100% OLT group and this may reflect the damage induced by shear stress and portal hypertension. Our histopathological, immunohistological and biochemical findings revealed that liver damage and apoptotic induction were observed in the early postoperative period after liver surgery, as in previous studies[3,12,13,18,29-31]. Paradoxically, the early postoperative period may have a therapeutic potential for a subsequent course after liver surgery.

OS causes DNA damage and subsequent apoptosis and is an imbalance between production of free radicals and antioxidant defenses[9-11]. From the viewpoint of production of free radicals, ROS/RNS can attack and damage a variety of critical biological molecules, including lipids, essential cellular proteins and DNA[9-11]. Products of lipid peroxidation can be easily detected in biological fluids and tissues and can reliably and rapidly reflect the sensitive and specific signals of lipid peroxidation that occur *in vivo*[35,36]. The compound 4-HNE is an end product of lipoperoxidation with antiproliferative and proapoptotic properties[35,36]. Our results with MDA and 4-HNE confirmed that OS occurred even in the early postoperative period.

With regard to DNA damage responses, the protein kinase ATM can be initiated through rapid intermolecular autophosphorylation induced by DNA damage, phosphorylate various proteins and subsequently amplify the responses to DNA damage[36,37]. This DNA damage-inducible kinase activates H2AX[38]. H2AX is required for cell cycle arrest and DNA repair following double-stranded DNA breaks[38,39]. DNA damage results in the rapid phosphorylation of H2AX by ATM[38,40]. Within minutes of DNA damage, H2AX is phosphorylated at the sites of the DNA damage[38]. This early event in the DNA-damage response is required for the recruitment of many DNA-damage response proteins. Therefore, histone H2AX is activated by ATM after DNA dam-

Figure 5 Protein expression and activities of matrix metalloproteinases and tissue inhibitor of metalloproteinases. A: Actual protein expression and activities of matrix metalloproteinase (MMP)-9; B: Normalized MMP-9; C: Normalized MMP-2; D: Normalized tissue inhibitor of metalloproteinase (TIMP)-1; E: Normalized TIMP-2; F: Relative MMP-9; G: Relative MMP-2; H: Relative TIMP-1; I: Relative TIMP-2. $^a P < 0.05$ *vs* control. NS: Not significant ($P \geqslant 0.05$); OLT: Orthotopic liver transplantation; SOLT: Split orthotopic liver transplantation.

Table 2 Statistical differences between groups

	Control *vs* 60%-hepatectomy	Control *vs* 100%-OLT	Control *vs* 40%-SOLT	60%-hepatectomy *vs* 100%-OLT	60%-hepatectomy *vs* 40%-SOLT	100%-OLT *vs* 40%-SOLT
Survival rate	$P < 0.05$	NS	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
Liver damage score	$P < 0.05$	$P < 0.05$	$P < 0.05$	NS	$P < 0.05$	$P < 0.05$
TUNEL positive ratio	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
Caspase-3 positive ratio	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
AST	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
ALT	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	NS
T-Bil	NS	NS	$P < 0.05$	NS	$P < 0.05$	$P < 0.05$
PT-INR	$P < 0.05$	$p < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
HA	$P < 0.05$	$p < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
Western blotting						
MDA	$P < 0.05$	$P < 0.05$	$P < 0.05$	NS	$P < 0.05$	NS
4-HNE	$P < 0.05$	$P < 0.05$	$P < 0.05$	NS	NS	NS
ATM	$P < 0.05$	$P < 0.05$	$P < 0.05$	NS	NS	NS
γH2AX	$P < 0.05$	$P < 0.05$	$P < 0.05$	NS	$P < 0.05$	$P < 0.05$
PI3K	$P < 0.05$	NS	$P < 0.05$	$P < 0.05$	NS	$P < 0.05$
Akt	$P < 0.05$	NS	$P < 0.05$	$P < 0.05$	NS	$P < 0.05$
SOD	NS	NS	NS	NS	NS	NS
Catalase	NS	NS	NS	NS	NS	NS
MMP-9	NS	NS	$P < 0.05$	NS	$P < 0.05$	$P < 0.05$
MMP-2	NS	NS	$P < 0.05$	NS	NS	NS
TIMP-1	$P < 0.05$	NS	$P < 0.05$	$P < 0.05$	NS	$P < 0.05$
TIMP-2	NS	NS	$P < 0.05$	$P < 0.05$	NS	$P < 0.05$
Zymography						
MMP-9	$P < 0.05$	$P < 0.05$	$P < 0.05$	NS	$P < 0.05$	$p < 0.05$
MMP-2	NS	NS	NS	NS	NS	NS
TIMP-1	NS	NS	NS	NS	NS	NS
TIMP-2	NS	NS	NS	NS	NS	NS

OLT: Orthotopic liver transplantation; SOLT: Split orthotopic liver transplantation; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; T-Bil: Total bilirubin; PT-INR: International normalized ratio of prothrombin time; HA: Hyaluronic acid; MDA: Malondialdehyde; 4-HNE: 4-hydroxynonenal; ATM: Ataxia-telangiectasia mutated kinase; PI3K: Phosphatidylinositol 3-kinase; SOD: Superoxide dismutase; MMP: Matrix metalloproteinase; TIMP: Tissue inhibitor of metalloproteinase.

age[38]. Thus, the ATM/H2AX signaling pathway is important in the response to and repair of DNA damage induced by OS[38,41]. Our results with ATM and H2AX clearly showed that OS after liver surgery caused DNA damage signaling and triggered subsequent DNA repair. In this study, groups with only CIWR injury (*i.e.*, 100%

OLT) caused OS-induced damage and subsequent apoptotic process. However, this group showed differences not in PI3K/Akt, but in ATM/H2AX. These results suggested that CIWR injury induce apoptosis due to OS *via* the ATM/H2AX pathway.

Akt also plays a critical role in controlling apoptosis

and promotes cell survival to prohibit apoptosis[42-44]. Apoptotic machinery is inhibited by the activation of Akt[42-44]. Akt is an integral component of the antiapoptotic process related to the activation of PI3K[42-45]. Our results clearly showed that groups with accompanying shear stress and portal hypertension (*i.e.*, 60% hepatectomy and 40% SOLT) had decreased PI3K and Akt. This suggested that a subsequent apoptotic process was triggered in these groups. Shear stress and portal hypertension due to insufficient liver volume induce apoptosis due to OS *via* the Akt/PI3K pathway.

With regard to antioxidant defense, scavenging enzymes of free radicals, such as SOD and catalase, also play an important role in reducing DNA damage and subsequent apoptosis[10,11]. Cells are normally able to defend themselves against OS-induced damage through this scavenging system[10,11]. Our results revealed that this scavenging system did not appear to be triggered, although these scavenging enzymes can cope with large amounts of ROS[46]. Shear stress with portal hypertension and/or CIWR injury after liver surgeries in this study caused considerable liver damage. A possible explanation is that this scavenging system failed to stimulate some reactive molecules because of considerable damage after liver surgery.

MMPs have been intensively studied and shown to play key roles in inflammation, carcinogenesis and regeneration and many researchers have already focused on MMP-2 and MMP-9 after liver surgery[12-21]. In the present study, 40% SOLT increased protein expression of MMP-2 in western blotting, although zymography did not show any differences. Contrary to MMP-2, postoperative MMP-9 clearly showed differences in protein expression and function. Additionally, MMP-9 showed high reproducibility in our previous studies[20,47,48]. The present results for MMP-9 suggested that MMP-9 clearly increased even in the early postoperative period after liver surgery and MMP-9 is a major therapeutic target after liver surgery.

TIMPs are also important after liver surgery. Many researchers have focused on TIMP-1 and TIMP-2 during liver regeneration[25-28]. Some researchers have focused on postoperative behavior of TIMP-1[28]. In particular, TIMP-1 has extrahepatic effects during liver failure[23,49-52] and therefore we initially expected that TIMP-1 would show differences in the liver samples. However, zymography for TIMP-1 did not show any differences, although groups with shear stress and portal hypertension (*i.e.*, 60% hepatectomy and 40% SOLT) showed increased protein expression of TIMP-1 in western blotting. TIMP-1 is an endogenous inhibitor of MMP-9 and a balance of MMP-9/TIMP-1 is linked[22,23]. However, the behavior of TIMP-1 in the postoperative liver is still unclear and further studies are required.

Liver damage and apoptotic induction are confirmed even in the early postoperative period after liver surgery but liver injury triggers the liver regeneration cascade after surgery. Once hepatic failure occurs after liver surgery, this damage is usually intractable and fatal.

Therefore, the early postoperative period may be a suitable time for treatment to achieve a good postoperative course after liver surgery and our lab focused on OS-mediated damage and the behavior of extracellular matrices after liver surgery[20,48,51,53-56]. The inhibition of apoptotic induction due to OS *via* the ATM/H2AX pathway may be important for a strategy against CIWR injury, even in the condition of sufficient liver volume. Under conditions with insufficient liver remnant, the prevention of apoptotic induction due to OS *via* the Akt/PI3K pathway may be key to improving postoperative course. Also, MMP-9 may be a reliable therapeutic target, especially in the condition of CIWR injury with insufficient liver volume. We hope that our results will be informative for researchers in the hepatology field.

COMMENTS

Background
After liver surgery, shear stress with portal hypertension and cold ischemia/warm reperfusion injury trigger the liver regeneration cascade and also cause fatal liver damage.

Research frontiers
Changes and behaviors of oxidative stress and extracellular matrices are still unknown.

Innovations and breakthroughs
Here, the authors investigate the oxidative stress-mediated damage and the behavior of extracellular matrices after liver surgery in various rat models.

Applications
Under conditions with insufficient liver remnant, prevention of oxidative stress-induced damage *via* the Akt/PI3K pathway may be key to improve postoperative course. MMP-9 may be also a therapeutic target after liver surgery.

Terminology
Regulations for oxidative stress and MMP-9 may have a therapeutic potential, in order to resolve the current problems after liver surgery.

Peer review
This is a very interesting paper about the pathophysiology of hepatic failure after hepatectomy and liver transplantation.

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BRIEF ARTICLE

Disease dependent qualitative and quantitative differences in the inflammatory response to ascites occurring in cirrhotics

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Abstract

AIM: To assess differing patterns and levels of ascitic fluid cyctokine and growth factors exist between those with a high risk and low risk of spontaneous bacterial peritonitis (SBP).

METHODS: A total of 57 consecutive patients with ascites requiring a large volume paracentesis were studied. Their age, gender, specific underlying disease conditions were recorded after a review of their clinical records. Each underwent a routine assessment prior to their paracentesis consisting of a complete blood count, complete metabolic profile and prothrombin time/international normalized ratio (INR) determination. The ascitic fluid was cultured and a complete cell count and albumin determination was obtained on the fluid. In addition, blood and ascitic fluid was assessed for the levels of interleukin interleukin (IL)-1A, IL-1B, IL-2, IL-4, IL-8, IL-10, monocyte chemotactic protein (MCP)-1, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) utilizing the Randox Biochip platforms (Boston, MA). A serum-ascites gradient, for each cytokine and growth factor was calculated. The results are reported as mean ± SEM between disease groups with statistical analysis consisting of the student t-test (two tailed) with a P value of 0.05 defining significance.

RESULTS: No clinically important demographic or biochemical differences between the 4 groups studied were evident. In contrast, marked difference in the cytokine and growth factors levels and pattern were evident between the 4 disease groups. Individuals with alcoholic cirrhosis had the highest levels of IL-1A, IL-1B, IL-4, IFNγ. Those with malignant disease had the highest levels of IL-2. Those with hepatitis C virus (HCV) associated cirrhosis had the highest value for IL-6, IL-8, IL-10, MCP-1 and VEGF. Those with cardiac disease had the highest level of TNF-α and EGF. The calculated serum-ascites gradients for the cardiac and malignant disease groups had a greater frequency of negative values signifying greater levels of IL-8, IL-10 and MCP-1 in ascites than did those with alcohol or HCV disease.

CONCLUSION: These data document important differences in the cytokine and growth factor levels in plasma, ascitic fluid and the calculated plasma - ascites fluid gradients in cirrhotics requiring a large volume paracentesis. These differences may be important in determining the risk for bacterial peritonitis.

Core tip: Previous studies have examined factors relative to the pathogenesis of spontaneous bacterial peritonitis (SBP) in patients with cirrhosis of the liver. This study was designed to examine the role of cytokines in decompensated cirrhotics requiring a large-volume paracentesis for ascites management and to compare the biomarker responses present in both the plasma and ascitic fluid of cirrhotics of differing etiologies. Factors likely to represent protective cytokines associated with a reduced risk for SBP include epidermal growth factor, tumor necrosis factor-α, interleukin (IL)-1A, IL-8, and IL-10. Those are more likely to be associated with potential for SBP include: IL-1B, IL-4, monocyte chemotactic protein -1, and interfero-γ.

INTRODUCTION

Large-volume ascites occurring in cirrhotic patients has been shown to manifest an inflammatory response characterized by increased levels of cytokines, interleukins and several growth factors[1]. The pathophysiologic mechanisms responsible for the development of cirrhosis and ultimately decompensated cirrhosis vary as a function of the underlying hepatic disease[2,3]. These differences in pathophysiology may be reflected in the cytokine, interleukin and growth factor induced response that occurs. Moreover, depending upon the site of inflammatory cell activation, differences in plasma and ascitic fluid levels of inducible cytokines and growth factors may exist. These differences may explain in part an increase rate of bacterial translocation and subsequent spontaneous bacterial peritonitis (SBP) development.

Previous studies have reported that cytokine characteristics of the Th1 response are increased in decompensated cirrhosis especially with infection[4]. Interleukin (IL)-4 which is a major cytokine of the Th2 response was not significantly different between decompensated cirrhotic patients with infected or non-infected ascites[4]. The current study was designed to confirm these findings and expanding it to study the role of growth factors in cirrhotics with non-infected ascites.

The aim of this investigation was: (1) to identify and quantitate the plasma and ascitic fluid biomarkers of inflammation in decompensated cirrhotics requiring a large-volume paracentesis for ascites management and (2) to compare and contrast the biomarker responses present in both the plasma and ascitic fluid of cirrhotics of differing etiologies.

MATERIALS AND METHODS

Subjects

A total of 57 consecutive cirrhotics requiring a large-volume paracentesis for clinical reasons were studied. Their age, gender and the specific disease etiology for their cirrhosis was determined by a review of their clinical records and, when necessary additional clinical testing procedures. Four distinct etiologic groups of cirrhotics were identified and the cytokine levels were compared between groups in an effort to examine the role of the etiologic factor responsible for cirrhosis in each subgroup.

Inclusion and exclusion criteria

Inclusion criteria: (1) cirrhosis documented by imaging (either an abnormal CT or US) or liver biopsy; (2) ascites requiring a large volume paracentesis because of tense ascites and failure to control the ascites with diuretics (furosemide and spironolactone); and (3) willingness to undergo a large volume paracentesis and sign an informed written consent documenting their participation and allowing for the additional studies required as a result of their participation.

Exclusion criteria: (1) no evidence for cirrhosis; (2) no ascites or adequate ascites control with diuretics; and (3) unwillingness to participate and sign an informed written consent.

Investigations

Each subject had the following routine laboratory studies determined: complete blood count, complete metabolic profile consisting of blood urea nitrogen, creatinine, glucose, total bilirubin, alkaline phosphatase, aspartate and alanine aminotransferases, total protein, albumin, and prothrombin time/INR. Each patient had a calculated Child-Turcotte-Pugh (CTP) score and the following studies were obtained on their ascitic fluid: cell counts for red blood cells, white blood cells and differential, albumin and ascitic fluid cultures. In addition to these routine measures, the plasma and ascitic fluid of each subject was assayed for a panel of biomarkers of inflammation to include the following: procalcitonin, IL-1A, IL-1B, IL-2, IL-4, IL-8, IL-10, monocyte chemotactic protein (MCP)-1, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF). Procalcitonin was assayed utilizing the BioMerieux Vidas assay (Lombard, Illinois). The interleukins, inflammatory cytokines and growth factors were assayed utilizing Randox Biochip assay Platforms (Boston, Massachusetts). All results were compared to that of a normal human plasma panel utilized as a control sample which was obtained commercially from Bioreclamation, LLC (Liverpool, NY, United States). In addition, the levels of the analytes

Table 1 Characteristics of the 57 subjects' studies and of procalcitonin cytokines, and growth factors in plasma (mean ± SEM)

Parameter	ETOH	HCV	Malignancy	Cardiac
n	25	20	8	4
Male/female	18/7	14/6	5/3	2/2
CTP score	9.1 ± 0.2	8.2 ± 0.1	8.1 ± 0.1	8.1 ± 0.1
Laboratory tests				
Creatinine (mg/dL)	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.3	1.3 ± 0.2
Prothrombin time (s)	14.0 ± 0.4	13.8 ± 1.0	13.6 ± 0.2	12.5 ± 0.2
Total bilirubin (mg/dL)	1.4 ± 0.1	1.2 ± 0.2	1.4 ± 0.2	1.9 ± 0.3
Albumin (g/dL)	3.1 ± 0.2	3.2 ± 0.2	3.0 ± 0.4	3.2 ± 0.3
PCT	0.375 ± 0.215	0.440 ± 0.230	0.954 ± 0.242	0.092 ± 0.70
IL-1A	0.26 ± 0.146	0.160 ± 0.070	0.182 ± 0.106	0.135 ± 0.065
IL-1B	4.710 ± 2.252	1.747 ± 0.800	1.982 ± 0.106	1.610 ± 0.990
IL-2	2.498 ± 1.333	1.203 ± 0.548	2.690 ± 1.905	1.025 ± 0.375
IL-4	3.157 ± 1.429	2.580 ± 1.005	1.508 ± 0.422	1.430 ± 0.090
IL-6	83.791 ± 47.204	164.430 ± 70.891	105.392 ± 60.511	129.700 ± 112.500
IL-8	92.790 ± 44.935	334.513 ± 184.222	104.165 ± 61.670	16.415 ± 6.815
IL-10	1.335 ± 0.454	1.620 ± 0.779	1.117 ± 0.297	1.415 ± 0.615
MCP-1	111.139 ± 17.746	326.407 ± 137.768	116.052 ± 32.101	88.350 ± 35.150
IFNγ	1.148 ± 0.650	0.303 ± 0.058	0.712 ± 0.201	0.725 ± 0.525
TNF-α	3.887 ± 1.218	5.843 ± 2.248	4.805 ± 1.304	13.600 ± 11.80
EGF	37.451 ± 11.642	92.453 ± 42.231	70.690 ± 36.431	126.00 ± 35.150
VEGF	11.658 ± 4.419	194.347 ± 130.788	20.523 ± 7.739	41.470 ± 32.870

No significant difference between any of these groups $P > 0.05$. HCV: Hepatitis C virus; PCT: Procalcitonin cytokines; IL: Interleukin; MCP-1: Monocyte chemotactic protein-1; IFN-γ: Interferon-γ; TNF-α: Tumor necrosis factor-α; EGF: Epidermal growth factor; VEGF: Vascular endothelial growth factor.

present in plasma were compared against those in the patients' ascitic fluid. Plasma- Ascites gradients were calculated for each analyte and the mean for each disease group was calculated.

Human research approval

The IRB of Cook County Health and Hospital System approved this study prior to its initiation. Each subject signed an informed written consent before their participation in the study. Moreover, the Cook County Health and Hospital System funded the study in its entirety.

Statistical analysis

The mean and standard error of the mean for each parameter was determined and the differences between the means of the various disease groups studies were calculated utilizing the students *t*-test. A *P* value < 0.05 was considered to be significant.

RESULTS

The characteristics of the 57 subjects studied are shown in Table 1. No clinically important differences between the 4 disease groups studied were evident. In contrast, the procalcitonin levels in plasma varied substantially between groups with the greatest values being present in the group with malignancy (Table 1). The lowest procalcitonin values were seen in those with cardiac cirrhosis. The alcoholic and hepatitis C positive groups had plasma procalcitonin levels that were midway between these two extremes (Table 1).

The ascitic fluid procalcitonin levels mirrored the plasma levels with the greatest values being found in the group with malignancy and the lowest levels being pres-

ent in those with cardiac disease. Again the other two groups had values midway between these two extremes (Table 2). Interestingly, however the alcoholic subgroup had an ascitic fluid procalcitonin value that was greater than that of the hepatitis C positive subgroup such that the relative position of the procalcitonin level in the two subgroups was reversed as compared to that found in plasma (Tables 1 and 2).

The cytokine and growth factor values varied markedly between groups for each parameter studied (Tables 3 and 4). The mean levels of the various factors measured in plasma aligned from the highest to the lowest for each disease group is reported in Table 3. Individuals with alcoholic liver disease had the highest IL-1a, IL-1b, IL-4 and interferon gamma levels. Individuals with malignant the liver disease had the highest values for IL-2. Those with hepatitis C had the highest levels IL-6, IL-8, IL-10, MCP-1 and VEGF.

The mean values for the ascitic fluid levels of the same 12 factors aligned from the highest to the lowest is presented in Table 4. The cardiac group had the greatest values for 5 of the 12 factors measured followed by the alcoholic group with 3 and the other 2 groups with 2 each. The malignancy group at the lowest value for 5 factors followed by the cardiac group with 3 and the other 2 groups with 2 each. Because of the variability in the measured values, the groups did not differ statistically, but when one examines the mean values per se considerable differences are seen between the various groups with mean plasma values ranging from 1.5-20 times the values of the lowest value for each parameter (Table 4). Similarly, when one examines the mean values in the ascitic fluid, the range of values for a given factor between groups ranged from 1.3-10 times the value

Table 2 Procalcitonin, cytokines and growth factors in the ascitic fluid (mean ± SEM)				
	ETOH	**HCV**	**Malignancy**	**Cardiac**
n	25	20	8	4
PCT	0.221 ± 0.129	0.125 ± 0.115	0.647 ± 0.497	0.043 ± 0.033
IL-1A	0.168 ± 0.042	0.200 ± 0.058	0.153 ± 0.052	0.700 ± 0.400
IL-1B	7.479 ± 4.813	4.3 ± 1.193	4.900 ± 1.021	5.000 ± 0.300
IL-2	1.368 ± 1.628	0.667 ± 0.067	1.990 ± 1.044	0.650 ± 0.050
IL-4	9.268 ± 1.628	15.833 ± 4.932	6.533 ± 0.984	10.555 ± 4.85
IL-6	687.177 ± 30.115	807.764 ± 0.867	707.525 ± 54.339	790.05 ± 39.750
IL-8	338.015 ± 91.838	329.567 ± 91.926	229.200 ± 105.057	718.25 ± 80.150
IL-10	74.686 ± 39.663	15.200 ± 3.5	9.043 ± 3.876	76.500 ± 44.700
MCP-1	919.608 ± 41.636	537.600 ± 88.357	576.728 ± 206.393	421.900 ± 82.6
IFNγ	2.114 ± 0.671	0.467 ± 0.267	1.488 ± 0.838	0.2 ± 0.1
TNF-α	22.818 ± 8.882	30.433 ± 20.055	9.672 ± 1.786	70.950 ± 61.750
EGF	1.318 ± 0.345	0.967 ± 0.167	2.407 ± 0.465	4.9 ± 3.4
VEGF	54.383 ± 12.143	129.733 ± 63.589	689.12 ± 499.836	119.450 ± 117.950

No significant difference between any of these groups $P > 0.05$. HCV: Hepatitis C virus; PCT: Procalcitonin cytokines; IL: Interleukin; MCP-1: Monocyte chemotactic protein-1; IFN-γ: Interferon-γ; TNF-α: Tumor necrosis factor-α; EGF: Epidermal growth factor; VEGF: Vascular endothelial growth factor.

Table 3 Mean levels of the plasma factors aligned from the highest to the lowest for each disease group			
Highest values → Lowest values			
IL-1A	ETOH	Malignancy	HCV	Cardiac
	0.260	0.182	0.160	0.135
IL-1B	ETOH	Malignancy	HCV	Cardiac
	4.710	1.082	1.747	1.61
IL-2	Malignancy	ETOH	HCV	Cardiac
	2.690	2.498	1.203	1.025
IL-4	ETOH	HCV	Malignancy	Cardiac
	3.157	2.580	1.508	1.43
IL-6	HCV	Cardiac	Malignancy	ETOH
	164.430	129.700	109.392	83.791
IL-8	HCV	Malignancy	ETOH	Cardiac
	334.513	104.265	92.790	16.415
IL-10	HCV	Cardiac	ETOH	Malignancy
	1.620	1.415	1.335	1.117
MCP-1	HCV	Malignancy	ETOH	Cardiac
	326.407	116.052	111.130	88.35
IFN-γ	ETOH	Cardiac	Malignancy	HCV
	1.148	0.725	0.712	0.303
TNF-α	Cardiac	HCV	Malignancy	ETOH
	13.600	5.843	4.805	3.887
EGF	Cardiac	HCV	Malignancy	ETOH
	126.000	92.453	70.690	37.451
VEGF	HCV	Cardiac	Malignancy	ETOH
	194.347	41.470	20.523	11.658

HCV: Hepatitis C virus; IL: Interleukin; MCP-1: Monocyte chemotactic protein-1; IFN-γ: Interferon-γ; TNF-α: Tumor necrosis factor-α; EGF: Epidermal growth factor; VEGF: Vascular endothelial growth factor.

of the lowest value (Table 5). The plasma-ascitic fluid gradients for each parameter were determined and are reported in (Table 5). A positive value for the plasma-ascitic fluid gradient identifies those factors wherein the plasma level was greater than the ascitic fluid level. In contrast, a negative value for the plasma-ascitic fluid gradient identifies those factors wherein the greater value was present in the ascitic fluid. A positive value suggests that the cytokine assayed arose from a systemic response while a negative value suggests that the response arose primarily in the abdominal cavity and that either a peritoneal or mesenteric origin for the cytokine.

DISCUSSION

This study extends the finding of an earlier study evaluating cytokine, and growth factor levels in the plasma and ascitic fluid of cirrhotics[1]. In both studies, the inflammatory cytokines IL-4, IL-6, IL-8, IL-10, TNF-α and MCP-1 have been shown to be increased in both the ascitic fluid and plasma of cirrhotics with large volume ascites. The present study performed in a completely different and slightly larger patient population extends the earlier study by documenting differences in the cytokine profiles based on the individuals underlying disease etiol-

Table 4 Mean values of the various factors in the ascitic fluid aligned from the highest to the lowest

	Highest values → Lowest values			
IL-1A	Cardiac	HCV	ETOH	Malignancy
	0.700	0.200	0.168	0.153
IL-1B	ETOH	Cardiac	Malignancy	HCV
	7.479	5.000	4.900	4.300
IL-2	Malignancy	ETOH	HCV	Cardiac
	1.990	1.368	0.667	0.650
IL-4	HCV	Cardiac	ETOH	Malignancy
	15.833	10.555	9.268	6.533
IL-6	HCV	Cardiac	Malignancy	ETOH
	807.764	790.050	707.525	687.177
IL-8	Cardiac	ETOH	HCV	Malignancy
	718.250	338.015	329.567	229.200
IL-10	Cardiac	ETOH	HCV	Malignancy
	76.500	74.686	15.200	9.043
MCP-1	ETOH	Malignancy	HCV	Cardiac
	919.608	576.728	537.600	421.900
IFN-γ	ETOH	Malignancy	HCV	Cardiac
	2.114	1.488	0.467	0.200
TNF-α	Cardiac	HCV	ETOH	Malignancy
	70.950	30.433	22.818	9.672
EGF	Cardiac	Malignancy	ETOH	HCV
	4.900	2.407	1.318	0.967
VEGF	Malignancy	HCV	Cardiac	ETOH
	689.120	129.733	119.456	54.383

HCV: Hepatitis C virus; IL: Interleukin; MCP-1: Monocyte chemotactic protein-1; IFN-γ: Interferon-γ; TNF-α: Tumor necrosis factor-α; EGF: Epidermal growth factor; VEGF: Vascular endothelial growth factor.

Table 5 Mean plasma- ascitic cytokine gradients segregated by disease etiology

	HCV	ETOH	Malignancy	Cardiac
IL-1A	-0.040	0.092	0.029	-0.565
IL-1B	-2.553	-2.769	1977.100	-3.390
IL-2	0.540	1.530	0.700	0.875
IL-4	-13.253	-6.111	-5.025	-9.125
IL-6	-643.337	-603.386	-602.133	-639.35
IL-8	-5.054	-245.225	-125.035	-701.835
IL-10	-13.580	-73.331	-7.926	-75.085
MCP-1	-201.193	-303.469	-460.673	-333.35
IFN-γ	-0.164	-0.966	-0.776	0.525
TNF-α	-24.590	-18.931	-4.777	57.050
EGF	96.486	36.176	-68.283	121.100
VEGF	64.614	-42.725	-668.649	-77.980

No significant difference between any of these groups $P > 0.05$. HCV: Hepatitis C virus; IL: Interleukin; MCP-1: Monocyte chemotactic protein-1; IFN-γ: Interferon-γ; TNF-α: Tumor necrosis factor-α; EGF: Epidermal growth factor; VEGF: Vascular endothelial growth factor.

ogy for the cirrhosis[1]. The current data suggests therefore that the pathophysiologic responses to the various hepatic disease etiologies in some way may determine, at least in part, the innate immune responses that occur and account for the differences in the cytokine and growth factor levels in the ascitic fluid and plasma[2,3]. The IL-6 and MCP-1 levels were universally increased in all four cirrhotic groups. VEGF levels were increased most markedly in those with malignancy and to a lesser degree in those with cardiac and alcohol induced disease. Individuals with cirrhosis due to hepatitis C had the lowest VEGF levels. In contrast, the hepatitis C positive group had the greatest levels of IL-4 present in both plasma and ascitic fluid.

The finding of an increase in VEGF levels in cirrhotics with malignancy is interesting but not particularly surprising as malignant disorders are known to be associated with increased VEGF levels[5,6]. The increase of VEGF levels in cardiac and alcohol induced liver disease is surprising and differs markedly from that seen in those with hepatitis C. This observation is consistent with the data reported in other studies wherein increased organ remodeling has been observed in individuals with cardiac and alcohol related disease but not so in those with hepatitis C virus[2,3].

These data also support the role of the peritoneal based immune response in the pathogenesis of both bacterial translocations spontaneous bacterial peritoni-

tis[1,7-14]. More specifically, they are consistent with the clinical observations that spontaneous bacterial peritonitis occurs less frequently in patients with cardiac and malignant ascites as contrasted to those with alcoholic liver disease and chronic viral induced liver disease.

As shown in Table 4, cardiac disease associated ascites has the highest ascetic fluid levels of IL-1A, IL-8, IL-10, TNF-α, and EGF. Conversely, the cardiac disease associated ascites has the lowest levels of IL-2 and MCP-1. Those with malignancy associated ascites have the highest levels of IL-2 and VEGF and the lowest levels of IL-1A, IL-4, IL-8, IL-10, and TNF-α.

The present findings for these two distinct etiologic groups suggest that the ascitic fluid immune response manifested in the ascitic fluid may account in some way for the lower rate of spontaneous bacterial peritonitis in individuals with ascites due to these two unique causes of cirrhosis.

Factors likely to represent protective cytokines associated with a reduced risk for SBP include EGF, TNF-α, IL-1A, IL-8, and IL-10. Those are more likely to be associated with potential for SBP include: IL-1B, IL-4, MCP-1, and IFN-γ (Table 4).

The data shown in Table 5 consisting of the serum-ascites gradient enables one to determine whether the primary source of the measured factor arose from the vascular space or the peritoneal cavity. Specifically, those with the positive value identify a primary vascular source of the measured factor while a negative value identifies these factors having their origin in the peritoneal cavity.

In summary, the present data suggest the well-recognized factors that include a reduced plasma oncotic pressure, increased splanchnic venous congestion and pressure, increased vascular permeability and an overwhelmed lymphatic mechanism for removing ascitic fluid account substantially for the development of clinical ascites. They suggest that unique immune related responses that differ between various hepatic disease states may also contribute to the development of ascites and the likelihood of developing spontaneous bacterial peritonitis. Further, these data suggest further that a better understanding of the different immune response characteristics present in cirrhotics of different etiologies may enable disease specific modulation of the immune response in each and thereby contribute to the development of improved therapies that control not only to the development of ascites but also overall disease progression.

COMMENTS

Background
Large-volume ascites occurring in cirrhotic patients has been shown to manifest an inflammatory response characterized by increased levels of cytokines, interleukins and several growth factors. The pathophysiologic mechanisms responsible for the development of cirrhosis and ultimately decompensated cirrhosis vary as a function of the underlying hepatic disease.

Research frontiers
This study extends the finding of an earlier study evaluating cytokine, and growth factor levels in the plasma and ascitic fluid of cirrhotics.

Innovations and breakthroughs
The current data suggests therefore that the pathophysiologic responses to the various hepatic disease etiologies in some way may determine, at least in part, the innate immune responses that occur and account for the differences in the cytokine and growth factor levels in the ascitic fluid and plasma.

Applications
The authors suggest that unique immune related responses that differ between various hepatic disease states may also contribute to the development of ascites and the likelihood of developing spontaneous bacterial peritonitis.

Peer review
The authors performed plasma and ascitic cytokines among various etiologies of cirrhosis.

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BRIEF ARTICLE

Association between inherited monogenic liver disorders and chronic hepatitis C

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Abstract

AIM: To determine the frequencies of mutations that cause inherited monogenic liver disorders in patients with chronic hepatitis C.

METHODS: This study included 86 patients with chronic hepatitis C (55 men, 31 women; mean age at diagnosis, 38.36 ± 14.52 years) who had undergone antiviral therapy comprising pegylated interferon and ribavirin. Viral load, biochemical parameter changes, and liver biopsy morphological data were evaluated in all patients. The control group comprised 271 unrelated individuals representing the general population of Latvia for mutation frequency calculations. The most frequent mutations that cause inherited liver disorders [gene (mutation): *ATP7B* (H1069Q), *HFE* (C282Y, H63D), *UGT1A1* (TA)7, and *SERPINA1* (PiZ)] were detected by polymerase chain reaction (PCR), bidirectional PCR allele-specific amplification, restriction fragment length polymorphism analysis, and sequencing.

RESULTS: The viral genotype was detected in 80 of the 86 patients. Viral genotypes 1, 2, and 3 were present in 61 (76%), 7 (9%), and 12 (15%) patients, respectively. Among all 86 patients, 50 (58%) reached an early viral response and 70 (81%) reached a sustained viral response. All 16 patients who did not reach a sustained viral response had viral genotype 1. Case-control analysis revealed a statistically significant difference in only the H1069Q mutation between patients and controls (patients, 0.057; controls, 0.012; odds ratio, 5.514; 95%CI: 1.119-29.827, $P = 0.022$). However, the H1069Q mutation was not associated with antiviral treatment outcomes or biochemical indices. The (TA)7 mutation of the *UGT1A1* gene was associated with decreased ferritin levels (beta regression coefficient = -295.7, $P = 0.0087$).

CONCLUSION: Genetic mutations that cause inherited liver diseases in patients with hepatitis C should be studied in detail.

Key words: Hepatitis C; Hepatolenticular degeneration (Wilson's disease); *ATP7B*; *SERPINA1*; *UGT1A1*; *HFE*

Core tip: This is the first study to evaluate the association between hepatitis C and the most frequently inherited monogenic liver diseases (hereditary hemochromatosis, alpha-1 antitrypsin deficiency, Gilbert's syndrome, and Wilson's disease) and their causative mutations. This case-control study revealed an association between hepatitis C and the mutation that causes Wilson's disease. In addition, biochemical data analysis

revealed an association between hepatitis C and the mutation that causes Gilbert's syndrome.

INTRODUCTION

Hepatitis C is an infectious disease caused by the hepatitis C virus (HCV), which primarily affects the liver. An estimated 130 to 200 million people worldwide are infected with HCV[1]. The most common monogenic inborn errors of metabolism associated with liver disease are hereditary hemochromatosis, alpha-1 antitrypsin deficiency, Wilson's disease, and Gilbert's syndrome. These diseases have a particularly high frequency in Northern Europe and Latvia[2-4]. Hereditary hemochromatosis is characterized by excessive iron overload and is most commonly caused by *HFE* gene mutations[2]. The frequency of the most common *HFE* mutation, C282Y, is 0.035 in Latvia and 0.026 Lithuania; however, the frequency of hereditary hemochromatosis is lower at 0.013[5]. Alpha-1 antitrypsin deficiency is caused by the absence of the proteinase inhibitor alpha-1 antitrypsin, and affected patients develop liver disease and emphysema in the third or fourth decade of life[3]. Wilson's disease is a progressive autosomal recessive disorder of copper metabolism. The carrier frequency of the causative mutation is 1:80 in Latvia and 1:90 in Europe[4]. Finally, Gilbert's syndrome is characterized by benign unconjugated hyperbilirubinemia with a frequency of 5.0% to 14.8% in Europe[6]. Most reports on the coexistence of monogenic liver diseases and HCV infection have focused primarily on hereditary hemochromatosis[7] because elevated iron levels are necessary for viral replication[8,9]. Although the associations of HCV infection with alpha-1 antitrypsin deficiency[10,11] and Gilbert's syndrome[12-14] have been investigated, the association of HCV infection with Wilson's disease remains unclear. Copper reportedly plays a potential role in the development of HCV infection[15,16].

The aim of the present study was to determine the frequency of mutations that cause inherited monogenic liver disorders in patients with chronic HCV infection who have undergone antiviral therapy and in whom the viral response status is known.

MATERIALS AND METHODS

Ethics

This study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the *Central Medical Ethics Committee* of *Latvia*. All study participants signed an informed consent form that was issued according to the regulations of the *Central Medical Ethics Committee* of *Latvia*.

Subjects

Eighty-six patients with HCV infection who had undergone antiviral treatment with ribavirin and pegylated interferon were included in this study. These patients comprised 55 men and 31 women with a mean age at diagnosis of 38.36 ± 14.52 years (men, 37.27 ± 15.69 years; women, 40.25 ± 12.26 years). All patients were of European descent. The pretherapeutic alanine transaminase level, iron level, ferritin level, viral load, and HCV genotype were evaluated in all patients. The Knodell histology activity index was used for morphological examination.

The control group comprised 271 unrelated individuals chosen to represent the general population of Latvia. Participants in the control group underwent polymorphism frequency determination only. Biochemical association analysis, clinical examination, and exclusion of HCV infection were not performed in this group.

Genotyping methods

Peripheral blood genomic DNA was purified by standard phenol: chloroform extraction and ethanol precipitation with slight modification as described elsewhere[17] using reagents from Sigma Aldrich, Inc. (St. Louis, MO, United States). A summary of the methods used in this study is presented in Table 1[4,18-20]. Reagents used for polymerase chain reaction (PCR) (buffers, dNTP mix, Taq polymerase, and agarose) were obtained from Thermo Fisher Scientific (Waltham, MA, United States). Synthetic oligonucleotides, the sequences of which have been previously published[4,18-20], were obtained from Metabion GmbH (Martinsried, Germany). Fluorescent PCR products were analyzed with an ABI Prism 310 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, United States) using the reagents described in the manufacturer's protocol.

Statistical analysis

PLINK software[21] was used for genotyping data analysis and quality control. Analysis adhered to a call rate of < 98% and Hardy-Weinberg equilibrium P value of $\leqslant$ 0.05. The chi-square test was used to compare the patient and control groups with a significance threshold of $P < 0.05$. SPSS software v.16.0 (SPSS Inc., Chicago, IL, United States) was used to compare mean biochemical marker values between the patient and control groups and between the two patient groups [with and without a sustained viral response (SVR), defined as the inability to detect viral RNA six months after therapy][22]. Parametric values were compared using ANOVA, and nonparametric data were evaluated with the Mann-Whitney test. Genotype association analysis with biochemical markers was conducted using a full linear model comprising

Table 1 Genotyping methods used in the present study

Disease	Gene	Mutation	rs[1]	Analysis method
Hereditary hemochromatosis	*HFE*	C282Y	rs1800562	PCR-RFLP with restrictase *Rsa*I[18]
		H63D	rs1799945	PCR-RFLP with restrictase *Mbo*I[18]
Gilbert's syndrome	*UGT1A1*	(TA)7, UGT1A1*28	rs8175347	Fluorescent PCR[20]
Alpha-1 antitrypsin deficiency	*SERPINA1*	PIZ	rs28929474	Bi-PASA[19]
Wilson's disease	*ATP7B*	H1069Q	rs76151636	Bi-PASA[4]

[1]Single nucleotide polymorphism database number (http://www.ncbi.nlm.nih.gov/snp/). PCR: Polymerase chain reaction; RFLP: Restriction fragment length polymorphism analysis; Bi-PASA: Bidirectional PCR allele-specific amplification.

Table 2 Allelic frequencies in patient and control groups

Gene	rs[1]	Mutation	Patients (*n* = 86)	Controls (*n* = 271)	OR	95%CI	*P* value
UGT1A1	rs8175347	(TA)7	0.371	0.350	1.098	0.643-1.871	0.796
HFE	rs1799945	C282Y	0.048	0.035	1.420	0.358-5.218	0.522
	rs1800562	H63D	0.096	0.121	0.740	0.301-1.760	0.563
ATP7B	rs76151636	H1069Q	0.057	0.012	5.514	1.119-29.827	0.022
SERPINA1	rs28929474	PIZ	0.012	0.016	0.363	0.013-5.158	0.576

[1]Single nucleotide polymorphism database number (http://www.ncbi.nlm.nih.gov/snp/). OR: Odds ratio.

three genetic effects: additive effects of allele dosage, dominance deviation from additivity (a negative value indicates a recessive allele), and the 2-df joint test of both additive and dominance. Beta was evaluated as the regression coefficient. Data were accepted as statistically significant at a *P* value of < 0.05. Sex and age (with confirmed HCV infection) were used separately as covariates. The adjusted beta coefficient and *P* value were applied for each covariate. The association of the HCV genotype with the therapy response was assessed using the χ^2 test.

RESULTS

Viral RNA was not detectable in 50 (58%) of the 86 patients (30 men, 20 women) in the third month of antiviral therapy. Sixteen (19%) patients (13 men, 3 women) did not reach an SVR. The HCV genotype was determined in 80 patients; genotypes 1, 2, and 3 were present in 61, 7, and 12 patients, respectively. All patients who did not reach an SVR had viral genotype 1; for this reason, the odds ratio (OR) and 95%CI were not calculated (*P* = 0.015).

Genetic marker analysis revealed a significantly higher frequency of the *ATP7B* H1069Q mutation in patients than in controls (0.057 *vs* 0.012, respectively; OR = 5.514; 95%CI: 1.119-29.827, *P* = 0.022). Further results of the genetic marker analysis are shown in Table 2.

The presence of inherited liver disease was confirmed in nine patients (eight had Gilbert's syndrome with genotype (TA)7/(TA)7, and one had hereditary hemochromatosis with genotype C282Y/H63D). The presence of inherited liver disease was confirmed in 30 (11%) controls; all had Gilbert's syndrome.

In the comparison of patients who had reached an SVR with those who had viral persistence (*i.e.*, nega-

tive response to treatment), a significant association was found between the iron level and the presence of viral persistence (Table 3). Neither the other biochemical markers nor the histology activity index showed statistically significant differences between the patient and control groups.

In the patient group, association analysis was performed between genetic markers and the biochemical markers alanine transaminase level, ferritin level, iron level, and viral load. A statistically significant association was found only between the ferritin level and the (TA)7 allele of the *UGT1A1* gene. The strongest model for the association of the *UGT1A1* gene with the ferritin level was dominance deviation from additivity (beta = -295.7, *P* = 0.0087), and the statistical significance remained after adjusting for age (beta$_{adjusted}$ = -264.4, *P*$_{adjusted}$ = 0.0219) and sex (beta$_{adjusted}$ = -249.3, *P*$_{adjusted}$ = 0.0305). The associations between the other biochemical indices and genetic markers were not statistically significant for any of the analyzed models.

DISCUSSION

Numerous studies have been conducted to identify host and viral factors that influence antiviral therapy efficiency in patients with HCV infection. Approximately 40% to 50% of individuals with viral genotype 1 and 80% with genotypes 2 and 3 reach an SVR[1]. Compared with these previously reported rates, a higher number of patients with genotype 1 in the present study reached SVR. In addition, all patients with genotypes 2 and 3 reached an SVR. These differences between our study results and those in the literature are likely due to our small patient group and relatively young patient age (38.36 ± 14.52 years) because increasing age is a risk factor for ineffective therapy[22]. Various risk factors are reportedly

Table 3 Characterization of the patient group

	Result of antiviral therapy	Mean	95%CI of mean		P value
			Lower bound	Upper bound	
Age in year at diagnosis	Sustained viral response	38.76	34.88	42.64	0.788
	Viral persistence	37.87	32.07	43.66	
	Total	38.43	35.24	41.61	
Alanine transaminase level	Sustained viral response	106.45	81.73	131.17	0.056
	Viral persistence	153.75	104.27	203.23	
	Total	123.65	99.86	147.44	
Iron level	Sustained viral response	20.84	18.19	23.49	0.015
	Viral persistence	29.92	22.05	37.80	
	Total	24.71	20.98	28.45	
Ferritin level	Sustained viral response	298.67	185.52	411.82	0.354
	Viral persistence	397.79	197.96	597.62	
	Total	336.09	231.98	440.20	
Viral load	Sustained viral response	1.91E + 06	1.12E + 06	2.70E + 06	0.115
	Viral persistence	4.21E + 06	5.15E + 05	7.90E + 06	
	Total	2.75E + 06	1.35E + 06	4.14E + 06	

associated with an individual patient's response to antiviral treatment, including the homocysteine level, vitamin D level, and many other parameters[23,24]. However, only sex, age, liver disease progression, viral genotype, and insulin resistance are included in the clinical guidelines as possible risk factors[22]. In the present study, the only markers that significantly influenced the efficacy of antiviral therapy were the alanine transaminase level (P = 0.056) and the iron level (P = 0.015). Iron is necessary for the replication of HCV; however, iron depletion therapy before antiviral therapy has not been proven to be effective[25]. The small size of our patient group is the main reason why the other data did not show a statistically significant impact on the efficacy of antiviral therapy.

Of all mutations that cause inherited liver diseases, the most extensively studied are those that cause hereditary hemochromatosis[26]. Although we did not detect a statistically significant association between HCV infection and the C282Y or H63D mutation in our study, the C282Y mutation was found to be more common in the patient group than in the control group (frequency of 0.048 *vs* 0.035, respectively) (Table 2). We also failed to detect an association between the iron or ferritin level with either the C282Y or H63D mutation. This result may have been due to our small patient group and/or the ages of our patients (men, 37.27 ± 15.69 years; women, 40.25 ± 12.26 years). Our patients may have been too young to manifest the symptoms characteristic of hereditary hemochromatosis because symptoms related to iron overload usually appear between the ages of 40 and 60 years in men and after menopause in women[27]. In addition, the higher serum iron levels seen in our patients with HCV infection may have been caused by various factors other than *HFE* gene mutations; *e.g.*, hepatocyte necrosis or increased intestinal iron uptake[28].

In contrast to previous reports[10,11], we did not detect an association between HCV infection and alpha-1 antitrypsin deficiency. Again, this may have been due to the small number of patients in our study and/or the fact that liver symptoms in patients with alpha-1 antitrypsin deficiency more commonly manifest in childhood or late adulthood. Advanced liver disease generally occurs around the age of 66 years in individuals heterozygous for the PIZ mutation[29]. The mean age of our patients was 38.43 years at the completion of analysis.

Gilbert's syndrome, also termed benign hyperbilirubinemia[30], was included in our study because an estimated 10% to 15% of European descent individuals are affected by this syndrome and because previous data have demonstrated anti-inflammatory and antioxidant functions of bilirubin[31,32]. The (TA)7 polymorphism of the *UGT1A1* gene was shown to be significantly associated with the ferritin level (beta = -295.7, P = 0.0087). Some studies have proposed that ferritin, being an acute-phase reactant, behaves as a marker of more active and advanced liver disease. Patients with chronic HCV infection and high serum ferritin levels reportedly have significantly more severe liver inflammation and fibrosis than do patients with normal serum ferritin levels[7,33]. In our study, patients with viral persistence had slightly elevated ferritin levels. Based on the analysis of the association of the ferritin level with genetic markers, the (TA)7 polymorphism could be associated with less prominent liver inflammation and lower ferritin levels in patients with HCV infection. This may in turn lead to a better antiviral treatment response, and future studies should address this notion. Our results also support the idea that more extensive liver inflammation can lead to viral persistence as evidenced by the fact that alanine transaminase levels were higher in patients with viral persistence.

Interestingly, the H1069Q mutation of the *ATP7B* gene was found to be associated with chronic HCV infection. We included this mutation in our analysis because a high rate of Latvians reportedly carry this mutation[4]. The *ATP7B* gene is involved in copper metabolism. Copper is well known to be critical for the proper functioning of both the humoral and innate immune systems; however, its precise mechanisms of action are unknown[34]. The spontaneous elimination or

persistence of HCV infection depends on the host's immune status[35]. Previous reports have stated that the host response to HCV infection may be primarily dependent on the human leukocyte antigen system. However, other factors, such as copper, may also influence the host response because changes in copper levels in patients with HCV infection have been reported[15,36,37]. We propose that the H1069Q mutation of the *ATP7B* gene may be an important modifier in patients with HCV infection. Future studies should investigate this in detail, especially considering the fact that Wilson's disease is treatable.

The main limitation of our study was the small number of patients and controls. No analysis was performed to exclude HCV infection in the control group. However, this was a pilot study. Research involving larger numbers of patients and controls in whom HCV infection has been excluded is warranted.

COMMENTS

Background
Inherited monogenic liver diseases and their causative mutations may represent genetic factors responsible for changing the host response to hepatitis C virus (HCV) infection. Although the association between HCV infection and hereditary hemochromatosis has been extensively studied, only a few studies on the associations between HCV and the mutations causing alpha-1 antitrypsin deficiency, Gilbert's syndrome, and Wilson's disease have been performed.

Research frontiers
Mutations that cause inherited liver diseases are highly distributed and associated with chronic inflammation and liver damage. This is one of the critical points in HCV infection.

Innovations and breakthroughs
Many studies have been performed in an attempt to identify host genetic factors that can influence the efficacy of antiviral therapy in patients with chronic HCV infection. This is first study to analyze all of the most common genetic disorders in one patient group. The results of this pilot study show that this research should be continued with a larger group of patients.

Applications
Therapy for inherited liver disorders is either already available or is currently under investigation. If the importance of such therapies with respect to alleviating liver damage in HCV infection is proven, the efficacy of antiviral therapy may be improved by establishing treatment that is more specifically targeted not only to the viral life cycle, but also to factors directly associated with the development of liver damage. Alpha-1 antitrypsin deficiency is a liver disease caused by the absence of the proteinase inhibitor alpha-1 antitrypsin. Wilson's disease is a progressive autosomal recessive disorder of copper metabolism. Gilbert's syndrome is characterized by benign unconjugated hyperbilirubinemia.

Terminology
Hepatitis C is an infectious liver disease caused by the HCV, that affects an estimated 130 to 200 million people worldwide. Inherited monogenic liver disorders are inherited diseases, caused by mutations in one gene (autosomal recessive inheritance), in which the primary manifestation is liver damage.

Peer review
This case-control study is the first to examine the association between HCV and frequently inherited monogenic liver diseases (hereditary hemochromatosis, alpha-1 antitrypsin deficiency, Gilbert's syndrome, and Wilson's disease) and their causative mutations. This study revealed an association between HCV and the mutation responsible for Wilson's disease. Biochemical experiments revealed an association between HCV and the mutation that causes Gilbert's syndrome. This is a well-designed study that brings new insight into the association between inherited liver diseases and HCV.

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BRIEF ARTICLE

Methylsulfonylmethane suppresses hepatic tumor development through activation of apoptosis

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Abstract

AIM: To investigate the effect of methylsulfonylmethane (MSM), recently reported to have anti-cancer effects, in liver cancer cells and transgenic mice.

METHODS: Three liver cancer cell lines, HepG2, Huh7-Mock and Huh7-H-*ras*[G12V], were used. Cell growth was measured by Cell Counting Kit-8 and soft agar assay. Western blot analysis was used to detect caspases, poly (ADP-ribose) polymerase (PARP), and B-cell lymphoma 2 (Bcl-2) expressions. For *in vivo* study, we administered MSM to H-*ras*[12V] transgenic mice for 3 mo.

RESULTS: MSM decreased the growth of HepG2, Huh7-Mock and Huh7-H-*ras*[G12V] cells in a dose-dependent manner. That was correlated with significantly increased apoptosis and reduced cell numbers in MSM treated cells. Cleaved caspase-8, cleaved caspase-3 and cleaved PARP were remarkably increased in the liver cancer cells treated with 500 mmol/L of MSM; however, Bcl-2 was slightly decreased in 500 mmol/L. Liver tumor development was greatly inhibited in the H-*ras*[12V] transgenic mice treated with MSM, compared to control, by showing reduced tumor size and number. Cleaved PARP was significantly increased in non-tumor treated with MSM compared to control.

CONCLUSION: Liver injury was also significantly attenuated in the mice treated with MSM. Taken together, all the results suggest that MSM has anti-cancer effects through inducing apoptosis in liver cancer.

Core tip: Methylsulfonylmethane (MSM) is an organic sulfur-containing compound. MSM suppressed hepatic tumor growth through activation of apoptosis. MSM could be a potential candidate as an anti-liver cancer agent.

Kim JH, Shin HJ, Ha HL, Park YH, Kwon TH, Jung MR, Moon HB, Cho ES, Son HY, Yu DY. Methylsulfonylmethane suppresses hepatic tumor development through activation of apoptosis. *World J Hepatol* 2014; 6(2): 98-106 Available from:

INTRODUCTION

Liver cancer is the sixth most common malignancy and the third most common cause of cancer-related mortality worldwide[1]. Approximately 560000 cases are diagnosed each year and 550000 deaths are due to liver cancer. In most countries, 75%-90% of liver cancers are hepatocellular carcinomas[2]. The main risk factors of liver cancer include infection with hepatitis B virus (HBV) or hepatitis C virus (HCV)[3]. Other risk factors include excessive alcohol consumption, nonalcoholic steatohepatitis, autoimmune hepatitis, primary biliary cirrhosis, particularly aflatoxin B and various genetic metabolic diseases[4]. However, mortality is diminishing with the development of vaccine and therapy methods. In particular, therapy using natural extracts with no side effects has been reported. It was recently reported that tetrandrine induces apoptosis in human hepatocellular carcinoma[5] and berbamine induces apoptosis and tumor growth inhibition[6].

Apoptosis is a physiological process for involution and atrophy of various tissues and organs during development and maintenance of tissue homeostasis[7]. The apoptosis pathway is mediated by death receptors that include tumor necrosis factor receptor (TNFR), Fas and TNF-related apoptosis-inducing ligand (TRAIL). These ligands lead to the recruitment and activation of initiator cysteine aspartic proteases (caspases) such as caspases-8 and 10. These lead to the activation of caspase-3. The active caspase-3 involves DNA fragmentation, nuclear fragmentation, membrane blebbing and other morphological and biochemical changes[8]. Otherwise, apoptosis is initiated by the stress-mediated release of cytochrome-c. The cytochrome-c activates initiator caspase, typically caspase-9, which leads to the activation of the executioner caspase-3. In response to apoptotic stimuli, pro-apoptotic members of the B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist killer (Bak) become activated and act on the mitochondria to induce the release of cytochrome-c[8].

Methylsulfonylmethane (MSM), an organic sulfur-containing compound, inhibits LPS-induced release of pro-inflammatory mediators in murine macrophages through downregulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling[9]. Moreover, the effect of MSM has been reported in cancer. MSM suppresses breast cancer growth by down-regulating signal transducer and activator of transcription 3 (STAT3) and signal transducer and activator of transcription 5b (STAT5b) pathways[10]. Apoptotic effects in other cancer cells, such as esophageal, gastric and liver cancer cells, were reported[11].

To further understand the effect of MSM in prevention of hepatic tumorigenesis, we have examined it in liver cancer cell lines and liver cancer mouse model.

MATERIALS AND METHODS

Cell culture and stable cell lines

HepG2 and Huh7 cell lines were maintained in a DMEM (HyClone, United States), supplemented with a 10% FBS (HyClone, United States), penicillin/streptomycin (HyClone, United States) in a CO_2 incubator at 37 ℃. Huh7-H-ras^{G12V} cell lines were generated by stably transfecting H-*ras*^{G12V} in Huh7 cells. The pCAG-HA-H-*ras*^{G12V}-neo was constructed as follows. The coding sequences for mutated H-*ras*^{G12V} were inserted by PCR cloning into the EcoR I site of the pCAG-HA-neo vector and confirmed by restriction mapping and DNA sequencing. Huh7 cells were plated in 6-well culture plates for 24 h prior to transfection. Cells were transfected with 3 μg of pCAG-HA-H-*ras*^{G12V}-neo construct using a Lipofectamine 2000 reagent (Invitrogen, United States), according to the manufacturer's instructions. After 48 h, cells were trypsinized and plated in a medium containing 400 μg/mL neomycin (G418). Following selection for 2 wk, total populations of neomycin-resistant cells were pooled and single-cells sorted into 96-well plates with a growth medium containing 400 μg/mL neomycin. Sorted single cells were grown under selection for an additional 2 wk and expanded into stable cell lines. The candidate clones were analyzed by Western blot analysis using a HA (Roche, Germany) antibody.

Animals

The generation of H-*ras*^{12V} transgenic liver cancer mouse model was previously described[12]. We used 3 mo old H-*ras*^{12V} transgenic male mice. H-*ras*^{12V} transgenic mice were divided into two groups and administered with PBS (control group, $n = 5$) and methylsulfonylmethane (MSM, 100 μg/g) (treated group, $n = 6$) every day for 3 mo. The genotyping of PCR primers for the H-*ras*^{12V} were 5'-CTAGCGCTGCAGGAATTC-3' and 5'-GTAGTT-TAACACATTATACACT-3'. The mice were housed in a pathogen-free animal facility under standard 12 h light/dark cycle. All animal procedures were conducted in accordance with the guidelines of the institutional Animal Care and Use Committee, Korea Research Institute of Bioscience and Biotechnology (KRIBB).

Reagents

MSM and crystal violet were purchased from Sigma-Aldrich Co. (United States).

Cell growth assay (anchorage-independent)

The cell growth after treatment with MSM was measured by Cell Counting Kit-8 (CCK-8) (Dojindo, Japan). HepG2 and Huh7 (Mock, H-*ras*^{G12V}) cells were suspended at a concentration of 5×10^3 cells/well and cultured in 96-well flat bottomed microplate. After exposure to MSM at different time points (0, 24, 48, 72 and 96 h),

CCK-8 (10 μL) was added to each well of a 96-well flat bottomed microplate containing 100 μL of culture medium and MSM (0, 200 μmol/L, 200 mmol/L, and 500 mmol/L) and the plate was incubated for 2 h at 37 ℃. Viable cells were counted by absorbance measurements at 450 nm using auto microplate reader (VERSAmax™, United States).

Soft agar assay (Anchorage-dependent)

HepG2, Huh7 - Mock, and Huh7-H-*ras*[G12V] (5×10^3 cells) were suspended in 1 mL of DMEM containing 0.3% agar in cell-growth medium and plated in triplicates over a first layer of 0.6% agar in cell-growth medium. The cells were grown at 37 ℃ and 5% CO_2. Then the viable colonies were stained with 0.01% crystal violet (Sigma, United States) for 2 h. We treated with MSM (0, 200 μmol/L, 200 mmol/L, and 500 mmol/L) on the top agar on day 0. The MSM contained medium was changed every day.

Flow cytometry analysis

Apoptosis was also evaluated by flow cytometry after Annexin V-FITC/PI (BD Bioscience, United States) staining. The cells were digested with trypsin and resuspended in 100 μL of binding buffer, 5 μL of Annexin V-FITC and added with 5 μL of PI, and the mixture was incubated at room temperature for 15 min in the dark. The cells were analyzed using a BD FACSCalibur (BD Bioscience, United States) and divided into four groups: normal cells (Annexin V negative and PI positive), early apoptotic cells (Annexin V positive and PI negative), late apoptotic cells (Annexin V positive and PI positive) and necrotic cells (Annexin V negative and PI positive). The percentages of the different cell groups were determined by a scatter plot analysis.

Western blot analysis

We homogenized liver cancer cell lysates in lysis buffer (20 mmol/L HEPES, 150 mmol/L NaCl, 2 mmol/L EGTA, 1 mmol/L EDTA, 20 mmol/L glycerol phosphate, 1% Triton X-100 and 10% glycerol) with protease (Sigma, United States) and a phosphatase-inhibitor cocktail (Roche, Germany). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. For Western blot analysis, 30 μg protein lysates were separated on 12% sodium dodecyl sulfate polyacrylamide gels and transferred onto nitrocellulose membranes (Millipore, United States). The membranes were primarily blotted with primary antibodies against GAPDH (Lab Frontier, South Korea), HA (Roche, Germany), cleaved caspase-3, cleaved caspase-8, cleaved PARP [Poly (ADP-ribose) polymerase] or Bcl-2 (Cell Signaling Technology Inc., United States) at 4 ℃ overnight. They were washed five times with 10 mmol/L Tris-HCl (pH 7.5) containing 150 mmol/L NaCl and 0.2% Tween-20 (TBST) and incubated with horseradish peroxidase conjugated goat anti-rabbit IgG or anti-mouse IgG (Pierce, United States)

for 1 h at room temperature. After the removal of excess antibodies by washing with TBST, specific binding was detected using a SuperSignal chemiluminescent substrate (Pierce, United States) according to the manufacturer's instructions.

Live cell counting by trypan blue stain

Huh7-H-*ras*[G12V] cells were suspended at a concentration of 0.3×10^6 cells/well and cultured in 6-well plate. After exposure to MSM for 24 h, cells were calculated by trypan blue stain through an electron microscope (Nikon, Japan).

Blood plasma analysis

Once a mo during the experimental period, blood samples were taken from orbital venous congestion. Plasma was prepared by centrifugation of the blood at 10000 rpm for 5 min at 4 ℃ and stored at -70 ℃ until analysis. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured with an automatic chemistry analyzer (Hitachi 7150, Japan).

Liver histology

The liver was removed from the mice and immediately fixed in a buffer solution of 10% formalin for pathological analysis. Fixed tissues were processed routinely for paraffin embedding and 5 μm sections were prepared and stained with hematoxylin and eosin (H and E). Stained areas were viewed using an optical microscope.

Statistical analysis

Data were analyzed using SigmaStat 3.1 software. All data are presented as the mean ± the standard error of the mean (SEM) from at least three independent experiments. Comparisons between groups were analyzed by Student's *t*-test for paired and unpaired measure. *P* value < 0.001 was considered statistically significant.

RESULTS

MSM inhibits cell growth in liver cancer cell lines

To investigate the effect of MSM in cell growth, cell lines such as HepG2, Huh7-Mock, and Huh7-H-*ras*[G12V] were exposed to MSM in a dose-dependent manner. Cell growth was analyzed at 0, 24, 48, 72 and 96 h using CCK-8. HepG2 cell growth was significantly inhibited with treatment of 500 mmol/L but the growth of Huh7-Mock and Huh7-H-*ras*[G12V] cells was significantly reduced with treatment of 200 mmol/L and 500 mmol/L of MSM (Figure 1A). To assess the effect of MSM in colony formation, we conducted a soft agar assay. As shown in Figure 1B, colony size and number of HepG2 cell were decreased in a dose-dependent manner. However, Huh7-Mock and Huh7-H-*ras*[G12V] cells were remarkably inhibited in 200 mmol/L and 500 mmol/L (Figure 1B). These results suggest that MSM treatment inhibits cell growth significantly in liver cancer cell lines.

A

B

Figure 1 Inhibitory effect of methylsulfonylmethane in liver cancer cell lines. A: Cellular proliferation effects of MSM were measured by CCK-8 assay. Cells were treated with MSM (0, 200 μmol/L, 200 mmol/L, and 500 mmol/L) at different time points (0, 24, 48, 72 and 96 h); B: Anchorage-independent growth assay was performed in liver cancer cells treated with MSM in a dose-dependent manner. [b]P < 0.001 *vs* control. MSM: Methylsulfonylmethane.

MSM induces apoptosis in liver cancer cell lines

To determine whether MSM induces apoptosis in liver cancer cell lines, we utilized Annexin V/PI staining, observed morphology and counted live cells. The Annexin V/PI staining was performed to examine the reversion of phosphatidylserine, a marker for apoptosis. Our results showed that the proportion of apoptotic cells was induced by treatment of 200 mmol/L and 500 mmol/L in liver cancer cell lines. Particularly, the apoptosis rate was increased 6-fold in all liver cancer cell lines treated with 500 mmol/L compared to control (Figure 2A). We examined the morphology and counted live cells of Huh7-H-*ras*[G12V]. Morphological changes were observed in 200 mmol/L and 500 mmol/L. Also, adherent cells were decreased in 200 mmol/L and 500 mmol/L treated with MSM. In addition, live cell number was significantly reduced in 500 mmol/L of MSM compared to control (Figure 2B). The data indicate that MSM treatment induces apoptosis in liver cancer cell lines.

MSM activates caspase-3, -8 and PARP in liver cancer cell lines

The expression of HA-H-*ras*[G12V] in Huh7-H-*ras*[G12V] was confirmed by Western blot. HA-H-*ras*[G12V] was overex-

Figure 2 Methylsulfonylmethane induces apoptosis in liver cancer cell lines. A: Detection of apoptotic cells by Annexin Ⅴ. Cells were treated with MSM in a dose-dependent manner for 24 h; B: Dose-dependent effects of MSM on the morphology and live cell counting of Huh7-H-*ras*[G12V] cell line for 24 h. [b]*P* < 0.001 *vs* control. MSM: Methylsulfonylmethane.

pressed in Huh7-H-*ras*[G12V] cell (Figure 3A). To understand the mechanisms involved in MSM-induced apoptosis in liver cancer cell lines, we first determined caspase activity. The protein levels of cleaved caspase-3, cleaved caspase-8 and cleaved PARP were significantly increased in liver cancer cell lines treated with 500 mmol/L (Figure 3B). To investigate whether mitochondrial anti-apoptosis proteins are involved in regulating MSM-induced apop-

Figure 3 Methylsulfonylmethane increases caspase-3, -8 and PARP activation in liver cancer cell lines. A: Expression levels of HA-H-*ras*[G12V] in Huh7-H-*ras*[G12V]; B: Expression levels of cleaved caspase-3, cleaved caspase-8 and cleaved PARP in liver cancer cells were determined by Western blotting analysis. Cells were treated with MSM in a dose-dependent manner for 24 h; C: Expression of Bcl-2 in liver cancer cells treated with MSM in a dose-dependent manner for 24 h. Numbers under each result indicate a fold increase of band density as compared to control, GAPDH. MSM: Methylsulfonylmethane; PARP: Poly (ADP-ribose) polymerase.

tosis of liver cancer cell lines, we examined the protein level of Bcl-2. The result indicated that Bcl-2 was decreased in liver cancer cell lines treated with 500 mmol/L (Figure 3C). It suggests that MSM induced apoptosis by regulating the expression of cleaved caspase-3, cleaved caspase-8 and cleaved PARP.

MSM inhibits hepatic tumorigenesis in H-ras[12V] transgenic mice

To investigate the suppression effects of MSM in hepatic tumorigenesis, H-*ras*[12V] transgenic mice were orally administered with MSM (100 μg/g) for 3 mo. Tumor volume and number were significantly reduced in the MSM treated group compared to the control group (Figure 4A). To determine histological changes, we examined the H and E staining in mouse liver. As shown in representative photomicrographs of liver histology, the tumor size in the MSM treated group was dramatically decreased compared to the control group. Moreover, necrosis was observed in the MSM treated group (Figure 4B). To confirm in vitro data on apoptosis, we examined the protein levels of cleaved PARP. Cleaved PARP was

increased in MSM treated non-tumors compared to PBS treated control; however, not in tumor (Figure 4C). To examine the effect of MSM in liver function of H-*ras*[12V] transgenic mice, we checked AST and ALT levels. AST levels were significantly lower in the MSM treated group for 3 mo than the control group. ALT levels were also lower in the MSM treated group for 1 mo than the control group (Figure 4D). These data suggest that MSM suppresses liver damage in H-*ras*[12V] transgenic mice.

DISCUSSION

MSM is naturally obtained from various species of fruits, vegetables, grains, animals and animal products. The chemical structure of MSM is a combination of oxygen in dimethyl sulfoxide (DMSO), so also called dimethyl sulfone (DMSO₂). The anti-inflammatory effects of MSM on lipopolysaccharide-induced inflammatory responses in murine macrophages[9] and effects of MSM in breast cancer by down regulating STAT3 and STAT5b pathways[10] were reported. However, the effect of MSM has not been studied in liver cancer. In the present study,

Figure 4 Methylsulfonylmethane inhibits hepatic tumorigenesis in H-*ras*[12V] transgenic mice. H-*ras*[12V] transgenic mice were administered with PBS and MSM (100 μg/g) every day for 3 mo. A: Liver morphologies of PBS (control) group (left) and MSM group (right) after administration for 3 mo; B: Hematoxylin and eosin stained section in livers of PBS and MSM treated H-*ras*[12V] transgenic mice (Scale bars, 500 μm); C: Expression levels of cleaved PARP in H-*ras*[12V] livers; D: The levels of AST and ALT in plasma of H-*ras*[12V] transgenic mice treated with MSM. MSM: Methylsulfonylmethane; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

we found that MSM dramatically inhibits hepatic tumor cell growth. We performed CCK-8 and soft agar assay. Anchorage dependent tumor cell growth inhibition was found in liver cancer cells, such as HepG2, Huh7-Mock and Huh7-H-*ras*[G12V] treated with 500 mmol/L of MSM (Figure 1A). Anchorage independent cell growth was also inhibited in the liver cancer cells treated with both 200 mmol/L and 500 mmol/L of MSM (Figure 1B). The results indicate that MSM is effective in inhibiting liver cancer cell growth.

To further investigate the apoptosis in liver cancer cells treated with MSM, we examined apoptotic cells by Annexin V/PI staining. Apoptotic cells were significantly increased by treatment of 500 mmol/L in liver cancer cell lines (Figure 2A) and the morphology of Huh7-H-*ras*[G12V] cells was changed in 200 mmol/L and 500 mmol/L. The numbers of live cells were reduced in 500 mmol/L (Figure 2B). The result indicated that MSM caused apoptosis in liver cancer cell lines.

Cancer chemotherapy is known to induce tumor cell death in a variety of cell types in part by promoting the intracellular ROS. Recently, salinomycin-induced apop-

tosis of human prostate cancer cells was due to accumulated ROS[13]. In our study, ROS levels were significantly increased in 500 mmol/L of MSM in Huh7 cell lines (Supplemental Figure 1), suggesting that MSM treatment regulated ROS levels in liver cancer cell lines.

To clarify the apoptotic mechanism stimulated by MSM, we studied both the death receptor pathway and the mitochondrial pathways[14]. Cell surface death receptors, such as Fas which bind their ligands, initiate signaling to activate caspase-8, caspase-3 to induce apoptosis, and signaling involved in mitochondrial release of cytochrome-c, which activates caspase-9 and caspase-3[15]. We performed Western blot. Bcl-2 was decreased in all of the cell lines treated with 500 mmol/L (Figure 3C) and MSM treatment led to an increased apoptotic response involving caspase-3, caspase-8 and PARP activation in liver cancer cell lines (Figure 3B). The results demonstrate that MSM induces apoptosis through activation of the caspase pathway.

We performed *in vivo* studies to investigate the liver tumor growth suppressive function of MSM. We orally administered MSM (100 μg/g) to H-*ras*[12V] transgenic

mice for 3 mo. During the administration, body weight ratio was not changed between MSM treated group and control group (Supplemental Figure 2). However, the AST and ALT levels of MSM treated group were lower than the control group (Figure 4D). In addition, tumor volume and number were noticeably reduced in the MSM treated group (Figure 4A). The expression of cleaved PARP was increased in MSM treated non-tumors compared to PBS treated control; however, similar in tumors between the PBS and MSM treated group (Figure 4C). As shown in photomicrographs of liver histology, tumor size of the MSM treated group was decreased compared to the control group (Figure 4B). All the data suggest that MSM improves liver function and suppresses hepatic tumorigenesis through activation of apoptosis.

MSM was efficacious with treatment of 500 mmol/L in inhibition of hepatic tumor cell growth. In addition, the apoptosis rate was increased 6-fold in all of liver cancer cell lines treated with 500 mmol/L compared to control. These results indicate that MSM is efficacious with treatment of the highest dose in liver cancer cells, consistent with the result that MSM suppresses breast cancer cell growth at 300 mmol/L[10]. MSM is an edible natural organic compound present in many food items and is not associated with any toxic effects, even at higher concentrations[16,17]. MSM administration with high dose (100 μg/g) to H-*ras*[12V] transgenic mice for 3 mo did not affect body weight ratio but improved liver function by showing lowered AST and ALT levels and remarkably retarded hepatic tumor growth in the MSM treated group. All the results suggest that MSM could be available for inhibition of hepatic tumor growth. Further research is needed to be feasible in humans.

In summary, we showed that MSM induced growth inhibition and apoptosis in hepatic tumorigenesis. Therefore, MSM could be a potential candidate as an anticancer agent.

COMMENTS

Background
Liver cancer is the third most common cause of cancer-related mortality worldwide. However, there are only a few effective ways to prevent or treat liver cancer. Therefore, studies are going on in the area of liver cancer. Methylsulfonylmethane (MSM), an organic sulfur-containing compound, is naturally obtained from various species of vegetables, grains, animals and animal products. Recently, a study has reported that MSM can be used to inhibit breast cancer growth.

Research frontiers
MSM is an edible natural organic compound present in many food items and is not associated with any toxic effects, even at higher concentrations. Research is focused on finding the efficacy of higher doses of MSM treatment in cells and mice with H-ras activated liver cancer.

Innovations and breakthroughs
MSM decreased the growth of Huh7-H-rasG12V cells in a dose-dependent manner. That was correlated with significantly increased apoptosis in MSM treated cells. Cleaved caspase-8, cleaved caspase-3 and cleaved PARP were remarkably increased in the liver cancer cells treated with 500 mmol/L of MSM. Liver tumor development was greatly inhibited in the H-ras12V transgenic mice treated with MSM compared to control, by showing reduced tumor size and number.

Applications
The results suggest that MSM could be a potential candidate for prevention of liver cancer.

Terminology
MSM is a very simple organic sulfur-containing compound with a molar mass of 94.13 g/mol. MSM contains only eleven atoms and is found in foods, including fruits, vegetables, grains and beverages.

Peer review
This study described the efficacy of MSM treatment in cells and mice with H-ras activated liver cancer well. The results are interesting and indicate that MSM could be used for preventing hepatic tumor growth.

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TOPIC HIGHLIGHT

Jaques Waisberg, PhD, *Series Editor*

Complications of radiofrequency ablation of hepatic tumors: Frequency and risk factors

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Abstract

Radiofrequency ablation (RFA) has become an important option in the therapy of primary and secondary hepatic tumors. Surgical resection is still the best treatment option, but only a few of these patients are candidates for surgery: multilobar disease, insufficient liver reserve that will lead to liver failure after resection, extra-hepatic disease, proximity to major bile ducts and vessels, and co-morbidities. RFA has a low mortality and morbidity rate and is considered to be safe. Thus, complications occur and vary widely in the literature. Complications are caused by thermal damage, direct needle injury, infection and the patient's co-morbidities. Tumor type, type of approach, number of lesions, tumor localization, underlying hepatic disease, the physician's experience, associated hepatic resection and lesion size have been described as factors significantly associated with complications. The physician in charge should promptly recognize high-risk patients more sus-

ceptible to complications, perform a close post procedure follow-up and manage them early and adequately if they occur. We aim to describe complications from RFA of hepatic tumors and their risk factors, as well as a few techniques to avoid them. This way, others can decrease their morbidity rates with better outcomes.

Core tip: This article is an interesting and updated compilation of the complications of radiofrequency ablation of liver tumors. Several complications are described, as well as their risk factors and incidence. Some strategies to avoid them from happening are also reported.

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INTRODUCTION

Radiofrequency ablation (RFA) has become an important option in the therapy of primary and secondary hepatic tumors. Surgical resection is still the gold standard treatment, but only 5%-15% of these patients are candidates for surgery[1]. For a few selected patients who have hepatocellular carcinoma (HCC), the most common primary cancer, liver transplantation is an option but the inclusion criteria are strict and organ donation is still insufficient. Inadequate liver function, multilobar lesions, extra-hepat-

ic disease, proximity to major hepatic vessels and the biliary tract, and co-morbidities are factors that make these patients not eligible for surgery[2].

Complications rates of RFA vary widely in the literature. They are divided into major and minor[3]. The former are those that need some type of medical intervention (*e.g.*, drainage), increase morbidity and mortality, increase hospital stay or require blood transfusions. All of the rest are considered minor[3]. Authors have reported rates as low as 2% to 5.7% for major complications[4-6]. Mortality related to the procedure is low, reported in the literature to be less than 1%[7-9]. Tumor type, type of approach, number of lesions, tumor localization, underlying hepatic disease, the physician's experience, associated hepatic resection and lesion size have been described as factors significantly associated with complications[9-12]. In one of their papers, Poon *et al*[10] concluded that after the physician's first 50 procedures, the incidence of complications is lower, as well as a shorter hospital stay and higher complete ablation rate.

In this article, we present the frequency and risk factors for complications after RFA. Complications are summarized in Table 1.

HEMORRHAGIC COMPLICATIONS

Intra-abdominal bleeding is the most common complication encountered in many studies[5,6,12,13]. In Mulier's review, it occurred in 0.7% of the procedures in 3670 patients[12]. Similar results were reported by Curley *et al*[9] (0.9% in 608 patients) and Livraghi *et al*[6] (0.5% in 2320 patients). It is believed to be a result of direct trauma from needle positioning rather than thermal injury (due to the protective "heat-sink" effect)[14,15]. Injuries to small vessels not visible on ultrasonography (US) are usually responsible for its origin. Increasing abdominal pain following the procedure is generally the most common symptom[9,15]. US or computed tomography (CT) confirms the diagnosis. Bleeding complications are more likely to happen in patients with HCC due to their underlying liver disease. In a study addressing this issue, tumor size, low platelet count and tumors located in segment VII were significant risk factors for intra-peritoneal bleeding[15]. Intra-hepatic bleeding may also occur and can be prevented by avoiding hepatic vessels while positioning the needle. This makes the imaging guidance essential. Both of them tend to have a benign course and stop spontaneously. Venous bleeding is usually treated conservatively or with blood transfusions only; arterial bleeding is more severe and may require surgical or endovascular intervention[9,14,16]. Tract cauterization by the withdrawal of the needle in high temperatures may prevent this kind of complication and should be performed in all cases[12]. Groups performing this have less or even no bleeding complications[7]. Rhim, in one of his articles, states that the open or the laparoscopic approach can decrease this kind of complication since needle positioning and withdrawal is under direct vision[16]. Transcatheter arterial embolization

Table 1 Complications of radiofrequency ablation	
Hemorrhagic	Intra-abdominal bleeding
	Intra-hepatic bleeding
	Hemothorax
	Hemobilia
	Subcapsular hematoma
	Abdominal wall hematoma
Infection	Hepatic abscess
	Wound infection
	Sepsis
Biliary tract	Bile duct injuries
	Biliary stricture
	Bilomas
	Bilioperitoneum
	Biliopleural fistula
Liver failure	
Pulmonary	Pneumothorax
	Pleural effusion
	Pneumonia
Skin burn	
Tract seeding	
Vascular damage	Portal vein thrombosis
	Hepatic veins thrombosis
	Hepatic artery damage
	Pseudoaneurysm
Visceral damage	Colon
	Stomach
	Gallbladder
	Kidney
	Diaphragm
	Abdominal wall
	Small intestine

is the treatment of choice for this hemorrhagic complication[9,14,17].

Several authors have also described hemothorax[8,12,15]. It is less frequent than intra-abdominal bleeding, with an incidence ranging from 0.1% to 0.3%[8,12,15,17]. It usually occurs due to injuries to intercostal arteries while percutaneously ablating tumors in the right liver through an intercostal approach. Chest pain and dyspnea are the most common symptoms[15]. US, chest CT and chest X-Ray confirm the diagnosis. Circulation stabilization and thoracic drainage are often necessary[15]. An open approach for these patients should prevent this from happening.

Another hemorrhagic complication is hemobilia, with an incidence from 0.1% to 0.5%[12,15]. It is caused by the puncture at the same time of the biliary tract and a vessel[15]. The most common symptoms are abdominal pain, hematemesis and melena. The main risk in these cases is biliary obstruction by blood clots, causing jaundice and liver failure. In this matter, the timing of drainage is essential. Goto *et al*[15] indicates bile duct drainage when bilirubin concentrations exceeds 4 mg/dL; they think that an early indication of the procedure may delay hemostasis. They also found that tumors in liver segment I was a significant risk factor for this type of bleeding. Avoiding puncturing dilated biliary radicles should prevent such complications to occur[14].

Subcapsular hematoma and abdominal wall hematoma have also been described. The first one occurs more often in subcapsular tumors, when tract cauterization is

not possible, due to its depth. The open or laparoscopic approach rather than the percutaneous is an option to avoid them.

This illustrates the need for vigilance for any signs of bleeding after the procedure and adequate screening for coagulation disorders, including the use of medications that affect the coagulation cascade[18,19]. Post procedure imaging is also essential since these complications usually occur in the first hours after the ablation.

INFECTION

Abdominal infection is also a common complication encountered[12,20]. This group of complications consists of hepatic abscess, wound infection and sepsis. Hepatic abscess is a potentially dangerous complication with an incidence ranging in the literature from 0.3% to 1.7%[6,9,11,12,21-23]. It can appear up to more than 60 d after the procedure[23]. Significant risk factors for its development are the presence of biliary abnormality or manipulation, prone to ascending biliary infection (bilioenteric anastomosis, endoscopic papillotomy and tumor with retention of iodized oil from a previous chemoembolization)[6,12,16,22,24]. In a study conducted by Elias *et al*[23] in 2006, the authors studied 11 patients with enterobiliary anastomosis or biliary stent and found an incidence of 44% of hepatic abscess in these specific subjects. They also stated an interesting issue: when the biliary procedure was synchronous with the RFA, no hepatic abscesses were observed; only when it was performed prior to the ablation was it considered a risk factor. Enteric bacteria coming from the injured colonized bile ducts contaminate the tumor necrosis generated by RFA[22]. Patients with hepatic abscess may present with fever and abdominal pain. The onset of these symptoms and signs usually occur within the first month after RFA[22]. Suspicion should arise when patients present with high body temperatures after the procedure, especially if it lasts longer than two weeks, although fever can be a symptom of the postablation syndrome. CT scan confirms the diagnosis; air bubbles are usually seen in the abscess. Thus, they may be seen in the ablated area after the procedure and this must not be misdiagnosed as an abscess[20]. Antibiotic prophylaxis is controversial in all patients, but in high risk cases it is recommended[6,12,22,23]. A question that comes up in these patients is if prolonged antibiotic prophylaxis is useful in reducing its incidence. Hoffmann *et al*[24] addressed this issue and tried to reduced this risk by maintaining the antibiotics for over 10 d after the procedure in 8 patients with prior bilioenteric anastomosis. The majority of the interventions (9/10) had prior administration of intravenous piperacillin/tazobactam and after the RFA, patients received Ciprofloxacin orally; 4 of the patients received additional antibiotics (metronidazole, cefpodoxime and cefazolin). Only one patient developed a hepatic abscess; he had a chemoembolization 8 d before the RFA. Despite the low number of patients and the lack of a control group, the authors suggest that this regimen may decrease the incidence of hepatic abscess. Elias *et al*[23] and de Baère *et al*[5] also debated this matter. Both groups administered prolonged antibiotics prophylaxis for 5 d (longer than usual) on these high-risk patients and a high incidence of hepatic abscess was encountered. Further studies with control groups and larger series of patients are necessary to resolve this question.

The most frequent organisms found in these abscesses were *Enterococcus*, *E. coli*, *Bacteroides fragilis*, *E. faecalis*, *C. perfringens* and *Klebsiella pneumonia*[5,21,23]. The best treatment option is percutaneous drainage in combination with systemic antibiotics[19-21,24]. Early suspicion, diagnosis and treatment are essential for a good outcome so the physician should be alert to the patient's clinical follow up, especially in those with risk factors.

BILIARY TRACT DAMAGE

Biliary tract damage includes bile duct injuries, biliary stricture, bilomas and, most rarely, bilioperitoneum and biliopleural fistula. Its incidence can be as low as 0.1% and up to 12%[9,12,25,26]. Bile ducts changes are expected and most of these changes have no clinical significance with the patient being asymptomatic with low rates of progression[9,12,26]. This explains its low and underestimated frequency since authors ignore those minor changes[12,26]. In a paper studying this matter, most of these changes seen on CT were mild dilatation of the upstream intrahepatic bile duct surrounding the ablation zone[26]. The authors did not mention the distance between the tumors and major bile ducts and stated that these changes are irreversible. In an Italian study, only two of 3554 patients required therapy after this kind of complication[6]. Another 15 patients presented with asymptomatic biliary tree abnormalities. These injuries are due to thermal damage from heating and direct mechanical damage from the needle. It is more likely to happen in hilar tumors or in tumors closer than 1 cm to major bile ducts when the safety margin is impossible to be obtained without injury. Biliary stricture is the most common complication in this group[12]. It may develop weeks to several months after RFA[26]. In a study where 28 high-risk patients were analyzed, the incidence of stenosis in this specific group of subjects increased up to 46% (13/28 patients with tumors closer than 5 mm to central bile duct on CT)[25]. Peripheral stenosis is usually asymptomatic, but central strictures may lead to serious complications. These strictures are believed to lead to liver atrophy and its consequent malfunction[25]. This is very important for cirrhotic patients because, due to their already impaired liver function, they may easily develop liver failure and cholangitis after bile duct stenosis[21,25]. Cholestasis and biliary infection may also occur.

Diagnosis is usually done by CT during follow-up and can also be detected by endoscopic retrograde cholangiography. The latter can also be used therapeutically by stenting the injured bile duct. The strictures are also well treated by endoscopic sphincterotomy[27].

Figure 1 Third-degree grounding pad skin burn on the right thigh.

The association of RFA with transarterial chemo-embolization (TACE) or percutaneous ethanol injection (PEI) is an option in these cases as these procedures, prior to the RFA, decrease tumor size and makes it possible for the ablation to be safer with a larger margin. Ohnishi *et al*[25] reported a method to prevent this complication by infusing intraductal chilled saline solution through an endoscopic nasobiliary drainage tube. Only one patient (2.5%) developed a stricture (left hepatic duct); the 39 remaining subjects were able to avoid thermal injury with this procedure. The incidence of this complication was significantly lower than the control group. This also significantly decreased the worsening of their liver function compared to the control group. The authors did not mention recurrence and other complications related to this procedure. Elias *et al*[23] also used this in 13 high-risk patients after the procedure. Two questions arise. The first one is if this protection is due to the low temperature itself or the heat sink effect caused by the solution's flow leading to inefficient ablation. The second one is if this procedure increases the incidence of hepatic abscesses. These questions need to be answered with future studies. Another concern regarding this issue is recurrence. This procedure also has a cooling effect on tumor cells near the cooled bile duct; thus, more insertions and more heat are necessary for adequate ablation which may lead to higher rates of complications[28]. Future studies are needed to address this. Curley *et al*[9] and Huang *et al*[29] suggested an open approach in these high-risk subjects for better needle placement with intra-operative ultrasonography. Patient selection is vital to avoid this type of complication.

Biloma is also encountered in this group of complications, with an incidence ranging from 0.1% to 5.8%[6,12,26,30]. It is defined as an encapsulated bile collection outside the biliary tree due to biliary leakage. This leakage can be caused by direct damage from the needle, direct thermal damage and by thermal damage to the microvasculature of the biliary tract caused by RFA. On CT, it is characterized as a circumferential fluid collection surrounding the ablation site or a communication between the bile duct and circumferential collection confirmed on cholangiography or CT[26,30]. Most bilomas develop within

the first 4 mo but can occur as late as 17 mo[30]. Almost all patients are asymptomatic and the fluid formation has spontaneous regression in half of the cases[30]. Percutaneous drainage is a good treatment option when required. Sphincterotomy should always be considered to exclude biliary stenosis and increased biliary pressure as a cause for biloma formation.

LIVER FAILURE

Liver failure is also a potentially fatal complication, especially in patients with cirrhosis whose liver function is often already impaired. Patients who have undergone previous hepatectomy are also at risk for this complication[14]. Its incidence ranges from 0.2% to 4.3%[4,9,11,12]. Child Pugh classification has been significantly related to post treatment liver failure[4,11]. Hepatic infarction due to injuries to major feeding vessels is believed to be responsible for its occurrence. Proper and careful needle placement is essential to avoid this from happening[14]. Other causes of liver failure are extensive ablation (overtreatment causes destruction of cirrhotic tissue around the lesions), portal vein thrombosis and extensive resection[6,12,16].

PULMONARY COMPLICATIONS

Pneumothorax, hemothorax (described in hemorrhagic complications), pleural effusions and pneumonias are in this group of complications. Its incidence varies from 0.8% to 2.1%[9,12]. Pneumothorax is more likely to happen in patients with tumors located directly under the diaphragm when an intercostal approach is chosen[12]. Some authors have described the use of artificial pleural effusion[28]. The idea is to separate the lung from the diaphragm and avoid these lesions. Inoue *et al*[28] published a series of 64 patients with 82 nodules near the diaphragm using this technique and encountered complications in 5 subjects. The treatment should be considered individually. Thoracentesis, underwater seal drainage and diuresis have been described[6,9,14]. Adequate needle positioning with a safe window (in the percutaneous approach) can avoid this complication[20]. Positioning the patient on the right side can also avoid it by limiting respiratory excursion[14]. Use of the epipericardial fat pad has also been described to avoid entering the pleural cavity[31]. Further investigation with CT is required if the patient experiences dyspnea or chest pain after RFA.

SKIN BURNS

Skin burns can occur at the point of needle entry and at the ground pad sites (Figure 1). This complication had a higher incidence in earlier studies due to smaller pads. In recent papers, it became a rarity because of their larger sizes and increased awareness, with a low incidence from 0.2% to 0.6%[6,14,20]. Third-degree skin burns are rare, but have been described, even leading to deaths[5,7,19,20]. Adequate pad placement and sizes are essential to avoid

Figure 2 Tumor seeding on needle entry site after percutaneous radiofrequency ablation.

this complication, as well as good contact with the skin. Large and sometimes multiple ground pads are necessary to disperse the high amount of energy generated by RFA. They should be equidistant from the needle due to the asymmetric distribution of the electrical current. This asymmetry makes the temperature beneath the pads not uniform, with greater heat on the edges and in the pads closest to the needle[19]. This was confirmed by de Baère *et al*[5], describing patients with first and third degree burns on the edges of one the pads facing the active electrode (needle).

TRACT SEEDING

Tumor seeding in the needle tract has an incidence from 0.2% to 0.9%[5,6,12,32]. Low rates of tumor seeding may be explained due to its underestimation in most papers due to a lack of follow-up. It usually occurs 3 to 12 mo after RFA[19]. Viable tumor cells that adhere to a biopsy needle or the electrode during its extraction, tumor cells carried into the needle tract with the bleeding and tumor cells forced into the tract by intratumoral hyperpressure are mechanisms that explain the seeding[12,33] (Figure 2). Decreasing the number of punctures and transversing a large amount of hepatic tissue before entering the tumor may avoid this complication[14,20]. Groups performing needle tract cauterization have not experienced tumor seeding or have very low rates[5,7,14]. Livraghi *et al*[32] reported their series with 1314 patients aiming to determine the risks of this complication in subjects with HCC treated by percutaneous RFA with a long follow-up (median 37 mo). They encountered seeding in 12 patients; tumors were located mostly in intercostal muscle and successfully treated by resection. The only significant risk factor described was a previous biopsy. They concluded that needle biopsy should be avoided. Other risks factors described by other authors are poorly differentiated, subcapsular location (where heating of the needle tract is not possible) and multiple needle insertions[5,6,33,34]. Optimal and meticulous first attempt electrode positioning is desirable[6]. Besides resection, RFA is also an option for treating tumor seeding. Some authors suggest the open approach in subcapsular lesions to avoid this complication[35].

HEPATIC VASCULAR DAMAGE

Portal vein thrombosis, hepatic vein thrombosis, hepatic artery damage and pseudoaneurysm represent this group of complications, with an overall complication rate from 0.5% to 1%[6,12,19,36].

Portal vein thrombosis is a potentially fatal complication, with a 0.2% incidence[12]. Thrombosis and coagulation of vessels larger than 3 mm are rare when normal flow is granted[37]. Most of these thromboses are asymptomatic even in larger vessels and no further therapy is required[5,36]. They are caused by heat damage to the endothelial cells of the portal or hepatic vein, leading to platelet aggregation and subsequent thrombosis[38]. It can be defined as being adjacent to the ablation zone and developing within 4 mo after RFA[36]. Liver function tests are usually normal but if elevated should normalize with no clinical significance[36]. Its occurrence should be avoided, especially in cirrhotic patients, as it may lead to liver failure in a patient with an already impaired liver function. Risk factors are the central location of the tumor, vein compression by the tumor and the Pringle maneuver. The latter stops blood flow into the liver and with that, vessels lose their cooling protection from the "heat-sink" effect, leading to vessels thrombosis. de Baère *et al*[5] showed in their paper that 30% of their procedures with balloon occlusion (for blood flow stop) led to complete thrombosis of the ballooned vessel. They also had more significant portal vein thrombosis in cirrhotic patients after performing the Pringle maneuver than in noncirrhotic subjects. It is suggested by the authors that it should be avoided in these patients, even for short durations[5].

Hepatic artery damage has a 0.2% incidence[12]. Small arterioportal shunts may occur after RFA and the majority of them heal spontaneously[12]. They can be successfully treated by endovascular or percutaneous therapies.

VISCERAL DAMAGE

Visceral damage is rare, with an incidence varying from 0.5% to 0.7%[6,12]. Damage to the colon, stomach, gallbladder, kidney, diaphragm, abdominal wall and small intestine has been described. Attention should be paid when tumors are closer than 1 cm to adjacent organs. Early diagnosis and adequate treatment are essential since it may lead to death. Risk factors are percutaneous approach, subcapsular tumors, previous abdominal surgery and chronic cholecystitis as the patient may have adhesions between the liver and the bowel[6,12,16]. Livraghi *et al*[6] suggest some issues in these patients: they should be treated by the open or laparoscopic approach for direct visualization of the organs, assuring they are in fact separated, and CT guidance is preferable for better adjacent bowel identification.

The colon is believed to be at greater risk of being

damaged due to its thin wall and fixed nature[5,6]. This complication has an incidence from 0.1% to 0.3%[5,6,12]. Some techniques have been developed to avoid bowel injuries: patient positioning in a steep oblique and prone position and breath holding during mechanical ventilation in patients under general anesthesia has also been described[14]. Another technique is creating a barrier between the liver and the colon, the hydrodissection. The use of 5% dextrose and saline solutions has been reported[14,28,39]. The former is preferred due to its properties since it does not conduct electricity and hence provides a thermal barrier around the organ[39]. Song *et al*[39] and Inoue *et al*[28] used artificial ascites and had no gastrointestinal injuries. The stomach and small bowel are less injured because adhesions along the gastrohepatic ligament are rare as the gastric wall is very thick and the small bowel has great mobility and peristalsis[6,19]. One should keep in mind that the onset of the symptoms of perforation is delayed; therefore, treatment is also usually delayed and the patient presents with a severe clinical status, eventually leading to death. A high level of suspicion is essential and close follow-up is important in these subjects.

Ribeiro *et al*[7] in their series routinely performed open cholecystectomy prior to RFA in tumors near the gallbladder, with the intention to avoid cholecystitis and incomplete ablation. Minimal wall thickening is expected on imaging after RFA, usually with no clinical significance. This probably happens due to the capacity of the fluid inside the gallbladder to dissipate the heat[16].

Injury to the diaphragm occurs in 0.1% of the cases[6,12]. It frequently results in severe shoulder pain[14]. Usually, RFA causes thickening of the muscle but perforation and hernia have been described[40]. Artificial ascites can also be used to decrease it.

CONCLUSION

Complication rates of RFA are low, making it a safe and feasible procedure. Every component of the treatment should be thoroughly analyzed. Proper patient selection is essential; subjects with exclusion criteria may lead to higher complication rates. Type of approach is also vital; depending on tumor location, one type may lead to a higher complication rate than another. This also fits for imaging guidance, where some tumors locations are better visualized by a specific method over another. The physician's experience is very important as well. Identification of high-risk subjects (with close follow-up), early diagnosis of known complications and a high level of suspicion are acquired with time and may lead to better outcomes and reduced risk of complications.

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REVIEW

Thyroid hormone analogues and derivatives: Actions in fatty liver

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Abstract

Fatty liver or nonalcoholic fatty liver disease (NAFLD), a problem of increasing clinical significance and prevalence worldwide, is associated with increased risk for the development of cirrhosis and hepatocellular carcinoma. Although several therapeutic approaches can be used in the context of NAFLD, dietary and physical activities are still the most frequently used strategies. Some pharmacological agents show promising results although no conclusions can be drawn from recent clinical trials. Thyroid hormones [THs; thyroxine (T4) and 3,3′,5-triiodo-L-thyronine (T3)] coordinate a diverse array of physiological events during development and lipid/energy homeostasis and have some potentially therapeutic actions which include inducing weight loss, and lowering plasma cholesterol levels and tissue adiposity. The thyroid hormones exert their physiological effects by binding to specific nuclear receptors [thyroid hormone receptors (TR)] of which the TRβ isoform is liver specific and has been considered a putative target for the treatment of dyslipidemia and fatty liver. In view of this, the aim of the review is (1) to provide an overview of the action of T3 on lipid metabolism with implications for liver steatosis and (2) to provide an update on the current knowledge concerning the administration of TRβ selective thyromimetics (GC-1 and MB07811), as well as of 3,5-diiodo-L-thyronine and its novel functional analogue TRC150094 in animal models of overweight and related disorders including primarily fatty liver.

Core tip: Fatty liver is associated with increased risk for the development of cirrhosis and hepatocellular carcinoma. Thyroid hormones have some potentially therapeutic actions by binding to specific nuclear receptors [thyroid hormone receptors (TR)] of which the TRβ isoform is liver specific and a putative target for the treatment of dyslipidemia and fatty liver. This review provides (1) an overview of the action of T3 on lipid metabolism and (2) an update concerning the administration of TRβ selective thyromimetics (GC-1 and MB07811), as well as of 3,5-diiodo-L-thyronine and its novel functional analogue TRC150094 in animal models of overweight and fatty liver.

INTRODUCTION

The liver plays essential roles in supporting many meta-

bolic processes and is critically involved in facilitating the maintenance of blood-glucose levels and energy homeostasis. Diet-induced obesity - commonly associated with diseases such as type 2 diabetes (T2DM), hypertension, heart failure, or cancer - also leads to fatty liver or steatosis, a histopathological condition characterized by an excess accumulation within hepatocytes of lipids, which are primarily triglycerides (TGs)[1]. Although the primary metabolic abnormalities leading to lipid accumulation within hepatocytes are still not fully understood, a decreased capacity to oxidize fatty acids, an increased delivery and transport of free fatty acids (FFAs) into the liver as well as an augmented hepatic fatty acid synthesis are likely to play significant roles in the pathogenesis of hepatic steatosis[2-4]. Moreover, steatosis is clearly, inextricably linked to modifications of mitochondrial functions[5,6]. Indeed, the mitochondrion plays an important role in the hepatocyte's metabolism because it is the primary site of fatty acid oxidation and oxidative phosphorylation. Multiple enzymes are involved in mitochondrial β-oxidation, and even partial deficiencies of these enzymes may lead to the development of hepatic steatosis[7,8] (Figure 1A). Two broad categories of hepatic steatosis have been recognized: alcoholic fatty liver disease (AFLD) and nonalcoholic fatty liver disease (NAFLD). In particular, NAFLD, commonly associated with insulin resistance (IR) and cardiovascular diseases[9], comprises a morphological spectrum of liver lesions ranging from simple triglyceride accumulation in hepatocytes (hepatic steatosis) to inflammatory and hepatocellular ballooning injury (non-alcoholic steatohepatitis; NASH), which eventually leads to fibrosis and cirrhosis[1]. The exact mechanism underlying the transition from steatosis to steatohepatitis is still unknown. According to the "two-hit" hypothesis[10]: the first hit involves the accumulation of TGs in hepatocytes that causes a vicious cycle of metabolic dysfunction; once the presence of hepatic steatosis is established, progression to steatohepatitis involves a "second hit" with oxidative stress playing a key role. Fatty liver is more susceptible to oxidative injury[1] and lipid peroxidation[11], and the chemical modification of biological molecules may be directly toxic to the cells or may stimulate host-immune response that leads to inflammation, collagen production and further disease progression[12-14].

Therapeutic interventions in NAFLD are mainly based on lifestyle changes, including diet and exercise[15,16]. Currently, there are no approved pharmacological therapies for NAFLD, but because IR is almost universally present in patients with this condition, drugs that increase insulin sensitivity are currently undergoing extensive evaluation and hold promise as therapeutically effective agents[17,18]. Several other agents, such as antioxidants and hepatoprotective compounds, have been evaluated, and the data was inconclusive or demonstrated no effects[16].

Thyroid hormones [THs; thyroxine (T4) and 3,3',5-triiodo-L-thyronine (T3)] exert a multiplicity of effects and are potent regulators of glucose and lipid metabolism and body weight. In particular, they play an important role in

hepatic lipid homeostasis. They exert their physiological effects by binding to specific nuclear receptors, the thyroid hormone receptors (TR) α and β that are widely distributed throughout the body. The β isoform is the major TR expressed in the liver. The beneficial effects of TRβ activation include lowering low-density lipoprotein (LDL) cholesterol, reducing whole body adiposity and weight[19], and increasing the metabolic rate in the liver which could potentially lead to reduced lipid content. However, to date, there is a lack of data available on the specific effects elicited by T3 on liver steatosis. In a recent study, T3 was shown to exert a strong inhibitory effect on the development of steatosis and to cause a rapid regression of fully established steatosis[20].

An excess of thyroid hormone is associated with unwanted effects particularly on the heart (including tachycardia and sudden death) and also on bone and skeletal muscle[21]. Because of these adverse effects of THs, several new TH analogs (generically termed as thyromimetics) have recently been developed to generate effective and safe treatments to counteract obesity and related disorders among which hyperlipidemia and liver steatosis. These either have selective effects on the liver *vs* the heart or bind selectively to TRβ rather than to TRα without cardiac side effects[22]. Such compounds could serve as powerful new tools to address some of the largest medical problems in developed countries-obesity and related disorders[23]. Interestingly, THs also exert non-genomic effects[24] and some are attributable to naturally occurring iodothyronines apart from T4 and T3[25,26]. These THs derivatives are currently being studied to elucidate their potential biological activities and application as anti-hyperlipidemic as well as anti-steatotic agents[22].

This review, including T3 action in the liver and fatty liver, will focus on the current understanding of the actions of thyroid hormone analogues and derivatives in fatty liver in view of the development of potential future therapeutic approaches for the prevention or counteraction of liver steatosis.

ACTIONS OF T3 ON LIPID METABOLISM IN THE LIVER: IMPLICATIONS FOR FATTY LIVER

The pleiotropic effects exerted by T3 includes the maintenance of lipid homeostasis *via* regulation of gene expression in target organs such as liver and adipose tissues. Most T3 effects are mediated by the canonical, or classic, pathway which requires the nuclear T3 receptors[27-29]. Actually, T3 can also signal through non canonical pathways by binding to cytoplasmic or mitochondrial TR isoforms[24]. In mammals, two distinct genes express the TRα and TRβ isoforms. The *TRβ* gene encodes three T3-binding TRβ isoforms (β1, β2, and β3) that share high sequence homology in the DNA and T3-binding domains but differ in length and amino acid sequences in the amino-terminal A/B domain. The *TRα* gene en-

Figure 1 Hepatic lipid partitioning and liver and systemic metabolic damages in nonalcoholic fatty liver disease (A) and a schematic representation of the anti-steatotic effect of 3,3',5-triiodo-L-thyronine (B). A: Hepatic lipid partitioning and liver and systemic metabolic damages in nonalcoholic fatty liver disease. Chronic overnutrition/hyperlipidemic feeding causes fat retention in hepatocytes that, in turn, results in alteration of fat uptake, de novo synthesis (lipogenesis) and oxidation with a significant imbalance of lipid homeostasis. This can subsequently induce insulin-resistance, metabolic syndrome and cardiovascular diseases; B: A schematic representation of the anti-steatotic effect of T3: An update. T3-administration associated adverse effects are also highlighted (for details see the text). T3: 3,3',5-triiodo-L-thyronine; TRs: Thyroid hormone receptor isoforms; FFA: Free fatty acid; TG: Triglyceride; L-FABP: Liver-type fatty acid-binding protein; COX2: Cyclo-oxygenase 2; JNK: c-Jun N-terminal kinases; STAT3: Signal transducer and activator of transcription 3.

codes one T3-high affinity binding TRα1 and two splice variants (TRα2 and TRα3) which differ from TRα1 in length and amino acid sequences in the C-terminal region starting at amino acid 370, and they have no T3-binding activity[30]. While TRα1 is preferentially expressed in the heart, TRβ1 is the major isoform in the liver, kidney and thyroid. However, TRβ2 is predominantly expressed in the brain, adipose tissue and anterior pituitary gland. The liver is an important T3 target tissue[31]. T3 increases the expression of several genes involved in hepatic lipogenesis including fatty acid synthase (FAS), hepatic product

spot 14 (which interacts physically and functionally with the TR to regulate malic enzyme gene expression[32]), acyl-CoA synthetase 5, fatty acid transporter protein, malic enzyme, glucose-6-P dehydrogenase (G6PDH)[33], sterol regulatory element binding protein-1c (SREBP-1c)[34]. T3 also induces genes involved in fatty acid oxidation, such as fatty acid transporter (FAT), fatty acid-binding protein (FABP), lipoprotein lipase (LPL)[33], and carnitine palmitoyltransferase-1alpha (CPT-1α), a key rate-limiting enzyme in mitochondrial fatty acid oxidation. In the liver, many of these genes (*e.g.*, malic enzyme, SREBP-1c, FAS

and CPT-1α) are directly regulated by T3/TR as the thyroid hormone response elements (TREs) have been reported in their promoters[34,35]. Importantly, T3 transcriptional activity also depends on several other factors including the type of TREs located on the promoters of target genes, the developmental- and tissue-dependent expression of TR isoforms, and a number of nuclear coregulatory proteins. TRs bind to TREs not only as homodimers but also as heterodimers with other members of the receptor superfamily, such as retinoic X receptors (RXRs), vitamin D receptor, and all subtypes of the retinoic acid receptors. Heterodimerization with RXR dramatically increases the binding of TRs to TREs, the responsiveness of TR to T3, transcriptional activation[30] and, due to promiscuity of RXR in heterodimerization with many members of the receptor superfamily, allows TR to crosstalk with other receptors. Crosstalk with peroxisome proliferator-activated receptor (PPAR) signaling *via* heterodimerization with RXR by TR is a well-known example[30,36].

Moreover, in the liver, activation of the NAD+-dependent deacetylase sirtuin 1 (SIRT1) facilitates fatty acid oxidation[37]. Indeed, in hepatocytes isolated from mice lacking SIRT1, fatty acid oxidation rates are reduced, and these mice accumulate lipids within the liver[38]. Recently, SIRT1 has been reported to interact directly with TRβ1, contributing to the T3-mediated stimulation of hepatic genes *via* the activation of several factors such as PPARα, estrogen-related receptor α (ERR-α), and peroxisome proliferator-activated receptor γ coactivator (PGC-1α)[39]. In the liver, PGC1-α promotes expression of genes involved in hepatic fatty acid oxidation[40] and drives the expression of genes involved in hepatic gluconeogenesis *via* interactions with HNF4α (hepatic nuclear factor-4) and FoxO1 (forkhead transcription factor)[41,42]. In particular, PGC-1α appears to be another key player in T3-signaling as it is able to coactivate the TRβ[43] and its overexpression enhances the induction T3-mediated of CPT-1α[44] and PDK4 (pyruvate dehydrogenase kinase, isozyme 4)[45,46]. Interestingly, T3 can also signal in a TRβ-independent manner by binding to surface receptor such as integrin αvb3 receptor, thus activating the MAPK/ERK and PI3K/Akt/mTOR-C1 pathways[24]. Recent studies have highlighted that such effects in hepatocytes have the potential to modulate lipogenesis and cholesterologenesis[47].

Normal serum THs levels are essential for the maintenance of a sufficient pool of cholesterol to meet the body's requirements and to regulate the critical steps of cholesterol synthesis, uptake and metabolism[19]. T3 signaling: (1) stimulates 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG CoA) reductase and farnesyl pyrophosphate (FPP), favoring cholesterol synthesis; (2) up-regulates the LDL receptor (LDLR; low density lipoprotein receptor), increasing cholesterol uptake; and (3) stimulates cholesterol 7α-hydroxylase (CYP7A1), enhancing the metabolism of cholesterol into bile acids[48-50].

Although the T3-mediated actions involved in the regulation of serum lipid homeostasis as well as in hepatic fat metabolism are quite well established, the T3-specific effects on NAFLD are still not fully elucidated. A study using a nutritional model of NAFLD [treating rats with high-fat choline-methionine deficient (CMD) diet] revealed that co-feeding T3 (4 mg/kg of diet) with a CMD diet exerts a strong inhibitory effect on the development of steatosis. Indeed, T3 prevents accumulation of TGs by inducing fatty acid oxidation with subsequent impairment of TGs hepatic synthesis/accumulation, and decreases the expression of liver-type fatty acid-binding protein (L-FABP), an abundant protein in the cytosol of hepatocytes that facilitates fatty acid transport and utilization[20]. Furthermore, the same study showed that T3 administration for only 1 wk, following 10 wk on a CMD diet, caused a rapid regression of fully established steatosis by: (1) dramatically reducing liver TGs levels and cyclooxygenase 2 (COX2) expression; (2) down-regulating pathways, such as JNK (c-Jun N-terminal kinase) and STAT3 (signal transducer and activator of transcription 3) pathways, usually activated in inflammatory processes; and (3) reducing the severity of liver injury as determined by serum levels of transaminases (AST and ALT; aspartate aminotransferase and alanine aminotransferase)[20] (Figure 1B).

Of note, T3 is also a potent mitogen that, in the liver, induces Cyclin-D1 expression[51]. Very recently, it has been shown that it exerts hepatocyte mitogenic response by PKA-dependent β-catenin activation, thus eliciting a potent liver regeneration action[52]. This has suggested that T3 can have therapeutic relevance in the treatment of selected cases of hepatic insufficiency.

However, long term treatment with T3, both in animals and humans, can produce several adverse effects including systemic thyrotoxicosis. Thus, extensive research is dedicated to the identification of new effective and safe active molecules (T3-derivatives and analogues) for the treatment of dyslipidemias, NAFLD, obesity and related disorders.

In particular, the development of synthetic thyroid hormone analogues which have tissue-selective hormone actions (*i.e.*, selective thyromimetics) has been pursued.

THYROMIMETICS AND LIVER STEATOSIS

As mentioned previously, many of the T3 actions are tissue-specific and are primarily mediated by a panel of TR isoforms that are expressed in different ratios in various tissues. Thus, there is a rationale to pursue approaches that selectively modulate TRs function, and several agents have been shown to have some β-selective, hepatic selective and/or cardiac sparing activities. The possibility of selectively targeting the TRβ was suggested by the findings that the TRα-forms may preferentially regulate the heart rate, whereas many other actions of T3 are mediated by the TRβ. X-ray crystal structures of the TRα and TRβ ligand-binding domains (LBDs) suggested that a

Figure 2 Chemical structure of thyroid hormones and thyromimetics/analogues with reported anti-steatotic effects.

single amino acid difference in the ligand-binding cavities of the two receptors could affect hydrogen bonding in the receptor region where the ligand's 1-position substituent fits and might be exploited to generate β-selective ligands[53].

The development of a TRβ-selective agonist has prompted a number of studies addressing whether such molecules could be used to trigger the metabolic effects of T3 while preserving the TRα-expressing tissues[54-58]. Essentially, these studies have been encouraging as it has been shown that the use of TRβ-selective agonists can prevent or improve metabolic parameters and/or complications resulting from high-fat feeding, NAFLD[20], or genetic hypercholesterolemia[59,60] with the liver being their major target. Indeed, tissue distribution analyses suggest that these molecules achieve TRβ selectivity by virtue of being concentrated predominantly in liver[55,61]. TRβ activation in the liver also favorably affects plasma cholesterol and lipoprotein levels by multiple mechanisms, which include increasing: (1) LDL clearance through increased expression of LDLR, (2) high-density lipoprotein (HDL) uptake through SR-B1 (scavenger receptor class B type 1); and (3) bile acid synthesis *via* CYP7A1[62].

To exploit the favorable consequences of hepatic TRβ activation, a variety of synthetic TRβ agonists have been prepared and tested on a variety of experimental models[63-73]. Ideally, these selective TRβ agonists, would cause modest increases in the metabolic rate without tachycardia[22]. However, it has been reported that most of thyromimetics could suppress thyroid axis and lower serum T4/T3 levels, especially at high doses.

Here, we will discuss two thyromimetics, namely GC-1[74] and MB07811[57,75,76] (Figure 2), for which these effects are less marked and which, at the same time, elicit anti-steatotic effects. In particular, MB07811, being specifically targeted to the liver, reduces serum T4 levels (-50% in Sprague-Dawley rats) probably by enhancing T4 to T3 conversion through deiodinase 1[53,57]. As far as it concerns GC-1, it has been shown a dose-dependent ability to reduce thyroid-stimulating hormone (TSH) levels, being this action 20-fold less potent than that of T3[55].

GC-1 (or sobetirome) is a halogen-free thyroid hormone agonist[74]. Although the structural changes it contains with respect to the natural hormone T3 (*i.e.*, replacement of the three iodines with methyl and isopro-

Figure 3 A summary of key events and molecular pathways underlying GC-1 (A) and MB07811 (B) anti-steatotic and hypolipidemic effects (for details see the text). TSH: Thyroid-stimulating hormone; T3: 3,3',5-triiodo-L-thyronine; TRβ: Thyroid hormone receptor β isoform; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; FFA: Free fatty acid; TG: Triglyceride; T4: Thyroxine; Apo-C3: Apolipoprotein C3; mGPDH: Mitochondrial glycerol-3-phosphate dehydrogenase; CPT-1α: Carnitine palmitoyltransferase-1α.

pyl groups, replacement of the biaryl-ether linkage with methylene linkage, and replacement of the amino acid side chain with an oxyacetic acid side chain)[77], it binds the TRβ with an affinity that is comparable to that of T3[22]. Functionally, when GC1 is administered to rats undergoing a CMD diet prevents and reverses the hepatic steatosis much like T3[20]. Moreover, similar to T3, GC-1 can reduce liver weight, liver weight/body weight ratio, and serum TGs levels. GC-1 also causes a reduction of CMD-induced TGs accumulation in the liver, with the

disappearance of hepatic TGs being accompanied by a concomitant decrease of lipoperoxidation, and of liver injury as indicated by the significant reduction in AST and ALT levels[20]. These findings made GC-1 an ideal molecule for therapies against fatty liver disease. Interestingly, GC-1 also stimulates energy expenditure[78] and mitochondrial oxidative processes but to a lesser extent compared to T3[79,80] (Figure 3A). Notably, animal studies[55] revealed that treatment with GC-1 also induces a reduction of cholesterol levels similar to that obtained

with equimolar doses of T3 and even higher than that achieved with the most common drugs currently available on the market for the treatment of hypercholesterolemia, such as the inhibitors of HMG CoA reductase (statins)[77]. In doing so, GC-1 regulates key steps in the reverse cholesterol transport pathway[62], increasing the expression of HDL receptor SR-B1 in the liver, stimulating the activity of CYP7A1 and inducing the expression of hepatic ATP-binding cassette proteins G5/G8 (ABCG5/G8), which promote biliary cholesterol secretion. Consequently, treated animals displayed an increased turnover of plasma HDL cholesterol, and an increased amount of fecal excretion of bile acids and cholesterol[70]. Phase 1 clinical studies tested the therapeutic concept of lowering cholesterol and found GC-1 to be generally well tolerated at all doses studied[81,82].

In a recent study, GC-1 has also been shown to be capable of markedly reducing serum cholesterol in mice devoid of functional LDLRs by inducing CYP7A1 expression[60]. These results, having elucidated the possibility that a LDLR-independent mechanism could underlie GC-1 action, potentiated the idea that GC-1 may represent a promising cholesterol-lowering therapeutic with a specific application for the treatment of diseases such as homozygous familial hypercholesterolemia. Currently, there are only limited treatment options for this disorder because most therapeutics are only minimally effective.

Another agent being studied is MB07811 which exhibits increased TR activation in liver relative to other tissues[57]. By using several experimental approaches, MB07811 was shown to have anti-steatotic activity and was able to reduce hepatic triglyceride levels in both normal and metabolically-challenged animal models, including ob/ob mice, Zucker rats, and mice with diet-induced obesity (rodent models of NAFLD)[83]. The main mechanism underlying MB07811 effects appears to be an augmented metabolic rate in the liver and, specifically, an increased rate of mitochondrial β-oxidation. MB07811 increases: (1) the levels of CPT-1α[35] and short and intermediate length acyl-carnitine species in plasma and (2) the liver mitochondrial respiration rates as well as the activity of hepatic mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH), an enzyme which is important for energy production and dissipation. Decreased mRNA levels of apolipoprotein C3 (Apo-C3), an inhibitor of hepatic lipase activity, might also contribute to the activation of fatty acid oxidation pathways. Additionally, MB07811 can also lower both serum cholesterol and triglyceride levels[83,84] (Figure 3B). These data in rodents confirm that MB07811 represents a novel class of liver-targeted TR agonists with beneficial LDL-lowering properties, and suggest that these compounds may provide additional therapeutic benefit to hyperlipidemic patients with concomitant NAFLD[83]. The human Phase 1b clinical trial showed reduced LDL cholesterol and TGs levels in both normolipidemic and hyperlipidemic subjects without severe adverse events[23].

Together, these data demonstrate that selective activation of hepatic TR prevents or reverses fatty liver and reveals a new approach to treat NAFLD based on selectively burning hepatic fat.

Now, the question is whether other molecules such as naturally occurring TH metabolites, even deprived of TR selectivity or characterized by low binding affinity for TRs, can have uses as therapeutic applications.

NATURALLY OCCURRING IODOTHYRONINES AND LIVER STEATOSIS: 3,5-T2

An increasing amount of data indicates that there are at least four natural iodothyronines with significant, but not identical, biological activities, namely T4, T3, rT3 (reverse T3), and 3,5-diiodo-L-thyronine (T2). T2 is particularly intriguing because of its effects on metabolism[22,25,26]. T2 has been estimated, in euthyroid rats, to reach serum concentrations of approximately 5 pM and is present in liver at concentrations of approximately 1.0 fmol/100 mg[85]. In humans, serum T2 levels consistently elevated in disease states, with the mean T2 serum level being 16.2 ± 6.4 pM in healthy subjects, 21.6 ± 4.8 pM in patients with brain tumors and 46.7 ± 48.8 pM in patients with sepsis[86].

T2 measurements are routinely taken using methods based on immunoassays, an approach with high sensitivity but which lacks specificity for many analytes[87,88]. In recent years, mass spectrometry (MS) techniques have drawn attention to the analyses of T4 and T3 because they provide high mass accuracy, structural information, and have the ability to quantify the hormones[89-93]. A recently developed methodology revealed that electrospray ionization tandem mass spectrometry (ESI-MS/MS) can be used for identification and quantification of mixtures of isomers and has been applied to identify and quantify T3 and rT3 isomers as well as T2 isomers[94,95]. Currently, however, intrinsic instrumental limits restrain the application of such approaches as routine tools for biological samples analysis and slow the advancements in understanding T2 metabolism[96,97].

T2 is a product of a currently unknown peripheral enzymatic process most probably utilizing T3 as its precursor[85] and has 50-1000 times lower affinity for TR than T3[98]. Thus, it is unlikely that TR activation represents a central mechanism in its effects on metabolism, at least in physiological conditions. However, more recent studies have reported new data concerning T2 binding to TRβ isoforms in teleosts[99,100]. Specifically, T2 has been shown to bind and transactivate both the human and the long tilapia TRβ1 isoform whereas T3 preferentially binds the short isoform. These results prompted a reevaluation of the mechanisms of action of thyroid hormone metabolites.

Several studies on T2 effects in mammals revealed its ability to stimulate cellular/mitochondrial respiration by pathways with mitochondria and bioenergetic mecha-

nisms being the major targets[26,80,101-103]. Outside the mitochondria, T2 also has effects on carriers, ion-exchangers, and enzymes, and may affect the transcription of some genes, but again the underlying mechanisms appear to be different from those elicited by T3[26].

In 1998, Arnold *et al*[104] identified the Va subunit of the mitochondrial respiratory chain complex cytochrome-*c* oxidase (COX) as a specific binding site for T2 using photoaffinity labeling procedures. T2 binding to the COX complex abolishes the allosteric ATP inhibition of COX which leads to a decrease in the respiratory control ratio of the complex[105] thus rendering the oxidative phosphorylation more inefficient.

The biological and pharmacological importance of T2 has become a topic of considerable interest to researchers during the past few years, and now represents a significant and promising issue in the field of metabolism and THs.

The effects and mechanisms underlining the beneficial actions of T2 have so far been studied with both *in vivo* and *in vitro*[106-108] models. *In vivo* studies from different laboratories have shown that acute or chronic administration of T2 to rats results in significant changes in mitochondrial activities and resting metabolic rate[22,26,109].

A recent study reported an increased basal metabolic rate and decreased body weight also in humans chronically administered with T2 with no deleterious side effects on the thyroid axis or at the cardiac level[110].

Of the currently described *in vivo* stimulatory and beneficial effects of T2, a particular physiological and pharmacological relevance appears to be associated with those effects that we can define as hypolipidemic and anti-steatotic effects which have been described in several animal models[111-114]. Among the methods to study liver metabolism and physiology, the variation in the nutritional status is a widely used approach because of its ability to affect several signaling pathways and regulatory mechanisms[115-118]. High fat feeding (HFD) in animals, in particular, has the advantage to mimic most features of human fat overload and overnutrition and allows the study of obesity and related disorders such as ectopic fat accumulation.

Specifically, the administration of T2 to rats subjected to HFD is able to prevent and reduce the visceral fat accumulation as well as hepatosteatosis, serum levels of triglycerides and cholesterol, and the onset of IR without inducing thyrotoxicosis[111,112,114,119]. Moreover, T2 has been reported to elicit additional beneficial effects on lipid metabolism by reducing LDL-cholesterol in a LDLR independent way in a mouse model of familial hypercholesterolemia[120].

The simultaneous administration of T2 (25 μg/100 g body weight) to rats receiving a HFD for 4 wk can prevent liver steatosis by stimulating hepatic fatty acid oxidation and increasing mitochondrial uncoupling[111]. This leads to a less efficient utilization of lipid substrates, and helps to prevent body-weight gain, hepatic fat accumulation, hypertriglyceridemia and hypercholesterolemia lev-

els without inducing changes in T3 and T4 serum levels or affecting the hypothalamus-pituitary-thyroid (HPT) axis[111,119].

The T2 effects on liver fatty acid oxidation are paralleled by an increased entry of activated fatty acids into the mitochondria *via* activation of the CPT system. This leads to a strong reduction in the fatty acids inside the cell and a strong activation of AMP-activated protein kinase (AMPK), enhancing the cycle of fat uptake and fat burning and the disappearance of lipid droplets (LDs)[111].

Importantly, T2 administration prevents the HDF-induced lipid peroxidation, as well as the increase in H_2O_2 metabolism counteracting both lipid accumulation and oxidative stress associated with increased fat metabolism[121].

In a more recent study, to further investigate how T2 affects lipid and glucose metabolism in HFD rats eliciting beneficial effects on liver, independently of AMPK, the involvement of another important regulator of metabolic balance[122-127], SIRT1, was studied[114]. In HFD rats, T2 was demonstrated to (1) rapidly increase hepatic nuclear SIRT1 activity and (2) through SIRT1-activation, deacetylate PGC-1α and SREBP-1c with a concomitant upregulation of genes involved in mitochondrial biogenesis and downregulation of lipogenic genes[114]. Moreover, the obtained data added new information on the time-latency of the anti-steatotic effect of T2 which within 6 h after administration, rapidly and directly activates hepatic SIRT1 (affecting β-oxidation and mitochondrial biogenesis) and later (4 wk) promotes AMPK phosphorylation/activation thus profoundly modulating liver expression pattern of genes and proteins.

A proteomic study[113] showed that the steatotic effect of HFD, and the anti-steatotic effect of T2-treatment are strictly associated with altered expression levels of several proteins and enzymes involved in key liver metabolic (canonical and non-canonical) pathways across different subcellular compartments (*i.e.*, cytoplasm, mitochondria and nuclei). These pathways included: fatty acid metabolism, ketone-bodies and energy metabolism, amino acid and nitrogen metabolism, the urea cycle and the stress response and protein turnover.

All the analyzed liver subcellular compartments were significantly affected, in terms of protein expression, by both HFD and long-term T2-treatment. However, mitochondria appeared to be a major target for the metabolic and energy adaptations induced by fat-overload, and displayed a significant response, in terms of their proteome, to T2-treatment. These data supported the concept that T2-supplementation while having hypolipidemic and anti-steatosis effects, may provide protection against diet-induced liver damage, possibly by counteracting the alterations in the expression of several cellular proteins, reducing oxidative stress and impairing the mitochondrial respiratory chain[113].

T2 administration to rats is also able to reduce pre-existing hepatic fat accumulation (that had already been induced by feeding with a HFD)[112] by eliciting systemic

and tissue specific effects. In particular, T2, without suppressing TSH, decreases body weight gain, metabolic efficiency and serum levels of cholesterol, triglycerides and ALT. In the liver, T2 increases hepatic mitochondrial oxygen consumption and fatty acid oxidation and activates mitochondrial proton leak reducing mitochondrial oxidative stress[112].

In vitro studies have been performed to address whether the anti-steatotic effect of T2 is due to the direct action of T2 on the liver or if it is a secondary effect due to upstream changes in endocrine or metabolic pathways. Primary cultures of rat hepatocytes overloaded with lipids ("fatty hepatocytes") and then treated with T2 showed a reduction in: (1) lipid content and LD diameter; (2) PPARs expression; and (3) activities of acyl-CoA oxidase (AOX) and antioxidant enzymes. These data support a direct role of T2 in reducing the excess fat in cultured hepatocytes[128]. The putative involvement of TRs in mediating such lipid-lowering effects of T2 has been elucidated using the rat hepatoma FaO cellular model, which is defective for functional TRs[128]. The addition of T2 to lipid-overloaded cells resulted in: (1) reduction in lipid content; (2) downregulation of PPARα, PPARγ, and AOX expression; (3) increase in PPARδ expression; and (4) stimulation of mitochondrial uncoupling[128]. These data demonstrate, for the first time that the *in vitro* lipid-lowering actions of T2 may be not mediated by TRs.

All the utilized approaches, both in animal models and humans, successfully highlighted metabolic actions and potential pharmacological use of T2. Notably, T2, without thyrotoxic side effects, increasing resting metabolic rate, decreasing adiposity and body weight, could be considered an active agent in preparation for treatment of metabolic disorders, such as T2DM, overweight and NAFLD (Figure 4A).

THYROID HORMONE FUNCTIONAL ANALOGUES: TRC150094 (TRC)

T2 research has been recently focused on the discovery and development of T2 functional analogues with therapeutic potentials. In particular, a series of novel substituted pyrazoles were designed and synthesized as T2 analogs with lower affinities toward TRs. Among these molecules, TRC150094 (TRC) has attracted particular attention emerging as a novel thyromimetic and functional T2-analogue linking fat consumption with the pathogenesis of hepatic steatosis. The chemical name for TRC is 3-[4-(7-hydroxy-6-methyl-indan-4-ylmethyl)-3,5-dimethyl-pyrazol-1-yl]-propionic acid, and its chemical structure is shown in Figure 2. TRC was synthesized by the Torrent Research Centre, Torrent Pharmaceuticals Ltd., Ahmedabad, Gujarat, India and has a much lower potency toward both TRα1 and TRβ1 isoforms' activation than T3. Therefore, it is devoid of adverse effects on the heart classically associated with TR hyperactivation [the rank order of potencies for TR (α1/β1) transcriptional activation being T3 > T2 >>TRC[129]].

When screened for its *in vivo* metabolic effects, TRC (injected, at a dose of 0.750 mg/100 g body weight, in rats simultaneously undergoing a HFD for 4 wk) counteracts the hepatic pathological condition observed in HFD rats without any thyrotoxic effects[129]. The anti-steatotic activity of TRC is due to increased rates of mitochondrial fatty acid uptake and oxidation, respiratory chain activity and resting metabolic rate, and to the activation of the CPT system[129,130]. Importantly, TRC significantly increases SIRT1 activity although the SIRT1 protein level remains unaltered. These results strongly suggested that a TRC-induced increase in SIRT1 activity might underlie the increase in fatty acid oxidation and the prevention of liver steatosis observed in the TRC-treated rats[129].

An integrated functional study performed by combining *in vivo* and *ex vivo* metabolic assays with proteomic and bioinformatic analyses showed that TRC administration to rats with pre-existing body fat accumulation significantly altered the expression levels of several proteins and enzymes involved in key liver metabolic pathways, including amino acid, nitrogen, fructose and mannose metabolism, and RXR activation and function[130]. Consistent with the increased oxidation of mitochondrial fatty acids and the unaltered mitochondrial efficiency, numerous mitochondrial enzymes associated with fatty acid oxidation and energy metabolism were increased in livers from HFD-TRC[130].

Oral administration of TRC to obese Zucker rats (obese ZSF1, spontaneously hypertensive fatty rats) decreased hepatic steatosis[131] by inducing a significant increase in mitochondrial respiration as well as an increased fatty acids oxidation. All the above mentioned studies have so far provided the characterization of the pharmacological/metabolic effects of TRC and highlighted its potential utility as a new compound with effectiveness for prevention/amelioration of certain key biochemical parameters altered during feeding on a HFD-regimen and a cluster of multiple cardiovascular risk factors associated with visceral obesity, steatosis and metabolic derangements (Figure 4B). If reproduced in humans, these results could determine whether TRC150094 represents an attractive therapeutic agent for the treatment of overweight dysglycemic patients.

Indeed, clinical trials are currently in progress to translate these effects into approaches for the treatment of human obesity.

THYROID HORMONES AND MITOCHONDRIAL FUNCTIONS

As already stated, mitochondria play a fundamental role in the development, perpetuation and worsening of liver steatosis and NAFLD. Indeed, the generation of NAFLD, apart from involving defects or polymorphisms in mitochondrial DNA, can be an important consequence of damages to the respiratory chain complexes impairing mitochondrial oxidative capacity, particularly critical when fatty acid supply to the hepatocytes is increased as
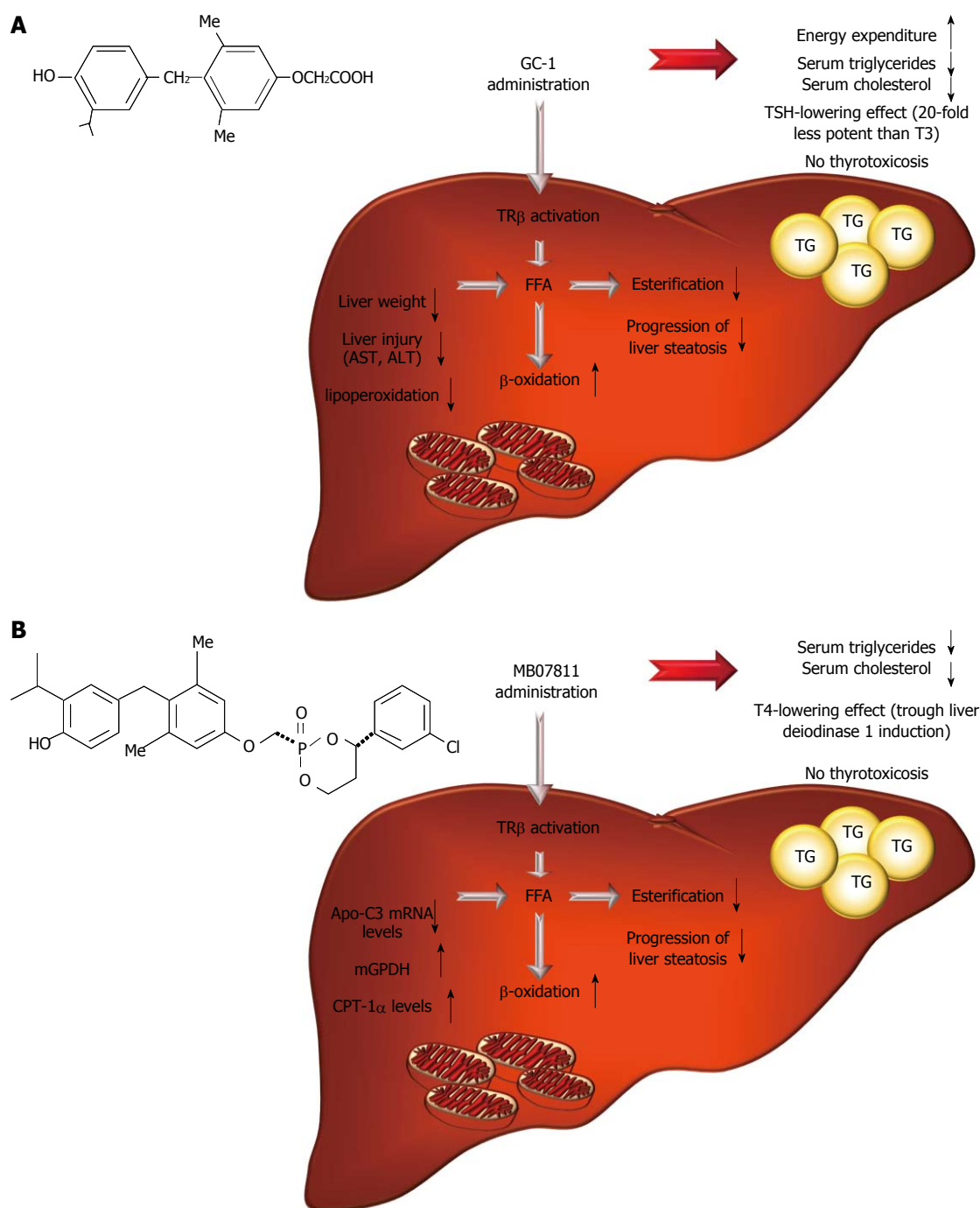
Figure 4 A summary of key events and molecular pathways underlying T2 (A) and TRC (B) anti-steatotic and hypolipidemic effects (for details see the text). T2: 3,5-diiodo-L-thyronine; TR: Thyroid hormone receptor; COX Va: Cytochrome-*c* oxidase Va subunit; FFA: Free fatty acid; TG: Triglyceride; P-AMPK: Phosphorylated AMP-activated protein kinase; SIRT1: NAD$^+$-dependent deacetylase sirtuin 1; PGC-1α: Peroxisome proliferator-activated receptor γ coactivator; SREBP-1c: Sterol response element binding protein-1c; CPT: Carnitine palmitoyltransferase system; OXPHOS: Oxidative phosphorylation system.

in calorie-rich diets[132].

Mitochondrial bioenergetics deficits may be the consequence of: (1) a progressive decay of oxidative capacity with impairments of β-oxidation; and (2) major changes in the redox balance with increased reactive oxygen species production.

It is widely recognized that mitochondria are central targets for THs actions. Indeed, mitochondria, providing about the 90% of the cellular energy supply, likely may be a major player of the so called calorigenic effects of THs[133]. In particular, T3 stimulates mitochondriogenesis and thereby augments cellular oxidative capacity and induces, at the same time, substantial modifications in mitochondrial inner membrane protein and lipid compositions, activating uncoupling of oxidative phosphorylation[133].

In terms of time latency, two types of effects of T3 on mitochondria have been described: (1) a rapid stimulation of respiration, which is evident within minutes/hours after hormone treatment; (2) a delayed induction

of mitochondrial biogenesis and changes in mitochondrial mass, which occur one to several days after hormone treatment. The first effect is probably due to extranuclear/non-genomic mechanisms; the second one involves both T3-responsive nuclear genes and a direct action of T3 at mitochondrial level[109,133]. In other words, this second effect allows T3 to modulate mitochondria activity in two different ways: direct or indirect. The direct action requires the presence inside the organelles of specific binding sites for the hormone[134-136] while the indirect one possibly requires T3 binding to extramitochondrial sites and the modulation of the expression of either nuclear-encoded mitochondrial proteins or intermediate factors (*e.g.*, nuclear respiratory factors 1 and 2; mitochondrial transcription factor A)[137,138].

T3, by the regulation of these pathways, allows the coordinated expression of both the nuclear and the mitochondrial genome that in turn modulates mitochondrial biogenesis, turnover and bioenergetics.

Although the network of factors and cellular events involved in T3 signaling remains incompletely understood, the so far described mechanisms can justify, at least in part, the above reported hypolipidemic and antisteatotic effects elicited by T3 in NAFLD.

CONCLUSION

Considering the impact of THs on the maintenance of lipid homeostasis but also of their adverse effects in TH excess states, recent efforts to identify effective and safe treatments for the counteraction of metabolic disorders (such as liver steatosis), has led to the development and characterization of thyromimetics. Some of these analogs are undergoing further examinations for possible clinical applications. However, despite their original promise, it is unlikely that any first generation synthetic ligands (*i.e.*, GC-1 and MB07811) which already reached human clinical trials will develop into therapeutics. Thus, attention should be focused on other molecules, such as T2 or TRC which could have therapeutic applications. Indeed, such compounds could serve as powerful new tools to address some of the largest over-nutrition associated medical problems as they are able to reduce, at least in animal models of diet-induced obesity, body adiposity, serum triglycerides and cholesterol. They also preserve glucose homeostasis without thyrotoxic side effects. Notably, the hypolipidemic effect of T2 is associated with a potent ability in both preventing and reducing fatty liver. Increasing evidence supports TH derivatives and analogues as attractive active agents that could be taken into consideration for the establishment of new treatments in the counteraction of metabolic disorders, such as T2DM, obesity and NAFLD, thus clinical trials are desirable.

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MINIREVIEWS

Management of gastric variceal bleeding: Role of endoscopy and endoscopic ultrasound

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Abstract

Gastric varices (GVs) are notorious to bleed massively and often difficult to manage with conventional techniques. This mini-review addresses endoscopic management principles for gastric variceal bleeding, including limitations of ligation and sclerotherapy and merits of endoscopic variceal obliteration. The article also discusses how emerging use of endoscopic ultrasound provides optimism of better diagnosis, improved classification, innovative management strategies and confirmatory tool for eradication of GVs.

Core tip: This mini-review addresses endoscopic management principles for gastric variceal bleeding. Endoscopic variceal obliteration (EVO) with tissue adhesives is the currently accepted strategy for controlling bleeding and eradicating gastric varices (GVs). EVO is deemed better than both variceal ligation and sclerotherapy in randomized controlled trials. One unsettled issue with EVO is if routine reinjection is better than reinjection in case of rebleeding. The experience with combination treatments is still premature. For secondary prophylaxis, EVO, transjugular intrahepatic portosystemic shunt or beta-blocker use is recommended. Emerging use of EUS provides optimism of better diagnosis, improved classification, innovative management strategies and confirmatory tool for eradication of GVs.

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INTRODUCTION

The natural history of gastric varices (GVs) is less understood than that of esophageal varices (EVs). GVs may be seen in 18%-70% of the patients with portal hypertension (PHT) and are probable source of bleeding in 10%-36% of patients with acute variceal bleeding (AVB)[1-3]. Isolated GVs (IGVs), without EVs, are seen in 5%-12% of patients with PHT[1-3]. They are also commonly seen in patients with non-cirrhotic portal hypertension (NCPHT),

especially with splenic vein thrombosis (SVT). They are more commonly associated with shunts than EVs, most commonly spleno-renal shunt, and their management is quite different from that of EVs.

CLASSIFICATIONS

GVs are commonly classified according to Sarin's classification[4,5] based on location and direction of blood flow: GOV1 (Gastro-Oesophageal Varices) are the most common (74% of all GV) and consist of the esophageal varices extending along the lesser curvature of stomach; GOV2 are the extension of the esophageal varices along the greater curvature near the fundus; IGV1 are isolated gastric varices localized to fundus, without any associated esophageal varices. These arise from spleno-renal or gastro-renal shunts where the feeding vessel arises from the splenic hilum and drains in to left renal vein through gastric cardia/fundus veins. GOV2 and IGV1 are sometimes together called "fundic varices". IGV2 are the isolated gastric varices present elsewhere other than the fundus, which drain in a similar fashion into left renal vein but with multiple tributaries. It is reported that fundal varices (GOV2 and IGV1), though less common than GOV1 varices, are noted to account for 80% of patients with bleeding GV.

Hashizume *et al*[6] proposed an alternate classification of GVs based on endoscopic findings, taking into account their shape (tortuous = F1, nodular = F2, and tumorous = F3), location (anterior = La, posterior = Lp, lesser curvature = Ll, greater curvature = Lg of the cardia, and fundic area = Lf) and color (white = Cw or red = Cr) and further emphasized on presence of glossy, thin-walled focal redness on the varix called as red color spot (RC spot) as a marker of impending bleeding risk[6].

BLEEDING RISK OF GVS

Although GVs are known to bleed less frequently than the EVs, however when they do, they bleed massively and are difficult to achieve primary hemostasis, with a mortality rate of 10%-30%[4,5]. Their chance of re-bleeding is high (35% to 90%) after spontaneous remission and 22%-37% with the glue technique[4,5]. The chance of variceal bleeding is driven by the pressure changes rather than hemostatic forces. The pressures in the GVs are lower than the in the EVs because of their larger size and more frequent presence of the shunts like spleno-renal[7,8]. Despite this, their rupture is more devastating because of the fact that the wall stress increases dramatically even with small rise in the portal pressures due to their larger radius. When there is increase in transmural pressure, the variceal size increases and wall thickness decreases, which leads to rupture[7,8]. The factors which predict hemorrhage in EVs also govern GVs: most importantly the size of the varices (15% in patients with large varices, which are defined as > 10 mm), decompensated cirrhosis and endoscopic presence of the red wale sign. Another factor

implicated in increase in incidence and/or size of fundic varices and possible bleeding is the treatment of EVs by either endoscopic variceal ligation (EVL) or endoscopic sclerotherapy (EST)[9]. The plausible explanation is that after treatment the existing collaterals are not sufficient enough to decompress the portal pressure causing an increased incidence of fundic varices.

GENERAL PRINCIPLES OF MANAGEMENT OF BLEEDING GVS

The preliminary management of bleeding GVs is the same as any other variceal bleeding[1-3]. Fluid resuscitation, airway protection, antibiotic administration for the bacterial peritonitis prophylaxis and use of vasoactive agents like octreotide and acid suppressant agents like proton pump inhibitors form cornerstone of initial management. Cautious administration of the blood products (to achieve a target of hemoglobin level between 7-8 g/dL) is advocated as there is potential risk of increased rebleeding if the portal pressures increase due to repeated transfusions. A schematic of management algorithm of GVs is presented in Figure 1.

Treatment options for acute GV bleeding are varied and include medical, surgical, endoscopic, and endovascular approaches[1-3]. Two general methods exist to deal with bleeding GVs: directly exclude the varices from the porto-systemic system or indirectly decrease the pressure in the varices by decompressing the portal system.

Direct approach
Variceal management by direct endoscopy or endoscopic ultrasound.

Role of endoscopy: Once the patient is deemed stable from airway and circulation standpoint, an esophagogastroduodenoscopy (EGD) should be performed, which might show active bleeding or reveal stigmata of recent bleeding, in addition to qualify type of GVs and concomitant presence of EVs or PHG[1-3].

Several endoscopic techniques have been tested to control acute gastric variceal bleeding with varying successes. However, the universal phenomena is that majority of the methods used in controlling the bleeding EVs are difficult to practice in GVs and are inconsistently successful. These include endoscopic injection sclerotherapy (EIS) and esophageal variceal ligation (EVL). The varying success of these methods may be owing to different physiology and size of GVs which pose technical problems.

GV ligation: The main indications for ligation in management of acute GV bleeding is banding of GOV1 varices, which are extensions of EVs into the stomach along the lesser curvature or as salvage strategy if other modalities are not available[10]. Studies suggest good hemostasis efficacy and comparable re-bleeding rates of GOV1 ligation to EVL of EVs. There is limited role for ligation

Figure 1 Proposed management algorithm for gastric varices. High risk patient: Child Pugh class B or C or endoscopic presence of red wale sign; Low Risk Patient: Child Pugh class A and no endoscopic high-risk features. GV: Gastric varices; EVO: Endoscopic variceal obliteration; EVL: Endoscopic variceal ligation; EIS: Endoscopic injection sclerotherapy; NSBB: Non specific beta blocker; BRTO: Balloon-occluded retrograde transvenous obliteration; TIPS: Trans-jugular intra-hepatic porto-systemic shunt; GOV: Gastro-oesophageal varices; IGV: Isolated gastric varices.

in management of bleeding fundic varices[1-3]. In head-to-head studies, EVL was less effective than endoscopic obturation by injection of cyanoacrylate for hemostasis of large GVs[11], and had higher re-bleeding rates too[12]. Smaller studies have attempted improvisation of ligation methods to increase its success in GV, like using detachable snares and elastic bands or in combination with sclerotherapy, however these experimental techniques are yet to be implemented universally[13,14].

Endoscopic injection sclerotherapy: Fundic varices (IGV1 and IGV2) are wider and have larger volume, needing large quantity of sclerosant which is susceptible to being washed away, potentially leading to systemic (esp. pulmonary) embolization, and may also lead to increased chances of ulceration at injection site. Before the advent of newer techniques, sclerotherapy of GVs using alcohol or tetradecyl sulfate was common and was associated with decent initial hemostasis rates (up to 67%-100%),

however the higher frequency of re-bleeding mainly due to post-procedure ulceration severely limited its long-term success[10]. Furthermore, the risk of complications including fever, retrosternal chest pain, temporary dysphagia and pleural effusions was unacceptably higher with EIS[15]. Overall, the success of EIS is questionable in management of acute GV bleeding[16] and hence is not the preferred method in any of the guidelines.

Endoscopic variceal obturation: Endoscopic variceal obturation (EVO) using tissue adhesives like glue, cyanoacrylate or histoacryl has provided a positive direction to management of fundic varices, which was always a challenge. Cyanoacrylate is a polymer which upon coming in contact with blood polymerises instantly leading to obliteration of varices. It is called "obliteration" and not "eradication" since the varices may be still visible post-treatment.

EVO with N-butyl-2-cyanoacrylate has been the advocated first-line method in managing the gastric varices especially fundic varices[1-3]. Kang *et al*[17] performed EVO with cyanoacrylate in 127 patients with GVs (100 active bleeding and 27 prophylactically) and reported a primary hemostasis rate of 98.4% (1 session-98 patients, 2 sessions-25 patients, ≥ 3 sessions-4 patients), with a recurrent bleeding rate of 18.1 % at 1 year[17]. Several studies have compared EVO head-to-head with EIS or EVL to conclude the favorable outcomes of EVO in terms of initial hemostasis, and lesser re-bleeding and complications[11,12,18-20]. Furthermore, re-bleeding rates after EVO were found to be comparable to transjugular intrahepatic porto-systemic shunt (TIPS) in patients with acute GV bleeding, suggesting this technique may be equally efficacious in secondary prevention and creating opportunity of therapy in patients in who TIPS is contraindicated for encephalopathy reasons[21]. Few studies have advocated using dynamic CT scan prior to EVO to increase the detection of feeding vessels, assessment of direction of blood flow, presence of shunts, in an attempt to increase efficacy and minimize complications of EVO technique[22], although this is not universally practiced.

Although EVO is clearly a superior technique than EIS or EVL for bleeding GVs, it is not free of technical difficulties (para-variceal injection, needle sticking in the varix, intra-peritoneal injection leading to peritonitis and adherence of the glue to the endoscope) or complications (fever, para-variceal injection with mucosal necrosis and bleeding, embolization into the renal vein, IVC, pulmonary or systemic vessels and retro-gastric abscesses)[12,18-20]. However, emerging literature supports preference of distilled water over saline to dilute cyanoacrylate to decrease coagulation and use of standardized techniques of tissue adhesive preparation and delivery to decrease rates of these complications[23]. In case of large gastric varix, it is advised to begin tissue adhesive injection from bottom to dome to minimize risk of bleeding if injected directly at high pressure-high flow dome area. Liu *et al*[24] reported an interesting scenario which devel-

oped when EVO of GVs led to hemorrhage from EVs due to embolism of the glue into the EV thus increasing the pressure. This was not amenable to EV ligation due to presence of foreign body (glue) and was managed with cyanoacrylate injection into EVs to achieve hemostasis and authors rightly cautioned endoscopists to treat EVs in the same setting as EVO of GVs to prevent such a complication[24].

Another major difference between EVO and other endoscopic techniques is that variceal obliteration of the GVs is not quite obvious after cyanoacrylate injection, and hence adequacy of EVO is controversial. Most often GVs are probed with an endoscope and the induration is accepted as a sign of inadequate obliteration with the need to inject more tissue adhesive till it is "hard" to palpate. Improved radiology (use of CT portography)[22] and newer endoscopic techniques have made this EVO adequacy assessment easier, as discussed later in this article. Notably, EVO has recently been shown to be superior to beta blocker therapy for primary prophylaxis of GVs and hence is being advocated[25]. Evidence regarding efficacy of the glue in pregnant females and in children is still emerging and premature, and so is data on newer combination EVO-sclerotherapy modalities[26].

Novel EVO materials: Endoscopists are trying several materials to achieve hemostasis in technically challenging situations, like successful use of hemostatic powder in situation with failed EVO with cyanoacrylate glue and contraindication to TIPS due to dilated cardiomyopathy[27]. Thrombin was used by Yang *et al*[28] and Ramesh *et al*[29] in separate studies to successfully achieve initial hemostasis 100% and 92% patients, with re-bleeding rates of 27% and 0% respectively. Thrombin helps in clotting by converting fibrinogen to fibrin and promotes platelet aggregation as well. Although these studies were limited by their patient size (12 and 13 patients respectively)[28,29], and did not report any untoward thrombo-embolic events, the concern for thrombin leakage into systemic circulation and potentially causing disseminated intravascular coagulation (DIC) or systemic embolization still remains. It is currently not being advocated due to lack of adequate data.

Role of endoscopic ultrasound: It is common knowledge that endoscopic ultrasound (EUS) enables the visualization of esophago-gastric varices and other venous collaterals viz. peri- and para-esophageal collateral veins and perforating veins, in patients with PHT, and can be useful to assess the patency of the portal venous system[30]. There has been an attempt in 1993 to classify gastric varices endosonographically by Boustière *et al*[31], which considered size of GVs and gastric wall abnormalities (Table 1), and inferred that while endoscopy graded EVs better, EUS was a better tool to classify GVs and early signs of portal gastropathy. The other EUS features of portal hypertension, in addition to EVs and GVs, may include dilatation of the azygos vein, splenic vein

Table 1 Endoscopic ultrasound classification of gastric varices: Proposed by Boustiere *et al* in 1993

Endoscopic ultrasound classification of gastric varices	
1: Size of gastric varices	Grade 0 (none)
	Grade 1 (small or non-confluent varies < 5 mm)
	Grade 2 (large or confluent varices ⩾ 5 mm)
2: Abnormalities of gastric wall	Grade 0 (none)
	Grade 1 (thickening and brilliance of the third hyperechogenic layer with or without fine internal anechogenic structures)
	Grade 2 (visible vessels in the third layer which deform the entire wall, with penetrating varices)

and portal vein, increased diameter of the thoracic duct, thickening of gastric mucosa and submucosa, presence of portal hypertensive gastropathy, and the presence of rectal varices[30,32]. In addition, EUS combined with color Doppler imaging enabled visualization of shunts viz. gastro-renal shunt in one report[33]. Furthermore, EUS doppler helps characterize gastric submucosal lesions better than EGDs before proceeding to the biopsy of potential GV.

Role of EUS in risk estimation for GV bleeding is a field of growing interest. EUS probes can be used to measure size of varices (diameter), and furthermore to estimate variceal wall thickness which is deemed as a better predictor of bleeding than varices diameter alone[34]. Intra-variceal pressure measurement may be a better surrogate for risk of bleeding, which can be accomplished by direct variceal puncture which is not practiced because of invasiveness. Although data is slim, there has been an attempt looking at EUS guided EV pressure recording, to better predict risk of bleeding, and it has been shown to have reasonable correlation with hepatic venous pressure gradient (HVPG)[35]. Finally, high risk stigmata like red hematocytic spot can be visualized with EUS[36].

EUS-assisted injection sclerotherapy for both gastric[37] and esophageal varices[38] is effective, achieving high eradication and low recurrence rates in long-term follow-up. In fact the risk of re-bleeding after EUS directed sclerotherapy is reportedly lower than endoscopic technique. Recently additional attention has been diverted towards EUS delivered therapies to control bleeding in acute variceal bleeding patients, using unique agents like adhesive tissue (histoacryl)[39], thrombin[40] and EUS-guided coil injection for gastric[41] and ectopic duodenal varices[42]. Last but not the least, EUS finds its utility in confirmation of adequacy of EVO of gastric varices, eliminating the need for inept endoscope probing assessment and thus increasing overall efficacy of EVO technique[43]. A recent study from Taiwan used miniature ultrasound probe (MUP) sonography in 34 patients who underwent cyanoacrylate EVO therapy for acute GV bleeding, during follow-up endoscopy session to assess adequacy of obturation and reinjection if necessary. The authors demonstrated a significantly greater

free-of-rebleeding rate and trend towards better survival for patients in MUP group compared with conventional endoscopy group[43]. Although these advances bring a sound of promise, EUS probe which has a larger diameter compared to conventional scope, in addition to GV intervention is certainly a high-risk procedure. Using a mini-probe may counter some of this added disadvantage but non-availability of pediatric sizes is still a limitation. Furthermore, future studies need to compare radial and linear EUS scopes in diagnosis and management of varices.

Indirect approach
Decreasing portal pressure - either surgically or percutaneously by establishing a TIPS.

Role of TIPS: Porto-systemic shunts such as TIPS are typically advocated as second-line acute therapy (after endoscopic management) to prevent re-bleeding of varices[1-3]. Although decreasing portal pressure is considered effective in reducing the bleeding rate of EVs, it is inconsistently effective for GVs, which tend to occur and bleed at lower portal pressures[21,44]. Also there is discordance between decreased hepato-portal gradient with TIPS and actual decrease in GV re-bleeding. In addition, TIPS has its own limitations including worsening of encephalopathy or shunt occlusion, which can lead to recurrence of hemorrhage, and surveillance for patency.

Role of advanced radiological procedures: If all endoscopic techniques and TIPS fail or if TIPS is contraindicated, then the next step would be balloon-occluded retrograde transvenous obliteration (BRTO)[1-3], which is a popular technique in Japan, and allowing modulation of flow within the varices. BRTO was popularized and named by Kanagawa *et al*[45] in 1996, this technique optimize the action of the sclerosing agent by inducing stagnation in the gastric varices, thereby allowing maximal sclerosant dwell time to cause endothelial sclerosis and vascular thrombosis. The discussion of technique, advantages and complications of BRTO is beyond the scope of current mini-review, but one of the emerging fronts in management of acute GV bleeding.

CONCLUSION
GVs are notorious to bleed massively and often difficult to manage with conventional techniques. EVO with cyanoacrylate glue injection is currently the most favored for being superior to variceal ligation or sclerotherapy in achieving hemostasis in acute gastric variceal bleeding. Endoscopists must remain cognizant about the possible complications of tissue adhesive injections and strive for standardization of EVO techniques to minimize them. Novel techniques like use of thrombin, coil embolization are under investigation as alternatives to cyanoacrylate aiming for improved outcomes. TIPS and BRTO are advanced radiological procedures available as

salvage techniques in uncontrollable bleeding situations or when patients are not candidates or have failed endoscopic management. The role of EUS in the therapeutic algorithm for GVs is still evolving. EUS is being used to confirm presence, size and location of GVs, to stratify the risk of re-bleeding, as a therapeutic tool to perform sclerotherapy or EVO, and to confirm eradication of GVs after EVO. Emerging use of EUS provides optimism of better diagnosis, improved classification, innovative management strategies and confirmatory tool for eradication of GVs.

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Role of intrahepatic innervation in regulating the activity of liver cells

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Abstract

Liver innervation comprises sympathetic, parasympathetic and peptidergic nerve fibers, organized as either afferent or efferent nerves with different origins and roles. Their anatomy and physiology have been studied in the past 30 years, with different results published over time. Hepatocytes are the main cell population of the liver, making up almost 80% of the total liver volume. The interaction between hepatocytes and nerve fibers is accomplished through a wealth of neurotransmitters and signaling pathways. In this short review, we have taken the task of condensing the most important data related to how the nervous system interacts with the liver and especially with the hepatocyte population, how it influences their metabolism and functions, and how different receptors and transmitters are involved in this complex process.

Key words: Liver innervations; Hepatocytes; Neurotransmitters; Cellular receptors

Core tip: Liver innervation comprises sympathetic, parasympathetic and peptidergic nerve fibers, organized as either afferent or efferent nerves with different origins and roles. Their anatomy and physiology have been studied in the past 30 years, with different results published over time. Hepatocytes are the main cell population of the liver, making up almost 80% of the total liver volume. The interaction between hepatocytes and nerve fibers is accomplished through a wealth of neurotransmitters and signaling pathways.

INTRODUCTION

Innervation of the liver comprises efferent and afferent nerves containing sympathetic, parasympathetic and peptidergic fibers. Sympathetic nerve fibers derive from splanchnic nerves and the parasympathetic counterparts have a vagal origin. Fibers derived from splanchnic, vagus and sometimes the phrenic nerves enter the liver through the hilum, together with the hepatic artery, portal vein and bile duct. Some nerve fibers do not accompany hepatic vessels and enter the liver *via* the small omentum or the hepatic vein. Sympathetic and parasympathetic nerves form two separate plexus but communicate with each other: the anterior plexus placed around the hepatic artery, consisting of nerve fibers with their origin in the celiac ganglion and posterior vagus nerve, and the posterior plexus located around the portal vein and bile duct,

formed by fibers from the celiac ganglion and the right vagus[1]. Nervous fibers which are distributed to the hepatic parenchyma derive from a corresponding nervous plexus and their intrahepatic distribution differ according to species[2,3].

In the human liver, nerve endings are located in the hepatic lobules[4], which consists of hepatocytes and non-parenchymal cells. Unlike hepatocytes, which occupy almost 80% of liver volume and have numerous functions, non-parenchymal liver cells occupy only 6.5% of the liver, although representing 40% of total liver cells[5].

Hepatocytes are arranged as cellular cords with a radial disposition that converges towards the centrilobular vein, being separated by sinusoidal capillaries. Between hepatocyte cell cords and sinusoid capillaries there is an interstitial space, a perisinusoidal called a Disse space. This space is formed by a fine network of reticulin fibers, a support for the sinusoids, non myelinated nerve fibers and mesenchymal type cells[6]. Non-parenchymal cells are located in the liver sinusoidal compartment. The hepatic sinusoidal wall consists of three cell types: sinusoidal endothelial cells (SECs), Kupffer cells (KCs) and hepatic stellate cells (HSCs)[5]. Most nerve endings from intralobular spaces are located in Disse spaces[4,7-12], where they make close contact with HSCs, SECs and hepatocytes[7,8,10].

NERVOUS INFLUX TRANSMISSION MECHANISM INTO HEPATOCYTES

Hepatocytes serve multiple functions, such as synthesis, storage, metabolism and transformation of carbohydrates, amino acids, proteins, lipids, vitamins and detoxification, conjugation and excretion of exo- and endogenous substances. During liver regeneration, hepatocytes initiate cell proliferation, maintain metabolic function of the liver, secrete interleukin-6 (IL-6), proteases, protease inhibitors and hepatocyte growth factor[13].

The liver receives both sympathetic and parasympathetic nerve fibers; however, the innervation that hepatocytes receive varies by species. Thus, in the cat, rabbit, guinea pig liver as well as primate liver, it appears that nerve endings are connected to all hepatocytes, unlike rats and mice in which only hepatic cells in the portal region appear to be in contact with intrahepatic nerve endings[14].

Nerve fiber communication with hepatocytes can be accomplished by several mechanisms (Figure 1): (1) hepatocyte direct innervation mediated by norepinephrine and acetylcholine, neuropeptides [neuropeptide Y, galanine (NPY), vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP), *etc*.], purines [adenosine triphosphate or adenosine (ATP)]; (2) signals intercellular transmission using gap type junctions; and (3) sinusoidal capillary cells innervation which interact with hepatocytes through eicosanoids (prostaglandins, leukotrienes), cytokines (necrotic factor, IL-6, IL-1) and other chemical mediators (endothelin, nitric oxide).

Direct innervation of hepatocytes

Nerve transmission to hepatocytes is achieved through neurotransmitters such as norepinephrine and acetylcholine, neuropeptides, such as NPY, galanine, VIP and CGRP, or purine derivatives as ATP and adenosine.

The liver is stimulated by norepinephrine and epinephrine released from intrahepatic nerve endings but also derived through the blood from the adrenal glands. Catecholamines act in the liver on α1-, α2- and β2-adrenergic receptors[15-17]. Norepinephrine is removed from the site of action by intrahepatic nerve ending uptake, being degraded by liver cells and diffused through the vascular bed[1].

Experiments on rat liver have shown that stimulation of the autonomic nerve plexus around the hepatic artery and portal vein causes increased production of glucose and lactate[1], urate and allantoin formation[17], decreased ketogenesis[18], increased ureogenesis and ammonia uptake[19], as well as increased oxygen utilization[20,21]. Also, hepatic nerve stimulation leads to decreased[16,17,20,22,23] and redistributed intrahepatic flow[21], as well as raised noradrenaline levels in the hepatic vein[15-18]. All these effects of hepatic nerves are only possible in the presence of extracellular calcium[22,24].

NPY, galanin, SP, CGRP, VIP and purine derivatives (ATP, adenosine) act as neurotransmitters, both in adrenergic and cholinergic nerve fibers, as well as in the related hepatic nerves. These neurotransmitters are released locally and are involved in regulating hepatic microcirculation. NPY and ATP act as vasoconstrictors, while VIP, CGRP, SP and adenosine produce vasodilation[14].

Some neurotransmitters also have a metabolic function. Thus, sympathetic hepatic nerve stimulation causes the release of noradrenaline but also of galanin[25], suggesting that galanin potentiates the action of norepinephrine to stimulate hepatic glucose production under stress[14].

Yamamoto *et al*[26] revealed the metabolic activity of ATP, which potentiates the action of hepatic sympathetic nerves suppression action on the formation of ketone bodies in the liver, the effect probably being due to ATP interaction with norepinephrine[14].

Intercellular transmission of signals through gap type junctions

Intrahepatic innervation varies by species. In some species, most hepatocytes are not directly innervated but there is an indirect mechanism for transmitting nervous inflow. One such mechanism is the intercellular communication carried out between adjacent hepatocytes *via* specific channels known as gap type junctions (GJ), which allow the passage of ions and small molecules[14].

GJ density is different among species[27]. Thus, hepatic GJ are more numerous in rats and mice compared to rabbits and guinea pigs[14].

GJ are membrane channels that allow intercellular communication between neighboring cells. GJ consists of two hemichannels, one hemichannel belonging to each

Figure 1 Schematic representation of the communication pathways between nerve fibers and hepatocytes. AG: Adrenal gland; IH NE: Intrahepatic nerve endings; ATP: Adenosine triphosphate; NPY: Neuropeptide Y; VIP: Vasoactive intestinal peptide; CGRP: Calcitonin gene-related peptide; IL: Interleukin; NF: Necrotic factor; NO: Nitric oxide; GJ: Gap junction.

of the two adjacent cells. A hemichannel consists of six subunits or connexins. Connexin 32 (Cx32) is the major protein component expressed in murine hepatocytes. Cx32 plays an essential role in signal propagation induced by the norepinephrine released from sympathetic nerve endings in hepatocytes[28].

GJ ensure the transmission of information to neighboring cells, achieving functional integration of hepatocytes, and thus functioning as a body and not as a mere cluster of cells[28].

GJ have an important role in transmitting nervous impulses from sympathetic nerve endings in parenchymal and non-parenchymal cells of the liver, in some species of mammals. There is an inverse relationship between sympathetic nerve fiber density and number of intrahepatic GJ[14]. Research on rat liver, which contains numerous GJ[15,29], showed that sympathetic nerves innervate only a part of parenchymal and non-parenchymal cells of the periportal area[15]. Metabolic and hemodynamic effects of hepatic sympathetic nerves are achieved by $\alpha 1$ receptor stimulation[17,23,30], are abolished by prostanoids synthesis inhibitors, and are mimicked by prostaglandins but not by thromboxanes[31]. Norepinephrine, as well as F2α prostaglandin, stimulates the release of glucose from isolated hepatocytes with increased inositol 1,4,5-triphosphate and glycogen formation[32].

Sending signals through GJ is involved in the metabolic effects of sympathetic nerves[14]. The norepinephrine released from sympathetic nerve endings binds to $\alpha 1$ receptors of parenchymal and non-parenchymal cells. The release of glucose from the proximal parenchymal cells from the periportal region under the action of norepinephrine initiates a signal which propagates through GJ to distal parenchymal cells, which in turn releases glucose. Norepinephrine stimulates contraction of proximal non-parenchymal cells (sphincters), reducing the flow in the sinusoid capillaries. It also causes the release of prostaglandins (PG) into the Disse space. PG, in turn, bind to prostanoid receptors of parenchymal and non-parenchymal cells. PG released from non-parenchymal cells are rapidly degraded in the liver[32] and so do not reach the distal cells. PG increase glucose release in the periportal parenchymal cells and perhaps initiate a signal that propagates to the other cells by GJ. PG stimulate contraction of proximal non-parenchymal cells, thus reducing the flow to sinusoid capillaries[15,33].

Sinusoidal capillary cells innervation
Sinusoidal capillaries are located between the cell cords and have a 9 to 12 micrometers diameter. The sinusoidal capillary wall is discontinuous and is formed out of a basement membrane and a capillary endothelium. Sinusoidal endothelium consists of flattened endothelial cells and phagocytic Kupffer cells in a ratio of 5/3. In addition to these two main types of cells in the sinusoid capillaries, there are also stellate cells and lymphocytes[6]. Capillary walls have an important role in the regulation

of the sinusoidal microcirculation[34,35].

Endothelial cells are flat and have elongated, hyperchromic nuclei and reduced cytoplasm. Junctional complexes are lacking. There are very small spaces of 0.5 micrometers between the cells. The apical membrane of the endothelial cells has transcytoplasmic fenestrations which are small holes arranged in nests with a diameter of 100 nm, with a role in controlling cholesterol, lipoprotein and vitamin A metabolism. In the cytoplasm of endothelial cells, a small number of cell organelles and pinocytosis vesicles were revealed. Endothelial cells produce prostaglandins, endothelin, IL-1 and IL-2[6,13].

Hepatic stellate cells (Ito cells, lipocytes) are located in the Disse space in small niches, among hepatocytes. They are in contact with the liver cells and through their microvilli and cytoplasmic processes have contacts with endothelial cell microvilli[6]. Well developed organelles and lipid droplets were found in the HSC cytoplasm. HSCs store vitamin A, containing factors related to retinoid acid and retinol, and produce extracellular matrix. Under normal conditions, HSCs have a deposit function, while in pathological ones it transforms into myofibroblastic type cells[6,13,36,37].

Research has shown that the distribution of intrahepatic innervation varies by species[29,38-43]. Guinea pig, cat and tupaia have an intralobular innervation similar to the human one[11,12,29,40], in contrast to mice and rats where it differs from the human[39,41-43].

In human liver, nerve endings are located in the Disse space[4,11,12], closely connected to hepatocytes and non-parenchymal cells, particularly HSCs[7,8,10,11].

Non-parenchymal cells are the only ones that can synthesize eicosanoids (prostaglandins, thromboxanes and leukotrienes) from arachidonic acid released from phospholipids by the action of phospholipase A_2 and converted to prostaglandins and thromboxanes *via* the cyclooxygenase path and leukotrienes *via* the lipoxygenase path[1]. Experiments on perfused rat liver have shown that the synthesis and secretion of prostanoids in non-parenchymal liver cells is influenced by a number of physiological stimuli, pathological and chemical. These stimuli also determine an increased release of glucose and lactate, as well as increased vascular resistance in the liver[1]. Of these stimuli, the most important are: extracellular nucleotides[44], nucleosides[45], zymosan[46,47], endotoxins[48], aggregates of immunoglobulins[49], anaphylatoxins[50,51], phorbol esters and calcium ionophores[44]. Norepinephrine and/or other chemical mediators released from nerve endings can stimulate the formation of prostanoids in non-parenchymal liver cells. Prostanoids, in turn, can modulate hepatocyte metabolism[1].

Of eicosanoids, only PG, without thromboxanes and leukotrienes, play a role in the events triggered by nerve stimulation[1].

PG participation in the chain of events initiated by nerve stimuli in the liver depends on hepatocellular receptors for PG. Research conducted so far confirms the existence of these receptors[1].

HSCs are indirectly involved in nerve fiber communication with hepatocytes, through PG. Noradrenaline may lead, by means of α1-adrenergic receptors, to increased synthesis of PG in the HSC, and PG, in turn, stimulate glycogenolysis in the hepatocytes. Unlike KC, producing predominantly PGD_2, HSC secret PGF 2 alpha released in increased amounts compared with PGD_2, as a result of sympathetic stimulation[52].

Intrahepatic nerve fiber terminations, often containing vesicles which contain neurotransmitters like substance P (SP) and vasoactive intestinal peptide (VIP), are closely related to HSC. It is considered that HSC that surround CECS, forming sinusoidal capillary walls, have a role in the contraction and relaxation of sinusoidal walls, thus intervening in the regulation of the sinusoidal microcirculation[4].

HSC contraction is stimulated by a number of substances such as endothelin-1 (ET-1), angiotensin II, norepinephrine, prostaglandin F_2, thromboxane A_2 and thrombin. In contrast, vasoactive substances such as acetylcholine, VIP, nitric oxide (NO), carbon monoxide, prostaglandin E_2 and adrenomedulin produce HSC relaxation[4].

ET-1 produces contraction of HSCs through ET receptor stimulation on autocrine or paracrine pathways. HSC contraction appears to be related to the increase of intracellular Ca^{2+} and inositol phosphate. For the sinusoid microcirculation control role of the HSC, the presence in the HSC cytoplasm of α smooth muscle actin, which is a contractile protein, also stands, such that the contraction of the HSC can be compared with that of smooth muscle cells in the vessel wall structure. On the other hand, prostaglandin E_2, adrenomedulin and other vasoactive substances determine HSC relaxation by increasing intracellular cAMP. In addition, HSC produces NO and inhibits contractility by an autocrine mechanism linked to NO[8].

Of the mentioned vasoactive substances, ET-1 and NO have an important role in the regulation of sinusoidal microcirculation. ET is a peptide consisting of 21 amino acids with a strong vasoconstrictive effect on the smooth muscle fibers. It has three isoforms, ET-1, ET-2 and ET-3[4,53].

Two receptors have been identified for ET: ET_A and ET_B, both belonging to the superfamily of G-protein-coupled receptors[54]. ET_A receptor has a higher affinity for ET-1 and ET-2 than for ET-3, while the ET_B receptor has a similar affinity for all three isoforms of ET. ET_A receptors stimulation increases intracellular cAMP levels, whereas ET_B receptor stimulation leads to inhibition of the adenylate cyclase system[55]. Also, ET_B receptor stimulation activates Ca^{2+}-dependent NOS[56]. Douglas *et al*[57] described two ET_B receptor subtypes: ET_{B1} and ET_{B2}. Stimulation of ET_A and ET_{B1} receptors causes contraction of smooth muscle fibers, while ET_{B2} receptor stimulation causes dilation by increased synthesis of NO[58].

ET-1 receptors from intralobular spaces predominate in the juxtaportal region. About 35% of ET-1 receptors

are located in the HSCs, a smaller number are located in the CECS and KCs[59]. Both ETA and ETB receptors are found in the liver. All cells of the sinusoidal capillary walls have ETB receptors but only HSCs have ETA receptors. Mallat *et al*[60] have identified 20% ETA receptors and 80% ETB receptors on activated HSCs.

NO is synthesized from L-arginine by the NO synthetase path (NOS). Three NOS isoforms have been identified: two are calcium-dependent, one produced by the neurons (nNOS or NOS I) and other by the vascular endothelial cells (eNOS or NOS III), and one calcium-independent isoform, cytokine-induced (iNOS or NOS II). Rat HSCs shrink under ET-1 or SP action and relax, causing vasodilatation, under NO action produced by HSC under the influence of IL-1[4,61].

In normal liver, sinusoidal contraction is inhibited by ETA receptor antagonists[62,63] but, according to some authors, not by ETB receptors antagonists[4,62-64]. Other researchers believe, however, that ETB receptor stimulation results in constriction of sinusoid capillaries[65]. It is possible that this discrepancy between the results obtained by different authors is due to coupling ETB receptors with NOS, which masks the vasoconstrictor effect[63,66].

The various subtypes of endothelin have different effects on hepatic microcirculation. The relationship between NO and endothelin is extremely important in the control of vascular tone[63].

Liu *et al*[58] have shown that ET-1 binding to ETB receptor leads to eNOS activation on the Akt phosphorylation path, thus reducing the phosphorylation of eNOS and NO synthesis. The same researchers highlighted the crucial role of βγ subunits of the G protein in triggering endothelin/NO reactions. The stimuli which regulate the ET expression and vascular sensitivity to ET also adjust the NOS and heme oxygenase-1 activity. Both enzymes catalyze the production of substances which, by guanylate cyclase activation, produce vasodilation[67].

CONCLUSION

Liver innervation is one of the most complex control systems in the human body; therefore, a better understanding of its inner workings is of paramount importance for developing future therapies and procedures for ameliorating the metabolic function of the liver. Being able to manipulate nerve impulses and synaptic mediators can possibly allow direct control over the functions of hepatocytes. Direct acting agents with excellent control over specific liver functions could become a reality, with direct implications for drug therapy, surgery or liver transplant.

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Hepatoprotective effect of silymarin

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Abstract

The use of medicinal plants in treating illnesses has been reported since ancestral times. In the case of hepatic diseases, several species such as *Silybum marianum*, Phyllanthus niruri, and *Panus giganteus* (Berk.) have been shown to ameliorate hepatic lesions. Silymarin is a natural compound derived from the species *Silybum marianum*, which is commonly known as Milk thistle. This plant contains at least seven flavoligands and the flavonoid taxifolin. The hepatoprotective and antioxidant activity of silymarin is caused by its ability to inhibit the free radicals that are produced from the metabolism of toxic substances such as ethanol, acetaminophen, and carbon tetrachloride. The generation of free radicals is known to damage cellular membranes and cause lipoperoxidation. Silymarin enhances hepatic glutathione and may contribute to the antioxidant defense of the liver. It has also been shown that silymarin increases protein synthesis in hepatocytes by stimulating RNA polymerase I activity. A previous study on humans reported that silymarin treatment caused a slight increase in the survival of patients with cirrhotic alcoholism compared with untreated controls.

Core tip: One of the mechanisms of liver damage caused by alcohol is the generation of free radicals formed by the metabolism of this xenobiotic. Silymarin is an antioxidant that protects the liver from the free radical damage produced by alcohol metabolism. Silymarin is the most used natural compound for the treatment of hepatic diseases worldwide due to its antioxidant, anti-inflammatory, and anti-fibrotic activities. Silymarin functions by stabilizing biological membranes and increasing protein synthesis.

INTRODUCTION

The liver is an important organ that has a key role in the

maintenance of homeostasis. The liver is responsible for multiple metabolic functions and physiological processes such as bile production, energy generation, vitamin storage, and the metabolism of carbohydrates, proteins, and lipids. After intestinal absorption is complete the blood is rich in nutrients and xenobiotics. The blood is then transported to the liver *via* the portal vein, which carries multiple toxic substances including ethanol (Et-OH), drugs, pharmaceuticals, and toxins to the liver. As a result, the liver is susceptible to toxicity and damage. Many people have been afflicted with some type of liver lesion. Examples of liver lesions include fatty liver, non-alcoholic steatosis, hepatitis A, B, or C, cirrhosis, and hepatocellular carcinoma (the third leading cause of cancer-related mortality worldwide)[1]. Hepatic diseases are primary causes of morbidity and mortality worldwide. The most recent surveillance report published by the National Institute on Alcohol Abuse and Alcoholism showed that liver cirrhosis was the 12th leading cause of death in the United States[2]. Liver disease is exacerbated by unhealthy lifestyles, obesity, and the excessive consumption of alcohol and drugs[3].

The use of medicinal plants has been reported since ancestral times. In the case of hepatic diseases, several species such as *Silybum marianum*[4], *Phyllanthus niruri*[5], and *Panus giganteus* (Berk.) have been shown to ameliorate hepatic lesions[6].

SILYMARIN

Overview

Flavonoids are polyphenol compounds that are also considered essential nutrients. Their basic chemical structure consists of two benzene rings bound by a three-atom heterocyclic carbon chain. The oxidation of the structure generates several families of flavonoids (flavones, flavonoles, flavanones, anthocyanins, flavanoles, and isoflavones). Chemical modifications of each family can lead to > 5000 individual compounds with different properties[7].

Silybum marianum is the scientific name for Milk thistle or St. Mary's thistle. It is a plant native to the Mediterranean region and belongs to the Asteraceae family. It is characterized by thorny branches and a milky sap, with its oval leaves reaching up to 30 cm. The flowers are bright pink and can measure up to 8 cm in diameter[8]. Milk thistle grows in its wild form in southern Europe, northern Africa, and the Middle East. The plant is cultivated in Hungary, China, and South American countries such as Argentina, Venezuela, and Ecuador. In Mexico, Milk thistle is consumed as a supplementary food[9].

Silymarin is a natural compound that is present in species derived from *Silybum marianum*, which is commonly known as Milk thistle. The plant contains at least seven flavolignans and the flavonoid taxifolin. The most important flavolignans present include silybin, silydianin, and silychristine. Silybin represents between 50% and 70% of the extract from silymarin. The following flavolignan iso-

forms are known (Figure 1): silybin A, silybin B, isosilybin A, and isosilybin B[10]. Silymarin has been used worldwide for many years as a complementary alternative medicine because of the beneficial effects associated with the treatment of hepatic diseases. Silymarin belongs to the Aster family (Asteraceae or Compositae). The mature plant has large brilliant-purple flowers and abundant thorns. The plant grows in places with sufficient sun exposure[11].

The low level of bioavailable flavolignans is known. For example, the level of silymarin absorption is between 20% and 50%. Silybin is the major compound of silymarin and limiting factors such as low solubility in water, low bioavailability, and poor intestinal absorption reduce its efficacy. New soluble silybin-derived biocompounds (silybin bis-hemisuccinate, β-cyclodextrin complex, silybin-*N*-methyl-glucamine, silybin 11-*O*-phosphate, and silybin-phosphatidylcholine) have thus been designed[10]. Chronic inflammation occurs in patients with hepatic damage. Thus, for patients with compensatory cirrhosis, hepatitis C, and non-alcoholic hepatic steatosis, the bioavailability of compounds present in silymarin may be affected, which may also explain the low effectiveness of treatment with flavonoids in these patients[12,13].

Sy-Cordero *et al*[14] isolated four key flavolignans and diastereoisomers (silybin A, silybin B, isosilybin A, and isosilybin B) from *S. marianum* on a gram-scale. These compounds and two other related analogues are present in extremely minute quantities. The compounds were evaluated for their antiproliferative/cytotoxic activity against human prostate cancer cell lines. Silymarin reduces the incidence of certain cancers[15]. Su *et al*[16] used silymarin on nasopharyngeal carcinoma cells (NPC-TW01) and found an increase in Bcl-2 expression and a decrease in the activated caspase-3 or apoptosis-inducing factor (AIF) with low-dose (80 μmol/L) treatment.

The molecular targets of silymarin for cancer prevention have been studied. Milk thistle interferes with the expression of the cell cycle regulators and proteins involved in apoptosis. Thus, it can modulate the balance between cell survival and apoptosis. Lee *et al*[17] reported that silybin inhibited the kinase activity of mitogen-activated protein kinase (MEK)-1/2 and ribosomal S6 kinase (RSK)-2 in melanoma cells. The treatment of melanoma cells with silybin attenuated the phosphorylation of extracellular signal-regulated kinase (ERK)-1/2 and RSK2, which is regulated by the upstream kinases MEK1/2. The blockade of MEK1/2-ERK1/2-RSK2 signaling by silybin resulted in the reduced activation of nuclear factor-kappa B (NF-κB), activator protein-1, and STAT3. These proteins are transcriptional regulators of several proliferative genes in melanomas. Silybin blocks the activation of these transcription factors and induces cell-cycle arrest at the G1 phase, which inhibits melanoma cell growth *in vitro* and *in vivo*. Silymarin suppresses ultraviolet radiation A-induced oxidative stress (OS), which can induce skin damage. Thus, the topical application of silymarin can be a useful strategy for protecting against skin cancer[18].

Silibyn A

Silibyn B

Isosilibyn A

Isosilibyn B

Figure 1 The chemical structure of silybin A, silybin B, isosilybin A, and isosilybin B, the extract of silymarin.

In previous studies, the inherent hepatoprotective and antioxidant activity of silymarin was shown to be caused by its control of free radicals (FR), which are produced by the hepatic metabolism of toxic substances such as Et-OH, acetaminophen (Paracetamol), or carbon tetrachloride. The FR damage cellular membranes and cause lipoperoxidation (LPO)[19]. The cytoprotective effect in liver is also caused by the inhibition of the cyclooxygenase cycle, leukotrienes, and the production of FR in Kupffer cells in mice. These affects reduce inflammation[20], and it has been suggested that silymarin also performs the following functions: protecting against genomic injury, increasing hepatocyte protein synthesis, decreasing the activity of tumor promoters, stabilizing mast cells, chelating iron, and slowing calcium metabolism, among other activities that have been described in the literature[21].

Silymarin has been reported to have antioxidant, immunomodulatory, anti-fibrotic, anti-proliferative, and antiviral properties. It also affects the synthesis of RNA and DNA. Furthermore, silymarin maintains the integrity of the hepatocyte membrane and impedes the entrance of toxic substances or xenobiotics. Due to its phenolic nature, it is capable of donating electrons to stabilize FR and reactive oxygen species (ROS). Silymarin also affects intracellular glutathione, which prevents lipoperoxidation of membranes[22].

Pure compounds extracted from silymarin have been examined in cell lines infected with the hepatitis C virus (HCV). Polyak *et al*[23] showed that silymarin inhibits the replication of an infectious HCV genotype 2a strain (JFH1) in hepatoma cell cultures. The most effective compounds were isosilbin A, taxifolin, and silybinin, and these compounds reduced virus infection. The OS level induced by HCV, the tumor necrosis factor (TNF)-α level, and the transcription factor NF-κB were affected by silbyn A and silbyn B treatment. In general, all of the compounds showed antiviral activity and reduced the OS level caused by HCV infection[24].

The use of a silymarin extract in 72 patients with non-alcoholic hepatic steatosis (non-alcoholic fatty liver disease, NAFLD) on a controlled diet led to significantly reduced levels of alanine aminotransferase (ALT) and aspartame aminotransferase (AST) (AST/ALT < 1). Another parameter evaluated was γ-glutamyl transpeptidase (γ-GT). In NAFLD patients, γ-GT is high because of obesity, hyperinsulinemia, inflammation, and changes in the membrane permeability of the hepatocytes. The level of γ-GT decreased due to the silymarin-mediated inhibition of toxins entering the cells. Additionally, silymarin permits the stabilization of hepatocyte membranes. It also reduced the level of TNF-α, which reduces inflammation. A favorable change in the hepatorenal clearance index was also observed, which suggests a reduction in the accumulation of lipids in the liver. All of these results were visible after 6 mo of treatment[4].

EVIDENCE FROM STUDIES OF SILYMARIN AS A HEPATIC PROTECTOR AGAINST ETHANOL

Silymarin has both hepatoprotective and regenerative actions. The mechanism of action is a reduction of the FR formed by toxins that damage the cell membranes (LPO) and competitive inhibition through hepatocyte external cell membrane modification. Silymarin forms a complex that impedes the entrance of toxins into the interior of liver cells. Additionally, silymarin metabolically stimulates hepatic cells and activates the RNA iosynthesis of ribosomes to stimulate protein formation[25-27]. In a study published by Sandoval *et al*[28], the authors observed a

silymarin protection effect in rat hepatic cells when they used it as a comparison factor to measure liver weight/animal weight % (hepatomegaly). The hepatomegaly was reduced compared to other groups that were administered antioxidant substances. There was no significant difference observed between the silymarin group and the silymarin-alcohol group. This result suggests liver protection by silymarin. Silymarin enhances hepatic glutathione generation by elevating cysteine availability and inducing cysteine synthesis while inhibiting its catabolism to taurine. The regulation of cysteine synthesis may subsequently contribute to the antioxidant defense[29]. Silymarin reduced collagen accumulation by 30% in biliary fibrosis induced in rats[30]. A study in humans reported a slight increase in the survival of patients with cirrhotic alcoholism compared with untreated controls[31]. Silymarin is perhaps the most frequently used natural compound for the treatment of hepatic diseases worldwide due to its antioxidant, anti-inflammatory, and anti-fibrotic activities[32].

Study conducted with guinea pigs (*Cavia porcellus*) examining hepatic fibrosis induced through the administration of Et-OH (4/kg of weight/d) for 90 d revealed a significant reduction of lesion markers such as ALT, AST, and γ-glutamyl after silymarin treatment. The gene expressions of cytochrome 450 2E1 (CYP2E1), TNF-α, transforming growth factor beta-1 (TGF-β1), and nuclear factor kappa-light-chain-enhancer of activated B cells-1 were also reduced. There was also a reduction in FR and reduced markers of fibrosis such as alpha smooth muscle actin, collagen α_1(I), and in the caspase cytotoxicity marker. However, silymarin was less effective than vitamin C in this study. This result indicates that vitamin C is more effective in reducing the markers of damage and the production of ROS during Et-OH-induced lesions[33]. Another study evaluated the hepatoprotective effect by measuring the level of antioxidants and the effect of body weight (*bw*) in rats exposed to Et-OH (1.6 g/kg of *bw* for 4 wk). The results revealed that intoxication by Et-OH influences the *bw* of rats and the levels of thiobarbituric acid reactive substances (TBARS). The activity of the enzymes superoxide dismutase (SOD) and glutathione-*S*-transferase (GST) increased significantly. Conversely, glutathione (GSH), the activity of glutathione reductase (GR), glutathione peroxidase, and catalase (CAT) were reduced by exposure to Et-OH. The rats that received silybin and ascorbic acid had attenuated lesion markers, although the effect was greater in the group that received ascorbic acid than in the group treated with silybin. The study also concluded that stopping alcohol intake favors hepatic regeneration. Thus, it is more effective to take preventive measures than to implement curative treatment[34]. A mouse study examining the antioxidant, immunomodulatory activity and vascular function of mice showed a significant increase in OS levels in animals that received ethanol (1.6 g/kg per *bw*/d during 12 wk). Ethanol increased the production of TBARS, nitrite levels, and the activity of GST. Ethanol also significantly diminished the content of GSH and the activity of SOD,

CAT, GPX, and GR. Mice that received Et-OH plus silymarin (250 mg/kg of *bw*/d for 12 wk) normalized the altered parameters. In addition, the silymarin-treated mice had reduced levels of interleukin-10 (IL-10), TNF-α, interferon (IFN), IFN-γ, vascular endothelial growth factor-A, and TGF-1β. The treatment also reduced the levels of IL-4 in the blood. The results of silymarin treatment were similar to mice that received vitamin C treatment[35].

The use of Sylubim β-cyclodextrin has been studied in non-insulin-dependent patients with diabetes and alcoholic hepatopathy. Treatment with a 135-mg/d dose did not influence insulin secretion but did significantly reduce the glucose ($P < 0.03$) and serum levels of triglycerides ($P < 0.01$) compared with the placebo. These results suggest that this treatment improves the response to insulin[36].

Clinical study conducted in 170 cirrhosis patients treated with 140 mg of silymarin three times daily for 41 months showed significant improvement, especially in the subgroups with alcoholic cirrhosis and initial "Child A" hepatic disease[31]. However, the results are controversial. A meta-analysis of 13 randomized clinical assays evaluated the beneficial or detrimental effects of Milk thistle and included patients with alcoholic and/or hepatitis B and C hepatic disease. The authors concluded that according to the data, Milk thistle did not significantly influence the improvement of these diseases. Conversely, it may have negatively affected the pathological condition[37].

CONCLUSION

There is substantial evidence suggesting that silymarin treatment improves hepatic diseases. However, some of the data are contradictory. Therefore, additional molecular studies investigating the mechanisms of action for these compounds are needed. It is known that silymarin does not possess adverse effects at high doses. Thus, it is a natural compound that is widely utilized in traditional medicine and has been investigated in formal scientific studies. Diverse hepatic damage models and ethanol injury have been utilized to study silymarin because ethanol is responsible for many cases of liver damage worldwide. The current data demonstrate that the use of silymarin treatment in alcoholic cirrhosis patients may attenuate the damage. However, silymarin treatment does not affect mortality.

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BRIEF ARTICLE

Khat (Catha Edulis) as a possible cause of autoimmune hepatitis

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Abstract

AIM: To investigate the potential role of khat in triggering auto immune hepatitis.

METHODS: Patients with a history of khat use and acute hepatitis were identified using the computer database in the hepatology department at the Royal Hallamshire Hospital. They were then assessed for probability of having autoimmune hepatitis using the revised autoimmune hepatitis scoring criteria.

RESULTS: Six patients were identified. All of them had presented with acute hepatitis on a background of khat. All were male and five of these patients were of Somali origin, while one patient was from Yemen. The patients were given points on the modified autoimmune hepatitis score which is based on their liver enzymes, autoimmune screen, exclusion of viral hepatitis alcohol and drugs, immunoglobulin levels and liver histology. The patients were given a score of -4 for khat use due to its potential to cause drug induced liver injury. Five of these patients scored between 10 and 15 points, placing them in the probable group for having autoimmune hepatitis. All of these patients were treated with prednisolone and demonstrated a good response to immunosuppression.

CONCLUSION: One possibile cause of hepatotoxicity with khat could be *via* triggering of autoimmune hepatitis in a genetically susceptible individual. Further studies are needed for confirmation.

Key words: Khat; Autoimmune hepatitis; Drug induced liver injury; Acute hepatitis; Herbs

Core tip: Khat causes hepatotoxicity. One possible mechanism could be by inducing autoimmune hepatitis.

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INTRODUCTION

Khat (Catha Edulis Celestrasae) is an evergreen shrub native to East Africa and Southern Arabia. It is chewed daily by over 20 million people in these countries. Chewing khat is a popular social habit, particularly in young males, that has spread to Yemeni, Somali and East African communities living in the United Kingdom and United States[1]. The use of khat as a stimulant is increasing primarily due to immigration. The Somali population in the United Kingdom is estimated to be as high as 90000, and

concerns over health and social problems associated with chewing khat have grown due to its potential side effects including hypertension, coronary vasospasm, myocardial infarction, delayed intestinal absorption, and mood disorders, which may result from its sympathomimetic action[2].

Various studies and case reports have suggested that khat is also hepatotoxic, leading to deranged liver enzymes and also histopathological evidence of acute hepatocellular degeneration[3-7]. Recent studies in Somali populations have shown that khat can cause acute severe liver injury in humans due to its hepatotoxic effects[8,9]. Certain drugs that are known to be hepatotoxic cause liver damage by inducing an immunological response leading to a clinical presentation similar to autoimmune hepatitis (AIH). D'Souza *et al*[10] have described atypical presentation of AIH in young Somali men, although any history of khat use was not reported. Our aim was to assess the possible relationship of khat and autoimmune hepatitis in patients presenting with acute hepatitis on a background of khat use.

MATERIALS AND METHODS

The Hepatology database at Sheffield Hospitals was searched for patients referred to the Hepatology department between 2005 and 2010 with liver problems and a history of khat use. All of the patients were tested for hepatitis A, B and C serology, autoimmune profile (including antinuclear antibodies, smooth muscle antibodies and LKM-1 antibodies), ceruloplasmin, alpha-1 antitrypsin, and serum ferritin, and underwent ultrasound scanning of the abdomen, which was normal. This was followed by a percutaneous liver biopsy. Each biopsy was reviewed with particular attention to features of interface hepatitis, lobular necroinflammation and biliary changes. The patients were categorized according to the probability of having autoimmune hepatitis: no evidence (scores < 10), probable (scores of 10-15) or definite (score of more than 15), according to the established international criteria for diagnosis of autoimmune hepatitis[11,12].

All patients were treated with prednisolone (0.5 mg per kilogram per day) initially. Complete response, partial response and no response were defined according to the original and revised international autoimmune hepatitis criteria[11,12].

RESULTS

Acute hepatitis was defined in accordance with the scheme established by the Council for International Organisations of Medical Sciences (CIOMS)[13], and by the USFDA Drug Hepatotoxicities Steering Committee[14]. Eight patients were identified, of which six had presented with acute hepatitis on this basis. All were male and five of these patients were of Somali origin, while one patient was from Yemen. The age range of these patients was 24 to 57 years (mean 42.3 years). All of the patients had

been using khat for several years. There was no history of herbal medication (other than khat) or alcohol use in any patient. All other causes of liver injury were excluded *via* non invasive liver screen. Five of the six patients went on to have a liver biopsy. The patients were scored according to the revised autoimmune hepatitis criteria - (Table 1). They were given -4 for khat use on the scoring system due to its potential hepatotoxicity. Despite this, five out of six patients had a pre treatment score of 10 to 15 which placed them in the probable group for autoimmune hepatitis.

The five patients that were in the probable group had at least a partial response to corticosteroids with a greater than 50% reduction in their ALT after one month of treatment. Only two patients had more than 1 year of follow up, with one showing complete response to treatment. The patient that had scored negative for AIH (< 10) showed the least improvement with prednisolone and continued to have raised liver enzymes after 1 year of treatment. Four out of the six patients were maintained on long term low dose prednisolone while the other two patients were lost to follow up after 1 year. Two patients were commenced on azathioprine with complete response at 1 year follow-up. There was no history of re-exposure to khat.

Five out of six patients met the criteria for probable diagnosis of AIH but none of the patients actually met the criteria for confirmed diagnosis (score > 15). It has been reported previously that Somalian patients with AIH present atypically. It is therefore suggested that the AIH in these patients may have been triggered by khat use.

DISCUSSION

Over 40 khat strains are grown and used in Southern Arabia and East Africa. It is consumed in the form of fresh leaves which may often be contaminated with pesticides. The leaves of khat contain the Pyrrolizidine alkaloids, Cathine, Cathidine, and Cathinone. The pleasure derived from khat chewing is attributed to the euphoric action of Cathinone which is a sympathomimetic amine, with properties similar to amphetamine[15]. Although Cathinone is restricted in the United Kingdom under the Misuse of Drugs Act 1971, khat possession and use are not[1].

The diagnosis of drug induced liver injury (DILI) *vs* AIH triggered by khat is challenging. Various causality methods have been used for herbal induced liver injury and can be broadly divided into retrospective and prospective methods[16-25]. Establishing with any degree of certainty as to whether the liver disease is drug-induced can be very difficult[26]. The issue is further compounded by the relatively rare incidence of DILI, under reporting and potential drug interactions, due to which establishing the identity of the culprit drug may be impossible[27,28]. Furthermore, histology is often unhelpful as it only provides the type and degree of liver injury rather than the

Table 1 Patient scores according to the revised criteria for diagnosing autoimmune hepatitis

Parameters	Score	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
ALP:ALT (or AST) ratio		125/1569	170/1223	187/1957	179/1005	298/1052	59/118
< 1.5	2	2	2	2	2	2	2
1.5-3.0	0						
> 3.0	-2						
Serum IgG above normal							
> 2.0	3	3		3	3	3	
1.5-2.0	2						2
1.0-1.5	1						
< 1.0	0		0				
ANA, SMA or LKM-1							
> 1:80	3			3		3	
Approximately 1:80	2				2		
Approximately 1:40	1						
< 1:40	0	0	0				0
AMA positive	-4	Negative	Negative	Negative	Negative	Negative	Negative
Viral hepatitis markers							
Positive	-3						
Negative	3	3	3	3	3	3	3
Drug History							
Positive	-4	-4	-4	-4	-4	-4	-4
Negative	1						
Average alcohol intake							
< 25 g/d	2	2	2	2	2	2	2
> 60 g/d	-2						
Liver histology							
Interface hepatitis	3	3	3	3	3	N/A	3
Lymphoplasmacytic infiltrate	1	1	1				1
Rosetting of liver cells	1	1	1	1			1
None of the above	-5						
Biliary changes	-3						
Other changes	-3						
Other autoimmune diseases	2	2 (IDDM)	0	0	0	0	0
Optional additional parameters							
Sero positivity-other antibodies	2					2 (ENA+)	
HLA DR3 or DR4	1	N/A	N/A	N/A	N/A	N/A	N/A
Pretreatment score		13	8	13	11	11	10

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; ANA: Antinuclear antibodies; SMA: Anti-smooth muscle antibody; LKM-1: Anti-liver/kidney microsomal type 1; AMA: Anti-mitochondrial antibodies; N/A: Not available.

aetiology. The key to causality is to assess the temporal relationship between drug initiation and development of abnormal liver tests and to diligently exclude other causes of liver diseases. This includes liver injury induced by alcohol, viral hepatitis (acute hepatitis A, B, C and E), autoimmune causes, metabolic disorders, biliary obstruction and sepsis.

The diagnosis of AIH alone is based on the characteristic clinical and histological features as well as the absence of other potential causes of hepatitis. The revised criteria for diagnosis of autoimmune hepatitis are considered the current gold standard[11,12]. Drugs can occasionally cause a clinical-serological picture similar to autoimmune hepatitis and may trigger autoimmune hepatitis in patients with an underlying genetic predisposition to autoimmune hepatitis, or the patients may develop AIH as a squeal of the drug itself. In a Swedish study of 23 patients who developed chronic DILI 23.1% were subsequently diagnosed with autoimmune hepatitis, the suspected drugs being ranitidine, enalapril, oestrogen, carbamazepine, and oestriol[29]. In a recent case series,

Peevers *et al*[9] described seven patients presenting with acute hepatitis who had a history of khat use. Two of those patients met the criteria for the diagnosis of probable AIH. It has been reported previously that Somalian patients with AIH present atypically. In a study of Somalian patients with a history of AIH, it was noted that all of the patients were male and scored in the probable group[10]. History of chewing khat was not mentioned in that particular study. However, in our series, all of the patients who presented with acute hepatitis had a history of khat use with five out of six patient meeting the criteria for probable AIH and demonstrating a good clinical response to immunosuppression. We therefore conclude that, in addition to producing DILI, khat may also trigger AIH in patients with a possible genetic pre-disposition.

Recently, Terschke *et al*[30] have validated the use of the CIOMS scale to be used with herbal induced liver injury (HILI) cases. Although the diagnosis of AIH is well founded in these patients, the causality assessment by means of CIOMS is not available. Also, the small number of patients in this series means that our hypoth-

esis of AIH being induced by khat can only be tentative and should be interpreted with caution. Whether these patients benefit from long-term immunosuppression after stopping khat remains unclear. Further studies of similar groups of patients are required to increase our understanding of this phenomenon and its management.

COMMENTS

Background
Khat is widely used in Southern arabia and East Africa. It is also known that autoimmune hepatitis presents atypically in these population as it is more common in males and presents at a younger age.

Research frontiers
Khat is well known to cause liver damage but the mechanism of this remains elusive. Patients in the areas where khat is consumed, present with atypical autoimmune hepatitis - the cause of which is not known.

Innovations and breakthroughs
The authors present an interesting observation of development of autoimmune hepatitis in a group of patients consuming Khat.

Applications
People can treat these group of patients more effectively by understanding the possible mechanisms of liver damage caused by Khat use.

Terminology
Khat (Catha Edulis Celestrasae) is an evergreen shrub native to East Africa and Southern Arabia. It is chewed daily by over 20 million people in these countries for its addictive and euphoric properties.

Peer review
This is an important case-report and the manuscript reads well.

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Nested stromal-epithelial tumour of the liver: An unusual liver entity

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Abstract

Nested stromal-epithelial tumours (NSETs) of the liver have been reported to be extremely unusual primary hepatic neoplasms. To date, few cases have been described in the literature. NSETs have been defined as non-hepatocytic and non-biliary tumours of the liver consisting of nests of epithelial and spindled cells, myofibroblastic stroma and variable intralesional calcification and ossification. Here, we report a case of a young female who underwent liver resection for a large hepatic lesion that proved to be a calcifying NSET on pathological examination. Details about the clinical and histopathological features of the tumour are reported.

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Key words: Nested stromal-epithelial tumour; Hepatic tumour; Liver resection; Hepatic mixed tumour; Hepatectomy

Core tip: Rare cases of hepatic nested stromal-epithelial tumours (NSETs), consisting of non-hepatocytic mixed stromal and epithelial neoplasms with associated calcification and ossification, have been previously described. To date, NSETs' behaviour and prognosis are completely unclear. We report the case of a 23-year-old female who underwent liver resection for a large hepatic, calcifying NSET. Details about preoperative imaging and the clinical and histopathological features of this very rare hepatic tumour are reported.

INTRODUCTION

Nested stromal-epithelial tumours (NSETs) of the liver are very rare primary tumours characterised by unexampled clinicopathological features. To date, only isolated cases[1] and small series have been reported in the literature[2-4]. NSETs have been defined as a non-hepatocytic and non-biliary tumour of the liver consisting of nests of epithelial and spindled cells with associated myofibroblastic stroma and variable intralesional calcification and ossification[2]. This rare liver malignancy appears solid on imaging and has been reported to be isolated or occasionally associated with hormone cortisol-related syndrome. Herein, we describe a patient who underwent radical hepatectomy for a large calcifying NSET of the liver.

CASE REPORT

Clinical history

In January 2012, a 23-year-old female was referred to our

unit for recurrent dull abdominal pain associated with abdominal distension and dyspepsia. The patient had a past history of being negative for hepatitis but positive for the consumption of oral contraceptives during the previous 5 years.

On physical examination, a palpable mass in the upper abdomen was revealed. No Cushingoid or other clinical features were evident.

Laboratory tests revealed an altered serum level of aspartate aminotransferase (36 IU/L; normal range: 5-30), alanine aminotransferase (297 IU/L; normal range: 5-35), alkaline phosphatase (62 IU/L; normal range: 4-150) and gamma-glutamyltransferase (285 IU/L; normal range: 6-32). Virological markers for hepatitis B and C yielded negative results. Tumour markers, including carcinoembryonic antigen (CEA) and alpha-fetoprotein (AFP), were negative, whereas the level of carbohydrate antigen 19-9 (CA19-9) was elevated (98 IU/mL; normal value: < 40).

Abdominal computed tomography (CT) revealed a well-circumscribed liver lesion of 16 cm in size involving the left hemiliver, part of the right anterior section (S5-8)[5] and the paracaval portion of the caudate lobe (S1pc). Extensive vascular invasion including the left hepatic vein (LHV) and middle hepatic vein (MHV) at the hepatocaval confluence and the left portal pedicle (LPP) was evident. The lesion appeared solid and heterogeneous, with a rim-like enhancement on the arterial phase and a gradual centripetal enhancement on delayed phases. Multiple intralesional calcifications were also evident.

On magnetic resonance imaging (MRI), the tumour showed hypointensity on T1-weighted images and hyperintensity on both T2- and diffusion-weighted images (DWI), with small central necrotic collections. An inhomogeneous pattern with subcentimetric calcifications showing mostly hypointensity on both T1- and T2-weighted images was depicted. On gadolinium-enhanced images, the lesion showed a heterogeneous enhancement pattern on the arterial phase and washout in the portal and parenchymal phases (Figure 1A). The hepatocyte-specific delayed phase (Primovist, Bayer-Schering, Berlin, Germany) showed a hypointense lesion on T1-weighted images, with well-defined margins and a hyperintense capsule.

The work-up was completed with total-body ^{11}C-choline positron emission tomography (PET), which showed only slight pathological uptake of the tracer into the liver (Figure 1B). Based on these preoperative findings, a percutaneous lesion biopsy was not considered, and the patient was candidated to liver resection with a presumptive diagnosis of fibrolamellar hepatocellular carcinoma (HCC) or hepatocholangiocarcinoma.

At laparotomy, peritoneal carcinomatosis was excluded. During liver exploration, a huge, hard liver tumour entirely occupying the left hemiliver, part of S5-8 and the S1pc was confirmed. On intraoperative ultrasonography (IOUS), no additional lesions were detected, and the tumour showed well-defined margins and a heterogeneous echogenicity, with several intralesional hyperechoic spots due to multiple calcifications. Extensive involvement of the LHV, MHV and LPP was confirmed. Once the intraoperative staging was completed, the patient underwent an extended left hepatectomy with left and middle hepatic vein resection for radical removal of the mass. The specimen weighed 2000 g. Intraoperative blood loss was 100 mL, and the patient did not receive a blood transfusion. The postoperative course was uneventful, and the patient was discharge on the 10th postoperative day. Currently, the patient is alive and disease free 21 mo after surgery.

Pathological features

Grossly, the tumour had well-defined margins, was arranged in yellow lobules with several granular and rasping foci and was 16 cm in size (Figure 2A). On histology, the tumour was composed of well-defined nests of epithelial cells separated by strands of stromal cells and focally admixed with calcifying material (Figure 2B). The epithelial component was represented by polygonal to spindle-shaped cells, devoid of overt cytological atypia; stained positive for CKpool, CK19 and CD56; and stained negative for Hep Par-1 (hepatocyte paraffin 1) and AFP. Scattered nuclei were also immunoreactive for WT1 (Figure 2C). The stromal component stained positive for vimentin and smooth muscle actin. The mitotic index was < 5 mitoses/10 HPFs, and 10%-15% of cells stained positive for Ki67.

Molecular genetic study

Several neoplastic nuclei were positive for the EWS-WT1 fusion transcript (Figure 2D) but negative for the SYT-SSX fusion transcript.

DISCUSSION

Stromal-epithelial tumours are extremely rare conditions of the liver, and very few cases have been previously described[1-4]. The unusual entity defined as an NSET typically displays an arrangement of cellular nests composed of spindled or epithelioid cells surrounded by desmoplastic stroma and associated with variable calcifications or ossifications. To our knowledge, few cases of NSETs have been reported[1-4,6]: of these, only one has been described in Asian descendants[6].

This primary liver lesion represents an unexampled clinicopathological entity with an unclear pathogenesis. However, based on immunohistochemical studies showing an intimate correlation of tumoural cell nests with bile ducts and the expression of specific antigens, as well as the shared expression of CD56 by both components, the origin of this tumour in a hepatic mesenchymal precursor cell with primitive differentiation along the bile duct lineage is strongly suspected[3,4]. The expression of WT1 is also in favour of this hypothesis, as this multifunctional protein is a requisite component of the mesenchymal-to-epithelial transformation during certain processes in organogenesis[7].

Figure 1 Radiological imaging. A: Abdominal magnetic resonance imaging scan showing a 16 cm diameter inhomogeneous and partially calcified (asterisk) mass of the left liver; B: Total-body ^{11}C-choline positron emission tomography showed a slight pathological uptake of the tracer in the peripheral part of the tumour (red arrows).

NSETs predominantly occur in young females and are more frequently located in the right hemiliver. Conversely, in the case described here, the tumour occupied the left hemiliver.

The development of this tumour may occur as an isolated condition or in association with a hormone-related syndrome, such as Cushing syndrome. Heerema-McKenney *et al*[3] reported two cases of NSETs occurring in paediatric patients that were associated with Cushing syndrome due to an elevated adrenocorticotropic hormone (ACTH) level[4]. Additionally, Rod *et al*[8] described a 17-year-old female patient affected by a large hepatic NSET causing mild Cushingoid syndrome secondary to moderate-to-high ACTH secretion. In our experience, Cushing-like symptoms were not evident.

From a clinical standpoint, one of the main differential diagnoses is a mixed epithelial and mesenchymal hepatoblastoma, although very few cases have been reported in adults[9]. However, this tumour shows components of foetal and/or embryonal hepatocyte differentiation and lacks the typical stromal architecture of NSETs. Synovial sarcomas and desmoplastic small round cell tumours (DSRCTs) are other diagnostic possibilities to consider that can be distinguished based on specific histologic features. Indeed, in the current study, the absence of a demonstrable carcinoma component and the SYT-SSX fusion transcript helped to exclude the diagnosis of synovial sarcoma. However, cases of synovial sarcoma with extensive calcification and osteoid formation have been reported[10,11]. In our case, the histologic features

of the NSET were slightly reminiscent of those of DSRCTs[12,13], as both tumours exhibited nests of WT1-positive cells and were positive for the EWS-WT1 fusion transcript. The NSET, however, can be distinguished from DSRCTs by the NSET's typical arrangement of myofibroblastic collars and fibrovascular supporting stroma, which differed from the fibrous stroma of DSRCTs. However, the NSET was not immunoreactive for desmin, whereas the opposite is observed in DSRCTs. In the case reported here, based on preoperative imaging features, a diagnosis of fibrolamellar HCC or eventually mixed hepatocholangiocarcinoma was considered.

Interestingly, the patient had taken oral contraceptive pills (OCPs) during the previous 5 years. As reported for hepatic adenoma[14], a possible role for OCPs in the occurrence of the NSET could be considered. However, no staining for hepatocyte antigens was demonstrated in any tumour cells, which led us to exclude a likely correlation with hepatic adenoma. Furthermore, a lack of progesterone and oestrogen receptors in tumoural cells contributed to doubt about the hypothetical correlation between NSET occurrence and OCP consumption. However, more studies on a larger number of cases are likely needed before a possible correlation can be determined.

NSET prognosis remains an unclear matter, but based on current information, this tumour seems to have low proliferation activity and an indolent course, behaving as a low-grade malignancy featuring unusual extrahepatic spread and a possible presentation since childhood. However, specific tumoural features, a large size and vascular

Figure 2 Pathological features of the reported calcifying nested stromal-epithelial tumor of the liver. A: At gross inspection the lesion shows well defined margins and is arranged in lobules; B: At histology the lesion is characterized by the presence of well defined nests of epithelial cells separated by strand of stromal cells, focally admixed with calcifying deposits (arrow); C: Scattered neoplastic cells stain positive for WT-1 immunostaining; D: Some neoplastic nuclei showed well separated green and orange probes signals (arrow), in keeping with a translocation involving *EWSR1* gene (FISH); some neoplastic nuclei (asterisks) did not show the same break apart pattern.

invasion seem to be associated with a higher probability of recurrence. Brodsky *et al*[15] reported experiences of recurrent NSETs of the liver with lymph node metastasis after partial hepatectomy. Hommann *et al*[16] reported a case of lung metastasis after liver transplantation for an unresectable NSET. Based on these experiences, although the risk of relapse seems negligible, a careful postoperative follow-up is recommended.

To date, the benefit of systemic chemotherapy and the most appropriate regimen to adopt remain poorly defined. In paediatric experience, preliminary results showing a minimal response for an unresectable NSET when a hepatoblastoma and sarcoma protocol regimen was adopted have been reported[3]. However, this topic remains completely unexplored.

Surgery seems to be the pivotal therapeutic approach, remaining the best strategy to guarantee longer survival and a better prognosis[1,3]. Our experience attempts to convey more information about the reliability of the surgical approach in the case of a resectable NSET. In this sense, our clinical experience confirms that liver resection allows the safe attainment of complete tumour clearance, even in advanced disease. Conversely, considering the low tendency of NSETs to relapse and previous unsuccessful experiences, at least for oncological control, liver transplantation generally should not be recommended, at least as a first choice[16]. Liver transplantation would be potentially useful for those patients with unresectable but

not extrahepatic disease.

In conclusion, this report aimed to clarify the clinical history, therapy, imaging pattern and histopathological features of a very rare primary liver tumour that is still poorly characterised. Awareness of hepatic NSET occurrence may help to identify additional cases, enlarging knowledge about NSETs' clinical behaviour and prognostic features and limiting the possibility that these tumours could be misdiagnosed and confused with other aggressive liver malignancies.

COMMENTS

Case characteristics
Symptoms were featured by dull abdominal pain associated with abdominal distension and dyspepsia.

Clinical diagnosis
Palpable mass in the upper abdomen. Cushingoid clinical features can be sometimes detected.

Differential diagnosis
Mixed epithelial and mesenchymal hepatoblastoma, synovial sarcoma and desmoplastic small round cell tumour are the main differential diagnosis. Histologic and immunohistochemical analysis help to distinguish them.

Laboratory diagnosis
Laboratory tests revealed altered serum level of AST, ALT, alkaline phosphatase and gamma glutamyltransferase. Virological markers for hepatitis B and C and tumour markers including carcinoembryonic antigen, alpha-fetoprotein were negative, while, the CA19-9 was elevated.

Imaging diagnosis
Preoperative imaging (Computed tomography, Magnetic resonance imaging

scan) revealed a well-circumscribed liver lesion involving the left hemiliver. The lesion appeared solid and heterogeneous with a rim-like enhancement at contrast phase with multiple intra-lesional calcifications. An extensive vascular invasion was evident.

Pathological diagnosis
Nested stromal-epithelial tumours (NSETs) are a non-hepatocytic and non-biliary tumor of the liver consisting in nests of epithelial and spindled cells with associated myofibroblastic stroma and variable intralesional calcification and ossification.

Treatment
Surgery seems the pivotal therapeutic approach remaining the best strategy to guarantee longer survival and a better prognosis.

Related reports
Heerema-McKenney *et al*, Rod *et al* reported cases of NSETs occurring in pedriatic patients and associated with a Cushing syndrome. Brodsky *et al* reported experiences of recurrent NSET of the liver with lymph-node metastasis after partial hepatectomy. Hommann *et al* reported a case of lung metastasis after liver transplantation for unresectable NSET.

Term explanation
WT1 is a mutifunctional zinc-finger protein involved in mesenchyme-to-epithelium transformation which suggests an origin of NSET in a hepatic mesenchymal precursor cell with primitive differentiation along the bile duct lineage. EWS-WT1 and SYT-SSX fusion transcript are genes can help to distinguish NSETs from other liver malignancy.

Experiences and lessons
NSETs occur predominantly in young female and their development may occur as an isolated condition, otherwise associated with hormone-related syndrome, such as Cushing syndrome. From a clinical standpoint, histologic and immunohistochemical studies are essential to distinguish NSETs from other malignancy because of lack of a typical imaging pattern.

Peer review
In the present study, the authors report a case with a nested stromal-epithelial tumor in the liver, which has been rarely reported worldwide. They fully examined the pathological features of the tumor, by using immunohistochemical analysis. This report has originality, figures are clear, and the discussion is well written.

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Drug-induced autoimmune liver disease: A diagnostic dilemma of an increasingly reported disease

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Abstract

The aetiology of autoimmune hepatitis (AIH) is uncertain but the disease can be triggered in susceptible patients by external factors such as viruses or drugs. AIH usually develops in individuals with a genetic background mainly consisting of some risk alleles of the major histocompatibility complex (HLA). Many drugs have been linked to AIH phenotypes, which sometimes persist after drug discontinuation, suggesting that they awaken latent autoimmunity. At least three clinical scenarios have been proposed that refers to drug-induced autoimmune liver disease (DIAILD): AIH with drug-induced liver injury (DILI); drug induced-AIH (DI-AIH); and immune mediated DILI (IM-DILI). In addition, there are instances showing mixed features of DI-AIH and IM-DILI, as well as DILI cases with positive autoantibodies. Histologically distinguishing DILI from AIH remains a challenge. Even more challenging is the differentiation of AIH from DI-AIH mainly relying in histological features; however, a detailed standardised histologic evaluation of large cohorts of AIH and DI-AIH patients would probably render more subtle features that could be of help in the differential diagnosis between both entities. Growing information on the relationship of drugs and AIH is being available, being drugs like statins and biologic agents more frequently involved in cases of DIAILD. In addition, there is some evidence on the fact that patients diagnosed with DIAILD may have had a previous episode of hepatotoxicity. Further collaborative studies in DIAILD will strengthen the knowledge and understanding of this intriguing and complex disorder which might represent different phenotypes across the spectrum of disease

Core tip: Drug-induced autoimmune liver disease (DIAILD) is a poorly defined and under-reported liver disorder, and, probably, a underestimated liver disease. A small number of drug-induced liver injury (DILI) cases exhibit features typical of autoimmune hepatitis (AIH). To differentiate between true AIH triggered by drugs (DI-AIH) and immune mediated DILI still remains a challenge. Patients diagnosed with DIAILD have frequently had a previous episode of hepatotoxicity. We consider that some basic requirements are needed to be considered before supporting a drug as a trigger of AIH and they should be taken into account by authors, reviewers and editors when cases are published and made available to the scientific community.

Castiella A, Zapata E, Lucena MI, Andrade RJ. Drug-induced autoimmune liver disease: A diagnostic dilemma of an increasingly reported disease. *World J Hepatol* 2014; 6(4): 160-168 Available from: URL: http://www.wjgnet.com/1948-5182/full/v6/i4/160.

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INTRODUCTION

The cause and pathogenesis of autoimmune hepatitis (AIH) is unknown[1]. AIH is characterised by the following clinical features: (1) presence of raised aminotransferases with normal or minimal elevations of alkaline phosphatase; (2) association with hypergammalobulinemia and raised immunoglobulin G; (3) female gender preponderance; (4) high titres of a variety of autoantibodies; (5) immunogenetic background; (6) good response to immunosuppressive treatment; and (7) the presence of extrahepatic autoimmune manifestations[2,3]. In liver biopsy specimens, the presence of interface hepatitis is characteristic[3]. In 1999, an international group developed a diagnostic score system[4]; which was later on simplified in 2008[5].

The aetiology of AIH is uncertain, but the disease can be triggered in susceptible persons by an external factor, such as viruses, drugs or herbal remedies[1,2].

Many clinical observations suggest that drugs are potential triggers in some patients[6,7]. Several drugs have been identified to cause AIH that may persist after discontinuation, suggesting that they triggered true autoimmunity. These include oxyphenisatin, nitrofurantoin, minocycline, chlometacin and alpha-methyl dopa[3,8]. Other drugs have been infrequently reported to lead to AIH, making the association less probable. Recently, a growing number of drug-induced autoimmune liver disease (DIAILD) reports meeting the established international criteria[4,5] and showing clinical data (hypergammaglobulinemia, ANA/ASMA), typical liver biopsy findings and HLA-DR status, which allow to establish a causal relationship[6,9-11].

For instance, Björnsson *et al*[12], in a series of 261 patients with AIH, reported 9.2% (24 patients) of patients with drug-induced AIH. A French group[10] searched for a potential causative drug in a consecutive series of 65 patients with AIH, and identified that 12% of the cases were drug-induced, highlighting the frequency of this disorder. In this line, 5 out of 29 (17%) consecutive patients with AIH from Spain were diagnosed with drug-induced AIH[13].

Very recently, Licata *et al*[14] reported 12 patients from a series of 136 drug-induced liver injury (DILI) subjects that were diagnosed as drug-induced AIH (8.8%). All were treated with corticosteroids and remission was achieved after six months in 10 of the cases (83%).

Although no particular drug has been definitively identified as a true aetiological trigger agent for AIH, it is interesting to note that drug-metabolizing enzymes of phase I and phase II are common targets of autoimmunity in idiopathic AIH and viral hepatitis[2].

It is important to distinguish drugs as triggers of a self-perpetuating autoimmune liver disease from immune-mediated drug-induced liver injury (IM-DILI). Immune-mediated DILI nearly always resolves[2,12] or becomes quiescent when drugs are withdrawn. Another possibility is that AIH was quiescent and remains undiagnosed until a drug triggered a new autoimmune process. Thus, to attempt to make a proper diagnosis of the type of immune process affecting the liver is challenging. Björnsson *et al*[12] reported 24 cases of drug-induced AIH (22 minocycline-nitrofurantoin cases, 11/11). Immunosuppressive therapy withdrawal was successful in all the 14 cases in which it was attempted. On the contrary of the AIH cases in this series 65% relapsed upon corticosteroid withdrawal. Heurgué *et al*[10], observed one spontaneous remission, three relapses and four remissions without relapse in 8 patients out of 65 with drug-induced AIH.

In the Spanish DILI Registry, out of 742 DILI cases, 16 were diagnosed as DIAILD[9] and 25% of these cases had another autoimmune associated disease that may require persistent immunosuppressive treatment. Such cases may preclude the therapy to be discontinued and, therefore, one cannot ascertain the course of AIH upon immunosuppressive drug withdrawal. Therefore, to state that relapse after corticosteroid discontinuation might distinguish DIAILD from classical AIH is a very attractive conclusion, but far away from the complex reality of this disorder since in many cases the coexistence of other autoimmune disorders does not allow for stopping immunosuppressive therapy[15,16]. To further complicate the differentiation between AIH and DILI, we must underline the fact that there does not seem to be any specific histological features for either of the processes and the pathological features may show only subtle differences, pointing toward an immune-mediated liver disease versus hepatic toxicity[17].

CLASSIFICATION

There are several possible combinations of DILI and AIH (see Table 1). In 2002 Liu *et al*[2] distinguished 2 types: drugs as a potential triggers of drug-induced AIH (DI-AIH), which supposes a self-perpetuating liver disease, and immune-mediated DILI (IM-DILI), which is an acute or chronic process depending of the duration of the exposure of the liver to the hepatic insult (viruses, herbal remedies, drugs) and disappears or becomes quiescent when the drug is withdrawn[2]. In 2011, Weiler-Normann and Schramm[18] established a classification of DILI and AIH proposing possible connections with suggested diagnoses and clinical characteristics. First, AIH with DILI: The reactivation of a known AIH upon introduction of a new drug is possible, but it is very difficult to demonstrate a causal relationship, as it might be coincidental (chance by association) Often, there is advance fibrosis on histology (see Table 1). Second, DI-AIH, reveals a patient that has not been diagnosed before, or even just the predisposition to AIH that is awakened by DILI. An immune reaction in a genetically predisposed individual may lead to a chronic process, perpetuating the AIH in these patients, with a permanent need for immunosuppression. Usually, they have typical HLA-DR associated with true AIH (Table 1). Third, IM-DILI: acute

Table 1 Classification of drug-induced autoimmune liver disease

AIH with DILI	Patients with known AIH
	AIH quiescent: the drug may be the trigger of a new bout
	AIH under IS or corticosteroids treatment: Reactivation of known AIH upon intro duction of a newdrug (very difficult to demonstrate a causal relationship as it might be coincidental
	Often advanced fibrosis on histology
DI-AIH	Patients with a low grade disease not diagnosed before or predisposition to AIH
	Drug produce an immune reaction that lead to a chronic process:
	Perpetuating the AIH
	Permanent need of IS
	Habitually typical HLA-DR associated
IM-DILI (Autoimmune hypersensitivity)	Fever, eosinophilia, lymphadenopathy, rush
	Indistinguishable from true AIH: Mandatory IS treatment
	Frequently spontaneous remission after drug cessation
	Usually complete response to treatment and sustained remission without relapse
	It is the most frequent drug-induced immune process in the liver attributable to drugs
Mixed autoimmune type	Patients with mixed clinical features of DI-AIH and IM-DILI
	Complete response to IS treatment but with chronic course after withdrawal
	Patients under IS treatment for another autoimmune disease. Withdraw IS drugs is not possible. Remission cannot be evaluated
DILI with positive autoantibodies	Patients with positive autoantibodies
	The probability of developing DIAILD increases in second DILI episodes indepe dently of the causal agent

AIH: Autoimmune hepatitis; DILI: Drug-induced liver injury; IS: Immunosuppressants; IM-DILI: Immunomediated DILI; DIAILD: Drug-induced autoimmune liver disease; HLA: Human leukocyte antigen.

or chronic liver injury (depending of the duration of the exposure to the drug), as has been explained by Liu and Kaplowicz[2], that resolves or becomes quiescent with drug withdrawal. This scenario can be caused by a number of drugs, such as minocycline and nitrofurantoin[2,12]. These individuals may have spontaneous remission of acute hepatitis after drug cessation, or have a hepatocellular or mixed type of liver damage that does not improve after drug-withdrawal. Fever, eosinophilia, lymphadenopathy and rash may be present, but not always. In these situations, which are indistinguishable from true AIH, the immunosuppressive (IS) treatment is mandatory and may be life-saving. Most of these patients do not need IS forever as they usually have a complete response to treatment and a sustained remission without relapse[12,16,18,19]. This type of adverse drug reaction, which can be viewed as autoimmune hypersensitivity, is the most frequent drug-induced immune process in the liver that is attributable to drugs (see Table 1)[2,12].

A second DILI episode (recurrent DILI) involving a different drug is a rare event, but if it happens, AIH or AIH-like DILI is frequent, and reported in up to 40% of cases in the series published from the Spanish DILI Registry[20]. Differentiating IM-DILI from DI-AIH in these cases is very difficult as the clinical presentation may be the same[20].

Nevertheless, we think that a fourth type must be added: mixed autoimmune type. These patients have mixed clinical features that belong to DI-AIH and IM-DILI types. Thus, there are published cases of IM-DILI induced by minocycline which have a complete response to IS treatment, but experience a chronic course after drug withdrawal[15,21,22]. Therefore, the same drug can be DI-AIH or IM-DILI, depending on the clinical charac-

teristics and outcome of the episode. Another possibility is that the patient requires IS treatment for an autoimmune disease, different to AIH, in which case we cannot withdraw IS drugs. In these cases, we cannot differentiate DI-AIH or IM-DILI because the patient needs chronic IS treatment for another autoimmune disease. Therefore, one cannot ascertain the course of AIH upon IS drug withdrawal. For example, chronic uveitis has been associated with AIH[23,24], and may require permanent IS treatment.In the Spanish DILI Registry[25], 16 out of 742 DILI cases, were diagnosed as DIAILD[9]; 25% of these cases had another associated autoimmune disease, which may require persistent IS treatment. In a French series of eight patients[10], 25% of them had another AI associated disease.

Finally, there are patients that present DILI with positive autoantibodies. However, its significance is unknown and requires further studies. In 2002, Ohmoto and Yamamoto studied 64 patients admitted to their hospital with DILI and identified 6 with positive ANA[26]. They found a higher prevalence of associated autoimmune diseases in the ANA positive group such as AIH, rheumatoid arthritis, Hashimoto's disease. The authors suggested that patients with DILI and ANA might also have autoimmune disease and should be followed over the long-term, even if liver function has recovered. On the other hand, Hinrichsen *et al*[27] reported a case of phenprocoumon-induced hepatitis with positive autoantibodies. After the drug was stopped, no clinical or laboratory features of autoimmune liver disease were present.

Recently, the Spanish-Latin American DILI Network has published a series of 73 DILI cases, in which 29% presented positive autoantibodies, mainly ANA. Six cases were DIAILD (AIH DILI) (8%) and 5 cases (7%) had

experienced a second DILI episode[28].

Therefore, this analysis further support that the probability of presenting positive antibodies increases in second DILI episodes, as previously shown by Andrade *et al*[29].

HISTOLOGY

Liver histology shows apoptosis, necrosis and inflammatory infiltrates including mononuclear cells, neutrophils, eosinophils and lymphocytes, or cholestasis and paucity of bile ducts with moderate portal inflammation. Granulomas may also be seen[2]. Distinguishing DILI from AIH is challenging histologically. Recently, Suzuki *et al*[17] showed that while a histologic overlap exists for these pathologies, sufficient differences exist for pathologists and they can use the pattern of injury proposed by Suzuki *et al*[17] to suggest a correct diagnosis. Interface hepatitis, focal necrosis and portal inflammation were present in all of the evaluated cases but were more severe in AIH than in hepatocellular DILI. Portal and intra-acinar plasma cells, rosette formation, and emperipolesis were features that favoured AIH. All Ishak inflammation scores were more severe in AIH than in cholestasic DILI. They did not identify any histologic features differentiating AIH from DIAILD (DI-AIH) in a small subgroup analysis (7 cases), but it might still be possible that detailed standardised histologic evaluation using a larger cohort of DIAILD (DI-AIH) *vs* AIH can identify histologic features that can be helpful in the differential diagnosis. Björnsson *et al*[12], in a recent work, revealed that similar histological grades and stages were present in patients with IM-DILI (DI-AIH) *vs* AIH. However, none of the IM-DILI (DI-AIH) patients had cirrhosis at baseline and it was present in 20% of the AIH patients. Czaja[16], based on the referred work, confirmed that cirrhosis is a rare histologic feature in nitrofurantoin AIH cases at presentation, which may help to distinguish it from classical AIH (0 *vs* 13%). In a recent review of the histological patterns found in cases of DILI[30], it was pointed out that, contrary to minocycline, significant fibrosis and cirrhosis occurs with nitrofurantoin. The French group[10,31] biopsied all of their cases (Minocycline *n* = 3; nitrofurantoin *n* = 2; atorvastatin *n* = 1; fenofibrate *n* = 1; isotretinoin *n* = 1), showing a fibrosis score F3-F4 in 57% *vs* 48% in the DIAILD (AIH-DILI) and AIH groups, respectively. Necroinflammatory activity A3, with Metavir, was revealed in 75% *vs* 65%. They concluded that the 2 groups showed similar histological lesions of fibrosis/cirrhosis. Recently, Licata *et al*[14] studied a series of 12 patients (4 nimesulide, 1 ketoprofen, 3 amoxicillin-calvulanate, 1 ceftriaxone, 1 epigallocatechin gallate and 1 hypericum perforatum -herbal drugs-, and dimethoate-toxic agent). All DIAILD patients were treated with corticosteroids and none developed cirrhosis. Appleyard *et al*[32] published a series of 3 cases, one of which had pre-cirrhosis/cirrhosis at diagnosis. The Spanish Registry of Hepatotoxicity[9] presented a case report of DIAILD that showed cirrhosis in the liver biopsy at presentation. Therefore, the absence of histological cirrhosis at presentation would not help to discern between these two entities.

Ju *et al*[33] have studied the histological features of DILI patients in Korea, with special focus on relevancy of AIH. The study showed that characteristic histologic features of AIH, as interface hepatitis and lymphoplasmocytic infiltrates, were present in up to one third of DILI patients.

FREQUENCY

Recently Czaja[16] stated that the best estimate of the frequency of drug-induced autoimmune-like hepatitis among patients with classical features of AIH is 9%[12]. There are other reports that show higher figures[15]. Heurgué *et al*[10], in France, identified 8 cases (12%) with drug-induced AIH in a consecutive series of 65 patients with AIH, fulfilling all requirements recommended in the review to confirm the diagnosis (Table 2). In Gipuzkoa, Spain, from 1994 to 2009, 29 cases of AIH were diagnosed, 5 of which were considered AIH-DILI, which resulted in a 17% frequency[13]. We believe that these differences in frequencies may be explained by the fact that AIH-DILI is often misdiagnosed.

If we study patients diagnosed with DILI, Licata *et al*[14] reported 8.8% of the patients presenting features of DIAILD (DI-AIH). In the Spanish DILI Registry[9], out of 742 recruited cases, 16 presented DIAILD criteria (2.15%).

Why is there a growing number of case reports?

Indeed, the diagnosis of AIH is often made in the setting of a patient being treated with multiple drugs. If the diagnostic scale points out probable AIH, the possible role of the drug is generally underscored, and immunosuppressive treatment is started. On the other hand, if the AIH scale is not conclusive and/or histology findings are more consistent with DILI, the case is assumed to be a DILI case, particularly if the clinical symptoms resolve after discontinuation of the suspected drug (DIAILD may be a self-limiting process), and the possibility of unmasking AIH by the action of a drug is disregarded. To further complicate the differentiation between AIH and DILI we must underline the fact that there is no specific histological features of either process and the pathological features may show only subtle differences pointing to the immune-mediated liver disease versus hepatic toxicity[17]. These considerations lead us to suggest that DIAILD might be underreported nowadays.

When a patient is admitted to hospital because of acute hepatitis and the drug that is thought to be responsible is identified, treatment is stopped.

If transaminases decreased more than 50% in 1 week to 1 month, and the DILI case fulfils other Council for International Organizations of medical Science (CIOMS) criteria such as a favourable temporal sequence, alternative pharmacological and clinical conditions have been ruled out, dechallenge with the drug is followed by improvement, and rechallenge if present is positive, then the DILI case is probably related to the drug[34,35]. Sponta-

neous recovery supports this possibility and, generally, if autoantibodies have been solicited to the laboratory and are positive, we do not consider the possibility of a drug as a trigger of IM-DILI.

If aminotransferases remain permanently raised, suffer an increase during follow-up, and autoantibodies are positive, AIH is assumed; if the AIH-scale reveals a probable AIH, treatment with IS is begun as soon as possible. There is complete exclusion of the trigger paper of a drug treatment if it is present prior to acute hepatitis, or if the drug is retired but without giving its importance to it; if true, AIH may resolve spontaneously.

New drugs: the growing use of drugs such as statins and biologic agents, which have been related to DILI with autoimmune features, which has prompted an increase in the diagnosis of DIAILD[36-42].

The diagnosis DIAILD represents a challenge to the clinician as there are neither histological, nor clinical features that are pathognomonic of true AIH, and HLA haplotypes do not convincingly distinguish between either entity. Indeed, as previously commented many cases of drug-induced AIH tend to be misdiagnosed as classical AIH cases. The CIOMS scale is unable to distinguish between AIH and DIAILD, as the scale fails to accommodate all relevant information for diagnosing DIAILD. Causality may also be challenged by the lack of spontaneous improvement after drug withdrawal in this form of DILI.

PREDICTION OF AUTOIMMUNE HEPATITIS-RISK FACTORS

Susceptibility

Autoantibodies: Many autoimmune diseases are chronic conditions that progress over the years, and are characterised by the presence of autoantibodies that may precede the overt disease by months or years[43]. In AIH, the hallmark is the presence of circulating autoantibodies. If ANAs are detected in female patients with DILI, the coexistence of an autoimmune disease is possible[26].

***HLA* genes and haplotypes:** Susceptibility to AIH, via a genetic predisposition, has been clearly associated with class II human leukocyte antigen (*HLA*) genes, more specifically to the DRB1 locus[44]. This is the major associated gene locus. Predisposition to AIH type I is associated with HLA-DRB1*0301, DRB3*0101, and DRB1*0401 alleles in European and North-American Caucasoid, encoding the HLA-DR3, DR52 and DR4 molecules, respectively. Czaja *et al*[45] determined in 1993 the following class II HLA associations with AIH: 44% DR3, 32% DR4, 9% DR3-DR4, and 15% other antigens. The study of different geographical areas and ethnic groups revealed different results. In Japan and Argentinean Caucasoid adults, susceptibility was associated with HLA-DRB*1-0405 (DR4): Mexican adults presented DRB1*0404 (DR4), and Caucasoid children and adults from Argentina presented DRB1*1301 (DR13)[45-49]. In Brazil, the haplotype DRB1*1301 is the most frequently

related to type 1-AIH[44]. A secondary association with HLA-DRB1*0301 has been detected in this country[50]. Another haplotype, DRB1*07, seems to produce susceptibility in German and Brazilian populations[51,52]. Type 2 AIH is associated with the haplotypes HLA-DRB1*0301 and HLA-DRB1*07[50]. Recently, Oliveira *et al*[50] searched for additional susceptibility factors in the extended MHC region. They have studied genes located in the MHC class III region, in addition to class I HLA-B and MICA genes, to verify if the specific haplotypes DRB1*1301 or DRB1*0301 could be involved in the susceptibility of paediatric patients in Brazil[53]. The ancestral haplotype comprising *TNFA*-308A, *TNFA*-238G, *LTA*+252G, *LTA*+80C, *NFKBIL1*-63A, *BAT1*-348C, *BAT1*-22C, *HLA-B*08 and *MICA*08 was more common in DRB1*03 positive patients than in controls (40% *vs* 14%), showing a seven-fold increased risk of disease. Finally, a variety of class III haplotypes was also present in HLA-DRB1*13 patients, without a predominant pattern. The most common of the 98 haplotypes present in patients were completely absent in controls. The extended haplotype analysis in this sample of AIH-1 patients highlighted not only the genetic diversity present in the Brazilian population, and was also in accordance with the previously documented microdiversity within the MHC region. The DRB1*1501 allele may protect from disease[46].

Triggers

Triggers may induce AIH. AIH may be induced by drugs[12], herbal remedies[11], different viruses and bacteria[54-58] and vaccines[59,60].

Exposure to all of these potential triggers may produce immune responses that especially target the liver, mainly in predisposed individuals, as we have seen previously; therefore, chronic hepatitis with autoimmune features might develop. If not recognised promptly and the responsible agent is not withdrawn, such responses can evolve to chronic hepatitis (resembling viral hepatitis) - alpha-metil dopa, halothane, hydralazyne, minocycline, nitrofurantoin, and oxyphenisatin - or to a chronic nonsuppurative cholangitis (resembling PBC) - chlorpromazine[1].

Identification of these risk factors might help us to think about this disease and halt treatment with the offending trigger as soon as possible. Withdrawal of the offending agent may lead to a rapid resolution of the process.

Genetic polymorphisms: Several genetic polymorphisms of drug metabolising enzymes, particularly CYP, have been identified, which may produce reactive metabolites[2]. Differences in the metabolism of drugs genetically conditions may produce protein adducts and susceptibility to DI-AIH. The occurrence of more than one case within a family supports the theory that genetic factors are involved[61]. It has been shown that a familiar sensitivity to the toxic effect of the metabolites exists, which may produce an inherited defect in the defence of

Table 2 Elements to be reported when a case of drug-induced autoimmune liver disease is suspected

Previously obtained ANA
Evolution:
　During the treatment with the suspicious drug
　After drug withdrawal
Check for the presence of HLA-DR:
　HLA-DRB1*0301,0401,07,1301
Drug type
Time to onset from the beginning of the treatment
AIH diagnosed:
　During the course of treatment
　After withdrawal of the drug
AIH scales for diagnosis
　International autoimmune hepatitis group report (4)
　Simplified score (5)
Previous DILI episodes
Response to corticosteroids
Autoimmune titres evolution
IgG values evolution

AIH: Autoimmune hepatitis; DILI: Drug-induced liver injury.

the liver from the injury produced by these toxic metabolites[2]. Anti-convulsants and sulphonamides have shown a familiar inheritance of *in vitro* lethal effects against the lymphocytes by their metabolites[62,63].

Sex

In AIH, there is a female sex predominance. Women are affected more frequently than men (sex ratio 3.6:1) and the disease is seen in all ethnic groups and across all age intervals[56]. Björnsson *et al*[12] published 78% (184/237) of females with AIH *vs* 92% in the DI-AIH (20/24); in the nitrofurantoin group the findings were 11/11, with numbers of 10/11 in the minocycline group. In the Spanish DILI Registry, sixteen cases out of the 742 cases (2.15%) of idiosyncratic DILI were identified[9]. There were 10/16 women. Heurgué *et al*[10] reported 8 patients with DI-AIH out of 65 AIH cases diagnosed consecutively. The female/male sex ratio was 87% *vs* 82%, revealing no statistically significant differences. Sugimoto *et al*[11] recently published a series of 7 patients with AIH that developed after a first DILI episode. Six out of the seven affected patients were women. In other smaller series, a female preponderance seems to be the general rule[32]. Czaja *et al*[16] reported that not only does DIAILD (DI-like AIH) occur almost exclusively in women, but that the drug injuries in the liver are more severe than in men[2,12,64,65]. Indeed, female sex has been found to be a risk factor for acute liver failure development after a DILI episode[25].

DILI

Multiple episodes of DILI in the same patient with drugs of similar structure or function as well as unrelated drugs may induce immune-related hepatotoxicity[20]. Between 1994 and 2009, Lucena *et al*[20] identified 9 patients out of 742 in the Spanish DILI Registry (1.21%), with evidence of two distinct DILI episodes produced by different drugs. In each individual, the type of injury was the same

in the two episodes, regardless of the causative drug. Second episodes were associated with features of AIH up to more than 40% (4/9) of cases, making it unclear whether this is drug-induced unmasking of true DI-AIH or IM-DILI (DILI with autoimmune features).

Age

Elderly individuals have an increased risk of drug toxicity[16]. The risk of DILI increases with age for certain drugs, such as those that are implicated in autoimmune-like hepatitis (nitrofurantoin, halothane, and isoniazid).

Association with other autoimmune diseases or induction by biologic agents

Biologic agents: Biologic agents are increasingly being used for rheumatological and systemic autoimmune diseases. The BIOGEAS project[39,40], created by the Spanish Society of Internal medicine, has retrieved more than 800 cases of AI diseases secondary to biological therapies. In this study, 19 cases of AIH have been reported. Statins[36,37] have been reported to induce AI diseases, typically in patients with other AI diseases.

Spontaneously: AI diseases frequently appeared to be associated between them[36,37]. Multiple examples are published in the literature[66].

Drugs

Nowadays, more than 900 drugs, toxins and herbal remedies have been reported to cause liver injury. Recently, it has been reported that at least 24 drugs, probably more, have been associated with AI chronic hepatitis mimicking AIH[67], but more and more new agents are being implicated[67-83]. With the appearance of new statins, biologic agents, and antibiotics, it will be quite normal to see more new agents being reported as being responsible for new drug-induced AIH cases (Table 3).

Elements to be reported when a case of DIAILD is suspected

Some basic requirements are needed to consider a drug as a trigger of AIH. These basic requirements must be debated and consensuated prior to the publication of a suspected new case. The elements that we think that must be included in a suspected DIAILD case before publication are outlined in Table 2.

CONCLUSION

DIAILD is still a poorly defined and under-reported liver disorder and is also a probably underestimated liver disorder. A small number of DILI cases exhibit features typical of AIH. To differentiate between true AIH triggered by drugs (DI-AIH) and IM-DILI still remains a challenge. Patients diagnosed with DIAILD have frequently had a previous episode of hepatotoxicity. The CIOMS scale has a limited value to ascertain causality in DIAILD. Hopefully, the collaborative efforts in DILI research will

Table 3 Drugs and drug-induced autoimmune liver disease
Well stablished drugs:
Minocycline
Nitrofurantoin
Oxyphenisatin, alpha-methyl-dopa, clometacin.
Emerging drugs:
Statins
Biologics agents:
Infliximab
Others: adalimumab, etanercept, efalizumab, ipilimumab
Other drugs:
Less compelling association (infrequent reports): atomoxetine, diclofenac, fenofibrate, pemoline, phenprocoumon, dihydralazine, tielinic acid, benzarone

enhance our knowledge of this intriguing hepatic disease. We consider that some basic requirements are needed to be considered before supporting a drug as a trigger of AIH and they should be taken into account by authors, reviewers and editors when cases are published and made available to the scientific community.

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Lipid lowering effects of iodothyronines: *In vivo* and *in vitro* studies on rat liver

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is emerging as one of the most common liver diseases, leading to the increasing interest for new therapeutic approaches for its treatment. NAFLD primarily depends on a hypercaloric and/or unbalanced diet leading to overweight and obesity. The liver, in fact, plays a central role in lipid metabolism by importing free fatty acids from the blood and synthesizing, storing, oxidizing and exporting lipids. Furthermore, the liver is the target for the thyroid hormones, thyroxine (T_4) and 3,3',5-triiodo-L-thyronine (T_3), that stimulate the basal metabolic rate and lead to body weight loss. In the last decade, other iodothyronines have been shown to possess biological relevance and play some thyromimetic activities; in particular, 3,5-diiodo-L-thyronine (T_2) gained large interest. The global effect of iodothyronines on liver lipid metabolism results from the balance between direct and indirect actions on the hepatocyte, leading to stimulation of lipid synthesis, oxidation and autophagy. In this review, the results so far obtained on both *in vivo* and *in vitro* models of hepatosteatosis are summarized in order to obtain an updated picture of the lipid-lowering effects of iodothyronines on mammalian liver.

Core tip: This review summarizes the recent insights about the mechanisms underlying the lipid lowering action of iodothyronines. In the last decades, extensive studies investigated the possible use of iodothyronines in the treatment of obesity and dysmetabolic syndromes. Since the pharmacological use of thyroid hormones has found severe limitations because of their thyrotoxic effects, the identification of iodothyronines retaining anti-obesity and hypolipemic efficacies, while being devoid of thyrotoxicity, gained great interest. The review discusses the recent studies employing both *in vivo* and *in vitro* models of hepatosteatosis, with particular attention to the *in vitro* studies demonstrating the direct anti-steatotic effect of iodothyronines.

IODOTHYRONINES AND METABOLISM

Thyroid hormones (THs) secreted by the thyroid gland comprise two main iodothyronines: 3,5,3',5'-tetraiodothyronine (thyroxine or T_4) and 3,5,3'-triiodo-L-thyronine (T_3) (Figure 1). T_4 is the major form secreted by the thyroid and the most abundant TH in circulation, while T_3, the active form, is mainly generated by peripheral deiodination of T_4. T_3 may be further deiodinated to yield different diiodothyronines such as 3,5-diiodo-L-thyronine (T_2) (Figure 1). In the past, T_3 was assumed to be the only active iodothyronine *in vivo*, but recent evidence suggested that other iodothyronines, such as 3',5',3-l-triiodothyronine (rT3) and T_2, may be of biological relevance[1,2].

THs influence a large number of physiological pro-

Figure 1 The chemical structures of three biologically active iodothyronines and one derivative: thyroxine, 3,3',5-triiodothyronine, 3,5-diiodothyronine and 3-iodothyronamine. T4: Thyroxine; T3: 3,3',5-triiodothyronine; T2: 3,5-diiodothyronine; T1AM: 3-iodothyronamine.

cesses in vertebrates, including growth, development and differentiation. THs have stimulatory effects on metabolic activity, thus inducing thermogenesis (the so-called calorigenic effect) that represents a major component of the energy expenditure in endotherms. Of particular interest is the effect of THs on lipid metabolism resulting from the balance between stimulation of lipid synthesis and lipid oxidation (Figure 2). The liver represents one of the main target tissues of THs. At the hepatic level, T_3 stimulates cholesterol synthesis and its metabolism into bile acids[3] and cholesterol uptake[4], and it induces lipogenic enzymes, including fatty acid synthase (FAS) and acetyl-CoA-carboxylase[5]. In addition to the lipogenic action, T_3 also leads to a general reduction in the hepatic triglyceride (TAG) content, likely through stimulation of lipolytic pathways[6]. Moreover, it has been suggested that TH-stimulated lipogenesis/lipolysis "futile" cycle may contribute to the calorigenic effect[7].

The metabolic effects of iodothyronines have long been investigated because of their potential use as drugs to treat obesity and lipid metabolism disorders[8]. However, due to the simultaneous undesirable side effects, such as the induction of a thyrotoxic state (tachycardia, muscle wasting, bone loss), the employment of T_3 or T_4 to stimulate body weight loss or treat metabolic syndrome has been limited. At the same time, the development of TH agonists/analogs retaining lipid-lowering and anti-obesity efficacies, while being devoid of thyrotoxic effects, has received great interest as a potential therapeutic advancement. The research of the last years has identified several iodothyronines other than T_3 and T_4 that display some thyromimetic activities. Among them, T_2 assumed a great interest as it mimics several effects of T_3 on energy

metabolism[9-11] without inducing thyrotoxic effects[12]. A single dose of T_2 (25 μg/100 g body wt) stimulated the resting metabolic rate (RMR) of hypothyroid rats and increased the liver oxidative capacity to the same extent as the same dose of T_3[13]. Moreover, T_2 significantly reduced serum triglyceride and cholesterol levels and increased liver oxygen consumption[10].

With regards to the calorigenic effects of THs, several cellular targets have been proposed but none has received universal acceptance. By virtue of their central role in the energy-transduction pathway, mitochondria are natural candidates to mediate the calorigenic activity of iodothyronines[14]. Single injections of T_2 or T_3 into hypothyroid rats stimulated RMR[15], in association with an increase in oxygen consumption[16]. It is widely accepted that iodothyronines may exert two kinds of effects on mitochondria: (1) a rapid stimulation of respiration (within minutes/hours); and (2) a long term effect leading to mitochondrial biogenesis and mitochondrial mass increase. The calorigenic activity of THs has long been ascribed to uncoupling of mitochondrial oxidative phosphorylation, but the mode by which they promote mitochondrial proton leak is still unresolved. Harper et al[17] related the T_3-induced increase in mitochondrial proton leak to an increased permeability of the phospholipid bilayer due to a change in the lipid composition of the inner mitochondrial membrane. On the other hand, T_2, and to a lesser extent T_3, was shown to bind the Va subunit of the cytochrome oxidase complex, thus abolishing the allosteric inhibition due to ATP binding and stimulating enzyme activity[18]. Recently, Yehuda-Shnaidman et al[19] reported that mitochondrial uncoupling by T_3 was transduced both *in vivo* (in rats) and *in vitro* (Jurkat cells) by gating of the

Figure 2 Schematic representation of the mechanisms underlying the control of lipid metabolism by iodothyronines in the hepatic cell: A summary of the possible signaling pathways involved in iodothyronine actions is presented. The classic "receptor-mediated" pathway describes the action of iodothyronines through the thyroid hormone receptors (TR). The "non receptor-mediated" pathway occurs through the interaction of iodothyronines with different cellular targets. FFA: Free fatty acids; LD: Lipid droplet; AOX: Acyl-CoA oxidase; ATGL: Adipose triglyceride lipase; CPT: Carnitine palmitoyl-transferase 1; FAS: fatty acid synthase.

mitochondrial permeability transition pore.

Interestingly, T_2 administration has been demonstrated to be able to stimulate RMR and to also reduce body weight in humans. In a pilot study, two euthyroid subjects were treated with increasing doses of T_2 (from 100 to 900 mcg/d, three times a day for 8 d) and for a further 3 weeks with 3000 mcg/d. A reduction in body weight of -4% was observed without effects at the cardiac level[20].

The pharmacological effects of some derivatives of thyronines called thyronamines have been also investigated. Scanlan et al[21] described the synthesis and biological properties of 3-iodothyronamine (T1AM), a novel thyronamine that was shown to be an endogenous component of biogenic amine extracts from rodents. T1AM has a carbon skeleton identical to that of T_4 and theoretically, it could be produced from T_4 by enzymatic decarboxylation and deiodination (Figure 1). T1AM treatment rapidly induced a hypometabolic state and hypothermia in rodents, with opposite effects compared with those typical of THs.

MECHANISMS OF ACTION OF IODOTHYRONINES

In the past, it was a common notion that TH actions were mediated by specific nuclear thyroid hormone receptors (TRs) acting as ligand-dependent transcription factors binding the "thyroid hormone response elements" (TREs) on the promoter region of thyroid hormone-responsive genes[22]. In the early 1960s, Tata and co-workers provided the first evidence for a "receptor-mediated" mechanism of T_3 action on energy metabolism[23]. In the 1980s, two distinct genes, *THRA* and *THRB*, were iden-

tified in humans and rodents, each encoding a different TR isoform (TRα and TRβ, respectively). The *THRA* gene was originally identified in chicken[24], while *THRB* was cloned from human and rat cDNA libraries[25]. Each isoform shows alternative splice variants (TRα1, TRα2, TRβ1 and TRβ2) with specific and distinct functions and tissue localization.

Although the "receptor-mediated" mechanism accounts for several actions of THs, other effects independent of TRs have been described, suggesting an alternative model for their action. Effects of iodothyronines that are not initiated by binding to TRs are termed 'non-receptor-mediated' mechanisms[26] and could involve a multiplicity of signaling pathways, such as phosphorylation of effector proteins[27], binding to surface receptors[28], Ca^{2+} mobilization[29], alteration of mRNA stability[30], modification of membrane fluidity and permeability[2]. The possibility that TH action was mediated by interactions with membrane surface receptors was confirmed by using cell impermeant agarose-conjugated T_3. The results clearly indicated that both free and conjugated hormones led to activation of extracellular signal-regulated kinase (ERK1/2s)[31] and affected Ca^{2+} homeostasis[32]. Moreover, THs were shown to interact with theαVβ3 integrin receptor triggering the ERK1/2 pathway[33]. Although the "non-receptor mediated" effects are sometimes called "non-genomic", this term is rather confusing as these pathways may also in turn affect gene transcription[34].

In conclusion, it is now widely accepted that TH effects may result from a synergism between "receptor-mediated" and "non-receptor mediated" mechanisms. Moreover, we can distinguish between early and late effects of THs (also called "short-term" and "long-term" effects), the first being evident within minutes or a few

hours, whereas the second occurs over several hours or days[34,35]. However, the latency of a response is not sufficient to discriminate between "receptor-mediated" and "non-receptor" mediated effects.

HEPATIC STEATOSIS: *IN VITRO* AND *IN VIVO* MODELS

With the rapidly growing prevalence of obesity throughout the Western countries, morbidity and mortality related to its complications are on the rise. Severe obesity is generally associated with TAG accumulation in non-adipose tissues like liver, muscle and pancreas and leads to a high risk of co-morbidities, including nonalcoholic fatty liver disease (NAFLD), cardiovascular disease and diabetes (for a review see[36]). NAFLD is a pathological condition associated with over-accumulation of TAGs in the liver and represents the most common of all hepatic disorders and the most frequent cause of chronic liver disease[37,38]. The earliest stage of NAFLD is hepatic steatosis characterized by the deposition of cytoplasmic TAGs as macro- and/or micro-vesicular lipid droplets in more than 5% of hepatocytes. Simple steatosis may progress to nonalcoholic steatohepatitis (NASH), cirrhosis and finally hepatocellular carcinoma[39]. NAFLD is now considered the hepatic manifestation of the metabolic syndrome and has insulin resistance as its hallmark. NAFLD is a syndrome with multifactorial etiology for which there is no effective treatment, although weight loss may halt disease progression and revert histological changes[36].

In hepatocytes, steatosis results from an imbalance between lipid availability (deriving from circulating lipid uptake or *de novo* lipid synthesis) and lipid disposal (through FFA oxidation or TAG secretion)[40]. Typically, the main cause of steatosis is an overflow of free fatty acids (FFAs) into the liver that may eventually trigger lipoperoxidative stress and hepatic injury[39,41]. In the liver, FFAs are stored as TAGs through their esterification with glycerol or, alternatively, catabolized by oxidation to generate adenosine triphosphate (ATP). Excess TAGs are accumulated inside lipid droplets (LDs) that regulate storage and traffic of lipids (for a review see[42]). Typically, LDs are composed of a core of neutral lipids surrounded by phospholipids and proteins of the PAT protein family (acronym referring to the first members identified)[43]. The main PAT proteins are the adipocyte differentiation-related protein (ADRP, also called PLIN2), the oxidative tissue-enriched PAT protein (OXPAT or PLIN5) and the tail-interacting protein (TIP47 or PLIN3)[44]. ADRP expression is increased in rat models of NAFLD and in isolated hepatocytes[45]. PAT proteins are under the control of peroxisome proliferator-activated receptors (PPARs), a subfamily of lipid-activated transcription factors[46] consisting of three members, PPARα, PPARγ and PPARδ, with distinct functional roles[47,48]. In the liver, PPARα enhances lipid catabolism and mobilization[49], PPARδ induces glycolysis/lipogenesis and PPARγ promotes lipid synthesis and LD formation[50]. In summary, PPARα and PPARδ mainly act in energy burning, whereas PPARγ regulates energy storage, although an overlapping in their function has been described[40-51]. Moreover, PAT proteins regulate action of hepatic lipases that mobilize TAGs stored in LDs towards oxidation or secretion[52], in particular, the adipose triglyceride lipase (ATGL) performs the first step in TAG hydrolysis.

In vivo models

Steatosis and steatohepatitis can be modeled in rodents by two main dietary protocols: a methionine and choline deficient (MCD) diet or a high-fat diet (HFD). Different dietary approaches produce different disease severities and work by specific mechanisms[53]. In rodents, a MCD diet quickly induces (2-4 wk) hepatic steatosis (mainly macrovesicular) that may progress to inflammation and fibrosis. MCD diet-induced NASH is reversible by switching to a diet with methionine and choline. Rodents fed MCD diets lose weight (due to the lower caloric intake) and do not show insulin resistance. By contrast, HFD increases body weight, body fat and induces insulin resistance in rodent models. In general, HFD feeding induces only mild steatosis (mainly microvesicular) and does not produce liver fibrosis. The term "HFD" encompasses a wide variety of diet formulas but in all of them about 30%-75% of total calories is derived from saturated fatty acids. This diet closely resembles the pathological and molecular alterations found in humans with NAFLD[53]. It can be emphasized that fatty liver is typically characterized by altered lipid metabolism, increased oxidative stress and abnormal pattern of cytokine production.

In vitro models

Hepatic steatosis in humans is typically associated with excess accumulation of oleic acid, a monounsaturated omega-9 fatty acid which represents the end product of *de novo* fatty acid synthesis. A number of studies using both primary cell cultures[54] and immortalized cell lines[55,56] proposed reliable cell models of hepatosteatosis in which the steatosis severity might be modulated and the TAG content was exactly quantifiable. These *in vitro* models represent a simple experimental system to investigate the mechanisms underlying the steatosis progression and the hepatocyte alterations by excluding the interference from the matrix and other non-hepatocytic cells. Over the past decade, several cellular models of hepatosteatosis have employed palmitate (C16:0) and oleate (C18:1) as exogenous fatty acids since these are common dietary long-chain FFAs and the most abundant FFAs in liver in both normal subjects and patients with NAFLD[57]. The human hepatoma cell line (HepG2) incubated with a mixture of oleate/palmitate (2:1 ratio) was used to study the cellular mechanisms involved in FFA-mediated lipotoxicity[55,58]. The same FFA mixture was used to induce steatosis in primary human hepatocytes[58]. In order to assess the different toxicity of saturated and unsaturated FFAs, primary mice hepatocytes and HepG2 cells were treated with various concentrations (0.05-0.5 mmol/L) of long

chain FFAs with different degrees of saturation; exposure to monounsaturated fatty acids resulted in lipid accumulation without changes in hepatocyte viability; in contrast, saturated fatty acids significantly decreased cell viability[59]. The effect of increasing concentrations of oleate alone (0.1-2.0 mmol/L) was also evaluated in order to clarify the pathophysiological changes associated with NAFLD[60].

LIPID-LOWERING EFFECTS OF IODOTHYRONINES ON *IN VIVO* MODELS OF HEPATOSTEATOSIS

In 1994, a first study reported that a daily intraperitoneal (*ip*) injection of T3 (from 0 to 25 μg/100 g b.w.) to *ob/ob* mice decreased body weight and body fat and increased oxygen consumption and oxidative metabolism[61]. About ten years later, Goglia and coworkers described similar effects for T2[10]. They showed that a daily ip injection of T2 (25 μg/100 g b.w.) to rats simultaneously receiving HFD reduced both adiposity (about -50%) and body weight gain (about -13%) when compared with rats receiving HFD alone. Moreover, T2 administration resulted in an almost complete disappearance of fat accumulation in the liver, a reduction in serum TAG and cholesterol levels (-52% and -18%, respectively), and a stimulation (about +42%) of FFA oxidation rate without inducing thyrotoxicity [10]. The effects of T2 on liver metabolism seemed to involve mitochondria, even although peroxisomes are the main site for fat oxidation. In fact, long chain FFAs enter mitochondria through the activity of carnitine palmitoyl-transferase 1 (CPT1) that was stimulated by HFD and further increased by T2 [10].

Interestingly, dietary administration of T3 was also able to both prevent and reverse hepatic steatosis in rats[62]. In fact, concurrent dietary administration of T3 and MCD diet resulted in prevention of fatty liver and decrease in lipid peroxidation in rats fed a MCD diet for 10 weeks and then co-fed T3 for 1 week. Similar effects were observed using the potent TR selective agonist GC-1[62].

The hepatic effects of T2 administration to HFD rats were investigated in more detail by Grasselli *et al*[63,64]. HFD feeding resulted in hepatic lipid accumulation under the form of numerous LDs, a condition resembling the microvesicular steatosis typical of NAFLD. Fat accumulation was associated with increased transcription of PPARα, a regulator for a number of genes involved in FFA catabolism, and of ATGL, a lipase mobilizing fat from LDs, together with a stimulation of anti-oxidant agents such as catalase and metallothioneins, in line with the increased production of reactive oxygen species (ROS) from mitochondria and peroxisomes as a consequence of fat accumulation[65]. In the liver of HFD rats, concomitant T2 administration was able to prevent lipid accumulation, but also oxidative stress conditions associated with the diet[63]. Moreover, T2 prevented the HFD-induced up-regulation of both PPARα and ATGL and stimulated - (AOX) expression, indicating a stimulation of peroxisomal FFA oxidation[64].

In addition to the above described reports demonstrating the ability of T2 to prevent liver steatosis when administered simultaneously to HFD, other studies demonstrated that T2 was also able to reverse hepatic steatosis after its induction through long term HFD and these effects were associated with a stimulation of mitochondrial uncoupling and a reduction in mitochondrial oxidative stress[66].

A recent paper investigated the changes in the rat liver proteome induced by T2 treatment. The proteomic approach allowed identification of which proteins were differentially expressed in the liver of HFD rats as a function of T2 treatment[67]. Upon T2 administration, the rat liver proteome resembled that typical of a non-steatotic condition. In particular, high-fat feeding led to changes in the expressions of enzymes involved in a multiplicity of pathways (*i.e.*, lipid metabolism, antioxidant defense, respiratory chain, oxidative metabolism). Mitochondria, in particular, appeared as the major target for the metabolic/energy adaptations induced by lipid overload in the liver and showed the more marked changes in terms of proteome as a response to T2 treatment. In mitochondria from HFD rats, enhanced activities of complexes I and V and reduced activities of complexes II and IV were detected, even although the protein levels for all the complexes were increased. T2-treatment stimulated complexes I and II and normalized complex IV activity. On this basis, the authors suggest that the T2-induced enhancement of oxidative capacity may actually be based on a stimulation of the individual respiratory chain complexes (I , II and IV)[67].

LIPID LOWERING EFFECTS OF IODOTHYRONINES ON *IN VITRO* MODELS OF HEPATOSTEATOSIS

The above described *in vivo* studies could not distinguish between the direct antisteatosic effects of THs on the liver and their secondary effects due to upstream changes in endocrine or metabolic pathways. The employment of isolated hepatocytes allowed overcoming these problems.

Grasselli *et al*[51] assessed *in vitro* the direct effects of T2 and T3 (10^{-7}-10^{-5} mol/L doses for 24 h) using primary cultures of rat hepatocytes overloaded of lipids ("steatotic" hepatocytes) by exposure to the classical oleate/palmitate (2:1 ratio) mixture. The use of supraphysiological doses of iodothyronines depends on both their rapid metabolism *in vitro* and on their binding to the high concentration (1%) of albumin present in the culture medium. In accordance with reports showing altered expression of PPARs in murine models developing fatty livers[47], isolated "steatotic" hepatocytes exhibited increased expression of both PPAR-γ and PPAR-δ, as well as of ADRP, a PPAR-regulated PAT protein. As in liver of HFD rats[64], also in isolated "steatotic" hepatocytes an increased activity of AOX, the enzyme catalyzing peroxisomal β-oxidation, as well as of SOD and catalase, two antioxidant enzymes protecting cells from the higher ROS production associ-

ated with FFA catabolism, was described. A reduction in the number and average sizes of LDs was observed after treatment with T2 or T3, suggesting that iodothyronines lead to dispersion/fragmentation of LDs, thus making the stored TAGs more accessible to enzymes acting on catabolism/secretion of FFAs. Moreover, both T2 and T3 were able to reduce the FFA-induced up-regulation of PPARγ and PPARδ, the stimulation of AOX, SOD and catalase activities. These results clearly indicate the lipid-lowering effect of iodothyronines mainly depends on a direct action on the hepatic cell[51].

The use of primary rat hepatocytes allowed verification that the lipid-lowering effect of iodothyronines was a direct action on the hepatocyte but the involvement of thyroid hormone receptors in mediating this action remained to be elucidated. To this end, the same experiments were repeated using the FaO rat hepatoma cell line defective for functional TRs. FaO cells were exposed to the classical oleate/palmitate (2:1) mixture and then treated with T2 or T3 for 24 h (10^{-7}-10^{-5} mol/L doses). In FaO cells, TAG accumulation was associated with an increase in number and size of LDs and in PPARγ mRNA expression. The addition of T2 or T3 to "steatotic" cells reduced both the TAG content and the number and size of LDs and down-regulated expression of PPARα and PPARγ. Moreover, iodothyronines stimulated the fuel-induced O2 consumption. Since iodothyronines prevented the ADP-induced transient stimulation of O2 consumption, this indicated a mitochondrial uncoupling action. In conclusion, this study demonstrated that the lipid-lowering actions of both T2 and T3 on the hepatocyte occur *via* "non-receptor-mediated" mechanisms and involve a short-term action by stimulation of mitochondrial O2 consumption[68].

IODOTHYRONINES AND AUTOPHAGY OF LIPID DROPLETS

Despite the advances in the understanding of the effects of THs on cellular metabolism, little is known about the mechanisms by which THs regulate energy consumption within the cell. This is particularly true for the events involved in the delivery of FFAs to mitochondria, a necessary step in converting stored intracellular triglyceride fuel into ATP.

Autophagy is a stress-induced catabolic process involving lysosome fusion that is conserved in almost all eukaryotes. Autophagy of lipid droplets, termed "lipophagy", has been shown to be a major pathway of lipid mobilization in hepatocytes[69] and its inhibition has been linked to development of fatty liver and insulin resistance[70]. The regulation of autophagy also appears to be important in the context of metabolic diseases, such as obesity. In a recent paper, Sinha *et al*[71] showed that T3 induced both lipophagy in cultured liver cell lines and hepatic autophagy in the mouse liver. The authors observed that the T3-stimulated autophagy of LDs depends on the presence of functional TRs and occurred before any

stimulation of hepatic lipases or oxidation enzyme[71]. Moreover, in animals with impaired autophagy, the effect of THs on FFA oxidation was abolished. Therefore, they propose that T3 may increase the delivery of FFAs to mitochondria for β-oxidation through induction of autophagy of LDs. In this light, T3 or its analogs, through their proautophagic action, may be useful in the treatment or prevention of NAFLD and its associated complications.

CONCLUSION

In the last decades, extensive studies investigated the possible use of iodothyronines as pharmacological tools in the treatment of obesity, hyperlipidemia and dysmetabolic syndromes. The possible pharmacological use of the thyroid hormones T3 or T4 to stimulate body weight loss has found severe limitations because of the thyrotoxic effects associated with their long-term administration. For this reason, the identification of TH agonists/analogs retaining anti-obesity and hypolipemic efficacies, while being devoid of thyrotoxic effects, would represent a potential therapeutic advance.

Recent *in vivo* and *in vitro* studies have accumulated evidence on the lipid-lowering action of iodothyronines in the liver (Figure 2). The first studies showed that systemic administration of iodothyronines to rats receiving HFD resulted in a significant reduction in body weight gain and in the serum levels of triglycerides and cholesterol. At the organ level, the effects on the liver were very interesting, where iodothyronines could lower the excess lipid accumulation associated with HFD. These studies prosecuted by investigating the mechanisms of iodothyronine action. The development of *in vitro* models of hepatosteatosis using both primary cultures of rat hepatocytes and rat hepatoma cell lines allowed demonstration that the lipid lowering effects of iodothyronines depend on a direct interaction with the hepatic cell and is not mediated by thyroid hormone receptors. In conclusion, all the data summarized in this review clearly indicates that T2 is able to reduce the lipid content of "steatotic hepatocytes", thus supporting the possible utilization of T2 as a pharmacological tool in the treatment of dysmetabolic syndromes, such as NAFLD, and also in the light of its lack of thyrotoxic effects.

Although a preliminary study on humans has been published, clinical trials are needed to translate these effects to the treatment of human obesity. If reproduced in humans, these results may offer an interesting perspective on the possible pharmacological approaches to the above mentioned lifestyle-related dysfunctions.

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Update on inflammatory bowel disease in patients with primary sclerosing cholangitis

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Abstract

Patients with primary sclerosing cholangitis (PSC) complicated by inflammatory bowel disease (IBD) represent a distinct subset of patients with unique characteristics, which have serious clinical implications. The aim of this literature review was to shed light to the obscure clinical and molecular aspects of the two diseases combined utilizing current data available and putting issues of diagnosis and treatment into perspective. The prevalence of IBD, mainly ulcerative colitis in PSC patients is estimated to be 21%-80%, dependent on screening programs and nationality. PSC-associated colitis is likely to be extensive, characterized by rectal sparing, backwash ileitis, and generally mild symptoms. It is also more likely to progress to colorectal malignancy, making it imperative for clinicians to maintain a high level of suspicion when tackling PSC patients. There is no optimal surveillance strategy but current guidelines advocate that colonoscopy is necessary at the time of PSC diagnosis with annual endoscopic follow-up. Random biopsies have been criticized and a shift towards targeted biopsies using chromoendoscopy, laser endomicroscopy and narrow-band imaging has been noted. Techniques directed towards genetic mutations instead of histological abnormalities hold promise for easier, more accurate diagnosis of dysplastic lesions. Chemopreventive measures against colorectal cancer have been sought in these patients. Ursodeoxycholic acid seemed promising at first but subsequent studies yielded conflicting results showing anticarcinogenic effects in low doses (8-15 mg/kg per day) and carcinogenic properties in high doses (15-30 mg/kg per day).

Core tip: Combination of primary sclerosing cholangitis (PSC) and inflammatory bowel disease (IBD) has recently arisen as a challenging research field. Recent data highlight the specific clinical and genetic traits that differentiate PSC-IBD from the two diseases individually. We reviewed the literature on colorectal neoplastic susceptibility in this subset of patients and the underlying pathogenetic mechanisms. We also emphasize the technological advances that have provided novel diagnostic tools for more accurate detection of dysplastic lesions. Finally, we present current guidelines on follow-up as well as all evidence available as to whether ursodeoxycholic acid should be used prophylactically against colorectal cancer.

INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic progressive disease characterized by inflammation and fibrosis of

medium size and large ducts in the intrahepatic and extrahepatic biliary tree[1,2]. This disorder results in multifocal intrahepatic and extrahepatic biliary strictures, leading to cholestasis, liver cirrhosis, portal hypertension, and ultimately, premature death from liver failure. It was first reported in the German literature in 1867 by Hoffman, but was described in more detail in the 1920s by two French surgeons, Delbet and Lafourcade. The term sclerosing cholangitis was first used in 1954 by Castleman and later by Schwartz and Dale in their review article[3]. Its etiology remains largely unknown, although it is strongly believed that autoimmunity is the main culprit. The differential diagnosis of PSC includes congenital diseases (*e.g.*, Caroli disease and choledochal cysts) and secondary cholangiopathy, as observed in patients with collagen vascular diseases (*e.g.*, systemic lupus erythematosus, rheumatoid arthritis, and systemic sclerosis) and in those with infiltrative diseases (*e.g.*, mediastinal fibrosis, Riedel thyroiditis, eosinophilic cholangitis, and histiocytosis X). Parasitic, fungal, viral or bacterial infections or recurrent cholangitis itself, especially in patients who are immunocompromised, can cause multifocal liver abscesses that lead to a PSC-like appearance of the bile duct. This disease is associated with many cancers including cholangiocarcinoma, gallbladder cancer, hepatocellular carcinoma and colorectal cancer (CRC), thus establishing a link between chronic inflammation and carcinogenesis.

THE PSC-INFLAMMATORY BOWEL DISEASE INTERPLAY

The overwhelming majority of PSC cases have underlying inflammatory bowel disease (IBD). IBD is defined as a chronic condition characterized by immune-mediated inflammation of the gastrointestinal system. The prevalence ranges from 21% to 80%, with the higher rates seen in settings where screening programs are more intense and rectal and sigmoid biopsies are routinely obtained. A geographical variation also exists, with northern European and American societies exhibiting higher rates of PSC-IBD than southern regions and Asia. About 85%-90% of patients with PSC and IBD are comprised of ulcerative colitis (UC) patients and the remainder involves patients with Crohn's colitis or Crohn's ileocolitis[4]. The association of PSC and Crohn's disease (CD) was first described by Atkinson and Carroll in 1964[5]. A year later, Smith and Loe described an association between PSC and UC[6]. Conversely, it has been estimated that PSC occurs in about 5% of UC patients and 3% of CD patients[7].

IBD may be diagnosed at any time during the course of PSC. Until recently, the diagnosis of IBD more frequently preceded that of PSC, even by several years[8-11]. Nowadays, there has been a shift in the timing of diagnosis of IBD and PSC. PSC is most commonly diagnosed first, or at least there is a concomitant diagnosis of the two diseases. It is intriguing that *de novo* IBD may present after liver transplantation for PSC[12], and PSC may present several years after proctocolectomy for IBD. The altered trend in diagnostic timing can be attributed to two factors. First, the advent of noninvasive imaging techniques such as magnetic resonance cholangiography has enabled early diagnosis of pathological liver biochemistry. Second, the increasing awareness among physicians of the PSC-IBD association has led to early routine endoscopic screening in patients diagnosed with PSC, even in the absence of symptomatic colitis. A study comparing the interval from PSC to IBD diagnosis in 1993-1997 and 2003-2007 showed a decrease from 9 to 7 mo respectively[13].

The genetic factors for PSC development are still poorly understood. There is an obvious geographic clustering with high prevalence in northern countries compared to Southern Europe and Asia. It has been shown that first-degree relatives of PSC patients have a disease prevalence of 0.7%, representing a nearly 100-fold increased risk of developing PSC compared to that in the general population[14]. In siblings the prevalence even reaches 1.5%[15]. Taken together, these epidemiologic data and heritability studies have revealed a strong genetic background for PSC. Genome-wide association studies have shed some light on the subcellular maze of PSC and its overlap with IBD. Human leucocyte antigen (HLA) and non-HLA haplotypes have been identified. The HLA-A1 allele[16], HLA-C7[17], major histocompatibility complex class I chain-related A (MICA)*002 and 008/5.1 alleles[18,19], as well as the tumor necrosis factor (TNF)α promoter -308 A allele[20] were identified as risk loci for PSC susceptibility. Data from five different European countries (United Kingdom, Italy, Norway, Spain and Sweden) demonstrate that PSC is positively associated with three different HLA class II haplotypes: DRB1*03, DQA1*0501, DQB1*02 (which confers the highest relative risk for PSC development); DRB1*15, DQA1*0102, DQB1*0602; and the DRB1*13, DQA1*0103, DQB1*0603[21]. However, non-HLA associations have also been confirmed. Of note, MMEL1 and TNFRS14 on chromosome 1p36 encoding a membrane metalloendopeptidase-like protein of unknown function and a receptor for cytokines and membrane-bound ligands, respectively, have been identified as risk loci for PSC[22]. The risk of PSC has also been associated with the *FUT2* gene encoding fucosyltransferase[22], which is an enzyme that regulates expression of the ABO blood group antigens on the surface of epithelial cells. To date, there is scarcity of molecular evidence regarding the shared susceptibility loci between PSC and IBD. A Scandinavian study comparing PSC with UC patients showed distinct HLA associations[23]. No significant differences were noted between PSC patients with concurrent UC and PSC patients without IBD. This study provides genetic evidence that UC in PSC patients follows a distinct course and demonstrates phenotypic uniqueness compared with UC in isolation. More recently, *REL*, *IL-2* and *CARD9* have been identified as genetic links between the two diseases[24].

Taking into consideration its associations with HLA haplotypes, autoimmune diseases and the presence of IBD in the majority of PSC patients, immunopathoge-

Table 1 Characteristics of inflammatory bowel disease associated with primary sclerosing cholangitis
Extensive colitis (with right-sided predominance)
Rectal sparing
Backwash ileitis
Mild or quiescent course
Increased risk of colorectal cancer
Increased risk of pouchitis in patients undergoing proctocolectomy with
Ileal pouch anal anastomosis
Increased risk of peristomal varices in patients undergoing
proctocolectomy with ileostomy

netic mechanisms have been sought in PSC pathogenesis. In this regard, two theories have been proposed: the leaky gut hypothesis and the gut lymphocyte homing hypothesis. According to the leaky gut hypothesis, bacteria or bacterial products enter the portal-venous system due to the increased intestinal permeability resulting from inflammation, and translocate to the liver. Bacteria trigger the release of cytokines by Kupffer cells and macrophages in the liver and lead to periductal fibrosis[8]. The gut lymphocyte homing hypothesis supports the notion that T lymphocytes primed in the inflamed gut may persist as long-lived memory cells, undergo enterohepatic circulation, and trigger portal inflammation in PSC *via* aberrantly expressed adhesion molecules in the liver and gut[25].

IBD IN PSC: A UNIQUE PHENOTYPIC EXPRESSION

There are many clinical and endoscopic features that differentiate patients with IBD and concomitant PSC and those with IBD in isolation (Table 1). Loftus *et al*[10] compared 71 patients with PSC who had IBD with a matched group of 142 patients with UC. Among the PSC patients, 86% had UC, 7% had CD, and 7% had indeterminate colitis. The PSC patients more frequently had pancolitis (87% *vs* 54%), rectal sparing (52% *vs* 6%), and backwash ileitis (51% *vs* 7%) than the control group. It is now commonly believed that the colitis associated with PSC is frequently extensive and characterized by rectal sparing and backwash ileitis[26,27]. These special traits impede the definitive classification of IBD. For instance, the presence of rectal sparing or ileitis may be misinterpreted for CD or indeterminate colitis, rather than UC. In addition, PSC-associated colitis runs a milder, quiescent course, sometimes with absent clinical manifestations, thus delaying diagnosis[28]. Another intriguing trait in PSC-IBD patients is the higher rate of colorectal neoplasia, which tends to be proximal, is diagnosed at a later stage and has a worse prognosis. The colorectal neoplastic potential in these patients will be discussed later.

Of note, PSC patients who have an ileal pouch anal anastomosis (IPAA) after colectomy have an increased risk of pouchitis compared to patients with UC without PSC[29,30]. The underlying mechanism for this complica-

tion remains obscure. There is also one report suggesting that patients with PSC and IPAA run an increased risk of development of dysplasia in the ileal pouch mucosa compared with UC patients without PSC, and that these patients consequently should be under intensive surveillance[31]. However, more studies are required to substantiate these claims. Interestingly, it has been suggested that PSC-IBD patients undergoing proctocolectomy with ileostomy develop peristomal varices more frequently than IBD patients without evidence of hepatobiliary disease[32]. Bleeding from these often is recurrent and is challenging to treat. This complication can be controlled with a portosystemic shunt or transjugular intrahepatic portosystemic shunt, but liver transplantation may be considered.

PSC in patients with IBD does not seem to run a different course when compared to patients without IBD. Nevertheless, one study has demonstrated that PSC in patients with concomitant IBD has a predilection for men, is more likely to manifest itself for the first time with abnormal liver biochemistry, and has intrahepatic and extrahepatic biliary tree strictures[33]. Proctocolectomy, as a surgical treatment for UC, has no effect on liver function tests, histology or survival of patients with PSC[34].

COLORECTAL NEOPLASTIC POTENTIAL IN PSC-IBD PATIENTS

The increased occurrence of CRC in IBD patients has been well documented since 1925, when it was first described by Crohn and Rosenberg[35]. The cancer risk involves both UC and CD[36-39], and has been linked with prolonged duration and extent of disease, associated PSC and active inflammation[40,41]. Data on the relative risk of CRC in IBD are not in agreement in different studies. The cumulative risk varies from 1.4% after 18 years[42] to 34% after 25 years from onset of disease[43]. Some studies even advocate that the risk is not increased at all[44].

Recent investigations have unveiled another relationship, that of PSC-IBD patients and CRC. The concept that PSC is associated with an increased risk of colorectal neoplasia in patients with UC was proposed by Broomé *et al*[45] in 1992. In a study of 17 patients with UC who were found to have dysplasia, carcinoma, and/or DNA aneuploidy, 28% had coexistent PSC. This led to the hypothesis that PSC is an independent risk factor for the development of colorectal neoplasia in patients with existing UC. There has been a wealth of studies since then reporting the connection of PSC-IBD and CRC, particularly highlighting the compounding neoplastic risk when the two disorders coexist as opposed to patients with IBD alone[46-53] (Table 2). Soetikno *et al*[54] performed a meta-analysis of 11 studies and described an odds ratio (OR) of 4.79 (95%CI: 2.89-5.76) when comparing patients with UC and PSC to UC patients without PSC. However, two studies (both from the Mayo Clinic but using different groups of patients) have yielded contradictory results rejecting the hy-

Table 2 Summary of studies evaluating primary sclerosing cholangitis as a risk factor for colorectal neoplasia in chronic ulcerative colitis

Ref.	UC case group (No)	Centre	End point (No)	Matched controls	Colectomy rate	Is PSC a risk factor?
Broomé et al[45]	Dys (17)	Hudding, Sweden	PSC (5)	Yes	0%	Yes
D'Haens et al[46]	Dys (29)	Chicago, United States	Cholestasis/PSC (10)	Yes	0%	Yes
Broomé et al[47]	PSC (40)	Hudding, Sweden	CRC/Dys (15)	Yes	30%	Yes
Brentnall et al[48]	PSC (20)	Seattle, United States	Dys (9)	No	0%	Yes
Leidenius et al[49]	PSC (45)	Helsinki, Finland	CRC/Dys (13)	Yes	29%	Yes
Marchesa et al[50]	PSC (27)	Clevelan, United States	CRC (4)/Dys (14)	Yes	All postop	Yes
Shetty et al[51]	PSC (132)	Clevelan, United States	CRC (17)/Dys (16)	No	0%	Yes
Loftus et al[52]	PSC (143)	Mayo Clinic, United States	CRC (8)	No	37%	No
Nuako et al[53]	CRC (171)	Mayo Clinic, United States	PSC (30)	Yes	14%	No

CRC: Colorectal cancer; CRN: Colorectal neoplasia; Dys: Dysplasia; PSC: Primary sclerosing cholangitis.

pothesis that there is an increased risk for CRC in PSC-IBD patients[52,53]. In general, the increased neoplastic potential in PSC-IBD patients could be ascribed to late diagnosis due to the subclinical course of colitis and the conservative treatment of mild flare-ups as opposed to colectomy, thereby increasing the duration and extent of colitis.

CRCs associated with PSC display a number of characteristics. They appear to have a more proximal localization with up to 76% right-sided distribution. A full colonoscopy is therefore mandatory for surveillance purposes. CRCs in this subset of patients are diagnosed at a more advanced stage and tend to be fatal. In a recent study, PSC patients with IBD and CRC were found to be younger at onset of IBD than patients who had IBD and CRC without PSC (19 *vs* 29 years; *P* = 0.04). The time interval from onset of colitis until diagnosis of CRC was, however, similar in the two groups (17 *vs* 20 years; *P* = 0.02)[55].

PATHOGENETIC MECHANISMS OF CRC IN PSC

The mechanisms underlying the pathogenesis of CRC in IBD patients have been rigorously investigated and many differences in comparison with sporadic CRC have been addressed[56,57]. Even though IBD-CRC usually follows a dysplasia-cancer pattern, as in sporadic cancer, molecular and genetic events seem to occur in an unconventional sequence. Alterations to the *p53* tumor suppressor gene occur earlier in colitis-related CRC[58], whereas adenomatous polyposis coli (*APC*) gene alteration is usually a later event[59]. The reverse applies to sporadic CRC. It is also noteworthy that p53 mutations can be present in nondysplastic mucosa in IBD-CRC, but only in dysplastic areas in sporadic cancer[60]. Another noteworthy difference is that low-grade dysplasia is often in flat lesions in CRC-IBD, which are difficult to detect endoscopically, whereas such dysplasia occurs within raised polyps in sporadic cancer. The role of microsatellite instability, hypermethylation, chromosomal instability, interleukin (IL)-23/IL-17 signaling and E-cadherin (CDH1) has been addressed in studies but is not yet fully understood[61-64]. Polymorphisms of the mismatch repair genes *MLH1* and *MSH2*

have been incriminated for the pathogenesis of IBD and related malignancy[65,66].

The direct impact of PSC on colorectal carcinogenesis has not yet been delineated. Another theory highlights the significance of the cholestasis-associated secondary bile salt pool in the colon[67]. Bile acids such as deoxycholic acid and lithocholic acid (LCA) are thought to contribute to tumorigenesis through disruption of the balance between colorectal crypt cell proliferation, differentiation and apoptosis[68-71]. Mucosa in carcinoma displayed an increased frequency of bile acid receptors compared with normal tissue[72]. A higher fecal bile acid concentration was found in patients with UC who developed neoplasia compared with those without UC[73]. Folate deficiency has also been implicated in the pathogenesis of CRC in IBD patients. Folate deficiency arises from sulfasalazine use to treat UC, which is a competitive inhibitor of folate absorption. Folate supplementation was associated with a 62% reduction in the incidence of neoplasia in patients with pancolonic UC compared with placebo[74]. This theory, however, contradicts epidemiological reports according to which maintenance therapy in UC reduces risk of carcinogenesis[75].

SURVEILLANCE RECOMMENDATIONS

Periodic surveillance colonoscopy is the milestone of cancer prevention in IBD[76,77]. PSC in combination with IBD further enhances the risk for CRC as described above and necessitates increased alertness. That along with the fact that IBD in PSC patients usually follows an asymptomatic, subclinical course raises the need for routine colonoscopy at the time of PSC diagnosis, which should be repeated on an annual basis[78]. However, a recent study by Imam *et al*[79] showed a low risk of colonic neoplasia in young patients with a combined diagnosis of PSC and IBD, with an estimated prevalence of 1.3% and an incidence of 0.4% per year. This finding raises the question whether annual surveillance is unnecessary in this selected group of patients.

Three categories of dysplasia have been identified according to the IBD Dysplasia Morphology Study Group[80]: (1) negative for dysplasia; (2) indefinite for dysplasia; and (3) positive for dysplasia, which is further subdivided into low-grade dysplasia (LGD) and high-grade

dysplasia (HGD). Each of these categories necessitates a different approach. A finding of indefinite dysplasia dictates a repeat colonoscopy in 3-6 mo. The management of LGD is debatable with no clear evidence of optimal approach. St Mark's Hospital[81] demonstrated a 54% cumulative probability of LGD progressing to HGD or CRC. Mayo Clinic reported a 33% 5-year progression[82], while other studies described an even lower rate[83,84]. Thus, in the case of LGD different options should be discussed with patients and informed consent for conservative or operative management should be obtained. Patients with multifocal flat LGD in one screening or unifocal LGD in more than one screening should prompt prophylactic total proctocolectomy. HGD, however, needs unquestionable referral for total proctocolectomy due to the increased risk of concurrent or subsequent malignancy[85].

There has been increased skepticism in the medical community about the random biopsies used for surveillance purposes. New methods enable targeted biopsies to be obtained from identifiable lesions. Chromoendoscopy, confocal laser endomicroscopy and narrow-band imaging (NBI) are new promising techniques that are likely to replace old-fashioned random biopsies that have proven their inadequacy in many studies[86,87]. Chromoendoscopy uses the application of indigo carmine or methylene blue to stain dysplastic areas on the colonic mucosa. Hurlstone *et al*[88] examined 700 patients in a prospective case-control trial and diagnosed 69 dysplastic lesions with chromoendoscopy and only 24 with random biopsies ($P < 0.001$)[88]. Confocal laser endomicroscopy enables the histological visualization of the mucosa in real time. It is easily inferred that this technique requires specialized training for histological interpretation. A randomized controlled trial was conducted by Kiesslich *et al*[89] showing an increase of 4.75 times in yield of neoplasia when using endomicroscopy ($P = 0.005$). NBI uses optical fibers to enable a clear visualization of vessels, pit pattern and soft tissue structures. A study showed that NBI cannot be recommended as a chromoendoscopy substitute because it detected fewer lesions than chromoendoscopy in chronic colitis, although most were not dysplastic[90].

Newer techniques that target genetic alterations rather than histological abnormalities have been proposed to increase detection efficacy. Deletions and point mutations of tumor-suppressor genes such as *p53*, *Rb*, *APC*, *mcc* and the Sialosyl-Tn antigen have been found in dysplastic lesions and could be a useful tool for early diagnosis[91,92]. DNA evaluation by flow cytometry could reveal aneuploidy and predict suspicious areas likely to progress to CRC. The main drawback is that aneuploidy is not always a prerequisite for cancer occurrence and its presence does not always lead to malignancy.

CHEMOPREVENTION

Many studies have investigated the potential protective effects of different drug agents against malignancy in patients with UC. Pinczowski *et al*[93] was the first to report

that CRC risk is diminished by therapy with 5-aminosalicylic acid (5-ASA) in 1994. Ever since, studies have failed to demonstrate a clear relationship between 5-ASA therapy and CRC, rendering its potential protective effect presumptive rather than definitive. Azathioprine and mercaptopurine have not been shown to have a beneficial effect with regards to CRC in IBD[85]. Likewise, research has yielded inconclusive results regarding the use of corticosteroids, nonsteroidal anti-inflammatory drugs or folates for chemopreventive purposes.

Ursodeoxycholic acid (UDCA) is a drug commonly used in PSC patients due to its safe profile and favorable effects on the biochemical parameters of the disease. *In vitro* and animal studies have revealed a chemoprophylactic effect of UDCA. UDCA appears to arrest proliferation of colon cancer cell lines *in vitro*[94]. CRC induced by N-methylnitrosourea[95] and azoxymethane[96,97] in rats appeared to respond to UDCA therapy with a decrease in size. UDCA also decreased fecal concentrations of deoxycholic acid in animals, suggesting a potential protective effect through control of bile acid concentration in colon[98]. Several molecular mechanisms have been proposed to explain the chemoprophylactic effect of UDCA, including downregulation of cyclo-oxygenase-2 expression[97], prevention of carcinogen-induced changes in protein kinase C isoforms[99], suppression of epidermal growth factor receptor[100], cell cycle modulation by inhibiting the expression of cyclin D1 and promoting that of E-cadherin[101], and stabilization of mitochondrial membranes against damaging free radicals[102]. These results triggered a number of investigations in humans in order to shed light on the real effect of UDCA. Two studies have confirmed the protective effect of the drug. Tung *et al*[103] conducted a retrospective review of 59 patients showing a reduced OR of 0.18 (95%CI: 0.05-0.61) of colonic dysplasia after ursodiol use. Pardi *et al*[104] also performed a randomized placebo controlled study evaluating the effect of UDCA in the subgroup of UC patients with concomitant PSC. Patients who received a low dose of UDCA (13-15 mg/kg per day) showed a relative risk of 0.26 for developing CRC or dysplasia. Wolf *et al*[105] showed in a retrospective study of 120 patients that there was no reduction of CRC or dysplasia in the UDCA group. A hallmark study by Eaton *et al*[106] has recently reversed the long-standing conviction that UDCA has a place in CRC prevention in PSC-IBD patients. Using high doses of UDCA (28-30 mg/kg per day), they showed an increased risk of CRC in the UDCA group. The majority of patients developed colorectal neoplasia after > 2 years of use. This association remained significant after adjusting for smoking history and UC duration. High-dose UDCA also resulted in an increased risk of liver transplantation and/or death[107]. The discrepancy between different studies can be attributed to their inherent limitations and their failure to adjust for confounding factors such as age at onset of colitis, extent of colitis, family history of CRC, cigarette smoking, use of other drugs such as 5-ASA and folate, and use of the same criteria for dysplasia classification.

There has been speculation with regard to mechanisms underlying the toxic and carcinogenic UDCA properties. The most prevalent theory implicates the alteration of colonic bile acid milieu when high doses are used. An increase in serum UDCA and LCA levels in the treatment group has been reported[108]. That combined with results from *in vitro* studies stating that bile acids stimulate cell invasion in a dose-dependent fashion and reduce apoptosis could possibly provide a plausible explanation of the differing effects when low and high UDCA doses are used[109-111]. It is therefore prudent to recommend UDCA chemoprevention only to a high-risk subset of patients, including those with a personal or family history of CRC, and those with long-standing extensive colitis. This rationale has been incorporated to recent European guidelines[112].

In conclusion, PSC-IBD patients represent an important public health concern. Significant steps have been made towards the elucidation of the pathogenetic mechanisms underlying this complex disease. HLA and non-HLA susceptibility genes have been thoroughly studied and proven their association with PSC-IBD. Further investigations are warranted to reveal PSC- and IBD-specific genes and clarify their real impact on the disease. Genome-wide association studies could be invaluable in this direction but are severely undermined by the rarity of the disease and therefore the limited number of PSC patients that can be recruited. In terms of diagnosis, biomarkers currently in use are liver function tests and histology. A couple of new methods have been introduced to facilitate the evaluation of PSC patients. Fibroscan and a breath test assessing the elasticity and metabolic capacity of the liver respectively have paved the way for rapid, non-invasive diagnosis. Their diagnostic accuracy in PSC, however, remains under scrutiny.

CRC is a well-established risk for PSC-IBD patients. Aggressive colonoscopic surveillance is therefore imperative, even in those who have undergone liver transplantation[113]. In an attempt to relieve the socioeconomic and medical burden that PSC-IBD poses, many studies have explored potential pharmaceutical agents that may retard disease progression and protect against colorectal neoplasia. Antibiotics, immunomodulators, UDCA and antifibrotic agents have attracted the attention of researchers but their full potential has not yet been unraveled. Recent meta-analyses have demonstrated that UDCA in low to medium doses seems to have a chemoprophylactic effect, whereas high doses are carcinogenic[114,115]. Further investigations are required to test the efficacy of existing drug agents and promote the development of new ones. Understanding and harnessing molecular events seems a pivotal step towards this direction.

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REVIEW

Effects of resveratrol in experimental and clinical non-alcoholic fatty liver disease

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Abstract

The prevalence of obesity and related conditions like non-alcoholic fatty liver disease (NAFLD) is increasing worldwide and therapeutic options are limited. Alternative treatment options are therefore intensively sought after. An interesting candidate is the natural polyphenol resveratrol (RSV) that activates adenosinmonophosphate-activated protein kinase (AMPK) and silent information regulation-2 homolog 1 (SIRT1). In addition, RSV has known anti-oxidant and anti-inflammatory effects. Here, we review the current evidence for RSV-mediated effects on NAFLD and address the different aspects of NAFLD and non-alcoholic steatohepatitis (NASH) pathogenesis with respect to free fatty acid (FFA) flux from adipose tissue, hepatic *de novo* lipogenesis, inadequate FFA β-oxidation and additional intra- and extrahepatic inflammatory and oxidant hits. We review the *in vivo* evidence from animal studies and clinical trials. The abundance of animal studies reports a decrease in hepatic triglyceride accumulation, liver weight and a general improvement in histological fatty liver changes, along with a reduction in circulating insulin, glucose and lipid levels. Some studies document AMPK or SIRT1 activation, and modulation of relevant markers of hepatic lipogenesis, inflammation and oxidation status. However, AMPK/SIRT1-independent actions are also likely. Clinical trials are scarce and have primarily been performed with a focus on overweight/obese participants without a focus on NAFLD/NASH and histological liver changes. Future clinical studies with appropriate design are needed to clarify the true impact of RSV treatment in NAFLD/NASH patients.

Key words: Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Steatosis; Resveratrol; AMP-activated protein kinase; Silent information regulation-2 homolog 1; Anti-oxidants; Anti-inflammatory agents; Animal studies; Clinical trial

Core tip: The prevalence of obesity and related conditions like non-alcoholic fatty liver disease (NAFLD) is increasing. Therapeutic options are limited and alternative treatment options are sought after. An interesting candidate is resveratrol (RSV), a known AMP-activated protein kinase and silent information regulation-2 homolog 1 activator with anti-oxidant and anti-inflammatory properties. Here, we review the current evidence

for RSV-mediated effects and address the different aspects of NAFLD and non-alcoholic steatohepatitis pathogenesis. We review the *in vivo* evidence from animal studies and clinical trials. Uniformly, animal studies report a decrease in hepatic triglyceride accumulation and improvements in histological fatty liver changes, whereas results from the few clinical trials are equivocal.

INTRODUCTION

The prevalence of obesity is increasing worldwide and consequently related conditions like non-alcoholic fatty liver disease (NAFLD) have increased. NAFLD now affects up to one-third of adults and a growing number of children in developed countries[1-3]. Early stages of NAFLD involve pathological accumulation of triglyceride (TG) in the liver, a fairly benign condition. However, some individuals elicit an inflammatory response that can progress to cirrhosis, cirrhosis complications and an increased risk of liver cancer[4]. NAFLD is now the third leading cause of liver transplantation in the United States[5]. Thus, NAFLD and especially the subtype non-alcoholic steatohepatitis (NASH) are thought to become a major health issue in the United States and throughout the world[6].

Therapeutic options are limited and include weight loss, which is hard to obtain and sustain[7], bariatric surgery, Vitamin E and glitazone treatment, especially the latter with a risk of significant side effects[8-11]. Alternative treatment options are therefore warranted and intensively sought after[12-15].

A potential new therapeutic option is the polyphenol resveratrol (RSV). RSV is found in a number of plants, although in low concentrations. It is known as an activator of AMP-activated protein kinase (AMPK) and silent information regulation 2 homolog 1 (SIRT1), thereby mimicking a condition of caloric restriction *in vivo*. In addition, RSV has anti-oxidant and anti-inflammatory properties. All of these effects could in theory be beneficial for the treatment of NAFLD and a number of experimental and clinical studies have been performed.

The aim of the present review is to provide a comprehensive description of the rationale for RSV treatment for NAFLD and to review the present evidence from RSV intervention in experimental and clinical NAFLD studies.

RSV ACTIONS

RSV is primarily recognized as an AMPK and SIRT1 activator[16-18]. AMPK and SIRT1 are both central in the metabolism of many different cell types, rendering RSV with pleiotropic effects in various tissues. To date, studies have been unable to determine if RSV activates AMPK, SIRT1 or both, directly or indirectly[19], a matter of ongoing debate[20]. Regardless, the effects of the enzymes are closely interdependent. Recently, Park *et al*[21] proposed a mechanism involving a direct RSV-mediated inhibition of cAMP-specific phosphodiesterases and identified the cAMP effector protein Epac1 as a key mediator, which may lead to activation of first AMPK[21] and then SIRT1 through the up-regulation of NAD$^{+[20]}$. Furthermore, RSV may also act independently of AMPK/SIRT1; however, the mechanisms are not clarified.

Through the activation of AMPK, SIRT1 and alternative routes including anti-inflammatory and anti-oxidant actions, RSV may inhibit the development or progression of steatosis and steatohepatitis. Former attempts to use the AMPK activator metformin in the treatment of NAFLD have largely been abandoned because clinical studies showed no effect on histological NASH changes, despite a general decrease in hepatic steatosis and transaminase levels[22-24].

AMPK

The AMPK pathway regulates energy homeostasis, both intracellularly and at the whole-body level. Through the action of upstream kinases, AMPK responds to changes in the AMP/ATP ratio and thus serves as an intracellular sensor of energy levels, *e.g.*, in the situation of fasting, calorie restriction or accelerated ATP consumption[25].

AMPK activation in the liver shuts down anabolic processes like cholesterol and TG biosynthesis by reducing the activities of, *e.g.*, sterol regulatory element-binding protein-1c (SREBP-1c) and fatty acid synthase (FAS). AMPK activation also promotes catabolic processes, *e.g.*, fatty acid (FA) β-oxidation by inactivation of acetyl-CoA carboxylase (ACC) and promotion of carnitine palmitoyltransferase-1 (CPT-1) activity[26-28]. *In vivo*, it has been shown that chronic AMPK activation limits TG accumulation in both high-fat and control diet fed rats[29]. These AMPK-mediated effects have been shown in *in vitro* and *in vivo* studies, using RSV as an AMPK activator[28]. As an example, RSV treatment of HepG2 cells in high glucose media dose-dependently attenuated enhanced FAS expression, increased ACC activity and elevated TG accumulation[30].

In adipose tissue, the AMPK effects are similar, impairing lipolysis and promoting mitochondrial β-oxidation, thereby decreasing the level of circulating FFAs and the FFA load on the liver[26]. Both hepatic and peripheral insulin sensitivity is augmented.

SIRT1

SIRT1 is a member of the sirtuin family and a NAD$^+$-dependent deacetylase that acts as a master metabolic sensor of NAD$^+$. Thus, it adapts gene expression and metabolic activity in response to the intracellular energy state. SIRT1 is mainly found in the nucleus, where it functions

as a transcriptional repressor through histone, transcription factor, co-factor and enzyme deacetylation[31]. Following the SIRT1 metabolic effects, the molecule is thought to link calorie restriction and healthy aging and/ or longevity. A number of studies have confirmed this *in vivo* and *in vitro*[32-34], while few have disputed it[35].

In the liver, SIRT1 is implicated in the control of energy metabolism through deacetylation and activation of especially peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and the lipid-sensing transcription factor peroxisome proliferator-activated receptor (PPARα), resulting in increased FA β-oxidation[36]. PGC-1α stimulates mitochondrial biogenesis, thereby increasing the mitochondrial content in hepatocytes[37]. In unison with AMPK, SIRT1 deacetylates and regulates SREBP-1c and liver X receptor (LXR), which govern lipid and cholesterol metabolism[38-41]. Adenovirus-mediated overexpression of SIRT1 specifically in mouse liver has been shown to reduce liver fat by downregulation of SREBP-1c and FAS and up-regulation of expression of genes that control FA β-oxidation[42].

SIRT1 is an inhibitor of inflammation, repressing especially NF-κB transcription and activation as shown in liver and adipose tissue[31,36,43]. Anti-inflammatory effects of RSV have also been demonstrated in *in vitro* and *in vivo* studies, however, better documented in adipose tissue[44-48] than in hepatic cells or tissue[18,49-52].

Targeting SIRT1 activation for treatment of NAFLD has been suggested[53] as SIRT1 expression is decreased in dietary NAFLD models and NAFLD patients[54-56] and moderate SIRT1 overexpression protects mice from developing NAFLD[57].

NAFLD PATHOGENESIS

The pathogenesis of NAFLD and NASH is far from clarified and especially the factors that drive disease progression towards a more progressive, inflammatory phenotype are not fully characterized. Recently, a "multiple parallel hit hypothesis" has been proposed by Tilg and Moschen. Here, TG accumulation is viewed as an "innocent bystander", while a number of different parallel hits lead to NASH development[58]. Thus, it appears that there are 3 types of NAFLD patients: the "Good Fat Storer" (the NAFLD patient with a benign course); "the Bad Fat Storer" (the patient who develops immediate NASH); and the "Unfortunate Good Fat Storer" (the NAFLD patient that experiences additional hits and becomes a NASH patient)[59]. Only the latter two may require pharmacological treatment however, bearing in mind that NAFLD may be an independent risk factor for type 2 diabetes[60,61].

STEATOSIS

Hepatic steatosis occurs most often in the setting of obesity and metabolic syndrome and is the result of lipid overload, primarily with increased free fatty acid (FFA)

flux and TG accumulation. Several mechanisms are involved[58,62] and some may be targeted directly or indirectly by RSV treatment. An illustration of the proposed RVS effects on NAFLD pathogenesis is shown in Figure 1.

Increased FFA supply due to increased lipolysis from adipose tissue

Insulin resistance (IR) results in increased lipolysis of TG in adipose tissue, resulting in elevated levels of circulating FFAs. Hepatic uptake of both diet- and lipolysis-derived FFAs is unregulated with limitless hepatocyte uptake *via* fatty acid transporters (*e.g.*, CD36). The bulk of hepatic TG (two-thirds) is derived from circulating FFA from lipolysis[62]. RSV may decrease lipolysis in adipose tissue through improvement of peripheral insulin sensitivity, as documented in several studies[63,64]. Furthermore, RSV may favorably modulate the expression of fatty acid transporters[65-67].

Overnutrition

Dietary fat contributes approximately 15% to the overall FFA load on the liver[62]. Increased fat intake increases circulating FFA, whereas elevated carbohydrate intake, especially in the form of fructose, increases *de novo* lipogenesis[68].

Increased de novo hepatic lipogenesis from dietary carbohydrates and amino acids

Normally, *de novo* synthesis accounts for 5% of hepatic fat content. However, in subjects with NASH, up to 25% of the hepatic fat content may be caused by *de novo* lipogenesis[62]. The process is regulated independently by insulin and glucose. In the postprandial and in the IR state, insulin stimulates the transcription factor SREBP-1c that promotes transcription of all genes involved in lipogenesis, among them ACC, FAS and peroxisome proliferator-activated receptor (PPARγ)[69,70]. Glucose stimulates lipogenesis through stimulation of the transcription factor carbohydrate response element binding protein (ChREBP)[71]. An upstream activator of both SREBP-1c and ChREBP is LXR, which is a transcription factor governing lipid and cholesterol metabolism[72,73].

Results from *in vitro* and *in vivo* studies show that RSV inhibits hepatic lipogenesis by AMPK/SIRT1-mediated inhibition of SREBP-1c, ACC and FAS activity[27,28,37,44,74].

Inadequate fatty acid oxidation

Under normal physiological conditions, the mitochondria handle FFA by β-oxidation. Inadequate β-oxidation may be involved in NAFLD development due to the increased FFA flux through the liver[62,68] and both de- and increased β-oxidation rates are reported[75]. Regardless, SREBP-1c inhibits FA oxidation by indirect inhibition of CPT-1.

RSV may increase FA β-oxidation by stimulating mitochondrial biogenesis through PGC-1α activation[37], increasing mitochondrial number[37,76], increasing uncoupling protein 2 expression[76] and by increasing CPT-1 expression and activity[27,77].

Figure 1 Proposed resveratrol effects on nonalcoholic fatty liver disease pathogenesis, AMP-activated protein kinase and silent information regulation-2 homolog 1 dependent and non-dependent mechanisms. Evidence of *in vivo* effect demonstrated especially a RSV-mediated inhibition of adipose tissue lipolysis, inhibition of hepatic de novo lipogenesis and an increase in FA β-oxidation. ACC: Acetyl-CoA carboxylase; CPT-1: Carnitine palmitoyltransferase-1; FAS: Fatty acid synthase; FFA: Free fatty acids; NAFLD: Non-alcoholic fatty liver disease; PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1 α; PPARγ/α: Peroxisome proliferator-activated receptor γ/α; ROS: Reactive oxygen species; SREBP-1c: Sterol regulatory element-binding protein-1c; TG: Triglyceride; TGF-1β: Tumor growth factor 1β.

STEATOHEPATITIS

Hepatic inflammation is the hallmark of NASH and the inflammation is driven by several inflammatory hits that may include both intra- and extrahepatic factors[58].

Among the intrahepatic factors are the excess cholesterol, FFA and lipotoxic intermediates, which elicit a number of damaging effects, collectively named "lipotoxicity"[78]. This is recently reviewed in comprehensive reviews[78,79]. Converging the harmful factors is the NF-κB pathway in immune cells, hepatocytes and hepatic stellate cells (HSC), resulting in an inflammatory, profibrogenetic and pro-apoptotic hepatic environment. NF-κB activation enhances transcription of pro-inflammatory cytokines[80,81], with increased hepatic transcription of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and the TNFα receptor, as shown in NASH patients[82]. Kupffer cells and damaged hepatocytes release, *e.g.*, transforming growth factor 1β (TGF-1β) that acts on quiescent HSC, inducing an activated state and thereby fibrosis. In addition, extracellular molecules, endotoxins, such as lipopolysaccharide (LPS) (*via* toll-like receptor 4), TNFα, IL-1, IL-6 and reactive oxygen species, can induce NF-κB-mediated activation of the HSCs.

The anti-inflammatory effects of RSV are also documented in the liver. Animal studies have shown reduced hepatic macrophage infiltration[51] and TNFα levels[49,52,83],

as well as inhibition of the NF-κB pathway[50,52,83]. Only one study has focused on NASH-like fibrosis[52], probably due to the lack of appropriate animal models. However, other hepatic fibrosis models have shown RSV-mediated mitigating effects on markers of hepatic fibrosis[84-87] and HSC activation[86,88,89].

A number of studies report endoplasmatic reticulum stress, lipid peroxidation and oxidative stress as causative or early events in NASH pathogenesis[90-93]. RSV is a known anti-oxidant compound and has been shown to lower hepatic oxidative stress in rodent diabetes and NAFLD models[49,51,94-98]. One potential mechanism is interference in the Keap1/Nrf2 pathway, leading to up-regulation of anti-oxidant enzymes[99,100].

The extrahepatic inflammatory factors include dietary factors (*e.g.*, trans-fatty acids and fructose), gut-derived factors (*e.g.*, microbiota composition and bacterial byproducts) and adipose tissue-derived factors (*e.g.*, hypoadiponectinemia, adipo and cytokines, namely leptin, resistin, IL-6, TNF-α and monocyte chemotactic protein-1 (MCP-1)), that induce a state of whole-body low-grade inflammation in obesity[101]. Several lines of evidence document the anti-inflammatory effects of RSV in adipose tissue, especially by inhibiting NF-κB activation[46,102,103], lowering IL-6 levels[47,104] and macrophage infiltration[103], and modulating circulating cytokines and adipokines[45,50,103,105]. RSV-mediated reduction of LPS-

induced liver pathology and oxidative stress has also been reported[106,107].

In summary, RSV has AMPK/SIRT1 activating, anti-inflammatory and anti-oxidant effects that may act in unison, combating the different hits in the pathogenesis of NAFLD and NASH development.

RESVERATROL *IN VIVO*

Animal studies of RSV effects on NAFLD are numerous and span a wide variety of models, intervention periods and RSV doses. The studies can be divided into low-dose [RSV doses 7-45 mg/kg bodyweight (BW) daily] and high-dose studies (RSV 45-300 mg/kg BW daily). Some of the studies have hepatic steatosis as the primary end-point, others as secondary. The RSV treatment is generally started from the beginning of the study and therefore most of the studies concentrate on the preventive effect of RSV and not the therapeutic effect. Almost uniformly, the studies report beneficial effects of RSV treatment on NAFLD pathology. In addition, RSV treatment in experimental models generally reduce circulating insulin and glucose levels[18,37,45,46,50-52,63,105,108-110] and, in some instances, weight[50,52,110-113], circulating transaminases[44,52,77,110] and lipids[45,50,65,96,108,111,112].

In Table 1 we present a list of published RSV animal studies with hepatic histological NAFLD/NASH data.

MOUSE STUDIES

In 2006, Baur *et al*[37] published a much-cited study on the effect of RSV on the health and survival of mice on a high-fat diet (HFD). HFD and low-dose RSV (10 mg/d) were fed to the mice from senescence to death. Besides increased survival and a number of beneficial metabolic effects, the study showed RSV-mediated hepatic AMPK activation, ACC inhibition, decreased FAS transcription and increased mitochondrial number. RSV also decreased liver weight and the degree of steatosis. This study triggered a number of other mouse studies, often using C57BL/6J diet-induced obese (DIO) mice.

Regarding NAFLD data, the studies report a decrease in hepatic TG[45,50,65,96,111,114] and/or cholesterol accumulation[45,108,111-113] and liver weight[37,65,112], along with improvement in histological fatty liver changes[37,46,51,52,65,96,108,111,113-115]. Only a few studies report NASH changes in the histological specimens. Ahn *et al*[65] and Li *et al*[52] find that RSV supplementation represses development of histological steatosis and steatohepatitis and also fibrosis in the latter study. Tauriainen *et al*[115] find that high-dose RSV represses steatosis and hepatocyte ballooning in a model with minimal hepatic inflammation.

Although tested in a few studies, only two mouse studies are able to verify RSV-mediated AMPK activation in liver tissue[105,114]. Also, no subsequent mouse studies have investigated hepatic PGC-1α deacetylation as a marker of SIRT1 activation. However, other markers of AMPK/SIRT1 activation have been documented in several mouse studies, among them inhibition of FAS expression

and activation[65,109,111], inhibition of ACC activation[97,114], augmentation of FA β-oxidation[111], inhibition of PPARγ and SREBP-1c expression and stimulation of PPARα expression[52,65]. In mouse models, RSV treatment inhibits NF-κB activation[50,52] and lowers hepatic expression of inflammation markers[52]. Furthermore, oxidative stress is alleviated by RSV treatment in a number of mouse models[51,52,97].

In a long-term study of high-dose RSV treatment alone and in combination with another polyphenolic compound, namely quercetin, transcriptomic and metabonomic data demonstrated that combination therapy results in a significant restoration of gene sets in functional pathways of glucose and lipid metabolism (glycolysis and FA β-oxidation), inflammation/immunity, liver function and the cardiovascular system, which were altered by HFD feeding[108].

Also, in mutant Werner syndrome mice (showing premature signs of aging, *e.g.*, fatty liver), RSV treatment reversed liver steatosis and lipid peroxidation[109]. Microarray and biological enrichment analyses on liver tissues suggested that RSV mainly decreases lipogenesis and increases genes involved in the insulin signaling pathway and glutathione metabolism. The authors also observed a lower prevalence of hepatocellular carcinoma, however, an increase in lymphomas and other solid tumors was observed.

RAT AND HAMSTER STUDIES

Numerous different rat models of NAFLD report on RSV effects on NAFLD relevant endpoints, along with a single hamster study. Similar to the mouse studies, the conclusions are positive overall. The studies show a decrease in liver weight[67,74,77], hepatic TG[27,44,66,67,74,76,77] and/or cholesterol accumulation[66,67], and histological fatty liver[49,66,67,74,76,77].

The first to describe RSV effects on NAFLD in rats was Shang *et al*[74], using a HFD rat model in which the HFD was started 6 wk prior to the high-dose RSV treatment (100 mg/kg BW daily). This study therefore focused on the therapeutic effects of RSV. Besides alleviating NAFLD changes, it demonstrated that high-dose RSV treatment promotes phosphorylation and activation of AMPK and suppresses expression of FAS and SREBP-1c. This is backed by a recent study in which the HFD was added to a high amount of sucrose. Alberdi *et al*[27] found that low-dose RSV treatment for 6 wk activates AMPK and PGC-1α, increases CPT-1 and decreases ACC activities with no change in the mRNA expression of SREBP-1c, PPARα, SIRT1 and PGC-1α. Yet, not all rat studies find AMPK activation either. At variance, our group found no increase in AMPK phosphorylation or expression of related genes in spite of improvement in fatty liver changes, along with an increase in the hepatic mitochondrial content[76].

Obese Zucker rats have been used in low-dose RSV studies[44,77], with a reduction in hepatic lipid content and alanine aminotransferase levels, along with activation of

Table 1 Rodent resveratrol studies with histological liver data

Ref.	Focus	Model	RSV dose	RSV exposure (wk)	Histology/ LW	TG/CH content	Liver AMPK activation	Suggested RSV actions/mechanisms
Baur *et al*[37] (2006)	Longevity	Middle-aged DIO mice	22.4 mg/kg BW = 0.04% in diet	27	+/+	ND	+	Elevated PGC-1α deacetylation as marker of enzymatic SIRT1 activity. Phosphorylation of ACC
Ahn *et al*[65] (2008)	NAFLD/NASH	DIO mice (1% CH in diet)	1.25 g/kg diet	8	+/+ Decrease in NASH	+/-	ND	Reduced hepatic expression of FAS, PPARγ, CD36. Increased PPARα expression
Kang *et al*[46] (2010)	Insulin signaling	DIO mice	30 mg/kg BW	2	+/ND	ND	-	Increased Akt phosphorylation, improving insulin signaling
Labbé *et al*[109] (2011)	NAFLD, metabolic profile, longevity	Werner syndrome mice	0.04% RSV in diet	Life-long (≤ 22.5 mo)	+/ND	ND	-	Decreased FAS expression Decreased HCC prevalence
Tauriainen *et al*[115] (2011)	Obesity, NAFLD	DIO mice	2 or 4 g/kg diet	15	+/ND	ND	ND	Increased SIRT1 expression in liver tissue. High-dose most effective
Cho *et al*[111] (2012)	NAFLD	DIO mice (1% CH in diet)	7 mg/kg BW or 30 mg/kg BW	10	+/ND	+/+	ND	Suppressed FAS activity. Activation of FA β-oxidation in liver. The lower dose is more efficient than the higher dose
Zhou *et al*[108] (2012)	Overall transcriptomic and metabolic profiling	DIO mice	0.04% or 0.02% RSV + quercetin 0.02%	26	+/ND	-/+	ND	Modulation of inflammation and FA β-oxidation. Combination with quercetin was more effective than RSV alone
Jeon *et al*[51] (2012)	Cognitive deficit	DIO mice	200 mg/kg BW	20	+/ND	ND	ND	Attenuation of hepatic lipid peroxidation and macrophage infiltration
Shiozaki *et al*[97] (2012)	Longevity	SAMP10 mice	0.04% RSV	20	+/ND	ND	ND	Inhibition of ACC. Improved mitochondrial number, redox status and activity
Gao *et al*[114] (2013)	NAFLD	T0901317-treated mice	200 mg/kg BW	< 1	+/+	+/ND	+	Inhibition of ACC. Unchanged expression SREBP-1c and related genes
Li *et al*[52] (2013)	NAFLD	HFS mice	50 mg/kg BW	3	+/ND Decrease in NASH	ND	ND	Inhibition of NF-κB-induced inflammation and MDA-induced oxidative stress. Protection against NASH fibrosis
Bujanda *et al*[49] (2008)	NAFLD	Fasting/ feeding special diet, rats	10 mg daily	4	+/-	ND	ND	Hepatic TNF-α decrease. Improved oxidant/antioxidant markers (MDA, NOS, SOD, *e.g.*, catalase)
Shang *et al*[74] (2008)	NAFLD	HFD rats	100 mg/kg BW	10	+/+	+/-	+	Suppressed *SREBP-1c* and *FAS* gene expression
Poulsen *et al*[76] (2012)	NAFLD	HFD rats	100 mg daily	8	+/-	+/ND	-	Increased UCP2 expression Increased mitochondrial number
Gomez-Zorita *et al*[103] (2012)	NAFLD	Obese Zucker rats	15 mg/kg BW or 45 mg/kg BW	6	+/+	+/?	ND	Increased CPT-1α and ACO No effect on activity of lipogenic enzymes
Bagul *et al*[94] (2012)	Oxidative stress	High fructose fed rats	10 mg/kg BW	8	+/ND	ND	ND	Attenuation of hepatic oxidative stress, *e.g.*, with increased level of NRF2
Franco *et al*[96] (2013)	Oxidative stress and NAFLD	Obese 'early weaned' rats	30 mg/kg BW	4	+/ND	+/ND	ND	Decreased markers of oxidative stress
Xin *et al*[66] (2013)	NAFLD	HFS rats	50 or 100 mg/kg BW	13	+/ND	+/+	ND	Decreased hepatic LDLr and SRB1 mRNA and protein express
Cho *et al*[67] (2008)	Hyperlipidemia	HFD hamsters	0.25 g/kg diet	8	+/+	+/+	ND	Decreased HMG-CoA expression and modulated lipoprotein expression
Burgess *et al*[110] (2011)	Metabolic syndrome	HFD mini-swine	100 mg/kg daily	11	(+)/ND	ND	ND	Improved insulin sensitivity

+ positive finding; - negative finding; ND: Not determined. LW: Liver weight; BW: Body weight; TG: Triglyceride; CH: Cholesterol; AMPK: AMP-activated protein kinase; PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1 α; SIRT1: Silent information regulation 2 homolog 1; ACC: Acetyl-CoA carboxylase; NAFLD/NASH: Nonalcoholic fatty liver disease/nonalcoholic steatohepatitis; DIO: Diet induced obesity; FAS: Fatty acid synthase; PPARγ/α: Peroxisome proliferator-activated receptor γ/α; SAMP10: Senescence-accelerated mouse P10; HCC: Hepatocellular carcinoma; FA: Fatty acids; SREBP-1c: Sterol regulatory element-binding protein-1c; TNF-α: Tumor necrosis factor-α; MDA: Malondialdehyde; NOS: Nitric oxide synthase; SOD: Superoxide dismutase; HFD: High fat diet; UCP2: Uncoupling protein 2; CPT-1α: Carnitine palmitoyl transferase-1α; ACO: Acyl-coenzyme A oxidase; NRF2: Nuclear factor-like 2; HFS: High fat/sucrose diet; LDLr: Low-density lipoprotein receptor; SRB1: Scavenger receptor class B member 1; HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzyme A.

AMPK and increased CPT-1 activity, which is important for the rate of FA β-oxidation. Resveratrol treatment also improved the inflammatory status of visceral adipose tissue[44] and reduced liver oxidative stress[77]. Here, the ef-

fect on lipogenetic enzyme activity was equivocal.

Similar results on inflammatory and oxidative status was found by Bujanda *et al*[49] in a model in which cycles of fasting and feeding with a high carbohydrate-fat free diet induced steatosis. Low dose RSV for 4 wk lowered hepatic TNFα levels and reduced markers of lipid peroxidation and hepatic oxidative stress.

To our knowledge, only one study reports no RSV effect on hepatic lipid levels. Andersen *et al*[116] used a dietary rat model and high-dose RSV treatment for 8 wk, yet found no decrease in liver TG, FFA or cholesterol content. Also, they found no effect on liver SIRT1 protein expression.

Taken together, the current evidence shows that RSV prevents NAFLD-like hepatic steatosis in rodent NAFLD models. This may be caused by inhibition of adipose tissue lipolysis, inhibition of hepatic *de novo* lipogenesis and an increase in FA β-oxidation. A graphic illustration of the proposed RVS effects on NAFLD pathology is shown in Figure 1. Development of steatohepatitis may be attenuated by an inhibition of adipose tissue and hepatic inflammation and reduction of oxidative stress. However, few studies have used appropriate animal NASH models and there is only one study documenting an alleviating effect on NASH fibrosis. RSV could exert some of these effects through AMPK activation but AMPK activation is not found in all studies. Also, hepatic SIRT1 activation has not been verified in an experimental NAFLD model. Studies focusing on the therapeutic effect as opposed to the preventive effect of RSV on NAFLD and especially NASH are few but warranted.

OTHER ANIMAL MODELS

In a porcine model of metabolic syndrome, Burgess *et al*[110] found that high-dose RSV treatment had mitigating effects on insulin resistance and transaminase levels. Oil red O staining showed a decrease in hepatic lipid accumulation. However, a HE stain found no difference in histology between the control, HFD and HFD with RSV groups, signifying that steatosis was not sufficiently induced in this model.

CLINICAL TRIALS

So far, only a few clinical RSV trials on efficacy outcomes have been concluded and none of these studies have focused on fatty liver disease *per se*. Two studies on obese but otherwise healthy male participants report liver data. In the study by Timmers *et al*[117], 11 participants received a daily dose of 150 mg RSV or placebo for 30 days in a double-blind, cross-over design. Results suggest a number of beneficial metabolic effects, among these a reduction of liver transaminases and liver fat by magnetic resonance (MR) spectroscopy. In another study, 24 participants received a dose of 1.5 g RSV or placebo daily for 4 wk[118] and there was no effect on liver fat content (MR spectroscopy) or transaminase levels. This is consistent with another study in 45 non-obese postmenopausal women receiving a dose of 75 mg for 12 wk where no effect on liver fat (MR spectroscopy) or any other physiological parameter could be demonstrated[119].

Future clinical studies should focus on patients with biopsy verified NAFLD and NASH to determine any efficacy of RSV treatment in this setting.

CONCLUSION

So far, clinical studies of RSV effects on steatosis are scarce and the overall positive effects seen in rodent studies are still missing. None of the studies have included verified NAFLD patients or histological data and the studies differ significantly in the RSV dose used. New clinical studies should focus on the RSV effects in patients rather than healthy or near-healthy individuals[120], in this case, histologically verified NAFLD/NASH patients. In this setting, the focus should be on the therapeutic effects of RSV and not its preventive effects, as reported in the majority of animal studies.

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Insulin sensitizers for the treatment of non-alcoholic fatty liver disease

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is the leading cause of liver disease in the Western world and is closely associated with metabolic syndrome, which includes hypertension, central obesity, dyslipidemia and insulin resistance. NAFLD includes a wide spectrum of liver alterations, ranging from simple hepatic steatosis to variable degrees of fibrosis, cirrhosis and even hepatocellular carcinoma. Although the etiology and progression of the disorder remain poorly understood, insulin resistance is considered to play a pivotal role in the pathogenesis. Insulin sensitizers such as biguanides, thiazolidinediones (TZDs), glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase 4 inhibitors have been studied as therapeutic approaches for NAFLD in recent years. Metformin improves insulin sensitivity and serum alanine transaminase and aspartate transaminase (ALT/AST) levels in the majority of subjects; however, it has no significant effect on liver histology. TZDs improve insulin sensitivity, serum ALT/AST levels and histology in some cases, but there are some concerns about the safety of long-term therapy. Selection of appropriate patients for avoiding side effects and the treatment of underlying disease are the main points. These drugs are the best choice for the treatment of NAFLD in patients with type 2 DM who are also candidates for treatment with an insulin sensitizer. The present review provides an overview of insulin sensitizers in the treatment of NAFLD.

Core tip: Non-alcoholic fatty liver disease (NAFLD) is increasing significantly due to the obesity epidemic. Insulin resistance, mainly caused by obesity, plays a primary role in NAFLD pathogenesis. Medications that improve insulin sensitivity are theorized to be useful in the treatment of NAFLD. Therefore, recent studies have explored the role of insulin sensitizers to improve biochemical and histological features of NAFLD.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) was first described by Ludwig *et al*[1] as a liver disease that mimicked alcoholic hepatitis in histopathological features, but without a history of excessive drinking. A recent definition of NAFLD consists of evidence of hepatic steatosis either by imaging or by histology and no secondary hepatic fat accumulation from causes such as alcohol consumption, hereditary disorders and steatogenic medication[2]. The

Figure 1 The relationship between obesity, insulin resistance and hepatic steatosis. VLDL: Very low-density lipoprotein; TG: Triglyceride.

spectrum of the disease reaches from simple hepatic steatosis to lobular inflammation (non-alcoholic steatohepatitis or NASH), fibrosis, cirrhosis and hepatocellular carcinoma[3,4]. It is the most common cause of chronic liver disease in all industrialized regions of the world[5-7].

BACKGROUND

The estimated prevalence of NAFLD varies in a wide range depending on the population studied and methods used for diagnosis. The disease has been reported in up to 10%-15% of normal weight individuals and 90% of obese persons[8-10]. The prevalence of NAFLD in patients with type 2 diabetes mellitus and hyperlipidemia is approximately 70% and 50%, respectively[11]. Ageing, male gender and ethnicity, such as being Hispanic, are associated factors that increase the prevalence of NAFLD. Elevated liver enzymes, histopathology of liver biopsy and imaging techniques such as ultrasound and magnetic resonance spectroscopy are different methods used for definition.

Previous studies have shown that 40% of patients with NAFLD may go on to develop NASH. The most common cause of cryptogenic cirrhosis is NASH and it progresses to advanced fibrosis in 32% to 37% of patients[12]. The patients with advanced fibrosis and cirrhosis also have higher risk of hepatocellular carcinoma[13-15]. Patients with NAFLD and NASH have increased cardiovascular mortality as well as liver-related mortality[16-18]. This is due to increased pericardial fat, increased carotid intima thickness and abnormal electrocardiogram changes[19-21]. Ramilli *et al*[22] showed that the prevalence of carotid plaques was close to 60% in patients with NAFLD, while it was 38% in patients without NAFLD.

Type 2 DM, obesity and the associated insulin re-

sistance have been shown as independent factors for fibrosis progression[23]. Although a lot of risk factors have been defined, the major underlying mechanisms of the disease progression have not been clearly understood. The pathogenesis of NAFLD is closely related to obesity that leads to insulin resistance and significant metabolic alterations in liver occur in the setting of insulin resistance[24] (Figure 1). The prevailing hypothesis for explaining the pathway and involved mechanisms is the so-called two hit model[25,26]. At the first hit, hepatic steatosis develops due to insulin resistance and liver fat accumulation induced by excessive free fatty acid production, increased fatty acid oxidation and decreased hepatic triglyceride export. Following this step, the second hit includes increased oxidative stress which is characterized by excessive reactive oxygen species (ROS) in the liver. Progression from NAFLD to NASH is promoted by ROS through lipid peroxidation, cytochrome P450 activation and pro-inflammatory cytokine production[27]. Considering the complexity and unexpected progression of the disease, environmental and genetic factors are approved as contributors of a third hit.

Insulin resistance is the most specific metabolic risk and pathophysiological feature of NAFLD, with most patients having insulin resistance. In diabetic patients, a correlation between the severity of insulin resistance and grade of hepatic steatosis has also been shown[28]. On the basis of these data, studies of NAFLD treatment are mostly focused on improving insulin resistance and a pharmacological approach targeting improving insulin resistance are the more promising therapeutic candidates among categories that include antioxidants, lipid-lowering agents and anti-obesity drugs.

INSULIN-SENSITIZING MEDICATIONS

Metformin

Metformin was first used in medical practice in the 1950s and has been considered the first-line treatment of type 2 diabetes after receiving approval by the United States Food and Drug Administration (FDA) in 1994. Metformin belongs to a class of insulin-sensitizer drugs and acts through reducing hepatic glucose output, increasing insulin-stimulated glucose uptake in peripheral tissue and stimulating fatty acid oxidation in adipose tissue[29]. Adenosine monophosphate-activated protein kinase is the main player in mediating metformin effects.

Animal studies demonstrated that metformin reverses aminotransferase abnormalities, steatosis and inflammation in mouse models of NAFLD and NASH[30,31]. During last decade, many clinical trials have evaluated the useful effects of metformin on patients with NAFLD and NASH[32-41] (Table 1). Only a few of these studies were randomized and the results are conflicting.

The first nonrandomized study was carried out by Marchesini in 2001 and it included 20 biopsy proven NASH patients. For 4 mo the patients were treated with 1.5 g/d metformin and they observed a decrease in ami-

Table 1 Summary of metformin trials in adult patients with non-alcoholic fatty liver disease/non-alcoholic steatohepatitis

Ref.	Study type	Subject number	Therapy	Compared with	Duration	NAFLD *vs* NASH	Liver enzymes	Histology
Marchesini *et al*[32]	Open label, single arm	20	Metformin	Baseline	4 mo	NASH	Improved	Not assessed
Nair *et al*[33]	Open label, Single arm	15	Metformin	Baseline	48 wk	NAFLD	Transiently improved	Mildly improved
Uygun *et al*[34]	Open label, RCT	36	Metformin	Diet/Exercise	6 mo	NASH	Improved	Not improved
Bugianesi *et al*[35]	Open label, RCT	110	Metformin	Vitamin E/Diet	12 mo	NAFLD	Improved	Improved
Duseja *et al*[36]	Open label, RCT	50	Metformin	Diet	6 mo	NAFLD	Improved	Not assessed
de Oliveira *et al*[37]	Open label, Single arm	20	Metformin and NAC	Baseline	12 mo	NASH	Improved	Improved
Loomba *et al*[38]	Open label, Single arm	28	Metformin	Baseline	48 wk	NASH	Improved	Improved
Haukeland *et al*[39]	Open label, RCT	48	Metformin	Diet/Exercise	6 mo	NAFLD	Improved	Not improved
Garinis *et al*[40]	Open label, RCT	50	Metformin	Diet	6 mo	NAFLD	Improved	Not assessed
Shargorodsky *et al*[41]	Open label, RCT	63	Metformin	Placebo	12 mo	NAFLD	Not improved	Not assessed

NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; RCT: Randomized controlled trials.

notransferase levels[32]. The limitation of the study was the lack of histological evaluation and a control group. In 2004, Uygun *et al*[34] conducted the first randomized control trial comparing dietary modification to dietary modification plus metformin for six months. Aminotransferase levels and insulin sensitivity improved in the metformin treated group but there was no significant differences in necroinflammatory activity or fibrosis between groups. Bugianesi *et al*[35] presented an open label trial consisting of 110 patients who were randomized to receive either metformin 2 g/d (55 patients), vitamin E 800 IU/d (28 patients) or dietary-induced weight loss (27) patients for 12 mo. Liver transaminase levels were significantly decreased in the metformin group and there was also a histological improvement in hepatic steatosis, inflammation and fibrosis in the subset of 17 patients taking metformin. Haukeland *et al*[39] demonstrated that treatment with metformin for 6 mo was no better than placebo in terms of improvement in liver histology in patients with NAFLD. Another recent randomized control trial showed that metformin only transiently improved aminotransferase levels in patients with NASH[41]. A meta-analysis including five randomized controlled trials (RCT) concluded that metformin did not improve steatosis, lobular inflammation, hepatocellular ballooning and fibrosis in patients with NASH[42]. These results were independent of drug dose, treatment duration or diabetic state. Therewithal, a recent guideline indicates that metformin has no significant effect on liver histology and therefore it is not recommended as a specific treatment for liver disease in adults with NASH[2]. The largest RCT, "The Treatment of NAFLD in Children" (TONIC), investigated the effects of metformin in a pediatric population. The results of this study demonstrated that metformin was not associated with improvement in histology and reduction in serum alanine transaminase (ALT) levels[43]. On the basis of all these data, the AASLD guideline for the diagnosis and treatment of NAFLD concluded that metformin has no significant effect on liver histology and is not recommended as a specific treatment of NASH.

A position statement on NAFLD/NASH based on an EASL special conference has not recommended metformin for specific liver-directed therapy of NASH[44].

Some studies demonstrated that high insulin and IGF levels have important roles in hepatic fibrosis and hepatocellular carcinoma[45,46]. An inverse association between cancer risk and long term metformin therapy has been found in previous studies. A recent meta-analysis showed that metformin was associated with an estimated 62% reduction in the risk of liver cancer among patients with type 2 diabetes[47]. Chen *et al*[48] demonstrated that each incremental year increase in metformin use results in 7% reduction in the risk of hepatocellular cancer. While the main anti-tumor effect of metformin is not clear in reducing lipogenesis and lipogenic expression, inhibition of hepatocyte proliferation and induction cell cycle arrest at G_0/G_1 phase *via* adenosine monophosphate (AMP)-activated protein kinase, reduction endogenous reactive oxygen species are possible estimated mechanisms.

In summary, metformin improves insulin sensitivity and serum ALT and aspartate transaminase levels in the majority of subjects; however, it has no significant effect on liver histology. The precise dose and duration of treatment is unknown and the beneficial effects on serum ALT only continued during treatment. Metformin has no apparent increase in the risk of lactic acidosis[49] and unlike the thiazolidinediones, it is not encumbered by weight gain or potential hepatotoxicity. According to current data, it cannot be suggested for the specific treatment of NAFLD or NASH but can be given in patients with both NAFLD/NASH and type 2 DM.

Thiazolidinediones

Thiazolidinediones (TZDs) are a class of oral antidiabetic drugs that induce a nuclear transcription factor, peroxisome proliferator activated receptor-γ (PPAR-γ), by binding selective ligands[50]. PPAR-γ is predominantly expressed in adipose tissue and leads to decreased hepatic fat content and improves glycemic control with insulin sensitivity. TZDs also increase plasma adiponectin levels,

Table 2 Summary of thiazolidinedione trials in adult patients with non-alcoholic fatty liver disease/non-alcoholic steatohepatitis

Ref.	Study type	Subject number	Therapy	Compared with	Duration	NAFLD *vs* NASH	Liver enzymes	Histology
Caldwell *et al*[53]	Open label, single arm	10	Troglitazone	Baseline	< 6 mo	NASH	Improved	Mildly improved
Neuschwander-Tetri *et al*[54]	Open label, single arm	30	Rosiglitazone	Baseline	48 wk	NASH	Improved	Improved
Promrat *et al*[55]	Open label, single arm	18	Pioglitazone	Baseline	48 wk	NASH	Improved	Improved
Sanyal *et al*[56]	Open label, RCT	20	Pioglitazone + vitamin E	Vitamin E	6 mo	NASH	Improved	Improved
Belfort *et al*[57]	Blinded, RCT	55	Pioglitazone	Placebo	6 mo	NASH	Improved	Improved
Idilman *et al*[58]	Open label, RCT	74	Rosiglitazone	Metformin/life style modification	48 wk	NASH	Improved	Improved
Ratziu *et al*[59]	Blinded, RCT	63	Rosiglitazone	Placebo	12 mo	NASH	Improved	Improved
Omer *et al*[60]	Open label, RCT	64	Rosiglitazone	Metformin	12 mo	NAFLD	Improved	Improved
Sanyal *et al*[61]	Blinded, RCT	274	Pioglitazone	Placebo and vitamin E	24 mo	NASH	Improved	Improved

NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; RCT: Randomized controlled trials.

activate AMP-activated protein kinase and induce fatty acid stimulation[51].

A lot of human and animal studies have investigated the effect of TZDs on liver enzymes and histology to date. In rat models, pioglitazone and rosiglitazone prevented activation of hepatic stellate cells *in vitro* and improved hepatic steatosis and fibrosis *in vivo*[52].

The first human study was conducted by Caldwell in 2001[53]. Troglitazone was studied in 10 patients who had biopsy-proven NASH and the results were associated with improved aminotransferase levels. There was no change in histology and the drug was withdrawn from clinical use due to severe idiosyncratic hepatotoxicity. Neuschwander-Tetri *et al*[54] showed that rosiglitazone (4 mg twice daily for 48 wk) significantly decreased liver enzymes and improved steatosis, ballooning and inflammation scores in 30 patients who had biopsy-proven NASH. In addition to no change in fibrosis, the liver enzymes levels reverted to pretreatment values 6 mo after withdrawal of the drug. Clinical trials evaluating the effect of thiazolidinediones on patients with NAFLD and NASH are summarized in Table 2[53-61]. Omer *et al*[60] conducted an open label RCT including biopsy-proven NAFLD individuals to compare rosiglitazone with metformin. After 12 mo treatment, study results reported that rosiglitazone was more effective in metabolic control and histological improvement but fibrosis did not change significantly.

An early study with pioglitazone in 18 patients who had biopsy-proven NASH resulted in a decrease in aminotransferase levels with histological improvement[55]. The first double-blind, placebo-controlled trial using pioglitazone compared with placebo included 55 patients with NASH for 6 mo[57]. Insulin sensitivity, serum ALT levels, steatosis and necroinflammation, except fibrosis, were significantly ameliorated in the pioglitazone group.

In the largest trial completed to date for evaluation of the role of pioglitazone, 247 subjects with biopsy proven NASH were randomized to vitamin E, pioglitazone or placebo for 96 wk[61]. Compared with placebo, both agents, pioglitazone and vitamin E, were associated with reductions in liver steatosis, lobular inflammation, hepatocellular ballooning and improvement in insulin resistance and serum aminotransferase levels. However, there was no improvement in fibrosis scores in the pioglitazone treated group. The "Fatty Liver Improvement with Rosiglitazone Therapy" (FLIRT) trial compared rosiglitazone with placebo in 63 patients[59]. Rosiglitazone improved serum aminotransferase levels, insulin sensitivity and hepatic steatosis. The two year extended trial (FLIRT2) demonstrated that improvement in liver enzyme levels continued but there was no further improvement in liver histology[62].

A meta-analysis including six trials demonstrated reduction in steatosis and hepatocyte ballooning but no improvement in inflammation or fibrosis compared with control[63]. In contrast to this study, Mahady *et al*[64] found improvement in inflammation and fibrosis in addition to reduction in steatosis and hepatocyte ballooning in a meta-analysis including seven randomized trials.

The largest meta-analysis that included 11 RCTs (862 participants, 38% diabetic) showed that TZDs improve steatosis, hepatocellular ballooning and necroinflammation, delay fibrosis progression and ameliorate hepatic, muscle and adipose tissue insulin resistance with more consistent cardiovascular benefits with pioglitazone[42].

Although the results of studies suggest some benefits from TZDs, a major problem also emerges: safety of long-term therapy and adverse effects. The use of rosiglitazone has been highly restricted in the United States and prohibited in Europe due to the increased risk of coronary events. On the other hand, pioglitazone is associated with adverse events such as bladder cancer, bone loss, weight gain, painful swollen legs and congestive heart failure. After evaluation of the overall results, it would be a good choice to use TZDs for the treatment of NAFLD only in patients with type 2 DM who are also candidates

for treatment with a TZD. The AASLD guideline recommended that pioglitazone can be used to treat only patients with biopsy-proven NASH; however, it also raised the concern about its long term safety and efficacy in patients with NASH[2]. The guideline also stressed that most of the clinical studies had been done in non-diabetic patients and thus the effect of TZDs on NASH of diabetic patients was not established. The position statement of a special EASL conference has recommended that pharmacological therapy of NASH could be a 1-2 year course of therapy with glitazone[44].

Dipeptidyl peptidase 4 inhibitors

Dipeptidyl peptidase 4 (DPP4) inhibitors are a new class of drugs and include sitagliptin, vildagliptin and saxagliptin. DPP4 is a membrane associated peptidase with a widespread organ distribution and deactivates a variety of bioactive peptides such as glucagon like peptide-1 (GLP-1). Inactivation of GLP-1 causes glucose intolerance, diabetes mellitus and hepatic steatosis. In a study including 31 NASH patients, Balaban *et al*[65] reported that serum DPP-4 levels were higher in patients with NASH compared to controls. Furthermore, the serum DPP-4 activity and staining intensity in liver were correlated with histopathological grade of NASH and hepatosteatosis.

In rat models, DPP-4 inhibitors improve hepatic steatosis by increasing insulin sensitivity and decreasing hepatic triglyceride levels[66,67]. To date, there is no published controlled trial with these agents in humans.

GLP-1, a hormone excreted by intestinal L cells, regulates blood glucose by stimulation of glucose-dependent insulin release. GLP-1 has a direct effect on hepatocytes by inducing genes responsible for fatty acid oxidation and insulin sensitivity[68]. GLP-1 analogs (exenatide, liraglutide) have been approved by the FDA for treatment of patients with type 2 diabetes mellitus. Ding *et al*[69] demonstrated that exenatide improves insulin sensitivity and reduces hepatosteatosis in rats with fatty liver. In another animal study liraglutide treatment reduced hepatic steatosis[70]. A case series including 8 patients with type 2 diabetes and biopsy-proven NAFLD showed that exenatide improves serum liver enzyme levels but has no effect on histopathology[71]. A recent meta-analysis including 4442 patients indicated that liraglutide decreased aminotransferase levels and that this effect was dose-dependent[72]. However, controlled studies are needed to show the efficacy of GLP-1 analogs in NAFLD and NASH treatment.

CONCLUSION

NAFLD is a complex, multifactorial and major public health problem with an increasing prevalence worldwide. Insulin resistance is very common in this disease and the goal of the therapy should include improving insulin sensitivity. Insulin sensitizing agents could be convenient drugs to reach this target. Metformin has been accepted to have no significant effect on liver histology and is not recommended as a specific treatment for liver disease in

adults with NAFLD. TZDs have been most extensively evaluated in published trials to date and they have modest effects on liver histology. The long term safety and efficacy of TZDs in patients with NAFLD is lacking. Selection of appropriate patients to avoid side effects and treatment of the underlying disease causing insulin resistance, such as obesity, are crucial main points. NAFLD patients with metabolic syndrome and obesity are likely to be the best candidates to be treated with TZDs. According to current data, unfortunately insulin sensitizers do not satisfy expectations for the treatment of NAFLD. Future RCTs with adequate size and duration are still needed to assess the clinical outcomes in patients with NAFLD.

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Mechanisms of fibrogenesis in liver cirrhosis: The molecular aspects of epithelial-mesenchymal transition

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Abstract

Liver injuries are repaired by fibrosis and regeneration. The cause of fibrosis and diminished regeneration, especially in liver cirrhosis, is still unknown. Epithelial-mesenchymal transition (EMT) has been found to be associated with liver fibrosis. The possibility that EMT could contribute to hepatic fibrogenesis reinforced the concept that activated hepatic stellate cells are not the only key players in the hepatic fibrogenic process and that other cell types, either hepatic or bone marrow-derived cells could contribute to this process. Following an initial enthusiasm for the discovery of this novel pathway in fibrogenesis, more recent research has started to cast serious doubts upon the real relevance of this phenomenon in human fibrogenetic disorders. The debate on the authenticity of EMT or on its contribution to the fibrogenic process has become very animated. The overall result is a general confusion on the meaning and on the definition of several key aspects. The aim of this article is to describe how EMT participates to hepatic fibrosis and discuss the evidence of supporting this possibility in order to reach reasonable and useful conclusions.

Core tip: The cause of fibrosis and diminished regeneration, especially in liver cirrhosis, is still unknown. Epithelial-mesenchymal transition (EMT) has been found to be associated with liver fibrosis. The possibility that EMT could contribute to hepatic fibrogenesis reinforced the concept that activated hepatic stellate cells are not the only key players in the hepatic fibrogenic process. The aim of this article is to describe how EMT participates to hepatic fibrosis and discuss the evidence of supporting this possibility in order to reach reasonable and useful conclusions.

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INTRODUCTION

Chronic liver damage can be triggered by different mechanisms (*e.g.*, viral hepatitis, metabolic liver diseases, or chronic alcohol consumption)[1] and are accompanied by changes in several key biochemical pathways involved in hepatic tissue homeostasis. One of the most important alterations is hepatic fibrosis, which is characterized by deposition of extracellular matrix (ECM) components around the sinusoidal layer in the space of Disse, together with molecular reorganization of the matrix components resulting in an altered composition[2].

Fibrosis is comparable with a wound-healing response

Figure 1 Mechanisms of hepatic fibrogenesis. A: The proposed sources of hepatic myofibroblasts: Resident cells (hepatic stellate cells and portal fibroblasts); bone marrow-derived mesenchymal cells, and EMT from hepatocytes and cholangiocytes. Different insults initiate inflammation and then cause hepatocyte stellate cells activation and hepatocyte and biliary cell damage, necrosis and EMT. Continuous insults will shift those EMT-like cells to complete EMT cells and finally myofibroblasts, the main producer of extracellular matrix, which may be one of the main causes of an early loss of regenerative capacity. A similar process also occurs in biliary cells. Some cytokines play an important role to affect the adjacent cells and promote EMTs, such as TGF-β1 (B); B: Schematic presentation of the major intracellular signal transduction pathways of TGF-β1 in liver fibrosis. TGF-β1 is a chief inducer of the EMT process, and p-Smad2/3, p38 MAPK and ILK function as mediators of the intracellular signaling pathway. TGF-β1 signals via heteromeric transmembrane complexes of type I and type II receptors that are endowed with intrinsic serine/threonine kinase activity (ALK activin receptor-like kinase). Upon type-II-mediated phosphorylation of the type I receptor, the activated type I receptor initiates intracellular signalling by phosphorylating receptor regulated-Smad2 and Smad3. Activated Smads form heteromeric complexes with Smad4 and these complexes accumulate in the nucleus where they mediate transcriptional responses. p-Smad2/3: Phosphorylated-Smad2/3; MAPK: Mitogen-activated protein kinase; GSK-3β: Glycogen synthase kinase-3β; ILK: Integrin-linked kinase; TCF/LEF-1 complex: T cell factor/lymphoid enhancer-binding factor-1 complex; EMT: Epithelial-mesenchymal transition; TGF: Transforming growth factor.

being out of control. Repair mechanisms aim at the replacement of injured cells. However, contrary to the pure regeneration of tissue in fibroplasia, connective tissue substitutes normal parenchyma[3]. The ultimate result is organ failure. In the liver, the final common pathway is cirrhosis, characterized by accumulation of ECM. In human beings liver fibrosis is associated with dysregulated growth of hepatocytes and results in the formation of regenerative nodules, dysplastic nodules, and hepatocellular carcinomas[4]. At present, no therapeutic concepts have been developed to treat and reverse fibrosis[5].

Striking increases in our understanding of the pathogenesis of liver fibrosis include the identification of the main cellular effectors, key cytokines regulating the EMT process, and determinants of ECM turnover[3].

FIBROGENESIS OF HEPATIC STELLATE CELLS, MYOFIBROBLASTS AND HEPATOCYTES IN LIVER CIRRHOSIS

Recent work regarding liver fibrosis centers on the myofibroblast as a pivotal cell type due to its contractile nature and synthesis repertoire[6]. The sources of myofibroblasts

are still matters of discussion. Undisputedly, a "myofibroblast" phenotype is observed with hepatic stellate cell (HSC) after exposure to profibrogenic cytokines[3].

Liver myofibroblasts stand for a wide repertoire of functions that emphasize the dynamic nature of the wound-healing response, including synthesis of fibrillar collagens, contractile and migratory activities, secretion of chemotactic and vasoactive factors, and the secretion of matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs)[3]. The origin of myofibroblasts in the injured liver is now under scrutiny, although evidence hints at HSCs as a major source[3]. Myofibroblasts can be derived from local mesenchymal cells recruited from the bone marrow or could derive from other cellular sources by EMT, a physiologic process in embryogenesis and of relevance for cancerous cell transformation[7] (Figure 1A).

Studies with animals and human tissue indicate that bone marrow stem cells infiltrate the liver and contribute to the myofibroblast population after damage. This may occur directly or through an intermediary cell, such as quiescent HSCs or CD45+ fibrocytes[8,9]. Several studies have indicated that bone marrow-derived mesenchymal stem cells could be a source of multi-lineage cells for

various organs. They have the capacity to differentiate into hepatocytes, biliary epithelial cells, sinusoidal endothelial cells and even Kupffer cells in the presence of a suitable hepatic microenvironment[10,11]. There is growing evidence to suggest that bone marrow-derived stem cells are recruited during both progression and regression of liver fibrosis[8,12-16].

Most studies in the past decade have focused on HSCs and analyzed characteristic features like plasticity and transdifferentiation to myofibroblasts, a phenotype that can be readily recapitulated in tissue culture[3]. Current concepts envision activated HSCs as a crucial profibrogenic source, while the majority of hepatocytes are believed to undergo necrosis or apoptosis, thereby providing space for proliferating cells. Besides the resident hepatic cells, infiltrating neutrophils, macrophages, T and B cells, and eosinophils participate in the inflammatory response and may perpetuate the damage, whereby activated macrophages and neutrophils clean up tissue debris, dead cells, and invading organisms[17].

HSCs are located in the space of Disse of hepatic sinusoids between hepatocytes and sinusoidal endothelial cells[1] as liver-specific pericytes interposed by sparse connective tissue and closely adhering to sinusoidal endothelial cells[18]. They also directly face hepatocytes and maintain a quiescent phenotype with the main function to store vitamin A[19]. During liver injury, HSCs lose their vitamin A content[20] and undergo activation triggered by exposure to cytokines and growth factors, such as platelet-derived growth factor (PDGF) and transforming growth factor (TGF)-β1, which derive from activated Kupffer cells and damaged hepatocytes. During activation, HSCs transform into a myofibroblast-like phenotype[21-23] losing their typical star-shape[20], characterized by the expression of α-smooth muscle actin (SMA)[24], by the production of ECM and matrix degrading enzymes, such as MMPs[21-23] and TIMPs[25,26]. Activated HSCs are thought to migrate from the sinusoids into necrotic areas and produce a variety of ECM[19].

Some authors[27] have proposed that HSC could be transitional cells derived from epithelial cells that have undergone partial EMT[28] or even a particular type of oval cell/hepatocyte precursor[29]. This hypothesis was based, at least in part, on the finding of an adult subpopulation of primary rat HSC expressing the progenitor cell marker CD133 and differentiating into either myofibroblasts or hepatocytes when cultured under different *in vitro* conditions[30].

Another source of myofibroblast may be portal fibroblasts that are described in fibrotic diseases with a portal component (*e.g.*, viral hepatitis and autoimmune conditions)[31]. In coherence with experimental data on hepatocytes, TGF-β1 is required for myofibroblast transdifferentiation of this cell type[32]. The portal connective tissue in healthy liver is surrounded by quiescent portal fibroblasts, which constitute a second population of liver cells implicated in portal fibrosis[33]. Derived from small portal vessels, they express markers distinct from HSC (*e.g.*, elastin)[32]. Proliferation of biliary cells is often accompanied

by proliferation of portal fibroblasts, which form onion-like configurations around biliary structures and acquire a myofibroblast phenotype, and are thus implied in the early deposition of ECM in portal zones[34]. It is generally believed that substantial signaling from biliary epithelial cells leads to portal fibroblast activation, although the key factors remain to be identified[20]. In complex studies employing the "lineage tracing" methodological approach have documented, in different animal models of liver fibrogenesis, that some hepatocytes or cholangiocytes acquire "mesenchymal markers" implicated in cell motility and survival, but are not involved in active fibrillar ECM deposition and, therefore, cannot be considered profibrogenic cells[35].

Dooley *et al*[3] have provided *in vitro* and *in vivo* evidence that profibrogenic TGF-β1 functions during liver damage are directed toward hepatocytes. A TGF-β1-induced gene expression profiling of hepatocytes indicates a minor role of apoptosis and induction of fibrogenesis- and EMT-related genes. While the definite occurrence of EMT in this cell type *in vivo* needs further investigation (*e.g.*, by double-transgenic animals expressing fluorescent proteins under the control of hepatocyte- and myofibroblast-specific promoters), their results suggest that hepatocytes and TGF-β1 signaling in this cell type play a prominent role for fibrogenesis. In patients with chronic hepatitis B virus (HBV) infection, the activation of the TGF-β1 pathway was also shown by the accumulation of phosphorylated Smad2 in hepatocyte nuclei. Furthermore, the induction of Snail, a transcription factor known to repress E-cadherin expression, and the co-expression of type I collagen and transferrin in HBV livers, indicated that hepatocyte EMT was a feature of human liver fibrosis[36]. Therefore, the hepatocytes may be a contributor to hepatic fibrosis, especially when they are chronically injured[4].

TGF-β/SMAD AND NON-SMAD SIGNALLING PATHWAY IN LIVER FIBROSIS

TGF-β1 is recognized as a major profibrogenic cytokine and is a potent inducer of HSC proliferation and collagen production[37]. Furthermore, TGF-β1 expression is also associated with morphologic alterations like EMT in fetal[38] and adult hepatocytes[39], and changes in survival signaling pathways[40].

TGF-β1 binds to TGF-β1 receptor type Ⅱ (TbR-Ⅱ), and it recruits the TGF-β1 receptor type I (TbR-I)[41]. TbR-I subsequently phosphorylates Smad2 and Smad3, which form hetero-oligomers with Smad4. They translocate from the cytoplasm to the nucleus, where they regulate transcription of target genes[42] (Figure 1B). R-Smad signaling is limited by the inhibitory effects of inhibitory Smad6 and 7[3].

Dysregulated TGF-β1 signaling is implicated in multiple developmental disorders and various human diseases, including cancer and autoimmune illnesses[43]. Its

over-expression is linked to liver fibrosis in diverse animal models[44] and in human patients with chronic liver diseases[45]. TGF-β1 crucially regulates ECM deposition by controlling the expression of ECM network components such as fibrillar collagens and fibronectin, ECM-degrading protease inhibitors, such as plasminogen activator inhibitor (PAI)-1 and TIMPs. Its activity is strongly induced during chronic liver damage with links between TGF-β1 and connective tissue growth factor in the HSC activation process[46], which in turn acquire myofibroblastic features and produce ECM proteins.

Epithelial cell transdifferentiation comprises alterations in cellular morphology characterized by changes in cell polarity and loss of adhesion protein expression[3]. TGF-β1 can initiate and maintain this process in a variety of biological systems and pathophysiological contexts by activating major signaling pathways and transcriptional regulators integrated in extensive cellular networks[47]. In MDCKII cells, claudin-1, claudin-2, occludin and E-cadherin disappear within 72 h of exposure to TGF-β1. It is suggested that this expression loss occurs through a Smad-independent mechanism, involving mitogen-activated protein kinase kinase and phosphatidylinositol 3-kinase pathways with expression of Snail. On the other hand, a complete loss of E-cadherin and transition to the mesenchymal phenotype additionally requires Smad signaling, which results in formation of β-catenin/lymphoid enhancer factor-1 complexes that induce EMT[48]. Participation of TGF-β1 in the regulation of Notch signaling has been reported previously at the onset of EMT in epithelial cells from mammary gland, kidney tubules, and epidermis[49]. A set of the previously mentioned genes and others described to be involved in EMT, including Snail and Notch2, were identified as TGF-β1 target genes in hepatocytes. Some studies[39,50] have suggested on hepatocyte plasticity showing up-regulation of α1(I) collagen mRNA expression and type I collagen deposition in mouse hepatocytes and α (alpha) mouse liver 12 cells as a result of Smad2/3/4-dependent induction of Snail-1.

EMT IN HEPATIC FIBROGENESIS

EMT is a process that is normally evident in embryonic stages of development and recently has been investigated as a mechanism of cancer cell migration and metastasis[51,52]. A classification of EMT has been recently proposed to distinguish between these different types of EMT[53]. It is characterized by the loss of epithelial characteristics (E-cadherin) and the acquisition of a mesenchymal phenotype (vimentin and fibronectin)[54,55]. According to the functional consequences and biological context, EMT is divided into three subtypes[53,55,56]: Type I EMT occurs during embryogenesis, in which it produces motile cells but does not lead to ECM deposition or intravascular invasion. Type II EMT induces a morphogenetic change during organ fibrosis or wound healing, which is associated with ECM production and muscle-like characteristics. Type III EMT is involved in carcinoma-metastatic transition.

Evidence of EMT in fibrosis was first demonstrated in the kidney. *In vitro*, adult renal tubular epithelial cells were shown to undergo EMT[57,58]. Thereafter, induction of renal fibrosis in mice by unilateral ureteral obstruction (UUO) showed that epithelial marker expression (*i.e.*, E-cadherin) was lost in tubular epithelial cells, while the mesenchymal marker α-SMA expression was increased[59]. A cell tracing method later established that mice submitted to UUO display EMT-derived fibroblasts that contribute to the fibroblastic population[60].

Because the liver is an organ prone to fibrosis and because the origin of fibroblastic cells in fibrotic liver is still debated, the possibility that liver epithelial cells participate to fibrosis by EMT is appealing. Such hypothesis was strengthened by the observation that HSC lines express E-cadherin, while hepatic epithelial progenitor cells are positive for α-SMA[61]. Kaimori *et al*[39] then demonstrated that freshly isolated hepatocytes were able to convert to mesenchymal cells *in vitro*. Hepatocyte EMT, characterized by a decrease in E-cadherin expression and concomitant acquisition of mesenchymal markers (vimentin and type I collagen), was observed when cells were incubated with the profibrogenic cytokine, TGF-β1[39]. Zeisberg and co-workers were the first to report *in vivo* evidence for hepatocyte EMT[50]. They demonstrated that hepatocyte EMT was observed in CCl4 induced liver fibrosis by developing transgenic mice specifically expressing a molecular tag in hepatocytes (*i.e.*, β-galactosidase). In these mice challenged with CCl4, 45% of the cells expressing the mesenchymal marker fibroblast-specific protein-1 (FSP-1) were also positive for β-galactosidase expression. Furthermore, the inhibition of the TGF-β1 pathway limited the extent of liver fibrosis in the CCl4-injected mice[50]. *In vitro* TGF-β1 treatment induced higher vimentin expression in cirrhotic liver-derived hepatocytes than in normal liver-derived hepatocytes[4]. Taken together, these results suggest that hepatocyte EMT is triggered by TGF-β1 and contributes to liver fibrosis. Some studies have proposed that EMT leads to myofibroblast accumulation through a two-stage process. In the first stage, epithelial cells adopt a mesenchymal phenotype, whereas in the second stage these mesenchymal cells further transition to myofibroblasts as part of what has been termed an epithelial-to-myofibroblast transition (EMyT)[62-64].

Substantial experimental evidence supports the occurrence of EMT in embryonic development and tumor metastasis, processes in which the motility phenotype of the transitioned cells is essential[56,65,66]. For tissue fibrosis, however, there are conflicting data on whether or not EMT occurs[67]. Many studies of EMT in fibrosis have failed to define EMT rigorously or to differentiate between the transition to a mesenchymal (EMT) *vs* a myofibroblast (EMyT) phenotype. Type I collagen expression is the most direct measure of fibrogenesis, and the literature suggests that α-SMA-positive cells are the primary effectors of fibrogenesis[55,62,68,69]. Nevertheless, surrogate fibroblast markers have often been used to identify EMT, most notably FSP-1, despite some data suggesting that it is nonspecific[68,70,71].

Mendez *et al*[72] examined the expression of four different mesenchymal markers, including FSP-1, vimentin, α-SMA, and procollagen I . Their lack of colocalization with yellow fluorescent protein (YFP) in the setting of fibrosis supports the conclusion that in these models EMT does not contribute to fibrosis. The complete absence of its colocalization with YFP in their study suggests that liver epithelial cells do not transition to either mesenchymal cells or myofibroblasts in the mouse models examined.

The hypothesis of hepatocyte EMT contributing to liver fibrosis has been also challenged by a cell lineage strategy in mice[67]. A lineage tracing study in which β-galactosidase was expressed under the control of the hepatocyte marker albumin in transgenic mice expressing a collagen marker provided strong evidence against hepatocyte EMT in the CCl4 model of fibrosis[73]. Triple transgenic mice with permanent cell labeling were produced to track hepatocyte-derived cells and type I collagen-expressing cells. Hepatocytes isolated from these triple transgenic mice were able to undergo EMT in culture when incubated with TGF-β1. However, in mice challenged by CCl4, no cells exhibited a double labeling specific for both hepatocytes and collagen expressing cells[73]. These observations also suggest that hepatocytes may not undergo EMT *in vivo*, while the observed transition *in vitro* might be an experimental artifact.

EMT IN CHOLANGIOCYTES

The assumption that liver epithelial cells undergo EMT in liver fibrosis cannot however be ruled out for biliary epithelial cells. Indeed, biliary epithelial cell EMT could represent a cellular mechanism supporting histological observations[70]. For instance, primary biliary cirrhosis (PBC), a prototypical biliary-type liver disease, is characterized by both the loss of biliary epithelial cells and the concomitant development of periportal fibrosis.

The bile duct basement membranes undergo degradation in fibrogenic liver diseases and that cholangiocytes, the other major hepatic epithelial cell type, assume fibroblast-like, non-cuboidal shapes. Therefore, it became obvious that the next step was to investigate whether or not biliary cells could undergo EMT in chronic liver disease[27]. It is well established that proliferating cholangiocytes within the so-called "ductular reaction" (*i.e.*, "reactive cholangiocytes"), detectable in all types of chronic liver disease, express a variety of pro-fibrogenic growth factors and cytokines and are likely to contribute to fibrosis and inflammation by promoting activation, proliferation, and collagen synthesis in the surrounding pro-fibrogenic cells[74-80]. Nevertheless, the possibility of a direct contribution of cholangiocytes to fibrosis *via* EMT was suggested by Omenetti and his colleagues[81] showing *in vitro* a complete EMT in an immature cholangiocyte cell line treated with activated HSC conditioned medium.

Biliary epithelial cell EMT was confirmed by another study analyzing liver of patients with PBC, primary sclerosing cholangitis or alcoholic liver disease. Irrespective of the underlying etiology, biliary epithelial cells from ducts associated with the ductular reaction were positive for FSP-1 and vimentin[82]. In biliary atresia, a disease defined by a destructive inflammatory obliterative cholangiopathy with portal tract fibrosis and ductular proliferation[83], biliary epithelial cells were shown to express FSP-1 and vimentin, while hepatocytes were not. Moreover, the authors of this study show that the expression of mesenchymal markers in biliary epithelial cells is observed in all liver disease with a ductular proliferation component[84]. The common bile duct ligation (BDL) is an experimental liver fibrosis model that induces strong ductular reaction. In mice submitted to BDL, biliary epithelial cells undergo EMT as shown by α-SMA and type I collagen expression[85].

Evidence of cholangiocyte EMT was recently challenged with the lineage-tracing methodology previously used for the investigation of hepatocyte EMT[27]. Along these lines, Scholten and the colleagues[86] employed the Cre-Lox technology for lineage tracing and studied several mouse strains expressing Cre under cholangiocyte-, HSC-, or FSP-1-specific promoters in two established models of liver fibrosis, *i.e.*, chronic CCl4 intoxication and common BDL. In this case the fundamental experiment was tracing the fate of cells expressing K19, a bile ductular cell-specific marker, after permanent genetic Cre-mediated labeling of cholangiocytes. The key result of this study was that, although myofibroblast markers were often found in the close proximity of the K19+ progeny of cholangiocytes, the two signals never overlapped in either CCl4 or BDL fibrosis. Based on these and other observations reported in the paper, the authors concluded that cholangiocyte EMT does not occur in their experimental models.

It may be that in human livers EMT occurs in cirrhosis, a state not well modeled in rodents, and may require a florid ductular reaction, which is also poorly mimicked by rodent models. Alternatively, this discrepancy may reflect the limitations of immunohistochemistry-based lineage-tracing methodology[67]. Future work should focus on better understanding the direct contribution of dysfunctional epithelial cells to liver fibrosis, as well as determining the mechanistic relationships between fibrogenesis and the progenitor cell activation characteristic of the ductular reaction. This will ultimately require the development of animal models of biliary fibrosis that better reflect human disease[67].

SPECIFIC CELLULAR MARKERS IN EMT

The most widely used marker identifying myofibroblasts is the cytoskeletal protein α-SMA that is a part of the contractile machinery and is involved in cell motility[27]. In adult normal tissue, α-SMA expression is mostly restricted to vascular smooth muscle cells, but in most chronic inflammatory and fibrogenic disease states, it is often found in myofibroblasts of different derivation, and this expression is interpreted as an active involvement of these cells in fibrogenesis (*i.e.*, "activated myofibroblast").

Table 1 Useful biomarkers for identifying epithelial-mesenchymal transition

Biomarkers	Myofibroblast	Hepatic stellate cell	Hepatocyte	Cholangiocyte
α-SMA[1]	+	+	-	-
Vimentin[1]	+	+	-	-
Desmin[1]	+	+	-	-
ICAM-1	+	+	-	-
Collagen type IV	+	+	-	-
Fibronectin	+	+	-	-
Fibulin-2	+	-	-	-
IL-6 mRNA	+	-	-	-
NCAM	+	-	-	-
Synaptophysin	+	-	-	-
Neurotrophin	+	-	-	-
Neural growth factor	+	-	-	-
αB-crystalline	+	-	-	-
Tyrosine kinase	+	-	-	-
FSP-1[1]	+	-	-	-
HSP47[1]	+	-	-	-
CD95L	-	+	-	-
α2-macroglobulin	-	+	-	-
P100	-	+	-	-
Reelin	-	+	-	-
Fascin	-	+	-	-
E-cadherin	-	-	+	+
Cytokeratin	-	-	+	+
K19	-	-	-	+
Albumin	-	-	+	-
Slug[1]	+	?	-	-
Twist[1]	+	?	-	-
Snail[1]	+	?	-	-

[1]Previously proven markers associated with epithelial-mesenchymal transition. α-SMA: α-smooth muscle actin; ICAM-1: Intercellular adhesion molecule-1; IL-6: Interleukin-6; NCAM: Neural cell adhesion molecule; FSP-1: Fibroblast-specific protein-1.

Accordingly, α-SMA cannot be a good lineage marker since its expression is activated by disease states and, in addition, does not denote function. Regardless of this, there is supportive evidence that epithelial cells express intermediate filaments such as α-SMA and vimentin following tissue injury[4,87].

A multitude of studies have shown that epithelial cells, including hepatocytes, when cultured *in vitro* retain epithelial features including polarity and specific protein expression (*i.e.*, albumin for hepatocytes), but when chronically stimulated with TGF-β1 or serum factors acquire a pattern of gene expression that is somehow typical of myofibroblasts *in vivo* and in the mesenchyme during development[39,88-91]. These genes are often represented by Slug, Twist, Snail, α-SMA, vimentin, desmin, FSP-1, and discoidin domain receptor tyrosine kinase 2. Some of these markers have been used to identify epithelial cells that are in the midst of undergoing an EMT associated with chronic inflammation. These cells continue to exhibit epithelial-specific morphology and molecular markers, such as cytokeratin and E-cadherin, but often show the concomitant expression of the FSP-1 and α-SMA. These aspects have been proposed to represent the intermediate stages of EMT, when epithelial markers continue to be expressed but new mesenchymal markers have already been acquired, and, overall, these observations have led to the notion of the so-called "partial EMT"[53].

Previous work has demonstrated in a model of fetal hepatocytes that TGF-β1 treatment induces EMT-like morphologic changes in 50%-60% of the hepatocyte population, whereas the remaining hepatocytes undergo apoptosis[38,40]. This means that EMT can be elicited by several oncogenic pathways (Src, Ras, integrin, Wnt/β-catenin and Notch)[27,92]. In particular, Ras-MAPK has been shown to activate two related transcription factors known as Snail and Slug[93]. Both of these proteins are transcriptional repressors of E-cadherin and their expression induces EMT.

Chronic liver damage leads to fibrotic degeneration of parenchyma, characterized by the formation of fibrotic septa. Except for HSCs, portal myofibroblasts can produce collagen in the liver[94]. Although both cell types show similar expression patterns of intercellular adhesion molecule-1, desmin, vimentin, collagen type IV, fibronectin, and α-SMA, several differences between them have also been observed[94]. For instance, cultured portal myofibroblasts are positive for fibulin-2 and interleukin-6 mRNA, whereas CD95L, α2-macroglobulin, P100, and reelin are exclusively expressed by activated HSCs[94-100]. In addition, neural cell adhesion molecule, synaptophysin, neurotrophin, neural growth factor, αB-crystallin, and tyrosine kinases are markers that distinguish HSCs from portal myofibroblasts[99,101]. Experiments using these markers have shown that myofibroblastic cells in fibrotic septa strongly resemble portal myofibroblasts, that they may originate and migrate from the portal tract, and that they are different from sinusoidal HSCs[19].

Uyama *et al*[19] found that fascin is present in intralobular sinusoidal areas, but not in the periportal areas or fibrotic septa of the human liver. In addition, the localization of fascin in the sinusoidal area is similar to that of vimentin. They concluded that fascin was localized in human HSCs, but not in (myo)fibroblasts of the periportal area or fibrotic septa.

Evidence favoring biliary EMT comes largely from immunohistochemical studies of fibrotic human and rodent livers that identified cholangiocytes coexpressing epithelial markers (especially the cholangiocyte marker K19) and mesenchymal markers (*i.e.*, FSP-1, vimentin, and HSP47)[64]. Table 1 shows the summary of useful biomarkers for identifying EMT.

CONCLUSION

The pathogenesis of liver fibrosis is now better understood than ever before. It is increasingly recognized that the fibrogenic cells in the liver are heterogenous in both their formation and their behaviour.

EMT is an established process in embryo development and plays an important role in liver fibrosis. Discussions now arise on the involvement of EMT in organ

fibrosis. The possibility that EMT could contribute to hepatic fibrogenesis in chronic liver diseases reinforced the concept that activated HSCs are not the only key players in the hepatic fibrogenic process and that other cell types, either hepatic or extrahepatic (bone marrow-derived cells and circulating fibrocytes) could contribute to this process. The presence of cells expressing both epithelial and mesenchymal markers suggests that EMT is a feature of liver fibrosis, however the ability of these cells to produce ECM *in vivo* has not yet been documented.

Therefore, the knowledge relative to the interpretation of what is defined as EMT in chronic fibrogenic disorders of the liver represents a scientific treasure that has prompted discussion, animated debates and has ultimately provided further maturity in this field of research. Definitely, there is now need for a more insightful analysis of the real pathophysiological meaning of these observations beyond their morphologic and biological features. Nevertheless, the future holds great promise for EMT as a viable therapeutic target.

EMT research in the next few years promises to be exciting, as new mouse models and molecular probes are identified to address the identities of the EMT-inducing microenvironmental signals, the nature of the cellular response of such signals and signaling machinery within epithelial cells.

Future research will surely be required on uncovering the origin of all fibrogenic cells within the liver and the molecular similarities and differences among the EMT programs.

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MINIREVIEWS

Pediatric non-alcoholic fatty liver disease: New insights and future directions

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Abstract

One of the most common complications of childhood obesity is the non-alcoholic fatty liver disease (NAFLD), which is the most common form of liver disease in children. NAFLD is defined by hepatic fat infiltration > 5% hepatocytes, as assessed by liver biopsy, in the absence of excessive alcohol intake, viral, autoimmune and drug-induced liver disease. It encompasses a wide spectrum of liver diseases ranging from simple steatosis to non-alcoholic steatohepatitis, which, in turn, can evolve into cirrhosis and end stage liver disease. Obesity and insulin resistance are the main risk factors for pediatric NAFLD. In fact, NAFLD is strongly associated with the clinical features of insulin resistance especially the metabolic syndrome, prediabetes and type 2 diabetes mellitus (T2D). In particular, it has been clearly shown in obese youth that the prevalence of metabolic syndrome, pre-diabetes and type 2 diabetes increases with NAFLD severity progression. Evidence that not all of the obese patients develop NAFLD suggests that the disease progression is likely to depend on complex interplay between environmental factors and genetic predisposition. Recently, a non-synonymous SNP (rs738409), characterized by a C to G substitution encoding an isoleucine to methionine substitution at the amino acid position 148 in the patatin like phospholipase containing domain 3 gene (*PNPLA3*), has been associated with hepatic steatosis in a multi-ethnic cohort of adults as well as in children. Another important polymorphisms that acts with *PNPLA3* to convey susceptibility to fatty liver in obese youths is the rs1260326 polymorphism in the glucokinase regulatory protein. The pharmacological approach in NAFLD children poorly adherent to or being unresponsive/partially responsive to lifestyle changes, is aimed at acting upon specific targets involved in the pathogenesis. There are some therapeutic approaches that are being studied in children. This article reviews the current knowledge regarding the pediatric fatty liver disease, the new insights and the future directions.

Core tip: The prevalence of hepatic steatosis is increased in the last three decades concomitantly with the increased prevalence of pediatric obesity. Non-alcoholic fatty liver disease (NAFLD) is the most common form of liver disease in children. The *PNPLA3* rs738409 and the glucokinase regulatory protein rs1260326 are the strongest variants associated with fatty liver in paediatrics. Important risk factors are obesity, insulin resistence, gender, ethnicity and excessive dietetic intake of n-6 polyunsatured fatty acids and fructose. New pharmacological approaches are object of study, in NAFLD children poorly adherent to or being unrespon-

sive/partially responsive to lifestyle changes.

INTRODUCTION

In the last three decades with the increased prevalence of childhood obesity, there has been an increase also of the obesity complications in paediatrics. One of the most common complications of childhood obesity is the non-alcoholic fatty liver disease (NAFLD), which is the most common form of liver disease in children[1].

NAFLD is defined by hepatic fat infiltration > 5% hepatocytes, as assessed by liver biopsy, in the absence of excessive alcohol intake, viral, autoimmune and drug-induced liver disease[2,3]. It encompasses a wide spectrum of liver diseases ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), which, in turn, can evolve into cirrhosis and end stage liver disease[3,4].

The prevalence of NAFLD has more than doubled over the past 20 years. According to a landmark study by Schwimmer *et al*[1] based on autoptic data obtained in 1138 children and adolescents of the San Diego county (CA), its prevalence in the general pediatric population is estimated to be nearly 13%, while among obese and overweight children and, particularly, adolescents it rises up to 46%[1]. Nevertheless, other studies report quite a wide range of steatosis prevalence, likely due to the different diagnostic methods used. In fact, although liver histology is important for NAFLD evaluation, performing biopsies is not always indispensable from a clinical point of view; therefore, surrogate markers are often used in epidemiological and clinical studies. One of the marker most commonly used is liver aminotransferase [aspartate aminotransferase, and alanine aminotransferase (ALT)] evaluation. Children with NAFLD typically have elevated liver enzymes values[5], which is why elevated serum levels of liver enzymes, even though may misrepresent the entity of intrahepatic damage, are used as a non-invasive test to screen for pediatric NAFLD[6].

RISK FACTORS FOR THE DEVELOPMENT OF PEDIATRIC NAFLD

Obesity and insulin resistance are the main risk factors for pediatric NAFLD[1,7,8]. In fact, NAFLD is strongly associated with the clinical features of insulin resistance especially the metabolic syndrome (MS), prediabetes and type 2 diabetes mellitus (T2D)[9-11]. In particular, it has been clearly shown in obese youth that the prevalence of metabolic syndrome, pre-diabetes and type 2 diabetes increases with progression of NAFLD severity[12].

This picture is strongly contributed by pubertal insulin resistance, a physiologic state characterized by an increased insulin resistance during the adolescence and resolving at the end of the pubertal development and probably consequent to the increase in growth hormone action during this stage of life[8,13]. In fact, although obesity is the most important cause of NAFLD among obese and adolescents, it is important to note that a transient insulin resistant state occurs during puberty[14], and that this state worsens the insulin resistance present in obese children in turn accelerating the progression to MS and type 2 diabetes. In healthy individuals this phenomenon is balanced by an increased insulin secretion by the beta cell, but in obese individuals the co-occurrence of obesity and puberty represents the perfect storm causing such a high degree of insulin resistance that the beta cell is not always able to produce enough insulin to maintain the glycemic control[15-17].

Two other critical risk factors for NAFLD development are represented by the gender and the ethnic background. In fact, NAFLD is more common in boys than in girls[15] with a male to female ratio of 2:1. This has been explained by the liver-protective role of estrogens, as well as by the potentially negative role of androgens in aggravating NASH[18,19]. The beneficial effects of estrogens on liver could be mediated by the beneficial effect on insulin action. Studies showed that insulin sensitivity is greater in premenopausal women compared with age-matched men, and metabolic-related cardiovascular diseases and type 2 diabetes are less frequent in premenopausal women[20,21]. Also, estrogens deficiency leads to increased fat mass and body weight in postmenopausal women, which has been associated with increased intraabdominal fat[22]. Moreover, Camporez *et al*[23] showed that, in mice, endogenous estrogens are important to protect against high-fat diet induced skeletal muscle insulin resistance, whereas E2 treatment in estrogen-deprived mice increased insulin sensitivity in both liver and skeletal muscle. Also, the estrogens effect is important in turn preventing diet-induced ectopic lipid deposition and hepatic and muscle insulin resistance.

The risk linked to the ethnic background has been investigated in large multiethnic populations. A cornerstone article by Browning *et al*[15] described for the first time that the prevalence of NAFLD is the highest in the American Hispanic population (45%) and the lowest among African Americans (24%), with the Caucasians showing an intermediate prevalence (33%). Ethnic differences could possibly be due to different degree of insulin resistance, and of visceral adiposity at equivalent body mass index, but may also be a result of genetics as well as socio-economic factors, including type of diet, exercise choice and living location[24].

The accumulation of fat, as triacylglycerol (TAG), in the hepatocyte is the fingerprint of fatty liver. The TAG accumulated in the liver mostly derive from adipose tissue lipolysis (60%) and hepatic de novo lipogenesis (26%) whereas only a small amount directly derives from the diet as chylomicron remnants (14%)[25]. A large body of

Figure 1 **Risk factors for non-alcoholic fatty liver disease development.** In this figure, all risk factors for non-alcoholic fatty liver disease (NAFLD) are summarized. We divided the risk factors in modifiable and not modifiable. Among not modifiable risk factors we listed *PNPLA3* rs738409, but as underlined in the text, the weight loss can modify the capacity of *PNPLA3* polymorphism to lead to hepatic steatosis. *PNPLA3*: Patatin like phospholipase 3 gene; *GCKR*: Glucokinase regulatory protein; *FDFT1*: Farnesyl-diphosphate farnesyltransferase 1; *NCAN*: Neurocan; *PPP1R3B*: Protein phosphatase 1 regulatory subunit 3B; *LYPLAL1*: Lysophospholipase-like 1; *GC*: Group-specific component; *LCP1*: Lymphocyte cytosolic protein-1; *LPPR4*: Lipid phosphate phosphatise-related protein type 4; *SLC38A8*: Solute carrier family 38 member 8; *APOC3*: Apolipoprotein C3 gene; *SAMM50*: Sorting and assembly machinery component; *PARVB*: Parvin beta.

evidence suggests that not only the amount, but also the quality of dietary fat plays a role in NAFLD development[26]. In particular, recently published literature provides clues that the dietary imbalance between omega-6 (n-6) and omega-3 (n-3) polyunsatured fatty acids (PUFAs) leads to development of an adverse cardiovascular and metabolic profile, thus contributing to the pathogenesis of NAFLD[27]. N-6 and n-3 are essential fatty acids; this means that they are not synthesized by human body. N-6 species are mainly represented by linoleic acid while n-3 are represented by alpha-linolenic acid, mainly found in plants and limited sets of seeds and nuts[28]. N-6 is readily converted by the body into other species such as omega-9 and so incorporated into triglycerides, or converted into arachidonic acid, which is the parent molecule of the main regulators of the inflammatory response including prostaglandins (ciclooxigenase pathway), leukotrienes (lipoxygenase pathway) and tromboxane[28]. It has been demonstrated that individuals with NAFLD have a lower dietary intake of n-3 PUFAs than healthy controls[29] and an increased n-6/n-3 PUFA ratio consumed in the diet[29,30]. Consistent with these data, lipidomic studies have shown that the intrahepatic fat in subjects with steatohepatitis is composed by an excess n-6 PUFA[31]. In particular, studying the three groups of subjects-NAFLD, NASH and healthy controls- it has been observed a progressive increase in the n-6/n-3 ratio from controls to NASH subjects[31].

Another dietary risk factor contributing to the development of NAFLD is the fructose. Nowadays, the majority of fructose consumption comes from the added sugars in the beverages more than from the fruit[32]. Strong evidence exists that high in fructose intake results in increased de novo lipogenesis (DNL), dyslipidemia, insulin resistance, and obesity in humans[33]. Stanhope *et al*[33], studying the effect of consumption of glucose- or fructose-sweetened beverages providing 25% of energy requirements for 10

wk in overweight and obese subjects, provided the evidence that the consumption of fructose, instead of glucose, specifically increases DNL, promotes dyslipidemia, decreases insulin sensitivity, and increases visceral adiposity in overweight/obese adults.

Progression from NAFLD to NASH

A recent study demonstrated that NAFLD in children is a progressive disease[34]. In that study the authors showed that 6% of subjects with early onset NAFLD develop cirrhosis and end-stage liver disease with the consequent need of liver transplantation.

The oxidative stress seems to explain the progression to NASH and liver fibrosis. Reactive oxygen species (ROS) can induce hepatocellular injury by the inhibition of the mitochondrial respiratory chain enzymes, the inactivation of glyceraldehyde-3-phosphate dehydrogenase and the inactivation of membrane sodium channels. ROS further cause lipid peroxidation, cytokine production, and induce Fas ligand, contributing to hepatocellular injury and fibrosis[12]. The risk of progression varies by ethnicity, in fact, as recently demonstrated, the African American obese children and adolescents show a lower degree of liver damage than Caucasians and Hispanics, independent of the degree of hepatic fat accumulation and insulin resistance. These data suggest that African Americans are protected from hepatic damage even in presence of high degree of hepatic fat accumulation and insulin resistance[35].

GENETIC PREDISPOSITION

Evidence that not all of the obese patients develop NAFLD suggests that disease progression is likely to depend on complex interplay between environmental factors and genetic predisposition (Figure 1).

Recently, a non-synonymous SNP (rs738409), char-

Table 1 Current and future non-alcoholic fatty liver disease treatment strategies

Present	Under development	
Weight loss	Vitamin E	Pentoxifylline
Physical activity	Metformin	Farnesoid X receptor agonists
Reduced dietary	Probiotics	Toll-like receptors modifiers
sucrose intake	Oral treatment	Glucagon-like peptid-1 receptor
Reduced dietary	with omega 3	agonists resistant to DPP-4
fructose intake		mediated degradation
Reduced dietary		DPP-4 inhibitors
omega 6 intake		
Increased dietary		
omega 3 intake		

DPP-4: Dipeptidyl peptidase-4.

acterized by a C to G substitution encoding an isoleucine to methionine substitution at the amino acid position 148 in the patatin like phospholipase 3 gene (*PNPLA3*), has been associated with hepatic steatosis in a multiethnic cohort of adults[36] as well as in children[37,38]. *PNPLA3* encodes for a triglyceride hydrolase expressed in the liver and adipose tissue[39]. Metabolic studies in transgenic mice revealed that high level expression of PNPLA3[I148M] in the liver, but not in adipose tissue, affected both hepatic triacylglycerol (TAG) synthesis and catabolism. A surprising finding was that the PNPLA3[I148M] transgenic mice have significantly increased fatty acid synthesis and an altered spectrum of TAG-fatty acids in the liver, with no evidence of insulin resistance[40]. It is interesting that PNPLA3[I148M] transgenic mice develop steatosis on a sucrose diet but not on a high-fat diet. Ingestion of sucrose stimulates de novo synthesis of fatty acids[41], whereas most of the hepatic fatty acids in livers of fat-fed mice are derived from circulating non esterified fatty acids (NEFAs). Perhaps PNPLA3 in hepatocytes is exposed preferentially to newly synthesized TAG and is shielded from fatty acids that enter the liver in lipoproteins or are synthesized from circulating NEFAs[40]. Alternatively, PNPLA3 may function specifically under conditions of insulin-stimulated lipid anabolism. The finding that PNPLA3 is virtually absent from livers of fasting animals and is strongly upregulated both transcriptionally[42-45] and post-translationally[39] by carbohydrate refeeding is consistent with the latter hypothesis.

Moreover, purified recombinant PNPLA3 has been found to have 5 enzymatic activities: triacylglycerol, diacylglycerol, and monoacylglycerol hydrolysis[42,46] as well as acyl-CoA thioesterase[42] and lysophosphatidic acid acyltransferase activity[47]. These different activities are not equally affected by the I148M substitution. In vitro assays, the I148M substitution results in a substantial loss of triacylglycerol, monoacylglycerols, and diacylglycerols hydrolytic activity[33]; a modest reduction in acyl-CoA thioesterase activity[33]; and an increase in lysophosphatidic acid acyltransferase activity[47]. None of these activities alone can explain all of the changes in TAG metabolism observed in the *PNPLA3* I148M transgenic mice. Therefore, some of the metabolic changes observed in these animals are likely to be secondary rather than direct consequences of altered *PNPLA3* activity.

The effect of this polymorphism on liver damage seems to be driven by the size of abdominal fat, expressed as waist to height ratio (W/Hr)[38]. More recently it has been demonstrated that weight loss reduce the effect of this polymorphism in obese children[48].

Other findings suggest also that the influence of *PNPLA3* on hepatic fat in obese children and adolescents might be modulated by dietary factors such as n-6/n-3 polynsatured fatty acids (PUFA) intake[49]. Finally, the rs738409 *PNPLA3* polymorphysm is considered, regardless of metabolic profile, a risk factor for liver disease. In fact, in a recent study, in a hepatitis C-infected population, the *PNPLA3* polymorphism influenced the development of liver steatosis[50]. Moreover, it was also demonstrated as a novel genetic marker associated with progressive ALD (alcoholic liver disease)[51].

Another polymorphism that acts along with the *PNPLA3* gene variant to convey susceptibility to fatty liver in obese youths is the rs1260326 polymorphism in the glucokinase regulatory protein (*GCKR*). This polymorphism is associated with hepatic fat accumulation along with large VLDL and triglyceride levels[52].

Speliotes *et al*[53], in addition to *GCKR,* identified variants in novel loci *NCAN* and *LYPLAL1* associated with both increasing computer tomography (CT) hepatic steatosis and histological NAFLD and identified variants in another locus, protein phosphatase 1 regulatory subunit 3B (*PPP1R3B*), associated with CT steatosis but not histologic NAFLD[53]. Recently Kitamoto *et al*[54] found that *PNPLA3, SAMM50* sorting and assembly machinery component (*SAMM50*), parvin beta (*PARVB*) genetic regions was significantly associated with NAFLD in the Japanese population. Adams *et al*[55] showed that SNPs in two genes expressed in liver were associated with NAFLD in adolescents: group-specific component (*GC*) and lymphocyte cytosolic protein-1 (*LCP1*). SNPs in two genes expressed in neurons were also associated with NAFLD: lipid phosphate phosphatise-related protein type 4 (*LPPR4*) and solute carrier family 38 member 8 (*SLC38A8*)[55].

TREATMENTS

Diet and lifestyle changes

The goal of lifestyle interventions is a gradual and controlled weight loss achieved by diet and physical exercise (Table 1). This aim is difficult to achieve and only a small percentage of individuals is able to steadily lose weight and exercise regularly[56]. Weight loss in NAFLD patients improves hepatic insulin sensitivity by reducing hepatic NEFAs supply, improves extra-hepatic insulin sensitivity through better glucose utilization and reduces ROS generation and adipose tissue inflammation[56].

Currently, there are no evidence-based guidelines establishing the optimal intervention. The only effective interventions are physical activity and dietary changes. In fact, reduction in sugar/sucrose and in soft drinks

rich in fructose, most probably not only acts through a reduction in IR and lipogenesis, but also counteracts the recently evidenced hepatic pro-inflammatory/fibrogenetic role of fructose[57]. It should be taken in mind that diet in childhood must be balanced to allow a healthy and harmonic growth, including wellness of bone structures. To intervene on dietary changes does not only mean to reduce the caloric intake, but also the single components of the diet (Table 1). In fact, also diet composition plays an important role in the development of NAFLD, as an increased dietary intake of monounsaturated and poly-unsaturated fatty acids (mainly omega 3 PUFA) has been associated with a reduction of hepatic fat content, representing a reasonable intervention especially in the pediatric population[58].

Pharmacological interventions for NAFLD

The pharmacological approach, in NAFLD children poorly adherent to or being unresponsive/partially responsive to lifestyle changes, is aimed at acting upon specific targets involved in etiopathogenesis (Table 1).

Antioxidants, by reducing oxidative stress, protect susceptible components of biological membranes from lipid peroxidation, and may, therefore, prevent the progression of simple steatosis to NASH. The most studied antioxidant in children with NAFLD is alpha tocopherol (vitamin E) and warrants consideration in obesity-related liver dysfunction for children unable to adhere to low-calorie diets[59]. Sanyal *et al*[60] showed that vitamin E therapy, as compared with placebo, was associated with a significantly higher rate of improvement in NASH (43% *vs* 19%, *P* = 0.001) in adults without diabetes. There was no benefit of pioglitazone over placebo for improvement of NASH but serum alanine and aspartate aminotransferase levels were reduced as well as with vitamin E.

For its pathogenic role, insulin resistance appears as an adequate therapeutic target. Metformin is the only insulin-sensitizing agent evaluated in children.

Lavine *et al*[61] in a more recent large, multicenter, randomised double-bind placebo-controlled trial (TONIC study), evaluated the effect of daily dosing of 800 IU of vitamin E (58 patients), 1000 mg of metformin (57 patients) or placebo (58 patients) for 96 wk of NAFLD course. The patients (aged 8-17 years) with biopsy-confirmed NAFLD and persistently elevated levels of ALT, without diabetes or cirrhosis, were randomly assigned to 1 of 3 groups. At 96 wk neither vitamin E nor metformin was superior to placebo in attaining the primary outcome of sustained reduction in ALT level in pediatric NAFLD; vitamin E and metformin groups, however, showed an improvement in histological hepatocellular ballooning in NAFLD and NASH.

Another, single-arm, open-label, small pilot study on metfotmin (500 mg twice daily for 24 wk), conducted in 10 non-diabetic children with biopsy proven NASH and elevated ALT levels showed reduction of hepatic steatosis, as evaluated with Magnetic Resonance Spectroscopy (MRS) and low serum ALT levels[62].

FUTURE DIRECTIONS

A growing body of evidence[63] shows that the gut microbiota controls obesity and visceral fat storage. Specific variations in gut microbiota in early life may determine a major risk factor of obesity and its complications later in life[64]. Small intestinal bacterial overgrowth (SIBO) (a frequent condition in obese individuals, mainly prompted by slowing of the oro-coecal transit time) may promote NAFLD progression to non-alcoholic steatohepatitis by enhancing intestinal permeability and by favouring absorption of endotoxins with pro-inflammatory and pro-fibrogenetic effects on the liver[65].

Probiotics are live microorganisms which when consumed in adequate amounts, confer an healthy benefit to the host[66]. Gut microbiota manipulation with probiotics in rodents with fatty liver reduces intestinal inflammation and improves the epithelial barrier function[67,68]. Therefore, probiotics could represent a new effective treatment also in NAFLD human patients (Table 1). Loguercio and colleagues have shown that probiotics may reduce NAFLD liver injury and may improve liver function tests[69].

Moreover, recent pharmacological studies in NAFLD animal models and in adult humans focusing on the effect of oral treatment with n-3 fatty acids, demonstrate that they have both anti-inflammatory and insulin sensitizing properties, suggesting a potential role in treatment of NAFLD[70]. In NAFLD children n-3-docosahexaenoic acid (DHA) treatment for 6 months improved ultrasonographic fatty liver and insulin sensitivity[71]. Because this treatment is well tolerated in pediatric population, DHA deserve further studies in the management of children with NAFLD.

A series of other interesting approaches, hitherto explored only in NAFLD animal models or in few pilot studies in adults will possibly become in future the object of study in pediatric population (Table 1), as well: (1) tumor necrosis factor-α (TNF-α) and other adipocytokines produced by adipose tissue are involved in NAFLD progression. Pentoxifylline, a phosphodiesterase inhibitor, exerts immunomodulatory functions by antagonizing the TNF-α pathway. In adults with NASH, pentoxifylline treatment showed good tolerability and could decrease serum ALT levels and improve histological features[72]; (2) the nuclear bile acid receptor, Farnesoid X receptor (FXR), strongly expressed in bowel and liver, is probably involved in NAFLD pathogenesis, by mediating control of lipids and glucose homeostasis, and controlling bacterial flora growth. Altogether, these effects may induce reduction of hepatic inflammation and fibrogenesis, through different mechanisms. Therefore, recently developed FXR agonists have a potential role in the pharmacological therapy of NAFLD/NASH[73]; (3) toll-like receptors (TLRs) are receptors sensing microbial components of gut microbiota. A number of recent evidences suggests the role of SIBO and increased intestinal permeability in NAFLD, by exposing *via* portal vein the liver to an high load of intestinal noxae including lipo-

polysaccharide and other pathogen-associated molecular patterns[74]. Furthermore, TLRs stimulation causes downstream activation of the inflammatory response. Proinflammatory patterns result in production of cytokines and chemokines implicated in progression from simple steatosis to steatohepatitis and fibro-cirrhosis; so therapeutic manipulation of innate immune system through TLRs modifiers, formerly evaluated for autoimmune diseases[75], might be a new potential therapeutic target for pediatric NAFLD, but further studies are necessary; and (4) glucagon-like peptid-1 (GLP-1) is an incretin secreted in response to food intake, allotted to multiple functions, including, the stimulation of glucose-dependent insulin secretion and inhibition of glucagon release. The enzyme dipeptidyl peptidase-4 (DPP-4) rapidly degrades circulating GLP-1 (half-life: 1-2 min). Recent animal model and NAFLD adults studies showed an effective role of GLP-1 receptor agonists resistant to DPP-4 (such as exenatide and liraglutide) or DPP-4 inhibitors (*e.g.*, some gliptins) as a promising new therapy in NAFLD for their ability in modulating fatty acid oxidation, decreasing lipogenesis, and improving hepatic glucose metabolism[76].

CONCLUSION

Non-alcoholic fatty liver disease, because of the rise in the prevalence of childhood obesity, is becoming one of the most important chronic liver disease among children. Evidence that only a sub-group of obese patients develop NAFLD suggests that disease progression is likely to depend on complex interplay between environmental factors and genetic predisposition (Figure 1). Recent researches led us to understand the genetic basis predisposing to NAFLD. Many genes have been identified and many other will be identified and, actually, the most important it appears to be *PNPLA3* gene. Probably, all the genetic polymorphisms implicated in NAFLD development could have a summarizing effect. In fact, if more predisposing NAFLD polymorphisms coexist in the same subject, the risk to develop NAFLD and to develop it more severely could increase. Other important findings are related to the diet. For example, strong evidence exists that high in fructose intake, usually present in beverages, results in increased de novo lipogenesis and then in increased risk of NAFLD. Moreover, also high n-6/n-3 PUFA ratio consumed in the diet could predispose the NAFLD development. All these findings must drive the clinical practice: the diet is the first and important approach for the NAFLD prevention and treatment. In fact, there is the striking evidence that the weight loss can reduce the effect of I148M polymorphisms on determining hepatic steatosis. In addition to weight loss, to reduce the fructose intake trough the beverages and increasing the n-3 fatty acids dietary intake could be also useful in contrasting the NAFLD.

In conclusion, waiting the new approaches, the dear and old diet is always a fundamental and irreplaceable NAFLD therapy.

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MINIREVIEWS

To treat or not to treat the "immunotolerant phase" of hepatitis B infection: A tunnel of controversy

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A thorough review of the updated published reports was carried out and a merge of the various management options, with a special point of view of the author, is stated.

Abstract

Hepatitis B virus (HBV) infection is a global public health problem, with an estimated 350 million people worldwide chronically infected and approximately 500000 who die annually from HBV-related liver diseases. Management of chronic HBV is challenging and waves of guidelines emerge every year. One of the hottest topics and a matter of debate is the management of patients in their early immunotolerant phase of infection. With the lack of evidence, dealing with this particular subset of patients creates a great conflict with opposing views. In this review, the author highlights the pros and cons of these views and proposes a reasonable solution to resolve this dilemma.

Key words: Liver biopsy; Hepatitis B Virus; Immunotolerant phase; Polymerase chain reaction; Nucleotide analogue

Core tip: In this mini review, the author discusses the management dilemma of this peculiar subset of patients suffering from chronic hepatitis B in the immunotolerant phase. As already known, the immunotolerant phase of hepatitis B virus may last for a long period and hence there may be a potential for subtle liver damage.

INTRODUCTION

Hepatitis B virus (HBV) infection is a global public health problem, with an estimated 350 million people chronically infected worldwide. Fifteen to forty percent of these individuals will develop serious sequelae during their lifetime, with greater evolution to cirrhosis or hepatocellular carcinoma (HCC). The estimated 5-year rate of progression from chronic hepatitis B (CHB) to cirrhosis was estimated to be 12%-20% and the 5-year cumulative risk of developing HCC was also estimated to be between 10%-17% in patients with cirrhosis. These figures vary from country to country according to the disease endemicity and prevalence[1-3]. The natural history of CHB is complex and described to run through different immunological phases that may overlap. In its early phases, HBV infection is characterized by minimal liver damage on liver biopsy, a high level of HBV replication and positivity for HBe-antigen (HBeAg). These patients are asymptomatic and have normal levels of serum alanine aminotransferase (ALT). This phase is described as the "immunotolerant phase"[3,4].

Managing these patients creates a great conflict with two opposing views. One view is optimistic, conservative and relies upon the long-term course of benignity of the disease. They adopt the view of "leave the patient alone on close follow up". On the other hand, the other view is

pessimistic and relies upon the great risk of cancer development, even without cirrhosis. This latter view adopts the view of "to treat the patient and why wait". Between these two views, there are no real evidence based guidelines.

In this review, an extensive online research for English reviews and articles that tackle this subject by using the key words "immunotolerant", "HBV" and "management" was carried out. The author highlights the pros and cons of all views regarding the management strategies of this subject and makes a reasonable proposed solution for this dilemma.

IMMUNOTOLERANT PHASE: CHARACTERISTICS AND IMMUNOLOGICAL INSIGHT

The natural history of HBV infection is perplexing and its net result is an interplay between the viral replication and the host immune response. After primary infection, an immunotolerant phase characterized by a very high rate of viral replication but without liver injury takes place. The mechanism of this tolerance is not yet fully understood[5]. These patients are infected early in life through vertical or early horizontal infection. Such infection most often occurs in areas with high rates of endemic infection, low rates of maternal screening, and lack of widely available neonatal prophylaxis with HBV vaccine and hepatitis B immunoglobulin[6,7].

It is believed that before birth, HBeAg acts as a "tolerogen viral protein" in the fetus, and thus virus specific T-cells undergo deletion. This phase lasts from weeks to years, depending on the age at acquisition. After years/decades, this tolerance is somehow ruptured and the immune attack against infected hepatocytes to clear them begins, causing liver damage. During this "immune clearance phase", ALT levels increase and HBV DNA levels begin to decrease. Immune attacks of infected hepatocytes result in HBeAg seroconversion and this seroconversion is usually associated with sustained remission of liver disease. Selection pressures for the virus come from either competition between viral variants, which are different in their replicative efficiency, and the host immune activity[8-10].

It was found that the majority of young children who presented in the immunotolerant phase have either minimal chronic hepatitis or, more commonly, non-specific reactive hepatitis, in spite of persistently normal ALT activity[11-13].

Wang *et al*[14] studied seven patients (age range between 7-25 years) by follow up for at least 17 years with serial sampling for ALT activity and viral load. They concluded that the interplay between viral replication and host immunity explains the pattern of HBV dynamics within the host during the early stages of infection. That is, without immune selection, competition between peers increases the viral load and decreases the nucleotide diversity; in contrast, host immunity accelerates viral evolu-

tion and decreases copy numbers but increases diversity. The fully infected liver can yield between 10^9 to 10^{10} viruses per milliliter of serum, a level of production that would be expected to persist if infection were benign and the host were truly immunotolerant. Virus titers in adolescent and young adult carriers in the immunotolerant phase of infection tend to be lower, ranging from 10^7 to 10^9 copies per milliliter[15,16]. Some studies explain the declining of virus titers during the time in the immunotolerant phase by a low but persistent immune destruction of infected cells by the cytotoxic T-cell, leading to an adaptive immune response over time[14].

IMMUNOTOLERANT PHASE: MANAGEMENT OPTIONS AND DEBATES

Of particular concern is the fact that until now there is no drug therapy that is actually effective in achieving a sustained response against HBV in the immunotolerant phase[17].

The currently approved treatment options for chronic HBV infection are interferon and nucleoside analogues (NA). Interferon acts primarily as an immunomodulatory agent, while NAs have essentially antiviral effects. According to current consensus and guideline statements, treatment candidates are patients with active liver disease characterized by persistently elevated ALT levels and detectable HBV DNA (10^5 copy/mL) by most commercial assays, irrespective of their HBeAg/Ab status. These statements also concluded that HBeAg-negative inactive carriers do not need any treatment because of the absence of viral replication and liver injury. Also, patients in the immunotolerant phase should be followed up without treatment[18,19].

However, and in the light of the Risk Evaluation of Viremia Elevation and Associated Liver Disease study, a baseline high HBV DNA level was associated with a significant risk of hepatocellular carcinoma[20]. These results led to the debate on whether a HBV infected person with normal liver enzymes, unremarkable liver histology, but with a detectable level of HBV DNA (high or low regardless the cutoff), should be treated with antiviral drugs or not[20,21].

As a rule, most of the current guidelines recommend that patients with moderate/severe inflammation or bridging fibrosis/cirrhosis must be treated. Also, they recommend liver biopsy for the grey zone of patients who do not meet the typical criteria, have a detectable level of HBV DNA and/or fluctuating or persistently elevated ALT. The presence of significant inflammation or bridging fibrosis/cirrhosis is an indication for treatment[22,23].

Hence, and in the light of the previous statements, we can assume that there are two options regarding the management of the immunotolerant phase; the "why wait" view and the "close follow up" view.

The "why wait" view adopts the option to treat all patients with a persistently high level of viral replication regardless of the phase of infection and relying only on the presence of detectable DNA levels. They rely on the

high risk of cancer/cirrhosis development, considering the infection as not totally benign[24]. Therefore, earlier treatment intervention may be beneficial in preventing disease progression. A recently published study aimed to break this tolerance in children by treating a group of HBV-infected children in the immunotolerant phase with lamivudine and interferon and comparing them to an untreated group. They reported a cure rate in more than one-fifth of the studied cohort, a figure that is still primitive and not high[25].

On the opposing side, another strong option exists and adopts the view of "wait and observe". This view relies on some evidence. The first is the evidence of the benign long term course of the immunotolerant phase[26]. The second is the pooled results of poor response to antiviral therapy in this unique phase, which hardly reaches 19%[27]. The third is the proved emerging resistance on long term therapy[28]. The last is a heavy cost burden of treatment.

Wong *et al*[29] studied the risk of liver fibrosis progression in HBeAg-positive patients at different phases by recruiting two hundred and forty-seven HBeAg-positive patients without advanced fibrosis at baseline. They found that liver fibrosis progression is uncommon in HBeAg-positive patients and hence their results enforce the follow-up strategy.

As is known, the degree of fibrosis or inflammation on liver biopsy cannot be predicted by the level of HBV-DNA and ALT is also considered an imperfect surrogate marker for liver disease[30]. Therefore, without evidence of normal liver histology, the definition of immunotolerant disease depends mainly on the persistence of a normal ALT level as a major determinant. Nevertheless and unfortunately, the definition of a "normal" ALT level has been redefined several times and was subjected to a strong debate. The study of Prati *et al*[31] modified the normal upper limit for ALT to be 30 IU/mL for men and 19 IU/mL for women. Re-introducing these relatively low figures will endorse many more patients under the umbrella of raised ALT levels.

The most appropriate way to make this miss clear cut is to perform a liver biopsy. However, there are still some unanswered questions; *e.g.*, what is the optimal timing of liver biopsy during the natural history of this phase, how many times and at what intervals should it be done, which drug is the best to start with, *etc.*

The use of therapeutic vaccines may also help to break the tolerance. In spite of its preliminary application, the published results of the study of Buchmann *et al*[32] carry a great hope for a wide future applicability. They evaluated the potential use of a novel vaccine formulation, comprising particulate hepatitis B surface and core antigen and the saponin-based adjuvant, for its ability to stimulate T and B cell responses in C57BL/6 mice. Their results were promising and future intense research in this subject is deemed to be mandatory.

CONCLUSION

The immunotolerant phase of chronic HBV is a chal-

lenging problem, with an increasing awareness of its occurrence, especially in endemic areas. More intense studies are required for a better delineation of the pathogenesis and whether it is better is to break the tolerance or to wait for the natural clearance. Until then, the most suitable solution is to perform liver biopsy to stand on solid ground in choosing the best option, to wait or to interfere.

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Melatonin attenuates cisplatin-induced HepG2 cell death *via* the regulation of mTOR and ERCC1 expressions

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Abstract

AIM: To elucidate the effects of melatonin on cisplatin-induced hepatocellular carcinoma (HepG2) cell death and to identify potential cross-talk pathways.

METHODS: Hepatocellular carcinoma HepG2 cells were treated with melatonin and/or cisplatin for 24 to 48 h. Cell viability and the 50% cytotoxic concentration (CC_{50}) were calculated by MTT assays. The effects and intracellular events induced by the selected concentrations of melatonin (1 mmol/L) and cisplatin (20 µmol/L) were investigated. Cell death and survival detection were primarily evaluated using a fluorescence microscope to assess 4',6 diamideno-2-phenylindol DNA staining and acridine orange lysosome staining and then further analyzed with immunocytochemistry using an anti-LC3 antibody. The potential molecular responses mediated by melatonin against cisplatin after the combined treatment were investigated by reverse transcription-polymerase chains reaction and Western blot analyses of the genes and proteins associated with cell survival and death. A cell cycle analysis was performed using a flow cytometry assay.

RESULTS: Melatonin had a concentration-dependent effect on HepG2 cell viability. At 1 mmol/L, melatonin significantly increased the cell viability percentage and decreased reactive oxygen species production due to cisplatin. Melatonin reduced cisplatin-induced cell death, decreasing phosphorylated p53 apoptotic protein, cleaved caspase 3 and Bax levels but increasing anti-apoptotic *Bcl-2* gene and protein expression. When combined with cisplatin, melatonin induced S phase (DNA synthesis) cell cycle arrest and promoted autophagic events in HepG2 cells. Melatonin also had a concentration-dependent effect on Beclin-1 and its autophagic regulator mammalian target of rapamycin (mTOR) as well as the DNA excision repair cross complementary 1 (ERCC1) protein. The expression levels of these proteins were altered in HepG2 cells during cisplatin or melatonin treatment alone. In the combination treatment, melatonin reversed the effects of cisplatin by suppressing the over-expression of mTOR and ERCC 1 and enhancing the expression levels of Beclin-1 and microtubule-associated protein-light chain3-Ⅱ, leading to intracellular autophagosome progression.

CONCLUSION: Melatonin attenuated cisplatin-induced cell death in HepG2 cells *via* a counter-balance between the roles of apoptotic- and autophagy-related proteins.

Core tip: Melatonin has anti-oxidative stress and anti-

proliferative effects on cisplatin-treated hepatocellular carcinoma cells through a counter-balance between the roles of apoptosis and autophagy proteins. Melatonin also reduced cisplatin-induced DNA damage by decreasing the activation of excision repair cross complementary 1 in the DNA repair system. Thus, co-treatment with melatonin to ameliorate cisplatin adverse effects might be beneficial for Hepatocellular carcinoma therapy.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the leading causes of human death worldwide. Aberrant gene expression and mutations induced by any genotoxic agents that can cause DNA damage contribute to its development. Effective treatment for HCC is not yet available because all anticancer strategies have limitations associated with the tumor grade and stage of the disease. For example, chemotherapy is limited by the side effects of cytotoxic drugs that also kill normal cells and by the long-term side effects of these drugs, which are carcinogenic and can cause secondary cancers[1]. Moreover, the mechanisms controlling the responses of pro-oncogenes and tumor suppressor genes in the effected cells are not well defined. In cancer research, the human hepatocellular carcinoma (HepG2) cell line has been used as a model system for studies of hepatocarcinogenesis because it is a permanent cell line derived from a well-differentiated hepatocellular carcinoma patient. The HepG2 cell line has also been used as an in vitro model for studying liver metabolism and in drug targeting assays[2].

To date, several anticancer drugs have been used clinically. Cisplatin, a platinum-based drug that has a known molecular mechanism of action, is a drug of choice that has been widely chosen for the treatment of solid cancers[3,4]. Cisplatin platinum can form complexes with DNA, inducing DNA adducts and damage[5]. Once produced, the complex mediates a series of intracellular responses that lead to apoptosis and also activates the DNA repair system, which is capable of inducing recovery of the damaged DNA and participates in the restoration of normal cell systems. However, if the damage is too extensive, the repair mechanism fails, and the cell undergoing apoptosis dies[6]. Through this pathway, cisplatin has no selective effects on cancers or normal cells. When receiving chemotherapy with cisplatin, some individuals respond by showing excessive side effects, and some cancers develop resistance to cisplatin by mechanisms that are only partially known. To improve chemothera-

peutic efficiency, investigators have emphasized the need to search for new drugs. An example would be to study the potency of cisplatin at low concentrations, which can protect against hepatotoxicity, and to study drug resistance mechanisms to identify a new drug to replace cisplatin.

Melatonin is a hormone that is produced in the pineal gland and has several normal physiological functions in the human body. Its known properties include circadian rhythm regulation[7], sleep induction[8], immunemodulation[9], neuroprotection[10], bone differentiation[11], and anti-microbial[12] and anti-oxidative effects[13]. Previous investigations have demonstrated that melatonin also possesses anti-proliferative effects, especially in cell lines derived from various malignancies such as lymphoma[14], prostate cancer[15], melanoma[16] and hepatocellular carcinoma[17]. Although most of the biological effects of melatonin are produced through the activation of melatonin receptors, some are due to its status as a powerful antioxidant that plays roles in the protection of nuclear and mitochondrial DNA[18]. The anti-proliferative effect of the human osteosarcoma cell line MG-63 was shown to be activated by melatonin if the concentration of melatonin reached an optimal value, which was a high concentration of 4-10 mol/L[19]. Further data have suggested that melatonin could be used as an adjuvant to increase responses to anticancer drugs and to ameliorate their side effects[20-22]. However, the effectiveness of melatonin as an adjuvant of chemotherapy most likely differs among cancer types, and the mechanisms of the action of melatonin are still unclear. Due to the selective effects of melatonin, the current oncology research aiming to enhance the apoptosis effect of cisplatin combined with melatonin has extended to alternative pathways in treated cells.

It is well accepted that cells under oxidative stress or exposed to DNA damage, hypoxia, nutrient deprivation, and intracellular pathogens can survive through an autophagy pathway[23]. This pathway involves the lysosomal degradation of cytoplasmic organelles or cytosolic components, allowing cells to eliminate damaged or harmful components and also to recycle the released amino acids and energy to maintain cellular homeostasis[24]. In mammalian cells, the pathway is regulated through a serine/threonine protein kinase called mammalian target of rapamycin (mTOR)[25]. Normally, mTOR is activated under nutrient-rich conditions and inhibits autophagy[26]. However, under stress conditions, mTOR plays a role in autophagy activation *via* an effector Beclin-1 that initiates core nucleation for autophagy formation[27]. Under the normal condition, Beclin-1 is inhibited by an interaction with the Bcl-2/Bcl-xL complex, but when p53 binds to Bcl-2, it frees Beclin-1, leading to autophagy[28]. In addition to losing collaborative proto-oncogenes and tumor-suppressor genes during DNA damage, the cells expressed several efficient DNA repair systems, such as the nucleotide excision repair (NER) pathway, to prevent cancer formation[29]. NER can eradicate a broad spectrum

of DNA damage lesions through the action of a specific endonuclease enzyme named excision repair cross complementary 1 (ERCC1), which functions at the incision step of the NER pathway. ERCC1 cleaves damaged DNA at upstream sites, leading to DNA re-synthesis and ligation to return the damaged DNA to its native state and configuration[30]. Therefore, increased or decreased levels of ERCC1 expression should indicate efficiency of the DNA repair system.

In this study, we hypothesized that autophagy is an important pathway that plays roles in the outcome of cell death or survival in HepG2 cell during cisplatin and melatonin chemotherapy. We explored the expression of genes and proteins that regulate autophagy processes and lead to cell death and also identified the possible mechanism or cross-talk pathways mediated by melatonin and cisplatin.

MATERIALS AND METHODS

Cell culture

A HepG2 cell line was purchased from the American Type Culture Collection (Rockville, MD, United States). The cells were cultured at 37 ℃ in a humidified 5% CO_2 incubator and maintained in DMEM supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) non-essential amino acids, 1% (v/v) sodium pyruvate, and 100 Units/mL penicillin-streptomycin were purchased from Thermo Fisher Scientific (Waltham, MA, United States).

Cell viability assay

HepG2 cells were seeded onto 96-well plates (2×10^4 cells/well) for 24 h and then treated with 0.5-5.0 mmol/L melatonin (Merck, Frankfurter , Germany), 2.5-80.0 µmol/L cisplatin (Sigma Aldrich, St. Louis, MO, United States), or the combination of both for 24 and 48 h. In the combination treatment, the selected concentration was based on the minimal concentration that induced the anti-proliferative effect of melatonin and the most tolerable concentration that induced the cytotoxic effect of cisplatin. Cell viability was measured using an MTT colorimetric assay; MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Sigma Aldrich (St. Louis, MO, United States), a working solution was added to each well and incubated at 37 ℃ for 2 h. The optical density of each well was measured using a microplate reader at 570 nm and the reference wavelength of 690 nm. Cell viability was calculated as the percentage of viable cells in the drug-treated group versus the untreated control group. The concentration of the compound that decreased cell viability by 50% cytotoxic concentrations (CC50) was calculated. Each experiment was performed in triplicate, and each result was presented as the mean ± SE.

Measurement of intracellular reactive oxygen species production

HepG2 cells were seeded onto 96-well black, flat, clear-bottom plates (2×10^4 cells/well). After treatment, the intracellular levels of ROS were measured by staining the treated and untreated cells with 50 µmol/L 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) that was purchased from Sigma Aldrich (St. Louis, MO, United States) after incubation in the dark at 37 ℃ for 30 min. The fluorescence intensity was quantified by the relative percentage of untreated cells using a fluorescence microplate reader and used 500 µmol/L H_2O_2-treated cells as the positive control. Each experiment was performed in triplicate, and each result was presented as the mean ± SE.

4',6 diamideno-2-phenylindol DNA stain and acridine orange lysosome stain

HepG2 cells were seeded onto 24-well plates (2×10^4 cells/well) for 24 h and then treated with 1 mmol/L melatonin, 20 µmol/L cisplatin, or both for 24 and 48 h.

DNA stain: Treated cells were fixed with cold methanol, air dried, and stained with 4'-6-diamidino-2-phenylindole, (DAPI) (Boehringer, Ingelheim, Germany) at 37 ℃ for 30 min. The primary signs of apoptosis induction, namely, chromatin condensation, fragmented DNA, and/or apoptotic body formation, were evaluated under an inverted fluorescent microscope.

Acridine orange stain: Treated cells were stained with 5 µg/mL acridine orange (Sigma Aldrich, St. Louis, MO, United States) in serum-free medium at 37 ℃ for 15 min. The cells were examined under an inverted fluorescence microscope. Positive acidic vacuoles or stained lysosomes were observed as orange or red foci in the cytoplasm, and DNA bound-acridine orange was detected as a green signal.

Immunocytochemistry

To determine an autophagic event, treated cells grown on coverslips were fixed in 4% paraformaldehyde for 5 min, permeabilized with methanol, and blocked with 3% bovine serum albumin in phosphate buffer saline (PBS) for 1 h at room temperature. The cells were then incubated with an indicator of autophagy, an anti-LC3 antibody was purchased from Cell Signaling Technology (Danvers, MA, United States) and was diluted 1:400 in PBS, overnight at 4 ℃. After incubation, the cells were washed with PBS and incubated with an Alexa Fluor 555-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA, United States) for 2 h at room temperature, washed with PBS, and counter-stained with DAPI. The immunofluorescently labeled cells were visualized under a confocal laser-scanning microscope (Olympus, Tokyo, Japan).

Semi-quantitative analysis (RT-PCR) of genes controlling apoptosis and autophagy

HepG2 cells were seeded onto 6-well plates (4×10^5 cells/well). After treatment with 1 mM melatonin, 20 µmol/L cisplatin, or both for 24 h, total RNA was extracted with TRI Reagent (Molecular Research Center Inc; Cincinnati, OH, United States). cDNAs were synthesized by RevertAid™ M-MuLV Reverse Transcriptase and amplified by polymerase chain reaction (PCR) with

Table 1 Genes of interests and the primer pairs for polymerase chain reaction

Genes	Function	Primers sequence (5'-3')	Size (bp)
Bax	Pro-apoptosis	F- AAAGCTAGCGAGTGTCTCAAGCGC R-TCCCGCCACAAAGATGGTCACG	366
Bcl-2	Anti-apoptosis	F-TTGTGGCCTTCTTTGAGTTCG R-TACTGCTTTAGTGAACCTTTT	332
mTOR	Autophagic inhibition	F-TCTCATGGGCTTCGGAACAA R-GTGAAGGCAGAAGGTCGGAA	318
ERCC1	DNA repair	F-CCCTGGGAATTTGGCGACGTAA R-CTCCAGGTACCGCCCAGCTTCC	273
GAPDH	House keeping	F-CATCACCATCTTCCAGGAGC R-CATGAGTCCTTCCACGATACC	307

mTOR: Mammalian target of rapamycin; ERCC1: Excision repair cross complementary 1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

specific primer pairs, as indicated in Table 1, using a High Fidelity PCR kit (Thermo Fisher Scientific, Waltham, MA, United States). The PCR cycles were adjusted accordingly to primer annealing. The PCR products were analyzed by 1.5% agarose gel electrophoresis and gel staining with ethidium bromide for 30 min. The gels were photographed using gel documentation (UVP Bioimaging System, United States). The experiments were performed in triplicate, and the relative band densities of cDNA from the treated cells were compared to the untreated cells.

Western blot analysis of proteins associated with apoptosis and autophagy

The treated cells were lysed in RIPA buffer (25 mmol/L Tris-HCl pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and protease inhibitor cocktail were purchased from Merck (Frankfurter, Germany) on ice for 30 min. The samples were then centrifuged at 24000 *g* at 4 ℃ for 15 min. The cell lysates were collected, and the total protein concentrations were determined by a BCA assay (Merck, Frankfurter, Germany). Equal amounts of protein (30 μg) from each sample were separated by 8%-15% SDS-polyacrylamide gel electrophoresis at 100 V and transferred onto nitrocellulose membranes (GE Healthcare, Buckinghamshire, United Kingdom). The membranes were blocked with 3% w/v bovine serum albumin (Sigma Aldrich, St. Louis, MO, United States) in Tris-buffered saline containing 0.1% Tween-20 for 2 h at room temperature. Then, the membranes were incubated with a 1:1000 dilution of primary antibodies against Bax (Santa Cruz, CA, United States), p53, phospho-p53, Bcl-2, pro-caspase3, cleaved-caspase3, p-mTOR, ERCC1, Beclin-1 or LC3 (Cell Signaling Technology, Danvers, MA, United States) overnight at 4 ℃, followed by two extensive washings with TBST. The membranes were incubated with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody for 2 h. The specific bands corresponding to the investigated proteins were visualized using enhanced chemiluminescence (ECL reagents, GE

Healthcare, Buckinghamshire, United Kingdom). The signal intensities were determined by densitometry using Image-J software (National Institutes of Health, Bethesda, MD, United States). After stripping off the first probe, each membrane was re-probed with a β-actin antibody (Santa Cruz, CA, United States) to confirm the equal loading of protein in each experiment. The levels of protein expression were presented in relation to β-actin.

Flow cytometry analysis of DNA synthesis and cell cycle activity

HepG2 cells were seeded onto 6-well plates (4×10^5 cells/well). After treatment with 1 mmol/L melatonin, 20 μmol/L cisplatin, or a combination of both for 24 h, the cells were trypsinized, centrifuged, washed twice with cold PBS, and fixed overnight with 70% ethanol at -20 ℃. The cells were resuspended in staining buffer containing 50 μg/mL propidium iodide (Merck, Frankfurter, Germany), 3.8 mmol/L sodium citrate, and 50 μg/mL RNase A (Sigma Aldrich, St. Louis, MO, United States) and incubated in the dark at 37 ℃ for 30 min. The stained cell suspensions were processed for flow cytometry analysis to determine the amount of DNA at different phases of the cell cycle using a FACScanto apparatus (BD Pharmingen, San Diego, CA, United States) with the loaded software. A total of 30000 cells from each sample were collected for evaluation of each data file.

Statistical analysis

All experiments were performed in triplicate ($n = 3$). Data are presented as the mean ± SE for each group, and these were compared for significant differences using a one-way analysis of variance test, followed by a post-hoc analysis (Tukey's multiple comparison test) using Prism 5 (GraphPad Software Inc; San Diego, CA, United States).

RESULTS

Effect of melatonin and cisplatin on HepG2 cell viability

As shown in Figure 1, melatonin (concentration 0.5-5 mmol/L) and cisplatin (concentration 2.5-80 μmol/L) reduced the viability of HepG2 cells in a time-concentration dependent manner. The 50% CC_{50} of melatonin were 6.25 mmol/L and 3.74 mmol/L and of cisplatin were 38.53 μmol/L and 17.53 μmol/L, at 24 h and 48 h, respectively. The concentrations of melatonin and cisplatin used in the combination treatment were selected from the minimal anti-proliferative effect of melatonin, which significantly decreased percent cell viability, and the most tolerable cytotoxic effect of cisplatin, which induced cell death at a rate below 50%. Therefore, 1 mmol/L melatonin was used in combination with 20 and 30 μmol/L cisplatin for 48 h. Melatonin increased the viability of HepG2 cells compared with cisplatin treatment alone. The combined treatment significantly reduced cell viability compared with the control and significantly increased cell viability compared with the cisplatin treatment alone

Figure 1 Effect of melatonin and cisplatin on cell viability analyzed using an MTT assay. Hepatocellular carcinoma (HepG2) cells were treated with various concentrations of melatonin and cisplatin for 24 h and 48 h. Both melatonin and cisplatin reduced the percent viability of HepG2 cells in a time and concentration-dependent manner ($P < 0.001$). The experiments were performed in triplicate, and the results are presented as the means ± SE.

Figure 2 Effect of the combined treatment of melatonin and cisplatin on cell viability. Hepatocellular carcinoma cells were treated with melatonin (1 mmol/L) and/or cisplatin (20 and 30 μmol/L) for 48 h and then analyzed by an MTT assay. Melatonin reduced the cisplatin cytotoxic effect at 20 μmol/L cisplatin. The results are presented as the mean ± SE. ([c]$P < 0.001$ compared with the control group, [d]$P < 0.05$, [f]$P < 0.001$ compared with cisplatin-treated group).

(Figure 2).

Potential mechanisms and pathways of the melatonin-mediated attenuation of cisplatin-induced cell death

Anti-ROS production: To determine whether melatonin reduced the ROS production due to cisplatin, the biochemical basis of intracellular ROS was explored using a fluorescein-labeled dye, DCFH-DA. The results showed significantly increased intracellular ROS in cisplatin-treated cells. However, the combined treatment with melatonin (1 mmol/L) reduced the intracellular ROS level (Figure 3).

Reduction of cellular damage and apoptosis formation: The combined effect of melatonin and cisplatin

Figure 3 Melatonin reduced cisplatin-induced intracellular reactive oxygen species levels. Hepatocellular carcinoma cells were treated with melatonin (1 mmol/L) and/or cisplatin (20 μmol/L) for 24 h. The results are presented as the means ± SE. ([c]$P < 0.001$ compared with the control group, [d]$P < 0.05$ compared with the cisplatin-treated group).

was evaluated in unstained in HepG2 cells under an inverted light microscope (Figure 4A) and in HepG2 cells stained with DAPI under a fluorescence microscope (Figure 4B). Melatonin treatment resulted in no morphological changes and the absence of apoptotic nuclei. Cisplatin treatment induced intense morphological changes, with cell shrinkage and typical apoptotic nuclear features, such as nuclear condensation and apoptotic bodies. The combined melatonin and cisplatin treatment revealed unchanged cell morphology and a reduction of apoptotic bodies.

Regulation of apoptosis *vs* anti-apoptosis genes and protein expression: To determine apoptotic events in cells, the expression of the genes and proteins involved in apoptosis were analyzed. As shown in Figure 5, Western blotting results indicated that melatonin (1 mmol/L) had no significant effect on proteins associated with apoptosis (p53, p-p53, pro-caspase3 and cleaved-caspase3), whereas cisplatin significantly increased p53 and p-p53 levels.

Figure 4 Histological changes. Hepatocellular carcinoma cells were treated with melatonin (1 mmol/L) and/or cisplatin (20 μmol/L) for 48 h. The cells were stained with DAPI for fragmented DNA (apoptotic bodies) and observed under an inverted microscope. A: Morphological changes showing vacuoles in the cytoplasm of cisplatin-treated cells (scale bar = 50 μm); B: DAPI staining showing nuclear condensation and apoptotic bodies (white arrows) in cisplatin-treated cells. The combined treatment showed less apoptotic effects (scale bar = 100 μm).

Gene and protein expression analyses of pro-apoptosis Bax and anti-apoptosis Bcl-2 levels revealed slight decreases in the Bax/Bcl-2 ratio with melatonin treatment while resulting in a significantly increased Bax/Bcl-2 ratio with cisplatin treatment. In the combined treatment, melatonin significantly ameliorated the pro-apoptotic effects of cisplatin.

Cell cycle regulation: To determine the effects of the treatments on HepG2 cell cycle control, DNA accumulation was measured by flow cytometry at different phases of the cell cycle. The phases of cell cycle arrest were determined at G_0/G_1 by melatonin and at $subG_0$, G_0/G_1, and S by cisplatin. In the combined treatment, melatonin reduced cisplatin cell cycle arrest at the $subG_0$ through G_0/G_1 phases but significantly increased cell cycle arrest at the S phase (Figure 6).

Autophagy regulation: The combined effect of melatonin and cisplatin in HepG2-treated cells was evaluated for lysosomal staining with acridine orange dye and observation under an inverted microscope to measure fluorescence. Both melatonin and cisplatin caused slight increases in the acidic lysosomal compartments; an increase in acridine orange intensity was highly apparent in the combined treatment (Figure 7A). In the immunofluorescence assay with the anti-LC3 antibody, melatonin and cisplatin treatment alone induced minor autophagy events in HepG2 cells compared with the control. However, the combination of melatonin and cisplatin treatment significantly enhanced autophagy, as indicated by the fluorescence intensity of LC3 scattered throughout the cytoplasm (Figure 7B).

Alteration of autophagy regulators and the DNA repair system: To explore the potential pathways of melatonin and cisplatin effects on autophagy regulation and DNA repair process in HepG2-treated cells, the relationship between the proteins Beclin-1, mTOR and ERCC1 were analyzed. Melatonin (0.5 and 1 mmol/L) increased Beclin-1, p-mTOR, and ERCC1 in a concentration-dependent manner (Figure 8). Conversely, cisplatin (10 and 20 μmol/L) decreased Beclin-1, p-mTOR, and ERCC1 in a concentration-dependent manner. At the selected concentrations (1 mmol/L melatonin and 20 μmol/L cisplatin), melatonin significantly increased p-mTOR and ERCC1 from the basal levels, whereas cisplatin decreased Beclin-1 and increased p-mTOR and ERCC1 from the basal levels.

Evaluation of the autophagy process in melatonin- and cisplatin-treated HepG2 cells showed changes in Beclin-1 (an initiator of autophagy) and LC3-Ⅱ (a mature marker of autophagy). Melatonin (1 mmol/L) treatment alone slightly increased both Beclin-1 and LC3-Ⅱ, but cisplatin (20 μmol/L) treatment alone decreased Beclin-1 and slightly increased LC3-Ⅱ (Figure 9). The combined treatment showed that melatonin prevented cisplatin from affecting the level of Beclin-1 but significantly increased the effect of cisplatin on the LC3-Ⅱ level.

The analysis of mTOR and ERCC1 mRNA and protein expression in the treated HepG2 cells revealed that melatonin (1 mmol/L) or cisplatin (20 μmol/L) treatment alone significantly increased p-mTOR and ERCC1 from the basal levels. However, the combined treatment resulted in the suppression of both p-mTOR and ERCC1 compared with the basal cellular levels (Figure 10). (See a schematic overview of the results in Figure 11)

DISCUSSION

Cisplatin chemotherapy has been used to kill several solid cancer cells with satisfactory results. However, due to

Figure 5 Reverse transcription-polymerase chain reaction and Western blot analyses of apoptosis regulators in treated hepatocellular carcinoma cells. Melatonin had minor effects on all apoptosis markers, whereas cisplatin significantly increased apoptosis via the activation of p53 and caspase3 and the Bax/Bcl-2 ratio. Melatonin reduced cisplatin effects in the combined treatment. ([a]$P < 0.05$, [c]$P < 0.001$ compared with the control group, [d]$P < 0.05$, [e]$P < 0.01$ compared with the cisplatin-treated group).

Figure 6 Effects of melatonin and cisplatin on the cell cycle, as evaluated using flow cytometric analysis. Hepatocellular carcinoma (HepG2) cells were treated with melatonin (1 mmol/L) and/or cisplatin (20 μmol/L) for 24 h. Melatonin induced DNA accumulation in the HepG2 cells at the G_0/G_1 phase, whereas cisplatin affected cell cycle arrest at the sub-G_0, G_0/G_1, and S phases. In the combined treatment, melatonin decreased the cisplatin effect on cell cycle arrest at the sub-G_0 through G_0/G_1 phases but significantly increased S phase cell cycle arrest.

Figure 7 Autophagic detection by acridine orange staining and immunofluorescence. Hepatocellular carcinoma (HepG2) cells were treated with 1 mmol/L melatonin and/or 20 μmol/L cisplatin for 24 h and evaluated for autophagy formation. The experiments were performed in triplicate, and representative micrographs are shown. A: Acridine orange staining showed lysosomal (red or orange) staining in the cells of all treatments. The increased acidic lysosomes in the combination treatment suggests potential lysosomal activation (scale bar = 20 μm); B: Confocal immunofluorescence micrographs of representative treated-HepG2 cells immuno-labeled with the anti-LC3 antibody (scale bar = 10 μm). Melatonin and cisplatin treatment alone induced some fluorescent immunoreactivity in the cytoplasm, but the combined treatment induced intense immunoreactivity.

its efficient cytotoxic effects, cisplatin produces adverse drug effects and chemo-resistance. Therefore, adjuvant therapy with melatonin has been proposed, and extensive studies were performed, with variable outcomes[22]. In the

Figure 8 Analysis of autophagy and DNA repair-related proteins. Hepatocellular carcinoma cells were treated with various concentrations of melatonin and cisplatin. At the selected concentrations, 1 mmol/L melatonin and 20 μmol/L cisplatin have a similar effect by significantly increasing p-mTOR and ERCC 1 from the basal levels. However, the effect on Beclin-1 was the opposite, with melatonin increasing Beclin-1 and cisplatin decreasing Beclin-1 from the basal level. The results are presented as the mean ± SE. ([a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ compared with the control group). ERCC1: Excision repair cross complementary-1.

Figure 9 Alteration of autophagy-forming proteins. Hepatocellular carcinoma cells were treated with melatonin and/or cisplatin for 24 h. Melatonin slightly increased Beclin-1 and LC3-Ⅱ, whereas cisplatin significantly decreased Beclin-1 and slightly increased LC3-Ⅱ. Melatonin inhibited the cisplatin effect on Beclin-1 but increased LC3-Ⅱ in the combined treatment. ([c]$P < 0.001$ compared with the control group, [f]$P < 0.001$ compared with the cisplatin-treated group).

present study, we set out to determine whether melatonin could mediate a protective effect against cisplatin-induced apoptosis using HepG2 cells and attempted to identify the potential molecular pathways of melatonin action. The results demonstrated that the overall effect of this adjuvant in chemotherapy depends on a number of melatonin effects on cells. When melatonin was combined with cisplatin in the HepG2 cell line, the expression of the apoptosis mediators phosphorylated p53, cleaved caspase 3, and Bax decreased, whereas anti-apoptotic Bcl-2 increased. These biochemical results correlated well with decreases in the intracellular ROS level and the recovery of cell morphology. The results indicated the anti-

oxidative stress property of melatonin, which directly scavenges reactive oxygen species induced by cisplatin anticancer drugs[31,32], thus reducing adverse cisplatin drug effects in hepatocytes.

In addition to the extended results from the alteration of the anti-apoptotic Bcl-2 level, we found that melatonin decreased the expression ratio of Bax/Bcl-2, which was affected by cisplatin. Thus, an apoptosis event in the combined treatment was switched to cell survival. The expression of p53 protein was also subjected to a switch by the melatonin and cisplatin combination, suggesting a direct effect of both on the cell cycle. The pro-death effect of cisplatin, which was mostly mediated by p53, indicated

Figure 10 RT-PCR and Western blot analyses of autophagy regulators. Both melatonin and cisplatin increased p-mTOR and ERCC 1 levels in the HepG2 cells, but the combined treatment resulted in the suppression of both proteins. ([b]$P < 0.01$, [c]$P < 0.001$ compared with the control group, [e]$P < 0.01$, [f]$P < 0.001$ compared with the cisplatin-treated group). ERCC1: Excision repair cross complementary-1.

Figure 11 Schematic overview of the major findings in the combination group.

that the cell cycle specificity of cisplatin in HepG2 cells

affected G_0/G_1 phase. In the combined treatment, the cell

cycle arrest was changed to the S and G₂/M phases. Therefore, melatonin must have increased the cisplatin effect on cell cycle arrest through inhibition at the S (DNA synthesis) and M (mitosis) phases. These are considered the most anti-proliferative (oncostatic) effects of melatonin. However, the cell cycle arrest by melatonin in the treated cells could also be mediated by other cell cycle control proteins, such as the calcium/calmodulin pathway[33] and autophagy regulation pathway. Both pathways are related to the anti-proliferation of cells and pro-survival.

In our study, DNA damage may have been induced in HepG2 cells by the cisplatin genotoxic chemical and ROS by-products of cell metabolism[34]. The subsequent response reactions include the activation of a DNA damage checkpoint, which arrests cell cycle progression and leads to apoptosis[35]. We demonstrated that there was a survival switch from cisplatin-induced apoptosis to autophagy in the combination treatment with melatonin. The indicators included acridine orange staining, an immunohistochemistry assay for autophagy, and the measurement of mRNA and proteins associated with autophagy [*i.e.*, mTOR, Beclin-1, and chain3-Ⅱ (LC3-Ⅱ)]. Compared with cisplatin treatment alone, the combination treatment showed a high intensity of positive fluorescence staining in the lysosomal compartment of the cell, suggesting increased autophagy activity. Cisplatin acted on p53 and Bcl-2 to induce apoptosis, but in the combined treatment, Bcl-2 was decreased to an extent lower than the basal level. This could occur through reduced binding between the Bcl-2/Bcl-xL complex and Beclin-1, followed by a release of Beclin-1 to initiate autophagy formation. Thus, the combined treatment could change apoptosis to autophagy.

The basal levels of Beclin-1 and mTOR, major regulators of autophagy, were evaluated in HepG2 cells. Cisplatin treatment alone up-regulated mTOR and down-regulated Beclin-1 expression compared with the basal levels, thus decreasing autophagy and promoting the apoptosis pathway. Melatonin treatment alone up-regulated both mTOR and Beclin-1 expression compared with the basal levels, and this up-regulation by melatonin played a significant role in the survival of HepG2 cells. In the combined treatment, melatonin caused autophagy through the final formation of the autophagosome and was shown by an increased microtubule-associated protein-light LC3-Ⅱ level via the up-regulation of p-mTOR and Beclin-1. This changed the apoptotic signal caused by cisplatin. Overall, melatonin attenuated cisplatin-induced HepG2 cell death by inducing an autophagy survival signal[36].

A novel autophagy regulation mediated by melatonin was identified in this study. Both melatonin and cisplatin exhibited concentration-dependent effects on mTOR, Beclin-1, and DNA ERCC1. ERCC1 is a marker of DNA repair, whereby ERCC1 is the rate-limiting enzyme in the NER system, a pathway known to remove cisplatin lesions from DNA[37]. The analysis of protein expression in this study revealed that increases in ERCC1 from the basal levels in HepG2 cells by each

treatment were associated with an increase in mTOR expression, suggesting a tight relationship between DNA repair and autophagy. We also found that both mTOR and ERCC1 were significantly decreased by melatonin induction in the co-treatment group compared with the cisplatin-treated group. However, the only slight reduction in ERCC1 in our assay using rapamycin as an inhibitor of mTOR demonstrated that the melatonin effect on ERCC1 was not directly to the mTOR activation pathway (data not included). Therefore, melatonin reduced cisplatin-induced DNA damage, resulting in decreased activation of the DNA repair capacity and might be involved in transcriptional regulation or an epigenetic mechanism. As previously shown, p-p53 is a transcription factor for apoptotic gene expression and also plays a role in DNA repair via the subsequent up-regulation of genes in the NER pathway[38-40]. AP-1 is a transcription factor for autophagy and ERCC1 regulation in DNA damage pathways[41]. In the combined treatment, melatonin and cisplatin may encounter transcription inactivation. That is, after cisplatin treatment, DNA damage occurs, and ERCC1 is induced. An increased level and activation of mTOR induced by melatonin counter-balances the role of p-p53 in the DNA damage and repair process induced by cisplatin. This finding is the first report that melatonin can activate ERCC1 in a concentration-dependent manner.

Interestingly, significant decreases in both mTOR and ERCC1 compared with the basal levels were identified in the combined treatment, suggesting a positive response of the cells. The expression level of ERCC1 has been previously suggested to be a prognostic marker of cancers, and ERCC1 over-expression has been associated with to cisplatin resistance[42]. In our research, ERCC1 decreased compared with the cisplatin-treated group, indicating an improvement in cancer prognosis.

In conclusion, melatonin exerted an oncostatic effect on cisplatin-treated hepatocellular carcinoma cells via a counter-balance between the roles of apoptosis-related p53 and Bcl-2 and autophagy proteins. The alteration of Beclin-1, mTOR, and ERCC1 levels determined the pro-death and pro-survival effects of treatment in this cancer cell line. Therefore, during cisplatin treatment of hepatocellular carcinoma, the administration of a high dose of melatonin as an adjuvant in cancer therapy should ameliorate the adverse effects of cisplatin.

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COMMENTS

Background

Liver cancer is one of the major causes of death in humans worldwide due to

the limitation of drugs for treatment. Frequently, chemotherapy with platinum-based cisplatin is provided during the late stage of the disease. This drug causes excessive side effects and sometimes induces cancer drug resistance.

Research frontiers

Melatonin is a hormone produced in our body to maintain several normal physiological functions, which may be altered during the course of cancer. To reduce cisplatin side effects and still maintain the drug's efficacy, we treated human hepatocellular carcinoma (HepG2) cells with cisplatin and a high concentration of melatonin. The results showed that melatonin could reduce the oxidative stress effects of cisplatin by altering the stages of cell cycle arrest and apoptosis mediators such as p53. Melatonin switched cisplatin-induced apoptosis in HepG2 cell to autophagy-induced survival via Bcl-2, Beclin-1 and mTOR. In addition, melatonin reduced cisplatin-induced DNA damage by decreasing the activation of excision repair cross complementary-1 (ERCC1) in the DNA repair system.

Innovations and breakthroughs

Melatonin can activate DNA ERCC1 in a concentration-dependent manner. An analysis of protein expression in this study revealed that increases in ERCC1 from the basal levels in HepG2 cells by each treatment were associated with an increase in mTOR expression, suggesting a tight relationship between DNA repair and autophagy. In the combined treatment, melatonin and cisplatin may result in transcriptional inactivation between mTOR and ERCC1.

Applications

During cisplatin treatment of hepatocellular carcinoma, the administration of melatonin as an adjuvant in cancer therapy should ameliorate the adverse cisplatin effects while maintaining the drug's efficacy.

Terminology

ERCC1 is the rate-limiting endonuclease in the nucleotide excision repair (NER) pathway, which is the major pathway for removing cisplatin from the DNA strand. ERCC1 is a good predictive biomarker for cancer treatment with cisplatin. ERCC1-negative tumor patients were associated with a higher survival rate than ERCC1-positive tumor patients treated with adjuvant platinum-based regimens.

Peer review

This study has a good structure and is easy to read despite many abbreviations, even for inexperienced readers in this field. It is a very important and up-to-date subject. The work is interesting, technically correct, and well written and describes an important role for melatonin as an adjuvant to cisplatin, a therapy known to cause severe toxicity in the body. The studies were well-designed, clearly presented, highly mechanistic, and demonstrated novel pathways regulated by melatonin to improve health outcomes associated with hepatic cancer and cisplatin-induced hepatotoxicity. This study will open up new research areas in this field. The authors used myriad techniques and approaches to tackle this deadly disease.

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BRIEF ARTICLE

Murine model to study brain, behavior and immunity during hepatic encephalopathy

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Abstract

AIM: To propose an alternative model of hepatic encephalopathy (HE) in mice, resembling the human features of the disease.

METHODS: Mice received two consecutive intraperitoneal injections of thioacetamide (TAA) at low dosage (300 mg/kg). Liver injury was assessed by serum transaminase levels (ALT) and liver histology (hematoxylin and eosin). Neutrophil infiltration was estimated by confocal liver intravital microscopy. Coagulopathy was evaluated using prolonged prothrombin and partial thromboplastin time. Hemodynamic parameters were measured through tail cuff. Ammonia levels were quantified in serum and brain samples. Electroencephalography (EEG) and psychomotor activity score were performed to show brain function. Brain edema was evaluated using magnetic resonance imaging.

RESULTS: Mice submitted to the TAA regime developed massive liver injury, as shown by elevation of serum ALT levels and a high degree of liver necrosis. An intense hepatic neutrophil accumulation occurred in response to TAA-induced liver injury. This led to mice mortality and weight loss, which was associated with severe coagulopathy. Furthermore, TAA-treated mice presented with increased serum and cerebral levels of ammonia, in parallel with alterations in EEG spectrum and discrete brain edema, as shown by magnetic resonance imaging. In agreement with this, neuropsychomotor abnormalities ensued 36 h after TAA, fulfilling several HE features observed in humans. In this context of liver injury and neurological dysfunction, we observed lung inflammation and alterations in blood pressure and heart rate that were indicative of multiple organ dysfunction syndrome.

CONCLUSION: In summary, we describe a new murine model of hepatic encephalopathy comprising multiple features of the disease in humans, which may provide new insights for treatment.

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Core tip: The study of hepatic encephalopathy is crucial for development of new therapies but has been dampened by the absence of murine models resembling the disease in patients. We showed that sequential thioacetamide injections cause extensive liver injury in mice, leading to increased ammonia levels, electroencephalography alterations and brain edema. In line with this, mice presented with poor psychomotor activity and survival rate. Liver injury and brain function impairment by thioacetamide resulted in systemic alterations such as coagulopathy, hemodynamic instability and lung inflammation, consistent with multiple organ failure. Therefore, this alternative model may provide tools for new therapeutic insights for hepatic encephalopathy.

INTRODUCTION

Acute liver failure (ALF) is a rare but severe clinical syndrome. It is typically characterized by jaundice, coagulopathy and encephalopathy resulting from sudden hepatic dysfunction without preexisting liver disease[1]. Drug-induced liver injury (DILI) is the main cause of ALF in the United States and Europe[2], whereas in developing countries viral hepatitis is the most important etiology. Although liver injuries may result from a wide range of situations, drug overdose may become the most important etiology of ALF worldwide in a few years[2] as life expectancy increases and the medicalization of the older population tends to increase[3]. Moreover, the worldwide cause of ALF tends towards DILI due to increasing public health measures (*e.g.*, vaccination)[1], making it an emergent widespread problem. ALF has a high mortality rate of approximately 30% due to multiple organ dysfunction, hepatic encephalopathy and sepsis. Unfortunately, therapeutic options are very limited since liver transplantation is the only definitive therapy available. Besides that, patients are kept under clinical management and supportive care until spontaneous liver recovery[1,4].

Hepatic encephalopathy (HE) is one of the major complications in ALF because of rapid brain edema, intracranial hypertension and cerebral herniation[5]. Clinically, HE is a neuropsychiatric syndrome that comprises a wide range of signs and symptoms from subtle altered mental status to stupor and coma[6]. The severity of HE has prognostic implications since patient outcome worsens as HE progresses. There is a chance of 65%-70% of spontaneous liver recovery in mild HE and less than 20% in severe HE[7]. Moreover, patients with ALF who manifest increased intracranial pressure during the course of illness are more likely to develop sepsis[8]. Hence, understanding HE pathophysiology is crucial for development of new and specific therapies that would slow down its progression and increase chances of better outcomes.

In this sense, a murine model that reproduces features of HE in humans would be key to improve our understanding in HE pathophysiology and may also be useful for drug testing and development. Here, we propose an alternative murine model of HE using sequential systemic thioacetamide (TAA) injections in a lower dosage instead of a single higher dose administration.

MATERIALS AND METHODS

TAA induced liver failure and hepatic encephalopathy

Female C57BL/6 mice and Lysm-eGFP (eGFP-expressing neutrophils) had free access to food and water. TAA-induced liver injury protocol was based on two consecutive days of treatment with thioacetamide (300 mg/kg) administered intraperitoneally. Treated groups received the first dose in time zero and an additional dose after 24 h. Controls received vehicle following the same chronogram. Mice were sacrificed after 24 or 48 h and liver, lung, blood and brain samples were collected for further analysis. All mice received glucose replacement (12/12 h; 5%; *s.c.*) and glycemia was monitored (12/12 h) with commercially available reactive strips and a glucometer (Accu-Chek, Performa). Body temperature was maintained at 37 ℃ by a thermal pad. Psychomotor activity was graded following an adapted clinical score, in which 0 = normal behavior; 1 = mild lethargy; 2 = decreased motor activity, poor gesture control, diminished pain perception; 3 = Severe ataxia, no spontaneous righting reflex; 4 = no righting reflex, no reaction to pain stimuli; and 5 = death[9]. All procedures were approved by Animal Care and Use Committee in UFMG (CEBIO n°051/2011). The investigation conformed to the standards of Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 85-23, 1996 revision). In a separate set of experiments, the liver was imaged using confocal intravital microscopy as described previously[10]. Lysm-eGFP mice received propidium iodide (200 μL of a 100 μmol/L stock solution; *i.v*) prior to the surgical procedure in order to visualize necrotic cells.

Serum and cerebral ammonia determination

Immediately after decapitation, the brain was rapidly removed from the cranial cavity and fast-frozen in liquid nitrogen. Subsequently, brain samples were macerated using a pestle in perchloric acid (ice-cold; 1mol/L) and centrifuged at 10000 *g* for 10 min at 4 ℃. The supernatant was collected for immediate dosage. Also, blood

samples were centrifuged for 7 min (7000 *g*) and plasma was collected. Ammonia concentration was estimated using Ammonia Assay kit (Sigma, United States) following manufacturer instructions.

Alanine aminotransferase

The alanine aminotransferase enzyme is present in the cytoplasm of hepatocytes and is highly specific for the liver. The measurement of serum alanine aminotransferase (ALT) is a gold-standard marker of liver damage. To determine the activity of ALT, blood samples were centrifuged and the serum was collected and dosed using a kinetic kit (Bioclin, Brazil).

Hemodynamic measurements using tail cuff method

Mean arterial pressure (MAP) and heart rate (HR) were evaluated by a volume pressure recording sensor and an occlusion tail-cuff, which measures mice blood pressure and HR noninvasively (Kent Scientific Corporation, Torrington, CT, United States)[11]. Mice were acclimated to the restraint and tail cuff inflation for two days before the beginning of the experiments. The restraint platform was maintained at 36-38 ℃. In each session, mice were placed in an acrylic box restraint, and the tail was inserted into a compression cuff that measured the blood pressure 10 times. Following the measurement cycle, the average of these values was considered for each mouse. MAP and HR were evaluated at 0, 24 and 48 h after TAA administration.

Procedures for electroencephalography recording

Surgery procedures: Mice (*n* = 8) were anesthetized using a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). Prophylactic treatment with antibiotics (enrofloxacin; 10 mg/kg; *s.c.*) was done in order to prevent post-surgical infections. The animals were submitted to surgery for electroencephalography (EEG) electrode implantation in the right and left parietal cortices. The electrodes for superficial EEG recordings were made with surgical screws (Fine Science Tools; model 19010-00; Foster City, CA, United States) previously soldered to Teflon-coated stainless-steel wires (A&M Systems; model 7916; Carlsborg, WA, United States) and introduced bilaterally in the parietal bones. A reference electrode, also made with surgical screws, was inserted in the nasal bone. All the electrodes were soldered to a common pin connector and anchored to the cranium with dental acrylic. Following surgery, animals were allowed to recover for at least 4 days before EEG recordings.

Experimental protocol: Mice were injected with thioacetamide in the same regime (*n* = 5) or saline (*n* = 3). Each mouse was recorded in three consecutive days, namely: day 1, before drug injection; days 2 and 3, after first and second drug injections, respectively. Mice were recorded for a period of 10 min each day. Bioelectrical activity was recorded with a video-EEG recording system (8:00-11:00 am). The EEG signal from both parietal cortices was amplified (1000 × gain) and filtered (1 Hz High

pass, 500 Hz Low pass) by a signal conditioner (Aisha4 - Kananda® Ltda). Data were sampled at 1 kHz (12 bit DI-148U A/D converter - DATAQ® Instruments) and recorded in a computer hard disk for offline analyses. Spectral analysis and 3D rendering of EEG spectrum were done using MatLab® scripts.

Magnetic resonance imaging: Acquisition and analysis

MR image experiments were acquired using 4.7T NMR system (Oxford Systems) controlled by a UNITY Inova-200 imaging console (Varian). The imaging protocol consisted of coronal T2-weighted (TR = 3000 ms, TE = 50 ms) spin echo multislice scans, 16 contiguous 1 mm thick slices. Mice (*n*= 12) were anesthetized with halothane (4% induction, 1.5% maintenance) and oxygen (1.5 l/min) delivered by a facemask in a head holder to minimize artifact movements. Animals anesthetized for the duration of an imaging experiment (50 min) recovered with no apparent difficulty and could be used for subsequent imaging studies. Each mouse was imaged in three consecutive days, namely: day 1, before drug injection; days 2 and 3, after first and second drug injections, respectively. Brain masks, based on the anatomical scans, were done using a tablet driver (Bamboo Tablet Driver, V5.2.5 WIN, WACOM Technology Corporation, United States) and MeVisLab software (MeVis Medical Solutions AG, Fraunhofer). Additionally, densitometry analysis was done using MatLab® scripts and repeated measures. ANOVA was used to compare densitometry values on different days.

Statistical analysis

Statistical analyzes were performed using one-way ANOVA (Tukey's post test) and Student's *t* test. *P* values less than 0.05 were considered statistically significant. All data are presented as mean ± SE.

RESULTS

Repeated TAA injections caused neuropsychomotor changes, brain edema and hyperammonemia, with EEG spectrum suggestive of metabolic encephalopathy

As shown in Figure 1A, around 30% of mice treated with a single dose of TAA died after 24 h and more than 40% succumbed due to a second TAA administration. Also, a progressive increase in neurological score (reflecting a decreased neuropsychomotor activity) was observed (Figure 1B) and, probably due to such reduced mobility, TAA-treated mice presented with a significant weight loss in comparison to controls throughout the experiment (Figure 1C). In addition, TAA treatment caused serum hyperammonemia after 24 h (Figure 1D), which was also detected in the brain in later timepoints (48 h; Figure 1E). Taking into account the edematogenic effects of cerebral ammonia accumulation, we imaged mice brains using magnetic resonance (MR) to investigate potential morphological alterations, including suggestive areas of fluid accumulation. MR revealed a discrete but diffuse brain edema in TAA-treated mice, which was not observed in

Figure 1 Thioacetamide treatment triggered neuropsychomotor changes, diffuse brain edema and hyperammonemia, with electroencephalography spectrum compatible with of encephalopathy. A: Survival rate following repeated TAA injections (arrows); B: Neurological score was assessed through all experimental procedure. Neuropsychomotor deficit increases as higher is the score average, with 1 = normal and 5 = death; C: Mice treated with TAA also presented with significant weight loss in comparison to controls; D, E: Serum ammonemia was detected following 24 h of TAA injection, while brain ammonia concentration increased gradually, reaching significant higher values after 48 h (E); F: In agreement, MRI from TAA-treated mice brains showed discrete, but diffuse edema as pointed by arrow heads. G, H: EEG analysis revealed that while controls had coincident EEG records during the experimental protocol (G), brain waves spectrum of TAA-treated mice (H) was compatible with metabolic encephalopathy; I: 3D rendering of 60 minutes EEG record (Z axis) plotted comparing energy (in Y axis) with frequency (X axis). Note the increase in higher frequencies waves (theta and alpha; 4-8 and 8-13 Hz, respectively) with a concomitant increase of lower frequency ones (mainly delta; up to 4 Hz). Best case results were depicted here; *N* = 8 for each group; J: Total EEG energy was also reduced in TAA-treated group. aIndicates statistical significance in comparison to controls and bin comparison to 24 h group. *P* < 0.05, analysis of variance (Tukey's post test). *N* ≥ 5 for each group. TAA: Thioacetamide; EEG: Electroencephalography.

controls (Figure 1F). Increased fluid accumulation was more evident following 48 h of TAA treatment; however, such a feature was not prominent. In fact, no significant differences were observed in brain densitometry during analysis of MRI images (data not shown), suggesting that a mild brain edema was occurring in this model. To further dissect the neurological effects of TAA poisoning, we submitted mice to daily EEG registration throughout the experimental protocol. Analysis of the EEG spectrum revealed that while untreated mice had coincident EEG records during all experimental protocol (Figure 1G), TAA administration caused a progressive decrease in higher frequencies waves (*theta* and *alpha*; 4-8 and 8-13 Hz, respectively) with a concomitant increase of lower frequency ones (mainly *delta*; up to 4 Hz; Figure 1H-I). Such an EEG profile, with concentration of energy mainly in *delta* waves region and significant reduction in total energy (Figure 1J), confirmed that repeated TAA administration led to a diffuse brain lesion and metabolic encephalopathy.

Severe liver necrosis and inflammation triggered metabolic encephalopathy, coagulopathy and remote lung injury

It is well established that impaired liver function caused by extensive hepatocyte death leads to a general deficiency in metabolism, including ammonia depuration and synthesis of coagulation cascade factors[3]. Liver intravital microscopy revealed a marked increase in necrotic cells (stained by propidium iodide; in red) following TAA exposure (Figure 2A), which was also confirmed by histology analysis (Figure 2B). Also, we observed an exuberant neutrophil accumulation in liver necrotic areas, which increased throughout TAA intoxication process (Figure 2A; eGFP-expressing cells). Macroscopically, livers from TAA treated mice displayed extensive areas of necrosis (Figure 2C), which became more obvious after repeated TAA administration (48 h). Significantly higher serum levels of alanine aminotransferase (ALT) also indicated massive liver injury, which was sustained during the whole experimental period (Figure 2D). In fact, a complete lack of hemostatic function also confirmed hepatotoxicity and organ failure, suggesting that synthesis of liver-derived coagulation factors was seriously impaired. While controls had normal hemostatic parameters (MAP and HR), TAA-treated mice had a prolonged (undetermined) prothrombin and partial thromboplastin time (Figure 2E, F). Moreover, while control mice had normal hemodynamic parameters, the TAA-treated group had a significant drop in MAP (approximately 35%) with concomitant tachycardia, suggesting that mice might be evolving to a hemodynamic shock (Figure 2G, H). In this direction, we hypothesized that in our model TAA might also cause multiple organ failure and remote injury.

We have previously shown that necrosis-derived products may reach systemic circulation and trigger remote inflammatory responses in organs including the lungs[12]. Here, we evaluated the potential of TAA in induction of lung inflammation. Histopathological analysis showed that TAA-induced liver injury also caused pulmonary injury. After 24 h, lungs from mice had increased cellularity in lung parenchyma, alveolar edema and hemorrhage in comparison to controls (Figure 1I; arrows). Those observations were found to be more pronounced after 48 h, showing increased tissue damage, alveolar hemorrhage and lung architecture disruption, suggesting that lethality induced by TAA can also be due to exacerbation of lung inflammatory response in this model.

DISCUSSION

The hepatotoxic ability of TAA is well described in the literature and this drug has been extensively used in rat models of ALF and HE in sequential repeated dose administration[9,13-16]. Once in circulation, TAA is absorbed and bioactivated by hepatocytes. This metabolite will finally modify aminolipids and proteins, causing cell damage and death[17]. In mice, acute TAA poisoning causes centrilobular necrosis and an increase in plasma transaminases and bilirubin. We have shown that following two daily TAA injections, mice developed a massive and progressive liver damage and failure, with hyperammonemia in serum and brain, with discrete signals of cerebral edema.

Hyperammonemia is a hallmark of HE pathophysiology. In patients with ALF, the arterial ammonia level is directly related to severity of HE and development of intracranial hypertension[18]. Also, persistent hyperammonemia seems to be more important than a transient increase in the ammonia level regarding the occurrence of intracranial hypertension[19]. Most studies in mice used a single dose of a hepatotoxic drug which caused a briefer period of illness[20-22]. To supersede this, we developed a novel protocol using a lower TAA dose (300 mg/kg) and repeated administration (2 daily doses), trying to mimic a more realistic scenario closer to severe HE. We found that under these conditions, mice progressively presented with neuropsychomotor deficiencies (as assessed by neurological score)[23], which was accompanied by persistent liver injury and failure. Interestingly, serum ammonia concentration peaked after the first TAA administration, decreasing at 48 h. However, cerebral ammonia gradually increased throughout the experimental protocol, reaching significantly higher levels 48 h after TAA, suggesting that probably excessive serum levels of ammonia might be transferred to the brain due to deficient liver ammonia clearance. The mechanisms responsible for ammonia-mediated encephalopathy and brain edema are still under debate; however, it is accepted that increased blood-derived ammonia intensifies glutamine synthesis via amidation of glutamate, generating a hyperosmotic environment within brain cells (mainly astrocytes)[24]. Consequently, cerebral liquid accumulation leads to intracranial hypertension, herniation and coma. In agreement with this, magnetic resonance imaging from mice treated with TAA revealed a discrete, but crescent-shaped and diffuse

Figure 2 Thioacetamide caused severe liver necrosis and inflammation, which may explain mice metabolic encephalopathy, coagulopathy and remote lung injury. A: Liver intravital microscopy showing crescent number of necrotic cells (in red, propidium iodide) with concomitant neutrophil infiltration (in green, Lysm-eGFP mice); B: Liver sections stained by hematoxylin and eosin (4 × increase). Arrows indicate necrotic areas; C, D: Liver macroscopic analysis confirmed extensive and diffuse necrosis, which is also reflected by elevated serum transaminase activity (D); E, F: Liver failure was confirmed by prolonged prothrombin and partial thromboplastin times; G, H: Also, significant drop in mean arterial pressure (MAP; G) and increased heart rate (H), as assessed by tail cuff method, suggested that TAA-treated mice also evolved to hemodynamic shock; I: After TAA treatment hours, lungs from mice had increased cellularity in lung parenchyma, alveolar edema and hemorrhage in comparison to controls (arrows, 4 x increase) aIndicates statistical significance in comparison to controls. [a]$P < 0.05$, ANOVA (Tukey's post test). N ≥ 5 for each group. TAA: Thioacetamide.

brain edema[25]. In addition, through analysis of the EEG spectrum we found diffuse a brain lesion compatible with metabolic encephalopathy[26], suggesting that our DILI model reproduces some of the main clinical manifestations of HE[27]. In conjunct, our data suggest that this model may be suitable for further studies involving ALF,

hyperammonemia and brain edema.

We also investigated the mechanisms involved in liver damage triggered by TAA in mice. Intravital microscopy revealed that large areas of liver necrosis were infiltrated by neutrophils and such sterile inflammation has been described as a key factor for injury amplification[12]. Furthermore, we also observed repercussions in hemodynamic functions and inflammatory infiltration in the lungs. In this sense, the innate immune response triggered by necrosis-derived products may add to direct TAA-mediated hepatotoxicity to establish not only ALF, but also remote organ injury. Accordingly, TAA-treated mice displayed a severe impairment in hemostatic function compatible with an end-stage liver failure, with coagulopathy, hypovolemic shock and possible multiple organ failure. Thus, these factors should be also evaluated in future studies primarily dedicated to the liver or central nervous system.

In conclusion, we have shown that repeated lower doses of TAA might constitute as a novel murine model of HE and ALF, which reproduce several features of human disease. Also, we provided read-outs for disease grade and severity that comprise behavioral changes, brain edema and ammonemia, hemodynamic parameters and inflammatory response.

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COMMENTS

Background
Acute liver failure (ALF) is a rare but severe clinical syndrome. ALF is the result of massive liver injury, which can be caused mostly by drugs or viruses. During the disease, patients may suffer hepatic encephalopathy, multiple organ dysfunction and sepsis, leading to a high mortality rate. The incidence of ALF is increasing worldwide and liver transplantation and supportive medical care are the only therapies available for ALF.

Research frontiers
Hepatic encephalopathy (HE) is one of the major complications in ALF because of rapidly progressing brain edema and hypertension. Hence, understanding HE pathophysiology is crucial for development of new and specific therapies that would slow down its progression and increase chances of better outcomes. In this sense, a murine model that reproduces most features of HE in humans would be key to improve our understanding of HE pathophysiology and may also be useful for drug testing and development.

Innovations and breakthroughs
This study provided a new model to study hepatic encephalopathy and acute liver failure in mice. In this model, mice are treated twice with a low dose thioacetamide, causing extensive liver injury and characteristic complications of human HE, including high ammonia levels, altered electroencephalography (EEG), brain edema and poor neuropsychomotor function. In addition, mice presented with a systemic response similar to multiple organ failure syndrome, where lung inflammation, coagulopathy and hemodynamic alterations were observed.

Applications
This is a new model to study ALF and HE in mice, the most used animal model. Also, this is the first animal model to present such similar symptoms to the human disease, making it a more useful research tool for this biomedical area.

Terminology
The most important terms in this article are: ALF, HE and thioacetamide.

Peer review
The authors describe a murine model of acute hepatic failure to study hepatic encephalopathy by sequential administration of thioacetamide intraperitoneally. The hypothesis is convenient, the methodology for acute hepatic failure was suitable, the evaluation of hepatic encephalopathy was convenient and sophisticated, the results were interesting and the discussion appropriate.

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CLINICAL PRACTICE

Nuclear medicine dynamic investigations in the diagnosis of Budd-Chiari syndrome

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Abstract

AIM: To investigate the hepatic hemodynamics in the Budd-Chiari syndrome (BCS) using per-rectal portal scintigraphy (PRPS) and liver angioscintigraphy (LAS).

METHODS: Fourteen consecutive patients with BCS were evaluated by PRPS between 2003 and 2012. Ten of them underwent LAS and liver scan (LS) with Tc-99m colloid. Eleven patients had clinical manifestations and three were asymptomatic, incidentally diagnosed at PRPS. The control group included 15 healthy subjects. We used new parameters at PRPS, the liver transit time of portal inflow and the blood circulation time between the right heart and liver. PRPS offered information on the hepatic areas missing venous outflow or portal inflow, length and extent of the lesions, open portosystemic shunts (PSS), involvement of the caudate lobe (CL) as an intrahepatic shunt and flow reversal in the splenic vein. LAS was useful in the differential diagnosis between the BCS and portal obstructions, highlighting the hepatic artery buffer response and reversed portal flow. LS offered complementary data, especially on the CL.

RESULTS: We described three hemodynamic categories of the BCS with several subtypes and stages, based on the finding that perfusion changes depend on the initial number and succession in time of the hepatic veins (HVs) obstructions. Obstruction of one hepatic vein (HV) did not cause opening of PSS. The BCS debuted by common obstruction of two HVs had different hemodynamic aspects in acute and chronic stages after subsequent obstruction of the third HV. In chronic stages, obstruction of two HVs resulted in opening of PSS. The BCS, determined by thrombosis of the terminal part of the inferior vena cava, presented in the acute stage with open PSS with low speed flow. At least several weeks are required in the obstructions of two or three HVs for the spontaneous opening of dynamically efficient PSS. The CL seems to have only a transient important role of intrahepatic shunt in several types of the BCS.

CONCLUSION: Dynamic nuclear medicine investigations assess the extent and length of hepatic venous obstructions, open collaterals, areas without portal inflow, hemodynamic function of the CL and reverse venous flow.

Core tip: Per-rectal portal scintigraphy (PRPS) and liver angioscintigraphy (LAS) are reliable investigations of the liver hemodynamics in the Budd-Chiari syndrome (BCS). Diagnosis of the number, length and succession in time of hepatic vein obstructions allows identification

of hemodynamic varieties and stages of the BCS. Our new PRPS parameters, liver transit time and right heart to liver time, are used to diagnose obstructed hepatic veins, areas missing venous outflow or portal inflow, open collaterals, reverse splenic vein flow and hemodynamic role of the caudate lobe. LAS is useful in the differential diagnosis with portal occlusions, highlighting arterial-venous shunts and reverse portal flow.

INTRODUCTION

The Budd-Chiari syndrome (BCS) is determined by hepatic venous obstruction localized from the small hepatic veins (HVs) to the terminal part of the inferior vena cava (IVC), resulting in increased sinusoidal pressure, hepatic congestion and portal hypertension (PHT)[1]. The natural outcome is poor in many cases, with a three year survival rate of about 10%[2,3]. Clinical manifestations are extremely varied and include ascites, jaundice, hepatomegaly, splenomegaly, collateral veins and upper right abdominal quadrant pain[4]. Ascites fluid has a characteristic high protein concentration (> 2.5 g/dL).

A widely accepted classification is based on etiology, site of obstruction, manifestations and duration of the disease[5]. Primary BCS is produced by thrombosis, its sequels or web obstruction[6]. Secondary forms are determined by malignant or parasitic obstruction of the lumen or by extrinsic compression, commonly produced by tumors[7]. The site involved by the obstruction and the affected HVs are commonly diagnosed through non-invasive imaging [ultrasound (US), magnetic resonance imaging (MRI), computed tomography (CT)] or by using venography[8].

Clinical approach accounts for the severity of disease (fulminant/non-fulminant) and its duration (acute, subacute or chronic). Subacute forms show signs or symptoms for less than six months and no evidence of liver cirrhosis. Chronic evolution is characterized by onset over six months, with evidence of PHT and cirrhosis[9]. However, the severity of liver damage may be inconsistent with apparent symptomatology. Recent clinical onset may be discordant with advanced liver fibrosis, suggesting a long course without clinical symptoms. Asymptomatic disease (10%-15% of cases) is usually associated with the obstruction of only one HV but also with spontaneous development of dynamically efficient intrahepatic collaterals and extrahepatic portosystemic shunts (PSS), incidentally diagnosed by imaging at surgery or necropsy[10]. For the patients with acute clinical onset but with advanced histological damage, a recent hepatic venous obstruction added to older obstructions of other

HVs has to be suspected. In acute forms, the liver area without physiological outflow is hypertrophied and PHT occurs. Because of the higher pressure (35 mmHg) in the hepatic artery (HA) than in the portal vein (PV) branches (3-6 mmHg), the portal flow may be reversed.

Chronic alterations include parenchyma atrophy and extended fibrosis, gaining a cirrhotic appearance and considerably reducing both portal and arterial inflow. Splenomegaly is found in a third of patients[11].

Thrombosis is the usual cause for the occlusion of large HVs, while the obstructions of the IVC or of the small HVs are rarely thrombotic[12].

The anatomical varieties of the HVs have to be accounted for, both for the diagnosis and surgical treatment. A common opening into the IVC of the left hepatic vein (LHV) and middle hepatic vein (MHV) was reported in around 55%-60% of cases[13]. Other varieties, such as a common trunk of the MHV and right hepatic vein (RHV), separate opening of the three HVs into the IVC or the existence of an accessory RHV, were identified in various percentages[14-16]. The caudate lobe (CL) hemodynamic status is important as it may be an anastomosis between the obstructed HVs and the IVC. CL hypertrophy is described in 65%-75% of cases, with good sensitivity but not specificity[17]. A caliber of the CL vein higher than 3 mm is considered diagnostic for the BCS[18].

US usually demonstrates altered HVs and hypertrophy of the CL[19]. Duplex Doppler sonography is widely used, offering a good assessment of the blood flow through the HVs[20,21]. Color Doppler sonography allows a more reliable identification of abnormalities of the HVs than conventional sonography and detects collateral vessels not visible with other techniques. The lack of flow signals in the HVs, intrahepatic and extrahepatic collaterals, together with reverse, slow or turbulent portal flow, are characteristic findings. US techniques should be the first line investigations, due to a low cost and a diagnostic sensitivity of more than 75%. There are, however, cases where the occlusion of HVs is difficult to demonstrate by US, even by color Doppler imaging[22].

CT scans offer a good assessment of thrombosis of the HVs or IVC, global liver enlargement, abnormalities of liver structure, size and direction of the venous flow. The contrast-enhanced helical CT allows a good dynamic visualization of HVs[23]. MRI provides useful images of the hepatic venous outflow and thrombosis of HVs, as second line investigations together with CT[24,25]. It can be difficult to diagnose spontaneous intrahepatic anastomoses and prominent azygos and hemiazygos veins (especially in IVC thrombosis) on MRI[26]. The liver biopsy has limited value due to the inhomogeneous distribution of liver lesions in the BCS[27].

Per-rectal portal scintigraphy (PRPS) was used over the last decades to investigate PHT and PSS in chronic liver disease (CLD) by evaluating a per-rectal portal shunt index[28,29]. Detailed information may be acquired about liver hemodynamics by using the parameters introduced by us in the interpretation of PRPS dynamic curves - liver transit time (LTT) and right heart to liver

time (RHLT)[30].

Liver angioscintigraphy (LAS) evaluates the contribution of arterial inflow to the total liver perfusion. It is especially useful in the differential diagnosis between the BCS and obstructions of the portal branches. Liver areas missing portal inflow have compensatory increased arterial inflow due to the hepatic artery buffer response (HABR)[31] and a characteristic pattern at LAS, with abrupt arterial entry and a flattened portal segment.

Liver scan (LS) with Tc-99m labeled colloid performed after LAS may show changes in radiotracer capture by liver, spleen and spine. Increased radioactivity on the CL area is considered a characteristic finding in the BCS[32].

The aim of this study is to underline the diagnostic possibilities in the BCS of the PRPS and LAS, with auxiliary use of the LS. The goals are assessment of liver areas missing venous drainage, length and order of the appearance of lesions, arterial and portal perfusion changes, existence of dynamically efficient collaterals and of supplementary drainage through the CL.

MATERIALS AND METHODS

We evaluated 14 consecutive patients with the BCS between 2003 and 2012, 9 females and 5 males, between 20 and 63 years old. Eleven patients had clinically manifested BCS and 3 were asymptomatic, incidentally identified at PRPS among over 400 patients explored for chronic liver disease (CLD) staging. The control group included 15 healthy subjects from the laboratory casework, 7 men and 8 women, between 19 and 67 years old. All 14 patients with BCS underwent PRPS. LAS and LS were performed in 10 of them. Anonymity of the patients was respected. All persons gave their informed consent prior to the investigations, accepting inclusion in research studies. The study was realized as part of routine clinical practice.

All the investigations were performed by using a single photon emission computed tomography (SPECT) Orbiter Siemens gamma-camera with high resolution, low-energy, parallel collimator, connected to a Power Macintosh computer, using ICON dedicated software. We used Tc-99m sodium pertechnetate, eluted from Drygen generators (General Electric, Amersham, United Kindom) and Fyton colloid (Institute of Isotopes, Budapest, Hungary).

PRPS was performed using the method developed by Shiomi *et al*[33]. A solution containing 2 mL (296-370 MBq/8-10 mCi) of Tc-99m sodium pertechnetate was instilled at PRPS into the upper part of the rectum through a Nelaton tube, followed by 15 mL of air under pressure. Serial scintigrams were recorded every 2 s for 5 min. Radioactivity curves were built thereafter by computer on liver and heart areas to analyze the dynamics of the radiotracer absorbed from the rectum. The patients were told to fast from the evening preceding the test. Two enemas were performed in each patient, the first the previous evening and the the second one 2 h prior to the PRPS. The patients were placed in a supine position, with

the camera detector in the anterior view, including in the field the liver and heart areas.

LTT and RHLT allow detailed assessment of liver hemodynamics in the BCS. These time parameters can be assessed for particular hepatic areas or for the whole liver. The portal inflow is missing in those areas where the tracer arrives (through the HA) with a delay equal to RHLT after entering into the right heart (RH). LTT increased between 25-40 s shows a higher resistance opposed to the portal inflow. Values over 50 s of the time interval between the entering of tracer into the liver and its arrival to the RH may occur in the acute stage of the BCS produced by obstruction of the terminal part of the IVC. This high delay is determined by the slow flow through the PSS open to the superior vena cava. LTT between 15-23 s reflects a decrease of the resistance opposed by the liver to the portal inflow due to the enlargement of intrahepatic small size shunts between terminal portal branches and small HVs. Open extrahepatic PSS of high flow are emphasized at PRPS by arrival of the portal tracer to the RH before entering into the liver. PRPS also detects alterations of portal and arterial perfusion in the liver areas which maintain their physiological venous outflow, highlighting the changes determined by the redistribution of flow from the affected areas.

The arterial upward inflexion (AUI) of the PRPS curve on a liver area is placed at a time interval equal to RHLT after the moment when the tracer entered into the RH. The beginning of the AUI segment on our figures is noted HA, marking the arrival of tracer to the liver through the HA. The moments when the portal tracer enters into the different areas of the liver are noted as Co, Lo, Mo and Ro. The entering of tracer into the RH is noted as Ho. The slope of the AUI gives information about the amplitude of arterial inflow. The portal segment of the PRPS curve (before the AUI) offers information about the portal inflow and the dynamic resistance encountered. Summed images at PRPS resulting from the overlapping of all the sequential images may highlight the normal aspect, increased accumulation or low quantity of tracer in a liver area. The presence of the spleen on the summed image highlights reverse flow in the splenic vein.

LAS was performed by rapid bolus injection of 8 to 15 mCi (300-450 MBq) of Tc-99m labeled colloid in a volume smaller than 0.5 mL, followed by computer dynamic recording of sequential images during 1 min at a 1 s rate. The detector's area included the heart, liver and kidneys. Six patients underwent LAS in the posterior-anterior view (P-A) and four in the anterior-posterior view (A-P). Patients were asked to fast 12 h before the LAS. Dynamic time-radioactivity curves for the liver, spleen and left or right kidney were built by computer. The moment of the peak of the kidney curve corresponds on the liver curve to the upward inflection point when the portal inflow of tracer adds to the arterial inflow. The interval of 8 s on the liver curve before the kidney curve's peak corresponds to the arterial segment, while the 8 s interval after the peak represents the portal segment[34].

Figure 1 Budd-Chiari syndrome with obstruction of the middle hepatic vein. A: Per-rectal portal scintigraphy; B: Liver angioscintigraphy; C: Liver scan.

The shape of the LAS curve built on a liver area may highlight amplitude changes of the arterial flow, a higher resistance opposed to the blood flow or open arterial-venous shunts. Hepatic perfusion index (HPI) calculated at LAS is used to estimate the ratio between the arterial inflow and total liver perfusion[35]. HPI > 45% on an area of the right liver lobe (RLL) without tumors diagnoses a decrease in portal inflow, with reactive increase in the arterial flow determined by the HABR[36]. HPI > 100% emphasizes reverse flow in the portal vein. HPI can be accounted for the RLL only, as the left liver lobe (LLL) normally has an increased arterial inflow.

LS images were acquired beginning at least 15 min after the LAS, based on the same administration of Tc-99m labeled colloid. P-A, A-P and right lateral (RL) views were used. Time consuming SPECT was not considered to bring significantly more information on hemodynamic status than planar LS and was not performed.

RESULTS

We highlighted three hemodynamic categories of the BCS using PRPS and LAS: (1) BCS debuted by obstruction of one HV: asymptomatic, incidentally diagnosed and old obstruction of one HV followed by recent obstruction of the other two HVs; (2) BCS started by obstruction of two HVs: old obstruction of two HVs, followed by recent obstruction of the third HV, and old obstruction of two HVs, also followed by old obstruction of the third HV; and (3) BCS with acute onset due to simultaneous obstruction of all three HVs caused by obstruction of the terminal part of the IVC.

BCS with one obstructed HV, asymptomatic, incidentally diagnosed

We had three cases of asymptomatic BCS in our study, two with obstruction of the MHV and one with obstruc-

Figure 2 Per-rectal portal scintigraphy in Budd-Chiari syndrome with obstruction of the middle hepatic vein. A: Curve on the territory drained by the middle hepatic vein; B: Caudate lobe curve; C: Left lobe curve; D: Curve on the territory drained by the right hepatic vein; E: Heart curve.

tion of the RHV. All three were incidentally identified at PRPS.

The case presented in Figures 1 and 2 had MHV obstruction. The patient was a 36-year-old male, known to have chronic alcoholic hepatitis. US described inhomogeneous liver with regenerative nodules and dilated portal branches.

Summed image at PRPS (Figure 1A) reveals a decreased amount of radiotracer in the medial part of the RLL. The dynamic PRPS curves built on the RLL show highly different slopes on the medial and lateral segments. The arrival of portal tracer to the medial area was delayed about 30 s after the entrance into the lateral segments. Initial segment of the PRPS curve built on the medial area of the RLL has a slow slope, denoting moderately increased resistance opposed to the portal inflow (Figure 2A). The PRPS curve built on the lateral part of the RLL has an increased slope, showing a rise of its portal inflow (Figure 2D). The dynamic curve built on the CL has a low initial slope, indicating that the portal flow blocked due to the obstruction of the MHV was not drained through the CL. The LLL has a simultaneous entry of tracer as the CL and as the lateral area of the RLL, with a normal initial slope of the PRPS curve.

LTT is prolonged to 30 s, most likely due to the subjacent CLD, with increased resistance opposed to the portal inflow. The LS images confirm the diagnosis of alcoholic CLD, showing splenomegaly and hypertrophy of the LLL, with a normal aspect of the spine. Normal LS aspect of the medial area of the RLL suggests a subacute stage of the BCS, without cirrhotic changes of the affected parenchyma. PSS were not open, as the tracer arrived at the RH by passing through the liver. The LAS curve on the medial segments of the RLL is significant for arterial-venous shunts. The differential diagnosis accounted for the value of LTT and missing of the HABR, excluding a portal branch obstruction.

BCS with old obstruction of one HV and recent obstruction of the other two HVs

The patient presented in Figures 3 and 4 was a 32-year-old male, without a previous diagnosis of CLD. The patient complained of flatulence, vomiting and severe abdominal pain which had started several weeks earlier. US highlighted ascites and venous dilations. No blood flow in the HVs and no portal thrombosis were observed at the US, while numerous intrahepatic arterial-venous and venous-venous collaterals were described. Esophageal varices and gastric injuries were not seen on endoscopy.

The summed image at PRPS presents a sharply increased radioactivity on the CL area (Figure 3A). The LLL only has arterial inflow, receiving tracer after the RH with a delay equal to RHLT (Figure 4B). This suggests an old obstruction of the LHV, with chronic alteration of perfusion of the LLL. The clinical onset in this case resulted from a recent obstruction of the RHV and MHV, while the LHV had been obstructed for a long time. The PRPS curve on the RLL has low slopes on

Figure 3 Budd-Chiari syndrome with old obstruction of the left hepatic vein and recent obstruction of the middle and right hepatic veins. A: Per-rectal portal scintigraphy; B: Liver angioscintigraphy; C: Liver scan.

the initial portal segment and on the AUI, showing that its portal and arterial inflows were decreased. The differential diagnosis excluded portal obstruction, accounting for small arterial inflow of the two liver lobes and missing the HABR.

Arrival of tracer to the RH at 30 s after entering into the CL shows that PSS were not open due to the earlier obstruction of one HV and the recent obstruction of the other two HVs (Figure 4). The tracer was detected in the RLL about 12 s after the CL, a slow portal inflow to the RLL being maintained.

Altered flow of the RLL was highlighted at the LAS (Figure 3B). After a short initial interval of about 1 s with normal arterial input, the LAS curve flattens on the rest of the arterial segment and has a low slope on the portal segment. The flattened curve on the RLL highlights the higher dynamic resistance encountered by the arterial inflow.

The ASH curve of the CL has an initial abrupt and high segment, due to the increased arterial inflow, followed by a flat portal segment and then by fluctuations of amplitude suggesting arterial-venous shunts (Figure 3B). The CL has a very high entering of portal tracer at the PRPS (Figure 4A). LTT on the CL is increased to 30 s due to the dynamic resistance opposed by the supplementary arterial and portal flows redirected from the RLL and LLL. The high amplitude of the PRPS curve on the CL was determined by the increased and slowed portal inflow. LS (Figure 3C) revealed increased radioactivity in the CL, inhomogeneous liver parenchyma and slightly increased radioactivity in the spleen.

The perfusion changes in the LLL and RLL suggest a subacute stage of the BCS. We underline that the old obstruction of the LLL together with more recent occlusion of the other two HVs did not open PSS. The arterial and portal blood flow of the whole liver was drained to the IVC through the CL.

Figure 4 Per-rectal portal scintigraphy in Budd-Chiari syndrome with old obstruction of the left hepatic vein and recent obstruction of the middle and right hepatic veins. A: Caudate lobe curve; B: Left lobe curve; C: Right lobe curve; D: Heart curve.

BCS started by the common obstruction of two HVs, also followed by old obstruction of the third HV

The scintigraphy investigations in a patient with BCS with an old obstruction of the MHV and RHV and subsequent but also old obstruction of the LHV are presented in Figures 5 and 6. This 20 year old woman, a user of an oral contraceptive, was hospitalized with an impaired general condition and abdominal pain. The first symptoms appeared two and a half years earlier when the BCS was diagnosed. US at admission showed abundant ascites and PV dilation to 15 mm. Doppler US could not detect flow in the HVs.

The RLL received tracer at PRPS at a time interval equal to RHLT after the RH, emphasizing that the portal inflow was missing and the blood supply of the RLL came from the HA only (Figures 6C, D).

The tracer entered the LLL and the CL (Figure 6A, B) at about 25 s after reaching the RH, showing that their perfusion was mainly (but not completely) arterial. The

initial low slopes of the CL and LLL curves were caused by increased resistance opposed to the portal inflow. The high slope of the AUI on the CL and LLL curves was determined by the large quantity of tracer that arrived through the HA. The slope of the AUI is smaller on the RLL than on the LLL and CL curves with about 50% (Figure 6C). Due to the older impairment of the RLL, its arterial perfusion per unit of area became lower than for the LLL and CL.

PSS were open, allowing the tracer absorbed from rectum to arrive faster to the RH than to the liver. The presence of the IVC on the summed PRPS image suggests the existence of open per-rectal PSS.

The ASH curve on the CL has a steep and biphasic entrance during the arterial segment. The portal phase has a drop in amplitude and subsequent fluctuations (Figure 5B). Increased arterial perfusion and existence of arterial-venous shunts are highlighted in the CL. The high arterial inflow suggests that the CL maintained part of its role of intrahepatic shunt to the IVC for the arterial inflow redirected from the rest of the liver. The ASH curve on the RLL shows increased arterial perfusion and reverse portal flow, with HPI = 115%.

The CL is visible on the LS, contrasting to the low colloid capture in the RLL (Figure 5C). The normal LS aspect of the spine and spleen argues against alcoholic or viral cirrhosis.

BCS with old obstruction of two HVs followed by recent obstruction of the third HV

We encountered one case of the BCS syndrome with old RHV and MHV obstruction and very recent occlusion of the LHV. One of these patients was a 32-year-old woman, an oral contraceptive user, with ascites in high quantity, increasing abdominal girth and without viral or alcoholic cirrhosis.

The time interval at PRPS (Figure 7) between the dynamic curves built on the heart and on the whole liver is equal to RHLT, showing that both the LLL and RLL missed portal inflow and the extrahepatic PSS of high flow were open. The high initial slope of the PRPS curve on the heart confirms that the PSS were hemodynamically efficient.

The highly increased radioactivity in the congested LLL on the summed PRPS image suggests that its deprivation of physiological venous drainage was very recent, while the obstruction of the MHV and RHV was old. The CL cannot be specifically distinguished on the PRPS summed image or by building dynamic curves on its area, therefore not playing a specific hemodynamic role. The image of the spleen on the summed PRPS image suggests inversion of flow in the splenic vein, while presence of the IVC suggests open per-rectal shunts.

BCS with acute onset by simultaneous obstruction of all three HVs (occlusion of terminal portion of the IVC)

Two of the BCS patients in our study had fulminant symptoms. US and CT scans argued for obstruction of

Figure 5 Budd-Chiari syndrome with old obstruction of the middle and right hepatic veins also followed by old obstruction of the left hepatic vein. A: Per-rectal portal scintigraphy; B: Liver angioscintigraphy; C: Liver scan.

the terminal portion of the IVC in both of them. The data shown in Figure 8 belong to a 39-year-old woman with myeloproliferative disease, admitted for recently appeared severe pain in the upper right abdominal quadrant, with vomiting and encephalopathy signs. US and CT scans showed abundant ascites and significant dilation of the HVs.

PRPS highlights a sharp increase of the transit time of the tracer from entering into the liver to reaching the RH, up to values over 50 s. The amount of tracer passing through the heart during the first 30 s after its arrival was very low, with a flattened PRPS cardiac curve. The normal slope of the PRPS curves built on the liver lobes

show that the dynamic resistance encountered by the portal flow was not significantly increased. The very late arrival to the RH of a small quantity of tracer was determined by the slow transit through the PSS open to the superior vena cava. PRPS highlights the completely blocked venous outflow on the physiological pathway between the liver and the RH, involving all the HVs. The slow flow through the PSS suggested that the obstruction of the terminal portion of the IVC had been recently installed.

DISCUSSION

We propose a new method to assess the liver hemodynamics

Figure 6 Per-rectal portal scintigraphy in Budd-Chiari syndrome with old obstruction of the middle and right hepatic veins also followed by old obstruction of the left hepatic vein. A: Caudate lobe curve; B: Left lobe curve; C: Right lobe curve; D: Heart curve.

in the BCS by performing the PRPS and LAS, with auxiliary use of the LS. PRPS is the main investigation, based on the parameters introduced by us, LTT and RHLT, offering information about the portal and arterial flows and the effects of the venous outflow obstruction.

The liver areas missing portal inflow with only arterial perfusion are highlighted at PRPS based on the time interval equal to RHLT between the origins of cardiac and liver curves. The increased arterial perfusion of a liver area causes a high slope of the AUI at PRPS and HPI > 45% at LAS. In old obstructions of the HVs, PRPS shows decreased portal and arterial flows of the parenchyma without venous outflow. In recent obstructions of the HVs, PRPS detects small or inverted portal flow, increased arterial inflow and accumulation of radioactivity on the summed image in the affected area. A high quanti-

ty of radiotracer appears on the summed PRPS image in the CL of the patients with recent common occlusion of two HVs. Inverted flow in the splenic vein is suggested by a visible spleen on the summed PRPS image.

ASH is useful in the differential diagnosis between the BCS and portal obstructions and also in highlighting arterial-venous shunts and increased resistance opposed to the arterial flow. Reverse portal flow is emphasized by HPI > 100%. LS was performed after ASH, showing increased radioactivity in the CL in particular types of BCS. LS aspect of the liver lobes, spleen and spine is useful when viral or alcoholic CLD is suspected.

Several hemodynamic varieties and stages of the BCS were described by using PRPS and LAS. Liver perfusion status is closely related to the initial number of obstructed HVs and to the lengths of occlusions. The category of the BCS debuted by obstruction of one HV included the patients actually affected by one obstructed HV and the patients affected by an old obstruction of one HV followed by recent obstruction of the other two HVs. For the BCS started by obstruction of two HVs we found different perfusion patterns in acute and chronic stages after the obstruction of the third HV. The BCS with acute onset due to the simultaneous obstruction of all the three HVs is commonly caused by obstruction of the terminal part of the IVC and specifically presents in the acute stage highly prolonged LTT at PRPS. Different hemodynamic patterns of the liver flows related to various types of hepatic venous occlusions underline the autonomous regulation of the perfusion of the two liver lobes.

It is important to know in all the varieties and stages of the BCS if PSS are open. PRPS highlighted that PSS were not open in patients with occlusion of one HV. The CL did not play a significant hemodynamic role in the subacute stage of such patients, suggesting that the blood flow redirected from the area without physiological venous outflow was drained through the unaffected HVs. PSS were not open even in the acute stage after common obstruction of two HVs following an old obstruction of the other HV.

Open PSS were emphasized in our cases with old obstruction of two HVs. This finding suggests that the outflow of two HVs is too high to be redirected in chronic stages only through the unaffected HV and through the CL, with having to leave the liver through extrahepatic PSS.

In acute stages after the occlusion of terminal part of the IVC, the PSS draining the blood to the superior vena cava allowed only a low speed flow. Our data suggest that spontaneous effective drainage through PSS requires at least several weeks to open after the obstruction of two or three HVs.

Hypertrophy of the CL is currently underlined in the diagnosis of the BCS. However, the hemodynamic role of the CL looks to be unimportant or transient in several varieties and stages of the BCS. The CL was not involved as an intrahepatic shunt in asymptomatic pa-

Figure 7 Per-rectal portal scintigraphy of a patient with Budd-Chiari syndrome with old obstruction of the middle and right hepatic veins and recent obstruction of the left hepatic vein.

Figure 8 Per-rectal portal scintigraphy in Budd-Chiari syndrome with obstruction of the terminal part of the inferior vena cava. A: Left lobe curve; B: Right lobe curve; C: Heart curve.

tients with obstruction of one HV and in IVC obstructions. In the patients with chronic obstruction of two HVs, the CL had a reduced role of a hemodynamic shunt for part of the arterial flow redistributed from affected areas, presenting arterial-venous collaterals. The CL was highly active in the subacute stage after the obstruction of two HVs following an old occlusion of the third HV. Our study suggests that the CL usually plays an efficient hemodynamic role of intrahepatic shunt to the IVC in

acute or subacute stages and in particular varieties of the BCS. The importance in each case of the caliber and morphology of the CL venous drainage for its functioning as hemodynamic shunt has also to be accounted for.

Due to the rarity of the disease, we did not explore several other theoretical varieties of the BCS so our classification will have to be detailed. Common obstruction of the LHV and MHV, acute stage after the debut of the BCS by obstruction of one or two HVs, and chronic stage after the occlusion of the terminal part of the IVC may bring more information about the opening of PSS and the role of the CL in draining the redirected flows.

To conclude, PRPS associated with LAS are able to play a useful role as second line investigations in the BCS, adding important data to the US, CT or MRI findings. These scintigraphic procedures have reliable costs, are non-invasive and easily reproducible. The accuracy of the method, however, is dependent on the operator's expertise.

ACKNOWLEDGMENTS

COMMENTS

Background

Impaired liver perfusion in the Budd-Chiari syndrome is determined by the obstruction of the hepatic blood outflow. Portal and arterial altered flows may be properly explored by combined use of two nuclear medicine dynamic investigations, per-rectal portal scintigraphy and liver angioscintigraphy. Radioisotope techniques allow a more precise diagnosis in different types and stages of the Budd-Chiari syndrome and highlight the changes of perfusion patterns during evolution of the disease.

Research frontiers

Scintigraphy investigations proposed by us to explore the Budd-Chiari syndrome highlight open portosystemic shunts, liver areas without portal inflow, hemodynamic involvement of the caudate lobe, inverted flow in the splenic or

portal vein and length of the obstructions of the hepatic veins or the terminal portion of the inferior vena cava. The authors described three hemodynamic categories of the Budd-Chiari syndrome with several subtypes and stages, based on the finding that perfusion changes depend on the initial number and succession in time of the hepatic veins obstructions.

Related publications

The authors previously described the use of dynamic nuclear medicine investigations to evaluate portal hypertension and portosystemic shunts in chronic liver disease. Clinical applications of the liver angioscintigraphy are commonly related to hepatic tumors evaluation.

Innovations and breakthroughs

The authors introduced a new method of interpretation for the per-rectal portal scintigraphy by proposing two new parameters, the transit time of the portal inflow through the liver and the transit time of the blood from the right heart to the liver. These time parameters allow an accurate description of hepatic hemodynamic changes determined by venous obstructions. The authors used liver angioscintigraphy in the differential diagnosis between the Budd-Chiari syndrome and portal obstructions, highlighting the absence of the hepatic artery buffer response in the Budd-Chiari syndrome. The authors showed that portosystemic shunts are not open after the obstruction of one hepatic vein, while at least several weeks are required in the obstructions of two or three hepatic veins for the spontaneous opening of dynamically efficient portosystemic shunts.

Applications

The hemodynamic data offered by per-rectal portal scintigraphy and angioscintigraphy of the liver are especially important for surgery and TIPS mounting. Diagnosis of the number, length and succession in time of hepatic veins obstructions allow identification of hemodynamic varieties and stages of the Budd-Chiari syndrome and support an adequate therapeutic approach.

Terminology

Per-rectal portal scintigraphy is a dynamic procedure performed by instillation into the rectum of a small quantity of radioactive tracer followed by recording its dynamics through the portal vein, liver and heart areas. Liver angioscintigraphy is performed by rapid antecubital intravenous bolus injection of a small quantity of radiotracer followed by recording its entrance into the liver through the hepatic artery and portal vein, allowing the assessment of an arterial to total liver perfusion (arterial plus portal) ratio. The Budd-Chiari syndrome is a vascular liver disease determined by hepatic venous obstruction localized from the small hepatic veins to the terminal part of the inferior vena cava, resulting in increased sinusoidal pressure, hepatic congestion and portal hypertension.

Peer review

This is a well thought and comprehensive manuscript on the relevance of using PRPS and liver angioscintigraphy techniques to investigate the liver hemodynamics in Budd-Chiari syndrome. The manuscript provides useful and interesting information. It also has potential for clinical application.

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Obesity and non-alcoholic fatty liver disease: Disparate associations among Asian populations

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Abstract

Obesity is a global epidemic contributing to an increasing prevalence of obesity-related systemic disorders, including nonalcoholic fatty liver disease. The rising prevalence of nonalcoholic steatohepatitis (NASH) will in the near future lead to end-stage liver disease in a large cohort of patients with NASH-related cirrhosis and NASH is predicted to be a leading indication for liver transplantation in the coming decade. However, the prevalence of obesity and the progression of hepatic histological damage associated with NASH exhibit significant ethnic disparities. Despite a significantly lower body mass index and lower rates of obesity compared to other ethnic groups, Asians continue to demonstrate a significant prevalence of hypertension, diabetes, metabolic syndrome and NASH. Ethnic disparities in central adiposity and visceral fat distribution have been hypothesized to contribute to these ethnic disparities. The current review focuses on the epidemiology of obesity and NASH among Asian populations.

Core tip: Non-alcoholic fatty liver disease (NAFLD) is rapidly becoming a major contributor of chronic liver disease worldwide. The increasing prevalence of NAFLD among Asians reflects both an increasing awareness and diagnosis and the increasing risk of obesity and obesity-related diseases among this population. Ethnic disparities in the impact of weight gain on the development of obesity-related diseases is especially important for Asian populations, who have greater rates of central obesity and visceral deposition of fat and therefore are at greater risk of obesity-related diseases, such as NAFLD, at a lower body mass index.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) spans a spectrum of liver diseases that ranges from simple steatosis of the liver to progressive inflammation and fibrosis, resulting in non-alcoholic steatosis (NASH) and cirrhosis[1]. While the definition of NAFLD relies heavily on the clinical exclusion of significant alcoholic liver disease as well as other concomitant chronic liver diseases that can mimic similar histopathological features, one of the major hallmark features of NAFLD is the consistent association with type 2 diabetes mellitus, hypertension, hyperlipidemia and obesity[1-5]. The rising epidemic of obesity and obesity-related diseases in many post-industrialized countries has been accompanied by a concurrent rise in

the prevalence of NAFLD. These emerging trends along with our better understanding of the pathophysiology of NAFLD clearly highlight the important role of obesity and obesity-related diseases in the increasing prevalence of NAFLD.

Several studies have reported the alarming increase in obesity and metabolic syndrome in western countries[6-12]. One recent large population-based study in the United States, utilizing data from the National Health and Nutrition Examination Surveys from 2009-2010 (NHANES), reported obesity rates of 35.5% among men and 35.8% among women[7]. Furthermore, population based studies utilizing United States census based data have demonstrated a concurrent rise in the prevalence of obesity-related diseases, such as hypertension and diabetes mellitus[13-18]. Among the same population, several studies have reported an increasing prevalence of NAFLD, suggesting that the rising rates of NAFLD are a consequence of the rising rates of obesity and metabolic syndrome in these populations. In fact, a recent study by Charlton *et al*[19] estimates that the rising prevalence of NAFLD in the United States population will soon lead to large cohorts of patients with decompensated cirrhosis from NASH and that NASH will soon become the leading indication for liver transplantation in the United States.

However, the epidemiology of obesity and obesity-related diseases demonstrates significant ethnic disparities. For example, several studies among both western and eastern cohorts demonstrate that Asians as a group consistently have a much lower body mass index (BMI) compared to other ethnic groups[20-23]. The relatively lower BMI is not protective in Asians. The rates of hypertension and diabetes mellitus, while somewhat lower, still continue to demonstrate rising trends among Asians[20]. In addition, cohort studies have demonstrated that despite having significantly lower BMI than other ethnic groups, Asians have a surprisingly high prevalence of NAFLD[24]. While not entirely elucidated, one emerging theory for this discrepancy between BMI and NAFLD prevalence may result from ethnic differences in the distribution of body fat, with more central adiposity and visceral fat deposition reported among individuals of Asian ethnicity[25-29]. Nevertheless, the increasing prevalence of obesity, metabolic syndrome and NAFLD among the Asian population will contribute to a large burden of chronic disease. The current paper reviews the concerning rise in obesity and NAFLD, with a focus on Asian populations.

OBESITY DISPARITIES

The global obesity epidemic has been associated with the increasing burden of obesity-related diseases such as coronary artery diseases, hypertension and diabetes mellitus[9-12]. In addition, a link has been established between obesity and NAFLD such that obesity increases the risk of progression of hepatic inflammation and fibrosis leading to NASH-related cirrhosis. However, one emerging theme in the study of obesity is the ethnic disparities in the prevalence of obesity as well as the impact of weight gain on the overall risk of obesity-related diseases.

Several studies have reported ethnic disparities in the prevalence of obesity, with higher obesity rates in minority groups such as blacks and Hispanics[20,30-34]. However, Asians as a group generally have a lower BMI and lower prevalence of obesity compared to other ethnic groups[20-23]. Despite lower obesity prevalence, higher rates of metabolic syndrome have been reported in Asians compared to other ethnic groups at similar BMI levels[20]. These findings demonstrate that BMI thresholds for defining overweight and obesity should not be applied uniformly to all ethnic cohorts.

Current BMI categories set forth by the United States Centers for Disease Control and Prevention (BMI > 25 kg/m^2 as overweight and BMI > 30 kg/m^2 as obese) were intended to predict an individual's risk of developing diseases associated with overweight and obese categories[35]. Two large population-based longitudinal studies, the San Antonio Heart Study and the Insulin Resistance Atherosclerosis Study, demonstrated a strong association of BMI with the risk of metabolic syndrome. Obese individuals (BMI > 30 kg/m^2) were three to eight times more likely to develop metabolic syndrome compared to individuals with BMI < 25 kg/m^2[31,36]. In addition, the association of obesity and metabolic syndrome with development of complications such as cardiovascular disease and diabetes mellitus is well established[37-40]. However, similar to ethnic disparities in the prevalence of obesity, the correlation of BMI with obesity-related diseases is not uniform across all ethnicities. For example, using data from NHANES, Palaniappan *et al*[30] demonstrated that fasting insulin levels, a marker of insulin sensitivity and risk of diabetes, was 19%-26% higher in blacks and 17-22% higher in Hispanics when compared to non-Hispanic whites with similar BMI. This disparity was also noted among Asians, with one study demonstrating significantly higher rates of metabolic syndrome in Asians compared to other ethnic groups with similar BMI. For example, Palaniappan *et al*[20] demonstrated that the predicted prevalence of metabolic syndrome in non-Hispanic white women aged 55 years with BMI 25 kg/m^2 was 12% compared to 30% in Asians with similar demographics and BMI. Furthermore, compared to white men with BMI 25 kg/m^2, comparable prevalence of metabolic syndrome was seen in Asian men with BMI 19.9 kg/m^2.

Using data from the California Department of Public Health and the United States Centers for Disease Control and Prevention, our group performed an in-depth analysis of ethnic disparities in obesity and obesity-related diseases with a focus on Asian populations. From 1985 to 2011, Asians as a group had the lowest BMI and lowest obesity prevalence (Asians: 22.6 ± 3.3 kg/m^2 in 1985-1990 to 24.4 ± 4.3 kg/m^2 in 2006-2011; non-Hispanic whites: 24.2 ± 4.1 kg/m^2 in 1985-1990 to 26.7 ± 5.5 kg/m^2 in 2006-2011; blacks: 25.4 ± 4.5 kg/m^2 in 1985-1990 to 29.0

Figure 1 Trends in body mass index over time stratified by ethnicity, 1985-2011, California behavioral risk factor survey database.

$\pm$ 6.9 kg/m^2 in 2006-2011; Hispanics: 25.0 $\pm$ 4.1 kg/m^2 in 1985-1990 to 28.3 $\pm$ 5.8 kg/m^2 in 2006-2011) (Figure 1). Despite lower overall BMI, Asians had comparable or even higher rates of hypertension and diabetes mellitus compared to other ethnic groups. To evaluate whether weight gain as measured by BMI affected ethnic groups similarly, we created a multivariate logistic regression model to assess the effect of each one unit increase in BMI on the risk of hypertension or diabetes mellitus (Table 1). In our cohort model, each one unit increase in BMI was associated with 15% increased risk of hypertension in Asians, compared with 11% increase among non-Hispanic whites and 8% increase among blacks and Hispanics. When evaluating the impact of weight gain on the risk of diabetes mellitus, each one unit BMI was associated with 15% increased risk of diabetes mellitus among Asians, compared to 11% increase among non-Hispanic whites, 7% increase among blacks and 8% increase among Hispanics. These data suggest that despite having lower BMI, weight gain as measured by BMI disproportionately affects Asians to a greater degree. Furthermore, similar risks of hypertension and diabetes mellitus among non-Hispanic whites and blacks were seen in Asians at significantly lower BMI. For example, risks of hypertension among Asians with BMI > 22 kg/m^2 were similar to non-Hispanic whites with BMI > 27 kg/m^2 and blacks with BMI > 28 kg/m^2 (Figure 2). While many theories have been proposed to explain these disparities, ethnic differences in body fat distribution may be a major contributing factor. Previous studies evaluating the correlation of BMI with percentage body fat demonstrated that blacks generally have more lean mass and less fat mass compared to whites. In contrast, Asians have more central adiposity and visceral fat distribution, which carries a greater risk of developing cardiovascular and metabolic diseases[25-29].

Acknowledging these disparities, several studies have suggested that current thresholds for defining obesity and overweight in Asians may not accurately reflect the risk of developing obesity-related diseases and BMI thresholds should be lowered for Asian cohorts[41,42]. In 2000, the World Health Organization Western Pacific Regional

Office proposed a lower cutoff of BMI > 25 kg/m^2 for obesity in Asian populations[43]. Several Asian countries have begun to adopt this modified BMI categorization[44-46]. Additional studies have attempted to incorporate additional anthropometric tools to better stratify the risk of metabolic diseases among Asians. Using a cross sectional population-based survey study of 2947 patients in China, Shao *et al*[47] demonstrated that waist-height ratio was significantly better at predicting risk of metabolic syndrome than BMI or waist circumference alone. Liu *et al*[48] performed a similar evaluation among a cross sectional cohort of 772 Chinese patients. BMI, waist circumference and waist-hip ratio were found to have similar predictive power for risk of metabolic diseases, such as hypertension, diabetes mellitus and dyslipidemia. While solely relying on BMI to predict risk of obesity-related diseases such as NAFLD has several limitations, these additional complementary anthropometric tools may improve risk stratification.

DISPARATE ASSOCIATION OF NAFLD AND BMI

While studies in western populations clearly indicate that NASH will be a leading cause of chronic liver disease, less is known about the epidemiology of NAFLD among Asian populations. Recent community-based studies from Asian countries, including Japan, China, Taiwan, and Korea, indicate that the overall NAFLD prevalence reaches as high as 45% with year-specific analyses, demonstrating a continued rise in NAFLD prevalence with time[49-55]. Additional studies from the Asia-Pacific region demonstrated similar trends of NAFLD prevalence in India, Malaysia, Singapore and Indonesia[56-62]. Wong *et al*[63] performed a large cross-sectional study in Hong Kong to assess the community prevalence of NAFLD using proton nuclear magnetic resonance (p-NMR) spectroscopy. A total of 922 patients randomly selected from the Hong Kong census database without chronic liver disease completed a full clinical assessment. Among this cohort, p-NMR was utilized to measure intrahepatic triglyceride content with a cutoff of 5% used to distinguish patients with and without fatty liver disease. Transient elastography was also utilized to assess for hepatic fibrosis with a cutoff of 9.6 kPa to define advanced fibrosis. Overall, the cohort was 42.2% men and average BMI was 22.8 $\pm$ 3.5 kg/m^2. A total of 264 patients (26.8%) met the cutoff for diagnosis of fatty liver disease. Average BMI among the fatty liver disease cohort was 25.3 $\pm$ 3.4 kg/m^2 and among the non-fatty liver disease cohort was 21.8 $\pm$ 3.0 kg/m^2. Prevalence of advanced fibrosis was 3.7% (*n* = 8) and 1.3% (*n* = 7) among fatty liver and non-fatty liver cohorts, respectively. A similar study was performed in Shanghai that included 3175 adults that assessed for prevalence of metabolic syndrome using criteria from the National Cholesterol Education Program - Adult Treatment Panel Ⅲ and for fatty liver with ultrasonography[64]. Overall, 22.9% and 20.8% of individuals had metabolic

Table 1 Increased odds of hypertension and diabetes associated with one unit increase in body mass index						
	Hypertension			Diabetes		
	OR	95%CI	*P* value	OR	95%CI	*P* value
Asian	1.15	1.13-1.18	< 0.001	1.15	1.13-1.18	< 0.001
Non-Hispanic White	1.11	1.10-1.11	< 0.001	1.11	1.11-1.12	< 0.001
Black	1.08	1.07-1.10	< 0.001	1.07	1.06-1.09	< 0.001
Hispanic	1.08	1.07-1.09	< 0.001	1.08	1.07-1.09	< 0.001

Figure 2 Odds of (A) hypertension and (B) diabetes by ethnicity and body mass index categories.

syndrome and fatty liver, respectively. The risk for fatty liver was increased among patients with abdominal obesity (waist circumference > 90 cm in men and > 80 cm in women: 32.8-fold increase), diabetes mellitus (31.6-fold increase), dyslipidemia (22.6-fold increase) and hypertension (22.3-fold). Patients that met the diagnostic threshold for metabolic syndrome had a nearly 40 times increased risk for fatty liver. When stratified by BMI, those with fatty liver disease and BMI < 25 kg/m^2 had 36.1% prevalence of metabolic syndrome. Furthermore, the presence of fatty liver disease was found to have the best positive predictive value and attributable risk percentage in detecting risk factor clustering for metabolic syndrome.

Variations in fat distribution have been implicated as one potential reason for disparate associations between BMI and NAFLD prevalence among Asian populations. It has been previously reported that the percent body fat as well as fat distribution differs significantly among Asian and non-Asian populations, such that greater central and visceral adiposity is commonly seen in Asians[25-29]. It has also been implied that as a result of this disparate distribution of fat, excessive amounts of visceral adipose tissue may occur in Asians not overweight or obese using BMI cutoffs. Greater central adiposity distribution is associated with higher risks of cardiovascular disease and metabolic syndrome[65-67]. Furthermore, ideal body weight may be different among different ethnicities and different world regions, such that while an individual does not meet BMI threshold for obesity, he may be significantly heavier than ideal body weight, and this translates into increased risk of insulin resistance and metabolic syndrome[68-70]. For example, Chang *et al*[68] performed a prospective South Korean study of 15,347 men to assess ultrasound-based diagnosis of fatty liver disease. Even among men with BMI 18.5-22.9, mild weight gains

of 0.6 to 2.3 kg were associated with 38%-73% increase in the risk for fatty liver disease. This phenomena, termed "metabolically obese", namely the increased risk of insulin resistance, metabolic syndrome and NAFLD despite normal or lean BMI, has been more commonly seen in Asian populations[68-70].

Another potential theory that may partially contribute to the rising prevalence of NAFLD among Asian populations centers on the role of diet. Carbohydrates in the form of rice are a central component of the Asian diet. However, significant amounts of carbohydrates in the diet can lead to accumulation of triglycerides within the liver, which is mediated by glucose stimulated activation of the liver transcription factor, carbohydrate responsive element-binding protein (ChREBP). This process over time leads to significant hepatic steatosis and eventual progression of disease towards NASH[71-73]. However, the impact of ChREBP on hepatic steatosis among individuals with significant carbohydrate exposure may not necessarily correlate with development of insulin resistance. A recent study by Benhamed *et al*[74] evaluated ChREBP over expressing mice fed a standard diet, demonstrating that despite having increased expression of genes involved in lipogenesis/fatty acid esterification and resultant hepatic steatosis, the mice remained insulin sensitive. In addition, ChREBP over expressing mice fed a high-fat diet also showed normal insulin levels and improved insulin signaling and glucose tolerance compared with controls, despite having greater hepatic steatosis.

NATURAL HISTORY OF NAFLD IN ASIANS

The progression of inflammation and fibrosis in patients

with NAFLD is not believed to differ significantly by ethnicity. However, some earlier studies have suggested that NAFLD may be less severe with slower progression among Asian populations[75,76]. This hypothesis is complicated by several potential confounding factors. NAFLD is a relatively more recent phenomenon in Asian countries and the expected progression of disease leading to cirrhosis may occur over the next several decades. Thus, the emergence of fatty liver disease observed in the recent era in Asian populations probably lags behind western populations by several decades and the impact of large cohorts of patients with chronic liver disease and cirrhosis from NASH is expected to flood our health care system in the coming years. Another potential contributing factor is the increasing awareness and subsequent diagnosis of NAFLD among these Asian Pacific regions. Furthermore, the previously reported disparate association between BMI and metabolic syndrome that results from ethnic disparities in central adiposity and visceral fat distribution may alter the natural history of NAFLD among this population.

Despite these potential caveats, it is generally agreed that the progression of disease among patients with simple steatosis is slow compared with other diseases, such as hepatitis C virus (HCV), whereas patients with histological evidence of NASH can progress more rapidly towards advanced fibrosis and cirrhosis[1,2,77]. Long-term longitudinal studies have demonstrated increased mortality among patients with both NAFLD and NASH when compared to controls without underlying liver disease[78-86]. Interestingly, the most common cause of death among patients with NAFLD and NASH was cardiovascular diseases, reflecting the close correlations of NAFLD with metabolic syndrome and cardiovascular disease outcomes. However, simple steatosis is not always benign and progression of disease, while slow, can occur. In a single centered Hong Kong cohort, Wong *et al*[87] conducted a prospective longitudinal study of 52 patients with biopsy proven NAFLD. Among patients with simple steatosis on histology at baseline (n = 13), 15% had normal histology, 23% still had simple steatosis and 62% had evidence of histological progression towards NASH at 36 mo. While the small sample size may limit the generalization of these findings, this study raises awareness of the dynamic nature of steatosis and that simple steatosis is not necessarily benign and may warrant closer follow up.

However, progression of NAFLD to NASH-related cirrhosis is clearly associated with increased risks of hepatic decompensation and liver-related mortality[88-91]. Hui *et al*[88] performed a prospective longitudinal cohort study of 23 patients with clinically/pathologically confirmed NASH-related cirrhosis compared with 46 age and gender matched HCV-related cirrhosis patients. Over a median follow up of 60 mo (range 5-177 mo), 9/23 NASH-related cirrhosis patients developed hepatic decompensation (8 with ascites or encephalopathy, 1 with variceal bleeding). The overall survival at 1, 3, and

10 years was 95%, 90% and 84%, respectively. After multivariate regression modeling, there was no significant survival difference between the NASH-related cirrhosis and HCV-related cirrhosis cohorts. A larger United States study compared 152 patients with NASH-related cirrhosis to 150 matched patients with HCV-related cirrhosis[89]. Over 10 years of follow up, NASH patients had significantly lower mortality compared to HCV patients but this mortality difference was primarily seen in patients with Child Pugh Turcotte (CPT) class A cirrhosis. Among patients with CPT class A cirrhosis, NASH patients had significantly lower rates of hepatic decompensation, development of ascites and hepatocellular carcinoma (HCC). Similar findings were reported in a large multi-center international study of 247 patients with advanced fibrosis or cirrhosis secondary to NASH compared to 264 chronic HCV patients with similar stages of fibrosis[91]. Among the NASH cohort, there were 19.4% liver-related complications and 13.4% deaths or liver transplantation over a mean follow up of 85.6 mo. Among the HCV cohort, there were 16.7% liver-related complications and 9.4% deaths or liver transplantations over a mean follow up of 74.9 mo. After adjusting for differences in baseline characteristics, cumulative incidence of liver-related complications was significantly lower in the NASH group compared to the HCV group. However, the incidence of cardiovascular events and overall mortality was not significantly different between NASH and HCV cohorts. The results of these studies indicate that while progression of NAFLD towards NASH cirrhosis is clearly associated with increased risks of hepatic decompensation and mortality, these increased risks may not be as high as that seen among the cohort of chronic HCV cirrhosis patients.

NAFLD AND HCC

While the risks of HCC from chronic liver disease secondary to hepatitis B and HCV are better defined, the risk of HCC among patients with NASH is less well known. NASH-related HCC occurs primarily in the setting of hepatic cirrhosis[1,92-95]. A large retrospective cohort study from South Korea evaluated 329 patients with fatty liver disease associated HCC and demonstrated an increase in NAFLD-related HCC from 3.8% in 2001-2005 to 12.2% in 2006-2010[96]. A United States based study evaluated 195 NASH-cirrhosis patients from 2003-2007 with serial abdominal computed tomography and serum alpha-fetoprotein every 6 mo with a median follow up of 3.2 years[97]. Among this cohort for NASH-related cirrhosis patients, 12.8% (n = 25) developed HCC with an annual cumulative HCC incidence of 2.6%. Several additional studies, both in western and Asia-Pacific regions, report on the progression of NASH-related cirrhosis towards HCC but this rate of progression is significantly lower than that seen among patients with cirrhosis secondary to chronic HCV. Yasui *et al*[98] prospectively evaluated 412 NAFLD patients from 1990 to 2006. Among this cohort,

Table 2 Etiology of liver disease among liver transplantation recipients in the United States, 1992-2012, United Network for Organ Sharing database *n* (%)

Liver disease etiology	Pre-MELD (1992-2002)	Post-MELD (2003-2007)	Post-MELD (2008-2012)
Acute liver failure	2390 (7.9)	1639 (6.9)	1285 (5.1)
Chronic HCV	9248 (30.7)	7970 (33.5)	7803 (31.2)
Chronic HBV	1419 (4.7)	802 (3.4)	604 (2.4)
HCC	466 (1.6)	1714 (7.2)	3423 (13.7)
ALD	5027 (16.7)	3704 (15.6)	3636 (14.6)
ALD + HCV	2495 (8.3)	1845 (7.8)	1529 (6.1)
NASH	8 (0.1)	796 (3.3)	2162 (8.7)
AIH	1277 (4.2)	715 (3.0)	693 (2.8)
Cryptogenic	3460 (11.5)	2115 (8.9)	1634 (6.5)
PBC	1992 (6.6)	1009 (4.2)	795 (3.2)
PSC	1648 (5.5)	1 (4.3)	922 (3.7)
Metabolic	729 (2.4)	478 (2.0)	491 (2.0)

Metabolic includes Wilson disease, alpha-1 antitrypsin disease and hemochromatosis. MELD: Model for end stage liver disease; HCV: Hepatitis C virus; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; ALD: Alcoholic liver disease; NASH: Non-alcoholic steatohepatitis; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; PSC: Primary sclerosing cholangitis.

68 patients with NASH-related cirrhosis were compared with 69 age and sex matched HCV-related cirrhosis patient controls to determine HCC risk. Overall, the 5-year cumulative HCC rate was 11.3% for NASH patients and 30.5% for HCV patients. This lower HCC risk among NASH-related cirrhosis patients compared with HCV-related cirrhosis patients was confirmed in additional studies.

While the majority of NASH-related HCC occurs in patients with cirrhosis, several studies have reported HCC development among non-cirrhotic NASH patients, with one Japanese study reporting rates of non-cirrhotic NASH-related HCC ranging from 10%-75% of cases[97-101]. The exact etiology for this non-cirrhotic pathway towards HCC is unclear. However, studies have demonstrated that obesity and diabetes mellitus, both of which are closely associated with NAFLD, are independently associated with increased risk of HCC among patients with chronic liver disease[102-104]. Furthermore, Welzel *et al*[105] utilized the National Cancer Institute's Surveillance, Epidemiology and End Results-Medicare database to evaluate the impact of metabolic syndrome on overall HCC risk among the general United States population. Among a cohort of 3649 HCC cases and 195953 comparison cohort, metabolic syndrome (as defined by National Cholesterol Education Program Adult Treatment Panel III criteria) was associated with a significantly increased risk of HCC (OR = 2.13, 95%CI: 1.96-2.13, $P < 0.0001$).

The implications of these findings on HCC screening among NAFLD patients are a major public health issue. While more studies evaluating the long-term HCC risk among patients with NASH-related cirrhosis are needed, it is reasonable to implement standard HCC screening programs in this cohort as one would for patients with cirrhosis from other chronic liver disease etiologies.

However, as with other chronic liver disease etiologies, only a fraction of NASH-related cirrhosis patients will develop HCC and the ability to better define the cohort of patients from those who will not develop HCC will be especially important in the management of this group of patients. More studies are needed to investigate risk factors for HCC development among this cohort that will allow a more targeted approach towards risk stratifications and earlier detection and treatment of HCC. However, the increasingly reported cases of HCC among non-cirrhotic NAFLD patients introduce an unexpected component to the commonly accepted pathogenesis of HCC. Clearly, these patients do not carry the same HCC risk as those patients with non-cirrhotic hepatitis B infection. However, what distinguishes those patients with non-cirrhotic NAFLD that develop HCC from those that do not? What are the important risk factors that should be incorporated into risk stratification models? How should HCC screening programs be implemented among this cohort? More studies are needed to better understand the risk factors associated with HCC development among NASH patients with and without cirrhosis.

NAFLD AND LIVER TRANSPLANTATION

The increasing prevalence of patients with NASH who develop cirrhosis and decompensated liver disease will undoubtedly lead to a major increase in the number of patients on the waiting list for liver transplantation. Several studies have already predicted that as a result of the obesity epidemic, the rising rates of NASH will become a leading indication for liver transplantation (Table 2)[19,106-108]. A recent study by Charlton *et al*[19] retrospectively evaluated liver transplantations in the United States from 2001-2009 utilizing a national liver transplantation database. This study demonstrated a significant increase in the proportion of patients undergoing liver transplantation for NASH from 1.2% in 2001 to 9.7% in 2009, making NASH the third leading indication for liver transplantation. Furthermore, the trajectory of increasing prevalence of NASH among liver transplantation recipients indicates that it will soon become the leading indication for liver transplantation. It has also been suggested that our current estimation of NASH prevalence is an underestimation, as many patients with cirrhosis secondary to cryptogenic cirrhosis may in fact be more accurately categorized as NASH. This hypothesis is supported by evidence demonstrating that cryptogenic cirrhosis patients share many similar characteristics to NASH patients, including risk factors associated with metabolic syndrome, and many patients with cryptogenic cirrhosis can in fact be more accurately categorized as NASH[109-113]. Furthermore, the outcomes associated with cryptogenic cirrhosis are also similar to those seen among patients with NASH[110-113]. Clearly, the rising prevalence of obesity and NASH patients who develop decompensated liver disease will soon become a significant cohort impacting the liver transplantation waiting list.

In Asia-Pacific regions, viral hepatitis and hepatocellular carcinoma are the leading indications for liver transplantation. Furthermore, unlike western countries, living donor liver transplantations play a more significant role in liver transplantation surgeries[114-116]. With the continued rising prevalence of NAFLD and NASH in this region, NASH may soon become a leading contributor of end stage liver disease and need for liver transplantation in the Asia-Pacific regions.

CONCLUSION

The global obesity epidemic is associated with the increasing prevalence of metabolic syndrome and NAFLD. This phenomenon will contribute to an increasingly large cohort of patients that will develop NASH-related cirrhosis, decompensated liver disease and HCC. The emergence of this cohort is on the horizon and will introduce a significant disease burden in the field of liver transplantation. However, there are significant ethnic disparities in the prevalence and association of obesity with development of NASH. Furthermore, it is not clear if the risk factors associated with development of NASH and progression to cirrhosis and HCC vary by ethnicity. Our current focus on Asian populations clearly indicate that despite having lower average BMI, Asians as a group still maintain significant risks of metabolic syndrome and NAFLD, resulting primarily from the disparately higher central adiposity and visceral fat distribution seen in this cohort. This may further contribute to relatively increased risk of NASH development. More studies are needed to identify factors that influence the ethnicity-dependent rate of hepatic histological damage and the risk of HCC in NASH patients.

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REVIEW

Gender and racial differences in nonalcoholic fatty liver disease

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Abstract

Due to the worldwide epidemic of obesity, nonalcoholic fatty liver disease (NAFLD) has become the most common cause of elevated liver enzymes. NAFLD represents a spectrum of liver injury ranging from simple steatosis to nonalcoholic steatohepatitis (NASH) which may progress to advanced fibrosis and cirrhosis. Individuals with NAFLD, especially those with metabolic syndrome, have higher overall mortality, cardiovascular mortality, and liver-related mortality compared with the general population. According to the population-based studies, NAFLD and NASH are more prevalent in males and in Hispanics. Both the gender and racial ethnic differences in NAFLD and NASH are likely attributed to interaction between environmental, behavioral, and genetic factors. Using genome-wide association studies, several genetic variants have been identified to be associated with NAFLD/NASH. However, these variants account for only a small amount of variation in hepatic steatosis among ethnic groups and may serve as modifiers of the natural history of NAFLD. Alternatively, these variants may not be the causative variants but simply markers representing a larger body of genetic variations. In this article, we provide a concise review of the gender and racial differences in the prevalence of NAFLD and NASH in adults. We also discuss the possible mechanisms for these disparities.

Key words: Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Race; Gender; Prevalence; Genetic polymorphism

Core tip: According to the population-based studies, nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) are more prevalent in males and in Hispanics. Both the gender and racial ethnic differences in NAFLD and NASH are likely attributed to interaction between environmental, behavioral, and genetic factors. In this article, we provide a concise review of the gender and racial differences in the prevalence of NAFLD and NASH in adults. We also discuss the possible mechanisms for these disparities.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is highly associated with obesity and insulin resistance (IR) and represents a spectrum of liver injury ranging from simple steatosis with a more benign course to nonalcoholic steatohepatitis (NASH) which may progress to advanced fibrosis and cirrhosis[1,2]. According to the National Health and Nutrition Examination Survey (NHANES), 33.8% and 23.7% of the United States (US) adults are obese and have metabolic syndrome, respectively[3,4]. Due to the worldwide epidemic of obesity, NAFLD has become the most common cause of elevated liver enzymes with

Table 1 Prevalence rates of nonalcoholic fatty liver disease from population-based studies

Ref.	Study population	*n*	Definition of NAFLD	Prevalence of NAFLD				
				Overall	NHW	Hispanic	NHB	Others
Ruhl *et al*[11]	NHANES Ⅲ (1988-1994)	5724	ALT[1]	2.8%	2.6%	8.4%	1.9%	3.1%
Clark *et al*[12]	NHANES Ⅲ (1988-1994)	15676	ALT or AST[2]	5.4%	4.8%	9.9%	4.2%	
Browning *et al*[13]	Dallas Heart Study	2287	MRS[3]	31%	33%	45%	24%	
Ioannou *et al*[15]	NHANES (1999-2002)	6823	ALT or AST[4]	8.1%				
Younossi *et al*[17]	NHANES Ⅲ (1988-1994)	11613	Ultrasound	18.8%				
Lazo *et al*[18]	NHANES Ⅲ (1988-1994)	12454	Ultrasound	19%	17.8%	24.1%	13.5%	
Schneider *et al*[19]	NHANES Ⅲ (1988-1994)	9675	Ultrasound		12.5%	21.2%	11.6%	
Smits *et al*[20]	NHANES Ⅲ (1988-1994)	3846	Ultrasound	30.2%	29.8%	39.4%	23.1%	
Liangpunsakul *et al*[22]	NHANES Ⅲ (1988-1994)	4376	ALT[2]	4.5%				

[1]ALT > 43 U/L; [2]ALT > 40 U/L and AST > 37 U/L for men; ALT and AST > 31 U/L for women; [3]Hepatic triglyceride content > 5.5%; [4]ALT > 43 U/L or AST >40 U/L. NHANES: National Health and Nutrition Examination Survey; NAFLD: Nonalcoholic fatty liver disease; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; MRS: Magnetic resonance spectroscopy; NHW: Non-Hispanic whites; NHB: Non-Hispanic blacks.

prevalence rates ranging from 2.8% to 46%[5,6]. Individuals with NAFLD and NASH, especially those with metabolic syndrome, have higher overall mortality, cardiovascular mortality, and liver-related mortality compared with the general population[7-9]. Liver cirrhosis secondary to NAFLD is now the second most common indication for liver transplantation in obese patients[10].

Among different racial and ethnic populations in the US, Hispanics (predominantly of Mexican origin) are at particular risk for NAFLD and tend to have a more aggressive disease course[11-20]. Hispanics accounted for nearly 50% of the US population growth from 2000 to 2010 and are projected to reach 30% of the US population within the next three decades[21]. Given the increasing prevalence and the expected growth in the Hispanic population, NAFLD poses a huge threat to the US health care system.

In this article, we provide a concise review of the gender and racial differences in the prevalence of NAFLD and NASH in adults. We also discuss the possible mechanisms for the racial/ethnic disparities, with a special focus on the Hispanics.

PREVALENCE OF NAFLD IN GENERAL POPULATIONS

The prevalence of NAFLD varies depending on the study population and the diagnostic tool used to determine the condition. The prevalence rates of NAFLD in the US based on population-based studies are summarized in Table 1. Most of these studies were based on the third NHANES (1988-1994) data. Defined as elevated alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST), NAFLD was prevalent in 2.8%-5.4% of the US population[11,12,22]. From 1999 to 2002, the prevalence of NAFLD in the US further increased to 8.1%[15]. The differences of the prevalence between the two periods could be due to differences in assay methodology. Serum specimens were initially frozen after collection and then thawed prior to assay during the earlier period (1988-1994) whereas sera were only refrigerated before testing during the later time (1999-2002). Freezing

serum specimens to -20 ℃ has been shown to lead to a 46% loss of ALT activity, whereas refrigerating serum specimens to 4 ℃ only led to a 6% loss[23]. Therefore, more individuals could have been falsely stratified as having normal liver enzymes and hence lower prevalence of NAFLD in the earlier period. On the other hand, true differences may exist as there was an increase in the prevalence of predictors for elevated ALT such as higher body mass index (BMI) and waist circumference between the periods 1988-1994 and 1999-2002[15]. Nevertheless, studies relying on elevated liver enzymes probably underestimate the true prevalence of NAFLD as normal ALT level provides little diagnostic or prognostic value when assessing persons for NAFLD. In the Dallas Heart Study, 79% of the subjects with hepatic steatosis had normal ALT levels (defined as ALT ≤ 40 U/L for men and ≤ 31 U/L for women)[13].

Using ultrasonography as the diagnostic tool for NAFLD, recent studies reported prevalence rates of 18.8%-30.2% in the US (Table 1)[17-20]. Ultrasonography has been used in two studies to assess the prevalence of hepatic steatosis in non-US populations. The first study performed 25 years ago reported that fatty liver was found in 14% of the population in Okinawa, Japan[24]. The second study reported that NAFLD was present in 20% of the residents who live in Northern Italy (the Dionysos study)[25]. The lower prevalence of hepatic steatosis found in the Japanese study likely reflects the low frequency or absence of obese or diabetic subjects in the study cohort[13]. Despite being more sensitive than liver enzymes for the detection of NAFLD, ultrasonography has its own limitation due to a low sensitivity for detection of mild hepatic steatosis (less than 30%)[26]. Therefore, ultrasonography also likely underestimates the true prevalence of NAFLD in general populations. Using a more sensitive magnetic resonance spectroscopy technique for measuring fat content, 31% of the participants in the Dallas Heart Study had hepatic steatosis, defined as hepatic triglyceride content greater than 5.5%[13].

NAFLD occurs in non-obese and non-overweight (defined as BMI < 25 kg/m^2) persons as well. Based on the third NHANES data, 7% of the lean individuals have

Table 2 Gender difference in the prevalence of nonalcoholic fatty liver disease from population-based studies

Ref.	Study population	n	Definition of NAFLD	Prevalence of NAFLD	
				Men	Women
Ruhl *et al*[11]	NHANES Ⅲ (1988-1994)	5724	ALT[1]	4.3%	1.6%
Clark *et al*[12]	NHANES Ⅲ (1988-1994)	15676	ALT or AST[2]	5.7%	4.6%
Browning *et al*[13]	Dallas Heart Study	734[5]	MRS[3]	42%	24%
Ioannou *et al*[15]	NHANES (1999-2002)	6823	ALT or AST[4]	13.4%[6]	4.5%[6]
Lazo *et al*[18]	NHANES Ⅲ (1988-1994)	12454	Ultrasound	20.2%	15.8%
Schneider *et al*[19]	NHANES Ⅲ (1988-1994)	4037[5]	Ultrasound	15%	10.1%

[1]ALT > 43 U/L; [2]ALT > 40 U/L and AST > 37 U/L for men; ALT and AST > 31 U/L for women; [3]Hepatic triglyceride content > 5.5%; [4]ALT > 43 U/L or AST >40 U/L; [5]Non-Hispanic white only; [6]Not adjusted for alcohol consumption or hepatitis C antibody status. NHANES: National Health and Nutrition Examination Survey; NAFLD: Nonalcoholic fatty liver disease; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; MRS: Magnetic resonance spectroscopy.

NAFLD compared to 28% of the overweight-obese population[17]. In the Dionysos study, hepatic steatosis on ultrasound was present in 16% of the non-obese participants[27]. In a Japanese study, ultrasonographic fatty liver was found in 11.2% of non-obese persons during voluntary health check-up[28].

PREVALENCE OF NASH IN GENERAL POPULATIONS

Liver biopsy is the current suboptimal standard for the diagnosis and staging of NASH, but invasiveness and cost preclude its use as a screening tool in general populations[29]. The population prevalence of NASH has therefore been difficult to establish since it is unethical to biopsy asymptomatic persons in the community. Among 351 apparently nonalcoholic patients, a Canadian autopsy study from the late 1980s found that NASH was present in 2.7% of lean patients and in 18.5% of markedly obese patients[30]. More recently, two Asian studies reported similar prevalence of NASH in 1.1%-2.2% of living donors before liver transplantation[31,32]. Based on the third NHANES data, 2.6% of the US population have NASH defined as the presence of moderate-severe hepatic steatosis by ultrasound and elevated aminotransferases in the presence of type 2 diabetes or IR[17].

GENDER DIFFERENCE IN THE PREVALENCE OF NAFLD

Some old studies reported that women were at higher risk for NAFLD, but these studies were not population based and were subject to potential ascertainment bias[11]. Based on the third NHANES data, most of the studies reported that NAFLD is significantly more prevalent in men than in women (Table 2). However after dichotomizing individuals into lean and overweight-obese groups, Younossi *et al*[17] reported that the lean NAFLD cohort was more commonly female. Using data from 698 patients from the well characterized NASH Clinical Research Network (CRN), patients with biopsy proven NASH were more likely to be female than male in a roughly 2:1 ratio; possibly reflecting a higher disease burden in women or, alternatively, sex differences among those pursuing and receiving healthcare[33]. Together, these findings highlight uncertainties regarding the influence of gender on NAFLD.

A number of mechanisms may contribute to gender differences in the prevalence of NAFLD.

The role of IR, which is closely associated with NAFLD[1,2], remains controversial. Ruhl *et al*[11] reported that NAFLD was more prevalent in men than in women (4.3% *vs* 1.6%, respectively), a finding essentially explained by the higher waist-to-hip circumference (WHR) ratio in men. WHR is correlated with visceral adipose tissue (VAT) and visceral adiposity is associated with both peripheral and hepatic IR[34,35]. In another study using the same database but different cohort size, Clark *et al*[12] also reported that men have higher prevalence of NAFLD than women (5.7% *vs* 4.6%, respectively), although there was no significant difference in either gender in IR as calculated by homeostasis model assessment (HOMA) or exercise level. Moreover, in the Dallas Heart Study, non-Hispanic white men had an approximately 2-fold higher prevalence of hepatic steatosis than white women. Differences in body weight or insulin sensitivity measured by HOMA did not explain these sex differences.

Alcohol use is another possible explanation for gender differences in NAFLD. In the Dallas Heart Study, white men who reported moderate ethanol intake had a significantly higher prevalence of hepatic steatosis than female counterparts (42% *vs* 20%, $P = 0.03$). In fact, moderate alcohol intake was associated with an decrease in the prevalence of hepatic steatosis in women[13]. Similarly, Schneider *et al*[19] reported that non-Hispanic white men, who were more likely to be self-defined as "low current drinkers" (men ⩽ 2 drinks/d; women ⩽ 1 drink/d), had a significantly higher prevalence of NAFLD than non-Hispanic white women (15% *vs* 10.1%, respectively), even after adjusting for BMI and waist circumference. Finally, in adult members of the Kaiser Permanente Medical Care Program in California, NAFLD was 3.5 times more common in Asian men than in Asian women ($P = 0.016$). There was no significant difference in BMI (> 28 kg/m^2), diabetes mellitus, dyslipidemia, or current alcohol use between Asian men and women, but 68% of Asian men were previous drinkers, compared with 17% of Asian

women ($P < 0.02$)[14]. Together, these studies suggest an effect of alcohol consumption on gender differences in the prevalence of NAFLD. Whether differences in hepatic metabolism of alcohol between men and women also contribute to the gender difference in not fully defined[19].

Other factors, including lifestyle and sex hormone may also influence the gender difference in the prevalence of NAFLD. In one study, individuals with NAFLD had similar degrees of IR and obesity to those without, but males with NAFLD consumed more non-diet soda on a weekly basis (54.4% *vs* 34%, $P = 0.037$)[16]. Another recent study showed that prevalence of NAFLD was similar in pre- and intrapubertal boys and higher in the postpubertal groups (51.2%), whereas in girls NAFLD was most common in the intrapubertal group (25.2%) and lower in the postpubertal group (12.2%)[36].

RACIAL/ETHNIC DIFFERENCES IN NAFLD AND NASH

Despite using different diagnostic tools, US population-based studies all found that Hispanics have the highest and non-Hispanic blacks have the lowest prevalence of NAFLD (Table 1). Echoing the racial/ethnic differences in the NAFLD prevalence, Younossi *et al*[17] recently reported that NASH was independently associated with being Hispanic [odds ratio (OR), 1.72; 95%CI: 1.28-2.33] and inversely associated with being African-American (OR, 0.52; 95%CI: 0.34-0.78). Each of these studies is limited by the fact that NASH was diagnosed by imaging and/or biochemical criteria rather than by histology.

Single center studies show that ethnicity may also influence NAFLD histology. For instance, African Americans were found to have less steatosis than whites. Asians and Hispanics showed higher grades of ballooning and Mallory bodies, respectively, than whites and other ethnicities combined[37]. Williams *et al*[16] also reported a significantly higher prevalence of NASH in Hispanics than Caucasians (19.4% *vs* 9.7%, $P = 0.03$) although comparison of demographics such as BMI between different ethnic groups were not available in this study. However, Kallwitz *et al*[38] found no significant differences in hepatic steatosis, NASH, or liver fibrosis ($\geqslant$ F2) between morbidly obese Hispanic and non-Hispanic white patients receiving bariatric surgery. Similar to the other reports, morbidly obese African American patients had a lower rate of NAFLD, NASH and less fibrosis than non-Hispanic whites and Hispanics. Moreover, in a NASH CRN study consisting mainly of Caucasian subjects (82%), subjects of Hispanic ethnicity overall had lower fibrosis scores and less advanced fibrosis[33]. Finally, in an analysis restricted to 3082 individuals with normal weight (BMI 18.5-24.9 kg/m^2), Schneider *et al*[19] found no significant racial differences in the fully adjusted logistic regression model for NAFLD; however, Mexican Americans remained significantly more likely to have NAFLD with elevated aminotransferases (OR, 3.4; 95%CI: 1.29-7.18). This finding was confirmed in a prospective study where

overweight or obese Hispanics and Caucasians had similar hepatic or adipose tissue IR and severity of NASH by histology when matched for major clinical variables, in particular for total body fat[39]. These findings suggest that a component of the higher prevalence of NAFLD and NASH observed in Hispanics may be attributed to differences in the frequency of major clinical variables such as components of metabolic syndrome or diabetes that influence the development of NAFLD.

MECHANISMS FOR THE RACIAL/ETHNIC DIFFERENCES IN NAFLD AND NASH

A number of potential factors have been implicated in racial and ethnic differences in NAFLD. These include differences in lifestyle, IR, distribution of adiposity and genetics. These factors are not mutually exclusive and may occur and act in concert.

Lifestyle
According to the "two hit" theory, steatohepatitis development requires a double hit, the first producing steatosis, and the second a source of oxidative stress capable of initiating significant lipid peroxidation[40]. Dietary habits may promote steatohepatitis directly by modulating hepatic triglyceride accumulation and antioxidant metabolism as well as indirectly by affecting insulin sensitivity and postprandial triglyceride metabolism[41]. Several studies have reported that different racial and ethnic groups have substantial differences in their diet. In an early US population-based study (1987 National Health Interview Survey), Hispanics reported higher energy and carbohydrate intakes and a lower percentage of energy from fat than blacks or whites (35.6%, 38.4%, and 38.7% of energy from fat for Hispanics, blacks, and whites, respectively). Whites had lower cholesterol intake than the other two groups, and blacks had a higher intake of sweets[42]. According to the San Antonio heart study published almost 20 years ago, when data were pooled across socioeconomic groups, Mexican Americans consumed more carbohydrate, saturated fat, and cholesterol, and less linoleic acid than Anglo Americans. However, there were no ethnic differences in total fat, saturated fat, or carbohydrate consumption when compared within a given socioeconomic status[43]. Data from the Stanford Five-City Project showed that low educated white adults consumed significantly more fat as measured by percentage of calories from total fat (37.7% *vs* 33.3%) and saturated fat (13.7% *vs* 11.8%), and consumed significantly less dietary carbohydrate (45.5% *vs* 49.7%) and fiber (17.1 g *vs* 26 g) than Hispanic adults. Interestingly, a graded relationship was found between acculturation and dietary measures, where more acculturated Hispanics (English-speaking) were intermediate between less acculturated Hispanics (Spanish-speaking) and whites in their dietary intake[44].

Common theme in these studies is that Hispanics consume more carbohydrates than other ethnic groups.

The role of excess carbohydrate intake in NASH has been shown in at least two other studies[45,46]. In the first study of a small series of Japanese adults, individuals with histology proven NASH had a higher intake of simple carbohydrates than those with simple steatosis[44]. In the second study from the NASH CRN, Hispanics with NASH had higher carbohydrate intake compared to non-Hispanic whites with NASH[45]. In addition to high carbohydrate diet, NASH is also associated with a low intake of zinc and lower ratio of intake of polyunsaturated fatty acid to saturated fatty acid[44].

Analysis of the NASH CRN data further showed that patients with NAFLD ate at fast-food restaurants ($\geq$ 1 per week) more often (70.9% *vs* 60.5%, *P* = 0.049) and exercised ($\geq$ 30 min per week) less frequently (56.3% *vs* 68.9%, *P* = 0.02) than their non-NAFLD counterparts. However, racial differences in these two measures was not studied[16]. A recent study based on the NHANES data reported that sedentary individuals had a significantly higher prevalence of NAFLD independent of other risk factors[18]. In a small series of 37 patients, Krasnoff *et al*[47] reported that patients with NAFLD of differing histological severity have suboptimal cardiorespiratory fitness, muscle strength, body composition, and physical activity participation. These findings establish the association between physical inactivity and NAFLD and support the current recommendation of regular exercise for patients with the condition.

IR

Several US and non-US population-based studies have shown that NAFLD is highly associated with central obesity, IR, and components of metabolic syndrome (high triglyceride, low high-density-lipoprotein cholesterol, hyperglycemia, and hypertension)[11-13,15,18,22,25,48]. NAFLD has therefore been suggested to be a hepatic feature of the metabolic syndrome[49]. However, Smits *et al*[20] recently challenged this popular notion. In their study, NAFLD was strongly related to the different components of the metabolic syndrome. However, adding hepatic steatosis to a mathematical model containing the traditional components of the metabolic syndrome did not improve goodness of fit and if anything resulted in a decrease in model fit. They thus concluded that NAFLD is not an independent additional component or manifestation of the metabolic syndrome.

In addition to being a lipid storage compartment, adipose tissue is also an endocrine organ[50]. Adipose tissue IR plays key role in the development of metabolic and histological abnormalities of obese patients with NAFLD. Liver steatosis was rare in metabolically healthy obese subjects with normal adipose tissue insulin sensitivity. Compared to patients without steatosis, patients with NAFLD were insulin resistant at the level of adipose tissue, liver, and skeletal muscle. Metabolic parameters, hepatic IR, and liver fibrosis but not necroinflammation deteriorated as adipose tissue IR worsened[51]. The coincident occurrence of hepatic steatosis and IR has led to the

hypothesis that excess triglyceride in liver causes IR[52]. This notion was challenged by a recent study by Lomonaco *et al*[39]. In that study, liver fat was slightly, but not significantly, higher in Hispanic than Caucasian patients. This slightly higher liver fat content was not associated with worse hepatic or adipose tissue IR[39].

As shown in Table 1, Hispanics have a higher prevalence and blacks have a lower prevalence of NAFLD than whites. According to the data from the third NHANES, both black and Mexican American women had higher cardiovascular disease risk factors such as hypertension, physical inactivity, higher BMI and diabetes than white women of comparable socioeconomic status[53]. While the higher prevalence of hepatic steatosis in Hispanics can be explained by the high prevalence of obesity and IR in this population, the lower prevalence of hepatic steatosis in blacks cannot be explained by the same reason. In the Insulin Resistance Atherosclerosis Study, African Americans were more insulin resistant than Hispanics. Hispanics however had higher prevalence of NAFLD than African Americans (24% *vs* 10%)[54]. Therefore an IR paradox may exist[55]. It has been hypothesized that differences in NAFLD and NASH by race may result from differences in the distribution of adiposity (*e.g.*, subcutaneous *vs* visceral) or differences in triglycerides because blacks have relatively less VAT and lower triglycerides than Hispanics[19,56]. In addition, African Americans may be more resistant to both the accretion of triglyceride in the abdominal visceral compartment (adipose tissue and liver) and hypertriglyceridemia associated with IR[55].

Distribution of adiposity

Several studies have reported racial differences in the distribution of adiposity, especially in women. In a small study of age- and weight-matched healthy women (8 black and 10 white), black women had 23% less VAT as measured by computed tomography (CT) than white women. In addition, black women had significantly lower plasma glucose and triglycerides and significantly higher plasma high-density-lipoprotein cholesterol[57]. Based on the Dallas Heart Study data, blacks had less intraperitoneal fat as measured by magnetic resonance imaging and more lower extremity fat than their Hispanic and Caucasian counterparts, despite controlling for age and total adiposity. In that study, the prevalence of IR was similar between blacks and Hispanics who had the highest levels of intraperitoneal fat and liver fat. Furthermore, insulin levels and HOMA values were the highest and serum triglyceride levels were lowest among blacks after controlling for intraperitoneal fat[55]. In a prospective study of healthy sedentary women, Casas *et al*[58] found that Hispanic women had greater total adiposity than white women, which was primarily the result of higher percentage fat and fat mass in the trunk. Within the trunk region, abdominal and subscapular skinfold thicknesses were 30%-40% significantly greater in the Hispanic women. Total fat-free mass was slightly but significantly lower in the Hispanic women primarily due to a smaller fat-free

mass in the trunk region. In a study involving healthy subjects, Asians despite of having lower BMI had more upper-body subcutaneous fat as measured by dual-photon absorptiometry than did whites. The magnitude of differences between the two races was greater in females than in males[59]. A later study with a smaller cohort reported that Asian American premenopausal women had higher VAT than European American women, after adjusting for age and total body fat. There was a significant age by race interaction such that race differences in VAT were more evident over the age of 30 years. No differences in VAT could be detected between Asian American and European American men, even after adjusting for potential covariates[60]. Visceral adiposity has been reported to be associated with both peripheral and hepatic IR, independent of gender, in diabetic patients[35]. Visceral fat has also been shown as an important site for interleukin-6 secretion and provides a potential mechanistic link between visceral fat and systemic inflammation in people with abdominal obesity[61]. Inflammatory activation within metabolic tissues such as white adipose tissue, liver, and skeletal muscle potentiates IR and metabolic disease[62].

Together, these results support that differences in distribution of adiposity may influence racial differences in the prevalence of NAFLD and NASH.

Genetic variations

Caldwell *et al*[63] previously proposed that obesity and IR are often "essential but not sufficient" in the development of NAFLD given the variable prevalence of steatosis in different ancestry groups. They further suggested a genetic basis for the variable presence of steatosis in the metabolic syndrome. Possible mechanisms to explain this variation include differences in hepatic fatty acid-binding protein (influencing fatty acid import to the liver), in the activity of microsomal triglyceride transfer protein (influencing *de novo* fat synthesis), or in other compensatory mechanisms that are active in insulin-resistant patients without steatosis. The findings of familial clustering of NAFLD and NASH suggest a hereditary component for the conditions. Struben *et al*[64] retrospectively examined 8 index patients who had either NASH with or without cirrhosis or cryptogenic cirrhosis and 10 of their relatives from 8 kindreds. They found that co-existence of NASH and/or cryptogenic cirrhosis in 7 of 8 kindreds studied. Willner *et al*[65] reviewed 90 patients with NASH and found that 16 (18%) of the patients came from 9 families with NASH. Two generations were involved in 6 families and siblings were involved in the other 3 families. Notably, cirrhosis was observed in 7 of these 9 families. A small case series from Japan reported 3 families each with 2 members with biopsy-proven NASH[66]. By studying overweight children with and without biopsy-proven NAFLD and their families, Schwimmer *et al*[67] reported that fatty liver was significantly more common in siblings (59% *vs* 17%) and parents (78% *vs* 37%) of children with NAFLD than those without NAFLD. In addition to genetic basis, sharing common environmental factors

and/or lifestyles could be alternative explanations for the familial nature of NAFLD and NASH.

In the landmark study from the Dallas Heart Study, Romeo *et al*[68] first reported that the rs738409[G] allele in patatin-like phospholipase domain-containing 3 (*PNPLA3*) gene was strongly associated with hepatic fat content even after adjustment for BMI, diabetes status, ethanol use, as well as ancestry. The variant is a cytosine to guanine substitution that changes codon 148 from isoleucine to methionine (I148M). Hepatic fat content was more than twofold higher in the G allele homozygotes than in noncarriers. The frequencies of the G allele were concordant with the relative prevalence of NAFLD in the three ancestry groups; the highest frequency of allele was in Hispanics (0.49), with lower frequencies observed in European Americans (0.23) and African Americans (0.17). In the Dallas Heart Study, rs738409(G) was significantly associated with ALT and AST levels only in Hispanics. Interestingly, rs738409(G) was not associated with BMI or indices of insulin sensitivity such as fasting plasma glucose and insulin concentrations or HOMA. Furthermore, *PNPLA3* genotype was not associated with concentrations of triglyceride, total cholesterol, high-density-lipoprotein cholesterol or low-density-lipoprotein cholesterol. Another variant of the *PNPLA3* [rs6006460(T), encoding S453I] was found to be associated with lower hepatic fat in African Americans. Regression analysis indicated that these two sequence variations accounted for 72% of the observed ancestry-related differences in hepatic fat content in the Dallas Heart Study.

Similar to the Dallas Heart Study, Wagenknecht *et al*[69] also found a higher frequency of *PNPLA3* rs738409(G) in Hispanics in a large US minority cohort (843 Hispanic Americans and 371 African Americans) study. The G allele was two times more common in Hispanic Americans than in African Americans (40% *vs* 19%), consistent with the greater prevalence of NAFLD in Hispanic Americans (24% *vs* 9%). The G allele was also associated with elevated ALT and AST but not metabolic phenotypes in both Hispanic- and African Americans. However, unlike the Dallas Heart Study, the *PNPLA3* genotype could only explain 4.4% of variation in liver fat content in Hispanic Americans and 5.6% in African Americans. Even with adjustment for the *PNPLA3* variation, a significant ethnic disparity in liver fat content persisted. It was therefore suggested that *PNPLA3* does not explain the unusually high prevalence of NAFLD in Hispanic Americans.

The *PNPLA3* genotype is associated with hepatic fat content and aminotransferase in non-US populations as well. In a Finnish study, 291 individuals were genotyped and had liver fat measured by magnetic resonance spectroscopy. The G allele was associated with increased liver fat content and AST independently of age, sex, and BMI. *PNPLA3* expression in the liver was positively related to obesity and to liver fat content in persons who were not morbidly obese (BMI < 40 kg/m^2)[70]. In another study, 678 obese (mean BMI = 41 kg/m^2) Italians were genotyped for the *PNPLA3* variant. It was found that ALT

and AST were significantly higher in carriers of the G allele; 50% of the individuals homozygous for the G allele had elevated ALT (> 40 U/L) compared with 25% of the carriers of two C alleles, whereas 30% of the heterozygotes had elevated ALT. Glucose tolerance and insulin sensitivity were similar in all three genotypes[71]. In a Latin American study, 172 Argentinians with NAFLD defined by ultrasonographic steatosis and 94 controls were genotyped. Similar to the previous reports, rs738409[G] was significantly associated with NAFLD, independent of age, sex, BMI, and HOMA index. Patients with CC genotype had a lower histologic steatosis score (14.9% ± 3.9%) in comparison with the CG genotype (26.3% ± 3.5%) and GG genotype (33.3% ± 4%) (*P* < 0.005). Similar to the previous US minority cohort study[69], the *PNPLA3* genotype could only account for a small amount (5.3%) of the total variation in hepatic steatosis[72].

The *PNPLA3* genotype exerts a strong influence not only on liver fat accumulation but also on the susceptibility of a more aggressive disease course. A recent meta-analysis of 16 studies concluded that the GG homozygotes had 3.24-fold greater risk of higher necroinflammatory scores and 3.2-fold greater risk of developing fibrosis when compared with the CC homozygotes (data from 1739 and 2251 individuals, respectively). NASH was more frequently observed in the GG than the CC homozygotes (OR, 3.488; 95%CI: 1.859-6.454; data from 2124 patients). In the meta-analysis, a negative correlation between the male proportion in the studied population and the effect of rs738409 on liver fat content was observed, suggesting that a sexual dimorphism might be involved in the effect of the single nucleotide polymorphism (SNP) on NAFLD development. The rs738409 GG genotype versus CC genotype was associated with a 28% increase in ALT levels. The *PNPLA3* rs738409 was therefore proposed as a strong modifier of the natural history of NAFLD[73].

In addition to *PNPLA3*, the Genetics in Obesity-related Liver Disease (GOLD) Consortium studied 7176 individuals of European ancestry and identified genetic variants in or near three novel loci [neurocan gene *NCAN* (rs2228603), glucokinase regulatory protein gene *GCKR* (rs780094), and lysophospholipase-like 1 gene *LYPLAL1* (s12137855)] that were associated with both increasing CT hepatic steatosis and histologic NAFLD. The genetic variant in or near glycogen binding subunit of protein phosphatase 1 gene *PPP1R3B* (rs4240624) was associated with CT steatosis but not histologic NAFLD. Variants at these 5 loci exhibited distinct patterns of association with serum lipids, as well as glycemic and anthropometric traits. Specifically variants in or near *NCAN*, *GCKR*, and *PPP1R3B* associated with altered serum lipid levels, whereas those in or near *LYPLAL1* and *PNPLA3* did not. Variants near *GCKR* and *PPP1R3B* also affected glycemic traits. These findings suggest development of hepatic steatosis, NASH/fibrosis, or abnormalities in metabolic traits are probably influenced by different metabolic pathways and may provide new insights that into

how obesity can lead to metabolic complications in some but not all individuals[74]. The observed genetic variants in European ancestry individuals were recently characterized in a multi-cohort study of African- (*n* = 3124) and Hispanic Americans (*n* = 849)[75]. In that study, variants in or near *PNPLA3*, *NCAN*, *GCKR*, *PPP1R3B* in African Americans and *PNPLA3* and *PPP1R3B* in Hispanic Americans were significantly associated with CT hepatic steatosis. *LYPLAL1* was not significantly associated with hepatic steatosis in either African- or Hispanic Americans despite comparable allele frequencies. The association of *NCAN* with hepatic steatosis was in an opposite direction in Hispanic Americans, suggesting it would have a small protective effect in this population. The allele frequency and effect size of each variant varied across ancestries. For example, the effect size of *PNPLA3* rs738409 was similar across the ancestries and the frequency of the G allele was higher in Hispanics. The effect size of *PPP1R3B* rs4240624 was twice in European ancestry individuals than other ancestries, whereas its frequency was roughly the same across the three ethnic groups. *GCKR* rs780094 had the same effect across ancestries but its frequency in African Americans was half of that in European ancestry individuals and Hispanic Americans, which were about equal[75].

Finally, in a recent multi-ethnic (*n* = 4804) study from the third NHANES, Hernaez *et al*[76] attempted to replicate the findings of the GOLD Consortium. Similar to the previous report by Palmer *et al*[75], the G allele of *PNPLA3* rs738409 was more prevalent in Mexican Americans than non-Hispanic whites and blacks. However, the T allele of *GCKR* rs780094 and the A allele of *PPP1R3B* rs4240624 were more common in non-Hispanic whites than the other two ethnic groups. In contrast to the GOLD Consortium, several discrepancies were noted. First of all, the *PNPLA3* variant was associated with hepatic steatosis diagnosed by ultrasonography only among Mexican Americans. Secondly, *NCAN* and *PPP1R3B* regions were associated with hepatic steatosis only in non-Hispanic whites. Thirdly, neither *LYPLAL1* nor *GCKR* were associated with hepatic steatosis in the third NHANES population. Fourthly, *PNPLA3* and *GCKR* were the only variants associated with elevated ALT (> 30 U/L in men and > 19 U/L in women) and the association in non-Hispanic whites only[76]. In an editorial comment, Browning called for the following considerations when interpreting the data of Hernaez *et al*[76]. The true prevalence of fatty liver in the study population might be higher than reported and/or that some individuals might have been mistakenly classified as having NAFLD since ultrasound is not as sensitive or specific for hepatic steatosis as other imaging modalities. In addition, the study appears to be underpowered to examine associations across ethnic/racial groups, especially for SNPs with a low allelic frequency. If underpowered, the analysis would be prone to false-negative results[77].

Variants in other genes such as cytochrome P450 2E1[78] and apolipoprotein C3[79] have been reported to be

implicated in NAFLD. To provide a detailed review of other genetic variants in NAFLD is beyond the scope of this review.

CONCLUSION

According to the population-based studies, NAFLD and NASH are more prevalent in males and in Hispanics. The gender differences in NAFLD and NASH can be probably explained by gender disparities in body fat distribution, lifestyle, and sex hormone metabolism. The racial/ethnic differences in NAFLD and NASH are likely attributed to interaction between environmental, behavioral, and genetic factors. Despite having similar or worse insulin sensitivity, non-Hispanic blacks are less likely to have NAFLD/NASH than non-Hispanic whites and Hispanics. Racial differences in body fat distribution and lipid metabolism may explain the IR paradox. By using genome-wide association study, several SNPs have been identified to be associated with NAFLD/NASH. These genetic variants however only account for a small amount of variation in hepatic steatosis among ethnic groups and may serve as modifiers of the natural history of NAFLD. As suggested by Browning[77], these trait-associated SNPs may not be the causative genetic variants but simply tags representing a larger body of SNPs. Further study is required to define how these variants alter normal physiology and/or identify the functional genetic variant in the haplotype block represented by the SNP[77].

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REVIEW

Chronic hepatitis B: Advances in treatment

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Abstract

Treatment of chronic hepatitis B (CHB) has markedly improved in the last 15 years due to the availability of direct antivirals which greatly increase therapeutic options. Currently, there are two classes of agents licensed for CHB treatment: standard or pegylated interferon alpha (IFN or Peg-IFN) and five nucleoside/nucleotide analogues (NAs). Long-term treatment with NAs is the treatment option most often used in the majority of CHB patients. Entecavir and tenofovir, the most potent NAs with high barrier to resistance, are recommended as first-line monotherapy by all major treatment guidelines and can lead to long-lasting virological suppression, resulting in histological improvement or reversal of advanced fibrosis and reduction in disease progression and liver-related complications. In this review, we focus on current treatment strategies of chronic hepatitis B and discuss the most recent efficacy and safety data from clinical trials and real life clinical practice. Recent findings of response-guided approaches are also discussed.

Key words: Chronic hepatitis; Antiviral therapy; Peg-interferon; Nucleos(t)ide analogues; Antiviral resistance

Core tip: Patients with chronic hepatitis B are a hetero-geneous population and require different management strategies. In clinical practice, several baseline factors, related to the patient, drug, stage of liver disease, comorbidities, lifestyle factors, coinfections and profile of hepatitis B virus infection, should be taken into consideration in order to individually optimize therapy. Surface antigen of the hepatitis B virus quantification is a potential new biomarker for treatment individualization and response-guided therapy. In the last two decades, the availability of potent oral antivirals changed the natural history of chronic hepatitis B; however, the risk of hepatocellular carcinoma (HCC) has not been abolished and thus regular HCC surveillance in high risk patients is required.

INTRODUCTION

Chronic infection with hepatitis B virus (HBV) is a major health problem worldwide, affecting approximately 350 million people, and is the leading cause of chronic liver disease, cirrhosis and hepatocellular carcinoma (HCC), accounting for over 1 million deaths annually[1].

The goal of chronic hepatitis B (CHB) treatment is to prevent or reduce the development of cirrhosis, end-stage liver disease, HCC and, ultimately, liver-related death. Several studies have shown that the risk of disease progression is reduced by means of sustained suppression of viral replication[2-4]. Furthermore, maintaining viral suppression increases the rate of hepatitis B surface antigen (HBsAg) clearance, which is the ideal end-point of antiviral treatment as it is associated with a definite remission of chronic hepatitis B activity and an improved long-term outcome. However, even if HBsAg loss occurs, HBV cannot be completely eradicated by treatment due to the persistence of the so-called covalently closed

Table 1 European Association for the Study of the Liver guidelines compared to other international guidelines

Criteria	EASL 2012[6]	AASLD 2009[7]	APASL 2012[8]
HBV DNA treatment threshold			
HBeAg(+) (IU/mL)	2000	20000	20000
HBeAg(-) (IU/mL)	2000	2000-20000	2000
ALT treatment threshold	> ULN	> 2 × ULN	> 2 × ULN
Liver biopsy	Moderate to severe necroinflammation or fibrosis	Not applicable (consider in certain groups)	

ULN: Upper limits of normal; HBeAg: Hepatitis B e antigen; ALT: Alanine aminotransferase; AASLD: American Association for the Study of Liver Diseases; APASL: Asia Pacific Association for the Study of the Liver; EASL: European Association for the Study of the Liver; HBV: Hepatitis B virus.

Figure 1 Management of chronic hepatitis B patient: decision making process. ALT: Alanine aminotransferase; HBeAg: Hepatitis B e antigen; ALT: Alanine aminotransferase; HBV: Hepatitis B virus.

circular DNA (cccDNA), the template for viral RNA transcription, in the nucleus of infected hepatocytes[5].

Since the introduction of interferon alpha as an initial antiviral therapy at the end of the 1980s, the treatment of CHB has markedly improved in the last 15 years due to the availability of nucleos(t)ide analogues (NAs), direct antiviral agents which have greatly increased therapeutic options and permitted the achievement of virological response in almost all patients.

In this review, we focus on current treatment strategies of chronic hepatitis B and discuss the most recent long-term NA efficacy and safety data from clinical trials and real life clinical practice.

ANTIVIRAL TREATMENT

Treatment indications

The complex interplay between viral replication and host immune response determines the natural course of chronic HBV infection which can generally be divided into four phases: immune tolerance, immune clearance, low/non replicative and reactivation phases. Liver disease is associated with immunoclearance and reactivation

phases; therefore, immunotolerant and inactive carriers do not require treatment, while antiviral therapy should be reserved for HBsAg carriers with active viral replication and biochemical or histological evidence of liver damage. The criteria for identification of candidates for antiviral therapy, according to current guidelines[6-8] are shown in Table 1.

In clinical practice, however, the decision-making process is more complex as it involves several factors related to the patient (age, sex, genetics), the drug (efficacy, side effects, resistance barrier), the liver disease (fibrosis, type and extent of inflammation), the liver disease cofactors (alcohol use, diabetes, insulin-resistance, obesity), the co-infections (HDV, HCV, HIV) and the profile of HBV infection (HBeAg-status, HBV DNA levels, genotype, viral heterogeneity) (Figure 1).

The assessment of hepatic fibrosis with liver biopsy or non-invasive methods is recommended since it can assist the decision to start antiviral therapy. Treatment is mandatory for patients with severe fibrosis or cirrhosis (F3-F4) and patients with compensated or decompensated cirrhosis and detectable HBV DNA should be considered for treatment, independent of ALT levels.

Table 2 Main advantages and disadvantages of pegylated interferon alpha and nucleos(t)ides analogues in chronic hepatitis B[6]

	Peg-IFN	Nucleos(t)ides analogues
Advantages	Finite duration (usually 48 wk)	Potent antiviral effect
	Higher rates of anti-HBe and anti-HBs seroconversion with 12 mo of therapy	Excellent tolerance, good safety
	Absence of resistance	Oral administration (once daily)
		No contraindication for treatment
Disadvantages	Moderate antiviral effect	Unknown (perhaps indefinite) duration of treatment
	Inferior tolerability	Rare HBsAg loss
	Risk of adverse events	Risk of viral resistance
	Subcutaneous injections	Unknown long-term safety
	Contraindications in specific patient subgroups	

Peg-IFN: Pegylated interferon; HBsAg: Hepatitis B surface antigen.

Therapy is indicated for patients with moderate fibrosis (F2), while in those with mild or no fibrosis (F0-F1), the indication for treatment should be assessed individually, taking into account patient age, comorbidities, presence of liver disease cofactors, HDV/HCV/HIV coinfections, family history of HCC or cirrhosis, and extrahepatic manifestations.

Anti-HBV drugs

At present, there are two classes of agents licensed for the treatment of CHB: standard or pegylated interferon alpha (IFN or Peg-IFN) and five nucleoside/nucleotide analogues.

Standard IFN has been largely replaced by Peg-IFN due to the more convenient administration schedule (once weekly versus a thrice weekly subcutaneous injection), the longer half-life without wide fluctuations in serum concentrations, and a more effective viral suppression. There are two pegylated-IFN formulations: Peg-IFN alpha-2a and Peg-IFN alpha-2b which have demonstrated a similar efficacy in clinical trials, but only the former is globally licensed for treatment of CHB, while Peg-IFN alpha-2b has been approved in only a few countries. Peg-IFN is a cytokine with a dual antiviral and immunomodulatory activity and therefore has the potential for an immune-mediated control of HBV infection, thus providing the opportunity to obtain a sustained virological response after treatment discontinuation, and the possibility of inducing HBsAg loss in patients who achieve and maintain undetectable HBV DNA. IFN-based treatment, however, is often complicated by the occurrence of side effects, such as influenza-like symptoms, fatigue, neutropenia, thrombocytopenia and depression, which sometimes require dose modification and cause premature cessation of treatment[9]. Moreover, Peg-IFN is contraindicated in patients with decompensated HBV-related cirrhosis or autoimmune disease, in patients with uncontrolled severe depression or psychosis, in patients receiving immunosuppressive therapy or chemotherapy, and in female patients during pregnancy[6].

NAs are oral direct antiviral agents which specifically inhibit the viral polymerase/reverse transcriptase, an enzyme with a crucial role in the HBV life cycle. As a result, NAs block the production of new virions and progressively reduce serum HBV DNA to undetectable levels, but they have little or no effect on the cccDNA present in the nucleus of the infected hepatocytes. The persistence of the intrahepatic cccDNA determines the reactivation of HBV replication after interrupting NA treatment, thereby justifying the need for a long-term (potentially life-long) therapy for a sustained viral replication control. After lamivudine (LAM), the first nucleoside analogue approved for the treatment of CHB, another two nucleosides, telbivudine (LdT) and entecavir (ETV), and two nucleotide analogues, adefovir (ADV) and tenofovir (TDF), have gradually become available in recent years. NAs are characterized by a different antiviral potency and drug-resistance pattern, while entecavir and tenofovir are the two most potent analogues with a high barrier to resistance development.

The main advantages and disadvantages of Peg-IFN and NAs for treatment of CHB are shown in Table 2.

Treatment strategies

There are two different therapeutic strategies for both HBeAg-positive and HBeAg-negative CHB patients: short-term or "curative" treatment and long-term or "suppressive" treatment. The first strategy aims to obtain a sustained suppression of viral replication off-treatment by inducing the immune-controlled status of HBV infection which corresponds to the profile of the inactive carrier, that is, normal ALT levels coupled with HBV DNA < 2000 IU/mL and anti-HBe positivity. This strategy is IFN-based (Peg-IFN administered for 48 wk); a finite treatment with NAs is possible only in HBeAg-positive patients. The second strategy aims to obtain a rapid and long-term maintained viral suppression (HBV DNA < 10-15 UI/mL). This strategy is exclusively based on NAs.

First-line monotherapy

Peg-IFN, entecavir or tenofovir are recommended as first-line monotherapy by all major guidelines in patients with CHB or compensated cirrhosis[6-8]. The most favorable candidates for Peg-IFN are those with low HBV DNA levels, high ALT and HBV, genotype A or B rather than C or D, and those without advanced liver disease.

Entecavir or tenofovir are the only therapeutic op-

tions in patients with decompensated liver disease, in those undergoing immunosuppressive treatment or with contraindications, and those unwilling to receive Peg-IFN. As Peg-IFN can achieve a sustained off-therapy response in only a minority of cases and a proportion of patients cannot tolerate or have IFN contraindications or do not wish to be treated with Peg-IFN, long-term treatment with NAs is the most commonly used treatment strategy.

IFN-BASED THERAPY

Published data have demonstrated that in patients with HBeAg-positive CHB, Peg-IFN achieves a more than 30% HBeAg seroconversion rate after one year of treatment[6]. In a registration trial, Peg-IFN alpha-2a provided a sustained immune control which increased post-therapy; in fact, the HBeAg seroconversion rate continued to increase from 27% at the end of treatment to 32% during the six months after discontinuing treatment, and to 42% 1 year post-treatment[10,11]. Moreover, the seroconversion remained stable over time in > 80% of Peg-IFN alpha-2b treated patients, achieving this end-point at the end of therapy[12]. Peg-IFN also determined HBsAg seroconversion in up to 30% of patients with a long-term follow-up[13].

In patients with HBeAg-negative CHB, Peg-IFN alpha-2a demonstrated a sustained immune control (HBV DNA < 2000 IU/mL) in 31% of patients 1 year post-treatment. Among these, 88% maintained this response up to 5 years follow-up and, remarkably, 28% achieved HBsAg clearance 5 years post-treatment[14].

Peg-IFN treatment remains an attractive therapeutic option since it provides higher rates of off-therapy immune control, including HBsAg clearance, when compared to NAs. However, IFN is effective in only a minority of patients (20%-30%), has a poor tolerability and significant costs. Therefore, the improvement of Peg-IFN efficacy is a major challenge. Several attempts have been made to optimize the cost-effectiveness of IFN-based therapy, including combination therapy, longer treatment duration and identification of pre-treatment and on-treatment predictors of response. *De novo* combination therapy with NAs did not improve sustained response in either HBeAg-positive or HBeAg-negative patients[10,15-17]. Regarding duration of therapy, the NEPTUNE study conducted in patients with HBeAg-positive CHB reported that dose and duration are important because the highest sustained response was obtained with 180 μg and 48 wk compared to 90 μg and 24 wk[18]. Recently, an Italian multicenter study demonstrated in 128 HBeAg-negative patients (mean age 45 years, 94% genotype D, 13% with cirrhosis) that extended treatment with Peg-IFN alpha-2a to 96 wk was well-tolerated and improved the rates of sustained virological response (29% *vs* 12%, P = 0.03) in HBeAg-negative genotype D patients when compared to the current standard of care of 48 wk. In addition, 1 year post-treatment, HBsAg clearance (6%) was observed only

in the extended therapy group[19]. Among pre-treatment predictors of response, ALT levels, low baseline HBV DNA and virus genotype were significant predictors[6-8]. When combining data from the two largest clinical trials regarding HBeAg-positive CHB patients[9,20], Buster *et al*[13] found that the best candidates for a sustained response to Peg-IFN were genotype A patients with high levels of ALT (ALT ⩾ 2xULN) or low levels of HBV DNA (< 9 $\log_{10}$ copies/mL), and genotypes B and C patients who have both high levels of ALT and low HBV DNA. Genotype D patients have a low chance of sustained response. However, these factors cannot accurately predict response at the individual level; furthermore, ALT and HBV DNA levels are time-dependent and thus their use in clinical practice is difficult.

To obtain additional insight into the individual patient's probability of achieving response to Peg-IFN, the presence of precore and basal core promoter mutants before treatment has been correlated to the serological and virological response in HBeAg-positive CHB patients. Data from this study demonstrated that the presence of a wild-type virus at baseline was an independent predictor of response to Peg-IFN and can assist in improving patient selection for this treatment option[21].

More recently, the role of IL28B polymorphisms, clearly indicated as a baseline host factor predictor of response in patients with chronic hepatitis C, has also been investigated in CHB patients. Studies in HBeAg-positive patients provided conflicting results[22-24]. The only existing data in HBeAg-negative patients are in 101 subjects treated with either conventional IFN or Peg-IFN alpha 2a for 24 mo and followed for 11 years after treatment. Patients with *IL28B rs12979860* genotype CC were shown to have higher EOT (69% *vs* 45%, P = 0.01) and higher SVR (31% *vs* 13%, P = 0.02) than non-CC patients. Interestingly enough, CC patients had a higher cumulative probability of clearing HBsAg during an observation period of 16 years (38% *vs* 12%, P = 0.039)[25]. Further studies are necessary to define the role of *IL28B* polymorphisms as a baseline factor to improve pre-treatment patient selection.

A promising approach to improve the cost-effectiveness of Peg-IFN therapy is a response-guided treatment based on serum HBsAg kinetics which permits early identification of either responders for whom continuation of treatment to week 48 could be beneficial or non-responders who should discontinue IFN treatment.

Two stopping rules at week 12 have been proposed for HBeAg-positive patients: (1) no HBsAg decline; and (2) HBsAg levels > 20000 IU/mL. The negative predictive value (NPV) for a sustained response ranged from 92% to 100% depending on HBV genotypes; thus, HBV genotype-specific stopping-rules may be considered at week 12. However, at week 24, treatment discontinuation is indicated in all patients with HBsAg > 20000 IU/mL, irrespective of HBV genotype[26,27].

In HBeAg-negative genotype D patients, no HBsAg decline and < 2 log copies/mL HBV DNA decline at

HBeAg-positive	HBeAg-negative (geno D)
Week 12: HBsAg > 20000 IU/mL No decline of HBsAg	**Week 12:** No decline in HBsAg plus < 2 log decline in HBV DNA
95%-100% negative predictive values	

Figure 2 Response-guided therapy using hepatitis B surface antigen levels in pegylated interferon-treated patients: stopping rules[26-29]. HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen.

week 12 has been proposed as a stopping rule and independently validated with a 100% NPV[28,29]. Overall, therapy with Peg-IFN could be discontinued at week 12 in the 20% of primary non-responders, who are therefore candidates for suppressive therapy with NAs (Figure 2).

Recently, it has been demonstrated that, even in HBeAg-negative patients, on-treatment HBsAg kinetics varied according to HBV genotype. In fact, for genotype A, the difference between responders and non-responders was greatest at week 24, while for genotypes B and D, the difference was evident at week 12; there was no significant difference for genotype C over time. Moreover, highly positive predictive values for long-term virological response was obtained by applying end-of-treatment genotype-specific HBsAg level cut-offs[30].

NA-BASED THERAPY

Entecavir and tenofovir are the third-generation NAs recommended as first-line therapy for CHB NA-naïve patients by all international guidelines. In registration trials, both antivirals demonstrated a long-lasting efficacy (viral suppression in more than 95% of patients over 5 years) associated with prevention of developing cirrhosis and, to a greater extent, with fibrosis regression[31-36]. Chang *et al*[31] first documented the histological reversal of cirrhosis in 4 of 10 cases who met the criteria for efficacy analysis while they were in a 3 to 7 year period of virological response to ETV. More robust evidence of cirrhosis reversion has been offered by Marcellin and colleagues who reported the effect of 5 years of viral suppression on histology in liver fibrosis and cirrhosis in 348 patients who had evaluable histology at baseline and at week 240. Of the 96 (28%) patients with cirrhosis (Ishak score ≥ 5) at baseline, 71 (74%) demonstrated a reduction in fibrosis at year 5 and were no longer cirrhotic[34].

Moreover, registration trials reported a minimal risk of drug resistance (1.2% with ETV and 0% with TDF after 6 years) and a favorable safety profile[31-36]. However, as registration trials are conducted under standardized conditions with strict enrolment criteria in well-selected and compliant patients, long-term efficacy and safety of ETV and TDF are still to be confirmed in real life patients who generally have a more complex clinical profile as they are usually older, with a higher prevalence of cirrhosis and comorbidities treated with several concomitant medications.

Efficacy and safety of entecavir in real life practice

In a retrospective/prospective multicenter Italian study, 418 consecutive NA-naïve patients initiating treatment with ETV 0.5 mg/d were studied. In this cohort, patients were older at baseline (median age 58 years), were predominately infected with HBV genotype D (90%), 49% had cirrhosis, approximately 46% had a body mass index over 25 kg/m^2, and 56% had concomitant diseases. Viral suppression was achieved in 99% of patients over 60 mo of therapy, independent of HBeAg status. Only one patient with a partial virological response at week 48 developed resistance at year 3 of treatment, with a cumulative rate of 0.2%. In HBeAg-positive patients, the 5 year cumulative probability of HBeAg seroconversion and HBsAg loss were 55% and 34%, respectively. HCC developed in six non-cirrhotics with a yearly rate of 0.8%. The 204 compensated cirrhotics remained clinically stable, yet 18 developed a HCC, a 5 year cumulative rate of 13% and a yearly rate of 2.6%, making continuous surveillance for liver cancer mandatory[37].

The single center Hong Kong cohort study prospectively included 222 NA-naïve patients (median age 45 years) who demonstrated a 97.4% 5 year cumulative rate of virological response. Only two cases of resistance (corresponding to a 1.2% cumulative resistance rate up to year 5) were reported in this patient cohort[38].

The European network of excellence for Vigilance against Viral Resistance performed a multicenter cohort study with over 10 European referral centers between 2005 and 2010. The study including 243 consecutive NA-naïve patients receiving ETV monotherapy; the cumulative probability of achieving a virological response at week 144 was 90% in HBeAg-positive patients and 99% in HBeAg-negative patients, and the proportion of HBeAg-positive patients with HBeAg loss was 34%[39]. In this cohort, 81% of patients with partial virological response at 48 wk reached a virological response during prolonged ETV monotherapy and no patient developed ETV resistance. When stratifying patients according to their viral load at week 48, 95% of patients with HBV DNA < 1000 IU/mL and 57% of patients with HBV DNA > 1000 IU/mL achieved a virological response without treatment adaptation during the prolonged treatment period beyond week 48. Therefore, the authors concluded that no treatment adaptation is needed in the majority of NA-naïve patients treated with ETV who reach a partial virological response, particularly in those with HBV DNA < 1000 IU/mL at week 48. In addition, data from the Virgil cohort demonstrated that in cirrhotic patients, virological response to ETV is associated with a lower probability of developing a clinical event and disease progression[40].

The safety profile of ETV in real life studies has been largely consistent with those of registration trials as there have been no reports of serious drug-related side effects, discontinuation or renal toxicity[37-41]. One retrospective study identified five cases of lactic acidosis among 16 ETV-treated patients with decompensated liver disease.

Table 3 Cross-resistance data for the most frequent resistant hepatitis B virus variants[43]

HBV variant	LVD	LdT	ETV	ADV	TDF
Wild-type	S	S	S	S	S
M204I/V	R	R	I	S	S
L180M + M204V	R	R	I	S	S
A181T/V	R	R	S	R	I
N236T	S	S	S	R	I
A181T/V + N236T	R	R	S	R	R
L180M + M204V/I ± I179T ± T184G ± S202I/G ± M250I/V	R	R	R	S	S

HBV: Hepatitis B virus; LVD: Lamivudine; LdT: Telbivudine; ETV: Entecavir; ADV: Adefovir; TDF: Tenofovir; S: Sensitive; I: Intermediate/reduced susceptibility; R: Resistant.

These patients all had highly impaired liver function, with model for end-stage liver disease scores of 22 or higher[42]. In two subsequent studies enrolling patients with hepatic decompensation, no cases of lactic acidosis were reported[43,44].

Efficacy of tenofovir in real life practice

In the multicenter European cohort study, 374 consecutive NA-naïve patients receiving tenofovir (245 mg/d) were retrospectively and prospectively followed for a median period of 39 mo. At baseline, median age was 55 years, 35% of patients had cirrhosis, and concomitant diseases were present in 47%[45]. Virological response rates increased over time, reaching 97% at year 4, independent of HBeAg status. Virological breakthrough was reported in 2% of patients, with no potential resistance-associated mutations identified to date. In HBeAg-positive patients, cumulative probability of HBeAg seroconversion at 4 years was 37%. Sixteen patients (17%) cleared HBsAg (11 HBeAg-positive patients), six of whom successfully interrupted tenofovir. Most partial virological responders at week 48 achieved undetectable HBV DNA during additional treatment. Serum creatinine and phosphorus median levels remained unchanged over time. The proportion of patients with an eGFR of < 50 mL/min (as calculated by the Modification of Diet in Renal Disease formula) increased from 2% to 3% (year 4). The TDF dose was reduced in 19 patients (5%) because of a decline in the estimated glomerular filtration rate in 17 and low serum phosphate levels in two. Therapy was discontinued in seven patients (2%) who were switched to ETV. Nine additional patients withdrew from TDF and switched to ETV because of non-renal-related side effects. HCC developed in 10 compensated cirrhotics (4 year cumulative probability: 17%, 4.2%/year) and in six non-cirrhotics (4 year cumulative probability: 4%, 1%/year), while no cirrhotics clinically decompensated[45].

Management of antiviral drug resistance

The management of treatment failure has changed significantly in recent years due to the availability of potent antivirals. An appropriate rescue therapy should be initiated with the most effective antiviral drug without cross-resistance to reduce the risk of selecting multiple drug-resistant viral strains (Table 3)[46]. In the past years, the add-on strategy was the therapeutic approach recommended by guidelines in order to prevent the emergence of multi-drug resistant strains and raising the resistance barrier. However, with the availability of more potent drugs, such as entecavir and tenofovir, there is a trend to recommend a switch to a complementary drug with a high barrier to resistance. Both options are considered in the recent EASL guidelines (Table 4)[6]. The switch strategy does not apply to patients who have been exposed to multiple monotherapies; these patients should be treated with add-on strategies in order to minimize the risk of subsequent treatment failure.

NA treatment discontinuation

In HBeAg-positive patients with documented HBeAg seroconversion, NA-treatment can be discontinued after 6-12 mo consolidation therapy[6-8], although the optimal duration of consolidation treatment is not clearly defined. However, the long-term durability of HBeAg seroconversion induced by NAs is controversial and high relapse rates have been reported, suggesting that long-term continuation of NA-treatment, irrespective of the occurrence of HBeAg seroconversion, appears to be necessary[47].

Overall, the ideal end-point for stopping NA-treatment is HBsAg loss; however, the likelihood of HBsAg clearance is very low in clinical practice[6-8]. Studies are underway to determine if it is possible to successfully combine the potent effects of NAs with Peg-IFN to increase the HBsAg clearance rates and allow more patients to stop therapy. Recent reports propose quantification of serum HBsAg levels together with serum HBV DNA levels for predicting the outcome after treatment discontinuation in individual patients and thus whether therapy can be safely stopped. Petersen and colleagues showed that stopping long-term NA-therapy in HBeAg-negative CHB patients without advanced liver disease might be an option for patients with HBsAg titers < 500 IU/mL since these selected patients developed a high rate of HBsAg loss off-therapy.

CONCLUSION

Chronic hepatitis B remains a serious clinical problem because of its worldwide distribution and potential adverse sequelae. Over the last decades, treatment of CHB has greatly advanced due to the availability of safe and effective drugs and new standards of care and guidelines have been developed. Both Peg-IFN and two NAs, entecavir and tenofovir, can currently be prescribed as first-line monotherapy for CHB.

Peg-IFN treatment is the only short-term treatment strategy which provides significant off-treatment sustained responses, including loss of HBsAg. However, as Peg-IFN is effective in 20%-30% of patients, it should

Table 4 European Association for the Study of the Liver 2012 Guidelines recommendations in resistant patients[6]

Resistance	Action
LAM resistance	Switch to TDF (add ADV if TDF not available)
ADV resistance	If patient was NA naive before ADV: switch to ETV or TDF;
	ETV may be preferred in such patients with high viremia
	If patient had prior LAM resistance: switch to TDF and add a nucleoside analogue
LdT resistance	Switch to or add TDF (add ADV if TDF not available)
ETV resistance	Switch to or add TDF (add ADV if TDF not available)
TDF resistance	TDF resistance not detected to date: add a nucleoside analogue
	Switch to ETV if patient had no prior LAM resistance or add ETV in patients with LAM resistance

LAM: Lamivudine; ADV: Adefovir dipivoxil; TDF: Tenofovir; LdT: Telbivudine; ETV: Entecavir.

be considered only for patients with an elevated possibility of response based on pre-treatment and on-treatment factors. In particular, quantitative serum HBV-DNA and HBsAg levels may be suitable to identify patients early who are unlikely to benefit from Peg-IFN early during the treatment course, thereby avoiding unnecessary therapy. Nevertheless, despite this individualized and response-guided approach, increasing the cost-effectiveness of Peg-IFN therapy remains a clinical challenge. Combining Peg-IFN with NAs appears to be the most appealing approach to increase the efficacy of antiviral therapy and new trials on a combination of Peg-IFN with ETV or TDF are required.

Currently, NAs represent the treatment option most often used in the majority of CHB patients. TDF and ETV suppress HBV replication in most treatment-naïve field practice patients with CHB but fail to prevent HCC development, independent of liver disease severity. NA long-term administration raises several concerns: the patient's commitment to lifelong treatment, adherence, long-term safety, drug resistance in the long-term and costs. Different strategies combining Peg-IFN with ETV or TDF might achieve an antiviral synergy and provide new opportunities to increase HBsAg clearance rates and shorten treatment duration.

Finally, development of new antiviral agents targeting other steps in the HBV replication cycle (viral entry, capsid assembly, viral RNA transcription and epigenetic control of cccDNA) and new immune therapies restoring immune response to HBV remain a major research challenge to improve the efficacy of current antiviral therapy and to achieve HBsAg loss and HBV eradication.

ACKNOWLEDGMENTS

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REVIEW

Cystic echinococcosis of the liver: A primer for hepatologists

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Abstract

Cystic echinococcosis (CE) is a complex, chronic and neglected disease with a worldwide distribution. The liver is the most frequent location of parasitic cysts. In humans, its clinical spectrum ranges from asymptomatic infection to severe, potentially fatal disease. Four approaches exist in the clinical management of CE: surgery, percutaneous techniques and drug treatment for active cysts, and the "watch and wait" approach for inactive cysts. Allocation of patients to these treatments should be based on cyst stage, size and location, available clinical expertise, and comorbidities. However, clinical decision algorithms, efficacy, relapse rates, and costs have never been properly evaluated. This paper reviews recent advances in classification and diagnosis and the currently available evidence for clinical decision-making in cystic echinococcosis of the liver.

Key words: Cystic echinococcosis; Hydatidosis; Clinical management; Diagnosis; Treatment; Surgery; Albendazole; Watch-and-wait; Follow-up; Percutaneous treatment

Core tip: Cystic echinococcosis (CE) is a neglected parasitic disease and echinococcal cysts are mostly located in the liver. Therefore, CE should always be included in the differential diagnosis of cystic lesions of the liver. However, diagnosis and clinical management can be difficult because of the combination of clinical variables (cysts stage, size, presence of complications, available expertise and three different treatments that have never been systematically compared). This review summarizes current knowledge and open issues in this field for those hepatologists who have limited or no experience with this complex condition.

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INTRODUCTION

Hepatologists may encounter cystic echinococcosis (CE) in their practice. However, due to its relatively low prevalence in many Western countries, this infection is poorly characterized and its complex management can be difficult for clinicians unfamiliar with this condition. Moreover, hepatic CE should be included in the differential diagnosis of focal liver lesions. In this paper, we summarize the current knowledge on clinical management of hepatic CE to increase hepatologists' awareness of this complex condition.

Figure 1 Life cycle of *Echinococcus granulosus*. Source: www.cdc.gov.

CE, or hydatidosis, is caused by the larval stage (metacestode) of *Echinococcus granulosus* (*E. granulosus*). Its life cycle develops in dogs and other canids, which harbor the adult tapeworm in the intestine, and herbivores (or humans as dead-end occasional host) as intermediate hosts, where the larval metacestode form develops in different organs (Figure 1).

Once eggs are ingested by the intermediate host, the oncosphere (also named exacanth larva), is released from the keratinized embryophore in the stomach and intestine where it penetrates the small intestine wall *via* its hook movements. The oncosphere is then carried *via* portal flow to the liver and other organs where the metacestode implants. Organs may also be reached through the lymphatic system[1]. This process results in primary echinococcosis, while secondary echinococcosis follows the spillage of protoscoleces (tapeworm heads) or small daughter cysts from the original cyst that ruptures following trauma or surgery and their seeding, primarily in the peritoneum for abdominal cysts[2].

The impact of CE on human health is significant, with an estimated 1.2 million people affected and 3.6 million DALYs (Disability Adjusted Life Years) lost globally[3]. Despite the low mortality rate (0.2/100000 population with a case fatality rate of 2.2%) morbidity is high[4]. Moreover, it has a major economic impact with an estimated annual livestock production loss of up to 2190 million US$[5].

Despite these figures, the infection is still under-reported and has received to date much less attention than infections of comparing burden[5]. In humans, its clinical manifestations range from asymptomatic infection to severe, potentially fatal disease.

The liver is the most frequent location of echinococcal cysts, representing approximately 70% of cases[4]. The lungs are the second most common location; however, CE can present in virtually any other organ, although this rarely occurs[1,2].

Echinococcal cysts consist of a periparasitic host tissue (pericyst or adventitia), which surrounds the larval endocyst, and an endocyst itself. The endocyst is composed of an outer, acellular laminated layer and an inner layer, the germinal layer, which gives rise, in fertile cysts, to brood capsules and protoscoleces[6]. Each protoscolex may develop into an adult tapeworm if ingested by a suitable definitive host. The cyst is filled with clear fluid containing molecules of both parasite and host origin, numerous brood capsules, and protoscoleces. Some cysts may also harbor daughter cysts of variable size (Figure 2). The fluid is clear in the early stages (Figure 3A), but can be yellowish and turbid, with fragments of endocyst in advanced stages (*e.g.*, in CE3b cysts) or after months of treatment with albendazole (Figure 3B).

E. granulosus occurs in a broad range of geographic areas and can be found on all continents except Antarctica, and in circumpolar, temperate, subtropical, and tropical zones. Eurasia, Africa, Australia, and South America show the highest prevalence[7]. Within endemic zones, the prevalence varies from sporadic to high, with recent studies showing an higher prevalence among females and with increasing age[8]. Only a few countries can be regarded as free of *E. granulosus* infection[3].

Figure 2 Diagrammatic representation of the metacestode of *Echinococcus granulosus*. Source: Eckert J, Gemmell MA, Meslin FX and Pawłowski ZS. WHO/OIE Manual on Echinococcosis in Humans and Animals: a Public Health Problem of Global Concern. Paris, France, 2001. *E. granulosus*: *Echinococcus granulosus*.

Figure 3 Appearance of cystic fluid. A: Clean and clear cyst fluid from a diagnostic puncture; B: Yellowish and turbid echinococcal fluid in a catheter bag after percutaneous catheterization.

E. granulosus parasites from different hosts show considerable phenotypic variation in terms of morphology, larval growth *in vivo* and *in vitro*, range of host infectivity, and biochemical features. Currently, 10 genotypic strains of *E. granulosus* have been identified (G1-G10), and the impact of these variations on CE epidemiology, pathology and control is being investigated. Genotypes are grouped into 4 species that constitute the *E. granulosus* complex: *E. Granulosus sensu strictu* (G1-G3), *E. equinus* (G4), *E. ortleppi* (G5) and *E. canadensis* (G6-G10). The great majority of *E. granulosus* isolates from humans thus far characterized have been of the sheep genotype (G1)[1,2].

COURSE OF INFECTION

Acute infection in humans has never been documented[9], thus all available data come from experimental studies in animal intermediate hosts. Cavity formation and the development of both germinal and laminated layers of the cyst wall occur 10 to 14 d post infection in the mouse model[10]. Formation of brood capsules and protoscoleces requires a longer time period in sheep, from 10 mo to 4 years[11].

Based on clinical observations using ultrasound (US), the cysts progress from a fluid-filled unilocular cavity to a pseudo-solid, eventually calcified lesion. The sequence of cyst development between these 2 stages is poorly understood[12]. Long-term clinical observation indicates that the early stages are CE1 and CE3a cysts, while final stages are represented by CE4 and CE5, referring to the standardized US classification (see "Imaging" below)[13]. Preliminary observations suggest that cysts that have reached the CE4 stage as a result of treatment may revert to CE3b more often than those reaching the inactive

stage spontaneously; this may occur many years after apparently successful treatment[14] (Junghanss, personal communication). The origin and fate of CE2 and CE3b stages are less clear. CE2 may represent a relapsed CE3a, and CE3b a relapsed CE4, but long-term observations of large cohorts of patients are needed to confirm this hypothesis.

The growth rate of cysts is variable. The average increase in cyst diameter is thought to be 1 cm/year, but data on the natural history of CE are scarce. Cysts may behave differently in different subjects and their growth rate also depends on the surrounding host tissue, with growth rates up to 5 cm/year reported for brain cysts[15-19].

DIAGNOSIS

The presentation of human CE is protean. Patients come to the clinician's attention for a variety of reasons. Potential presentations may be due to the mechanical effect of a large cyst on surrounding tissues, rupture of a cyst causing an acute hypersensitivity reaction, or complications such as biliary obstruction or embolism. The cyst is often asymptomatic and diagnosed accidentally during radiographic examination, surgery, or during evaluation of other clinical diagnoses.

Common symptoms are upper abdominal discomfort and pain and poor appetite. Physical findings are hepatomegaly, presence of an abdominal palpable mass and abdominal distension. Cysts in the liver should be included in the differential diagnosis of several conditions, such as jaundice, colicky pain, portal hypertension, ascites, compression of the inferior vena cava and Budd-Chiari syndrome and can be misdiagnosed as non-parasitic cysts, single or multiple hemangiomas, pyogenic or amebic liver abscess, hematoma, adenoma, adenocarcinoma, hepatocellular carcinoma, metastases, focal or diffuse lymphoma, alveolar echinococcosis, and textiloma[20,21].

As the infection may remain silent for years before the enlarging cysts cause symptoms, the clinical diagnosis of CE is often difficult and requires a combination of physical examination, imaging techniques, in particular

Figure 4 World Health Organization Informal Working Group on Echinococcosis standardised classification of echinococcal cysts. Source: World Health Organization Informal Working Group on Echinococcosis. CE: Cystic echinococcosis; CL: Cystic lesions.

US, and serology; the latter plays a supportive role in diagnosing CE despite the development of sensitive sero-diagnostic tests and the use of different antigen sources.

Imaging

Imaging techniques have revolutionized the diagnosis and clinical management of CE. Gharbi *et al*[22] developed the first US classification for CE in 1981. Other classifications were subsequently produced but were not widely adopted. In 1995, the WHO Informal Working Group developed an international standardized US classification that could be universally applied to replace the plethora of classifications in use.

This classification, published in 2003[23], differs from Gharbi original classification by introducing a cystic lesion (CL) category to include cysts of unclear origin, and by reversing the order of CE types 2 and 3 (Figure 4). The number of cyst types remains unchanged from Gharbi's classification and the types are categorized into active, transitional, and inactive stages. CL cysts are not included as a type of CE, as they require further evaluation before being classified as CE[24]. CE1 and 2 are active, usually fertile cysts containing viable protoscoleces. CE3 are cysts entering a transitional stage where the integrity of the cyst has been compromised either by the host or by chemotherapy. CE4 and CE5 are inactive cysts that have lost their fertility and are degenerating. A more recent amendment to the WHO classification clarifies that calcifications are not limited to CE5 cysts, but may be present to a various extent in all cystic stages and are therefore not indicative of cyst death[25].

Data on long-term follow-up of cysts treated with albendazole and percutaneous treatment provide ground for a further sub-classification of CE3 (transitional) cysts into CE3a (with detached endocyst) and CE3b (predomi-

nantly solid with daughter vesicles). This has important implications for clinical decision-making and prognosis[26]. The sub-classification of CE3 into CE3a and CE3b is supported a recent work using high-field ^{1}H magnetic resonance spectroscopy evaluating the metabolic profile of cysts contents *ex vivo*[27]. This study confirmed findings from optical microscopy that CE3a are equally likely to be viable or non-viable, whereas CE3b are consistently viable. Of note, CE3a and CE3b also respond differently to non-surgical treatments[28,29]. In light of these features, CE3b cysts should be considered as active, while CE3a are the transitional cysts *sensu stricto*.

The same study confirmed the biological activity of CE1 and CE2 and the inactivity of CE4 and CE5. Another study showed how a CE1 brain cyst, in *in vivo* magnetic resonance spectroscopy matched the profile of an active stage before the medical treatment with albendazole (ABZ) and that of an inactive one after ABZ[30]. CE2 and CE3b cysts tend to relapse both after PAIR (puncture, aspiration, injection of a scolecidal agent, and reaspiration) and ABZ[26,28,29], and several studies suggest that a strong Th2 response correlates with susceptibility to disease (active cyst), whereas a Th1 response correlates with protective immunity (inactive cyst), however this is not clear cut[31-36].

Computed tomography (CT), including spiral or multidetector CT, with multiplanar reformations, and magnetic resonance imaging (MRI), with at least a T2-weighted imaging sequence, and if necessary cholangio-pancreatography, have distinct indications: (1) impaired US visualization due to obesity or subdiaphragmatic location of the cyst; (2) disseminated disease; (3) extra-abdominal location; (4) complications (cyst infection, cysto-biliary fistulae); and (5) pre-surgical evaluation and follow-up (Figure 5). Whenever possible, MRI is pre-

Figure 5 Computed tomography and magnetic resonance imaging of hepatic cystic echinococcosis. A and B: Contrast enhanced computed tomography (CT) abdominal scan of a 59-year-old male patient with a CE3b cyst in the Ⅶ liver segment; C: Disseminated peritoneal echinococcosis in 64-year-old male patient, 30 years after surgery for CE without albendazole prophylaxis[1]; D: Abdominal CT scan of a 59-year-old female patient with a CE3a cyst in the Ⅳ-Ⅷ liver segments; E: Abdominal CT scan of a 47-year-old male patient with a CE5 calcified cyst in the Ⅷ liver segment; F: Abdominal magnetic resonance imaging scan of a 52-year-old male patient with a CE3b cyst in the Ⅷ segment.

ferred to CT for pre-treatment assessment[37,38].

Serology

Routine blood tests are not specific for CE, and with liver involvement they can be normal or suggestive of cholestasis with or without hyperbilirubinaemia or raised transaminases or γ-glutamyltransferase (γ-GT)[1,39,40]. Transient elevation of γ-GT and alkaline phosphatase, in association with hyper-transaminasemia and eosinophilia, may indicate cyst rupture in the biliary tree. Despite CE being a helminthic infection, eosinophilia is usually moderate or absent.

Despite the development of sensitive laboratory tests and the use of different antigen sources, serology remains complementary to imaging in the diagnosis of CE. Currently, lipoprotein antigen B (AgB) and Ag5, the major components of cystic fluid, have received the most attention with regard to diagnosis, but purified cyst hydatid fluid is still the most widely used in current assays for immunodiagnosis of CE, which are not standardized[41,42]. In clinical practice, usually two tests are performed (for example ELISA and indirect hemagglutination (IHA), with immunoblotting (IB) as a confirmatory test. IHA and ELISA show sensitivity for hepatic cysts ranging between 85% and 98%[42-46]. The result of a single test is not considered diagnostic, and the two tests are generally run in parallel. IB is performed as a confirmatory test when ELISA and IHA are inconclusive or for the differential diagnosis with other infections, although *E.*

granulosus specific bands may be also be detected in the serum of patients affected by *E. multilocularis* and rarely *T. solium* cysticercosis[47]. False positives result from cross-reactivity, most commonly with other cestode infections (*E. multilocularis, Taenia solium* cysticercosis) and some other parasitoses (schistosomiasis, liver flukes, filariasis), but also from non-infectious diseases such as malignancies and cirrhosis[1,41,48-50].

Serologic testing for CE is hampered by many problems[41-43,45]. These include low sensitivity, partially dependent upon the location of the cysts in the body and the cystic stage, and the inability of serology to clearly distinguish between active and inactive cysts when US is inconclusive[43]. Up to 20% of patients with single hepatic cysts and up to 50% of those with lung cysts may be seronegative at diagnosis, while patients with cysts in other locations are often seronegative. In addition, patients with multiple cysts are generally seropositive. In the case of hepatic cysts, patients with CE1 and CE4-CE5 cysts are often seronegative (30%-58% and 50%-87% respectively), while rates of negativity are lower in the presence of CE2 and CE3 cysts (5%-20%). It is also worth noting that serodiagnostic tests may be persistently positive for > 10 years even after radical surgical removal of the cysts, and are often positive in the presence of inactive cysts[17,51,52]. This may lead inexperienced clinicians to prescribe unnecessary treatment and cause unjustified anxiety to the patient. New antigens are under investigation which promise to have higher diagnostic performances in

these situations[53].

Microscopical examination

When US and serology are inconclusive, a direct analysis of the material obtained by percutaneous aspiration is needed. The procedure must be performed with the assistance of an anesthesiologist because of the very low but nonetheless present risk of anaphylaxis[54]. The presence of protoscoleces or their components or of antigens specific to *E. granulosus* indicates the parasitic nature of the cyst[55].

TREATMENT

There is no standard treatment for hepatic CE. The appropriate treatment depends on individual patient factors, cyst characteristics, the therapeutic resources available, and the physician's preference[56]. Matters are further complicated by the dearth of randomized clinical trials evaluating treatment options, and the ensuing low level of evidence to support one therapeutic modality over another[57,58].

Surgery has long been considered the best, if not the only, option in the treatment of CE. However, in the past two decades, medical treatment, percutaneous procedures, and a "watch and wait" approach have been successfully introduced and have replaced surgery as the treatment of choice in selected cases[59].

Surgery

While surgery is increasingly being replaced by other options in uncomplicated cysts, it maintains a central role in complicated cysts (*i.e.*, rupture, biliary fistula, compression of vital structures, superinfection, hemorrhage), cysts at high risk of rupture, or large cysts with many daughter vesicles that are not suitable for percutaneous treatments.

Surgery can be performed as an open procedure, with either radical or conservative techniques, or laparoscopically. There are still controversies as to the safest and most effective technique, and in which cases it should be applied[57,60,61]. As a rule, perioperative ABZ prophylaxis, from 1 wk prior to surgery until 4 wk postoperatively, is necessary to minimize the risk of secondary echinococcosis from seeding of protoscoleces in the abdominal cavity[59].

Radical surgery aims to remove the entire pericystic membrane and the parasitic contents with or without hepatic resection, and can be performed with either the "open-cyst" or "closed-cyst" method. In conservative procedures, only the parasitic material is removed while part or all of the pericyst is left in place and the residual cavity is managed with different techniques, such as omentoplasty, capitonnage, or external drainage.

A cleavage plan between the inner layer of the host's reaction towards the parasite and the cyst outer layer, or "adventitia", as described by Peng *et al*[62], limits damage to liver parenchyma when dissecting around the cyst and

allows for safer removal. Based on these anatomical considerations, such an operation should be more adequately termed "total cystectomy." Mortality ranges between 0.8% and 6.5%, morbidity between 12% and 84%, and relapse rate between 2% and 30%[39,60,63,64].

It is commonly perceived that the more radical the surgery, the higher the operative risk but the lower the risk of relapses and *vice versa*. However, results of meta-analyses and single center studies indicate that radical surgery is superior to conservative surgery, with lower morbidity (3%-24% *vs* 11%-25%), mortality (1%-1.8% *vs* 2%-5%) and recurrence rates (2%-6.4% *vs* 10.4%-40%)[61,64-66], although the type of surgery was not found to be a predictive factor of post-surgery complications in the study of El Malki *et al*[60]. Other factors associated with surgical outcome are large cyst size, more than 3 hepatic cysts, presence of biliary fistulae, age > 40 years, repeated surgery due to recurrence, capitonnage alone as a measure of residual cavity management, and cyst rupture during surgery[60,61,64,67,68].

Recurrence, both local and as secondary echinococcosis, is associated with spillage during removal of the cyst, incomplete removal of the endocyst, and possibly the presence of unnoticed exophytic cyst development[63,69]. For the latter, intraoperative US has been shown to be an important tool to improve the quality of hepatic surgery[70].

Infection and biliary communication with the cyst (*i.e.*, leakage or rupture with cholestasis) are the most common complications of echinococcal cysts and can occur before or after surgical or percutaneous interventions[71,72]. Cyst diameter is a factor associated with a high risk of biliary-cyst communication in clinically asymptomatic patients. A recent study reported that cyst diameter > 7.5 cm had a specificity and sensitivity for biliary-cyst communication of 73% and 79%, respectively[73]. Thus, surgeons operating on cysts larger than 7.5 cm should be prepared to deal with this complication and should perform preoperative retrograde cholangiopancreatography or MR imaging[73,74].

Several methods have been proposed for the management of cyst-biliary communications. When intrabiliary rupture is diagnosed pre- or intra-operatively, a simple suture of the orifice is sufficient if there are no cystic contents in the biliary tree and the common bile duct has a normal caliber. When cyst contents are found in the biliary tree or the common bile duct has an abnormal caliber, evacuation of the cystic content and a T-tube drainage placement or even a coledochoduodenostomy are needed[71,75]. Alternatively, endoscopic treatment with sphincterotomy and placement of a nasobiliary catheter has been performed[76,77]. Postoperative bile leakage resulting in symptomatic bilomas or high-output biliary fistulae can be managed endoscopically by sphincterotomy with nasobiliary drainage or biliary stenting[78,79].

Surgical interventions other than segmentectomies can result in a number of residual cavities that may be mistaken for recurrences or other conditions[20]. Some groups have evaluated these findings and attempted to

categorize them relative to the type of surgical procedure performed[80].

A recent review on management of post-surgical complications concluded that " the evidence level is low" and that "there are many questions and few answers"[81].

Percutaneous treatments

Percutaneous treatments for abdominal CE were introduced in the mid-1980s, with the adoption of minimally invasive procedures made possible by new imaging tools, particularly CT and US[82-85]. These treatment modalities aim either to destroy the germinal layer with scolecidal agents or to evacuate the entire endocyst.

The most popular method is PAIR[13]. Several modified catheterization techniques are used to evacuate the endocyst, and are generally reserved for cysts which are difficult to drain or tend to relapse after PAIR, such as multivesiculated cysts or cysts with predominantly solid content and daughter cysts[26].

Catheterization techniques are based on the aspiration of the "solid" content of the cyst, the endocyst surrounded by pseudocaseous inflammatory material, through a large-bore catheter or other device. Several variants of these techniques have been proposed, in particular percutaneous evacuation (PEVAC)[86], a modified catheterization technique[87], and dilatable multi-function trocar[88].

Puncture of echinococcal cysts has long been discouraged because of the risk of anaphylactic shock and spillage of the fluid; however, as experience with US-guided interventional techniques has increased since the early 1980s, a growing number of articles have reported its safety in treating abdominal, especially liver, echinococcal cysts. In a recent systematic analysis on percutaneous aspiration of echinococcal cysts, only 2 cases of lethal anaphylaxis (0.04%) and 99 reversible anaphylactic reactions (1.8%) were reported[54]. This study divided the complications related to cyst puncture into major (0.5% of cases with anaphylactic shock and peritoneal liquid seeding, liver or intra-abdominal abscess, sepsis, biliary fistulas) and minor (10%-30% of patients with fever, hypotensive reactions, nausea, vomiting, skin rash, respiratory symptoms). Peritoneal seeding has never been reported, but it is difficult to assess the true rate because many reported series have a short follow-up time. Prophylactic administration of ABZ starting 4 h before the puncture and for at least 30 d after puncture is a cautionary measure that should always accompany PAIR[59].

PAIR is performed with several variants of the standard protocol and is generally successful at inducing permanent solidification of medium-sized CE1 and CE3a cysts[13]. A few reports with long-term follow-up indicate that multivesiculated cysts (*i.e.*, CE2 and CE3b) tend to relapse repeatedly after PAIR[26,29,89,90]. Reported morbidity and mortality range from 8.5%-32% and 0%-1% respectively[89,91-94]. Mean hospital stay is 1-4 d compared to 12 d in case of surgery[89,91,93]. PAIR has also been performed in remote, resource-poor areas using portable US ma-

chines[95]. Overall response rates range from 72%-97%, with relapse rates from 1.6%-5%[89,91,92,94,96]. However, these figures vary greatly when cyst stages are taken into account. Indeed, unilocular CE1 and CE3a cysts respond very well to percutaneous treatment (> 80% response), while multi-vesiculated CE2 and CE3b cysts have a success rate lower than 40%[29,89,90]. Giant CE1 and CE3a cysts of 10 cm or greater, should preferably be treated with a large catheter left in place until the daily drainage is less than 10 mL, on average 3 wk[97].

The experience with catheterization techniques in CE2 and CE3b cysts is more recent and less extensive than that with PAIR, and results from series with long-term follow-up are needed before their efficacy can be determined. Data available for PEVAC in cysts with cysto-biliary fistulas are less than satisfying, given the long hospitalization and catheter times, up to 128 and 55 d, respectively[86]; in these cases PEVAC does not compare favorably with surgery.

The use of percutaneous techniques should be reserved for referral or specialized centers where teams are prepared to deal with possible complications and an anesthesiologist should always be present during the procedure.

Use of scolecidal agents in surgery and percutaneous treatments: Scolecidal agents should be applied only after having excluded the presence of cysto-biliary fistulae, either with intraoperative cystoscopy or evaluating bilirubin content in the cyst fluid. Although chemical sclerosing cholangitis, due to contact of the scolecidal agent with the biliary ducts, has never been reported using PAIR, several reports are present in the literature after surgery[98-100] and damage to the biliary epithelium has been shown in animal models[101,102]. While hypertonic (15%-20%) saline and 95% ethanol are the most widely used scolecidal agents for percutaneous treatments, a range of other compounds have been tested or are being investigated in the attempt to find an agent that does not damage the biliary epithelium[103-106].

Direct intracystic injection of mebendazole (MBZ) has been successfully performed in animals and humans, and ABZ sulfoxide, the active metabolite of ABZ, has been successfully injected in cysts in animals, but not in humans[107-110]. However, little difference has been found in *in vitro* studies between the effect of hypertonic saline and that of ABZ sulfoxide or sulfone[103]. Unfortunately, ABZ sulfoxide is not available as an injectable formulation and this prevents its clinical use.

Chemotherapy

The use of benzimidazole (BZD) carbamates in the treatment of CE was introduced in the 1970s. While both albendazole and MBZ have been proven effective against the larval stage of *E. granulosus*, ABZ is the current treatment of choice due to better absorption[111]. ABZ is administered orally at a dose of 10-15 mg/kg per day generally for 3-6 mo; administration should be continu-

ous without treatment interruptions, in contrast to the recommendation in the 1980s[26,112]. However, the optimal dose and duration of treatment with ABZ have not been formally assessed.

The comparative rarity of CE in many industrialized countries where BZD is available and affordable is such that only a few centers are able to follow sufficient numbers of patients within a reasonable period of time. Thus, most studies are small, and few have adequate controls.

In the largest series published thus far, 848 patients with 929 cysts received 3-6-mo continuous cycles of MBZ or ABZ treatment[113]. Long-term follow-up showed that 74.1% of the cysts developed degenerative changes. These were more frequent in ABZ-treated than in MBZ-treated cysts (82.2% *vs* 56.1%; *P* < 0.001). During follow-up, 104 cysts (22%) had degenerative changes, whereas 163 cysts (25%) relapsed. In other series, reported outcome rates for hepatic cysts are: 28.5%-58% cure/marked improvement, 10%-51% partial response, 13%-37% no change, and 4%-33% worsened[112,114-120]. Relapse rates range from 9%-25%[112,113,116,121], and, although responsive to subsequent treatments, cysts tend to relapse multiple times[28]. Factors associated with treatment outcome include cyst stage, size, and localization. Unilocular (CE1 and CE3a) cysts and small cysts (< 6 cm) respond better and faster to ABZ treatment compared with multivesciculated (CE2 and CE3b) and larger cysts, with a lower relapse rate[28,113,117,120,122], as clearly shown in the systematic review by Stojkovic *et al*[28]. A recent study highlighted the importance of at least 12 mo of follow-up, since it is difficult to predict cyst behavior after treatment[28].

Adverse effects of BMZ include headache (10% of cases), gastrointestinal symptoms (56%), hepatotoxicity, severe leukopenia, neutropenia or thrombocytopenia (< 1%), and alopecia (2%)[59,123]. Increases in aminotransferases (15% cases) may be due to drug-related efficacy or to real drug-related toxicity. Risks observed in laboratory animals include embryotoxicity and teratogenicity. While teratogenicity is theoretical, it is nonetheless good practice to avoid use during pregnancy whenever possible. Thus, the treatment should be delayed until after delivery[124]. Hospitalization is not necessary, but regular follow-up is required with a monthly check of the hemogram and liver enzymes.

If ABZ is not available or not tolerated, MBZ, the first BMZ tested against *Echinococcus*, may be used at a dosage of 40-50 mg/kg body weight, in three divided doses during fat-rich meals. Costs of BMZ and repeated examinations may be prohibitive in countries with limited resources. Praziquantel (PZQ) 40 mg/kg once a week in combination with ABZ seems more effective in killing protoscoleces than ABZ alone[125]. Other clinical studies evaluating this combination are available but they do not clarify whether PZQ has a pharmacological effect in its own right or acts only by enhancing ABZ absorption[126]. The usefulness of PZQ to avoid secondary echinococcosis needs confirmation[127].

Watch and wait

Recent expert opinion recommends that inactive CE4-CE5 cysts that are asymptomatic and uncomplicated should be left untreated and monitored regularly by imaging techniques, using the so-called "watch-and-wait" approach[56,59]. The rationale of leaving uncomplicated, inactive cysts untreated and solely monitored over time follows the observation that up to 20% of cysts become spontaneously inactive without any treatment and such cysts are likely to remain stable over time[18,26,118,120,128-130].

Follow-up

In chronic conditions such as CE, follow-up is crucial in order to evaluate the efficacy of treatment. The follow-up should start with a short interval (every 6 mo for the first 2 years) and continue with a longer interval (once a year), but this needs to be adjusted to the patient's setting. In referral centers, follow-up includes US imaging and serology; for specific patients (*e.g.*, with abdominal gas, obesity, multiple cysts, and so on) it may also include CT or MRI.

Long-term follow-up, generally longer than 5 years, is required to evaluate local recurrences which have been reported up to 10 years after apparently successful treatment[14].

CLINICAL DECISION-MAKING IN HEPATIC CE

CE can be very difficult to treat and even more difficult to cure for a number of reasons. The disease is complex and dynamic, with an evolving phase and quietly growing cysts, followed by an involution process during which the parasite is gradually dying, leaving behind a solidified, often calcified cyst or a scar.

Each successive active cyst stage carries its own risks for serious and even life-threatening complications. This variation during the CE disease process leads to a wide range of treatment modalities with an equally wide range of technological and training backgrounds necessary for implementation and delivery. As a result of all of these issues, no "one size fits all" management approach is available, and a stage-specific approach currently appears to be the best way to manage this condition[26].

Technical and economic difficulties are encountered in countries with limited resources where the patient load is greatest: here CE is defined as a *neglected disease*. Problems in acquiring clinical competence in countries where few patients suffer from the disease are also an obstacle: in these settings CE is an *orphan disease*[26]. Further complicating matters is the fact that CE is a chronically neglected disease. Investment in research is very low compared to what is needed based on estimated burden of disease[5]. The latter is very difficult to gauge because the true incidence is unknown. Acute cases have never been recorded because they are clinically silent and only the prevalence can be assessed, although often with great difficulties due to poor access to healthcare

Figure 6 Schematic representation of the natural history of hepatic cystic echinococcosis and suggested treatments. Solid black arrow indicates natural evolution toward inactivation; black dashed arrows indicate evolution of therapy-unresponsive chronic stages. US images: cyst ultrasound classifications according to World Health Organization Informal Working Group on Echinococcosis[23] (in bold) and Gharbi et al[22]. Gray boxes: suggested stage-specific approach to uncomplicated hepatic CE[59]. ABZ: Albendazole; PAIR: Puncture, aspiration, injection of scolecidal agent, re-aspiration; PC: Permanent catheterization.

and underreporting[3]. The best solution to this problem is likely the setting up of national CE registries modeled on the European Register for Alveolar Echinococcosis. We have recently set up the Italian Register for Cystic Echinococcosis: http://www.iss.it/riec/), and preliminary results of systematic enrolment of CE patients seen in Italian hospitals will be published in the near future. However, such initiatives require resources and funding, both difficult to come by when dealing with a neglected disease[5].

OPEN ISSUES IN STAGE-SPECIFIC APPROACH

Although the evidence base for clinical decision-making is still at the level of expert opinion, clinical management of hepatic CE patients is facilitated by the standardization of US classification, enabling clinicians to identify the most rational option on the basis of cyst stage[26,131]. The stage-specific treatment approach for uncomplicated cysts of the liver can be summarized as follows (Figure 6).

Small (< 5 cm) univesicular CE1 and C3a cysts tend to respond well to ABZ treatment, while larger cysts are treated preferentially with PAIR plus ABZ. Giant cysts (> 10 cm) should be treated with a catheter left in place until the drainage is minimum, usually about 3 wk.

Surgery should be reserved for complicated cysts, including those with rupture or high risk of rupture, fistulization, compression of vital organs or vessels, hemorrhage, or bacterial infection. Surgery is also an option for

cysts poorly responsive to medical or percutaneous treatment when a "watch and wait" approach is not viable because of poor access to healthcare.

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REVIEW

Metabolic syndrome and non-alcoholic fatty liver disease in liver surgery: The new scourges?

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Abstract

The aim of this topic highlight is to review relevant evidence regarding the influence of the metabolic syndrome (MS) and its associated liver manifestation, non-alcoholic fatty liver disease (NAFLD), on the development of liver cancer as well as their impact on the results of major liver surgery. MS and NAFLD, whose incidences are significantly increasing in Western countries, are leading to a changing profile of the patients undergoing liver surgery. A MEDLINE search was performed for relevant articles using the key words "metabolic syndrome", "liver resection", "liver transplantation", "non alcoholic fatty liver disease", "non-alcoholic steatohepatitis" and "liver cancer". On one hand, the MS favors the development of primary liver malignancies (hepatocellular carcinoma and cholangiocarcinoma) either through NAFLD liver parenchymal alterations (steatosis, steatohepatitis, fibrosis) or in the absence of significant underlying liver parenchyma changes. Also, the existence of NAFLD may have a specific impact on colorectal liver metastases recurrence. On the other hand, the postoperative period following partial liver resection and liver transplantation is at increased risk of both postoperative complications and mortality. These deleterious effects seem to be related to the existence of liver specific complications but also higher cardio-vascular sensitivity in a setting of MS/NAFLD. Finally, the long-term prognosis after curative surgery joins that of patients operated on with other types of underlying liver diseases. An increased rate of patients with MS/NAFLD referred to hepatobiliary units has to be expected. The higher operative risk observed in this subset of patients will require specific improvements in their perioperative management.

Core tip: The metabolic syndrome (MS) and its hepatic manifestations, non-alcoholic fatty liver disease (NAFLD), are increasingly observed in Western countries. Both MS and NAFLD could favor the development of primary liver malignancies and may also lead to end-stage liver disease. These patients are at higher operative risk because of underestimated postoperative liver related complications but also specific increase in cardio-vascular complications. Specific improvements in the perioperative management of these patients are required in order to improve the operative results.

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INTRODUCTION

The prevalence of the metabolic syndrome (MS) is reaching epidemic levels in Western Europe and Northern America, where it is reported to be as high as 25% in the general population[1]. The MS is a constellation of clinico-biological features closely related to insulin-resistance and includes dyslipidemia, hypertension, glucose intolerance and central obesity[1]. Non-alcoholic fatty liver disease (NAFLD) represents the hepatic manifestation of the MS. NAFLD pathological alterations, which range from simple steatosis to steatohepatitis, may lead to fibrosis and end stage liver disease[2]. As its incidence parallels that of the MS, NAFLD is currently becoming one of the first chronic liver diseases in Western countries and therefore has a major health impact[3]. Also, both MS and NAFLD have been suggested to be directly or indirectly associated with the development of primary liver malignancies[4-7]. For all these reasons, it is likely that more and more of these patients will be referred to hepatobiliary (HPB) and liver transplant units in upcoming years[8].

The increasing prevalence of MS/NAFLD and MS/NAFLD-related liver tumors is not the only issue related to these disorders. Despite numerous advances in the fields of hepatology, perioperative management and liver surgery, the impact of both MS and NAFLD on the postoperative course of patients undergoing liver surgery has long been neglected. As a matter of fact, it is only recently that evidence suggesting a specific and underestimated risk regarding postoperative morbidity and mortality in the setting of liver surgery has been released[8-13]. In that sense, it seems crucial that gastroenterologists and surgeons should be fully aware of the existence of MS and NAFLD as well as their negative impact on the postoperative course in order to optimize the perioperative management of concerned patients and to prevent any avoidable morbidity/mortality.

The objectives of this review are therefore: (1) to provide comprehensive insights regarding the current standards and issues in the diagnosis of both MS and NAFLD; (2) to clarify their respective impact on tumor progression as well as their influence on postoperative outcome; and (3) to discuss the measures which should be undertaken in upcoming years in order to improve the results of surgery.

DEFINITIONS AND ISSUES

Metabolic syndrome

The definition of MS has evolved during the past decade. Current consensual criteria for its diagnosis are summarized in Table 1. These include central (or android) obesity, hypertension, dyslipidemia, with either increased triglycerides level or decreased high density lipoprotein cholesterol level, and glucose intolerance[1]. Even although the presence of at least 3 out of 5 criteria of the consensual definition are required to define the MS[1], both liver histological manifestations and influence on surgical outcomes after liver surgery may occur in patients presenting with individual components of the MS. Indeed, fatty liver disease may also occur in patients with isolated diabetes mellitus (DM)[14], hypertriglyceridemia[15] and obesity[16,17]. Likewise, higher perioperative morbidity or mortality rates after liver resection are reported in patients with only DM[18,19] or overweight/obesity[20,21], whereas our groups found the association of just 2 disorders to be related to poor outcome of surgery[13,22].

Interestingly, most of the medical and surgical studies do not always gather all these consensual criteria but rather use substitutes for convenience. Such substitutes may lead to a certain degree of confusion. For example, it is frequently assumed that patients receiving statin or fenofibrate medication have dyslipidemia[8,11] and that patients receiving antihypertensive therapy have hypertension. However, some of these patients may receive such medications for primary cardiovascular prevention or renal protection. In the same way, central obesity, which reflects visceral adiposity, it is often measured using the BMI and various cut off values are proposed[8,12,13]. Yet, BMI does not allow distinguishing central obesity, which is a metabolic disorder included in the MS, from peripheral obesity. In that sense, circumferential waist appears to be more reliable and should be preferred[23,24]. Finally, the terms hyperglycemia and insulin-resistance are often used indiscriminately, whereas some authors suggest that they should not. Hence, the presence of insulin-resistance should be routinely assessed using the homeostasis model assessment of insulin resistance[25] whenever hyperglycemia is found.

NAFLD

NAFLD has emerged as one of the most frequent forms of chronic liver disease in Western countries[5,6] and should be considered in cases of fatty infiltration exceeding 5% of the liver parenchyma at histology in the absence of previous or ongoing significant alcohol consumption[26]. Although NAFLD is considered the hepatic manifestation of the MS, other conditions, including chronic hepatitis B and C infection[27,28], irinotecan based chemotherapy[29,30] and several other medications, including methotrexate, tamoxifen or amiodarone[31,32], may also lead to fatty liver disease and should be meticulously ruled out. NAFLD, which encompasses a wide spectrum of diseases ranging from simple steatosis to non-alcoholic steatohepatitis (NASH)[26], can progress to cirrhosis and may lead to end-stage liver disease[5,6]. Histological analysis remains the gold standard for the assessment of NAFLD and should be performed by a trained pathologist[33]. Several histological scores might be useful for diagnosis. The most frequently used score is the non-alcoholic liver disease activity score (NAS) proposed by Kleiner *et al*[26], which is a semiquantitative,

Table 1 Diagnostic criteria of the metabolic syndrome

Criteria	Consensual criteria definition[1]	Other non-consensual criteria
Central obesity	Abdominal waist[2]	Different cutoff values of BMI
	> 102 cm (United States) or 94 cm (Europe) in men	$\geqslant 28$ or $\geqslant 28.8$ or $\geqslant 30 \ kg/m^2$
	> 88 cm (United States) or 80 cm (Europe) in women	
Dyslipidemia	Triglycerides $\geqslant 150 \ mg/dL$ (1.7 mmol/L)	Statin or fenofibrate medication[3]
	HDL cholesterol	
	< 40 mg/dL (1.03 mmol/L) in men	
	< 50 mg/dL (1.29 mmol/L) in women	
Hypertension	Blood pressure > 135/85 mmHg	Any antihypertensive therapy[3]
Glucose intolerance	Hyperglycemia	Any diabetes
	Fasting glucose $\geqslant 110 \ mg/dL$,	Any antidiabetic therapy (oral or insulin)
	or type Ⅱ diabetes	

[1]Diagnosis of metabolic syndrome (MS) requires at least 3 out of 5 criteria; [2]Other cut off values have been established for Asians and Latin Americans; [3]These treatments can be taken in account for the diagnosis of MS unless if given in preemptive purpose.

histology-based score system including three parameters, namely steatosis (on a scale of 0-3), lobular inflammation and hepatocellular ballooning (on a scale of 0-2 each) and therefore ranges from 0 to 7. Likewise, Bedossa *et al*[34] recently published a histopathological algorithm and scoring system for evaluation of liver lesions in morbidly obese patients.

NASH

NASH is considered the result of long-lasting inflammation. It is characterized by several histological alterations, including steatosis, lobular inflammation and ballooning, and may also be associated with fibrosis. Even although the diagnosis of NASH was initially suggested for NAS values of 4 or 5[26], there is an ongoing debate regarding the accuracy of NAS in assessing NASH. Interestingly, Brunt *et al*[33] have emphasized that the diagnosis of NASH based on evaluation of patterns as well as individual lesions on liver biopsies did not always correlated with threshold values of the semi quantitative NAS. Moreover, NAS does not include other histological alterations often present in NAFLD, such as microcirculation modifications, which are not routinely reported by pathologists[35]. Thus, rather than being based on the NAS value alone, the differentiation between NASH and no-NASH should rather take into account the pathologist report[33].

Identification of NASH in patients with MS/NAFLD

Since the increasing incidence of both MS and NAFLD in Western populations de facto puts a great amount of patients at risk of developing NASH, any large scale screening policy aimed at obtaining histological diagnosis of NAFLD does not seem reasonable. Furthermore, the accuracy of histology in identifying NASH is suboptimal as both inter-observer variations[36] and discrepancies from one sample to the other within the same parenchyma may occur[37]. In order to increase cost/effectiveness and accuracy of diagnosis, and also to avoid the intrinsic invasiveness of biopsy, there has been significant interest in identifying non-invasive methods of predicting liver histology in patients with suspected NASH. Hence, numerous biological (alanine aminotransferase/aspartate

aminotransferase ratio, FIB-4, analysis of organic compounds in breath)[38] and imaging techniques (magnetic resonance imaging (MRI) for quantification of liver steatosis[39] or magnetic resonance spectroscopy) have been proposed for the detection of underlying parenchymal changes among patients with MS, but none has become the "gold standard". In particular, although MRI has shown high accuracy in detecting steatosis, its effectiveness in evaluating (and possibly ruling out) fibrosis is questionable in the presence of fat[40].

MS/NAFLD INFLUENCE ON CARCINOGENESIS

The association between individual components of the MS, such as diabetes[41] and being overweight[42], and an increased risk of cancer has long been known. More recently, it has been suggested that the MS itself was implicated in carcinogenesis, especially in the liver[4]. Indeed, two recent series have shown that the MS itself was associated with an increased risk of developing of both HCC[3] and intrahepatic cholangiocarcinoma[43]. In particular, HCC incidence in patients with MS is reportedly 2-4 fold higher than in general population[7].

How the MS acts to promote carcinogenesis remains to be fully elucidated. Several genetic mechanisms are supposed to be involved in MS-related carcinogenesis. Firstly, direct oncological effects may play a role in the carcinogenesis by loss of tumor suppression genes, deregulation of IL-6 signal or inhibition of JNK1 phosphorylation[22]. This mechanism is supposed to be at the origin of malignant transformation of liver cell adenoma in men[44]. Secondly, the MS has been reported to be associated with low-grade, chronic systemic inflammation, implying a serum increase of inflammatory cytokines, such as TNF-α and IL-6[5], and a decrease in anti-inflammatory ones, including adipocytokines[45].

Interestingly, most studies focusing on HCC occurring in patients with MS (or arising in a context of NAFLD) have consistently reported that 30%-60% of the patients displayed no feature of severe underlying fibrosis[7,8,22,46]. More surprisingly, almost 20% of the

patients had a normal underlying liver parenchyma after conventional pathological examination. In this setting, HCCs furthermore tended to be isolated and of large size[8,22]. These findings seem to indicate that several different pathways may be implicated in liver carcinogenesis in patients with MS, as suggested by the inconstant presence of various histology alterations.

Although not always present, NASH related cirrhosis may possibly be considered a precancerous lesion as it is associated with a yearly incidence of HCC as high as 2.6%[5], leading to a cumulative 5-year incidence ranging from 7.6%[47] to 11%[48]. In the event of NASH related cirrhosis, both presence and pattern of hepatic iron deposition[49] have been incriminated to further accentuate parenchymal changes, thus promoting liver carcinogenesis.

Virus infection may also play an indirect role in tumor development in patients with MS. In particular, the specific subset of patients with chronic hepatitis C virus (HCV) infection developing an HCC is worth being mentioned. Several authors have emphasized that chronic HCV infection was associated with fatty infiltration of the liver parenchyma in 50%-70% of cases, including massive steatosis and NASH[27,28,50,51]. A non-negligible number of the latter display the so-called "viral steatosis" as a consequence of virus interference with fat metabolism (in the absence of pre-existing metabolic disorders). Thus, in this setting, steatosis itself could be responsible for the occurrence of secondary insulin-resistance and systemic inflammation. Even although the "viral steatosis" has been shown to regress after viral eradication[52], its existence has been incriminated in recurrence of HCV related HCC[53] after curative surgery. However, since steatosis and lobular inflammation may be found in HCV infection regardless of MS/NAFLD, the supposed association between HCC, HCV and NAFLD could be more a statistical artifact than a real oncogenic mechanism. Taken together, the supposed pathway from viral infection to viral steatosis and HCC, as well as the possible mechanisms finally leading to HCC development (fibrosis, inflammation or induced insulin-resistance), still remain to be assessed.

Finally, the association between MS, NAFLD and colorectal liver metastases (CLM) has to be considered. Indeed, whereas several studies on colorectal cancer patients analyzed the impact of 5FU + irinotecan based chemotherapy on the development of steatohepatitis[28,30], it is only recently that studies have focused on the specific oncological influence of both MS and NAFLD on CLM, with various results. On one hand, Hamady *et al*[54] found that liver steatosis was associated with a 1.3 fold risk of local recurrence following liver resection for CLM, regardless of the chemotherapy regimen used. On the other hand, Viganò *et al*[55], studying the impact of chemotherapy-related liver injuries, pathological tumor regression grade and micrometastases on long-term survival, found that higher grade (2-3) steatosis was significantly associated with improved 5 year overall survival compared to lesser steatosis (grade 0-1) after resection of CLM (52.5% *vs* 35.2%, *P* = 0.002). Even although these studies lacked specific histological assessment of NAFLD and precise identification of metabolic disorders, the observed results clearly reflect the growing enthusiasm of surgeons in exploring the impact of NAFLD on the long-term outcomes of patients with CLM.

MS/NAFLD IMPACT ON OUTCOME OF LIVER SURGERY

The impact of individual components of the MS and liver steatosis on the postoperative course following liver resection has been extensively investigated[18,56-60]. Accordingly, it has been established that liver surgery provided poorer results in patients affected by diabetes[18] or obesity[56,57] than in otherwise healthy patients. Similarly, several studies have highlighted that steatosis per se was a risk factor for postoperative complications after major hepatectomy[58-60]. In experimental models, liver fatty infiltration, such as mild or severe steatosis, has been found to be associated with lower regenerative ability following portal vein occlusion, elevated sensitivity to ischemia-reperfusion injury and higher hepatocellular injury after partial liver resection[61]. Nevertheless, it is only recently that surgeons have focused on the results of surgery, liver resection and transplantation in the specific subset of patients with MS or NASH.

Liver resection
Table 2 summarizes the results of recent series analyzing the early outcome of patients undergoing liver resection in a setting of MS/NASH[8-13]. Of these six series, three aimed at assessing the influence of the MS on outcome[8,12,13], whereas the remaining three aimed at evaluating the impact of histological modifications, including NAFLD and NASH[9-11]. The fact that data concerning metabolic disorders (and MS) and liver histology were gathered together in only half of the series[8,11,13] emphasizes the absence of clear understanding of the relationship between MS and MS-related liver disease. In these studies, mortality after liver resection varied from 3% up to 30% and was related to the primarily studied parameter, *i.e.,* MS, NAFLD or NASH. In this setting, it has been recently suggested that MS patients with a NAS > 2[8] or those with an histological diagnosis of NASH[11] had a 2.7-fold higher risk of experiencing liver related but also cardio-respiratory complications than those with normal underlying parenchyma. Hence, it seems that steatohepatitis rather than simple steatosis was a risk factor for postoperative complications[11]. Even if these recent findings may appear to be in opposition with previously published results maintaining a negative impact of steatosis on outcome[58-60], it is likely that the poor assessment of inflammatory changes in the underlying steatosic parenchyma may have biased older series. On the opposite hand, the progressively increasing degree of parenchymal change, damage and inflammation from steatosis to steatohepatitis is nowadays considered as a continuum, which progressively and proportionally increases overall

Table 2 Studies focusing on liver resection in a context of metabolic syndrome, non-alcoholic fatty liver disease and non-alcoholic steatohepatitis

Ref.	Endpoint	Underlying parenchyma	Assessment of metabolic factors	Morbidity			Mortality
				Overall	Liver related	CV and respiratory	
Wakai *et al*[9]	Influence of the underlying liver on liver resection	NAFLD (*n* = 17)	BMI	59%	47%	6%	12%
Neal *et al*[10]	Influence of the underlying liver on right trisectionectomy	NASH (*n* = 9)	All factors	NA	NA	NA	22%
Reddy *et al*[11]	Influence of the underlying liver on liver resection	Simple steatosis (*n* = 72)	All factors	35%	19%	28%	4%
		NASH (*n* = 102)		57%	28%	13%	4%
Bhayani *et al*[12]	Influence of the MS on liver resection	NA	MS (*n* = 256)	29%	NA	22%	6%
			No MS (*n* = 3.717)	23%		15%	2%
Zarzavadjian Le Bian *et al*[13]	Influence of the MS on right trisectionectomy	NAFLD (*n* = 27)	> 2 MS factors (*n* = 30)	60%	53%	NA	30%
			⩾ 3 MS factors (*n* = 13)	NA	NA	NA	54%
Cauchy *et al*[8]	Influence of the MS on liver resection	NASH (*n* = 16)	MS (*n* = 62)	58%	21%[1]	17%[1]	11%

[1]Major complications: Clavien Ⅲ-Ⅴ. MS: Metabolic syndrome; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; CV: Cardiovascular; NA: Not applicable.

postoperative morbidity/mortality.

Intuitively, not only the "quality" but also the "quantity" of liver remnant should be considered. In fact, it has been recently suggested that NASH was independently associated with both higher postoperative liver insufficiency and mortality following right hepatectomy (including extended right hepatectomy)[13], and trisectionectomy[10], although a (usually) "safe" amount of liver parenchyma was left in place. This result clearly emphasizes the worse tolerance to extended resection of fatty and inflammatory livers. This feature may be of particular importance in the case of HCC developing in a MS/NAFLD context, where large lesions often require major resections[8,22].

Considering cardiovascular morbidity/mortality, it has been shown that NASH was an independent riskfactor for the development of coronary artery disease and calcifications regardless of the degree of visceral adiposity[62,63], thus leading to a higher incidence of cardiorespiratory events following liver resection. Possibly, the recently described hemorheological alterations occurring in MS patients, including increased erythrocyte aggregation[64,65], may play a role in ischemic cardiac events.

Liver transplantation

NASH can progress to cirrhosis[2,4] and may lead to end-stage liver disease requiring liver transplantation (LT). During the last decade, the rate of LT performed for NASH related end-stage liver disease has dramatically increased from about 3% in the early 2000s up to 19% in 2011[2]. Currently, non-alcoholic steatohepatitis is the third most common cause of LT in the US and is on pace to become the most common within the next two decades in Western countries[66].

LT in NASH patients has peculiar aspects. Compared with other patients undergoing LT, recipients with NASH tend to be older[67] and obviously have a higher frequency of metabolic disorders[62]. In this setting, procedures significantly last longer and are associated with higher blood loss and longer post-transplantation hospital stay[62]. Accordingly, 30 d mortality after LT in patients with NASH tends to be higher than that for other indications[68]. Several studies have reported increased liver related morbidity rates in NASH patients, such as acute rejection rates[67], but also extra-hepatic complications, including sepsis and renal dysfunction[69]. Similarly to patients undergoing liver resectional surgery, NASH patients also have a higher likelihood of developing cardio-vascular complications after LT[62,67,69]. These events, which mainly occur within the first year after LT, have been reported to be responsible for as high as 50% of the total mortality following LT[62]. The relationship between MS/NASH and cardiovascular morbidity seems more complex than a generic multi-organ vascular disorder due to MS, as suggested by the significantly higher occurrence of cardiovascular events associated with MS whenever NASH is present[70]. In fact, similarly to what has been observed after LR, NASH is nowadays thought to put patients at an even higher risk of cardio-vascular complications, regardless of comorbidities and patient-specific cardiac risk[62]. Here again, it is likely that the degree of inflammation in the underlying liver represents a key factor in the occurrence of increased cardiovascular sensitivity.

Long-term results of LT following transplantation for NASH are encouraging. One, three and five year survivals after LT for NASH range from 84%-87.6%, 75%-82.2% and 70%-76.7%, respectively, and are at least similar to that observed for LT for other traditional indications[2,62,67,68,71]. Even more remarkable, LT for HCC developed in patients with NASH seems to provide excellent long-term outcomes with higher survivals compared with patients transplanted for HCV related HCC[72]. These observations could be the result of less aggressive tumors in NASH patients with lower micro vascular invasion and decreased rates of poorly differentiated lesions[8,72].

LT in patients with NASH related cirrhosis presents peculiar issues, including cirrhosis recurrence, to be discussed separately. Recurrent disease after LT for NASH

related cirrhosis has been reported to occur in as high as 34% of recipients[68,73]. There is little information detailing the occurrence and histological evolution of NAFLD recurrence after LT and the long-term natural history of NAFLD recurrence itself is unclear[74]. Nevertheless, in these patients, recurrence is often associated with the presence of the MS or its individual components[73]. Accordingly, recurrence should be further evaluated in larger studies, with special emphasis on management of MS and secondary prevention strategies[73].

WHICH IMPROVEMENTS SHOULD BE UNDERTAKEN IN UPCOMING YEARS?

Both MS and NAFLD/NASH adversely affect short and long-term results of liver surgery. Considering that the rate of patients presenting with such conditions will keep on increasing in upcoming years, it appears crucial that specific measures should be undertaken in order to improve those unsatisfactory results. Above all, the worse tolerance to extended resection of fatty and inflammatory livers (as a consequence of lower regenerative ability) requires that this issue should be attentively pondered in the preoperative planning of surgical strategy whenever a major resection is needed. Unfortunately, the culture of considering just MS or steatosis (even without liver biopsy confirmation) a potential risk factor for major surgery has not already entered clinical practice, even in specialized environments. Addressing this issue, our group has recently shown that MS patients operated on for HCC less frequently underwent preoperative PVE when they displayed a NAS > 2 without severe fibrosis compared to those with severe underlying fibrosis, suggesting that these latter patients would probably benefit from a better anticipation of their operative risk, especially in cases of planned major LR[8].

In general, preventative measures to reduce MS/NAFLD related morbidity/mortality should include: (1) better characterization of the underlying parenchyma using invasive or non-invasive means knowing that patients with inflammatory fatty liver even without severe fibrosis are at similar operative risk as those with severe underlying fibrosis; (2) targeted perioperative management, including complete preoperative cardio-vascular work-up and intraoperative cardio-vascular and pulmonary monitoring; and, finally (3) specific "NAFLD-tailored" perioperative surgical care, such as parenchymal sparing resections, wide use of liver volume modulation techniques, including portal vein embolization and portal vein ligation, but also targeted medical therapies developed in order to improve the tolerance of LR. Concerning this latter issue, a recent experimental study has highlighted the benefits of omega-3 acids in reducing severe steatosis in a preoperative setting leading to improved liver regeneration and functional recovery following partial hepatectomy[75]. These encouraging preliminary results still require confirmation in a clinical setting but may already

be considered a promising future field of research.

Concerning the relationship between MS/NAFLD and neoplastic disease, several strategies should be developed in order to prevent both occurrence and recurrence of primary liver cancer in MS/NASH patients. Even although it is generally recommended that overweight and obese patients with NAFLD lose 7%-10% of their body weight by dietary modification and exercise over the course of 6-12 mo, the paucity of data makes it difficult to make evidence-based recommendations about dietary modification and exercise to treat NAFLD and NASH[76]. In fact, medical research has mainly focused on reducing NASH in MS patients using medical therapies. Several randomized controlled trials have shown significant downstaging of NASH following the administration of specific medications, including vitamin E and pioglitazone[77-79]. Retrospective studies have shown that the use of biguanides, such as metformin, was associated with HCC risk reduction among diabetic patients[80,81]. Experimentally, metformin has been shown to provide antineoplastic effects through deregulation of the m-TOR pathway[82,83]. Hence, in a context of MS/NAFLD related HCC, metformin would theoretically represent an ideal preventative therapy reducing both incidence of HCC following parenchymal alterations or systemic inflammation and also providing inherent antitumoral properties. Nevertheless, despite the encouraging results of all these medications and the possible future development of others that are even more effective, it should be kept in mind that none have currently been tested in a surgical context. In fact, the prolonged time interval required by medications to obtain relevant effects on liver parenchyma possibly reducing morbidity definitely questions its applicability in a surgical environment prior to (or after) surgery. This consideration gains interest if one considers that the great majority of patients undergoing major liver surgery (LR and LT) presents with cancer or end stage liver disease, needing prompt management. Obviously, any medical/preventative strategy should ideally require a large-scale evaluation in a surgical setting.

CONCLUSION

Both the pro-oncogenic effect on the underlying liver and the rising incidence of MS/NASH imply that an increased number of patients with such conditions referred to HPB units has to be expected. The higher operative risk observed in these patients can be partially explained by both underestimated liver related risk and also high perioperative cardio-vascular and respiratory susceptibility. These unsatisfactory postoperative results will require targeted perioperative management. Such actions are justified by the observed favorable long-term outcomes.

ACKNOWLEDGMENTS

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REVIEW

Management of patients with hepatitis B and C before and after liver and kidney transplantation

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Abstract

New nucleos(t)ide analogues (NAs) with high genetic barrier to hepatitis B virus (HBV) resistance (such as entecavir, tenofovir) have improved the prognosis of patients with HBV decompensated cirrhosis and have prevented HBV recurrence after liver transplantation (LT). NAs are considered the most proper approach for HBV infection in patients under renal replacement therapy but their doses should be adjusted according to the patient's creatinine clearance. In addition, physicians should be aware of the potential nephrotoxicity. However, patients with chronic hepatitis C and decompensated cirrhosis can receive only one therapeutic option before LT, as well as for Hepatitis C virus (HCV) recurrence after LT, which is the combination of subcutaneous Peg-IFN and ribavirin. Generally, therapy for HCV after renal transplantation should be avoided. Although the optimal antiviral therapy for HCV infection has not been established, attention has turned to a new, oral direct acting antiviral treatment which marks a promising strategy in prognosis and in amelioration of these diseases.

Core tip: While nucleos(t)ide analogs (NAs) offer a benign course in patients with hepatitis B virus before and after liver and renal transplantation, there is still scope for improvement. The administration of high genetic barrier NAs such as entecavir or tenofovir pre-transplant and the careful patient selection for hepatitis virus immunoglobulin-free regimens post-transplant contribute to improved medical care and facilitate its provision from a practical standpoint. Concordantly, attention has turned to new treatment strategies regarding hepatitis C virus recurrence after liver and renal transplantation. The addition of oral direct acting antivirals to the existing treatment marks a promising strategy for prognosis amelioration of these patients.

INTRODUCTION

The major breakthrough in the field of transplantation for patients with hepatitis B virus (HBV) and hepatitis C virus (HCV) is the application of nucleos(t)ide analogs (NAs) and direct acting antivirals (DAAs). NAs form the mainstay in the treatment of patients with HBV in the non-transplant setting as well as before and after liver and kidney transplantation. The preliminary data regarding the DAAs application favored its use in treatment of HCV recurrence after liver transplantation (LT) and in HCV renal transplant candidates.

Table 1 Recommendations for the management of hepatitis B and C infection before and after liver or renal transplantation

	Chronic hepatitis B		Chronic hepatitis C	
	Liver transplantation	Kidney transplantation	Liver transplantation	Kidney transplantation
Pre transplant		NAs[1]	Peg IFN ± RBV ± DAAs[3] (depending on genotype and type of DAA)	PegIFN plus very low dose RBV (plus DAAs)[4]
Post transplant	Prophylaxis and treatment		No prophylaxis[2]	
	Short term HBIG plus NA	NAs[1]	Peg IFN ± RBV ± DAAs[3] (depending on genotype and type of DAA)	RBV+ sofosbuvir (?)

[1]Entecavir or tenofovir are the advisable NAs in adjusted doses regarding creatinine clearance; [2]Therapy is indicated in fulminant cholestatic hepatitis and de novo glomerulonephritis; [3]Records for boceprevir, telaprevir and sofosbuvir. Triple combination is preferable in cases of cirrhosis (METAVIR fibrosis stages 3 and 4), cholestatic hepatitis, previous virological failure and in the presence of predictors of poor response; [4]There are only cases for the use of DAAs in renal transplant candidates. HBV: Hepatitis B virus; HCV: Hepatitis C virus; NA: Nucleos(t)ide analog; HBIG: Hepatitis virus immunoglobulin; DAAs: Direct oral acting antivirals; Peg IFN: Peg interferon; RBV: Ribavirin.

In general, the induction of immunosuppressive therapy carries the risk of HBV reactivation, leading to liver graft loss and fatal complications[1,2]. NAs have dramatically improved the clinical course of patients with HBV decompensated cirrhosis, reducing the need for LT, and have further improved the prognosis of HBV transplant patients[3,4]. At present, due to high rates of resistance[3,4], lamivudine is preferable only when short term immunosuppression is scheduled. Entecavir and tenofovir are the first choice NAs because they have very high efficacy and low resistant rates[5,6]. NAs administration implies detailed renal function monitoring. Telbivudine may improve renal function but it has an unfavorable resistance profile[7].

In regards to transplantation in patients with chronic hepatitis C (CHC), improvements in the understanding of the viral cycle have led to development of the first generation DAAs (telaprevir and boceprevir) which belong to HCV NS3/4 protease inhibitors[8,9]. Their addition to the standard of treatment [pegylated interferon (Peg-IFN) and ribavirin (RBV)] has improved the response rates in a small number of liver transplant recipients and in a few cases of renal transplant candidates. When DAAs are used, calcineurin inhibitors doses should be adjusted[10] and the hemoglobin levels should be regularly monitored. The present review focuses on the current treatment of patients with HBV and HCV before and after liver and kidney transplantation.

MANAGEMENT OF PATIENTS WITH HEPATITIS B AND C BEFORE AND AFTER LT

The management of patients with hepatitis B and C listed for LT contains a three step approach. Targeted therapy should start before transplantation and continue after transplantation, becoming more intensive immediately post-transplant when the immunosuppression is higher. Therapy escalation in an early post-transplant stage is imperative mainly for avoidance of HBV recurrence, while routine prophylactic therapy for HCV recurrence is not recommended[11,12] (Table 1).

HBV positive and HCV positive liver transplant candidates

Before transplantation, treatment aims to eliminate viral load, to keep it undetectable at the time of transplantation in order to lower the risk of HBV recurrence and improve the outcome[13,14]. HBV DNA clearance pre-transplant has reduced the rate of HBV recurrence in patients with HBV infection[15]. Similarly, HCV RNA eradication pre-transplant resulted in amelioration of fibrosis and long term survival in patients with HCV infection[13,15-18]. The suppression of HCV viremia in LT candidates and the undetectable HBV DNA at the time of LT are the most important goals for each particular infection.

The current management of patients with cirrhosis and CHB before LT is based on NAs[3,15] and modification of lifestyle, comorbidities and drug interactions[19]. Generally, the institution of NAs has ameliorated the transplant prognostic scores to such a high level that many LT candidates with CHB have delisted[20-22], presenting with great clinical improvement and better survival[23-25]. NA administration as monotherapy or in combination are the current guidelines for LT candidates with HBV decompensated cirrhosis[26-28]. The high genetic barrier antivirals entecavir and tenofovir are recommended as monotherapy. Entecavir has reduced the HBV DNA in patients with decompensated cirrhosis, has improved their underlying liver function up to 70% and has presented with very low resistant rates[22,29-31]. Tenofovir has also been an effective initiation therapy, accounting for high fibrosis resolution over five years administration[32-34] with almost negligible resistance. When administered in patients with decompensated cirrhosis, it led to virological, biochemical and clinical improvement with very good tolerance[35].

Nevertheless, entecavir should not be applied in patients with proven lamivudine-resistance because there are high chances of resistance and treatment failure[30], while tenofovir should be used with caution because of potential tubular injury and osteomalacia[34]. However, recent trials have doubted tenofovir nephrotoxicity after ten years of therapy in large groups of HIV infected patients[36,37] or even the nucleotide nephrotoxicity in LT recipients[38].

Furthermore, it has been hypothesized that an antiviral combination might achieve higher virological response rates and lower resistance rates compared to monotherapy. However, emtricitabine plus tenofovir (200 mg and 300 mg daily respectively) have not been found to have superior antiviral potency compared to entecavir and tenofovir monotherapy[35]. In addition, the higher cost of antiviral combination compared to monotherapies limits its use in clinical practice. In conclusion, all patients on NA therapy should be monitored every three months for virological response and possible virological breakthrough with serum HBV-DNA testing[39,40].

Contrary to LT candidates with CHB, patients with CHC and decompensated cirrhosis have only one therapeutic option before LT. This is the combination of subcutaneous Peg-IFN and RBV, leading to reduced cirrhosis-related complications and improving histological changes[41], but in an unsatisfactory percentage of patients (5%-33% in genotype 1 and 14%-100% in genotypes 2/3)[12,42-45]. IFN-based regimens have also been related to poor tolerability and many side effects[42,44], such as anemia, infections and neuropsychiatric disorders[46], which require either erythropoietin and granulocyte colony-stimulating factors or antibiotics to sustain drug regimen optimal doses[47]. The aim is undetectable HCV RNA or SVR at LT to reduce the frequency of HCV recurrence[13].

HBV positive and HCV positive liver transplant recipients

The primary goal in this step is the prevention of HBsAg appearance in a patient with erased HBV infection (HBV recurrence) and a new HBV DNA finding in a patient with negative HBsAg (virological breakthrough)[48]. The combination of HBV immunoglobulin (HBIG) with high genetic barrier NA (entecavir or tenofovir) for the long term is the most effective prophylactic approach for HBV recurrence prevention[49,50]. However, the high cost of HBIG and the fact that the majority of patients receive a transplant with undetectable or minimal HBV DNA since they have been on NAs before LT led to the use of short term HBIG or HBIG-free regimens in the post-transplant period. In the first case, LT recipients take a combination of HBIG and NA for a short period post-transplant, continuing with NA monotherapy long term[49]. In a group of LT recipients with low risk for HBV recurrence (only 4.5% had detectable HBV DNA at the time of LT), entecavir or tenofovir monoprophylaxis after HBIG discontinuation was similarly effective, with no difference in renal adverse events[38,49].

Regarding the HBIG-free prophylactic regimens, dual NAs such as tenofovir and emtricitabine or tenofovir plus entecavir[51-54] accounted for undetectable HBV DNA after 26 mo treatment post LT, but they did not eliminate cases of recurrence[49,52]. Entecavir and tenofovir should be the first-line options for HBIG-free prophylaxis. It is advisable that entecavir not be used in patients with previous lamivudine resistance who should be preferably treated with tenofovir. Until the optimal HBIG-free prophylactic regimen is determined, the combination of

HBIG (at least for a short period) and one high genetic barrier NA appears to be the most reasonable post-transplant approach[49,55].

Physicians should individualize the therapeutic regimen according to the pre-transplant type of liver disease, the patient's viremic status and the risk of reactivation[56,57]. HBV DNA clearance and HBeAg negativity at the time of LT, fulminant HBV and hepatitis D virus coinfection may allow HBIG reduction or withdrawal strategies[48]. At present, more and more patients maintain HBV DNA undetectable peritransplant so their prognosis has improved[3]. In our clinical setting, we use maintenance therapy with entecavir or tenofovir monoprophylaxis after a short course with low dose HBIG plus entecavir or tenofovir as antiviral prophylaxis against HBV recurrence after LT. Striking techniques such as covalently closed circular DNA (cccDNA) could detect occult HBV (HBV infection with negative HBsAg test) in hepatic and extrahepatic sites early. Nevertheless, Lenci *et al*[58] showed that many patients had a recurrence after cessation of any anti-viral prophylaxis despite negative cccDNA.

Regarding HCV positive liver transplant recipients, recurrence of HCV infection occurs in virtually all patients transplanted for HCV-related liver disease after LT. Additionally, three years post-transplant, decompensation developed in 70% of recipients compared with other immunocompetent groups in which the same proportion was less than 10%[59]. Post-transplant prophylaxis (preemptive) against HCV recurrence is not recommended because randomized trials have not confirmed its superiority regarding treatment when there was recurrence and it was associated with high cost and poor tolerability[11,12,60]. Interferon (IFN) use on the basis of high immunosuppression has not been effective and has been related to sepsis and rejection episodes[13,61]. Indications for antiviral therapy are fibrosing cholestatic hepatitis and significant fibrosis[61] [METAVIR score > F1[62], hepatic venous gradient > 6[63] and liver stiffness > 8.7 kPa[64]], but not fibrosis level > 3 because those patients cannot tolerate therapy[18]. Emphasis should be given to prompt diagnosis of histological evidence of HCV recurrence. Patients with female gender, steatosis of the graft, older donor age[65-67], cytomegalovirus and human herpes virus 6 infection[68] require sustained attention with protocol graft biopsies, regardless of normal liver function tests and good clinical condition. Non-invasive diagnostic methods such as elastography, serum and molecular fibrosis markers should also be used simultaneously[47].

The combination of Peg-IFN with RBV is again the standard of care for HCV recurrence after LT. Likewise, the regimen's efficacy is frustrating because after 72 wk of administration, SVR stabilization was achieved in only 30% of recipients[69-71]. The currently used DAAs, boceprevir and telaprevir, on the top of the old regimen have shown very promising results for the treatment of HCV reinfection in LT recipients with CHC[61]. Five studies[72-76] have demonstrated that the institution of the triple regimen obtained SVR in 50%-89% of LT

Table 2 Safety and efficacy of the combined regimen, interferon, ribavirin and protease inhibitors to treat hepatitis C after liver transplantation

	Boceprevir (*n*)	Telaprevir (*n*)	Complete virological response	Side effects
Coilly *et al*[72]	18	19	58% TVR	Anemia 92%
			89% BOC	Infections 27%
				Fatal events 8%
Pungpapong *et al*[73]	31	35	86% TVR	Anemia 95%
			48%BOC	Infections 10%
				Renal dysfunction 76%
				Fatal events 3%
Werner *et al*[74]	-	9	88.80%	Anemia 75%
				Rash 33%
				Renal dysfunction 33%
Stravitz *et al*[75]		50	62%	Anemia 82%
				Renal failure
				Fatal events 7%
Ann Brown *et al*[76]	-	46	60%	Anemia 48%
				Rash 35%
				Pruritus 22%
				Renal failure 22%
				Infections 22%
				Anorectal symptoms 41%
Forns *et al*[77]	Sofosbuvir 115		78%	Fatal events 16%

BOC: Boceprevir; TVR: Telaprevir.

recipients with CHC, mostly with genotype 1, when administered for 12 to 66 wk (Table 2). Serious side effects, fatal events, were recorded in two studies[73]. Although the place of DAAs in the management of LT recipients has not been totally clarified, two reported algorithms[19,61] may guide therapy. According to them, the triple regimen should be applied in cases of cirrhosis (METAVIR fibrosis stages 3 and 4), cholestatic hepatitis, previous virological failure and in the presence of predictors of poor response. Interestingly, sofosbuvir (NS5B inhibitor) combined only with RBV with or without PEG IFN demonstrated strong antiviral potency and disease improvement of CHC recurrence in LT recipients[77]. These are all very promising data but need to be tested in large multicenter prospective trials to become the standard of care.

In line with reducing severity of HCV recurrence after LT, immunosuppression is one of the major factors that accounts for accelerated HCV recurrence. For example, both steroid boluses as well as their very rapid tapering have been associated with aggressive HCV recurrence and graft loss. Interestingly, the long term maintenance immunosuppression with azathioprine, tacrolimus and prednisolone delayed the appearance of histologically proven severe fibrosis, while the sirolimus therapy led to HCV RNA elimination without antiviral treatment[78,79].

MANAGEMENT OF PATIENTS WITH HEPATITIS B AND C BEFORE AND AFTER KIDNEY TRANSPLANTATION

HBV positive and HCV positive renal transplant candidates

Antiviral therapy advances for HBV and HCV infection on renal transplantation (RT) have indicated great benefits in pre-transplantation and post-transplantation management and results[80-83]. However, antiviral therapy for HCV is hardly tolerated by RT candidates, especially if they have comorbidities and dialysis-related complications. It may not be wise for HCV positive patients with congestive heart failure, uncontrolled diabetes and with short life expectancy to receive antiviral therapy[84,85].

HBV and HCV positive candidates for RT should preferably undergo liver biopsy. The transjugular route is preferable since coagulation abnormalities are very common[86]. Fibroscan and other noninvasive techniques are supplementary. The presence of cirrhosis precludes patients from sole RT, while in patients with decompensated cirrhosis combined liver and kidney transplantation is the recommended option[87-90].

HBV positive RT candidates should initiate antiviral therapy when HBV DNA > 2000 IU/mL or HBV DNA ≤ 2000 IU/mL two weeks before RT[39,91,92]. Therapy should be instituted as long as immunosuppressive therapy lasts whatever the HBV DNA level is[2] or for at least the first 2 years when immunosuppressive therapy is most intense[91]. HCV positive RT candidates should receive therapy when there is active viral replication (HCV RNA positive) and a biopsy proven chronic hepatitis[93]. Before transplantation, the goal is the accomplishment of HBV DNA clearance to prevent post-transplant virological relapse and liver-related complications[26,80,94]. The disappearance of viral load is a prerequisite for a HBV or HCV positive patient on hemodialysis to be enrolled in the RT list. Therapy with entecavir, tenofovir or lamivudine on adjusted doses for renal function is included in the current guidelines for prophylaxis of HBV positive RT candidates[26,80,91,94]. The NA optimal regimen has not been pro-

posed yet, so prophylaxis may start before or at the time of RT and continue thereafter[91,92]. Entecavir should be the first line option for avoidance of short term resistance and adefovir nephrotoxicity[91], while tenofovir had better be applied in case of lamivudine resistance.

Guidelines for HCV positive RT candidates recommend treatment with interferon α (α-IFN) in adjusted doses for renal function[93,95], although studies in this population[96,97] have shown the advantage of IFN and RBV to provide persistent SVR. The very severe anemia and heart failure caused by the combinative regimen avert clinicians from using it in clinical practice[97]. However, the addition of very low doses RBV (200-400 mg three times weekly) under thorough monitoring (weekly measure of hemoglobin, application of high erythropoietin doses and iron supplementation) could result in HCV RNA clearance and allow more patients to get on to the list[93]. The preliminary results for five RT candidates with CHC treated with the triple regimen of IFN, RBV and DAAs (four received telaprevir and one boceprevir)[98,99] are very promising. Telaprevir and boceprevir has not required dose adjustment to renal function so far. After 12 to 48 wk of triple therapy, viral load disappeared in 4/5 of patients, while moderate, almost expected side-effects were noted. These were dysgeusia, diarrhea and anemia, leading to the increase of the doses of erythropoietin and the modification of RBV doses.

HBV positive and HCV positive renal transplant recipients

The high doses of immunosuppressants (steroids and anti-CD3 antibody) required to avoid graft rejection post-transplant may be responsible for rapidly progressive liver disease and fibrosing cholestatic hepatitis[100,101]. Initially, HBV positive RT recipients should be under close surveillance and continue the same treatment started before RT. Entecavir is again the therapy of choice. It has been tried in naïve, lamivudine or adefovir resistant RT recipients for 33 mo[102-106], providing excellent results regarding HBV DNA reduction, without aggravation of creatinine clearance, microalbuminuria or allograft rejection. Discontinuation of applied NA is desirable in cases of fibrosing cholestatic hepatitis and resistance, which may occur as hepatic flare and rarely as hepatocellular carcinoma (HCC) and fatal liver decompensation[105,107]. Tenofovir (245 mg daily) adapted to creatinine clearance could be a safe alternative subsequent to resistance[108] on the condition that tubular injury is of great concern. If renal allograft dysfunction is in progress and the HBV positive RT recipient presents with a low viral load, the inception of telbivudine could potentially lead to renal function recovery[20,109-111].

Therapy of HCV after RT should only be considered in RT recipients with fibrosing cholestatic hepatitis or de novo glomerulonephritis[93,112]. α-IFN alone or α-IFN plus RBV post-transplantation are contraindicated because a high percent of irreversible and steroid resistant acute allograft rejection and low efficacy levels have been recorded[113,114]. In our clinical setting, HBV positive and HCV positive RT recipients are screened for liver enzymes, bilirubin and prothrombin time at each visit. Ultrasonography with triplex of splenoportal axis and/or transient elastography is monitored annually. HBV DNA and HCV RNA as well as a-fetoprotein are tested every year. In patients with cirrhosis, endoscopy for detection or monitoring of varices is performed every 1-2 years. All HBV positive and HCV positive RT recipients should avoid alcohol and hepatotoxic drugs. In the case of fever, effective antibiotics are started immediately. Liver biopsy and modulation of antivirals is considered in patients with abnormal liver function and/or increased viral load.

SELECTION OF PATIENTS WHO NEED CLOSE MONITORING

High HBV viral load pre and peritransplant predispose to closer patient surveillance and stronger prophylactic antiviral regimens. This group of patients is more likely to progress to decompensation and to HCC[115]. It is preferable that they be treated with entecavir or tenofovir and often be monitored for signs of decompensation. In a case of severe decompensation, patients receiving antivirals are at higher risk of lactic acidosis so physicians should be vigilant. LT candidates with HCV compensated cirrhosis are more vulnerable to IFN-related hematological toxicities since the splenomegaly caused by portal hypertension magnifies the risk for cytopenias[116]. Therefore, IFN dose modification and close regular monitoring is recommended. Furthermore, therapy in patients with Child-Turcotte-Pugh (CTP) score C (or MELD score > 18) is challenging and should be carried out in dedicated and experienced centers because IFN may cause sepsis and is associated with a low sustained virological response (SVR) rate[61]. Careful monitoring should also be applied to patients with CTP score B. They need individualization of treatment decisions regarding non-genotype 1, high viral load, treatment naïve or relapse from previous antiviral therapies[13].

RT recipients with severe liver disease should receive non aggressive immunosuppressive protocols (cannot always be applied in immunologically high risk patients) and a selected immunosuppressive regimen with minimal or preferably no steroid use. All antivirals should be modified continuously regarding current renal function. Additionally, HBsAg-positive RT recipients with cirrhosis are at risk for hepatic decompensation after isolated RT and therefore they require simultaneous liver and kidney transplantation[86]. In conclusion, we should be on the alert for all HCV positive RT recipients which means screening them regularly for HCC[117], emergence of diabetes, renal thrombotic microangiopathy[118], glomerulonephritis[119,120], renal graft nephropathy[118] and sepsis[121].

SPECIAL TREATMENT CONSIDERATIONS

Generally, entecavir and tenofovir are the preferable an-

Table 3 Prophylactic schemes against hepatitis B virus recurrence after liver and renal transplantation when grafts are from hepatitis B virus positive donors

	Donor	Recipient	Prophylaxis
Liver transplantation	Anti-HBc positive	HBsAg positive	HBIG plus NA
		HBsAg negative	No prophylaxis
		Anti-HBc positive	
		Anti-HBs positive	
		Anti-HBc negative	Long term lamivudine
		Anti-HBs negative	
Kidney transplantation	HBsAg positive	Anti-HBc positive	HBIG plus NA
	Anti-HBc positive	HBsAg negative	No prophylaxis
			Treatment when HBV DNA increases
	HBsAg positive	HBsAg positive	HBIG plus lamivudine
	(HBV DNA negative)	HBsAg negative	Long term lamivudine
		(HBV DNA) negative	

HBV: Hepatitis B virus; NA: Nucleos(t)ide analog; HBIG: Hepatitis virus immunoglobulin.

tiviral treatment for post LT or RT recipients due to its high resistance barrier, the favorable safety profile and the regression of fibrosis with long term application[34,122]. However, entecavir is not advisable for patients with lamivudine resistance and it should be applied carefully in patients with decompensated cirrhosis with MELD score ≥ 22 because cases of lactic acidosis have been recorded with its administration[115]. Tenofovir is the only effective choice practically for patients with resistance to lamivudine or any nucleoside analogue[39] but there are concerns about creatinine clearance decline and acute tubular nephropathy during long term therapy, especially in group of patients with high risk for renal dysfunction over time such as RT and LT recipients[122]. Telbivudine is the only NA with a renal protective effect but its use in the RT and LT setting has still not been tested. Other NA limitations are the indefinite course and the relatively high cost of therapy which complicate patient compliance. Similar concerns exist about the cost and efficacy of HBIG continuation in HBV positive LT recipients. The duration of HBIG prophylaxis in HBV positive LT recipients is controversial and challenging and should be discussed on a case-by-case basis. The access to optimal therapy for HCV positive RT and LT recipients is limited by the low tolerance or contraindication of IFN-based regimens and the lack of experience of the efficacy and safety of first generation DAAs. Newer DAAs (such as sofosbuvir) need further evaluation in this setting.

HBV AND HCV POSITIVE DONORS

Many studies have shown that liver grafts from anti-HBc positive donors can be used safely in: (1) HBsAg negative but anti-HBc/antiHBs positive recipients without antiviral prophylaxis[123]; (2) in HBsAg positive recipients on the condition that dual therapy HBIG and NAs is applied[123]; and (3) anti-HBc and/or antiHBs negative recipients when receiving long term prophylaxis with lamivudine, dual therapy or no prophylaxis[123,124]. Heterogeneity of data exists regarding the use of liver grafts from HBsAg positive donors[125-128].

Similarly, renal grafts from anti-HBc positive donors can be used in HBsAg negative recipients without prophylaxis[129]. It is acceptable practice for renal grafts from HBsAg positive donors to be used in HBsAg positive or HBsAg negative recipients with subsequent long term NA administration with or without HBIG[130,131]. In all cases, serial HBV DNA measurements regardless of normal liver biochemistry are required. In particular, in LT or RT recipients who are not on any antiviral prophylaxis, an increase in viral load indicates NA initiation. On the other hand, when immunosuppression is reduced and complete viral clearance has been achieved, NA interruption could be considered[103] (Table 3).

LT candidates with HCV-related cirrhosis can undergo LT from HCV positive donors if they are not HCV RNA positive because early hepatitis C recurrence may occur[132,133]. Renal grafts from HCV positive donors are acceptable only for HCV positive RT candidates[93]. In this setting, the survival of HCV positive RT recipients increases compared to their survival rates if they remain on hemodialysis[134,135]. Renal grafts from HCV positive donors should not be distributed to HCV negative recipients because many fatal liver complications have been recorded[136,137].

CONCLUSION

Current knowledge on the management of patients with HBV offers effective and safe options for liver or renal transplantation. Individualization and determination of less nephrotoxic and finite duration antiviral treatment will enhance the quality of their treatment and prognosis. Various types of vaccinations (S and pre-S antigen vaccines, DNA vaccination, T cell vaccines) and some monoclonal antibodies (exbivirumab and libivirumab) are promising for preventing HBV recurrence and are being evaluated in clinical trials. Subcutaneous HBIG and hyperimmune anti-HBs plasma may prove to be alternative options with a lower cost and the same efficacy levels. The optimal antiviral therapy has not been established yet for LT or RT candidates with CHC. The DAAs in-

stitution marks a bright new era for treatment approach of these patients. Control randomized studies involving DAAs use in patients with decompensated cirrhosis and in RT candidates and recipients are in high need. Moreover, the optimal use and benefits of granulocyte growth factors and erythropoietin in improving SVR rates should be further researched and become established practice.

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MINIREVIEWS

NS3 protease inhibitors for treatment of chronic hepatitis C: Efficacy and safety

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Abstract

A new treatment paradigm for hepatitis C is that the treatment must include an existing direct-acting antiviral agent, namely, a protease inhibitor (PI) combined with PEGylated interferon-α and ribavirin. The currently marketed PIs and PIs in clinical trials have different mechanisms of action. The development of new PIs aims for an improved safety profile and higher effectiveness. This article reviews NS3/4A protease inhibitors, focusing on major criteria such as their effectiveness and safety. Specific attention is paid to dosing regimens and adverse event profiles of PIs administered in clinical settings.

Key words: Protease inhibitor; PEGylated interferon-α; Ribavirin; Antiviral treatment; Adverse event; Response-guided therapy; Hepatitis C virus

Core tip: This article reviews NS3/4A protease inhibi-

tors, focusing on major criteria such as effectiveness and safety. Specific attention is paid to dosing regimens and adverse event profiles of protease inhibitor administered in clinical settings.

INTRODUCTION

Since 2011, increased attention has been given to "direct" antiviral agents for chronic hepatitis C (HCV). Combined treatment with PEGylated interferon-α (PEG-IFNα) and ribavirin cannot be considered a standard treatment for type 1 HCV anymore. A new treatment paradigm is that the treatment must include an existing direct-acting antiviral agent (DAA), namely, protease inhibitor (PI) combined with PEG-IFNα and ribavirin.

The currently marketed PIs and PIs in clinical trials (CTs) have different mechanisms of action. The development of new PIs aims for an improved safety profile and higher effectiveness[1]. An ideal combination is an interferon-free therapy with oral once-daily agents that are highly effective and well tolerated, do not interact with the majority of well-known therapeutics, and can be used to treat concomitant disorders. The recent evolution of DAA has included a considerable improvement in their effectiveness since 2011, and in most cases, antiviral treatment (AVT) duration has decreased.

This article reviews NS3/4A protease inhibitors, focusing on major criteria such as effectiveness and safety. Specific attention is paid to the dosing regimens and adverse event (AE) profiles with PIs administered in clinical settings.

Table 1 Direct-acting antivirals approved or at the computed tomography stage (phase II or III)

NS3/4A protease inhibitors	Polymerase inhibitors		Inhibitors	
	Nucleotides/nucleosides	Non-nucleoside	NS5A	Cyclophilin
Generation I:	PSI-7977 (Pharmasset)[1]	Filibuvir (Pfizer)[1]	Daclatasvir (Bristol-Myers	Alisporivir (Novartis)[1]
Boceprevir (Merck)[1]	PSI-938 (Pharmasset)[1]	VX-222 (Vertex)[1]	Squibb)	SCY-635 (Scynexis)[1]
Telaprevir (Vertex)[1]	Mericitabine (Roche/	Tegobuvir (Gilead)[1]	GS-5885 (Gilead)[1]	
	Genentech)[1]	ANA-598 (Anadys)[1]		
	IDX-184 (Idenix)	ABT-072 (Abbott)[1]		
		ABT-333 (Abbott)[1]		
Generation II:				
Simeprevir (Tibotec)				
BI 201335 (Boehringer Ingelheim)				
Danoprevir (Roche/Genentech),				
studied with Ritonavir;				
Vaniprevir (Merck)[2]				
BMS-650032 (Bristol-Myers Squibb)[2]				
GS-9451 (Gilead)[1]				
GS-9256 (Gilead)[2]				
ACH-1625 (Achillion)[1]				
ABT-450 (Abbott)				
MK-5172 (Merck)[1]				

[1]Approved by the United States Food and Drug Administration; phase II CT; phase III CT; [2]Not studied. From Short Guide to Hepatitis C 2013, Mauss S, Berg T, Rockstroh J, Sarrazin C, Wedemeyer H.

Table 2 General protease inhibitor description with computed tomography results since 2011

PI	Genotype	PI treatment duration (wk)	Treatment duration (wk)	Treatment regimen	PEG-IFNα	Publication date
Telaprevir	1a/1b/1c/unknown	12/8	20/24/44/48	750 mg TID	(+)	2011
Boceprevir	1a/1b/unknown	24/32/44	28/36/48	800 mg TID	(+)	2011
Daclatasvir	1a/1b	24	24	60 mg/d	(+/-)	2012
Asunaprevir	1a/1b	24	24	600 mg BID	(+/-)	2012
ABT-450	1a/1b	12	12	250/150 mg/d	(-)	2013

PI: Protease inhibitor; PEG-IFNα: PEGylated interferon-α.

GENERAL STATEMENTS

Since 2011, we have seen an increase in the value placed on "direct" antiviral agents for HCV. Most of these agents are at various CT stages, with some already being integrated in routine clinical practice as a treatment standard for type 1 HCV patients (Table 1). In 2011, FDA and EMA approved the first DAA-telaprevir and boceprevir - for HCV treatment in patients infected with type 1 HCV. Randomized CTs have shown that triple therapy is not only significantly more effective for type 1 HCV patients but that it is also the only alternative for patients with previous AVT failure. One should note that in Russia, telaprevir was approved in December 2012 and boceprevir in May 2013.

NS3/4A serine protease inhibitors are divided into two classes. The first generation includes the well-studied telaprevir and boceprevir. By the time their phase III CT was completed, these agents were already acknowledged as new AVT standards for type 1 HCV patients.

NS3/4A protease has a crucial role in the replication cycle of hepatitis C virus. It cleaves polyprotein in four sequential active sites, forming the N-terminal proteins NS4A, NS4B, NS5A and NS5B. Regarding its chemical properties, this enzyme is related to the serine protease group. For instance, it can cleave and inactivate the host proteins Trif and Cardif. Both of these proteins are important in the responses to interferon (IFN) mediated by the receptors TLR3 and RIG-I, respectively[2,3]. Additionally, NS3 is not only a protease but also a component of the replication complex for viral RNA, acting as an RNA-helicase and nucleotide triphosphatase (NTPase). Due to its impressive set of functions, NS3 protease is an attractive target for HCV therapy. The HCV RNA replication cycle and targets for direct-acting antivirals have been thoroughly described in publications by Moradpour and Pawlotsky[4,5]. Clinical trials have studied several promising molecules that inhibit HCV protease.

Table 2 reviews published CT results for ultra-novel NS3/4a PIs, including their efficacy and safety parameters[6].

FIRST-GENERATION NS3/4A PROTEASE INHIBITORS

Telaprevir efficacy

Telaprevir efficacy was studied in phase II and III CTs (Table 3)[6-8].

Table 3 Telaprevir: clinical parameters

RCT	Dose frequency	Duration	SVR	Possible AE
Prove 1	Each 8 h, 6 t/d	24 wk: 12 wk of triple therapy, 12 wk of conventional treatment	61%	Rash, anemia, nausea, diarrhea
Advance	Every 8 h, 6 t/d	24-48 wk: 8-12 wk of viral response-based treatment followed by conventional treatment	69%-75%	Rash, anemia, nausea, diarrhea
Illuminate	Every 8 h, 6 t/d	24-48 wk: 12 wks of viral response-based treatment: 12 wk of triple therapy followed by conventional treatment	64%-92%	Rash, anemia, nausea, diarrhea
Optimize	Every 12 h, 6 t/d	24-48 wk: 12 wk of viral response-based treatment: 12 wk of triple therapy followed by conventional treatment for 12 to 36 wk	58%-81% (depending on fibrosis stage)	Rash, anemia, nausea

AE: Adverse event.

Table 4 Adverse events under telaprevir-based therapy

Agent RCT	Telaprevir				
	Advance		Realize		Illuminate
	PR	T8/12PR	PR48	(lead-in) T12PR48	T12PR24/48
Serious AE	7%	9%	5%	12%	9%
Discontinued AVT due to AE	7%	10%	3%	15%-11%	18%
Anorectal symptoms	4%	8%-13%	7%	15%-12%	-
Taste disturbances	-	-	6%	12%	-
Anemia	19%	39%-37%	15%	30%-36%	39%
Severe neutropenia	19%	17%-14%	11%	14%-13%	-
Rash	24%	35%-37%	19%	37%-36%	37%
Fatigue	57%	58%-57%	40%	55%-50%	68%
Pruritus	36%	45%-50%	27%	52%-50%	51%
Nausea	31%	40%-43%	23%	35%-33%	47%
Diarrhea	22%	32%-28%	14%	25%-26%	30%

AE: Adverse event.; AVT: Antiviral treatment.

Treatment mode

For telaprevir-based AVT, the following regimens are used: Treatment-naive patients and relapsers: Telaprevir is started from treatment day 1 and is always combined with conventional treatment of PEG-IFN/RBV for 12 wk. If no viremia is present (HCV RNA-negative) at 4 and 12 wk, treatment duration is 24 wk. If viral load is detected (HCV RNA-positive) at 4 or 12 wk, treatment duration is 48 wk. Null responders or partial responders as well as liver cirrhosis patients: The only option for triple therapy is telaprevir for 12 wk, with a total AVT duration of 48 wk. Telaprevir-based regimens have clear algorithms for AVT early discontinuation[9]. Triple therapy must be totally canceled in the following cases: HCV RNA above 1000 IU/mL at 4 and 12 wk on triple therapy; HCV RNA-positive at treatment week 24; Viral breakthrough and/or viral load increase.

The above rules are unambiguous and must be strictly followed because they are evidence-based results that were developed after multicenter randomized CTs. If HCV RNA is present in the titers above, it indicates that the AVT is ineffective when continued treatment has no clinical or cost-effective rationale. Moreover, ongoing treatment might result in resistant strain development, as indicated by phase Ⅱ and Ⅲ CTs showing relapses and no viral response.

Safety

The AE control algorithm is important for telaprevir-based treatment. The safety profile of triple therapy has a higher AE number *vs* conventional treatment, which in the future, may be a limiting factor for first-generation PI use (Table 4).

Triple-therapy AEs have been reviewed in CT results and other recent publications[10]. Therefore, it seems necessary to dwell on some of them because developing AEs might require changes in patient management (PI or AVT discontinuation) or may be difficult to control in clinical settings.

Telaprevir has the following common AEs: rash, anemia and anorectal signs (as shown by the ADVANCED and REALIZE trials).

Telaprevir-based triple therapy increases the anemia rate by 15% to 21% *vs* control. The severe anemia rate is comparable among study arms and results in discontinued treatment in 2%-4% of cases (Table 5).

Anemia development in the compared arms is not a negative prognostic criterion for SVR. Currently, the main method for anemia control is ribavirin dose adjustment. Some experts consider that an Hb below 7.5 g/dL implies complete triple therapy discontinuation. However, the CUPIC study showed that AVT can be continued if erythropoietin and blood transfusions are used[11,12].

Rash is considered a specific AE for telaprevir-based therapy and results in 5%-7% of treatment discontinuation cases. In 50% of cases, rash appears within the first 4 wk of treatment, but rash can develop during the whole course of treatment. In some rare cases, skin signs can be classified as serious AEs.

The rash treatment algorithm depends on its severity (evaluated on the body surface involved). Mild to moderate rash is an indication for antihistamine agents, local steroid ointments and avoiding sunlight. It does not require stopping triple therapy. For severe rash, it is recommended to stop telaprevir, and conventional treatment can be continued with the provision of effective treatment with steroids (locally) and antihistamines. In case of progression and severe skin signs, treatment must be canceled.

Table 5 Boceprevir: clinical parameters

RCT	Dose frequency	Duration	SVR	Possible AE
SPRINT 1	12 pills for 3 intakes	28-wk triple therapy *vs* 4-wk lead-in phase	54%-56%	Metal taste, anemia
		48-wk triple therapy *vs* 4-wk lead-in phase	67%-75%	
SPRINT 2	12 pills for 3 intakes	28-48 wk: "viral response-based treatment"; "lead-in period"; if HCV RNA (-) by week 8 and 24, to stop at week 28; if HCV RNA (+), 20 wk of double therapy	67% And 44% were given abridged AVT	Taste disturbances, anemia, neutropenia

AE: Adverse event; AVT: Antiviral treatment; HCV: Hepatitis C virus.

Figure 1 Hepatitis C virus RNA drop after lead-in phase (treatment week 4).

Figure 2 SVR rate after the lead-in period in patients with F3/4 fibrosis. PR: Conventional therapy (PEGylated interferon-α and ribavirin). BOC RGT: Boceprevir-based, viral response-dependent triple therapy; BOC/PR48: Boceprevir-based triple therapy for 48 wk; the results are from the SPRINT-2 and RESPOND-2 studies.

BOCEPREVIR

Efficacy

Boceprevir's efficacy was studied in phase Ⅱ/Ⅲ CTs (Table 5)[1,6]. Considering a new term, *i.e.*, the lead-in period or lead-in phase, several issues should be discussed. Triple therapy development has provided data on new sensitive response predictors. Assessing the viral load decrease after a 4-wk lead-in can allow for an accurate assessment of a patient's chances to reach SVR (Figure 1): A lead-in period enables researchers to assess conventional treatment tolerance and prognosis if PEG-IFN/RBV is safe to use. RCT results (RESPOND-2 and PROVE-2) show that a lead-in period lowers the viral load before the onset of triple therapy and delineates a patient group that should receive a shorter AVT duration. These diminish the probability of mutant, PI-resistant HCV strain development. If the viral load drops by more than 2 log10, this indicates high patient sensitivity to IFNα and ribavirin, which is a rationale to continue AVT as a standard treatment. However, it seems that the main objective of the lead-in period is to discern the patient groups in which conventional AVT appears to be less effective in a prognostic sense, and triple therapy makes it possible to avoid unjustified treatment costs and a non-mandatory pharmaceutical load when double therapy is continued (Figure 2).

Treatment mode

An important issue is strict compliance with discontinuation rules for triple therapy. For instance, ineffective boceprevir-based triple AVT should stopped in time to prevent the development of boceprevir-resistant HCV

strains. The entire triple therapy should be canceled in the following cases (algorithm for early treatment discontinuation): (1) if the HCV RNA is above IU/mL at week 12 of AVT (triple therapy week 8); (2) if there is no aviremia at week 24 of AVT (triple therapy week 20); and (3) in case of viral breakthrough and/or viral load increase by 1 log10.

Safety

The SPRINT-2 and RESPOND-2 data show that boceprevir use increases the rate of taste disturbances, anemia and neutropenia[6,13] (Table 6).

Second-generation NS3/4A protease inhibitors

The development of second-generation NS3/4A protease inhibitors resulted in some hopes of improving treatment outcomes in type 1 HCV patients. This group of agents has some advantages compared to the 1st-generation NS3/4A protease inhibitors (telaprevir and boceprevir): first, the dosing mode (once a day) and second, a better tolerance profile (fewer adverse events). However, the fact that both groups of agents have both a common viral genotype as their target and similar resistance profiles restrain us from considering 2nd-generation NS3/4A protease inhibitors to be a new class of HCV protease inhibitors. Nevertheless, modern publications still use this term for a range of new therapeutic agents with improved pharmacokinetics. It is possible that after

Agent	Boceprevir			
RCT	SPRINT-2		RESPOND-2	
	PR48	PR4/PRB24/44	PR48	PR4/PRB32/44
Serious AE	9%	11%-12%	5%	10%-14%
Discontinued AVT due to AE	16%	12%-16%	2%	8%-12%
Anorectal symptoms	-	-	-	-
Taste disturbances	18%	37%-43%	11%	43%-45%
Anemia	29%	49%	20%	43%-46%
Severe neutropenia	14%	24%-25%	9%	19%-20%
Rash	23%	25%-24%	5%	17%-14%
Fatigue	60%	53%-57%	50%	53.7%-57.1%
Pruritus	27%	24%-26%	17.50%	18.5%-19.3%
Nausea	42%	48%-43%	37.50%	43.8%-39.1%
Diarrhea	22%	22%-27%	15%	22.8%-23%

Table 6 Adverse events under triple therapy

AE: Adverse event; AVT: Antiviral treatment.

some CTs are completed, 2nd-generation NS3/4A protease inhibitors will replace the 1st-generation agents when combined with PEG-IFN/RBV, thereby becoming the 1st generation of DAA in regimens of so-called interferon-free HCV treatment.

Simeprevir

Simeprevir (TMC435; Tibotec, Beerse, Belgium; Medivir Pharmaceuticals, Stockholm, Sweden; Janssen, Beerse, Belgium) is one of the 2nd-generation NS3/4A protease inhibitors. Simeprevir has passed phase I to III trials in patients with 1a and 1b HCV genotypes.

Efficacy

Phase I and II trials demonstrated potential antiviral activity for TMC435, as well as its efficacy and tolerability. TMC435's pharmacokinetic properties enable its use in once-a-day dosing[14].

To study TMC435's efficacy and safety, a phase II b trial, PILLAR [Protease Inhibitor TMC435 trial assessing the optimaL dose and duration as once daiLy Antiviral Regimen] (TMC435-C205; NCT00882908), was organized. Conducted in 13 countries in Europe, North America and Australia, it enrolled 368 naive patients with genotype 1 treated with simeprevir combined with PEG-IFN/RBV for 24 or 48 wk. Two doses of simeprevir (75 mg *vs* 150 mg) and treatment durations (12 wk *vs* 24 wk) were compared. The final analysis of the PILLAR study showed that TMC435 given in combination with PEG-IFN/RBV to naive patients with genotype 1 HCV resulted in a SVR rate that significantly exceeded that observed in patients treated with the placebo + PEG-IFN/RBV combination (Figure 3).

In 2 patient arms treated with TMC435 at 75 mg/d, the patient percentage reaching SVR in varied from 75% (12 wk) to 82% (24 wk); with TMC435 at 150 mg/d, this percentage varied from 81% (12 wk) to 86% (24 wk). In the comparison arm, the percentage of placebo-treated patients reaching SVR in 24 wk or less amounted to 65%[15].

A new publication[16] analyzing the PILLAR study

Figure 3 Portion of patients treated either with TMC435 at 75 mg/d or 150 mg/d or with placebo, combined with PEGylated interferon/ribavirin, who reached SVR24 (%) in the PILLAR study[15].

results indicated that SVR determined in 24 wk after planned treatment completion (SVR24) varied in the range of 74.7%-86.1% for all simeprevir arms *vs* 64.9% in the control. All arms treated with simeprevir for 12 or 24 wk showed a significant difference in SVR24 parameters, excluding the arm given 75 mg/d for 24 wk. Rapid virologic response (HCV RNA < 25 IU/mL, undetermined level at treatment week 4) was reached in the SMV-treated arms in 68.0%-75.6% of cases, compared to 5.2% in the placebo-treated controls. The criteria for a shortened treatment course (RGT criteria) were met in 79.2%-86.1% of the SMV-treated patients completing treatment in 24 wk. SVR24 was reached in 85.2%-95.6% of patients in these groups[16].

An international, randomized, double-blinded, controlled phase II b study, ASPIRE (TMC435-C206; NCT00980330), aimed to evaluate the efficacy, tolerability, safety and pharmacokinetics of TMC435 given in combination with PEG-IFN/RBV[17]. ASPIRE enrolled 462 treatment-experienced genotype 1 HCV patients. This arm included partial responders, prior relapsers, and patients with significant fibrosis or cirrhosis (Metavir, F4 stage). Patients were randomized to receive 100 or 150 mg of simeprevir OD or placebo for 12, 24 or 48 wk. The study arms with simeprevir treatment for 12 or 24 wk later continued treatment with PEG-IFN/RBV (control) only up to 48 wk. The SVR rate was significantly higher in all simeprevir-treated arms compared to those treated with PEG-IFN/RBV only. The best results were reached in patients treated with simeprevir 150 mg/d. For instance, SVR in prior relapsers reached 85% *vs* 37% in the control, 75% and 19% in the two subgroups of partial responders, and 51% and 19% in the two subgroups of non-responders[18]. It is important to note that a high SVR24 (31%) was found with simeprevir-based therapy in subgroups of liver cirrhosis patients and those with a previous null response, *i.e.,* those who traditionally are considered difficult to treat.

Viral breakthrough (in 42 of 43 patients) or infection relapse (in 34 of 36 patients) was associated with viral resistance development. Viral breakthrough has been noted in most studies when using protease inhibitors in

combination with conventional treatment[18]. Genotype 1a patients more often have mutation R155 as the only mutation or with other mutations, while genotype 1b patients have mutation D168V[18].

At the moment, 3 Phase Ⅲ clinical studies to study simeprevir's efficacy are known: (1) in treatment-naive HCV patients: QUEST-1 and QUEST-2; (2) in relapsers after previous PEG-IFN/RBV treatment: PROMISE; and (3) in null-responders: ATTAIN.

QUEST-1 enrolled 394 genotype 1 HCV patients with F0-F4 fibrosis (METAVIR scale) stratified by HCV subtype and genotype *IL28B*[19]. Patients were randomized to a simeprevir dose of 150 mg/d or placebo combined with PEG-IFN/RBV for 12 wk followed by PEG-IFN/RBV monotherapy. A treatment duration of 24 or 48 wk in the simeprevir arm and the placebo arm depended on treatment response at wk 4 and 12. If virus was undetected in the blood (HCV RNA < 25 IU/mL) at weeks 4 and 12, the patient met the short treatment criteria (RGT-criteria), and the treatment was finished at week 24. Treatment for 48 wk was recommended for patients who were treated with placebo instead of simeprevir. The majority of simeprevir-treated patients were compliant with the RGT criteria (85%) and completed treatment at week 24. The rapid response rate (RVR) reached 80% in the patients treated with simeprevir in combination with PEG-IFN/RBV and 12% in the patients treated with placebo and PEG-IFN/RBV. Simeprevir combined with PEG-IFN/RBV resulted in HCV elimination in more patients compared to the combination of placebo and PEG-IFN/RBV (80% *vs* 50%, P < 0.001). The relapse rate in the simeprevir/PEG-IFN/RBV arm was less than that in the placebo/PEG-IFN/RBV arm (9% *vs* 21%), as was the percentage of treatment failures (9% *vs* 34%). The QUEST-1 study showed that simeprevir 150 mg/d OD given along with PEG-IFN/RBV provided a high SVR12 rate, making it possible to decrease the treatment duration to 24 wk in a majority of patients (85%).

QUEST-2, a randomized, double-blinded, placebo-controlled study (NCT01290679), enrolled approximately 400 treatment-naive patients with genotype 1 HCV[20]. Patients were stratified according to genotype 1 subtype and host genotype (*IL28B*). They were given simeprevir (150 mg OD) combined with PEG-IFN/RBV (both of PEG-IFN type) or placebo combined with PEG-IFN for 12 wk followed by a PEG-IFN/RBV regimen. A treatment duration of 24 or 48 wk in both patient arms depended on the treatment response at weeks 4 and 12. If virus was undetected in the blood (HCV RNA < 25 IU/mL) at weeks 4 and 12, the patient met the short treatment criteria (RGT-criteria) and the treatment was finished at week 24. Treatment for 48 wk was recommended for patients who were treated with placebo instead of simeprevir. A majority of simeprevir-treated patients complied with the RGT criteria (91%) and completed treatment at 24 wk. The rapid virologic rate (RVR) was 79% in simeprevir/PEG-IFN/RBV patients and 13% in patients treated with placebo/PEG-IFN/RBV. Simeprevir combined with PEG-IFN/RBV provided HCV elimination in more patients than did the combination of placebo with PEG-IFN/RBV (SVR12 rate: 81% *vs* 50%, P < 0.001). The relapse rate in the simeprevir/PEG-IFN/RBV arm was lower than that in the placebo/PEG-IFN/RBV arm (13% *vs* 24%), as was the rate of treatment failure (7% *vs* 32%). The QUEST-2 study showed that simeprevir 150 mg/d OD given along with PEG-IFN/RBV provides a high SVR12 rate, making it possible to decrease the treatment duration to 24 wk in a majority of patients (91%).

The objective of the phase Ⅲ trial PROMISE (TMC435-HPC3007) was to study the efficacy, safety and tolerability of simeprevir combined with PEG-IFN/RBV in patients infected with genotype 1 HCV and treatment failure. The study enrolled 393 prior relapsers after treatment. Approximately 40% of patients had the 1a subtype of the HCV genotype, approximately 75% of them had the unfavorable genotype IL28B, 15% had considerable liver fibrosis (stage F3), and 15% had diagnosed liver cirrhosis (stage F4). Patients were given 150 mg of simeprevir combined with PEG-IFN/RBV for 12 wk, followed by PEG-IFN/RBV only for another 12 wk. At this point, the patients either stopped treatment based on the RGT criteria (no virus in blood at treatment week 4 and 12) or continued PEG-IFN/RBV treatment up to week 48. In the control arm, placebo combined with PEG-IFN/RBV was given for 12 wk; up to week 48, they were given basic treatment, *i.e.,* PEG-IFN/RBV. A total of 77% of the simeprevir-treated patients and 3% of the control group developed rapid treatment response in 4 wk (RVR). At the end of treatment, the responses were very high: 97% in the simeprevir group and 72% in the control. The majority of patients (93%) complied with the RGT criteria (treatment termination at week 24); in this group, 83% of patients reached SVR during the following 12 wk of basic treatment (SVR12). Among the remaining 7% of simeprevir-treated patients who did not comply with the RGT criteria and continued treatment to week 48, SVR12 was reached in only 32%. Among patients with HCV subtype 1a, SVR12 was reached in 70% of the simeprevir-treated group and in 28% of the placebo group; for subtype 1b, these values were 86% and 43%, respectively. The IL28B CC genotype was associated with a better response to simeprevir-based triple therapy *vs* control (SVR12 was 89% *vs* 53%). SVR12 for the CT genotype was 79% *vs* 34%. SVR12 for the TT genotype was 65% *vs* 19%. Regardless of fibrosis severity, the SVR rate in the simeprevir-treated arms was higher than in control. For instance, with fibrosis stage F0-F2 (absent to moderate), the SVR rate was 82%; with significant fibrosis, it was 73%; and with liver cirrhosis, it was 74%. In the control group, SVR reached 41%, 20% and 26%, respectively. Ineffective treatment was noted in 3% of simeprevir-treated patients and in 27% of control patients. Relapse after treatment completion was found in 19% and 48%, respectively. Thus, in case of relapse after conventional therapy with PEG-IFN/RBV in genotype 1 HCV patients, treatment with simeprevir and PEG-IFN/

RBV provided a high cure rate: 79% of patients reached SVR12.

The phase III trial ATTAIN (NCT01485991) is studying the efficacy of simeprevir plus PEG-IFN/RBV and telaprevir plus PEG-IFN/RBV in patients with a failed attempt at HCV eradication after conventional therapy (PEG-IFN/RBV). ATTAIN is projected to be finished in 2014.

Safety

The profile of adverse events recorded in the PILLAR study[15,16] was similar between the group treated with simeprevir and the group with conventional treatment. For instance, comparing patients treated with simeprevir/PEG-IFN/RBV *vs* placebo/PEG-IFN/RBV, an adverse event rate > 10% was recorded for fatigue (42.4% and 48.1%, respectively), flu-like syndrome (31.7% and 37.7%, respectively), itching (31.1% and 45.5%, respectively), headache (46.0% and 51.9%, respectively), nausea (27.8% and 27.3%, respectively), rash (21.0% and 23.4%, respectively), anemia (20.4% and 20.8%, respectively), neutropenia (24.3% and 20.8%, respectively).

The majority of adverse events recorded in patients treated with simeprevir/PEG-IFN/RBV in the ASPIRE study were also observed in patients treated with disease-modifying therapy PEG-IFN/RBV (fatigue, flu-like syndrome, itching, headache, nausea) and were similar to the patient control group[17,18]. Adverse events requiring the discontinuation of at least one of the therapeutics in the study were reported in 4%-10.4% of the patients treated with simeprevir/PEG-IFN/RBV combination compared to 13% of the control group. Serious adverse events (SAEs) were detected at similar rates in patients treated with the combination of simeprevir and disease-modifying therapy (3.8%-11.5%) and in the patients treated with placebo combined with disease-modifying therapy (13%). Anemia developed in 19.0%-22.1% of patients treated with simeprevir + PEG-IFN/RBV and in 20.8% of patients treated with placebo combined with PEG-IFN/RBV. In both arms, anemia did not result in discontinued treatment. Skin rash of any type was reported in 23.4%-30.8% of patients treated with simeprevir + PEG-IFN/RBV and in 20.8% of in patients treated with placebo + PEG-IFN/RBV. Rash resulting in discontinued treatment was noted in only 3 cases (2 patients of the simeprevir + PEG-IFN/RBV arm and 1 patient of the placebo + PEG-IFN/RBV arm). Insignificant, isolated and reversible increase of both bilirubin types (direct and indirect) in blood serum was found in patients treated with simeprevir + PEG-IFN/RBV. Because elevated plasma activity of alanine aminotransferase (ALT) and alkaline phosphatase (ALP) was not associated with the simultaneous elevation of bilirubin, the elevated serum ALT in the majority of patients was interpreted as a developed biochemical response during the treatment.

In the PILLAR and ASPIRE studies, fatigue as a treatment-related adverse event was reported in 63%-65% of treatment-naive and 97% of treated patients[21,22]. In both trials, fatigue severity according to the Fatigue Se-verity Scale increased with treatment duration. However, fatigue disappeared more quickly in treatment-naive simeprevir-treated patients than it did in patients treated with PEG-IFN/RBV only. Considering the follow-up period of 72 wk after treatment completion, these differences were statistically significant ($P < 0.001$).

These trials also showed lower the quality of life in patients according to the health-related quality of life (HRQoL) scale. Treatment-naive simeprevir-treated patients showed a faster quality of life improvement compared to the group treated with PEG-IFN/RBV only[21,22].

Simeprevir was well tolerated by patients enrolled in the QUEST-1, QUEST-2, and PROMISE studies[19-23]. The total AE incidence was similar in the arms treated with simeprevir + PEG-IFN/RBV and placebo + PEG-IFN/RBV.

Discontinued treatment due to adverse events in both arms was found in 3% of patients[19]. The grade 3-4 adverse event rate was 23% in the simeprevir + PEG-IFN/RBV patient arm and 29% in the PEG-IFN/RBV arm. The most common adverse events in the simeprevir and placebo arms were fatigue (40% and 38%, respectively), headache (31% and 37%, respectively), and itching (21% and 11%, respectively). Simeprevir intake was associated with transient moderately elevated bilirubin that was not associated with elevated aminotransferases or alkaline phosphatase. Rash and photosensitivity were slightly more common in patients who were treated with simeprevir compared to the patients receiving placebo (27% *vs* 20% and 4% *vs* 1%, respectively). During the PROMISE study, a shorter treatment duration resulted in lower fatigue intensity and a faster return to normal activity among patients treated with simeprevir and PEG-IFN/RBV.

Summary

Simeprevir seems to be preferable when choosing HCV treatment compared to telaprevir or boceprevir because it is advantageous with regard to dosing regimen (once a day), tolerance and safety (no rash or anemia). All three phase III trials, QUEST-1, QUEST-2, and PROMISE, showed a high infection cure rate (79%-81% SVR12). Importantly, the addition of simeprevir to PEG-IFN/RBV was associated with a higher SVR rate without a significant increase in fatigue severity or decrease in quality of life. Moreover, the shorter-duration antiviral treatment with simeprevir was associated with a higher SVR rate and a shorter period of worsened quality of life.

FALDAPREVIR

Faldaprevir (BI 201335, Boehringer Ingelheim Pharmaceuticals, Ingelheim, Germany) is a 2nd-generation NS3/4A protease inhibitor with once-a-day dosing.

Efficacy

Faldaprevir's efficacy, tolerance and safety were studied

in genotype 1 HCV patients in multiple phase II and III clinical trials (SILEN-C1, SILEN-C2, SILEN-C3, STARTVerso™1).

SILEN-C1 and SILEN-C2 were phase II randomized clinical studies with the objective of examining BI 201335's efficacy and safety in combination with PEG-IFN/RBV in treatment-naive patients[22] and in treatment failures (partial or non-responders)[24] infected with genotype 1 HCV. Both trials studied the effectiveness of a 3-d lead-in phase with PEG-IFN/RBV. The lead-in period was used when studying boceprevir's efficacy in combination with PEG-IFN/RBV in genotype 1 HCV patients[13,25]. This treatment phase was expected to lower the probability of developing HCV resistance during the treatment.

SILEN-C1 enrolled 429 treatment-naive patients infected with genotype 1 HCV[22]. Four study arms were made: for 24 wk, patients were administered a combination of PEG-IFN/RBV with placebo (control group), faldaprevir 120 mg OD with a 3-d PEG-IFN/RBV lead-in (LI) phase, faldaprevir 240 mg OD with LI, or faldaprevir 240 mg OD without LI followed by PEG-IFN/RBV therapy up to the total 24 wk. If a patient taking faldaprevir 240 mg complied with the RGT criteria (HCV RNA < 25 IU/mL at week 4, undetectable viral load at weeks 8-20), the treatment was discontinued at week 24. The rest of the patients continued PEG-IFN/RBV therapy up to week 48. The SVR rate was 56%, 72%, 72% and 84%, respectively, for the four arms. In total, 92% of patients with the RGT in the faldaprevir 240 mg OD arms reached SVR, irrespective of the PEG-IFN/RBV duration, and 82% of patients with genotype 1a treated with faldaprevir 240 mg OD reached SVR, compared to 47% in the placebo group.

SILEN-C2 enrolled 288 patients without liver cirrhosis who were partial or null-responders to previous HCV treatment[24]. All three arms were treated with faldaprevir combined with PEG-IFN/RBV for 48 wk: 240 mg OD with a 3-d PEG-IFN/RBV lead-in, 240 mg OD without LI, or 240 mg BID with LI. Patients treated with faldaprevir 240 mg OD/LI and reaching HCV RNA < 25 IU/mL by week 4 and undetectable HCV at weeks 8-20 were randomized again. Some of them stopped treatment at week 24, and the others continued PEG-IFN/RBV therapy up to week 48. The SVR rate in prior partial responders was 32%, 50% and 42% in the arms given faldaprevir 240 mg OD with LI, faldaprevir 240 mg OD without LI, and 240 mg BID with LI, respectively. The SVR rate in prior null responders was 21%, 35% and 29% in the respective arms. In patients given faldaprevir 240 mg OD with lead-in (LI) and an AVT duration of 24 wk, the percentage of patients with RGT who reached SVR24 was 43%, while in those continuing treatment up to week 48 it was 72% ($P = 0.035$).

Summarizing the results of these two trials, we should note that due to unclear reasons, the patient arms treated with a 3-d lead-in phase (lead-in arms) experienced a treatment effectiveness that was significantly lower, which

was a basis for refusing such management to limit the chances of developing faldaprevir resistance.

SILEN-C3 enrolled 159 treatment-naive patients with genotype 1 HCV. Patients were randomized into two arms: 12 and 24 wk of treatment with 120 mg of BI 201335 OD combined with PEG-IFN/RBV. Liver cirrhosis was found in approximately 12% of patients at treatment onset; 48% of the first arm patients and 37% of the second arm patients had subtype 1a HCV, and 46% and 53%, respectively, had subtype 1b HCV. Both patient arms had a lead-in period of 3 d of PEG-IFN/RBV prior to starting BI 201335 therapy. Patients with an early rapid virologic response (eRVR), meaning unquantifiable HCV RNA at week 4 and undetectable load at weeks 8-18, stopped therapy. The rest of the patients continued treatment with PEG-IFN/RBV only up to week 48. SVR rates were similar for both AVT types (65% *vs* 73%) and in patients with eRVR (82% *vs* 81%).

The STARTVerso™ (placebo-controlled, double blinded, phase III) trial studied the efficacy and safety of faldaprevir combined with PEG-IFN/RBV in 652 patients previously not treated with AVT and with HCV subtypes 1a and 1b, including patients with compensated liver cirrhosis[26]. The patients were divided into three arms: placebo combined with PEG-IFN/RBV for 24 wk, faldaprevir 120 mg OD combined with PEG-IFN/RBV for 12 or 24 wk (RGT arm), and faldaprevir 240 mg OD combined with PEG-IFN/RBV for 12 wk. Patients complying with the RGT criteria (HCV RNA < 25 IU/mL at week 4 and undetectable load at week 8) and treated with faldaprevir combined with PEG-IFN/RBV stopped treatment at week 24. Patients who did not comply with the RGT criteria who were treated with placebo/PEG-IFN/RBV were given PEG-IFN/RBV treatment only up to week 48. The primary endpoint was reaching SVR within 12 wk of the planned treatment completion (SVR12). Patients given faldaprevir OD in combination with PEG-IFN/RBV (120 and 240 mg) reached SVR12 in 79% and 80% of cases, respectively. Compared with these 2 arms, the placebo/PEG-IFN/RBV arm had a SVR12 rate of 52% ($P < 0.0001$). In the RGT arm, early rapid response was seen in 87% and 89% of faldaprevir-treated patients (120 and 240 mg, respectively). Those patients were fully compliant with the criteria for treatment shortening. Treated for 12 wk with faldaprevir and 24 wk with PEG-IFN/RBV alone, 86% and 89% of this patient arm (120 and 240 mg, respectively) reached SVR12. Thus, the STARTVerso™ trial showed that it is possible for a majority of patients (88%) to shorten the treatment to 24 wk with considerable HCV elimination compared to patients treated with PEG-IFN/RBV only for 48 wk.

Safety

All phase II studies of the SILEN-C series reported that the differences in AE patterns and rates were not significant, including rash, photosensitivity, nausea, vomiting and diarrhea. As in the trials of other PIs, faldaprevir for HCV treatment was associated with transitory elevation

of non-conjugated bilirubin. With a faldaprevir OD regimen, significant AEs developed less frequently compared to a BID regimen.

In the phase III trial STARTVerso™[26], all drugs were discontinued in 4% of patients in the placebo arm, 4% in the faldaprevir 120 mg arm, and 5% in the faldaprevir 240 mg arm. Faldaprevir only was discontinued in 1% of patients in the 120 mg arm and 3% in the 240 mg arm. Serious adverse events developed in in 6%, 7% and 7% of patients in the respective study arms. Grade 3 rash (severe) was reported in < 1% in each of the study arms. The rate of Hb drop within first 24 wk (Hb ≤ 8.5 g/dL) was similar in all arms (2%, 3% and 3%, respectively).

Summary
Faldaprevir OD combined with PEG-IFN/RBV provides a high SVR rate in HCV patients along with good tolerance and safety.

DANOPREVIR

Danoprevir (RG7277; Roche, Basle, Switzerland; Inter-Mune Pharmaceuticals, Brisbane, CA, United States) is a 2nd-generation NS3/4A protease inhibitor of macrocyclic origin with the same activity toward HCV genotypes 1, 4 and 6 (*in vitro*)[27,28]. Phase I clinical studies showed the high antiviral activity of danoprevir for genotype 1 HCV. Danoprevir in that category of patients was administered as monotherapy, combined with PEG-IFN/RBV, or combined with a HCV polymerase inhibitor, *i.e.*, mericitabine, in an interferon-free regimen[29-32].

Efficacy
The phase II trial DAUPHINE[33] studied the efficacy of three danoprevir doses (50, 100 and 200 mg) boosted with ritonavir 100 mg taken BID in combination with PEG-IFN/RBV (RGT). Ritonavir addition is known to increase the PI blood concentration, thereby suppressing CYP3A activity. Twelve weeks after treatment completion, the SVR12 rate was 93% in genotype 1 HCV patients treated with danoprevir 200 mg BID combined with PEG-IFN/RBV. Danoprevir 100 mg/d provided a SVR12 rate of 83%, and 50 mg/d 67%. The effectiveness of danoprevir 200 mg BID combined with PEG-IFN/RBV was not affected by HCV genotype subtype (1a *vs* 1b) or *IL28B* genotype (CC *vs* non-CC).

The objective of the randomized, placebo-controlled, parallel-group phase II trial ATLAS (NCT00963885) was to study the efficacy and safety of RGT danoprevir combined with PEG-IFN/RBV for 12 wk compared to PEG-IFN/RBV in naive genotype 1 HCV patients[34]. It was an international study, with sites in North America (31 sites), Europe (8 sites) and Australia (3 sites). Patients who had not previously been treated for HCV (treatment-naïve patients) were randomized into 4 groups. For 12 wk, patients were given danoprevir (300 mg every 8 h, 600 mg every 12 h or 900 mg every 12 h) or placebo in combination with PEG-IFN/RBV. Follow-up treatment

included PEG-IFN/RBV therapy only. Patients with an extended rapid virologic response (eRVR) (RNA < 15 IU/mL for 4-20 wk) stopped treatment at week 24. Patients without eRVR continued PEG-IFN/RBV therapy for 48 wk. The main criterion for assessing efficacy was SVR within 24 wk after treatment completion. The SVR rate was 68% in patients treated with danoprevir 300 mg, 85% in danoprevir 600 mg and 76% in danoprevir 900 mg, compared with 42% in placebo-treated patients. RGT was found in 71 patients given danoprevir 600 mg combined with PEG-IFN/RBV, and SVR was found in 96%.

Safety
In the ATLAS study, serious adverse events were reported for 7%-8% of danoprevir-treated patients and for 19% of placebo-treated patients. Four danoprevir-treated patients had transient ALT elevation. The highest danoprevir dose (900 mg) resulted in grade 4 ALT elevation that, in turn, required therapy discontinuation in the relevant patient arm.

Summary
Danoprevir combined with PEG-IFN/RBV resulted in a high SVR rate in genotype 1 HCV patients. However, high danoprevir doses can result in prominent ALT elevation requiring AVT discontinuation.

ASUNAPREVIR

One of the most considerable achievements in AVT development with PI combinations (if not the most important) is the phase IIa trials with asunaprevir (ASV, BMS-650032, 600 mg; BID) and daclatasvir (DCV, NS5A inhibitor, 60 mg; OD) combined with conventional therapy and a comparison group (with no conventional treatment).

Efficacy
A phase II CT with the above combination, the AI447-011 study, showed its efficacy for one of the most complex HCV-infected patient groups: non-responders with zero prior virologic response (HCV RNA decreased less than 2 $\log_{10}$ by week 12 of conventional therapy). This group appears to be the most complex from the point of view of antiviral regimen selection because null responders should be considered insensitive to IFN-based agents. That study's results show that a combination of direct-acting antivirals is the only therapeutic option for this patient category. The design of the AI447-011 study involved an efficacy comparison in 2 patient arms: Arm 1 was given the combination of ASV + DCV, and arm 2 was given ASV+ DCV + conventional therapy. The treatment duration was 24 wk; one of the important exclusion criteria was liver cirrhosis[35] (Figure 4). The study reported the seemingly unreachable SVR of 90% among null responders under complex therapy (ASV+ DCV + conventional therapy).

Figure 4 Asunaprevir rate (%) after 12- and 24-wk follow-up in the AI447-011 study. ASV: Asunaprevir; DCV: Daclatasvir; PEG-IFN: PEG-IFNPE-Gylated interferon; RBV: Ribavirin.

Figure 5 Undetectable hepatitis C virus RNA rate depending on *IL28B* polymorphism.

Table 7 Effectiveness of combined therapy with asunaprevir *n* (%)		
	Null response (*n* = 21)	Contraindications to IFN-based therapy (*n* = 22)
RVR4	20 (95.2)	15 (68.2)
RVR12	19 (90.5)	14 (63.6)
SVR24	19 (90.5)	14 (63.6)

Data of BMS CT, Suzuki *et al*[36], 2012. IFN: Interferon.

Even more impressive results were shown by Japanese researchers, Suzuki *et al*[36] (2012), who also used an ASV + DCV regimen in null-responders (*n* = 21); their comparison group included patients (*n* = 22) with contraindicated IFN-based therapy (Table 7). Their data showed an SVR of 90.5% in group 1. Additionally, this CT showed the prognostic value of EVR: all EVR patients reached SVR.

The study of the ASV + DCV combination drew the following conclusions: *IL28B* polymorphism appears to lose its SVR predictive value; the majority of patients with aviremia after 2 wk treatment had the CC-genotype, without significant SVR differences in patients with different *IL28B* genotypes (Figure 5).

Safety
The study of the ASV+DCV combination reported relatively more often AEs related to moderate headache, nasopharyngitis exacerbation elevated aminotransferases, and diarrhea. Laboratory AEs were moderate and severe [grade 3-4 (G3-4)] impairments related to elevated transaminase activity. Serious adverse events were found in 6 patients: mild and moderate pyrexia (G2-3), moderate gastroenteritis (in 2 patients); hyperbilirubinemia (G4). In all patients pyrexia disappeared in 4 to 10 d after AVT cancellation. Hyperbilirubinemia and cytolytic syndrome resolved within 4 wk after treatment discontinuation (Table 8). We should note that in the studies of ASV + DCV + conventional therapy combinations, the AE structure showed a prevalence of disorders caused by conventional treatment.

Summary
Asunaprevir-based AVT regimens are highly effective

Table 8 Adverse event structure for asunaprevir				
	Lok *et al*[35] AI447-011		Suzuki *et al*[36]	
	ASV + DCV + conventional therapy (*n* = 10)	ASV + DCV (*n* = 11)	Null response (*n* = 21)	Contraindications to IFN-based therapy (*n* = 22)
Diarrhea	70.0%	72.7%	43%	9%
Fatigue	70.0%	54.5%	-[1]	-[1]
Headache	50.0%	45.5%	38%	27%
Nausea	50.0%	18.2%	-[1]	-[1]
Coughing	20.0%	27.3%	-[1]	-[1]
Subfebrile temperature	27.3%	10.0%	14%	23%

[1]Adverse events are not shown because they were observed in fewer than 3 patients. AE: Adverse event; ASV: Asunaprevir; DCV: Daclatasvir; IFN: Interferon.

(above 90%) in the most challenging patient category (null responders); the safety profile of the given AVT regimen was mainly not different from PEG-IFN/RBV.

ABT-450
Agent ABT-450 (AbbVie) is used only in combination with the non-nucleoside inhibitor NS5B (ABT-333), ribavirin and ritonavir. Therefore, ABT-450 efficacy and safety should be considered only a multicomponent "achievement".

Efficacy
The clinical efficacy of an ABT-450-based AVT treatment was published as the results of the AVIATOR study, a phase II a CT, by Poordad *et al*[37] (2013). The genotype 1 patient population mainly included treatment-naive patients (66%, *n* = 33), while partial responders and null responders comprised 34% of the population (*n* = 17). ABT-450 was not used as monotherapy. The dosing of the inhibitor combination depended on the studied population. The given study used a combination of NS5B (ABT-333), ribavirin and ritonavir coupled with various ABT-450 doses for 12 wk. The results showed that ABT-450 was effective at 150 mg OD: the SVR rate

Table 9 AVIATOR, phase Ⅱa study (combination of ABT-450 + ritonavir + ABT-333 + ribavirin)

Study arm	*n*	Genotype	Status	Combination	Duration	Treatment regimen	SVR
Total: group 1 + 2	33	1a/1b (28/5)	Naive	ABT-450 + ritonavir + ABT-333 + ribavirin	12 wk	ABT-450, 250 mg/d or 150 mg/d + ritonavir, 100 mg/d; ABT-333, 400 mg BID; ribavirin, body weight-based	93%-95%
3	17	1a/1b (16/1)	partial virologic response, null response	ABT-450 + ritonavir + ABT-333 + ribavirin	12 wk	ABT-450, 150 mg/d; ritonavir, 100 mg/d; ABT-333, 400 mg BID; ribavirin, body weight-based	47%

Table 10 SVR rate depending on *IL28B* polymorphism

Study arm	Status	CC-genotype	CT-genotype	TT-genotype	SVR
1	Naive	10/9	7/7	2/2	95%
2	Naive	5/4	7/7	2/2	93%
3	Partial virologic response, null response	0/0	12/6	5/2	47%

Table 11 Adverse event incidence of ABT-450 + ritonavir + ABT-333 + ribavirin combination

AEs with incidence above 20%	AE incidence
Headache	14%-26%
Fatigue	35%-47%
Insomnia	0-26%
Nausea	21%-24%
Rash	6%-21%

AE: Adverse event.

Table 12 SVR rate in genotype 1 hepatitis C virus patients *n* (%)

	GS-9256 + tegobuvir (*n* = 15)	GS-9256 + tegobuvir + ribavirin by weight (*n* = 13)	GS-9256 + tegobuvir + ribavirin by weight + PEG-IFNα (*n* = 14)
Week 4, RVR	1/15 (7)	6/13 (46)	10/14 (71)
Week 12, EVR	3/15 (20)	8/13 (62)	14/14 (100)
Week 24, SVR	10/15 (67)	13/13 (100)	13/14 (94)

PEG-IFNα: PEGylated interferon-α.

GS-9256

GS-9256 was used only in combination with the non-nucleoside inhibitor tegobuvir (GS-9190). Therefore, GS-9256's efficacy and safety should be considered only in a multicomponent treatment.

Efficacy

Another representative of the PI class, GS-9256 (Gilead) was studied in combination with the non-nucleoside inhibitor tegobuvir (GS-9190) and ribavirin. The combination of 4 agents (GS-9256 + tegobuvir + ribavirin + PEG-IFNα) was used as a comparison group. According to the phase Ⅱ study by Zeuzem *et al*[39] (2010), the SVR rate was comparable in the absence *vs*. the presence of PEG-IFNα: 100% *vs* 94%. The SVR rate of the two-component regimen (only with direct-acting antivirals) amounted to 67% (Table 12).

Safety

Patients taking GS-9256 + tegobuvir 40 mg showed good tolerance, and the majority of AEs were of medium severity. No specific AEs were found. Conversely, the comparison arm (GS-9256 + tegobuvir + ribavirin by weight + PEG-IFNa) developed common AEs typical for PEG-IFN/RBV regimens (Table 13). During the CT period, 2 cases of serious AEs were reported: bursitis (infected) and vasovagal attack, which the investigators interpreted as not related to the studied agent. No laboratory impairments of serious AE (G4) type were found.

Summary

In general, GS-9256 + tegobuvir + ribavirin is highly effective in treatment-naive genotype 1 HCV patients. An analysis of the phase Ⅱ study results indicated a good safety profile for combinations including GS-9256.

amounted to 93%, compared to 95% in the comparison group with ABT-450 250 mg OD (Table 9). The phase Ⅱb study of the combination (2013) showed comparable efficacy in similar study arms: treatment-naive 89% and 96%, null responders 89 and 95%, respectively. It should be noted that the SVR rate for ABT-450-based AVT did not depend on *IL28B* polymorphism (Table 10).

Safety

There were no noted specific AEs from ABT-450-based AVT. The most common were headache, fatigue, nausea (Table 11). However, good tolerance in its totality is related not only to ABT-450 but also to other combination constituents[38]. It is worth mentioning that despite the presence of ribavirin in the combination, anemia was not a frequent AE deserving special attention.

Summary

ABT-450 + ritonavir + ABT-333 + ribavirin in phase Ⅱa and Ⅱb studies was highly effective in HCV patients with the following criteria: genotype 1, both treatment-naive patients and null responders, no liver cirrhosis. At the moment, studies for optimal treatment duration are ongoing. Regarding safety, this combination was well tolerated, and possible AEs were mostly related to asthenia syndrome. Specific AEs were not detected in the studies.

Table 13 Most common adverse event after 4 treatment weeks			
	GS-9256 + tegobuvir	GS-9256 + tegobuvir + ribavirin by weight	GS-9256 + tegobuvir + ribavirin by weight + PEG-IFN[1]
AE incidence	50%	93%	81%-100%
Anemia	0	0	0-13%
Eye pain	0	0	0-13%
Diarrhea	19%	20%	6%-40%
Nausea	13%	20%	6%-40%
Flu-like syndrome	0	0	44%-80%
Fatigue	6%	33%	13%-33%
Headache	31%	47%	13%-40%
Insomnia	0	20%	6%-13%
Dry skin	0	13%	0%
Pruritus	6%	20%	0%-7%

[1]Mean value of two CT phases. AE: Adverse event; PEG-IFN: PEGylated interferon.

CONCLUSION

The appearance in clinical settings of the first direct-acting antivirals to treat HCV provided improved effectiveness and decreased AVT duration in a majority of genotype 1 patients.

The addition of protease inhibitors has been beneficial for HCV patients. The SVR rate of 40%-50% obtained using conventional treatment was significantly improved (up to 80%) through the addition of telaprevir or boceprevir in triple therapy regimens with the provision that AVT is administered following all main approaches and principles (*e.g.,* accounting for contraindications, potential drug-drug interactions, adherence to AVT regimens depending on initial patient parameters)[10].

Another solid argument for the development of new combined regimens that include direct-acting antivirals is the high rate of hematological AEs, especially ribavirin-induced anemia. When new AVTs for genotype 1 HCV are introduced, most attention should be paid to the regimens, such as by excluding PEG-IFNα to expand the treatment groups and including patients with contraindications to IFN and IFN intolerance.

Our review shows that combinations of direct-acting antivirals can become a novel therapeutic standard not only for patients with contraindicated IFN therapy. PI combinations with other direct-acting antivirals can improve the SVR rates in non-responders with prior partial virologic response and, more importantly, in those with prior null virologic response (Table 14).

When triple therapy appeared, it was the only combination available for null responders. However, the evident barriers were interferon intolerance and issues with concomitant treatment selection to avoid drug interactions. Currently, asunaprevir-based combinations are the treatment of choice for null responders. They have a SVR rate of 90%, and in case of interferon intolerance, patients can be offered antiviral ABT-450-based regimens. In any case, the era of direct-acting antivirals assumes interferon-free therapy. Once, supposedly ideal regimens

Table 14 SVR dependence in null responders for various direct-acting antiviral combinations				
Ref.	*n*	Combination	Duration	SVR
Zeuzem *et al*[39]	37	Telaprevir-based triple therapy	48 wk	33%
Bacon *et al*[25]	58	Boceprevir-based triple therapy	48 wk	52%
Lok *et al*[35]	11	DCV + ASV	24 wk	36%
Lok *et al*[35]	10	DCV + ASV + conventional therapy	24 wk	90%
Suzuki *et al*[36]	21			
Poordad *et al*[37]	7	ABT-450 + ritonavir + ABT-333 + ribavirin	12 wk	43%

DCV: Daclatasvir; ASV: Asunaprevir.

for HCV treatment implied interferon-free combinations. Now, the emergence of direct-acting antivirals makes it possible to develop optimally dosed treatments and completely exclude clinically significant AEs related to interferon use.

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RESEARCH REPORT

CYP2E1 immunoglobulin G4 subclass antibodies after desflurane anesthesia

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Abstract

AIM: To investigate CYP2E1 IgG4 autoantibody levels and liver biochemical markers in adult patients after anesthesia with desflurane.

METHODS: Forty patients who were > 18 years old and undergoing elective surgery under general anesthesia with desflurane were studied. Alpha-glutathione-S-transferase (αGST) and IgG4 antibodies against CYP2E1 were measured preoperatively and 96 h postoperatively, as well as complete blood count, prothrombin time (PT), activated partial thromboplastin time (aPTT), international normalized ratio (INR), aspartate aminotransferase (SGOT), alanine aminotransferase (SGPT), g-glutamyl-transpeptidase (gGT), alkaline phosphatase, total serum proteins, albumin and bilirubin. A separate group of 8 patients who received regional anesthesia was also studied for calibration of the methodology used for CYP2E1 IgG4 and αGST measurements. Student's t-test and the Mann-Whitney U test were used for comparison of the continuous variables, and Fisher's exact test was used for the categorical variables. All tests were two-tailed, with statistical significance set as $P < 0.05$.

RESULTS: None of the patients developed postoperative liver dysfunction, and all patients were successfully discharged from the hospital. No statistically significant difference was observed regarding liver function tests (SGOT, SGPT, γGT, bilirubin, INR), αGST and CYP2E1 IgG4, before and after exposure to desflurane. After dividing patients into two subgroups based on whether or not they had received general anesthesia in the past, no significant difference in the levels of CYP2E1 IgG4 was observed at baseline or 96 h after desflurane administration ($P = 0.099$ and $P = 0.051$, respectively). Alpha-GST baseline levels and levels after the intervention also did not differ significantly between these two subgroups ($P > 0.1$). The mean αGST differences were statistically elevated in men by 2.15 ng/mL compared to women when adjusted for BMI, duration of anesthesia, number of times anesthesia was administered previously and length of hospital stay. No significant difference was observed between patients who received desflurane and those who received regional anesthesia at any time point.

CONCLUSION: There was no difference in CYP2E1 IgG4 or αGST levels after desflurane exposure; further

research is required to investigate their role in desflurane-induced liver injury.

Core tip: Several case reports of hepatotoxicity following anesthesia with desflurane have been published in the literature, implicating cytochrome P450 2E1-IgG4 autoantibodies. This study investigates the possible changes in CYP2E1 IgG4 autoantibody levels and other biochemical markers of liver injury in 40 adult patients who received anesthesia with desflurane for elective surgery. Samples were obtained before and 96 h after exposure to desflurane, and no significant difference was observed in levels of CYP2E1 IgG4, a-glutamyl-S-transferase, aspartate aminotransferase, alanine aminotransferase, g-glutamyl-transpeptidase or alkaline phosphatase levels, regardless of patients' previous exposure to volatile anesthetics.

INTRODUCTION

Volatile anesthetics, mostly halothane, have been implicated as causative agents of drug-induced liver injury (DILI)[1-5], which has been reported to occur in approximately 1 out of 10000 adult patients receiving general anesthesia with halothane[4,5]. Three main mechanisms have been implicated in the development of hepatotoxicity: (1) a hypersensitivity reaction; (2) the production of hepatotoxic metabolites; and (3) hypoxia[4]. A hypersensitivity reaction is believed to be the most important of these mechanisms, it is also known as idiosyncratic drug-induced hepatitis (IDDIH) and is caused by the production of trifluoroacetylated (TFA) hepatic proteins during the metabolism of halothane by the cytochrome P450 2E1 (CYP2E1)[4]. These proteins act as neoantigens and are responsible for the production of autoantibodies against liver tissue[6-8]. In 1996, it was demonstrated that autoantibodies that react with CYP2E1 were significantly elevated in 45%-70% of patients with halothane hepatitis[7,9], particular including CYP2E1 IgG4 and 58 kDa endoplasmic reticulum protein (ERp58) autoantibodies[10,11].

With the development of newer volatile anesthetics, the risk of developing DILI has decreased, mainly due to the reduced metabolism of newer agents by CYP450 2E1 (20%-30% for halothane, compared to 2% for enflurane, 1% for sevoflurane, 0.2%-0.6% or less for isoflurane and 0.02% for desflurane)[12,13]. Njoku *et al*[14] (1997) demonstrated that desflurane produces lower levels of TFA hepatic proteins, reflecting the decreased metabolism of the agent by CYP2E1. Despite the fact that desflurane seems to have the best safety profile in this regard and is believed to have minimal, if any, hepatotoxic effect, there have been several case reports published implicating desflurane as a causative agent of DILI[11,15-20].

Aspartate aminotransferase (SGOT), alanine aminotransferase (SGPT), g-glutamyl-transpeptidase (gGT), alkaline phosphatase (ALP), albumin, bilirubin and coagulation status are all classical biochemical markers of liver injury, and they have all been reported to change significantly in all published case reports of hepatocellular damage due to desflurane anesthesia[11,15-20]. Additionally, alpha-glutathione S-transferase is believed to be a sensitive marker of hepatocellular injury because it is distributed equally in centrilobular and periportal hepatocytes, in contrast to SGOT/SGPT[21,22]. This distribution is important because liver biopsies of patients with halothane hepatitis have demonstrated that the main histological characteristic of this condition is centrilobular hepatic necrosis[4].

Based on case reports of desflurane hepatotoxicity and the possible mechanisms of DILI in the case of volatile anesthetics, the aim of this study was to investigate the possible production or alteration of CYP2E1 IgG4 autoantibody levels in addition to other biochemical markers of liver injury in patients who received general anesthesia with desflurane.

MATERIALS AND METHODS

The Institutional Review Board and Ethics Committee of "Attikon" University Hospital, where the study was conducted, approved the study, and written informed consent was obtained from all patients. Forty randomly selected adult patients who had received general anesthesia with desflurane for elective surgery between 01/2008 and 07/2011 were included. The exclusion criteria included the following: age < 18 years old; American Society of Anesthesiologists classification > III; history of liver disease of any etiology; history of chronic hepatitis B or C, recent viral disease of unknown etiology; history of exposure to hepatotoxic doses of acetaminophen, nonsteroidal anti-inflammatory drugs or antiepileptic drugs; current treatment with hepatotoxic drugs or immunosuppressants; autoimmune or connective tissue disorders; history of alcohol or illicit drug use; pregnancy; malignancies; intra-abdominal procedures; and operating time of more than 6 h.

Patients' complete medical history and medications were recorded during preoperative assessment. Samples of serum were collected from all patients preoperatively, immediately postoperatively and every 24 h until the 4th postoperative day (96 h after the completion of the surgery). At these time points, complete blood count, prothrombin time (PT), activated partial thromboplastin

time (aPTT), international normalized ratio (INR), aspartate aminotransferase (SGOT), alanine aminotransferase (SGPT), gGT, alkaline phosphatase, total serum proteins, albumin and bilirubin (direct/indirect) were measured. At baseline and 96 h postoperatively, alpha-glutathione-S-transferase (αGST) and IgG4 antibodies against CYP2E1 were measured. A time period of 4 d was selected because the latency period between the exposure and the clinical evidence of liver damage is variable in the current literature on halothane; its approximate value is estimated to be at least 6 d after first exposure and 3 d after multiple exposures[4]. The same applies to the case reports on desflurane-induced hepatotoxicity, in which the period between exposure and clinical presentation varied between 2-17 d[11,15-20]. Therefore, it was assumed that a time period of 4 d would be appropriate to detect early biochemical changes.

Serum was centrifuged at 1000 rounds/min for 10 min and immediately stored at -80 ℃ until the time of measurements. Complete blood count, coagulation status, SGOT, SGPT, gGT, ALP, serum proteins, and bilirubin were measured directly after sampling, according to the standard perioperative protocol.

Anesthesia in all patients was induced using propofol (2-2.5 mg/kg), fentanyl (3 μg/kg) and cis-atracurium (0.2 mg/kg). Maintenance of anesthesia was achieved with desflurane 6% (in an oxygen/air mixture of 50%) to achieve bispectral index values of 40-50, in addition to a remifentanil infusion as required. Intraoperative monitoring of all patients included systemic blood pressure measurement (invasive when needed), continuous ECG, pulse oxymetry, ETCO2 measurement and bispectral index. Postoperative analgesia included intravenous patient-controlled analgesia with morphine (bolus 1 mg, lockout 10 min) and acetaminophen (1 gr three times daily). In cases of minor surgery without significant postoperative pain, only an acetaminophen-codeine combination was administered (500 mg/30 mg), three times daily. Patients were closely monitored by the anesthesiology team for the efficacy of postoperative analgesia, hemodynamics, oxygenation, nausea and vomiting, two times a day, until the 4th postoperative day. All side effects and complications that occurred during that period were recorded.

A separate group of 8 patients who underwent orthopedic procedures under regional anesthesia was also studied to calibrate the methodology of CYP2E1 IgG4 and αGST measurements. All patients received combined spinal-epidural anesthesia using ropivacaine 0.75% combined with fentanyl, as required. The same serum sampling methodology was followed for these patients. Postoperative analgesia included only morphine administered epidurally (initial dose immediately after surgery) and subsequent acetaminophen-codeine administration (as for the general anesthesia patients).

Serum analysis for CYP2E1 IgG4 antibodies
The determination of serum CYP2E1 IgG4 antibody levels was performed according to a previously described

sandwich enzyme-linked immunosorbent assay[21] with slight modifications. Briefly, Immulon 2HB 96-well microtiter plates (ISC BioExpress) were incubated overnight with 0.5 μg/100 μL human CYP2E1 (human CYP2E1 plus P450 reductase plus cytochrome b5 Supersomes (BD Biosciences, Woburn, MA, United States). After washing, plates were incubated with 100 μL serum for 3 h at room temperature and subsequently with 100 μL of a 1:1000 diluted mouse anti-human IgG4 HRP-conjugated second antibody for 2 h at room temperature. The final product was determined by incubation for 20 min with 100 μL of a 1:1 mixture of Color Reagent A (H2O2) and Color Reagent Tetramethylbenzidine (R&D Systems, Minneapolis, United States). The reaction was stopped by the addition of 2 N H2SO4, and the optical density was determined at 450 nm.

Serum analysis for αGST
The determination of serum αGST was performed using a commercially available ELISA according to the manufacturer's instructions (Eagle Biosciences, Inc, Boston, United States).

Statistical analysis
The Shapiro-Wilk test was performed to test for normal distribution of continuous variables. The results are given as the mean ± SD or as the median and interquartile range (IQR) according to normality of continuous variables. All qualitative variables are presented as absolute and relative frequencies. Student's *t* test or its non-parametric equivalent, the Mann-Whitney *U* test, was used for comparison of continuous variables. Fisher's exact test was employed for comparison of categorical variables.

Mean differences in the liver function tests being studied before and after the intervention (general anesthesia) were investigated by the application of multivariate linear regression models. A stepwise backward-forward technique was applied for the selection of the dependent variables.

All tests were two-tailed, and statistical significance was established at 5% ($P < 0.05$). Data were analyzed using Stata ™ (Version 10.1 MP, Stata Corporation, College Station, TX, United States).

RESULTS
Characteristics of study population
Of the 40 patients included in the study, one was excluded due to the diagnosis of malignancy. The demographic characteristics of patients, such as their age, height, weight and body mass index, are presented in Table 1. Previous anesthetic exposure (to general anesthesia), type and duration of anesthesia, and total length of hospital stay after the operation, are also presented in Table 1. All patients remained hemodynamically stable throughout the procedure, without periods of sustained hypotension (of more than 20% below baseline values) or hypoxia that might interfere with liver function. No patient in this

Table 1 Characteristics of the study population (*n* = 39)		
Demographic characteristics	Mean ± SD/median (IQR) or frequencies[1]	Range[2]
Age (yr)	42 (29-62)	20-75
Gender (M/F)	22(56%) 17(44%)	-
Height (cm)	172.62 (10.55)	152-192
Weight (Kg)	82.7 (20.55)	35-140
BMI (Kg/m^2)	27.66 (6.14)	12.85-41.91
ASA Ⅰ / Ⅱ / Ⅲ	16 (41%)/22 (56%)/1 (3%)	-
Anesthetic data		
Previous anesthesias (no/yes)	12 (31%)/27 (69%)	-
No of previous anesthesias	1 (0-2)	0-6
Duration of anesthesia (min)	150 (90-180)	30-360
Length of stay (d)	5 (4-7)	2-15
Type of operation (n)	Orthopedic: 35	
	Fractures: 18	
	Arthroscopies: 12	
	Knee/hip arthroplasties: 5	
	Thyroidectomies (non-malignant): 3	
	Saphenectomy: 1	

[1]Data are presented as mean ± SD or as median [interquartile range (IQR)] according to normality of continuous variables; [2]Qualitative variables are presented as absolute and relative frequencies.

study developed postoperative hepatotoxicity, and all patients were successfully discharged from the hospital.

Comparison of blood test measurements before and after general anaesthesia

No statistically significant difference was observed in the liver function tests (SGOT, SGPT, γGT, bilirubin, INR), αGST or CYP2E1 IgG4 before and after exposure to desflurane. A significant decrease was only observed in the hematocrit, hemoglobin and albumin levels, postoperatively. All data are presented in detail in Table 2.

Multivariate linear regression models were applied using the mean differences of IgG4 and αGST adjusted for gender, BMI, duration of anesthesia, number of times anesthesia was administered previously, and length of hospital stay. A stepwise backward-forward technique was applied for the selection of dependent variables. No significant differences were detected in the mean differences of CYP2E1 IgG4 (before and after general anesthesia) or SGOT, SGPT, γGT, bilirubin and INR (*P* > 0.05). However, significant differences were detected between the mean differences of αGST according to gender, length of hospital stay and duration of general anesthesia. Alpha-glutamyl-S-transferase mean differences were statistically elevated in men by 2.15 ng/mL compared to women when adjusted for BMI, duration of anesthesia, number of times anesthesia was administered previously, and the length of hospital stay. An increase in the duration of anesthesia by one minute was associated with a mean increase in the αGST mean difference of 0.0323 ng/mL. These results are presented in Table 3.

Of the 8 patients who received regional anesthesia, one was excluded due to a malignancy that was diagnosed

Table 2 Comparison of blood test measurements before (baseline) and 96 h after exposure to desflurane			
	Baseline measurements	Measurements after intervention	*P* value[1]
Blood count			
HCT (%)	41.2 (37.4-43)	36.75 (31.65-41.7)	0.016
HBG (g/dL)	13.56 (1.944)	12.01 (1.941)	0.002
WBC	7907.94 (2218.9)	8330 (1826.7)	0.446
PLT (x 10^3)	251 (217-284)	241 (205-276)	0.259
Liver function tests			
SGOT (IU/L)	20 (17-25)	21.5 (17-28)	0.489
SGPT (IU/L)	19 (14-39)	19.5 (13-30)	0.987
ALP (IU/L)	64 (46-77)	54 (47-63)	0.222
γGT (IU/L)	22.5 (13-35)	23 (13-30)	0.532
Direct bilirubin (mg/dL)	0.2 (0.13-0.21)	0.185 (0.1-0.2)	0.277
Indirect bilirubin (mg/dL)	0.5 (0.3-0.7)	0.4 (0.3-0.6)	0.840
Total bilirubin (mg/dL)	0.6 (0.5-1)	0.6 (0.4-0.7)	0.610
Proteins (g/dL)	6.3 (6-6.7)	5.9 (5.35-6.45)	0.082
Albumin (g/dL)	4.1 (3.7-4.3)	3.5 (3.15-3.95)	0.013
PT (s)	11.9 (11.23-13)	12.55 (11.6-13.5)	0.219
aPTT (s)	27.97 (3.09)	29.83 (5.09)	0.125
INR	0.97 (0.92-1.08)	1.02 (0.9-1.1)	0.346
IgG4 (g/L)	0.04 (0.02 -0.5)	0.065 (0.02-0.415)	0.576
αGST (ng/mL)	3.5 (1.69-4.38)	3.03 (1.11-4.87)	0.489

[1]Statistical tests applied: Student's *t* test, Mann-Whitney *U* test. Data are presented as the mean ± SD or as the median [interquartile range (IQR)] according to normality of continuous variables. SGOT: Aspartate aminotransferase; SGPT: Alanine aminotransferase; ALP: Alkaline phosphatase; GT: Glutamyl-transpeptidase; PT: Prothrombin time; aPTT: Activated partial thromboplastin time; INR: International normalized ratio; αGST: Alpha-glutathione-S-transferase.

Table 3 Multivariate linear regression model of the mean difference of alpha-glutathione-S-transferase adjusted for gender, body mass index, duration of anesthesia, number of times anesthesia was administered previously, and length of stay				
αGST	Coefficient	Standard error	*P* value	95%CI
Gender	2.15	1.02	0.049[1]	0.0111-4.284
Length of stay	-0.445	0.202	0.04[1]	-0.868-(-0.0229)
Duration	0.0323	0.008	0.001[1]	0.0157-0.0489

[1]Statistical tests applied: Student's *t* test, Mann-Whitney *U* test. Data are presented as the mean ± SD or as the median (IQR) according to normality of continuous variables. αGST: Alpha-glutathione-S-transferase.

after the operation. Four women and 3 men were studied, of median age 70 years old (range 65-70) and ASA Ⅱ and Ⅲ. Three of them had received general anesthesia in the past [median 1.5 (range 1-2)], and 4 had not. Although the number of patients who received regional anesthesia was too small to serve as a control group, we compared the median differences (before and after anesthesia, general or regional) of the liver enzymes (SGOT, SGPT, ALP, γGT, αGST) and CYP2E1 IgG4, using the Mann-Whitney *U* test. There were no significant differences observed for any of the variables studied in this analysis.

Previous anesthetic exposure
Twelve of the patients studied had not received general

Table 4 Comparisons of IgG4 and alpha-glutathione-S-transferase levels according to medical history of receiving or not receiving anesthesia previously			
	No previous anesthesia (*n* = 12)	**Previous anesthesia (*n* = 27)**	***P* value[1]**
IgG4 levels			
Baseline	0.025 (0-0.04)	0.08 (0.02-0.66)	0.099
96 h after anesthesia	0.035 (0-0.07)	0.105 (0.03-0.74)	0.051
αGST levels			
Baseline	2.94 (1.69-3.94)	3.54 (1.42-5.87)	0.505
96 h after anesthesia	3.825 (3.02-5.4)	2.65 (1.05-4.33)	0.147

[1]Mann-Whitney *U* test. Data are presented as the median interquartile range; αGST: Alpha-glutathione-S-transferase.

Figure 1 Box plot of anti-CYP2E1 IgG4 values at baseline and 96 h after general anesthesia according to medical history of having received or not received general anesthesia previously.

anesthesia previously. In this subgroup analysis, mean values for CYP2E1 IgG4 levels, at baseline and after desflurane exposure, were 0.025 (0-0.04) and 0.035 (0-0.07), respectively (*P* = 0.73). Twenty-seven patients had received general anesthesia previously; of these patients, 19 had surgery in childhood. Their CYP2E1 IgG4 levels before and after desflurane exposure were 0.08 (0.02-0.66) and 0.105 (0.03-0.74), respectively (*P* = 0.59). The changes in IgG4 levels after a new exposure to desflurane for patients who had and had not previously received general anesthesia were not significantly different (*P* = 0.79). However, CYP2E1 IgG4 levels in patients who had a medical history of previously receiving general anesthesia compared to those who had not, showed borderline statistical significance 96 h after anaesthesia (with *P* = 0.099 at baseline and *P* = 0.051 96 h post anesthesia) (Table 4, Figure 1). Alpha-GST baseline levels and levels after the intervention did not differ significantly between patients who had previously received general anesthesia and those who had not (*P* > 0.1) (Table 4).

DISCUSSION

Severe postoperative liver injury has been reported as a rare complication after anesthesia with halogenated anesthetics[1-4,14-21,23]. The main mechanism responsible is believed to be the production of trifluoroacetylated proteins through the metabolism of halogenated anesthetics from CYP2E1, which act as neoantigens and induce the production of autoantibodies against liver tissue[14,21]. Although newer volatile anesthetics, desflurane especially, are reported to be safe due to their low metabolism by CYP2E1[14], there are several case reports implicating desflurane as the causative agent of IDDIH[11,15-20]. In some cases, it was assumed that the mechanism might be prior sensitization of patients from previous exposures to halothane or other halogenated volatile anesthetics[16].

Njoku *et al*[14] showed that patients with IDDIH had significantly increased CYP2E1 IgG4 autoantibodies compared to control subjects who had never been exposed to halogenated agents. Similarly, in another study, it was observed that the levels of CYP2E1 autoantibodies were asymptomatically increased in pediatric anesthesiologists compared to general anesthesiologists, due to an in-

creased environmental exposure to volatiles[24]. In another study by the same researchers[21] a comparison was made between the levels of CYP2E1 IgG4 in four separate groups: patients with anesthetic-induced IDDIH, pediatric anesthesiologists, general anesthesiologists and healthy controls; CYP2E1 IgG4 levels were significantly higher in patients who developed IDDIH than in all of the other groups. However, no comparison with our results can be made, because there was no direct quantification of the CYP2E1 IgG subclasses. In case reports of desflurane hepatotoxicity, hepatotoxicity occurred between the 2nd and 17th postoperative day and was related to at least one prior administration of anesthesia[11,15-20]. The problem is that CYP2E1 IgG4 levels were quantified and reported to be elevated in only two of these cases[18,20].

In the present study, no patient developed clinical or biochemical indications of liver injury, and there was no statistically significant increase in CYP2E1 IgG4 96 h after receiving anesthesia with desflurane. The hypothesis that previous exposure to halogenated anesthetics may sensitize patients to desflurane, leading them to develop IDDIH after subsequent desflurane exposure, has been stated by various researchers[14]. A cross-sensitization theory between inhalational anesthetics, in addition to the possibility of "immunological memory" of patients who were sensitized to an antigen, may be possible mechanisms to explain this effect[14]. To investigate this possibility, we also performed a subgroup analysis of patients, according to their previous exposure to halogenated anesthetics. Patients who had previously received general anesthesia were studied for their baseline values of CYP2E1 IgG4 and for their tendency to increase these levels after a new exposure to desflurane. The findings did not demonstrate any differences in the degree of increase of CYP2E1 IgG4 between naïve patients and those who had previously received any type of general anesthesia.

Regarding αGST, a multivariate regression model analysis showed that an increase in the duration of anesthesia by one minute was associated with a mean increase in αGST mean difference of 0.0323 ng/mL. This finding may be related to hepatic blood flow alterations and low-grade centrilobular hypoperfusion associated with

the longer duration of anesthesia[25-27]. This possibility is in accordance with the findings of another study of patients undergoing partial hepatectomy under anesthesia with desflurane versus propofol, where it was found that αGST levels 120 min after hepatic vascular declamping were significantly higher in desflurane patients[25]. Further research is required on this subject to investigate alterations of αGST with respect to liver blood flow and duration of anesthesia.

Limitations of this study include the small sample size of patients and the absence of detailed data regarding previous anesthetic exposure. Although the number of patients who previously received general anesthesia is known, the exact agents used (inhalational or intravenous) are unknown. However, most of the patients received anesthesia in childhood, where the use of inhalational agents is a common practice. Additionally, the lack of a longer period of measurement after anesthesia and the absence of more frequent measurements (*i.e.,* more time points) for CYP2E1 IgG4 and αGST are also limitations because there are cases of patients developing alterations after a longer time.

Our results suggest that there was no statistically significant difference between the levels of CYP2E1 IgG4 autoantibodies before and after anesthesia with desflurane. There was no evidence that the levels of αGST or any of the other biochemical markers of liver function were altered after anesthesia with desflurane and no signs that previous anesthetic exposure might affect these levels.

In conclusion, our findings suggest there are no significant differences between the levels of CYP2E1 IgG4 antibodies and αGST before and 96 h after anesthesia with desflurane and no difference between patients with previous anesthetic exposure versus naïve patients. Further research is required to investigate the actual role of CYP2E1 IgG4 in the pathogenesis of halogenated anesthetic-induced liver injury, especially in patients with multiple anesthetic exposures.

ACKNOWLEDGMENTS

COMMENTS

Background

Volatile anesthetics, mostly halothane, have been implicated as causative agents of Drug Induced Liver Injury (DILI). The most important mechanism for DILI is believed to be a hypersensitivity reaction, which is due to the production of trifluoroacetylated hepatic proteins during metabolism of halothane by the cytochrome P450 2E1 (CYP2E1). With the development of newer volatile anesthetics, the risk of developing DILI has been decreased, due to the minor metabolism of newer agents by the CYP450 2E1 (20%-30% for halothane, in comparison to 0.02% for desflurane). Despite the fact that desflurane seems to have the best safety profile with this regard, and is believed to have minimal, if any, hepatotoxic effect, there have been several case reports published, impli-

cating desflurane as the causative agent of DILI.

Research frontiers

Desflurane is a widely used volatile anesthetic which seems to have the best safety profile regarding liver injury. Despite that, there have been some cases of DILI after desflurane anesthesia and in all of them there was a history of at least one previous general anesthesia. It is very important to identify persons at risk of developing DILI in order to avoid this serious complication. IgG4 CYP2E1 autoantibodies have been implicated in the development of DILI although their specific role is not clear. The measurement of IgG4 CYP2E1 autoantibodies before and after anesthesia with desflurane in patients with and without previous exposure to volatile anesthetics could give some useful information.

Innovations and breakthroughs

In the present study, no patient developed clinical or biochemical indications of liver injury and it was demonstrated that there was no statistically significant increase of CYP2E1 IgG4, 96 h after receiving anesthesia with desflurane. Patients who had previously received general anesthesia were studied as for their baseline values of CYP2E1 IgG4, as well as for their tendency to increase these levels after a new exposure to desflurane. Findings did not demonstrate any significant differences in the degree of increase of CYP2E1 IgG4 between naïve patients and those who had previously received any kind of general anesthesia.

Applications

The study results suggest that desflurane anesthesia is not associated with an increase of IgG4 CYP2E1 autoantibodies and that their role has to be further investigated.

Peer review

Dr. Batistaki C and her colleagues investigated the levels of CYP2E1 IgG4 autoantibody levels and conventional biochemical variables in adult patients before and after anesthesia with desflurane, and found that there was no significant difference in hepatic biochemical variables and CYP2E1 IgG4 levels in patients who received general anesthesia with desflurane. This was an interesting study and the findings also provided them some useful reference in real clinical practice.

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SYSTEMATIC REVIEW

Central hepatectomy for centrally located malignant liver tumors: A systematic review

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Abstract

AIM: To study whether central hepatectomy (CH) can achieve similar overall patient survival and disease-free survival rates as conventional major hepatectomies or not.

METHODS: A systematic literature search was performed in MEDLINE for articles published from January 1983 to June 2013 to evaluate the evidence for and against CH in the management of central hepatic malignancies and to compare the perioperative variables and outcomes of CH to lobar/extended hemihepatectomy.

RESULTS: A total of 895 patients were included from 21 relevant studies. Most of these patients who underwent CH were a sub-cohort of larger liver resection studies. Only 4 studies directly compared Central *vs* hemi-/extended hepatectomies. The range of operative time for CH was reported to be 115 to 627 min and Pringle's maneuver was used for vascular control in the majority of studies. The mean intraoperative blood loss during CH ranged from 380 to 2450 mL. The reported morbidity rates ranged from 5.1% to 61.1%, the most common surgical complication was bile leakage and the most common cause of mortality was liver failure. Mortality ranged from 0.0% to 7.1% with an overall mortality of 2.3% following CH. The 1-year overall survival (OS) for patients underwent CH for hepatocellular carcinoma ranged from 67% to 94%; with the 3-year and 5-year OS having a reported range of 44% to 66.8%, and 31.7% to 66.8% respectively.

CONCLUSION: Based on current literature, CH is a promising option for anatomical parenchymal-preserving procedure in patients with centrally located liver malignancies; it appears to be safe and comparable in both perioperative, early and long term outcomes when compared to patients undergoing hemi-/extended hepatectomy. More prospective studies are awaited to further define its role.

Key words: Central hepatectomy; Segment orientated liver resection; Mesohepatectomy; Middle hepatic lobectomy; Central bisegmentectomy

Core tip: Central hepatectomy, defined as anatomical segment 4, 5, 8 ± 1 liver resection, is a promising parenchymal-preserving procedure in patients with centrally located liver malignancies. Based on current evidence, it appears to be safe and comparable in both perioperative, early and long term surgical and oncological outcomes when compared to patients undergoing traditional resections such as hemi-/extended hepatectomy.

INTRODUCTION

Surgical resection is the optimal treatment of choice and potential cure for most malignant tumors of the liver if possible[1,2]. With recent improvements in surgical techniques of liver resection, anesthesia and postoperative care, morbidity ranges from 5% to 25% and mortality has improved significantly and has approached zero[3-6]. Centrally located malignancies of the liver such as hepatocellular carcinoma (HCC), Cholangiocarcinoma (CCA) and liver metastases in segments 4, 5, 8 may require extensive resections because of their relationship to major vascular and biliary structures and deep location[7,8]. Traditionally, these centrally located tumors are resected by major resections such as right, left, extended right or extended left hemihepatectomies. Extended or anatomical resections are recommended for oncological reasons; however, these carry a risk of not only significant blood loss, longer operative time but also postoperative liver failure in patients with cirrhosis or poor liver functional reserve or even in patients without cirrhosis[9-11]. Non-anatomical resection is an alternative approach for parenchymal preservation, but it is hindered by intraoperative hemorrhage and betrays oncological principles evident by higher rates of margin positivity and poorer survival outcome[12,13]. With the perpetual lack of donor organs, long waiting time, along with other limitations of liver transplantation, anatomical parenchymal-preserving procedures have an increasing role in treatment of primary and secondary liver malignancies[14].

Central hepatectomy (CH), also known as meso-hepatectomy, was first performed for gallbladder cancer in 1972 and is used to describe the operative procedure to resect segments 4, 5, 8 ± 1 (Figure 1)[15-20]. Other synonymous terms used in the literature include central hepatic resection, middle hepatectomy, middle hepatic lobectomy, central bisegmentectomy and central bisectionectomy[20-27]. Regardless of the technical term used, the principle behind this procedure is the same and was not commonly carried out till more recently. In the Brisbane Terminology of Liver Anatomy and Resections by the International Hepatopancreatobiliary Association (IHPBA) in 2000, there was no definition of this surgical procedure[15,16].

The theoretical risks of CH compared to traditional major liver resections such as extended- or hemihepatectomy are obvious. These include a longer operating time, greater intraoperative blood loss, higher risk of biliary and vascular complications, all mainly attributed to the proximity to the hilar structures and the presence of 2 significant resection planes instead of a single plane. Despite this, previous reports showed that CH is safe and achieves comparable complication rates and overall survival rates as conventional major hepatectomies but harbors the advantages of: (1) preserving liver parenchyma with the aim of decreasing the risk of postoperative liver failure; (2) no proven oncological compromises as long as margins are negative and adequate; and (3) increases the opportunity for future repeat resection, if warranted,

Figure 1 Central hepatectomy-segment orientated resection. Couinaud segments are labeled 2-8; Caudate lobe (Segment 1) is not labelled in this diagram. here) Area shaded blue are the Central segments of 4, 5, 8 which are the segments resected in central hepatectomy (CH). Red rings indicate where the vascular outflow is encountered and ligated during CH.

in cases of recurrent malignancies such as colorectal or neuroendocrine liver metastases[7,8,22,23,25].

The current literature directly comparing patients undergoing extended hemi-hepatectomy and CH is lacking[9,15,17,23,25]. There is only one study in the current literature with more than 100 patients (Table 1)[3,7-9,17-20,22,23,25,27-37]. The aim of this review is to analyze and compare the perioperative, early and long- term results of patients with centrally located liver malignancies, between those treated with CH and those treated with hemi-/extended hepatectomy.

MATERIALS AND METHODS

A systematic literature search was performed in MEDLINE (PubMed) from January 1983 to June 2013 to evaluate the evidence supporting CH as a safe procedure for the management of central hepatic tumors and to compare the perioperative, short and long term results of CH to extended/lobar hemihepatectomy. The search used the following medical subgroup headings (MeSH) terms combined with Boolean operators: mesohepatectomy, central hepatectomy, central liver resection, segmentectomy, bisegmentectomy, central trisegmentectomy, bisectionectomy, segmental liver resection and segment oriented liver resection. References of the identified articles were reviewed to identify additional relevant studies. Only full articles published in the English language reporting a series of more than 10 cases were included in the review and effort was made to exclude studies with significant overlap of the same patient cohorts. The literature search was conducted according to PRISMA (preferred reporting items for systematic reviews and meta-analyses) recommendations(Figure 2)[38]. Definitions and nomenclature for liver resections was based on the 2000 IHPBA Brisbane terminology[15,16]. Namely, resection of segments 5 and 8 was named right anterior sectionectomy, while resection of segments 4a and 4b was named left medial sectionectomy. The procedure of segments 4, 5 and 8 resection has now predominantly been termed

Table 1 Characteristics and results of studies on central hepatectomy (1983-2013) *n* (%)

Ref.	Year	*n*	Diagnosis	Operative time (min)	Vascular control	Blood loss (mL)	Morbidity	Mortality	Survival outcome	
Hasegawa et al[27]	1989	16	HCC, CRM	300-660	Pedicle occlusion	600-7500	7 (50)	1 (6)	Median OS 34 mo	
Makuuchi et al[28]	1993	17	HCC, CCA, CRM	412	NA	1482	7 (41.1)	1 (5.8)	NA	
Nagino et al[29]	1998	15	Hilar CCA	NA	NA	NA	NA	NA	NA	
Wu et al[17]	1999	15	HCC	474	Pringle's maneuver or pedicle occlusion	2450	3 (20)	0 (0)	1-yr OS 67% 3-yr OS 44% 6-yr OS 30%	1-yr DFS 53% 3-yr DFS 31% 5-yr DFS 21%
Scudamore et al[19]	2000	18	HCC, CRM, GBC	238	Pringle's maneuver	914	11 (61.1)	0 (0)	NA	NA
Yamashita et al[30]	2001	16	HCC, CCA, metastases, hemangioma, others	NA	NA	NA	NA	NA	NA	NA
³Jarnagin et al[3]	2002	15	Benign and malignant lesions	NA	NA	NA	NA	NA	NA	NA
Wu et al[22]	2002	58	HCC, CCA, metastases	409	Total hepatic flow clamping (28 patients)	1685	8 (8.5)	0 (0)	NA	NA
				399	Selective clamping of ipsilateral blood flow (30 patients)	1159	10 (33.3)	0 (0)	NA	NA
Hu et al[23]	2003	52	HCC	265	NA	1030	9 (17)	0 (0)	Median OS 51 mo	Median DFS 23 mo
Chouillard et al[25]	2003	19	HCC, metastases, CCA, benign tumor	280	Pringle's maneuver ± hepatic vein clamping	NA	NA	0 (0)	NA	NA
¹Chen et al[18]	2006	118	HCC	128	Pringle's maneuver ± IVC occlusion	592	36 (30.5)	1 (0.8)	NA	NA
Kim et al[31]	2006	35	HCC, CCA, hepatic sarcoma	331	Extraglissonian approach and parenchymal Kelly crushing	516	2 (5.7)	1 (2.8)	1-yr OS 94% 2-yr OS 72% 5-yr OS 62%	NA
Giuliante et al[32]	2007	18	HCC, metastases	448	Intermittent pedicle clamping	NA	6 (33.3)	0 (0)	NA	NA
¹Chen et al[33]	2007	246	HCC	177 (with preoperative TACE)	Pringle's maneuver ± IVC control	790	31 (34.8)	3 (3.4)	1-yr OS 87.1% 3-yr OS 62.9% 5-yr OS 46.2%	1-yr DFS 75% 3-yr DFS 46.2% 5-yr DFS 31.8%
				115 (without)		420	38 (24.2)	1 (0.6)	1-yr OS 82.2% 3-yr OS 54.4% 5-yr OS 31.7%	1-yr DFS 69.6% 3-yr DFS 38% 5-yr DFS 16.5%
¹Chen et al[7]	2008	256	HCC	174	Pringle's maneuver ± IVC control	750 (Pringle only); 380 (Pringle with IVC control)	72 (28.1)	1 (0.4)	1-yr OS 77.0% 3-yr OS 49.8% 5-yr OS 35.1%	1-yr DFS 59.1% 3-yr DFS 28.8% 5-yr DFS 17.0%
Mehrabi et al[34]	2008	48	HCC, metastases, CCA, GBC, hemangioma, other	238	Pringle's maneuver in 9 patients	1120	13 (27.1)	1 (2)	NA	3-yr DFS 47.9%
Lee et al[20]	2008	27	HCC	330	Pedicle ligation	1400	12 (44.4)	2 (7.9)	NA	NA

Arkadopoulos et al[35]	2012	36	HCC, metastases	180	Selective hepatic vascular exclusion/ Pringle's maneuver (16 patients)	650	6 (37.5)	0 (0)	NA	NA
				150	Sequential hemihepatic vascular control (20 patients)	400	9 (45)	0 (0)	NA	NA
Gallagher et al[36]	2013	21	HCC	627	Intermittent Pringle's maneuver	1590	4 (19)	1 (4.8)	1-yr OS 90.5% 3-yr OS 66.8% 5-yr OS 66.8%	1-yr DFS 65% 3-yr DFS 34.8% 5-yr DFS 34.8%
Cheng et al[8]	2012	63	HCC	230	Pringle's maneuver	500	8 (12.7)	5 (7.9)	1-yr OS 87.5% 5-yr OS 53.1%	1-yr DFS 50% 5-yr DFS 15%
[3]Yang et al[37]	2013	150	HCC	NA	Pringle maneuver	NA	NA	NA	NA	
Total number		895[2]	range	115-627		380-2450	Overall morbidity: 27.5% (range: 12.7%-61.1%)	Overall mortality: 2.3% (range: 0%-7.1 %)		

[1]Indicates multiple studies with overlapping study periods from a single institution; [2]Total number calculated after excluding studies with repeat patients; [3]Limited data available as CH patients were a subset of a larger group of patients undergoing various types of hepatic resections. Overall morbidity and mortality was calculated by considering the number of events as a percentage of the total number of patients in included studies. HCC: Hepatocellular carcinoma; CRM: Colorectal metastases; CCA: Cholangiocarcinoma; GBC: Gallbladder carcinoma; IVC: Inferior vena cava; NA: Not available; OS: Overall survival; DFS: Disease-free survival; TACE: Transarterial chemoembolization; CH: Central hepatectomy.

Figure 2 Identification and screening of articles according to preferred reporting items for systematic reviews and meta-analyses recommendations.

central hepatectomy or mesohepatectomy[9,14,17].

When calculating the overall morbidity and mortality, this was calculated as the number of events reported as a percentage of the total number of patients in included studies. In considering individual complications, the number of events or incidences was calculated as a percentage of the total number of complication events in which this data was available. Weighted means were calculated when outcomes were expressed as a mean value.

RESULTS

A literature search on MEDLINE (PubMed) and review of references of relevant articles yielded a total of 90

Table 2 Summary of case-control studies comparing central hepatectomies and lobar/extended hepatectomies _n_ (%)

Ref.	Year	Resection type	_n_	Operative time (min)	Blood loss (mL)	Mortality	Morbidity	Complications (_n_)
Wu _et al_[17]	1999	Central hepatectomy	15	474	2450	0 (0)	3 (20)	Bile leak (1), pleural effusion (1), ascites (1)
		Extended hepatectomy	25	348	1863	1 (4)	6 (24)	Bile Leak (2), pleural effusion (1), ascites (1), prolonged jaundice (2), intra-abdominal abscess (2), wound infection (1)
		_P_ value		0.09	0.23	0.43	0.99	-
Scudamore _et al_[19]	2000	Central hepatectomy	18	238	914	0 (0)	1_intra_ (5.5)	Intraoperative bleeding (1), pneumonia (2), intra-abdominal fluid collection (2), fever longer than 48 hours (2), transient fever (2), bile leak (1), late intra-abdominal fluid collection (1)
		Lobar hepatectomy	71	222	1025	1 (1.4)	9_early_ (50) 1_late_ (5.6) 6_intra_ (8.5) 29_early_ (40.8) 3_late_ (4.2)	NA
		Extended hepatectomy	43	304	1628	0 (0)	2_intra_ (4.7) 21_early_ (48.9) 9_late_ (20.9)	NA
		_P_ value		< 0.001	0.009 for extended _vs_ central	-	0.05 for late complications	-
Hu _et al_[23]	2003	Central hepatectomy	52	265	NA	0 (0)	9 (17)	Bile leak (3), pleural effusion (3), 1 postoperative massive ascites (1), subphrenic abscess (1)
		Conventional or extended hepatectomy	63	264	NA	2 (3.1)	12 (19)	Bile leak (2), wound infection (4), pneumonia (2), liver failure (2), subphrenic abscess (1), pleural effusion (1)
		_P_ value		0.953		0.408	0.491	-
Cheng _et al_[8]	2012	Central hepatectomy	63	230	500	5 (7.9)	8 (12.7)	Liver failure (2), bile leak (1)[1]
		Hemi-/extended hepatectomies	41	316	750	3 (7.3)	6 (14.6)	Liver failure (2), bile leak (1)
		_P_ value		< 0.001	0.004	1.000	0.777	-
Total		Central hepatectomy	148	Mean 268	Mean 882	3.4% (0%-7.9%)		20.90% (range: 20%-66.7%)
		Hemi-/extended hepatectomies	243	Mean 299	Mean 1352	2.9% (0%-7.3%)		38.70% (range: 14.6%-74.4%)

[1]Other complications described included gastrointestinal bleeding, intra-abdominal hematoma, abscess, intra-abdominal bleeding, pneumonia, wound infection, but did not specify the treatment group in which it occurred. Weighted means were calculated for operative time and blood loss. NA: Not available.

articles on central hepatectomy after exclusion of irrelevant articles (Figure 2). Of these, 22 articles were published in English and included more than 10 patients. Of these, one study was excluded from analysis because results of these patients were also reported in a later series by the same author[17,39]. Three articles by Chen _et al_[7,18,33] reported on patients undergoing CH at a single institution with overlapping study periods, with the latest 2008 study of 256 patients constituting the largest reported series of CH at a single institution to date. Of the 21 articles included for analysis, 3 studies were comparative studies between 2 different methods of vascular control during CH, 4 studies compared the results of CH _vs_ conventional forms of hepatectomies (hemi- or extended hemihepatectomy), 1 looked at results of CH in patients who had pre-operative transarterial chemo-embolization (TACE) as compared to those who did not have preoperative TACE and 1 compared the outcomes of patients who had huge HCC of 10 cm or greater in size, as to compared to that of HCC measuring 5 to 10 cm[19,33,37]. The remaining papers were descriptive and non-comparative studies. The results of these 21 studies, including number of patients, operative time, method of vascular control, operative blood loss, mortality, morbidity and survival data (where available) are summarized in Table 1. The 4 studies which compared the outcomes of CH _vs_ conventional hepatectomies are summarized in Table 2.

Table 3 Type and frequency of complications reported following central hepatectomy

Type of complication[1]	Number reported (n = 200)	Frequency (%)
Surgical complications		
Bile leakage/biloma	36	18
Intra-abdominal abscess	16	8
Wound infection	7	3.5
Bleeding	4	2
Intestinal perforation	1	0.5
Medical complications		
Pleural effusion/empyema	62	31
Ascites	29	14.5
Pneumonia/pulmonary infection	13	6.5
Pulmonary edema	6	3
Fever	4	2
Upper gastrointestinal bleeding	3	1.5
Urinary tract infection	3	1.5
Transient hepatic dysfunction/ prolonged jaundice	3	1.5
Hepatic necrosis/liver failure	3	1.5
Acute renal failure	2	1
Stroke	1	0.5
Arrhythmia	1	0.5
Deep vein thrombosis	1	0.5
Other (not specified)	4	2

[1]Calculated out of a total of 200 events.

Study characteristics and operative details

A total of 1259 patients from 21 studies were reported after undergoing CH in the literature. However, after excluding studies with potential repeat patients (due to recruitment with significant overlapping study periods at a single institution), a total of 895 unique patients who had CH were obtained. The indication for central hepatectomy was detailed in 14 studies comprising a total of 659 patients. The main indication for CH was HCC, occurring in 565 patients (85.7%), but was also performed in conditions such as centrally-located liver metastases which accounted for 58 cases (most commonly colorectal in origin). Other diagnoses included CCA/ gallbladder carcinoma (25 cases), hepatic sarcoma (1 case), hemangiomas (2 cases) and occasionally for other benign lesions (4 cases).

The range of operative time for CH was reported to be 115 to 627 min, and Pringle's maneuver was used for vascular control in the majority of studies. The mean intraoperative blood loss during CH ranged from 380 to 2450 mL.

Mortality and morbidity

Data on mortality was available in 16 articles with mortality rates ranging from 0.0% to 7.1%. In nearly half of these studies (7 studies) had zero postoperative mortality following CH. A total of 16 deaths in 17 unique studies comprising 689 patients have been reported, giving an overall mortality of 2.3% following CH. The most common cause of death following CH was liver failure. Concomitant contributing factors that have been reported included sepsis, pneumonia, post-operative bleeding,

disseminated intravascular coagulopathy and multi-organ failure. Data on complications following CH were available in 15 studies, with reported morbidity rates ranging from 5.1% to 61.1%. After excluding repeat studies, there were a total of 187 patients reported to have morbidity following CH, out of a total of 680 patients. This gave an overall complication rate of 27.5% (including early and late complications) following CH. A detailed breakdown of the type of complications experienced was available in 13 studies, which accounted for a total of 200 complication events. The most commonly reported surgical complication was bile leakage or biloma formation, which accounted for 18% (36 events) of all complications reported. Bile leakage resolved with conservative treatment in the majority of cases. Other surgical complications reported included wound infection, intra-abdominal abscess, intra-abdominal bleeding/hematoma, and intestinal perforation. Medical complications described following CH included transient hepatic dysfunction/prolonged jaundice/liver failure, ascites, pneumonia/pulmonary infection, pleural effusion/empyema, urinary tract infection, fever, upper gastrointestinal bleeding, renal failure, stroke and deep vein thrombosis (Table 3).

Overall survival

Overall survival (OS) data was available in 8 studies[7,8,17,23,27,31,33,36]. Median survival for HCC treated with CH was reported in 2 studies: this was 34 mo in Hasegawa et al[27] (patient diagnosis included both HCC and colorectal liver metastases) and 51 mo in Hu et al[23] and Hasegawa et al[27] (HCC patients only), Wu et al[17] reported a 6-year OS of 30% (HCC, CCA and other liver metastases); Chen et al[33] reported a 5-year OS of 31.7% in the group of patients with HCC who did not have preoperative TACE, as compared to the group who had TACE prior to CH with a significantly better 5-year OS of 46.2% (P = 0.043). Overall, the CH group for HCC had 1-year OS ranging from 67% to 94%, with 3-year and 5-year OS having a reported range of 44% to 66.8%, and 31.7% to 66.8% respectively.

Disease-free survival

Seven studies reported disease-free survival[7,8,17,23,33,34,36]. The median Disease-free survival (DFS) in Hu et al[23] was 23 mo for HCC. In the remaining HCC studies with DFS data, the range for 1-, 3- and 5-year DFS was 50% to 75%, 28.8% to 46.2% and 15% to 31.8% respectively.

Comparative studies of CH vs hemi-/extended hepatectomies

Of the 21 studies on CH, 4 of these were case-control studies that compared the outcomes following CH vs hemi- or extended hepatectomies at their institutions (Table 2)[8,17,19,23]. A total of 148 patients underwent CH and 243 had hemi- or extended hepatectomies performed for their disease in these 4 studies.

Operative time for CH was shown to be not significantly different from that of extended hepatectomies in

the series by Hu *et al*[23] and Wu *et al*[17], while in 2 studies, Scudamore *et al*[19] and Cheng *et al*[8], patients who underwent CH had significantly shorter operative time (238 and 230 min respectively) as compared to extended hepatectomies (304 and 316 min respectively). The overall weighted mean operative time in these 4 studies for CH was 268 min *vs* 299 min for lobar/extended hemihepatectomy. Also, significantly less blood loss was experienced in CH as compared to extended hepatectomies in Cheng *et al*[8] (500 mL in CH *vs* 750 mL for extended hepatectomies, *P* = 0.004) and in Scudamore *et al*[19] (917 mL *vs* 1628 mL, *P* = 0.009). The overall weighted mean intraoperative blood loss in these 4 studies for CH was 882 mL *vs* 1352 mL for lobar/extended hemihepatectomy.

The morbidity rates between CH and hemi-/extended hepatectomy groups were also not significantly different individually in these studies, with the exception of late complications in Scudamore *et al*[19] which was studied as a subgroup. In study by Scudamore *et al*[19], the rate of late complications in extended hepatectomies (20.9%) was found to be higher than that in CH or lobar hepatectomies (*P* < 0.05). The overall morbidity in our review of CH was comparable: 20.9% (range: 20% to 66.7%) for CH *vs* 38.7% (range: 20% to 66.7%) for the hemi-/extended hepatectomy group. There was no statistically significant difference in mortality of patients who underwent CH and hemi/extended hepatectomy groups in any of the 4 studies. Notably, there was zero mortality in 3 out of 4 CH groups, however, there was mortality in the corresponding control groups (hemi-/extended hepatectomy). The overall mortality in the 2 groups were also similar: 3.4% (range: 0.0% to 7.9%) for CH group *vs* 2.9 % (range: 0.0% to 7.3%) for the hemi-/extended hepatectomy group (Table 2).

DISCUSSION

Advances in imaging technology have contributed to the improvements in the understanding of liver anatomy that is based on functional segmental anatomy and forms the foundation for segment-orientated liver surgery[9,12]. Central hepatectomy removes most or the entire left medial sector (segments 4a and 4b) and all or most part of the right anterior sector (segments 5 and 8) with or without segment 1 (Figure 1). It represents an alternative and attractive option for those patients with limited functional liver reserve especially those with liver cirrhosis or those with chemotherapy associated steatohepatitis, because it removes the tumor-bearing segments in entirety while preserving the rest of the liver without necessarily compromising on recurrence or survival outcome[9,40].

In the presence of a large and/or a deep seated tumor located in the central part of the liver (Couinaud segments 4, 5, 8), the resection is more technically challenging due to its proximity to important hilar structures. Central hepatectomy is more surgically daedalean than the conventional anatomical major liver resection because it has 2 resection planes instead of one, the need

for preservation of the bilateral peripheral segments and its vasculature and the potential need for 2 bilioenteric anastomoses (*e.g.* for perihilar CCA). It involves the resection of liver territory drained by the middle hepatic vein (HV) along 3 lines of transection planes: the right intersectional plane (to the left of the Right HV), the left intersectional plane (falciform ligament) and the coronal transection plane being above the hilum and anterior to the right posterior sectoral pedicle, the root of the middle HV vein is divided at the bottom of the right and left plane of the parenchymal division; as such, it may require longer vascular occlusion time and alternative pedicle clamping may be required (Figure 1)[22,25]. This is especially pertinent in a cirrhotic liver for parenchymal preservation to minimize the risks of post-operative liver failure. Injury or improper division of these important structures during parenchymal division in CH may result in ischemia or necrosis of the residual peripheral liver leading to liver failure and increased mortality[23].

Preoperative evaluation of hepatic functional reserve includes clinical assessment, liver function test, platelet count, coagulation profile and Child-Pugh classification[14]. The Indocyanine Green (ICG[15]) test has been found to be helpful in predicting the safe limit of liver resection along with Computed tomography volumetric evaluation of the adequate remnant liver volume to minimize post-hepatectomy liver failure[14]. For patients with chronic liver cirrhosis being considered for major resection ($\geq$ 3 segments), pre-operative portal vein embolization (PVE) is a reasonable option to hypertrophy the future liver remnant to minimize risk of post-operative liver failure[14]. Ipsilateral PVE is a feasible preoperative strategy facilitating extended or staged resections, however, with centrally located tumors it is often difficult to determine which side of the portal vein should be embolized; in addition, livers with limited functional reserve will also have lower than expected response to PVE, further limiting its role[41].

Does the preservation of an extra 20% to 25% of liver (40% to 60% parenchymal resection by CH *vs* 60% to 85% resection by extended/lobar hemihepatectomies) justify the increased technical demands of CH? Liver surgery has evolved significantly in past decades. Increased cumulative experience in hepatectomy, improved techniques such as different techniques of vascular occlusion(*e.g.*, Pringle's, total vascular occlusion, sequential vascular occlusion) and the liver hanging maneuver, aided by the advent of advanced surgical technology such as the cavitational ultrasonic surgical aspirator (CUSA, Integra LifeSciences Corporation) and various energy devices (*e.g.*, LigaSure™, Covidien Ltd; Aquamantys®, Medtronic Advanced Energy; Harmonic Scalpel™, Ethicon Endo-Surgery, Inc. Johnson and Johnson Medical Ltd) during liver resection, surgical morbidity and mortality rates have declined markedly as compared with historic data[4,14,23,42-47]. Major vessels and bile ducts in the resection plane can be well visualized, skeletonized, and controlled meticulously during division of the liver parenchyma with these de-

vices (CUSA, LigaSure™, Aquamantys®, Harmonic Scalpel™) as an adjunct to traditional methods such as Kelly clamps, surgical clips and staples. The additional routine use of intraoperative ultrasonography further allows the major vessels and intrahepatic bile ducts to be identified and controlled confidently, as a result, minimizing unexpected major blood loss from vessel injury and avoiding major bile duct injuries[48]. In view of the complexity of CH, sequential or alternative hemihepatic vascular control has been advocated by some authors to minimize clamping time *i.e.*, warm ischemic time of the remnant liver. Arkadopoulos and colleagues demonstrated in a recent study looking at CH comparing 16 patients with selective hepatic vascular exclusion (Pringle's maneuver with hepatic vein outflow occlusion) *vs* 20 patients with sequential hemihepatic vascular control, in which they demonstrated that patients with sequential vascular control received fewer blood transfusions, had less intraoperative blood loss, shorter liver warm ischemic time and lower postoperative transaminitis[35]. Chen *et al*[18] had similar results when they compared 2 cohorts of patients (*n* = 58 *vs* 60) undergoing CH: they demonstrated that utilizing Pringle's maneuver with IVC occlusion resulted in less blood loss, lower transfusion requirements and less liver damage than Pringle's maneuver alone. In the hands of experienced hepatobiliary surgeons, operative times were also not increased during these procedures, as seen in our review (Table 2: 256 min in CH group *vs* 305 min in the lobar/extended hepatectomy group). Application of a modified Belghiti's liver hanging maneuver (Double liver hanging maneuver) has also been described to help guide the transection planes for CH[44,49]. These newer techniques make central hepatectomy a feasible and safe procedure, especially in experienced hands[9,23,42,50]. More recently, central hepatectomy has also been reported to be performed *via* minimally invasive techniques (laparoscopic or robotic approach)[51-54].

There are 2 main techniques for performing CH. The first involves ligation and division of the central pedicles supplying segment 4, 5 and 8 of the liver segments during liver parenchyma transection under a Pringle's maneuver, while the second involves extrahepatic individual ligation and division of the vessels supplying the segments 4, 5, and 8 prior to parenchyma transection of the liver with or without temporary total hepatic inflow/outflow occlusion[54,55].

Stratopoulos *et al*[9] in 2007 performed a literature review of major series of central hepatectomies. That review encompassed studies published till 2006, and this current review expands on those studies and includes more recent publications on this topic. The surgical mortality rate reported in this earlier review was between 0% and 6.25%. Our review revealed similar mortality rates of 0% to 7.1 % with an overall mortality rate of 2.3%. Consistent with our review, Stratopoulos *et al*[9]'s review reported that the most common cause of perioperative death was liver failure followed by hemorrhage. In our review, the most common surgical complication was bile leak/biloma. Postoperative early morbidity rates were as high as 61% contributed in most part by surgical events such as bile leakage/biliary fistula, hemorrhage/intra-abdominal hematoma, wound infection, intra-abdominal abscess and intestinal perforation as well as medical complications such as liver failure/dysfunction leading to ascites and hepatic encephalopathy, pulmonary infection/pleural effusion/empyema, urinary tract infection, sepsis, upper gastrointestinal bleeding, renal failure, stroke and deep vein thrombosis.

Biliary leakage is one of the most frequently reported intra-abdominal complications after liver resection[30,45,56-58]. The rate of bile leakage in the literature after liver resection has been reported to range from 0% to 11%[23,46,47,59-61]. In hepatectomy without bilioenteric anastomosis, the principal causes of bile leakage are bile oozing from the transected liver surface and intraoperative biliary injury. There are reports that identified central hepatectomy as an independent risk factor for bile leakage because of the presence of two transection planes and exposure of the hepatic hilum[30,56]. In the 4 comparative studies comparing CH *vs* lobar or extended hemihepatectomies, there were no statistically significant differences in bile leak.

In previous reports, CH has been associated with a higher risk of bleeding than conventional major resections[17,32]. Our review revealed that median intraoperative blood loss during CH ranged from 380 to 2450 mL, which is comparable in large liver resection series with major conventional resections[3,4,62].

Most of the patients with reported survival data in this review underwent CH for diagnosis of HCC. The 1-year OS for these patients ranged from 67% to 94%, with 3-year and 5-year OS having a reported range of 44% to 66.8%, and 31.7% to 66.8% respectively. This is comparable to the results from a recent review of survival outcomes looking at 17 studies with more than a total 13000 patients with HCC treated by liver resection, they reported a 1- year survival rate ranging from 67% to 97%, a 3- year survival rate ranging from 34% to 84% and a 5-year survival rate of 17 to 72% was reported[14,63]. A recent study on CH for HCC by Jeng *et al*[40] reported that even with narrow margins (< 5 mm) after CH, there was no negative impact on recurrence and overall survival. Conversely, Nagino *et al*[64] recently commented in a review of over three decades of their experience in the evolution of surgical treatment for perihilar CCA; they reported that limited resections such a CH for perihilar CCA decreased significantly from almost 30% to less than 3% over almost 4 decades with a corresponding improved survival due to more R$_0$ resections from extended resections. The role of CH for central HCC and perihilar CCA remains to be defined.

Because of the breadth of the current review and the limited available literature of CH as a procedure, the types of studies and the highly descriptive nature of much of the data reviewed, no assessment of the grade quality of individual studies or presence of bias and con-

founders were performed in this review. These studies have inherent selection biases and there is a degree of publication bias as well that is not avoidable due to the retrospective nature of these studies. In addition, because of the lack of high-level evidence and prospective or randomized studies, no objective grading was performed for any specific intervention. The oncological outcomes of HCC in these studies of CH are generalized and not conclusive when compared to HCC undergoing conventional resections due to the lack of detailed clinical and pathological data and risk factors in these studies that determines prognosis and provide strong valid comparison. The literature search was limited to English-language studies within the defined search period and included more recent papers in the past decade that remain highly relevant today. The review nonetheless serves as an updated collection and summary of the cumulative experiences in this relatively novel procedure and approach to segment-orientated liver resection.

Further high-quality and prospective studies with large sample sizes or randomized controlled trials to ascertain the utility and feasibility of central hepatectomy would be important in defining its role in the treatment of centrally-located liver tumors.

In conclusion, this review shows that central hepatectomy can achieve similar overall patient survival and disease-free survival rates as conventional major hepatectomies. Our findings suggest that central hepatectomy may be considered an acceptable procedure for treatment of centrally located malignances and may be the procedure of choice in patients with compromised liver function. It has the advantages of preserving parenchyma and seemingly without oncological compromise however, validation of CH as an oncologically safe procedure requires further prospective studies.

COMMENTS

Background
Central hepatectomy (CH) has similar perioperative outcomes with lobar and extended liver resections and achieves similar overall patient survival and disease-free survival rates as conventional major hepatectomies. Current evidence suggests that CH may be considered an acceptable procedure for treatment of centrally located malignances and may be the procedure of choice in patients with compromised liver function.

Research frontiers
More prospective studies including randomized controlled trials need to be conducted to validate the procedure as an oncologically equivalent and safe operation, specific to different cancer such as hepatocellular carcinoma and colorectal liver metastases, when compared to standard, traditional hepatectomy

Innovations and breakthroughs
Improved surgical and anesthesia experience and better technology has improved the morbidity and mortality of major liver resection and paved the way for safe parenchymal preserving surgery especially in patients with limited liver reserve.

Applications
CH may be applicable in selected patients with central tumors where parenchymal preserving surgery is indicated or preferred.

Terminology
CH also known as mesohepatectomy or central liver resection is used to describe the operative procedure for anatomical liver resection of segments 4, 5,

8 ± 1.

Peer review
In the present manuscript the authors performed a meta-analysis to evaluate the implication of CH for the management of central hepatic malignancies and to compare the perioperative, short and long term results of CH to lobar/extended hemihepatectomy. The authors concluded that CH is a promising option for anatomical parenchymal preserving procedure in patients with centrally located liver malignancies; it is safe and comparable in both perioperative, early and long term outcomes when compared to patients undergoing hemi-/extended hepatectomy.

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CASE REPORT

Ductal paucity and Warkany syndrome in a patient with congenital extrahepatic portocaval shunt

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Abstract

An eleven-year-old clinically dysmorphic and developmentally retarded male child presenting with complaints of 5 episodes of recurrent cholestatic jaundice since 3 years of age was evaluated. Imaging revealed features consistent with congenital extrahepatic portocaval shunt (Abernethy type 1b), multiple regenerative liver nodules and intrahepatic biliary radical dilatation. The presence of ductal paucity and trisomy 8 were confirmed on liver biopsy and karyotyping. The explanation for unusual and previously unreported features in the present case has been proposed.

Key words: Congenital extrahepatic portocaval shunt; Ductal paucity; Warkany syndrome; Trisomy 8

Core tip: This study highlights the association between the congenital extrahepatic portocaval shunt with hepatic ductal paucity and trisomy 8 for the first time in the world literature. Although the exact pathophysiology remains uncertain, plausible explanations are proposed.

INTRODUCTION

An eleven-year-old male child was admitted with recurrent episodes of cholestatic jaundice since age three. Each episode was associated with fever, pruritus and clay colored stool, lasting for 10-15 d. There was no history of variceal bleeding, respiratory difficulty, altered sensorium, abdominal distension, oliguria, loose stool, blood in stool, rash, joint pains or any other autoimmune phenomena.

CASE REPORT

The patient was born preterm at 8.5 mo gestation and had indirect hyperbilirubinemia without kernicterus, and was managed with phototherapy. The antenatal period was uneventful. Developmentally, his motor development coincided with his chronological age, but mental age lagged by 5 years.

On examination, his vital parameters were stable. Anthropometry revealed severe malnutrition. There was no pallor, icterus, clubbing, cyanosis, edema, or any peripheral symptoms of chronic liver disease. Dysmorphic features were present and included triangular facies, deep set eyes, antimongoloid slant, bulbous upturned nose tip, mal-aligned upper teeth, retrognathia, camptodactyly, single crease on little finger, multiple finger webs and

Figure 1 Photograph showing deep plantar furrows (A) and finger webs with camptodactyly (B).

Table 1 Laboratory parameters of the child at various time periods

Parameter	Age			
	6 yr	**10 yr**	**11 yr** **First contact**	**11.25 yr** **Follow-up (episode of cholangitis)**
Hb (g/dL)	-	10.7	11	13.6
TLC (cells/mm^3)	-	11300	11600	22200
Platelets (cells 1000/mm^3)	-	329	253	213
Bilirubin (T/D) (mg/dL)	0.8/0.5	1.1/0.5	1.5/0.6	3.18/1.92
AST (IU/L)	100	71	60	242
ALT (IU/L)	104	43	71	148
SAP (IU/L)	410	513	550	604
GGT (IU/L)	-	-	165	224
Albumin (g/dL)	3.7	3.5	3.7	3.3
INR	-	-	1.3	1.1
Ammonia (µg/dL)	-	-	155	229
AFP (ng/mL)	-	6.5	-	1.81
Fasting blood sugar (mg/dL)	-	-	42	72
pO$_2$ (mmHg) on room air	-	-	79.3	80
A-aO$_2$ gradient (mmHg)	-	-	26.4	21
BMI Z-score	-	-	-3.5	-3.07
Height Z-score	-	-	-1.7	-1.9

A-aO$_2$: Alveolar to arterial oxygen gradient; AFP: Alpha-fetoprotein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; GGTP: Gamma glutamyl transpeptidase; Hb: Hemoglobin; INR: International normalized ratio; pO$_2$: Partial pressure of oxygen; SAP: Serum alkaline phosphatase; T/D: Total/direct; TLC: Total leukocyte count.

deep plantar furrows (Figure 1). Ophthalmological examination revealed clear lens with normal cornea, iris and fundus. Orthopedic assessment revealed pectus carinatum and elbow contractures with restriction of extension movements. The spine was normal. Abdominal examination revealed no organomegaly or free fluid. Neurological examination was normal with preservation of higher mental, motor and sensory functions. Cardiovascular and respiratory systems were normal.

Laboratory parameters revealed conjugated hyperbilirubinemia with elevated liver enzymes. Synthetic function markers (albumin and prothrombin time) and lipid profile were normal. Serology for hepatitis A, B and C viral infection was negative (Table 1). Doppler ultrasonography (USG) of the abdomen revealed coarse liver with anomalous drainage of the extra-hepatic portal vein (PV) into the inferior vena cava (IVC) with non-visualization of intra-hepatic PV radicles. These findings were confirmed on contrast-enhanced computerized tomography

(CT) (Figure 2A), magnetic resonance imaging (MRI) and cholangiopancreaticography (MRCP). Imaging also revealed bilateral nephromegaly and dilatation of intrahepatic biliary radicles (IHBRD, left > right), without evidence of stricture, beading, mass or calculi in the biliary tree (Figure 2B). On MRI, numerous nodules were seen in both liver lobes which were hyper-intense on T1 and showed variable signal on T2 (Figure 2C). These imaging features were consistent with congenital extrahepatic portocaval shunt (CEPS or Abernethy type 1b), multiple regenerative liver nodules and IHBRD.

Subsequent evaluation revealed fasting hypoglycemia and hyperammonemia. Arterial blood gas analysis showed high alveolar-arterial gradient with hypoxemia on room air. Echocardiography showed structurally normal heart with severe shunting on injection of saline contrast, thus suggesting moderate hepatopulmonary syndrome (HPS). Alpha-fetoprotein concentration was normal. Esophagogastroduodenoscopy revealed no varices. A skeletal

Figure 2 Congenital extrahepatic portocaval shunt (Abernethy Type 1b), multiple regenerative liver nodules and dilatation of intrahepatic biliary radicles.
A: Reconstructed 3-D images of contrast-enhanced computerized tomography (CECT) scan showing congenital extrahepatic portosystemic shunt with drainage of the portal vein (arrowhead) into the inferior vena cava-splenic and superior mesenteric veins are shown by vertical and horizontal arrows, respectively; B: Magnetic resonance cholangiopancreaticography images showing dilatation of bilobar intrahepatic biliary radicles (arrow); C: T1-weighted axial images on MR showing well defined, round, hyper-intense lesions in both liver lobes (arrows); D: Reconstructed 3-D CECT images of lumbar spine showing spina bifida at L1 and L2 levels; E, F: Liver biopsy specimen showing preserved acinar architecture; portal tract showing prominent hepatic arteriole (arrowhead) with absence of portal venule and bile ductules (E, hematoxylin and eosin stain, × 400) with periportal fibrosis (F, Masson-trichrome stain, × 40).

survey revealed spina bifida (Figure 2D). Brain imaging was normal. Karyotyping showed mosaic trisomy of chromosome 8. Liver biopsy revealed maintained acinar architecture with mild cholestasis; portal tracts showed total absence of PV profiles, ductal paucity (bile duct: portal tract ratio of 0.3), conspicuous hepatic arteries and periportal fibrosis (Figure 2E and F).

The child was started on sodium benzoate, uncooked cornstarch diet and pentoxiphylline. His fasting hypoglycemia, hyperammonemia and hypoglycemia got corrected after one month of follow-up. He had another episode of jaundice with fever four months later which was managed conservatively. The patient's family was counselled regarding the prognosis including the possible need for liver transplantation (LTx).

DISCUSSION

Congenital extrahepatic portosystemic shunts (CEPS), or Abernethy malformation, is a rare congenital anomaly characterized by shunting of portal venous blood to the systemic circulation. On the basis of the presence of the PV and its intrahepatic radicles and the type of shunt, these malformations were classified by Morgan and Superina as (1) Type 1 (85%), with complete diversion of portal venous blood into the systemic circulation and total absence of intrahepatic PV branches; this was further subclassified as Type 1a, where splenic (SV) and superior mesenteric veins drain separately into the IVC, and Type 1b, when these veins drain after the formation of a short PV trunk, which drains into the IVC in an end-to-side fashion; and (2) Type 2, with intact PV and the presence of a side-to-side portocaval shunt[1]. A recent review suggested a classification based on anatomical site of origin and termination, type of communication with the systemic vein and number of communications[2]. CEPS are known to be associated with various congenital anomalies, particularly cardiovascular, gastrointestinal, genitourinary, skeletal and neurological, and genetic syndromes (Turner's and Goldenhar's)[3]. Our case was Type 1b CEPS without any other major structural malformations, except dysmorphism and spina bifida. He also had nephromegaly with structurally normal kidneys on imaging.

Clinical manifestations in CEPS occur either due to direct entry of intestinal blood and toxins into the systemic circulation or due to long-standing deprivation of PV blood supply with subsequent effects on liver regeneration and metabolism; some features are secondary to associated congenital abnormalities. As per recent reviews, the median age at presentation is 3.7 years with common modes being hypoxemia secondary to HPS or portopulmonary hypertension (26%-34%),

galactosemia (raised blood and urinary concentrations of galactose without enzyme deficiency) (26%), hepatic encephalopathy (HE) or hyperammonemia (9%-34%), neonatal cholestasis (13%) and incidental detection on imaging (9%-21%). Less common presentations are liver dysfunction, hypoglycemia, hepatocellular carcinoma and gastrointestinal bleeding[2,4]. Our patient developed growth failure, hyperammonemia, hypoglycemia, HPS (low partial pressure of oxygen and high alveolar to arterial oxygen gradient on room air) as well as recurrent jaundice (Table 1).

Focal hepatic lesions are common (35%-50%) in CEPS. As the liver is deprived of hepatotrophic factors such as insulin and glucagon, it undergoes atrophy with concomitant regeneration. Regenerative changes manifest as focal nodular hyperplasia, nodular regenerative hyperplasia, hepatocellular adenoma, and rarely cirrhosis, hepatocellular carcinoma and hepatoblastoma[2-4]. Our case had multiple regenerative nodules on imaging with normal AFP.

The diagnosis is established with USG Doppler and CT/MR angiography. Liver biopsy is needed for classification and subsequent management, as well as to establish the nature of the focal lesions. Liver histology in CEPS usually show absence or hypoplastic PVs, thickened hepatic arteries, minimal or moderate portal fibrosis, proliferation of thin vascular, capillary, or lymphatic structures in portal and periportal regions, and occasionally focal ductular proliferation[2]. Our case showed the absence of PV with prominent hepatic arteries and F2 fibrosis on Metavir staging. In addition, our case had ductal paucity, a finding which has not been previously described in CEPS.

Trisomy 8 or Warkany syndrome is a rare disorder. The majority of cases are mosaic as complete trisomy is usually lethal in fetal life. Deep plantar furrow is pathognomonic. Patients also have corneal clouding, strabismus, low set or abnormally shaped ears, bulbous nose tip, cleft palate, camptodactyly, clinodactyly, deep palmar furrow, anomalies related to the skeletal, cardiovascular and urogenital system, and mild to moderate mental retardation[5,6]. Most of these findings were present in our case, and to the best of our knowledge this is the first report of an association between CEPS and trisomy 8.

The presence of recurrent jaundice with bile ductal paucity and IHBRD were unusual features in our case. Recurrent jaundice in CEPS has been described in the literature, but no explanation has been provided[7]. During embryogenesis, PV plays a crucial role in the formation and remodeling of the ductal plate. Conversely, lack of remodeling is frequently associated with abnormalities in the ramification pattern of PV. Thus, both ductal plate malformation (DPM) and PV abnormalities may be interrelated. DPM is also associated with Caroli's syndrome and thus may also explain IHBRD seen in our case[8]. Another possible explanation is a pressure effect on the biliary system by the prominent dilated hepatic artery leading to recurrent cholangitis and ductal paucity secondary to chronic biliary obstruction. Another genetic defect, Alagille's syndrome, associated with mutations in Jagged1 or NOTCH2 genes, is also associated with ductal paucity and dysmorphic features, but our child didn't have other phenotypic features to suggest the disorder[9].

An algorithm for the management of CEPS has been suggested depending on the type of abnormality, symptoms and the presence of liver tumor[2]. Acceptable therapeutic options include LTx in type 1 and shunt occlusion (radiological or interventional) in type 2 cases. Common indications for LTx include HE or hyperammonemia (38%), pulmonary complications (32%) and tumor (15%)[4]. Authors have also proposed shunt occlusion for type 1 cases, presuming that the miniature hepatopetal vessels may enlarge after the procedure[2]. Our patient had moderate subclinical HPS, hyperammonemia and hypoglycemia along with total absence of PV radicles on liver histology, thus mandating LTx.

We describe a case of CEPS type 1b with trisomy 8 with an unusual presentation manifesting as recurrent jaundice, cholangitis and ductal paucity. We discuss the possible cause of recurrent jaundice and ductal paucity in CEPS.

COMMENTS

Case characteristics
Recurrent episodes of cholestatic jaundice, dysmorphism, developmental delay.

Laboratory diagnosis
Conjugated hyperbilirubinemia with elevated liver enzymes, hypoglycemia, hyperammonemia, and hypoxemia with high alveolar-arterial gradient.

Imaging diagnosis
Congenital extrahepatic portocaval shunt (Abernethy Type 1b), multiple regenerative liver nodules and dilatation of intrahepatic biliary radicles.

Pathological diagnosis
Ductal paucity, conspicuous hepatic arteries and periportal fibrosis.

Treatment
Anti-hyperammonemia measures, uncooked cornstarch diet and pentoxiphylline.

Related reports
This is the first report highlighting the association between congenital malformation of portocaval shunt and trisomy 8. The cause of the unusual and previously unreported features in our case such as recurrent jaundice with bile ductal paucity and IHBRD remains unknown.

Term explanation
Congenital extrahepatic portosystemic shunts (CEPS), or Abernethy malformation is a congenital anomaly characterized by shunting of the portal venous blood to the systemic circulation.

Experiences and lessons
CEPS should be considered in the differential diagnosis of a child presenting with developmental delay and liver dysfunction. A simple bedside Doppler ultrasound by an experienced radiologist can help in the identification of this rare anomaly.

Peer review
Article reports the association between a very rare but known congenital malformation of portocaval shunt with not yet reported hepatic ductal paucity and trisomy 8 and also highlights vascular anomalies as one of the causes of recurrent cholestatic jaundice.

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TOPIC HIGHLIGHT

Christos Triantos, MD, PhD, *Series Editor*

Primary prevention of bleeding from esophageal varices in patients with liver cirrhosis

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Abstract

Variceal bleeding is a life threatening situation with mortality rates of at least 20%. Prophylactic treatment with non-selective beta blockers (NSBBs) is recommended for patients with small varices that have not bled but with increased risk for bleeding. The recommended treatment strategies on primary prevention of variceal bleeding in patients with medium and large-sized varices are NSBBs or endoscopic band ligation. Nitrates, shunt surgery and sclerotherapy are not recommended in this setting. In this review, the most recent data on prevention of esophageal variceal bleeding are presented. Available data derived from randomized-controlled trials suggest both treatment strategies, and according to Baveno V consensus in portal hypertension "the choice of treatment should be based on local resources and expertise, patient preference and characteristics, side-effects and contra-indications".

Key words: Cirrhosis; Portal hypertension; Esophageal varices; Primary prevention; β-Blockers; Endoscopic band ligation

Core tip: The significance of primary prevention of bleeding from esophageal varices in patients with liver cirrhosis is major, considering the high mortality rates that accompany the acute bleeding episode. Current management guidelines suggest the use of either non-selective beta-blockers or endoscopic band ligation with same efficacy between them. In this review, we summarize data from randomized clinical trials or prospective studies together with meta-analytical data, when applicable, to present the most updated recommendations on primary prevention of esophageal variceal bleeding.

INTRODUCTION

Bleeding from esophagogastric varices is a life-threatening condition with an incidence of 5%-15% in patients with liver cirrhosis and mortality rates of at least 20%[1,2], despite improvements in the management of these patients. The term pre-primary prophylaxis is used to define the prevention of development and growth of varices. The term primary prophylaxis refers to the prevention of the first variceal bleeding in patients with liver cirrhosis and consists of two main treatment strategies, non-selective beta blockers (NSBBs) aiming to reduce hepatic venous pressure gradient (HVPG) below 12 mmHg or by 20% from baseline levels, and endoscopic band ligation (EBL) performed until variceal eradication[3].

In this review, we discuss the most recent data on primary prevention of variceal bleeding using data from randomized controlled trials (RCTs), prospective studies or meta-analyses focusing mainly on probability of bleeding, mortality and adverse events. We searched MEDLINE

database, Scopus, and ISI Web of Knowledge search system using the textwords "esophageal varices", or "primary prevention of variceal bleeding", or "management of varices" and major Gastroenterology and Liver meetings.

DIAGNOSIS OF VARICES

The esophagogastroduodenoscopy (EGD) is the gold standard for the diagnosis of esophageal varices and should be performed every 2-3 years in patients with compensated cirrhosis and no varices at initial endoscopy, and every 1-2 years in patients with small varices[4]. In patients with decompensated cirrhosis, EGD should be performed yearly[4]. There is a great interest in identifying non-invasive factors to diagnose esophageal varices but currently there is no evidence that their predictive accuracy is equal to that of EGD. Such factors include platelet count, spleen size, portal vein diameter, Child-Pugh score, presence of ascites, albumin levels and transient elastography[5].

In a recent prospective study[6], spleen stiffness (SS) and liver stiffness (LS) were measured by transient elastography in 200 patients with liver cirrhosis of whom 124 (71%) had esophageal varices. There was a significant difference in median LS ($P = 0.001$), SS ($P = 0.001$), LS-spleen diameter to platelet ratio score (LSPS) ($P = 0.001$), and platelet count to spleen diameter ratio (PSR) ($P = 0.001$) between patients with and without esophageal varices. LS $\geq$ 27.3 kPa had a sensitivity of 91%, specificity of 72%, and a diagnostic accuracy of 86% in predicting esophageal varices. LSPS $\geq$ 3.09 had sensitivity and specificity of 89% and 76%, respectively, and a PSR cut-off value of 909 or less had sensitivity of 64%, specificity of 76%, and diagnostic accuracy of 68% in predicting esophageal varices. SS $\geq$ 40.8 kPa had a sensitivity of 94%, specificity of 76%, and diagnostic accuracy of 86% for predicting esophageal varices. SS was significantly higher in patients who had large varices (56 *vs* 49 kPa, $P = 0.001$) and variceal bleeding (58 *vs* 50.2 kPa, $P = 0.001$).

Capsule endoscopy (CE) has been shown to be an accurate prognostic method for diagnosis of esophageal varices but there is no consensus to recommend its use in this setting. In a meta-analysis of 9 studies including 631 patients[7], the pooled sensitivity and specificity of PILL-CAM ESO capsule was 83% and 85%, respectively with positive and negative likelihood ratios of 4.09 and 0.25, respectively. In a recent, prospective study[8], the overall diagnostic yield of CE for esophageal varices was 72% (51 of 71 esophageal varices detected by EGD). The diagnostic yield was significantly greater for F2/F3 esophageal varices than for F1 (87% *vs* 61%, $P = 0.03$) and for varices located at locus superior or locus medialis than those located at locus inferior (85% *vs* 55%, $P = 0.01$). The diagnostic accuracy of CE for gastric varices was low (1 of 29 gastric varices detected by EGD), whereas for portal hypertensive gastropathy was 69% (24 of 35). EGD is superior to CE in grading of esophageal varices because capsule lacks air insufflation.

RISK OF FIRST VARICEAL BLEEDING EPISODE

The major predictive factors of first variceal bleeding episode are the size of varices, the severity of liver dysfunction and the endoscopic presence of red wale marks[9]. However, the combination of these, fails to predict all episodes of bleeding. Thus, new and more accurate predictive factors are needed to predict the first bleeding episode considering the importance to identify the cohort of patients who are mostly in need for prophylactic therapy. A significant factor associated with rupture of varices is an HVPG higher than 12 mmHg[10], considering that a high HVPG relates directly to a high variceal wall tension. Goulis *et al*[11] have proposed that, in patients with large varices and a high wall tension, the release of endotoxin into the systemic circulation during episodes of bacterial infection results in a further increase in portal pressure through the induction of endothelin and possibly vasoconstrictive cyclo-oxygenase products. Furthermore, endotoxin-induced nitric oxide and prostacyclin could inhibit platelet aggregation, thus resulting in variceal rupture. Patients with cirrhosis and bacterial infection demonstrate a heparin effect using heparinase I-modified thromboelastography and have anti-Xa activity[12,13]. A heparin effect was reported immediately after the bleeding episode in patients with liver cirrhosis suggesting a possible association with continued variceal bleeding or early rebleeding[14].

PRE-PRIMARY PROPHYLAXIS

The rate of development of varices in patients with cirrhosis and no varices at initial endoscopy is 8% per year[4] and the strongest predictor for their development is a HVPG higher than 10 mmHg[4]. In a large RCT[15] of 213 patients with cirrhosis and portal hypertension (minimal HVPG of 6 mmHg), the effect of NSBBs (timolol) on the development of esophageal varices or the occurrence of variceal bleeding was assessed (timolol-group, $n = 108$; placebo-group, $n = 105$). During follow-up (mean 54.9 mo), no significant difference was observed between the timolol-group and the placebo-group, regarding development of varices (39% *vs* 40%, respectively; $P = 0.89$). Serious adverse events were more common in the timolol group (18% *vs* 6%, $P = 0.006$). However, the development of varices was less frequent in patients with a baseline HVPG lower than 10 mmHg and in those with a decrease of HVPG $\geq$ 10% at one year. Thus, NSBBs reduce portal pressure; however, they seem to have no effect on the development of varices. According to current evidence, the use of NSBBs in patients with cirrhosis and no varices is not recommended for the prevention of their development[4]. Treatment of the underlying liver disease may decrease portal hypertension and prevent its clinical complications, according to the recent Baveno consensus[3].

Development of large varices in patients with small

varices at initial endoscopy occurs at a rate of 8% per year[4]. The factors associated to the growth of small varices are decompensated liver cirrhosis (Child-Pugh class B or C), alcoholic etiology of cirrhosis and the presence of red wale marks at initial endoscopy[4]. The efficacy of NSBBs on preventing the progression of small to large varices is debated[16,17]. In a randomized double-blind controlled trial[16] aiming to evaluate propranolol in the prevention of the development of large varices in patients with cirrhosis and small or no varices, 102 patients were randomized to receive propranolol (160 mg/d) and 104 to receive a placebo. The proportion of patients with large varices was 31% in the propranolol group and 14% in the placebo group (*P* < 0.05), at 2 years. However, one third of patients were lost to follow-up after 2 years. In a placebo-controlled trial[17], 161 patients with cirrhosis and small esophageal varices were randomized to nadolol (*n* = 83) or placebo (*n* = 78). The dose of nadolol was adjusted to decrease heart rate by 25%. During follow-up (mean: 36 mo), 9 and 29 patients from nadolol and placebo group respectively, developed large varices. At the end of follow-up, the cumulative risk was 20% *vs* 51% (*P* < 0.001). In addition, the cumulative probability of variceal bleeding was lower in the nadolol group (*P* = 0.02), but there was no difference in survival between groups (*P* = 0.33). Treatment withdrawal because of adverse effects was higher in the nadolol group (*P* = 0.01).

According to current treatment guidelines[4], in patients with cirrhosis and small varices that have not bled but with increased risk of bleeding, NSBBs are recommended. In cases of low risk for variceal bleeding, NSBBs can be used, although their long-term benefit has not been well established[4].

PRIMARY PREVENTION OF VARICEAL BLEEDING

Both shunt surgery and sclerotherapy have been abandoned for primary prevention, mainly because of the high incidence of complications[18-21]. According to Baveno V consensus[3], the current treatment strategies for medium/large-sized varices are NSBBs or EBL, which are both effective in decreasing rates of bleeding and mortality. NSBBs are splachnic vasoconstrictors which reduce portal pressure and increase portal resistance through a decrease in portal venous inflow[4]. Endoscopic treatments have no effect on portal circulation as they act locally by obliteration of varices.

NSBBs vs no intervention
Nine randomized clinical trials enrolling 966 patients compared NSBBs with a non-active treatment[22]. The incidence of bleeding was significantly reduced (OR = 0.54, 95%CI: 0.39-0.74), particularly in patients with medium-sized or large varices or in patients with varices and HVPG higher than 12 mmHg. The number needed to treat (NNT) to prevent one bleeding episode was 11. However, only a trend towards reduced mortality was observed (OR = 0.75, 95%CI: 0.57-1.06). In another meta-analysis[23] which analyzed data from four randomized trials (286 patients received b-blockers-propranolol in 203 and nadolol in 83-and 303 patients received placebo), the mean percentage of patients without upper gastrointestinal bleeding after two years was 78% ± 3% in the treatment group and 65% ± 3% in the placebo group (*P* = 0.002), whereas the 2-year survival rate was 71% ± 3% and 68% ± 3%, respectively (*P* = 0.34). The efficacy of b-blockers in the prevention of bleeding or bleeding-related mortality was the same, independently of the cause and severity of cirrhosis, ascites and size of varices. However, when propranolol is discontinued, the risk of variceal hemorrhage returns to what would be expected in an untreated population[24].

The hemodynamic response to treatment with b-blockers is considered appropriate when HVPG is decreased below 12 mmHg or by ≥ 20% of baseline values, 1-3 mo after initiation of treatment. The acute hemodynamic response to b-blockers (20 min after administration of propranolol) was shown useful to predict the long-term risk of first bleeding by reducing HVPG ≥ 10% from baseline values[25,26].

In a recent study[27], patients with esophageal varices with HVPG measurement before and during propranolol treatment were included. HVPG responders were kept on propranolol (PROP group), and non-responders were treated with carvedilol (CARV group). HVPG responders were 36% (37/104), whereas 56% (38/67) non-responders achieved hemodynamic response with carvedilol (the remaining patients were treated with EBL). Carvedilol achieved a greater decrease in HVPG compared to propranolol (-19% ± 10% *vs* -12% ± 11%, respectively, *P* < 0.001). During a 2-year follow-up, bleeding rates were 11%, 5% and 25% for PROP, CARV and EBL, respectively (*P* = 0.0429). Hemodynamic responders showed lower mortality compared to the EBL group patients (PROP 14%/CARV 11% *vs* EBL 31%, *P* = 0.0455). Thus, it seems that carvedilol is more efficient than propranolol to decrease HVPG and it was recently suggested that it might be the beta blocker of choice for portal hypertension[28].

NSBBs have also the potential to protect against spontaneous bacterial peritonitis (SBP) in cirrhotic patients, considering that infection is a risk factor for variceal bleeding[11]. In a meta-analysis of three RCTs and three retrospective studies[29] (including 644 patients, 257 treated with propranolol and 387 receiving no treatment), b-blockers were evaluated against no treatment for the prevention of SBP. There was a statistically significant difference of 12.1% (95%CI: 5.5-18.8; *P* < 0.001) favoring propranolol. The NNT to prevent an additional episode of SBP was 8. In addition, NSBBs can protect against bleeding from portal hypertensive gastropathy by reducing cardiac output and inducing splachnic arterial vasoconstriction[30], whereas endoscopic treatments have no effects on portal inflow or resistance.

However, there are safety issues on the use of NSBBs in patients with cirrhosis and refractory ascites[31,32]. In a

self-control cross-over study[32], 10 patients with cirrhosis and refractory ascites treated with beta-blockers were evaluated regarding the development of paracentesis-induced circulatory dysfunction (PCID defined as an increase in plasma renin concentrations 1 wk after paracentesis). Patients underwent two clinical and biological assessments: first while receiving NSBBs and second after NSBBs discontinuation. Eight patients (80%) treated with NBBs developed PCID whereas only one patient developed PCID after beta-blocker discontinuation. Thus, a RCT comparing EBL and NSBBs in patients with refractory ascites is needed to determine the use of EBL as preferred prophylactic treatment in this subgroup of patients.

EBL vs no intervention

EBL has substituted sclerotherapy and it is the endoscopic procedure of choice in primary prevention. Meta-analysis of eight RCTs[33] showed that EBL is superior to no intervention in reducing both the risk of first variceal bleeding (OR = 0.3, 95%CI: 0.17-0.53) and mortality (OR = 0.42, 95%CI: 0.3-0.6). However, there are safety issues concerning EBL in primary prophylaxis. In a trial by Triantos *et al*[33], EBL *vs* no treatment was compared in cirrhotics with intolerance or contraindications to b-blockers. The trial had to stop prematurely due to increased bleeding. Sixty percent of the bleeding was probably iatrogenic and the authors suggested that EBL might be as harmful as sclerotherapy regarding primary prevention. However, in a prospective cohort study[34], patients with contraindications, intolerance or not responding to beta-blockers who were treated with EBL achieved protection from variceal bleeding comparable to that of good responders to beta-blockers. Furthermore, in another RCT[35,36], which compared EBL (*n* = 75) with propranolol (*n* = 77) for primary prophylaxis in cirrhotic patients with varices > 5 mm, 5 patients (6.7%) bled from ligation ulcers and the treatment-related mortality was 2.6% (*n* = 2/5).

EBL vs NSBBs

A recent meta-analysis[37] included 19 RCTS with 1504 patients (731 treated with EBL and 773 with NSBBs-propranolol in 17 trials, nadolol in one and carvedilol in one). In total, 24% (*n* = 176) randomized to EBL *vs* 23% (*n* = 177) randomized to NSBBs died and meta-analysis showed no difference in mortality between the two treatment groups (RR = 1.09, 95%CI: 0.92-1.30). Upper gastrointestinal bleeding was diagnosed for 14% (*n* = 103) with EBL and 20% (*n* = 158) with NSBBs. EBL appeared to be superior to NSBBs for this outcome (RR = 0.68, 95%CI: 0.52-0.90). EBL also had lower rate of variceal bleeding compared to NSBBs [13% (75/590) *vs* 19% (113/611), RR = 0.66, 95%CI: 0.45-0.96]. However, when analysis included trials with adequate randomization or full papers, EBL showed no superiority to NSBBs for gastrointestinal or variceal bleeding. No difference was seen between the two interventions regarding bleeding-related mortality [5.1% (29/567) *vs* 6.3% (37/585); RR =

0.85, 95%CI: 0.53-1.39]. Treatment with NSBBs was associated with dizziness, hypotension, impotence, lethargy, and peripheral edema, whereas EBL was associated with clinically important bleeding and retrosternal pain.

Combined treatment strategies

Gheorghe *et al*[38] randomly assigned 72 patients with high-risk esophageal varices listed for liver transplantation to combined treatment of EBL plus propranolol or propranolol monotherapy. During a mean follow-up of 8 mo, bleeding occurred in 6% patients in the combination group and 31% in the monotherapy group (*P* = 0.03), with 96% and 69% actuarial probability of bleeding-free survival after follow-up, respectively (*P* = 0.04). The authors suggested that combined treatment was superior to propranolol monotherapy regarding both bleeding and bleeding-related mortality. On the contrary, Lo *et al*[39] found no differences in upper gastrointestinal bleeding [26% (*n* = 18) *vs* 18% (*n* = 13), *P* = not significant], variceal bleeding [14% (*n* = 10) *vs* 13% (*n* = 9), *P* = not significant], and mortality [22.9% (*n* = 16) in both treatment groups] between patients treated with EBL combined with nadolol (*n* = 70) and nadolol alone (*n* = 70). Patients in the combination group showed a higher rate of adverse events than in nadolol monotherapy (68% *vs* 40%, *P* = 0.06). Two episodes of variceal bleeding were induced by EBL.

One RCT[40] of 144 patients (11.8% non-cirrhotic portal hypertension), has compared EBL combined with propranolol with EBL monotherapy. In this trial, the probability of bleeding, overall mortality and bleeding-related mortality were comparable between groups. Therefore, according to current evidence, combination treatment of EBL and NSBBs is not recommended for primary prevention.

Isosorbide mononitrate

Isosorbide mononitrate (IsMn) decreases portal pressure by lowering the intra-hepatic resistance through vasodilation and has been evaluated in cirrhosis considering the large number of patients with contraindications or intolerance to b-blockers[41]. The evidence concerning the use of IsMn for primary prevention of variceal bleeding is debatable[42,43]. In a recent meta-analysis[44] the effect of IsMn in primary prevention of variceal bleeding was assessed, comparing IsMn alone *vs* placebo or beta-blockers or EBL and IsMn plus beta-blockers *vs* beta-blockers or EBL. No differences in mortality were observed between IsMn and beta-blockers *vs* β-blockers (49/277 *vs* 50/275; RR = 0.95; 95%CI: 0.68-1.32), or EBL (6/31 *vs* 8/30; RR = 0.73; 95%CI: 0.29-1.84). IsMn increased the risk of bleeding compared to placebo (RR = 2.34; 95%CI: 1.10-4.97) or EBL (RR = 4.33; 95%CI: 1.57-11.92). There were no apparent differences between bleeding rates of patients randomized to IsMn alone or with beta-blockers *vs* beta-blockers or EBL. Meta-analyses of variceal bleeding found a negative effect of IsMn compared to EBL (RR = 3.31; 95%CI: 1.01-10.84), but no apparent dif-

Table 1 Summary on primary prevention of esophageal variceal bleeding			
		Management	Goal of treatment
Cirrhosis		Diagnostic endoscopy for the presence of varices	
No varices		Endoscopic surveillance	Surveillance for development of varices (every 2-3 yr in compensated cirrhosis/yearly in cases of decompensation)
Small varices	Low risk of bleeding	Endoscopic surveillance Or NSBBs	Surveillance for progression of varices (every 1-2 yr in compensated cirrhosis/yearly in cases of decompensation)
	Increased risk of bleeding[1]	NSBBs	Decrease in HVPG of at least 20% from baseline or $\leqslant$ to 12 mmHg or resting heart rate of about 55 to 60 beats/min
Medium-large varices		NSBBs Or EBL[2]	NSBBs: Decrease in HVPG of at least 20% from baseline or $\leqslant$ to 12 mmHg or resting heart rate of about 55 to 60 beats/min EBL: Variceal obliteration

[1]Child-Pugh B/C cirrhosis or red signs at initial endoscopy; [2]The choice of treatment should be based on local resources and expertise, patient preference and characteristics, side effects, and contra-indications. NSBBs: Non-selective beta blockers; EBL: Endoscopic band ligation; HVPG: Hepatic venous pressure gradient.

ference in variceal bleeding for the remaining treatment comparisons was observed. No effects on bleeding-related mortality were seen for any of the treatment comparisons assessed. Combination of IsMn and beta-blockers increased the risk of adverse events, compared to beta-blockers monotherapy (RR = 1.65, 95%CI: 1.25-2.17), as well as the number of treatment withdrawal (RR = 2.60, 95%CI: 1.55-4.38). Consequently, current evidence does not support the use of nitrates in primary prevention of variceal bleeding.

CONCLUSION

Baveno V[3] recommends both EBL and NSBBs for the prevention of first variceal bleeding (Table 1); however, there is a controversy on which one should be the first choice. Both therapies are equally effective and have no survival difference. Thus, other issues should be considered in order to determine the best therapeutic approach. Prophylactic treatment should have few adverse events, be easy to administer and inexpensive. EBL can cause fatal iatrogenic bleeding, is accompanied by increased expense, needs specialized staff and cannot prevent bleeding from portal hypertensive gastropathy. NSBBs could probably be the first choice in primary prevention, whereas EBL could be reserved for patients with contra-indications, not response, intolerance to NSBBs or lack of compliance to life-time use of drugs. The potent benefit of EBL on patients with refractory ascites should be further investigated.

Lastly, there are issues on the primary prevention of variceal bleeding that require further study including the use of carvedilol, the advancement in ligation devices with better endoscopic field of view and the evaluation of novel therapeutic agents.

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Management of cytomegalovirus infection and disease in liver transplant recipients

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Abstract

Cytomegalovirus (CMV) is one of the most common viral pathogens causing clinical disease in liver transplant recipients, and contributing to substantial morbidity and occasional mortality. CMV causes febrile illness often accompanied by bone marrow suppression, and in some cases, invades tissues including the transplanted liver allograft. In addition, CMV has been significantly associated with an increased predisposition to acute and chronic allograft rejection, accelerated hepatitis C recurrence, and other opportunistic infections, as well as reduced overall patient and allograft survival. To negate the adverse effects of CMV infection on transplant outcome, its prevention, whether through antiviral prophylaxis or preemptive therapy, is an essential component to the management of liver transplant recipients. Two recently updated guidelines have suggested that antiviral prophylaxis or preemptive therapy are similarly effective in preventing CMV disease in modest-risk CMV-seropositive liver transplant recipients, while antiviral prophylaxis is the preferred strategy over preemptive therapy for the prevention of CMV disease in high-risk recipients [CMV-seronegative recipients of liver allografts from CMV-seropositive donors (D+/R-)]. However, antiviral prophylaxis has only delayed the onset of CMV disease in many CMV D+/R- liver transplant recipients, and such occurrence of late-onset CMV disease was significantly associated with increased all-cause and infection-related mortality after liver transplantation. Therefore, a search for better strategies for prevention, such as prolonged duration of antiviral prophylaxis, a hybrid approach (antiviral prophylaxis followed by preemptive therapy), or the use of immunologic measures to guide antiviral prophylaxis has been suggested to prevent late-onset CMV disease. The standard treatment of CMV disease consists of intravenous ganciclovir or oral valganciclovir, and if feasible, reduction in pharmacologic immunosuppression. In one clinical trial, oral valganciclovir was as effective as intravenous ganciclovir for the treatment of mild to moderate CMV disease in solid organ (including liver) transplant recipients. The aim of this article is to provide a state-of-the art review of the epidemiology, diagnosis, prevention, and treatment of CMV infection and disease after liver transplantation.

Key words: Cytomegalovirus; Outcome; Hepatitis; Transplantation; Valganciclovir; Prophylaxis; Treatment

Core tip: This paper summarizes the current state in the management of cytomegalovirus disease after liver transplantation, including a review of recently updated guidelines for diagnosis, prevention and treatment.

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INTRODUCTION

Cytomegalovirus (CMV) is the single most common viral pathogen that influences the outcome of liver transplantation[1,2]. CMV is a ubiquitous herpes virus that, depending on the population studied, infects 50%-100% of humans[1,2]. Primary CMV infection in immune competent individuals presents most commonly as an asymptomatic illness or less commonly as a benign infectious mononucleosis-like syndrome. When CMV infection occurs in individuals with compromised immunity, such as liver transplant recipients, clinical disease with high morbidity may develop and, occasionally, may lead to death if untreated[1,2].

Primary infection results in viral latency in various cells, and ensures the persistence of the virus throughout the life of the host[1,2]. Such characteristic plays an important role in how liver recipients develop CMV infection. First, cellular sites of viral latency become reservoirs for reactivation during periods of inflammation (such as allograft rejection and critical illness). And second, cellular sites of viral latency serve as vehicles for transmission to susceptible hosts (*i.e.*, during blood transfusions and transplantation of liver allografts latently infected with CMV)[1-5].

CLINICAL IMPACT OF CMV ON LIVER TRANSPLANTATION

Direct CMV effects

The classic illness caused by CMV after liver transplantation is manifested most commonly as fever and bone marrow suppression (most commonly, leukopenia and neutropenia, termed CMV syndrome). CMV syndrome accounts for over 60% of CMV diseases after liver transplantation. Less commonly, CMV infection may clinically manifest as tissue-invasive disease (which may involve any organ system) (Table 1)[1]. The most common organ system involved is the gastrointestinal tract (in the form of CMV gastritis, esophagitis, enteritis, and colitis). Gastrointestinal CMV disease accounts for over 70% of tissue-invasive CMV disease cases in liver and other solid organ transplant recipients[6]. The transplanted liver allograft is also predisposed to develop tissue-invasion by CMV (*i.e.*, CMV hepatitis), and this is often manifested with symptoms that may be clinically indistinguishable from acute rejection[7].

CMV disease among liver recipients who are not receiving antiviral prophylaxis occur most commonly during the first 3 mo after transplantation[8]. Overall, it is estimated that 18%-29% of all liver transplant recipients will develop CMV disease in the absence of prevention strategy (Table 2)[4,5,9-11]. However, this incidence varies depending upon donor and recipient CMV serologic status; it may be as high as 44%-65% in CMV D+/R-, or as low as 1%-2% among CMV D-/R- patients (who may still acquire the virus from natural transmission or through blood transfusion). The incidence is between

Table 1 Direct and indirect clinical effects of cytomegalovirus after liver transplantation

Direct effects	Indirect effects
CMV syndrome	Acute allograft rejection
Fever	Chronic allograft rejection
Myelosuppression	Vanishing bile duct syndrome
Malaise	Chronic ductopenic rejection
Tissue-invasive CMV disease[1]	Hepatitis C virus recurrence
Gastrointestinal disease	Allograft hepatitis, fibrosis
(colitis, esophagitis, gastritis,	Allograft failure
enteritis)	Opportunistic and other infections
Hepatitis	Fungal superinfection
Pneumonitis	Nocardiosis
CNS disease	Bacterial superinfection
Retinitis	Epstein-Barr virus and PTLD
Mortality	HHV-6 and HHV-7 infections
	Vascular thrombosis
	New onset diabetes mellitus
	Mortality

[1]Any organ system may be affected by cytomegalovirus (CMV). Data adapted from Ref. [104]. PTLD: Post-transplant lymphoproliferative disease; HHV: Human herpes virus.

Table 2 Estimated incidence of cytomegalovirus disease during the first 12 mo after liver transplantation

	Use of anti-CMV prophylaxis for 3-6 mo	
	Yes[1]	No
CMV D+/R-	12%-30%	44%-65%
CMV D+/R+	2.70%	18.20%
CMV D-/R+	3.90%	7.90%
CMV D-/R-	0%	1%-2%
All patients	4.80%	18%-29%

[1]Most cases occur as delayed-onset cytomegalovirus (CMV) disease. CMV disease occurs rarely during prophylaxis with oral valganciclovir. Data adapted from Ref. [4,5,92,104]. D: Donor; R: Recipient.

8%-19% among CMV-seropositive (CMV R+) liver transplant recipients[4,9,11].

The incidence of CMV disease is markedly reduced in liver transplant recipients who received 3 mo of valganciclovir or oral ganciclovir prophylaxis. The CMV disease incidence rates are 12%-30% in CMV D+/R-, and < 10% of CMV R+ liver transplant recipients who received 3 mo of antiviral prophylaxis[3,4,9,11-13]. The onset of disease in these patients occurs during first 3-6 mo after completing antiviral prophylaxis; hence, the term late-onset CMV disease[3]. To reduce the incidence of late onset CMV disease, there have been efforts to prolong prophylaxis to 6 mo in CMV D+/R- liver recipients. There is limited data available on the incidence of late-onset CMV disease after 6 mo of prophylaxis, although this is estimated to be further reduced by half (*e.g.*, about 15% of CMV D+/R- liver recipients).

Indirect CMV effects

CMV has a variety of indirect effects that are believed to be mediated by the ability of the virus to modulate the immune system (Table 1)[1,2]. CMV is a potent up-regu-

Table 3 Actors associated with increased risk of cytomegalovirus disease after liver transplantation

CMV D+/R- > CMV R+
Allograft rejection
High viral replication
Mycophenolate mofetil
Anti-thymocyte globulin
Alemtuzumab
Human herpesvirus-6
Human herpesvirus-7
Renal insufficiency
Deficiency in CMV-specific CD4+ T cells
Deficiency in CMV-specific CD8+ T cells
Toll-like receptor gene polymorphism
Mannose binding lectin deficiency
Chemokine and cytokine defects (IL-10, MCP-1, CCR5)
Expression of immune evasion genes
Programmed cell death 1 expression
Others[1]

[1]Others include re-transplantation, volume of blood transfusion, sepsis and other factors associated with high tumor necrosis factor-α secretion. D: Donor; R: Recipient; IL-10: Interleukin-10; MCP-1: Monocyte chemotactic protein-1; CCR5: Chemokine (C-C motif) receptor 5; CMV: Cytomegalovirus.

lator of alloantigens, which increases the risk of acute rejection and chronic allograft dysfunction[14]. CMV has been associated with vanishing bile duct syndrome and ductopenic rejection that leads to chronic cholestasis and allograft failure[15-17]. A higher incidence of vascular and hepatic artery thrombosis has been reported in liver recipients with CMV disease, and this effect is postulated to result from infection of the vascular endothelial cells[18,19].

The immunomodulatory effects of CMV may account for a higher predisposition to develop opportunistic infections due to fungi, other viruses, and bacteria[20,21]. CMV-infected transplant recipients are more likely to develop Epstein-Barr virus-associated post-transplant lymphoproliferative disorders, or develop co-infections with other viruses such as human herpesvirus (HHV)-6 and HHV-7[20-22]. Co-infection with HHV-6 and HHV-7 is significantly associated with an increased predisposition to CMV disease[23-25]. Similarly, there is a significant association between CMV and hepatitis C virus (HCV) recurrence after liver transplantation[26-31], and this is clinically manifested as a more accelerated clinical course of HCV recurrence[29,31]. A recent retrospective study of 347 HCV-infected liver recipients observed that CMV infection increased by 1.5 times the risk of allograft fibrosis, while CMV disease increased by 3.4 times the risk of allograft inflammation[32]. A significant association between CMV infection and metabolic disease such as post-transplant diabetes mellitus has been reported. In a recent study of 169 non-diabetic liver recipients, CMV infection was a significant risk factor for development of new-onset diabetes after transplantation[33].

Impact on mortality
Through direct, indirect and possibly immunomodulatory mechanisms, CMV is associated with higher risk of death after liver transplantation[20,34,35]. The use of intravenous (IV) and oral ganciclovir has reduced the incidence of CMV disease and the risk of death due to CMV[20,36-38]. Despite these improvements in CMV prevention with use of antiviral drugs, late-onset CMV disease continues to occur, particularly among CMV D+/R- liver transplant recipients. Notably, late-onset CMV disease remains significantly associated with increased risk of mortality after liver transplantation[35]. In an analysis of 437 liver transplant recipients, CMV disease occurred in 37 patients (8.5%) and its occurrence was independently associated with a 5-fold increased risk of all-cause mortality, and 11-fold increased risk of infection-related mortality[35].

RISK FACTORS FOR CMV DISEASE AFTER LIVER TRANSPLANTATION

Lack of pre-existing CMV-specific humoral immunity
The most important risk factor for CMV disease after liver transplantation is a lack of effective CMV-specific immunity. In the clinical setting, this is best measured by serology to detect immunoglobulin G against CMV. Specifically, CMV D+/R- patients are at highest risk of CMV disease[4,20], while CMV R+ patients have modest and CMV D-/R- have the lowest risk of CMV disease after liver transplantation (Table 3).

Drug-induced suppression of immune function
Drug-induced immunosuppression impairs the ability of liver recipients to mount an effective immune response against CMV, thereby predisposing to higher risk of CMV disease[4,20]. Immune dysfunction is particularly intense with the use of lymphocyte-depleting drugs, as either induction or rejection therapy[39,40]. When alemtuzumab, an anti-CD52 lymphocytic antibody, is used for short-course induction therapy, the risk of CMV disease is not significantly increased[41,42]. However, when alemtuzumab is used as treatment for rejection, the risk of CMV disease is higher suggesting that rejection per se also increases the risk[42]. Basiliximab and daclizumab are associated with lower risk of CMV disease compared to anti-thymocyte globulin[43].

The combined effects of drugs for maintenance immunosuppression have been associated with CMV disease[1,2,20], although specific agents such as mycophenolate mofetil, when used at high doses has also been implicated to increase the risk[44,45]. In contrast, some of the newer immunosuppressive drugs such as sirolimus and everolimus [mammalian target of rapamycin (mTOR) inhibitor] have been associated with lower risk of CMV disease[46,47]. These observations have generated special interest in the use of the mTOR agents for patients at high risk of CMV disease.

Defects in innate immunity
Inherent defects in innate immunity, such as mutations

in innate immunity-associated genes, increase the risk of CMV disease (Table 3). In a pilot study in 92 liver recipients with chronic HCV, the R753Q single nucleotide polymorphism (SNP) in the Toll-like receptor 2 (*TLR2*) gene was associated with a higher CMV replication and higher incidence of CMV disease. TLR2 is a pattern recognition receptor that senses the presence of CMV and signals the immune cells to produce antiviral peptides and cytokines; the R753Q SNP impairs this immunologic cascade[48]. A larger study of 737 liver recipients confirmed that TLR2 R753Q SNP was significantly and independently associated with CMV disease after liver transplantation, especially for tissue-invasive disease[49].

The lectin pathway of complement activation is also important in the innate immune response to CMV. Mannose binding lectin levels or mutation in its gene has been assessed as prognostic indicators of CMV disease after transplantation[50]. In a study of 295 liver recipients, whose donors were also genotyped for SNPs in mannose-binding lectin (MBL2), Ficolin-2 (FCN2) and MBL-associated serine protease genes, the risk of CMV infection was 2.77 fold higher with the gene profile of the donor and 4.57 fold higher for the combined MBL2 and FCN2 donor-recipient mismatch profile. These results were independent from donor-recipient CMV serostatus[51].

Other immune measures, such as programmed death-1 expression[52] have also been assessed for their association with CMV infection. In one study, programmed death-1 receptor up-regulation was significantly associated with incipient and overt CMV disease and with CMV viremia[52].

Lack of CMV-specific cell-mediated immunity
Cell-mediated immunity are the most essential components to the control of CMV after liver transplantation[40]. Hence, measuring CMV-specific cell-mediated immunity is a promising strategy in CMV management after transplantation[53]. In one study, secretion of interferon-γ by CD8^{+} T cells during *in vitro* stimulation with CMV peptides was associated with a lower incidence of CMV disease in solid organ transplant recipients (including liver recipients)[54]. A variety of CMV-specific T-cell assays are currently being developed including QuantiFERON-CMV assay, ELISpot assay, and intracellular cytokine staining for IFN-γ using flow cytometry. The principle of these assays relies on the detection of cytokine (most commonly interferon-γ) production following *in vitro* stimulation with CMV antigens[55]. Recently, QuantiFERON-CMV assay was studied in a multi-center study that enrolled 124 high-risk (D+/R-) solid-organ transplant (including liver) recipients. Twenty five percent of patients had positive result, 65.3% had a negative result, and 9.7% had an indeterminate result. At 12 mo follow-up, patients with a positive QuantiFERON-CMV assay had a significantly lower risk of CMV disease (6.4%) compared to those with negative (22.2%) and indeterminate result (58.3%). The assay provides a positive and negative predictive values for protection from CMV disease of 0.90 (95%CI: 0.74-0.98)

and 0.27 (95%CI: 0.18-0.37), respectively[53,56]. Collectively, these studies indicate that immune monitoring of CMV-specific T-cell responses may have a potential to predict individuals at increased risk of CMV disease, and may be useful in guiding the use of prophylaxis.

Allograft rejection
Allograft rejection can trigger CMV reactivation after transplantation[13]. The cytokines released during acute rejection, particularly tumor necrosis factor-α[57], could transactivate CMV from latency[58,59]. Subsequent therapy for allograft rejection (intensified immunosuppression with the use of high doses of steroids or lymphocyte-depleting drugs) enhances viral replication by impairing the generation of an effective CMV-specific cell-mediated immunity[60]. In a bidirectional relationship, CMV increases the risk of allograft rejection[61].

Virus-to-virus interactions
Interactions among reactivated viruses have been proposed to enhance the risk of CMV disease after liver transplantation[22,23,27-31]. HHV-6 increases the risk of CMV disease after liver transplantation[22,23,25]. Likewise, HCV-infected liver transplant patients have a higher incidence of CMV disease[62], although the data in the era of valganciclovir prophylaxis has refuted this observation[26].

Viral burden and other factors
The risk of CMV disease after liver transplantation is associated, in direct proportion, with viral burden and the degree of CMV replication[9,24,63,64]. Other factors associated with CMV disease after liver transplantation include cold ischemia time, bacterial and fungal infections and sepsis, the amount of blood loss, fulminant hepatic failure as the indication for liver transplantation, age, female gender, and renal insufficiency[2,3,20,65].

PREVENTION OF CMV DISEASE AFTER LIVER TRANSPLANTATION

There are two major strategies for CMV disease prevention after liver transplantation: (1) preemptive therapy; and (2) antiviral prophylaxis. For preemptive therapy, patients are monitored for evidence of CMV replication by sensitive assays, most commonly using quantitative nucleic acid amplification tests by PCR and less commonly by detection of pp65 antigenemia, and upon the detection of asymptomatic CMV replication, antiviral therapy is administered preemptively to prevent progression to symptomatic clinical disease. In contrast, antiviral prophylaxis entails the administration of antiviral drugs such as valganciclovir to all patients at risk of CMV disease after liver transplantation[20]. Both of these strategies are similarly effective in preventing CMV disease after liver transplantation[4,5,66-69]. However, there has not been a large prospective well-controlled randomized trial directly comparing preemptive therapy and prophylaxis in liver

Figure 1 Suggested algorithm for preemptive therapy. Figure adapted from Ref. [62].

transplant recipients. In a retrospective study comparing the two approaches in liver transplant recipients, antiviral prophylaxis was more effective in prevention of CMV disease in high risk D+/R-, but there were no differences in acute rejection, opportunistic infections, or rate of mortality[40,70]. Another retrospective study reported the incidence of CMV viremia was 4.9% and 50.0% ($P < 0.001$) at 3 mo in the antiviral prophylaxis and preemptive therapy groups, respectively, but the rates were expectedly reversed, at 24.6% and 8.3% ($P = 0.026$), respectively at 6 mo; the reversal of the rates during the latter period accounts for the higher rates of late onset CMV disease with antiviral prophylaxis[71]. An NIH-sponsored prospective study is being conducted in six transplant centers in the United States to compare the efficacy and safety of antiviral prophylaxis *vs* preemptive therapy in CMV D+/R- liver transplant recipients.

According to the recently updated American Society of Transplantation (AST) and The Transplantation Society (TTS) guidelines, preemptive therapy may be an option in CMV D+/R- liver transplant recipients, however, many authorities prefer to use antiviral prophylaxis in this high-risk population and reserve preemptive therapy for lower-risk populations[39,72]. The main reason for this preference for antiviral prophylaxis is the rapidity of CMV replication in CMV D+/R- liver recipients, which may escape detection with once weekly CMV surveillance. Indeed, antiviral prophylaxis has been used by the majority of American and European transplant

centers in preventing primary CMV disease in high-risk CMV D+/R- liver transplant recipients[73,74]. Moreover, primary antiviral prophylaxis has the added benefit of reduction in bacterial and fungal opportunistic infections and mortality[34,35,37,75].

Preemptive therapy

The basic principle of preemptive therapy is to detect the presence of early CMV replication prior to the onset of clinical symptoms, so that antiviral therapy is administered early in order to prevent the progression of asymptomatic infection to clinical disease[64,66,67,69,76]. An example of a preemptive algorithm is shown in Figure 1. Preemptive therapy has the potential advantage of targeting therapy to the highest risk patients and thereby decreasing drug costs and toxicity. The success of this approach relies on several aspects including: (1) the optimal laboratory test and frequency and duration of monitoring; (2) selection of the appropriate population for preemptive therapy; and (3) choosing the type, dose and duration of an antiviral drug.

The two laboratory methods used for CMV surveillance for preemptive therapy are pp65 antigenemia assay and nucleic acid testing (NAT). During the past decade, clinical laboratories have been moving towards preference for NAT over antigenemia, mainly for assay sensitivities, performance and logistics. The pp65 antigenemia assay, a semi-quantitative assay based on detection of CMV pp65 antigen in infected leukocytes, has comparable sensitivity to CMV NAT[77], but it needs to be processed within 6-8 h of blood collection, it requires a large sample volume, it has subjective interpretation of results, and is labor-intensive. Accordingly, quantitative NAT is now the preferred method for detecting CMV after transplantation[78]. The assay has a better precision and faster turnaround time[79]. Because of its quantitative ability, the assay can distinguish between active viral replication (typically with high-level viremia) from latent virus (low-level viremia if using highly sensitive tests)[78]. In the past, NAT lacked standardization, and this prevented the generation of widely applicable viral load thresholds for various clinical applications. In 2011, CMV viral load standardization was made possible with the release of the World Health Organization (WHO) calibrator standard. A recent study applied this assay in the plasma samples of 267 solid organ (including liver) transplant recipients. This study demonstrated that patients with pretreatment CMV DNA of less than 18200 [4.3 log (10)] IU/mL have 1.5 fold higher chance for CMV disease resolution. Likewise, CMV suppression to less than 137 [2.1 log (10)] IU/mL is predictive of clinical response to antiviral treatment[80].

The optimal interval and duration of monitoring for preemptive therapy is still unknown, but guidelines recommend once weekly CMV NAT for 12 wk after liver transplantation. If a patient shows viremia above a defined threshold during the surveillance period, antiviral therapy (with oral valganciclovir or intravenous ganciclovir) should be initiated and continued until CMV

Table 4 Currently available antiviral drugs for cytomegalovirus prophylaxis and treatment in liver transplant recipients

Drug	Route	Usual adult prophylaxis dose	Usual adult treatment dose	Comments on use and major toxicity
Ganciclovir	Intravenous	5 mg/kg once daily	5 mg/kg twice daily	Intravenous access; leukopenia
Ganciclovir	Oral	1 g three times daily	Not applicable	Low oral bioavailability; high pill burden
Valganciclovir	Oral	900 mg once daily	900 mg twice daily	Ease of administration; leukopenia
Foscarnet	Intravenous	Not recommended	60 mg/kg every 8 h (or 90 mg/kg every 12 h)	Second-line drug Intravenous access; nephrotoxicity
Cidofovir	Intravenous	Not recommended	5 mg/kg once weekly × 2 then every 2 wk thereafter	Third-line drug Intravenous access; nephrotoxicity

viremia is no longer detectable[55,72]. Several studies have reported the success of IV ganciclovir or oral valganciclovir for preemptive treatment of CMV infection in liver transplant recipients, including high-risk CMV D+/R- patients[68,76]. However, some studies have indicated that preemptive therapy may not be completely effective in CMV D+/R- liver recipients since the replication kinetics of CMV in immune-deficient individuals is so rapid[63] that it may escape detection with once weekly surveillance[9,58,66]. Indeed, in our clinical experience, nearly 25% of CMV D+/R- liver recipients who developed CMV disease were not identified early despite weekly CMV PCR assay[9,58,66]. Accordingly, the recently updated AST and TTS guidelines prefer antiviral prophylaxis in CMV D+/R- liver recipients. In contrast, preemptive therapy is recommended for preventing CMV disease in CMV-seropositive liver recipients[55,72].

Clinical trials have demonstrated the efficacy of preemptive therapy in CMV disease prevention[66-68,76]. Three meta-analyses that collectively analyzed data from prospective clinical trials demonstrated the benefits of preemptive therapy in preventing CMV disease[35,36,68]. When conducted properly, preemptive therapy, with the use of IV ganciclovir or oral valganciclovir resulted in the reduction of CMV disease by about 70%[37,38,75]. Moreover, preemptive therapy is much less likely associated with late onset CMV disease (unlike in antiviral prophylaxis, as discussed below)[66,67]. Currently, valganciclovir is the most commonly used drug for preemptive therapy[73], and in one non-controlled study, it was demonstrated to be as effective in terms of clinical and virologic response, when compared to IV ganciclovir)[66,67]. In addition, preemptive therapy may be beneficial in reducing the indirect effects of CMV, although to a much lesser degree compared to antiviral prophylaxis. In one study, the incidence of major opportunistic infections, bacteremia, bacterial infection, HCV recurrence, and rejection were not significantly different between liver transplant patients who received preemptive therapy and those who did not have CMV reactivation[81].

Antiviral prophylaxis

Antiviral prophylaxis is highly effective in preventing the direct effects, and there is increasing evidence that it reduces the indirect effects of CMV after liver transplantation[4,5,37,38,75]. Compared to placebo or no treatment, patients who received antiviral prophylaxis had lower incidence of CMV disease (58%-80% reduction) and CMV infection (about 40% reduction)[75]. In one meta-analysis, a 25% reduction in the incidence of acute allograft rejection was observed[37]. In two studies, a reduction in all-cause mortality was observed[37,75], mainly due to a decline in CMV-related death[75]. A reduction in the incidence of other herpes viruses, bacterial, and protozoan infections were also observed[37]. Because of these additional benefits, liver transplant centers prefer the use of antiviral prophylaxis over preemptive therapy in the prevention of CMV disease, particularly in CMV D+/R- liver transplant recipients[73]. Table 4 shows the currently available antiviral drugs for CMV prophylaxis and treatment in liver transplant recipients.

Valganciclovir vs ganciclovir prophylaxis

Ganciclovir-based regimen is more effective than acyclovir or immunoglobulins in reducing the incidence of CMV disease after liver transplantation. In one study, the administration of IV ganciclovir for 90-100 d reduced the incidence of CMV disease in CMV D+/R- liver transplant recipients to 5.4% (compared to 40% in patients who received less than 7 wk of prophylaxis)[44]. Oral ganciclovir, administered at 1000 mg PO three times daily, was compared to placebo, and there was a significant reduction in the 6-mo incidence of CMV infection (51.5% *vs* 24.5%, $P < 0.001$), and CMV disease (19% *vs* 5%, $P < 0.001$) in liver transplant recipients[4], including CMV D+/R- patients (44% *vs* 15%, $P = 0.02$) and patients who received antilymphocyte antibodies (33% *vs* 5%; $P = 0.002$)[4]. Among CMV R+ liver transplant recipients, oral ganciclovir for 12 wk reduced the incidence of CMV disease to 1% (compared to 7% in patients who received acyclovir)[82]. Oral ganciclovir, however, is poorly absorbed, and its oral administration results in low systemic ganciclovir levels[83].

Valganciclovir provides systemic ganciclovir levels that are comparable to IV ganciclovir[83,84]. Pharmacokinetic studies indicate that a 900 mg dose of valganciclovir achieves a similar daily area under the concentration time curve (AUC_{24}) as an IV dose of 5 mg/kg of ganciclovir[83]. The role of valganciclovir in the prevention of CMV disease after liver transplantation was evaluated in a multicenter randomized non-inferiority clinical trial that compared it with oral ganciclovir in a cohort of 364 CMV D+/R- solid organ (including liver) transplant recipients (Figure 2). Among all solid organ transplant

Figure 2 Time to the onset of cytomegalovirus disease in solid organ transplant recipients who received three mo of oral ganciclovir or valganciclovir prophylaxis. Data obtained from the study by Paya *et al*[5].

recipients, the 6-mo incidence of CMV disease was 12% and 15% in the valganciclovir and oral ganciclovir groups, respectively. Follow-up at one year, demonstrated that the incidence of protocol-defined CMV disease in all patients was 17% and 18% with valganciclovir and oral ganciclovir, respectively[5].

However, in a subgroup analysis of the 177 liver transplant recipients, the incidence of CMV disease was 19% in the valganciclovir group as opposed to only 12% in the ganciclovir group. There was also a higher incidence of tissue-invasive CMV disease in the valganciclovir group[5]. As a result of these findings, valganciclovir did not gain approval from the United States food and drug administration (US-FDA) for prophylaxis against CMV disease after liver transplantation. A recent meta-analysis of 5 controlled clinical studies, including 380 liver transplant recipients who received valganciclovir (450 or 900 mg daily) prophylaxis, showed the overall CMV disease rate was 12%, and the rate among D+/R-patients was 20%. The risk of CMV disease with valganciclovir was 1.8-fold higher than oral ganciclovir. For CMV D+/R- patients, the risk of CMV disease was 2-fold higher than oral ganciclovir. The risk of CMV disease remained significant with valganciclovir 900-mg daily dose, but not with the 450 mg dose. The risk of leukopenia with valganciclovir was 1.9-fold higher than those using oral ganciclovir[85]. Despite these findings, and even if not FDA-approved for this indication, valganciclovir remains as the most widely used drug for CMV prophylaxis after liver transplantation[73].

Maribavir prophylaxis

Maribavir, an investigational oral benzimidazole riboside with *in vitro* activity against CMV, was compared to oral ganciclovir, for prophylaxis in 303 high-risk liver transplant recipients. In this randomized, double blind, multicenter controlled trial, maribavir was less effective than oral ganciclovir for the prevention of CMV disease. Significantly fewer patients who received oral ganciclovir prophylaxis had confirmed CMV disease or CMV infection compared to maribavir at 100 d (20% *vs* 60%, *P* < 0.0001) and at 6 mo (53% *vs* 72%, *P* = 0.0053) after liver transplantation. Because of this finding (and the results of the bone marrow transplant trial), the clinical development of maribavir for CMV management is on hold[86].

CMV immunoglobulin

A combination of anti-CMV drugs and CMV immunoglobulin has been used in a clinical practice for prophylaxis. A pooled analysis of previous studies revealed a combination regimen may reduce severe CMV disease and mortality in solid organ transplant recipients; however the finding has been debated[87,88].

Late-onset CMV disease

In many high-risk CMV D+/R- individuals, the use of antiviral prophylaxis for 100 d has only delayed the onset of CMV disease to 3-6 mo after liver transplantation[3,5,13]. In our analysis of 67 CMV D+/R- liver transplant recipients who received 3 mo of oral ganciclovir and valganciclovir prophylaxis, the two-year incidence of CMV disease was 29%, and was similar between the two drugs (22% *vs* 28%, *P* = 0.63)[3]. The most common presentation of late-onset CMV disease was CMV syndrome, with fever and bone marrow suppression[3]. In less than half of the patients, CMV manifested as tissue-invasive disease, and frequently affected the gastrointestinal tract[3]. Factors such as age[3], female gender[3,89], renal dysfunction[77], and allograft rejection[13] predisposed to the development of late-onset primary CMV disease. Late-onset CMV disease appears to be clinically less severe, although it is associated with significant mortality after liver transplantation[35].

Because of the negative effect of late-onset CMV disease on overall outcome, a better method for CMV prevention is needed among CMV D+/R- liver transplant recipients. The recently updated AST and TTS guidelines suggest that the duration of antiviral prophylaxis may be prolonged from the standard 3 mo to 6 mo in CMV D+/R- liver transplant recipients[41,81]. This recommendation is based on the trial that investigated the approach in CMV high-risk D+/R- "kidney" transplant recipients. In the Improved Protection Against Cytomegalovirus in Transplantation study, the incidence of CMV disease was significantly lower in the 200 d *vs* 100 d of prophylaxis at the end of 1 year (16.1% *vs* 36.8%, *P* < 0.0001) and the result was persistent up to 2 years after transplantation (21.3% *vs* 38.7%, *P* < 0.001)[90,91]. In a retrospective study on 203 liver transplant recipients who received valganciclovir 900 mg daily for 3 to 6 mo, the overall incidence of CMV disease was 14%. The incidence was highest in D+/R- (26%) compared to 16% in D+/R+ group and 7% in D-/R+ group[92]. However, it is emphasized that 6 mo of antiviral prophylaxis has not yet been studied prospectively in the liver transplant recipients, and that valganciclovir is not FDA-approved for the prevention of CMV disease after liver transplantation. In addition, there are theoretical concerns about ganciclovir resistance and drug toxicity particularly with leukopenia with prolonged prophylaxis, although these were not demonstrated in the clinical trial. The cost of prophylaxis will need to be evaluated with the use of prolonged prophylaxis.

In summary, the duration of prophylaxis in D+/R- liver transplant recipients should generally be between

3 and 6 mo. For seropositive patients with either donor seropositive or seronegative, a majority of the experts suggested that 3 mo of prophylaxis is sufficient[55].

Hybrid approach

A new strategy has been utilized in some transplant centers to prevent late-onset CMV disease is hybrid strategy in which preemptive monitoring is initiated after completing prophylaxis. A retrospective study of 199 liver transplant recipients [including 23 (11%) high-risk D+/ R- patients] who received 3 mo of valganciclovir prophylaxis and were monitored by CMV antigenemia after prophylaxis (twice a month up to month 6, and monthly until one year). The results were modest at best[93], possibly due to difficult and non-standardized logistics of this approach[94].

TREATMENT OF CMV DISEASE AFTER LIVER TRANSPLANTATION

The first line treatment of CMV disease after liver transplantation is IV ganciclovir or valganciclovir[62,76,93]. In contrast, oral ganciclovir should not be used for the treatment of CMV disease because of its poor bioavailability[20]. In addition, the degree of pharmacologic immunosuppression should be reduced if possible[20].

In a multi-center non-inferiority trial, 321 solid organ (including liver) transplant recipients with non-severe CMV disease were randomized to valganciclovir (900 mg twice daily) or IV ganciclovir (5 mg/kg twice daily) for a fixed 21-d course, followed by valganciclovir (900 mg once daily) maintenance treatment for 4 wk; the proportion of patients with viral eradication at 21 and 49 d were comparable in the IV ganciclovir and valganciclovir groups[93]. The overall time to viral eradication was 21 d with valganciclovir and 19 d with IV ganciclovir. The calculated viral decay was 11.5 d with valganciclovir and 10.4 d with IV ganciclovir. Likewise, clinical resolution was not different between the two groups. It was noted that patients enrolled into this trial were mostly CMV-seropositive, the majority were kidney recipients (although there were good number of liver transplant recipients), and patients with severe CMV disease were excluded. Despite these limitations, this pivotal trial now supports the use of valganciclovir for oral treatment of CMV disease, at least in selected transplant patients[93]. IV ganciclovir is preferable to valganciclovir in patients with severe or life-threatening disease, or in patients who may have a problem with gastrointestinal absorption of oral drug. In many instances, valganciclovir is used as a step-down treatment when the clinical symptoms have resolved after an initial induction treatment with IV ganciclovir.

The duration of treatment of CMV disease should be individualized[62,77]. The persistence of the virus at the end of therapy (by PCR or pp65 antigenemia) is associated with a higher risk of clinical relapse[78]. In the recent study that evaluated the role of viral load using a WHO standard calibrated assay, the degree of viral load at the time of CMV disease diagnosis and the presence or absence of viral load at the end of treatment were significantly associated with CMV disease resolution. It is now generally accepted that multiple (at least two) weekly negative CMV PCR results should be obtained before antiviral therapy is discontinued. Although this may be true for non-tissue invasive CMV syndromes, the utility of such an approach may not necessarily apply to some tissue-invasive disease, which may manifest as "compartmentalized disease"[20].

Treatment of compartmentalized CMV disease

Compartmentalized CMV disease refers to clinical syndromes wherein the virus is detected in the affected tissues but is minimally detectable or undetectable in the blood[20]. In the current era, gastrointestinal CMV disease constitutes the vast majority of tissue-invasive cases[3,8,20], and in a number of cases, especially in CMV R+ patients, this type of CMV disease is "compartmentalized". In a retrospective study, the sensitivity of pp65 antigenemia assay (defined as detection of ≥ 1 positive cells/2×10^5 leukocytes) for diagnosis of CMV gastrointestinal disease was only 54%[79]. Such a clinical presentation is reminiscent of CMV retinitis, a very rare manifestation of tissue-invasive CMV disease after transplantation, that is often not accompanied by viremia[75,80]. This dilemma brings to the forefront the limitation of viral load monitoring in assessing duration of treatment. In our clinical practice, it is not uncommon to have negative blood PCR assay even when there remains histologic evidence of tissue invasion. Accordingly, it has been suggested to perform colonoscopy or upper endoscopy to document clearance of gastrointestinal CMV disease prior to discontinuation of therapy. However, our retrospective review of this practice suggests that this should not be generalized to all patients with gastrointestinal CMV disease. We observed that relapse of gastrointestinal CMV disease was significantly associated with extensive involvement of gastrointestinal tract at the time of diagnosis[81]. In contrast, CMV serologic conversion, degree of viral load, treatment duration, maintenance therapy, and endoscopic findings at the end of therapy were not significantly predictive of CMV relapse. Our experience indicates that endoscopic evidence of resolution of gastrointestinal disease may not be necessary in mild to moderate disease as long as sufficient therapy is provided[81].

Treatment of ganciclovir-resistant CMV disease

Ganciclovir-resistant CMV is now emerging as an important complication of prolonged antiviral drug use after transplantation[2,20,44]. Currently, ganciclovir-resistant CMV is very rarely seen in liver transplant recipients (while it is relatively more common after kidney-pancreas and lung transplantation). The estimated incidence of ganciclovir-resistant CMV after liver transplantation is < 0.5%[95,96]. Several studies have identified risk factors for ganciclovir-resistant CMV[2,20,44], including CMV D+/R- status, high levels of viral replication, potent

immunosuppressive therapy, and suboptimal ganciclovir levels. The vast majority of drug-resistant cases involve the selection of viral strains with UL97 (kinase) mutation[2,20,44,83,84]. UL97 mutation generally confers resistance to ganciclovir, although in some cases, a concomitant UL54 mutation (CMV DNA polymerase) is also observed, in which case, cross-resistance with cidofovir and/or foscarnet is likely.

Drug-resistant CMV is associated with significant morbidity and mortality, and there is a very limited number of antiviral drugs (which are often toxic) available for treatment[82]. Drug-resistant CMV should be suspected when viral load or antigenemia rises or does not decline to undetectable levels despite IV ganciclovir treatment. In our retrospective study of 225 CMV D+/R- solid organ transplant recipients who received 3 mo of valganciclovir prophylaxis, CMV disease occurred in 65 patients (29%), including four (8%) caused by drug-resistant CMV, judged by the failure of the viral load to decline to undetectable levels while on IV ganciclovir treatment. The diagnosis is confirmed by genetic analysis to demonstrate mutational changes in UL97 and UL54 genes encoding for kinase and polymerase, respectively[40,82]. In patients where foscarnet or cidofovir was used, nephrotoxicity was a major and common adverse effect[85].

Other potential drugs for treatment of multi-drug resistant CMV include the off-label use of CMV Immunoglobulin (Cytogam®), adoptive infusions of CMV-specific T cells, leflunomide (an immunosuppressive drug), and artesunate (anti-malaria drug), although data supporting their use are only anecdotal[20,86]. Leflunomide acts at the stage of viral capsid assembly, not DNA replication, and therefore there is a potential use against ganciclovir-resistant strains. A single center retrospective study including 15 solid organ transplant recipients (but not including liver recipients) with drug-resistant[20,86], CMV infection treated with leflunomide monotherapy or in combination with other drugs showed some potential utility. At least half of patients (53%) had long-term responses in terms of control of CMV viremia and recurrences. The common side effects from this medication included diarrhea, anemia, and hepatic dysfunction[97].

Maribavir has also been used for treatment of drug-resistant CMV[98]. Anecdotal use in a small case series of 9 solid organ transplant recipients infected with resistant CMV showed the individual changes varied from a rapid decrease in viral load ($n = 4$) to no response ($n = 3$) with some late response slowly decreasing CMV viremia ($n = 3$)[99]. It has been used as salvage therapy at a higher dose (400 mg twice daily) for drug-resistant CMV infection, with mixed results including success in treating lower initial viral loads[97]. A new phase II trial of maribavir for salvage treatment of refractory and resistant CMV infection was launched in 2012 (ClinicalTrials.gov ID: NCT01611974).

Other investigational drugs being developed for CMV management are CMX001 and AIC246 (Letermovir). CMX001 is an orally bioavailable derivative of cidofovir with lipid acyclic nucleotide converted intracellularly to the active antiviral to avoid the high renal concentrations and nephrotoxicity[100]. It has demonstrated *in vitro* activity against CMV. It has successfully completed phase II clinical development for the prevention of CMV infection. There is an ongoing open-label, expanded-access study, CMX001-350 (ClinicalTrials.gov ID: NCT01143181), to provide access to CMX001 for patients who had no other treatment options[101]. Optimal dosing has yet to be determined, and diarrhea is a dose-limiting adverse effect. Letermovir (AIC246) is a small-molecular-weight compound with both *in vitro* and *in vivo* anti-CMV activity. It has distinct mechanism which acts late in the CMV replication cycle *via* a mechanism by not involving polymerase. Due to a lack of a human counterpart of the viral terminase complex, target-related toxicities are not expected. It also does not affect human blood precursor cells, and thus may allow the generation and expansion of CMV specific immunity during treatment. Theoretically, this may result in a lower rate of relapse after treatment of CMV infection or disease. Antiviral efficacy of letermovir was reported in phase II prophylaxis studies in HSCT recipients[102]. The successful use of letermovir in decreasing viral load has been reported in one case report of lung transplant recipient with drug-resistant CMV disease[103].

CONCLUSION

Remarkable advances in molecular diagnostics and therapeutics led to marked reduction in the incidence and severity of CMV disease after liver transplantation, and a parallel decline in associated morbidity and mortality. However, despite these improvements, CMV remains a common infectious complication and continues to negatively influence the outcome of liver transplantation. In addition to viral factors and pharmacologic immunosuppression, the role of innate and adaptive immune deficiencies is being recognized in the pathogenesis of CMV disease after liver transplantation. Such novel findings should provide additional avenues and opportunities for improving our management strategies. Indeed, there have been increasing evidence to support the use of immunodiagnostics, by measuring CMV-specific T cells, as a tool to predict the risk of CMV disease. Prevention of CMV with antiviral prophylaxis and preemptive therapy is effective, and a clinical trial assessing and comparing these two strategies in a head-to-head comparison in liver transplant recipients is currently being performed in the United States. The international standard for CMV viral load testing has allowed for standardization of viral load reporting, hence permitting the derivation of thresholds for preemptive and diagnostic protocols. Currently, valganciclovir prophylaxis is the most common approach for the prevention of CMV disease in CMV D+/R- and R+ liver transplant recipients. Hybrid approach of prevention (antiviral prophylaxis followed by preemptive therapy) has been utilized in some institutions among high-risk D+/R- liver transplant patients, but the efficacy is debatable due to inconsistency in the monitoring lo-

gistics. The practice of prolonging antiviral prophylaxis in D+/R- liver transplant recipients from 3 to 6 mo has been extrapolated from studies in kidney transplant recipients. IV ganciclovir and oral valganciclovir are the standard drugs for treatment of established CMV disease, although valganciclovir should be limited to patients with mild to moderate CMV disease. Oral valganciclovir should be avoided as initial therapy for patients with severe CMV disease and those with questionable gastrointestinal absorption. The duration of treatment should be individualized, depending upon clinical and laboratory parameters such as the decline of CMV load in the blood as measured by rapid and sensitive molecular standardized testing. In this context, it is generally recommended that treatment be continued until all evidence of active infection, such as positive CMV viral load, has resolved. Ganciclovir-resistant CMV and compartmentalized tissue-invasive disease (most commonly with gastrointestinal CMV disease) are emerging challenges to the management of CMV after liver transplantation. These, together with the common occurrence of late-onset CMV disease in high-risk patients, should serve as catalysts to the ongoing search for the optimal management strategy for CMV disease after liver transplantation.

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Clinical impact of occult hepatitis B virus infection in immunosuppressed patients

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Abstract
Occult hepatitis B infection (OBI), is characterized by low level hepatitis B virus (HBV) DNA in circulating blood and/or liver tissue. In clinical practice the presence of antibody to hepatitis B core antigen in hepatitis B surface antigen (HBsAg)-/anti-HBs-negative subjects is considered indicative of OBI. OBI is mostly observed in the window period of acute HBV infection in blood donors and in recipients of blood and blood products, in hepatitis C virus chronic carriers, in patients under pharmacological immunosuppression, and in those with immunodepression due to HIV infection or cancer. Reactivation of OBI mostly occurs in anti-HIV-positive subjects, in patients treated with immunosuppressive therapy in onco-hematological settings, in patients who undergo hematopoietic stem cell transplantation, in those treated with anti-CD20 or anti-CD52 monoclonal antibody, or anti-tumor necrosis factors antibody for rheumatological diseases, or chemotherapy for solid tumors. Under these conditions the mortality rate for hepatic failure or progression of the underlying disease due to discontinuation of specific treatment can reach 20%. For patients with OBI, prophylaxis with nucleot(s)ide analogues should be based on the HBV serological markers, the underlying diseases and the type of immunosuppressive treatment. Lamivudine prophylaxis is indicated in hemopoietic stem cell transplantation and in onco-hematological diseases when high dose corticosteroids and rituximab are used; monitoring may be indicated when rituximab-sparing schedules are used, but early treatment should be applied as soon as HBsAg becomes detectable. This review article presents an up-to-date evaluation of the current knowledge on OBI.

Core tip: In occult Hepatitis B infection (OBI), hepatitis B virus reactivation is more common in anti-HIV-positive subjects, in those in onco-hematological settings, in patients who undergo hemopoietic stem cell transplantation and in those treated with anti-CD20 or anti-CD52 monoclonal antibody. Reactivation may be severe and in nearly 20% of cases it may take a life-threatening course. The use of nucleot(s)ide analogues to prevent this reactivation is mandatory in hepatitis B surface antigen-negative/anti-hepatitis B core-positive patients in all conditions of strong and/or prolonged immunosuppression. We describe the characteristics of OBI in onco-hematological and rheumatological diseases, in solid cancers and in HIV infection.

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INTRODUCTION

Hepatitis B virus (HBV) infection is a major health problem in most countries, with approximately 2 billion people worldwide with serological evidence of previous exposure to the virus, of whom nearly 300 million have HBV chronic infection and over 1 million deaths per year are due to HBV-related cirrhosis and/or hepatocellular carcinoma (HCC)[1-6].

HBV infection is identified in most cases by the presence of circulating hepatitis B surface antigen (HBsAg), but an HBsAg-negative HBV infection has also been described [Occult B infection (OBI)][7], characterized by low levels of HBV DNA in circulating blood[8,9] and/or in liver tissue[10]. OBI has also been described as a serological condition characterized by the presence of hepatitis B core antigen (anti-HBc) in the absence of HBsAg and anti-HBs (isolated anti-HBc)[7,11-15]. OBI may be observed in the window period of acute HBV infection[16] in blood donors and in recipients of blood and blood products[9,17,18], in patients with HCV chronic infection[7,19], in cryptogenic chronic hepatitis, in patients under pharmacological suppression of the immune system[20,21] and in those with immunodepression due to HIV infection; it has also been associated to the development of hepatocellular carcinoma[22-30].

It has been shown that the hepatitis B virus maintains its pro-oncogenic properties in OBI[31] and that its presence in patients with chronic hepatitis C is associated with a higher risk of disease progression and HCC development[32-36] and with a reduced response to alfa interferon treatment[37-39]. The clinical importance of OBI is also underscored by the need for nucleot(s)ide treatment to prevent the recurrence of HBV infection in HBsAg-negative/anti-HBc-positive patients in various immunosuppressive settings[40-42].

This review article presents an up-to-date evaluation of the current knowledge on OBI, focusing in particular on the clinical approach in onco-hematological and rheumatological diseases, solid cancers and HIV infection.

OCCULT HBV INFECTION AND IMMUNOSUPPRESSION

Reactivation of HBV infection in patients under immunosuppressive treatment is a well-known, life-threatening event described in HBsAg-positive patients (overt HBV infection) and in subjects with OBI[20,21,42-50]. The reactivation of HBV infection, overt or occult, is characterized by a marked enhancement of viral replication during immunosuppressive therapy, with a wide spread of HBV to uninfected hepatocytes and a substantial increase in the HBV DNA serum level followed by the restoration of the immune function after treatment withdrawal and consequent cytotoxic-T-cell-mediated necrosis of HBV-

Figure 1 Virological and biochemical dynamics of reactivation of occult hepatitis B infection. HBV: Hepatitis B virus; ALT: Alanine aminotransferase; HBsAg: Hepatitis B surface antigen.

infected hepatocytes usually responsible for a hepatic flare and in some instances for liver failure and even death[42]. A schematic representation of the dynamics of serum HBV DNA and alanine aminotransferase (ALT) before and during the reactivation of OBI is shown in Figure 1.

Both in overt and occult infection the risk of HBV reactivation is estimated as high when immunosuppression is marked, particularly in onco-hematological patients (21%-67%), in those receiving hematopoietic stem cell transplantation and in those treated with the anti-CD20 monoclonal antibody rituximab or with the monoclonal anti-CD52 antibody alemtuzumab, both responsible for profound, long-lasting immunosuppression[20,21,43,51-59]. Under these conditions HBV reactivation has a mortality rate close to 20%, due to a hepatic failure or to the progression of the underlying disease due to the discontinuation of specific treatment[51,60,61]. Besides host factors, also some virological characteristics have been described as possibly associated with HBV reactivation. In 7 of 84 HBsAg-negative/anti-HBc-positive patients treated for hematological diseases or solid cancer, HBV reactivation was due to non-A HBV genotypes, core promoter and/or precore HBV mutants. In these 7 patients mutations known to impair HBsAg antigenicity were also detected[62]. A precore stop mutation (A1896) was detected in one patient with genotype Bj who developed fulminant liver failure[63]. Also sub-genotype D1 has been described as possibly associated with HBV reactivation in two studies, one from Egypt and one from Italy[21,64].

There is general agreement for the use of nucleos(t)ide analogues to prevent HBV reactivation in HBsAg-positive immunosuppressed patients, whereas it is still a matter of debate whether subjects with occult HBV infection should be treated or closely monitored for early treatment once HBsAg positivity has developed.

PHARMACOLOGICAL PROPHYLAXIS OF OCCULT HBV INFECTION IN DIFFERENT CLINICAL SETTINGS

Hematological diseases

A crucial role in the reactivation of OBI is played by the

severity and duration of immunosuppression, which in turn reflects the extent of immunodepression due to the hematological disease and of the degree of immunosuppression induced by chemotherapy. The drugs commonly responsible for HBV reactivation are those used in hematological malignancy, such as fludarabine, anthracyclines, high dose corticosteroids[51,52] and, more recently, rituximab (anti-CD20) and alemtuzumab (anti-CD52)[53].

Evidence has become available in hematological malignancy that the reactivation of occult HBV infection is frequent in patients treated with rituximab or fludarabine in the absence of lamivudine prophylaxis[21,60,61]. However, due to the retrospective nature of most studies published, the geographical differences in HBV epidemiology and the genetic differences in HBV and the host have not been investigated, and the prevalence of HBV reactivation varies widely (from 3% to 45%)[21,48,52,65-69]. The first prospective study[65] on 244 occult HBV carriers with malignant lymphoma showed a reactivation in 8 (3.3%) cases, with a higher risk of reactivation in patients receiving rituximab plus corticosteroids than in those under a rituximab-sparing schedule. In a prospective study on patients with diffuse large B-cell lymphoma (DLBCL), Yeo *et al*[20] reported reactivation of HBV infection in 5 of 21 (23.8%) patients treated with rituximab plus cyclophosphamide, adriamycin, vincristine and prednisone (R-CHOP) and in none of the 25 patients receiving only CHOP. Recently Fukushima *et al*[48] observed reactivation in 2 (4.1%) of 48 HBsAg-negative/anti-HBc-positive patients. In addition, in 150 patients with lymphoma and a resolved HBV infection who received rituximab-based chemotherapy, Hsu *et al*[70] described an incidence of HBV reactivation and of HBV hepatic flares of 10.4 and 6.4, respectively, per person per year. Matsui *et al*[71] followed up for a median period of 20.5 mo 59 patients with isolated anti-HBc and lymphoma treated with rituximab-based chemotherapy and observed HBV reactivation in 4 (6.8%).

Lower prevalences of HBV reactivation in HBsAg-negative patients after rituximab-based therapy have been reported in two studies from eastern Asia, 1.5% and 4.2%, respectively[46,72]. In another Asian study only one (2.3%) of 43 DLBCL patients treated with an R-CHOP regimen showed reactivation of HBV replication[73], for which a remission was obtained with antiviral therapy with no need to discontinue chemotherapy. Koo *et al*[74] described HBV reactivation in two (3%) of 62 HBsAg-negative/anti-HBc-positive patients treated with rituximab-based chemotherapy who did not undergo anti-HBV prophylaxis. More recently, the Asia Lymphoma Study Group investigated for HBV reactivation HBsAg-positive patients and HBsAg-negative/HBcAb-positive patients who received rituximab-based chemotherapy; the study was retrospective and performed on 340 patients, with a reactivation rate of 2.4% in subjects with OBI and 27.8% in HBsAg-positive patients[75].

The different frequency of cases with reactivation of occult HBV infection in different countries may explain, at least in part, the discordance in different national guidelines on lamivudine prophylaxis, some of which indicate the use of this nucleoside analogue for a pharmacological prophylaxis of HBsAg-negative/anti-HBc-positive patients undergoing highly immunosuppressive treatment for onco-hematological diseases[76], and others conclude that the information available does not allow any routine prophylaxis to be recommended for these patients[77].

The data of a recent meta-analysis, however, suggest that rituximab-based chemotherapy increases the risk of HBV reactivation in HBsAg-negative/anti-HBc-positive patients with non-Hodgkin lymphoma[78], an observation of clinical importance to be taken into consideration for future guidelines.

Rheumatological disease

Biological therapies targeting tumor necrosis factor-alpha (TNF-α) have been used increasingly over the last decade to treat various immune-mediated inflammatory diseases such as rheumatoid arthritis, psoriasis, psoriatic arthritis, ankylosing spondylitis and Crohn's disease[42]. Studies carried out over this period showed that monoclonal antibodies against TNF-α (anti-TNF-α) and high doses of steroid treatment may induce HBV reactivation in patients with overt HBV infection[79-82], thus suggesting the need for anti-HBV pharmacological prophylaxis for inactive HBsAg carriers[83] and treatment for patients with HBsAg-positive chronic hepatitis.

The reactivation of OBI during anti-TNF therapy has not been extensively investigated and the data available are anecdotal and mostly from case reports. In a recent evaluation of the literature data, HBV reactivation was found in only 8 (1.7%) of 468 HBsAg-negative/anti-HBc-positive patients with rheumatological diseases treated with anti-TNF[82]. In addition, none of 20 HBsAg-negative/HBV DNA-negative/anti-HBc-positive patients receiving anti-TNF-α for rheumatoid arthritis and spondyloarthropathy experienced reactivation of OBI during a 4-year follow up[84].

Solid cancers

The literature data give evidence of HBV reactivation in HBsAg-positive patients treated with chemotherapy for solid tumors[85-87], and, consequently, pharmacological prophylaxis for inactive HBsAg carriers and therapy for patients with HBsAg-positive chronic hepatitis is recommended. Instead, the studies on HBV reactivation in patients with OBI are not conclusive, but so far no evidence of reactivation of OBI in these patients has emerged. The longitudinal study by Saitta *et al*[88] on 44 HBsAg-negative patients with solid tumors undergoing chemotherapy did not find cases with HBV reactivation. Further prospective studies are needed to improve our knowledge of the clinical importance of OBI in patients with solid cancers.

STRATEGIES TO PREVENT REACTIVATION OF OCCULT HBV INFECTION

In patients with OBI, pharmacological prophylaxis with

Figure 2 Management of occult hepatitis B infection in hematological and rheumatological diseases and in solid cancers. [1]Entecavir instead of Lamivudine, when appropriate. HBsAg: Hepatitis B surface antigen; ALT: Alanine aminotransferase; Anti-HBc: Hepatitis B core antigen; TNF-α: Tumor necrosis factors-alpha.

nucleot(s)ide analogues should be based on the HBV serological status (anti-HBc-positive or -negative), the underlying diseases (onco-hematological diseases, hematopoietic stem cell transplantation or others) and the type of immunosuppressive treatment (rituximab, high doses of corticosteroids, anthracyclines, or others). In anti-HBc-positive patients, the prophylaxis with anti-HBV nucleos(t)ide analogues is indicated in hematopoietic stem cell transplantation and in onco-hematological diseases when high doses of corticosteroids and rituximab are used, whereas monitoring is indicated in all other clinical conditions or when rituximab-sparing schedules are used (Figure 2). The literature data have shown the efficacy of lamivudine in preventing HBV reactivation in these subsets of patients[17,43,61]. Also entecavir has been proposed in the prophylaxis of reactivation of OBI. In a randomized controlled trial[89] 80 patients with CD20+ lymphoma and resolved hepatitis B were randomly assigned to a prophylactic schedule with entecavir, started before rituximab-based chemotherapy and stopped 3 mo after its discontinuation, or to be treated with entecavir once HBV reactivation and reversion to HBsAg positivity had occurred (control group). During an 18-mo follow up, HBV reactivation occurred in 2.4% of patients who underwent entecavir prophylaxis and in 17.9% of cases in the control group ($P < 0.05$).

Although the efficacy of lamivudine and entecavir in preventing the reactivation of OBI has never been compared in published studies, we can conclude, in agreement with current international guidelines[2,76], that lamivudine, despite of its low genetic barrier, remains the nucleos(t)ide analogue of choice for the prophylaxis of reactivation of OBI because of its low cost and of the low or absent HBV viremia in OBI. Instead, entecavir should replace lamivudine for patients with advanced liver diseases for whom reactivation of OBI might be life threatening.

Monitoring of pharmacological prophylaxis is not

standardized and the widespread habit of determining HBsAg at three-monthly intervals is not the optimal strategy in all clinical conditions. In addition, it is not fully understood how long the pharmacological prophylaxis should last in order to prevent the reactivation of HBV infection. Observational studies suggest extending the prophylaxis to the 12th month after the discontinuation of immunosuppressive treatment, but in some case reports HBV reactivation occurred later, especially in patients treated with rituximab[39,90]. Recently, Tonziello *et al*[39] described a reactivation of OBI in an HBsAg-negative/anti-HBc-positive woman with non-Hodgkin lymphoma occurring 20 mo after rituximab discontinuation despite lamivudine prophylaxis covering the 4 mo of rituximab administration and the 12 mo after its discontinuation. Concluding on this point, prospective studies are needed to ascertain whether the pharmacological prophylaxis should be extended to the 18th month after the discontinuation of immunosuppressive treatment in patients receiving rituximab-based chemotherapy.

MANAGEMENT OF REACTIVATION OF OCCULT HBV INFECTION

Once reactivation has occurred, effective antiviral treatment should be immediately administered. Lamivudine monotherapy has been demonstrated to be ineffective in reducing mortality[21]. Consequently, patients should be treated with drugs of high potency and high genetic barrier such as entecavir or tenofovir.

OCCULT HBV INFECTION IN HIV-POSITIVE SUBJECTS

As a consequence of the availability of highly active antiretroviral therapy (HAART), which has determined a substantial improvement in the patients' survival, vi-

Figure 3 Management of occult hepatitis B infection in anti-human immunodeficiency virus-positive subjects. HCV: Hepatitis C virus; ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HAART: Highly active antiretroviral therapy; HBsAg: Hepatitis B surface antigen.

ral hepatitis has become the leading cause of morbidity and mortality in HIV-infected subjects. In these patients particular attention should be paid to OBI since it may have a strong clinical impact because of damage to the immune system and its frequent occurrence in HIV-HCV coinfected patients.

EPIDEMIOLOGY OF OBI IN HIV-POSITIVE SUBJECTS

The prevalence of OBI in HIV-infected patients is controversial, and the associated risk factors and the effect of HAART undefined. Also controversial is the role of the immune system in the genesis of OBI in HIV-positive patients. Some investigators never observed OBI in patients with CD4 counts > 500 cells/μL and concluded for a significant association of OBI with lower CD4 counts[91]. Other investigators, however, described no association of OBI with the CD4 count[92].

The prevalence of OBI in HIV-HCV coinfected patients varies in different studies from less than 1% to 40%[22,93-102].

OBI may also be observed in anti-HIV-positive patients with chronic HBV/HCV coinfection, due to an HBsAg serum clearance consequent to a strong inhibitory effect of the HCV genome on HBV replication[103].

In HIV subjects a strong association between OBI and HCV infection has been observed in several studies[28,101,104-106]. In contrast, Jardim *et al*[107] reported no significant difference in the rate of OBI in HIV-positive patients with or without HCV coinfection.

The discrepancies in the rate of OBI in the different studies most probably reflect differences in HBV, HCV and HIV epidemiology in different countries, a variation in the sensitivity of the assays used to detect HBV DNA and the retrospective nature of some of the studies.

Cassini *et al*[108] proposed a new approach to the detection of HBV DNA. By the genomic amplification of the partial S, X and precore/core regions, these Authors analyzed for the presence of HBV DNA the circulating blood, liver tissue and peripheral blood mononuclear cells

(PBMC). HBV DNA was never found in serum samples of the 24 HBsAg-negative patients investigated, but was detected in the liver tissue in 7 (29%) and in PBMC in 6 (86%) of these 7. The clinical value of these data should be confirmed in larger studies, but they suggest that the detection of HBV DNA in PBMC offers a useful tool to identify OBI. Morsica *et al*[104] analyzed 1593 anti-HIV-positive patients enrolled in the Italian Cohort of Antiretroviral Naïve patients and found 175 (11%) HBsAg-negative/anti-HBc-positive patients: 27of these 175 (15%) patients had detectable HBV DNA in plasma. This prevalence was significantly higher (21%) in the 101 anti-HCV-positive than in the 74 (8%) anti-HCV-negative, regardless of the immune status, HIV load, or ART regimen.

CLINICAL SIGNIFICANCE OF OBI IN HIV-POSITIVE SUBJECTS

The impact of OBI on the prognosis of HIV-positive patients is still unclear. In our previous study[22] on the clinical and virological impact of OBI in HIV-positive patients, we analyzed 115 HBsAg-negative patients, 86 of whom were observed in a long-term follow-up. A hepatic flare occurred more frequently in the 17 patients with occult HBV infection than in the 69 without (64.7% *vs* 24.6%; *P* < 0.005). These preliminary data still await confirmation in larger studies.

Lamivudine-based HAART is effective in suppressing HBV replication even in anti-HIV-positive patients with OBI, as most of these cases clear HBV DNA during treatment. However, in approximately half of the lamivudine-treated patients, occult HBV replication became detectable again after 12-40 mo of lamivudine treatment, always associated with a hepatic flare. Although the presence of *YMDD* mutants in patients who became HBV-DNA-positive under lamivudine was not detected, most probably because of the low levels of plasma HBV DNA, the hypothesis that lamivudine induced the selection of *YMDD* mutants in these anti-HIV-positive subjects with OBI cannot be ruled out. In another study the ALT and aspartate aminotransferase levels showed a tendency to increase more frequently in patients with OBI than in those without[104].

Concluding on this point, OBI seems relatively frequent in anti-HIV-positive patients, particularly in cases with HIV/HCV co-infection. This makes the clinical condition of HIV/HCV co-infection more complex since OBI may unfavorably affect the outcome of the liver disease. Lamivudine seems inadequate for a long-term prevention of hepatic flares in anti-HIV-positive patients with OBI and possibly in reducing the risk of HBV oncogenicity. Therefore, for these patients a high potency, high genetic barrier nucleos(t)ide analogue should be preferred (Figure 3).

CONCLUSION

Clinicians should pay careful attention to OBI since it has

been demonstrated that it occurs with some frequency and may have clinical consequences.

Further studies are needed to better define the biological and clinical role of OBI and to identify new measures to prevent or limit its unfavorable clinical action. It would be of particular benefit to investigate the oncogenicity of OBI, particularly in anti-HIV-positive subjects, in order to devise new strategies for the prevention of HCC.

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REVIEW

Challenge of liver disease in systemic lupus erythematosus: Clues for diagnosis and hints for pathogenesis

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Abstract

Systemic lupus erythematosus (SLE) encompass a broad spectrum of liver diseases. We propose here to classify them as follows: (1) immunological comorbilities (overlap syndromes); (2) non-immunological comorbilities associated to SLE; and (3) a putative liver damage induced by SLE itself, referred to as "lupus hepatitis". In the first group, liver injury can be ascribed to overlapping hepatopathies triggered by autoimmune mechanisms other than SLE occurring with higher incidence in the context of lupus (*e.g.*, autoimmune hepatitis, primary biliary cirrhosis). The second group includes non-autoimmune liver diseases, such as esteatosis, hepatitis C, hypercoagulation state-related liver lesions, hyperplasic parenchymal and vascular lesions, porphyria cutanea tarda, and drug-induced hepatotoxicity. Finally, the data in the literature to support the existence of a hepatic disease produced by SLE itself, or the occurrence of a SLE-associated prone condition that increases susceptibility to acquire other liver diseases, is critically discussed. The pathological mechanisms underlying each of these liver disorders are also reviewed. Despite the high heterogeneity in the literature regarding the prevalence of SLE-associated liver diseases and, in most cases, lack of histopathological evidence or clinical studies large enough to support their existence, it is becoming increasingly apparent that liver is an important target of SLE. Consequently, biochemical liver tests should be routinely carried out in SLE patients to discard liver disorders, particularly in those patients chronically exposed to potentially hepatotoxic drugs. Diagnosing liver disease in SLE patients is always challenging, and the systematization of the current information carried out in this review is expected to be of help both to attain a better understanding of pathogenesis and to build an appropriate work-up for diagnosis.

Core tip: The existence of liver disease associated with lupus itself, or increased susceptibility to concomitant liver diseases, either autoimmune or non-autoimmune ones, is still somewhat controversial, and difficult to diagnose. Data in the literature are scarce, and often based on case reports or clinical studies with limited patient size or histological evidence. The pros and cons to support the existence of such pathological entities, and the still preliminary studies on the mechanisms involved, are critically discussed here. We concluded that liver is often a target of systemic lupus erythematosus, and biochemical liver tests should be systematically carried out in these patients.

Bessone F, Poles N, Roma MG. Challenge of liver disease in systemic lupus erythematosus: Clues for diagnosis and hints for

INTRODUCTION

Systemic lupus erythematosus (SLE) is a systemic auto-immune disease with variable clinical presentation, usually characterized by several immunological signs and symptoms[1-3]. It primarily affects women under 50 years of age, and is diagnosed on the basis of presence of at least 4 out of 11 criteria identified by the American College of Rheumatology (ACR), either sequentially or simultaneously, namely malar rash, discoid rash, photosensitivity, oral ulcers, nonerosive arthritis, pleuritis or pericarditis, renal disorders (proteinuria or cellular casts), neurologic disorder (seizures or psychosis), hematologic disorder (hemolytic anemia, leukopenia or thrombocytopenia) and immunologic disorders (anti-DNA, anti-Sm or antiphospholipid antibodies)[4-6].

The most common symptoms are fever, weight loss, and a general lack of wellbeing and athralgia, while the most frequent signs are skin rashes. Biochemical exams typically present anemia, and increased rates of erythrosedimentation. Treatment includes nonsteroidal anti-inflammatory drugs (NSAIDs), corticoids, and immunomodulators. Death is generally caused by progressive renal insufficiency, severe impairment of the central nervous system, or multi-organic failure after systemic infection[4].

Even though, as above mentioned, alterations of skin, joints and kidney, as well as of the cardiovascular, hematological and central nervous systems, are part of the criteria indicating morbidity, the liver can also be affected[1-5]. Although a true liver disease triggered by SLE itself is a controversial issue, 25% to 50% of patients may present alterations in the liver function tests (LFTs)[7]. The for and against data in the literature to support the existence of the multiple associations of SLE with liver disease will be discussed in detail in this review. Our literature inclusion criteria limited the citation of clinical cohort studies to those written in English language and published in peer reviewed journals; only very exceptional studies in other languages were included, when dealing with topics with extremely scarce information. The quotation studies in abstract form, when equivalent full papers were unavailable, was also very exceptional, and limited to peer reviewed, highly prestigious meetings.

PREVALENCE OF BIOCHEMICAL AND HISTOLOGICAL HEPATIC ALTERATIONS IN PATIENTS WITH SLE

Subclinical liver disease is common in SLE, and 25%-50% of patients with lupus may develop abnormal liver function at some point[8,9]. The more common laboratory abnormalities associated with the different kinds of liver disease related to lupus are summarized in Table 1. In addition, an overview of the main biochemical and histological findings reported in the literature is depicted in Table 2.

Hepatomegalia is detected in 12%-55% of SLE patients, depending on the analyzed series[10]. In an original article by Mackay *et al*[11], the authors observed hepatomegalia and/or alterations in LFTs in 19 SLE patients, normal liver biopsies in 6 cases, and minimal histological changes in another 11 ones (fatty liver, portal fibrosis, and mild to moderate portal infiltrate). Histological changes compatible with chronic hepatitis with progression to cirrhosis were confirmed in the remaining 2 patients. Similar findings were obtained by Polish researchers in a study of 18 SLE patients; whereas 5 of them showed normal liver histologies, the other 13 ones showed only minimal hepatocellular changes[12]. These results do not agree with those observed by Runyon *et al*[13] who, in a retrospective review of 238 patients with SLE, observed hepatomegalia in 39% of patients, splenomegaly in 6% and jaundice in 24%. Twenty one percent of patients were defined as carriers of liver disease based on abnormal liver histologies or, in some cases, elevation of liver enzymes 2 times over the upper limit of normal (ULN).

In the same study, liver histology of 33 patients showed steatosis (36%), cirrhosis and chronic active hepatitis (12%), hepatic granulomatosis, centrilobular necrosis (9%), and chronic hepatitis and microabscesses (6%). These findings were very challenging for the common view at the beginning of 80 s, and prompted other researchers to replicate these results. However, only one year after this report, Gibson *et al*[14] failed to reproduce such a high rate of severe liver disease associated with SLE. They reported 55% of patients with increase in transaminase levels among 81 patients with SLE, and identified SLE as the only explanation for this abnormality in 29% of the cases. Histological analysis of 7 of these patients revealed portal inflammation in 5, fatty liver in 1, and active chronic hepatitis in the remaining one. They also reported a 23% increase in the levels of alanine aminotransferase (ALT)/aspartate aminotransferase (AST) and alkaline phosphatase (ALP) ($\leqslant$ 2 times ULN), with a notable predominance among patients that presented active clinical signs of SLE. All of these abnormalities normalized with steroid treatment.

A prospective analysis by Miller *et al*[15] recruited 260 patients with SLE that were followed up for a 12-mo period. In the follow-up examinations, liver enzymes levels were high in 23% of them. Clinical liver disease was observed in only 2% of the cases, while causes for liver compromise unrelated to SLE were verified in only 15% of the cases. No specific cause for liver disease other than SLE could be identified in 8% of the patients. The histological analysis carried out on 14 patients found only minimal and non-specific changes. It is noteworthy that the increase in transaminase levels in 12 out of 15 patients appeared concomitantly with lupus activity.

Ref.	Study type	Patients with SLE	NO. of patients with biochemical alterations and alteration types	Liver histological findings
Mackay *et al*[11]	Retrospective	19	(*n* = 19) ↑ AST, ALT	Minimal changes, portal fibrosis, steatosis, inflammation (*n* = 11) Normal (*n* = 6) Chronic hepatitis (*n* = 2)
Chwalińska-Sadowska *et al*[12]	Retrospective	18	NA	Minimal changes (*n* = 13) Normal (*n* =5)
Runyon *et al*[13]	Retrospective	238	(*n* = 124) ↑ AST, ALT, total bilirubin, ALP, GGT, LDH (⩾ 2 × ULN)	(*n* = 33) Steatosis (*n* = 12) Others: cirrhosis, chronic hepatitis, granulomatosis, chronic hepatitis, steatosis, cholestasis, centrilobular necrosis
Gibson *et al*[14]	Retrospective	81	(*n* = 64) ↑ AST, ALT, ALP	(*n* = 7) Portal inflammation (*n* = 5) Steatosis (*n* = 1) Chronic hepatitis (*n* = 1)
Miller *et al*[15]	Prospective	260	(*n* = 84) ↑ AST, ALT, ALP	Minimal changes (*n* = 14)
Matsumoto *et al*[17]	Retrospective	73	NA	Hepatic arteritis (*n* = 11) Steatosis (*n* = 53) RNH (*n* = 5) Viral hepatitis (*n* = 2) SLE-PBC overlap syndrome (*n* = 1) SLE-AIH overlap syndrome (*n* = 1)
Luangjaru *et al*[9]	Retrospective	225	(*n* = 80) ↑ AST, ALT (⩽ 4 × ULN)	NA
Chowdhary *et al*[7]	Retrospective	192	(*n* = 40) ↑ AST, ALT	HCV (*n* = 3) Steatosis (*n* =5) SLE-AIH overlap syndrome (*n* = 4) SLE-PBC overlap syndrome (*n* = 3) Cryptogenic cirrhosis (*n* = 1)
Piga *et al*[3]	Retrospective	242	(*n* = 59) ↑ AST, ALT (⩾ 2 × ULN)	NA
Her *et al*[138]	Retrospective	141	(*n* = 46) ↑ Total bilirubin, AST, ALT, LDH, ALP (⩾ 2 × ULN)	NA
Huang *et al*[90]	Retrospective	1533	(*n* = 134) ↑ AST, ALT (⩾ 2 × ULN during 2 yr)	Chronic Hepatitis (*n* = 6) Minimal changes (*n* = 4) Normal (*n* = 3)
Zheng *et al*[2]	Retrospective	504	(*n* = 47) ↑ Total bilirubin (13%), ALT (98%), ALP (42%), GGT (49%)	(*n* = 10) Portal blood cell infiltration (*n* = 8) Hydropic degeneration (*n* = 8) Steatosis (*n* = 2) Mild cholestasis (*n* = 2) Focal necrosis (*n*= 1) Nodular cirrhosis (*n* = 1)
Takahashi *et al*[18]	Prospective	206	(*n* = 123) ↑ AST, ALT (99%) ↑ ALP and GGT (81%)	(*n* = 25) Lupus hepatitis (*n* = 16): Unspecific reactive hepatitis (88%) Active hepatitis (12%) SLE-AIH overlap syndrome (*n* = 6): Interface hepatitis (100%) Cirrhosis (33%) SLE-PBC overlap syndrome (*n* = 3)

SLE: Systemic lupus erythematosus; ULN: Upper limit of normal; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; LDH: Lacto dehydrogenase; ALP: Alkaline phosphatase; GGT: Gamma glutamil transferase; HCV: Hepatitis C virus; PBC: Primary biliary cirrhosis; AIH: Autoimmune hepatitis.

A much lower frequency of liver abnormalities was reported by Fox *et al*[16] in a retrospective cohort of 200 patients, where an increase of liver enzymes was documented in only 2.5% of the cases. These biochemical changes were associated with liver clinic manifestations only in few cases, and had no relationship with plasmatic ribosomal-P antibodies.

Very interesting findings were published by Matsu-

Table 2 Laboratory abnormalities in the different hepatic manifestations associated with systemic lupus erythematosus

Hepatic alteration	Laboratory abnormalities
Hepatic steatosis	GGT, ALT/AST
Viral hepatitis	ALT, AST, HCV, cryoglobulinemia
Toxic hepatitis	ALP, GGT, AST/ALT, bilirubin
Nodular regenerative hyperplasia	ALT, AST, thrombocytopenia
Primary biliary cirrhosis	ALP, GGT, AMA
Autoimmune hepatitis	ANA, ASMA, gammaglobulin
Hepatic venous thrombosis	Antiphospolipidic antibodies
Lupus hepatitis	Anti-ribosomal P autoantibodies

AMA: Antimitocondrial antibody; ANA: Antinuclear antibody; ASMA: Antismooth muscle antibody; GGT: Gamma glutamil transferase; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

moto *et al*[17], who analyzed liver histology of 73 patients with SLE. They identified fatty liver as the major feature in 72% of the cases, while nodular regenerative hyperplasia, viral hepatitis, primary biliary cirrhosis (PBC), and autoimmune hepatitis (AIH) were identified as the main cause of liver disease only in few cases (6.8%, 4.1%, 2.7%, and 2.7%, respectively).

Finally, Takahashi *et al*[18] reported recently that liver dysfunction was apparent in 123 (59.7%) out of 206 patients. They identified different causes of liver dysfunction as follows: induced by drug (30.9%), caused by SLE itself (28.5%), fatty liver (17.9%), AIH (4.9%), PBC (2.4%), cholangitis (1.6%), alcohol (1.6%), and viral hepatitis (0.8%). The liver dysfunction tends to be mild, except when caused by AIH.

From the studies reported above, it is readily apparent that the published data linking liver diseases with SLE during the last four decades are highly heterogeneous, and that a high number of cases lack adequate histological documentation.

LIVER DISEASES IN THE SLE CONTEXT

The frequent association between SLE and LFT alterations may be accounted for by three possibilities, namely: (1) the existence of some kind of liver parenchymal injury associated with SLE alone, often referred to as "lupus hepatitis"; (2) the occurrence of an overlap syndrome by which SLE shows additional features of another autoimmune liver disease; and (3) the concurrency of comorbility of SLE with a non-autoimmune hepatopathy, *e.g.*, drug-induced liver damage, viral hepatitis or thrombotic liver disease, among others.

Lupus hepatitis

Although it is still a controversial issue, there is compiling evidence in the literature that lupus itself is not associated with a specific, severe and progressive liver injury. However, several authors have pointed a role for SLE in triggering an often subclinical hepatopathy, referred to as "lupus hepatitis". They described this disease as an asymptomatic hypertransaminasemia frequently associated with exacerbations of the lupus disease, which returns to normal values after corticosteroid therapy[2,14,15].

May be a part the confusion begun in the early 50's, when AIH was wrongly referred to as "lupoid hepatitis"[11]. Subsequent studies added more confusion when no serology was available to rule out overlapping chronic viral diseases [hepatitis C virus (HCV), hepatitis B virus (HBV), cytomegalovirus, *etc.*] in SLE patients with hyper-transaminasemia.

In the early 80's, Runyon *et al*[13] reactivated the debate publishing a very controversial study describing both a "canalicular cholestasis" profile and SLE-related cirrhosis as diseases triggered by lupus itself. As mentioned before, the sample analyzed in this study consisted of 33 lupus patients presenting different types of liver damage that were documented by liver biopsy, namely steatosis, chronic hepatitis, hemochromatosis, granulomatose hepatitis, cholestasis and cirrhosis. Serological and virological markers to rule out hepatitis C did not exist at this time.

As was also stressed above, another condition that is needed to rule out among SLE patients with hypertransaminasemia is an overlap with AIH, which represents a separate disease from lupus, both because of its distinct pathogenic mechanism (specific organ) and its distinctive biochemical, serological, and histological characteristics that allow for a clear differentiation.

Hypergammaglobulinemia, autoantibodies [antinuclear antibody (ANA), antismooth muscle antibody (ASMA), anti-liver-kidney microsome antibodies], a histological profile characterized by piecemeal necrosis (interface hepatitis), and a rich plama cells infiltrate are highly distinctive aspects of AIH. On the other hand, if a lupus patient presents evidence of progressive non-autoimmune chronic hepatitis characterized by persistent severe inflammatory damage, we need to consider first other probable diagnosis of chronic liver injury, such as hepatitis B or C, or other autoimmune diseases overlapping with lupus. The discrimination is further complicated by the fact that liver histopathological features in patients with lupus hepatitis are miscellaneous and non-specific, similar to those in other liver diseases. It is therefore important, before diagnosing lupus hepatitis, to rigorously rule out other liver diseases, including drug-induced liver injury, alcohol liver disease, viral hepatitis (hepatitis A, B, C, D, E, Epstein-Barr virus or cytomegalovirus), and other autoimmune-associated liver diseases [AIH, PBC, primary sclerosing cholangitis (PSC)].

A recent study by Zheng *et al*[2] based on this strict discrimination criteria reported a 9.3% lupus hepatitis incidence among 504 SLE patients evaluated. However, the prevalence reported in the literature is rather variable, with both lower[4,8,17,19] and higher[14,18,20] rate values.

Zheng *et al*[2] also reported that the prevalence of lupus hepatitis in patients with active SLE was higher than those with inactive SLE (11.8% *vs* 3.2%). The patients with lupus hepatitis mostly showed mild to moderate elevations of serum transaminase levels, though 6 patients had jaundice as the predominant feature. ALP and Gamma

glutamil transferase elevations were far less frequent. Only 12.8% had liver injury-related clinical manifestations. Lupus hepatitis responds well to moderate to high doses of corticosteroids[3].

In patients suspected to have lupus hepatitis, it has been often reported a correlation between hepatic enzymes abnormalities and autoantibodies to ribosomal P proteins (anti-ribosomal P), a highly specific marker for SLE[19,21,22]. Indeed, several reports suggest that SLE-related hepatitis may be associated with, or even caused by this autoantibody.

Anti-ribosomal P occurs in 12%-16% of patients with lupus[21-24], although this proportion increased to 30% when more sensitive methods were employed [enzyme-linked immunosorbent assay (ELISA) based upon the combination of different ribosomal-P antigens], with Caucasian ethnicity having lower values[25]. The proportion of serum anti-ribosomal P occurrence raised to 44% among SLE patients with liver dysfunction and, from them, 70% had SLE-associated hepatitis, a far higher value as compared with SLE patients suffering from other hepatic alterations, such as fatty liver (29%), drug-induced hepatitis (17%), or SLE-AIH overlap syndrome (20%)[26]. Furthermore, Koren *et al*[27] reported the development of chronic active hepatitis in a patient with SLE followed several months later by the appearance of high serum levels of anti-ribosomal P antibodies, and suggested a possible causal relationship. As for the mechanism explaining this causal relationship, anti-ribosomal P positive sera from SLE patients were found to react strongly "*in vitro*" with a polypeptide antigenically related to a 38 kD ribosomal P_0 protein present on the plasma membrane of hepatoma cells[28], thus further strengthening the possibility that anti-ribosomal P antibodies could be directly detrimental in lupus patients by inducing hepatocellular lysis, and further transaminase release. Finally, anti-ribosomal P antibodies up-regulate the expression of proinflammatory cytokines by peripheral monocytes in SLE, which may be a contributing factor for hepatitis development[29].

Given that auto-antibodies directed against eukaryotic P proteins are highly specific to SLE, they can be used as diagnostic markers of the disease. However, there is no standard methodology for its detection and titration in clinical practice. The plasma titers of this antibody often fluctuate in relation to lupus activity, and were formerly associated with neuropsychiatric kidney and liver failure[22,26].

Several isolated cases have been reported of association of anti-ribosomal P antibody occurrence with hepatitis, and also with kidney failure[27,30]. However, it was Arnett *et al*[19] the first to report this association in a cohort study in 1995. They found lupus-related hepatitis in 3% of 131 lupus patients in a retrospective study that analyzed the hepatic manifestations of SLE. The clinical outcome for these patients was variable, from a minimum, subclinical increase of transaminases to acute hepatitis and overt liver failure. Unfortunately, histological studies were not carried out in this study to correlate the degree of liver injury associated with lupus hepatitis and the levels of anti-ribosomal P antibodies.

Although these lines of evidence link anti-ribosomal P antibodies to liver damage in SLE patients, the association is still highly controversial. For example, lack of a clear association between lupus hepatitis and anti-ribosomal P levels was reported in a recently published retrospective study of 73 patients with SLE, where 12 of them (16%) were reported to have lupus hepatitis. In this group, 6 patients had a concurrent liver involvement with the diagnosis of SLE, and it occurred later during an exacerbation of the disease in the remaining 5 patients[19]. Clinical manifestations were as follows: hepatomegaly (*n* = 4), jaundice (*n* = 4), abdominal pain (*n* = 3), ascitis (*n* = 2), portal hypertension (*n* = 1), and hepatic failure with encephalopathy (*n* = 1). Despite elevated liver enzymes were noted in 11 cases and cholestasis in 8 ones, the presence of anti-ribosomal P antibodies was observed only in one case, and therefore an association between lupus hepatitis and any kind of specific antibody could not be documented. Liver biopsy in 5 patients showed chronic active hepatitis in 3 cases, chronic hepatic granulomas in 1 case, and nonspecific inflammation in another one. Although the authors showed clear evidence of immunosuppressive therapy response in most patients, liver biopsy was performed in less than half of them, and their description was not detailed enough to clearly differentiate lupus hepatitis from AIH.

In part, disagreements on the association between anti-ribosomal P antibody and lupus hepatitis can be explained by different features of the studied populations (*e.g.*, ethnicity), environmental factors affecting autoantigen expression, and distinct degrees of sensitivity/specificity of the methods used to detect anti-ribosomal P antibodies. Usually, associations between anti-ribosomal P antibody levels and hepatitis were investigated by using not well-standardized, or even "in-house" immunological methods[19,26]. Unfortunately, large cohort studies where lupus hepatitis or other SLE hepatic manifestations have been reliably documented, and where well-standardized, high sensitivity/specificity immunological methods are employed to detect anti-ribosomal P antibodies (*e.g.*, those using a mixture the ribosomal P antigens P_0, P_1, and P_2), are lacking, and we eagerly await them to confirm or deny the existence of this association.

To complicate the picture further, Calich *et al*[31] reported recently the presence of anti-ribosomal P antibodies in patients having AIH not associated with lupus (9.7%; 9/93), and suggested that this antibody predicts worse prognosis of the disease, with follow-up data showing higher prevalence of cirrhosis in anti-ribosomal P antibody-positive AIH patients (100%, 7/7). This finding suggests that anti-ribosomal P antibodies can be involved in the pathogenesis of other hepatic autoimmune diseases, apart from lupus hepatitis. The debate is still open, and it is apparent that we need more data to support the role and impact of anti-ribosomal P antibodies in both SLE and AIH pathogenesis.

Overlap of SLE with autoimmune liver diseases (overlap syndromes)

The existence of overlap syndromes linking SLE with other autoimmune liver diseases is matter of controversies since, again, the data in the literature are scarce.

According to the so called "theory of the mosaic of autoimmunity"[32], each of these associations may represent a particular variant of a major underlying autoimmune disease, which can shows up under the form of multiple autoimmune liver diseases coexisting in the same patient. Other good examples of such variants are more typical hepatic overlap syndromes, such as AIH-PBC and AIH-PSC[33].

Although AIH or PBC are rare among SLE patients taken as a whole[34], the co-existence of SLE with either of these liver diseases is not uncommon among the subgroup of SLE patients with liver enzyme abnormalities. Chowdhary *et al*[7] reported a strong association between SLE and autoimmune liver disease. They found that 8 out of 40 SLE patients (20%) were AIH carriers, while 6 (15%) showed evidence of PBC.

In another study by Efe *et al*[35], 36 SLE patients out of 147 (25%) had liver enzyme abnormalities, and 7 of them (4.7%) had SLE associated with another autoimmune liver disease. The rate rose to 19.4% when the subset of SLE patients having HLTs altered was considered and, from them, 72.3% fulfilled the criteria for AIH proposed by the International Autoimmune Hepatitis Group. The therapy with ursodeoxycholic acid, prednisone, immunosuppressive thiopurine analogs, or a combination of them, was successful in these patients.

SLE-AIH overlap syndrome

There have been very few reported cases of AIH associated with SLE. It is therefore apparent that AIH and SLE overlap syndrome is a rare condition, although its exact incidence is unclear.

Oka *et al*[36] reported 5 (3%) patients with AIH in an analysis of 162 cases of SLE meeting the ACR criteria. Similar findings were documented by Tamai *et al*[37], who found 10% of AIH in a series of 21 SLE cases.

There is evidence in the literature suggesting that SLE and AIH are different diseases, even when clinical, biochemical and serological characteristics may show overlapping features, such as the presence of polyarthralgia, hypergammaglobulinemia, and positive ANA, ASMA and anti-ribonucleoprotein[38]. In these cases, liver histology is the decisive tool to define diagnosis. The presence of cirrhosis or periportal hepatitis associated with lymphocytes and plasma cell infiltration, as well as rosette formation of liver cells, tips the scales towards AIH. On the other hand, the presence of mainly lobular and occasionally portal inflammation with a paucity of lymphoid infiltrates is more compatible with SLE. Finally, a mixed histological pattern is expected in SLE-AIH syndrome, displaying chronic hepatitis with severe inflammatory activity characterized by focal necrosis of hepatic cells, erosion of the lobular limiting plate, periportal hepatitis, infiltration by lymphocytes and plasma cells, presence of fibrosis in the portal areas and, eventually, cirrhosis[39,40]. In this context, positivity for anti-Sm antibodies, which are highly specific though relatively insensitive to SLE, helps to confirm SLE-AIH overlapping. In addition, presence of antibodies to double-stranded (ds) DNA, another hallmark of SLE, were found to be associated with poorer immediate response to corticosteroid treatment in AIH[41].

SLE-PBC overlap syndrome

PBC is also an autoimmune liver disease, and overlapping with PBC is likely to some extent. However, the co-existence of PBC and SLE is the subject of few reports in the literature, mostly based upon single case reports[42,43]. A large-scale study reported that, among 1032 PBC patients, 27 (0.03%) had also SLE[44]. Interestingly, anti-dsDNA and anti-ribosomal-P antibodies, two serological markers of SLE, were detected in 22% and 5%, respectively, of "pure" PBC patients[45].

SLE-PBC association has been documented mainly in patients with arthritis, polyserositis, and high titers of anti-native DNA and anti-mitochondrial antibodies (AMAs), two pathognomonic signs of SLE and PBC, respectively. Again, PBC can appear in a pre-existing lupus as an expression of an immunological disorder that has not been totally clarified. Osteopontin, a soluble ligand with pleomorphic immunologic activities that plays an important role in inflammation and immunity, may be a link. Osteopontin was reported to be highly expressed in the murphy roths large/lpr mouse[46], a well recognized models of SLE, and it is involved as a chemoattractant cytokine in the recruitment of macrophages and T lymphocytes in the liver granulomas in PBC[47]. Interestingly, Han *et al*[48], in a large cohort of 1141 SLE patients, confirmed the association between osteopontin and SLE.

Finally, AIH-PBC overlap syndrome has been reported to occur in 2.8% of SLE patients, suggesting the association of not only two but even three autoimmune diseases (SLE-AIH-PBC overlap syndrome)[49]. Furthermore, anti-dsDNA antibodies, which are known to be strongly associated with SLE, were detected in 60%[50] or 56%[51] of patients with AIH-PBC overlap syndrome.

SLE-PSC overlap syndrome?

Evidence for SLE-PSC overlap syndrome is limited at best, and only based upon few case reports[52-55]. Whether this clinical association indicates that some immune disorders are common to the two autoimmune diseases or whether they were casual associations remains to be ascertained.

Association of SLE with non-autoimmune liver diseases (comorbidity)

SLE patients often present comorbidity with a number of non-autoimmune liver diseases. In many cases, the prevalence of the concomitant hepatopathy is higher when associated with SLE than alone, indicating either increased susceptibility to the concomitant disease trig-

gered by SLE or *vice versa*.

Association of SLE with hepatitis C

Autoimmunity and viral infections are closely associated fields, and viruses have been proposed as a likely etiological, contributing or even triggering factor of systemic autoimmune diseases[56]. This holds true also for SLE, since some hypotheses have identified viruses as potential agents that trigger SLE, with a close relationship to the pathogenic mechanism of damage[57].

Very little association has been found between SLE and patients infected with HCV. Most reports linking the two diseases refer to the presence in these patients of skin lesions, anti-DNA antibodies, hypocomplementemia and cryoglobulinemia[57].

In a study of 134 patients carrying SLE, the presence of anti-HCV antibodies (ELISA) was observed in 18 patients (13%), while the prevalence among voluntary blood donors in a large number of countries ranges from 0.5% to 2%, only. Active infection by HCV was confirmed in 15 (11%) of the patients with positive ELISA HCV[57]. Similar results were obtained in other study where HCV was detected in 4 out of 40 SLE patients (10%), whereas prevalence among voluntary blood donors was only of 0.13%[58]. Steroid therapy in these patients did not seem to alter the HCV course[59]. Whether this reflects a true higher HCV prevalence associated to SLE or it is a mere consequence of the multiple admissions and blood transfusions that these patients are subjected remains to be defined. Large-scale studies avoiding these potential bias are awaited.

It should be on the other hand acknowledged that HCV chronic infection is associated with different biochemical and histological manifestations of autoimmunity that, in certain cases, can mimic SLE[60]. Different types of non-organ-specific autoantibodies can be detected in chronic hepatitis C (*e.g.*, anti-soluble liver antigen, ANA, AMT, rheumatoid factor) and, less frequently, it is associated with low anti-DNA titers; for example, about 20% with hepatitis C patients are ANA positive[61]. In addition, chronic hepatitis C can occur with cryoglobulinemia, which can lead to a wrong SLE diagnosis, due to the simultaneous occurrence of ANA, dermathological and renal lesions and plaquetopenia; this is why, in patients suspected to have SLE, HCV infection must be excluded using routine anti-HCV serology and, HCV-RNA tests. Several factors lead to the production of autoantibodies in HCV patients, including leakage of intracellular components due to the persistent destruction of infected cells[61], the molecular mimicry between HCV and auto-antigens[62], and the functional abnormalities of infected B lymphocytes, with production of excessive autoantibodies and cryoglobulins[63].

Fukuyama *et al*[64] reported for the first time in the literature the development of an SLE profile after interferon α-2 therapy. There are over 10 currently published cases that link the use of interferon to treat hepatitis C with the appearance of SLE associated with different levels of severity, including one patient with a serious lupus cardiomyopathy that threatened his/her life.

Although chronic infection with HCV can induce clinical and serological changes that can be confused with an autoimmune disease (arthritis, nephropathy, and cytopenias), the appearance of malar rash, discoid lesion, photosensitivity, neurological damage, high titers of ANA or anti-DNA antibodies, and anti-Sm antibody occurrence usually constitute sufficient evidence to diagnose SLE[7].

The clinician must consider three situations in the context of a HCV antibody in a patient with SLE, namely: (1) it may be a false positive HCV ELISA test due to the high levels of autoantibodies that are frequently presented in SLE patients; (2) could be true association between SLE and hepatitis C; and (3) HCV can trigger the occurrence of low levels of ANA and/or anti-DNA, associated with cryoglobulinemia, without typical skin changes[19].

One common complication of SLE patients is the so called "lupus nephritis", and HCV may play a role. Few cases of lupus nephritis coexisting with HCV infection have been described[65,66]. Although speculative, it is likely that the altered immune response in SLE facilitates HCV infection, and *vice versa,* that different autoantibodies associated with HCV infection facilitate the development of lupus nephritis due to formation of immune complex deposits in the kidneys. The increase in serum B-lymphocyte activating factor levels in chronic HCV patients with infection and SLE may be a contributing factor, by reinforcing B-cell activation and autoantibody production[67].

Association of SLE with hypercoagulation state-related liver lesions

SLE patients have a high potential to develop thromboembolic disorders that can impact on hepatic circulation[68]. The frequent presence of anti-phospholipid antibodies among these patients can include thrombotic manifestations in different territories of the splachnic vasculature, both in arterial and venous areas (thrombosis of the hepatic artery, portal thrombosis, and Budd-Chiari syndrome)[69]. Portal hypertension profiles and esophageal varices have also been reported in several cases as secondary events linked to thrombosis of the portal vein, triggered by the presence of anti-cardiolipin antibodies[10].

Regenerative nodular hyperplasia (RNH), which follows hepatic vein thrombosis and hepatic circulation disorders, has also been reported in association with SLE (Figure 1)[70]. The pathogenesis of RNH complicating SLE is believed to be related to vasculitis of intrahepatic arteries, leading to secondary portal venous obliteration and thrombosis of the adjacent portal veins[1]. Alternatively, occlusion of intrahepatic small vessels may result from coagulopathy in patients with associated anti-phospholipid syndrome[9]. It has been suggested that anti-phospholipid antibodies play a pathogenic veno-occlusive role in the pathogenesis of RNH[71].

One of the most attractive theories regarding RNH

Figure 1 Regenerative nodular hyperplasia. A: Magnetic resonance image of RNH (axial T1 FSE). Note the two hyperintense, solid nodules localized in the right hepatic lobe (arrows); B: Typical findings of a RNH lesion revealed by reticulin staining to highlight the sinusoidal architecture of the liver. Note the liver sinusoidal shrinking, mimicking a pseudonodule. RNH: Regenerative nodular hyperplasia.

origin involves the storage of immune complexes in small caliber intrahepatic vessels, and the further appearance of obliterative venopathy[70]. The liver histology pattern is characterized by the presence of multiple hepatic nodes that do not have their own walls and that, in the absence of fibrosis, are circumscribed by thin bands formed by the flattening of hepatocyte columns emulating thin fibrous membranes. This condition is another component of a long list of diseases linked to non-cirrhotic portal hypertension. It is often associated with hematological diseases and various conditions that typically present systemic impairment (rheumatoid arthritis, CREST syndrome, Felty's syndrome)[72]. Another theory suggests that the association between RNH and anti-phospholipid antibodies is due to the cellular regeneration process that begins in the liver to maintain its functional capacity after the ischemic injury induced by these antibodies in the hepatic microcirculation[68].

RNH should be suspected in any patient with both SLE and portal hypertension in the absence of cirrhosis. The diagnosis can be established after a liver biopsy. Due to the large size of the regenerative nodes, there is a chance for the needle to be positioned in an area with no histological damage, which accounts for sampling error. When RNH is to be diagnosed, laparoscopic wedge biopsy is a safe and efficient way to obtain enough tissue to preserve the hepatic architecture required for analysis, avoiding in turn the morbidity associated with an unnecessary open resection[73].

Hepatic imaging of RNH shows several additional findings, including focal nodular hyperplasia (FNH), hepatocellular adenoma, regenerative nodules, and liver metastatic disease. Computed tomography can show normal liver, numerous small nodules, or larger coalesced nodules spanning several centimeters. On nuclear medicine imaging, these lesions may take up sulfur colloid, but will remain iso- or hypodense in both arterial and portal venous phases; this helps to distinguish RNH from FNH[74]. The use of magnetic resonance imaging (MRI) to enhance diagnostic accuracy is still controversial. RNH lesions appear hyperintense on T1-weighted imaging and iso- or

hypointense on T2 images (Figure 1). However, the sensitivity and specificity are variable, according to a recent report[75].

RNH may be differentiated from large regenerative nodules (LRN) by either tomography or MRI. LRN can have a distinct presentation, and very often results in enhancing liver nodules, whereas RNH usually does not[76].

The spontaneous rupture of the liver has also been reported in patients with SLE as a serious consequence related to the occurrence of a large area of infarction, due to a thrombotic phenomena of the hepatic artery[77].

Focal disturbance of the hepatic blood supply associated with lupus might also facilitates the hyperplastic development of benign lesions in the liver, such as FNH and hemangiomas[78]. In a recent study analyzing a cohort of 35 SLE patients, FNH was observed at higher rates (5.7%) than in the normal population (0.6%-3.0%), and the same holds true for hemangiomas (54.2% *vs* 0.4%-20% in the general adult population)[79]. Whereas FNH is thought to be part of an abnormal adaptive regenerative response of the liver parenchyma to local hemodynamic disturbances[80], hemangioma formation may be also favored by an increase of angiogenic factors whose circulating levels are increased in SLE patients, such as estrogens[81], vascular endothelial growth factor, and interleukin-18[82,83]. Confirmation of an increased incidence of these kinds of hepatic benign lesions in SLE patients awaits large-scale studies.

Association of SLE with porphyria cutanea tarda

The association of SLE with porphyria cutanea tarda (PCT), the most frequent type of porphyria, is rare, and data defining whether this concomitance is pure coincidence or true association are still lacking[84-86].

Common features in both diseases may be a confusing factor. SLE is similar to PCT regarding photosensitivity, but the presence of blisters involving crusts and miliae in sun-exposed areas of PCT patients, which is characteristic of PCT but rare in SLE (< 5% of the cases)[87], can help to differentiate both diseases.

Co-existence of PCT is usually associated with an-

Table 3 Hepatotoxicity induced by drugs used in lupus treatment

Drug	Liver injury and clinical significance
Corticosteroids	Hepatomegalia
	Fatty liver
NSAIDs	Asymptomatic ALT increase
	Hepatocellular, cholestatic, or mixed injury
ASA	Acute and chronic hepatocellular injury
	(resolve with withdrawal)
Methotrexate	Asymptomatic ALT increase at high doses
	Esteatosis, fibrosis, or cirrhosis
Anti-malarial drugs[1]	Rare hepatotoxic effects
	Porphyria cutanea tarda
Azatioprine	Cholestasis, peliosis, SOS, RNH
Thioguanine	SOS, RNH, portal hypertension
Ciclophosphamide	Rare case reports at conventional doses
	SOS at high doses (resolve with dose reduction)
Mycophenolate mofetil	Asymptomatic ALT increase
	(resolve with dose reduction)
Rituximab	No liver reactions have been reported
Belimumab	No liver reactions have been reported

[1]Anti-malarial drugs: chloroquine, hydroxychloroquine. ALT: Alanine aminotransferase; NSAIDs: Non-steroidal antiinflamatory drugs; ASA: Acetylsalicilic acid; RNH: Nodular regenerative hyperplasia; SOS: Sinusoidal obstruction syndrome.

timalarial drugs for treating lupus (*e.g.*, chloroquine, hydroxychloroquine), and the regular use of these drugs in SLE patients should be considered a risk for PCT. This usually represents a diagnostic problem, given the frequent association of PCT with a long list of drugs apart from antimalarial agents, which makes the diagnosis of the cause even more complicated[88,89]. The risk associated with antimalarial drugs is dose-dependent; this is why several authors have contraindicated the daily intake of these drugs for SLE due to the risk of massive porphyrinuria, which is often associated with fever, nausea and hepatocelular injury, leading eventually to hepatic necrosis[78-81].

Association of SLE with drug-induced hepatotoxicity

Patients with SLE seem to have a relatively high rate of drug-induced hepatotoxicity (Table 3). For example, Huang *et al*[90] reported 35 cases of drug-induced hepatotoxicity among 1533 SLE patients reviewed. In another study by Takahashi *et al*[18], liver damage could be ascribed to drug-induced liver injury in 31% from a total of 123 SLE patients with overt liver dysfunction.

At the moment, it is impossible to know with certainty whether this high incidence is due to the chronic use, at relatively high doses, of different drugs commonly prescribed to treat this disease, or whether there is any kind of particular susceptibility that makes these patients prone to drug-induced hepatotoxicity. Of note, SLE patients have been shown to have elevated levels of systemic oxidative stress, which well correlated with liver enzyme elevations[91]. This relationship can be tentatively explained by drug-induced oxidative stress in the liver of these patients, with consequent liver injury. The elevated pro-oxidant liver status associated with a pro-

inflammatory conditions like SLE may also make the organ prone to develop hepatotoxicity by drugs exerting detrimental effects *via* oxidative mechanisms. Indeed, several drugs used in autoimmune disease may themselves be converted into free radicals "*in vivo*", thus aggravating oxidative damage[92,93]. Controlled, comparative studies on differential susceptibility to the same drug in patients with SLE and other autoimmune disease (*e.g.*, rheumatoid arthritis) are lacking, but they would be useful to establish whether SLE is indeed a peculiar prone condition for drug-induced liver injury.

Around 80% of SLE patients are treated with analgesic and NSAIDs, prescribed for febrile syndrome, athralgia/arthritis, serositis and/or cephale[94]. Hepatitis, fulminant hepatic failure, cholestasis, and mixed damage were reported to be caused by these compounds[95-98].

Lupus patients usually present a higher rate of NSAID-related complications than SLE-negative subjects. The most common complications are increased transaminase levels, skin rashes triggered by sun, increased retention of body fluids with arterial hypertension, gastric ulcers, and aseptic meningitis. NSAIDs should not be indicated over the counter in SLE, and prescription must always be accompanied by recommendations related to strict clinical and laboratory vigilance[94].

For many years, aspirin was the most common drug associated with SLE-related liver damage. Increments of ALT, AST and ALP have been reported in up to 25% of the SLE patients consuming high doses of aspirin (> 2 g/d)[94].

In the early 70's, the first publications appeared identifying aspirin as responsible for the hepatic damage in SLE patients[99,100]. It was not however until 1981 that Zimmerman, in a review focused on this issue, showed with certainty that aspirin generates both acute and chronic dose-dependent liver damage[101].

The onset of aspirin-induced liver disease is marked by the appearance of anorexia, nausea and non-specific pain in the upper abdomen. The patient usually does not present jaundice, and ALT and AST values are usually not more than 10 times ULN values. It is very common that AST levels are higher than ALT, and that these alterations are associated with normal ALP levels[102].

Although hepatotoxicity can occur with low levels of plasma salicylate, the mechanism is often dose-dependent, and the biochemical abnormalities revert when the drug is discontinued. In 3% of the cases, the lesion can be severe enough to lead to fatal hepatic failure. Chronic liver damage observed in the hepatic histology as a chronic active hepatitis pattern is much less common, and also returns to normality when the drug is withdrawn[103].

There is also controversial evidence that rheumatic patients usually have underlying conditions that increase the risk of aspirin-induced hepatic failure. However, SLE-related hypoalbuminemia and juvenile rheumatoid arthritis are well documented risk factors as well[104-106].

Thiopurine analogues, such as azathriopine (AZA) and 5-mercaptopurine, are immunosuppressive drugs often employed in autoimmune diseases, including their

Figure 2 **(A) Macrosteatosis, (B) microsteatosis and (C) megamitochondria (arrows), in a non-alcoholic 27-year-old patient with active systemic lupus erythematosus, treated with steroids and methotrexate (H and E staining).**

use to gain or maintain remission in SLE. Hepatotoxicity induced by thiopurine analogues occurs very often with increase in serum transaminase levels. It is associated generally with not severe liver injury, which responds to dose reduction in most patients. RNH is also a very rare but potentially severe complication of thiopurine-based therapies. It is often asymptomatic, and neither biochemical nor molecular markers are indicative of RNH. The suspicion should arise when there are clinical symptoms of portal hypertension, increments of transaminase levels, or thrombocytopenia. A liver biopsy is essential in this case to confirm diagnosis[107].

A recent review by Musumba[108] reports that inflammatory bowel disease patients treated with AZA have a cumulative incidence of RNH at 5 and 10 years of 0.6% and 1.3%, respectively, whereas those treated with high TG doses (> 40 mg/d) have an incidence of RNH of up

to 62%; this rate is even higher in patients with elevated liver enzymes and/or thrombocytopenia, as compared with those lacking these abnormalities (76% *vs* 33%).

Methotrexate (MTX) is currently the first-line therapy for early and chronic rheumatic and psoriasic arthritis, but it is also indicated to symptomatic patients with SLE[109]. The recognition of risk of chronic liver damage with MTX has prompted the need for intensive biochemical monitoring from several decades ago onwards. The frequency of hepatotoxicity varies widely according to differences in sampling, definitions of damage, dose regimens, and presence of other risk factors[110]. Although one study showed transaminase elevations higher than twice the upper limit of normal in 13% of patients[111], another report assessing 6000 patients receiving MTX, transaminase elevation was described in only 0.6% of patients[112]. Despite this wide difference, most studies concluded that prolonged use of low-dose MTX monotherapy (10 mg/wk for 2-15 years) has favorable long-term safety, and that the development of significant liver fibrosis and cirrhosis is very low[113]; rather, steatosis was the main finding when biopsies were carried out for surveillance dictated by cumulative MTX dose (Figure 2)[114]. Due to this disparity, Society's guidelines differ on how patients on MTX should be monitored to prevent MTX-induced liver fibrosis[115,116].

Although liver biopsy is still suggested in these patients in case of persistent elevation of transaminase after drug discontinuation, and for ruling out other potential cause of chronic liver disease, there is robust evidence that Fibroscan Elastography may become in a near future the gold standard for fibrosis investigation in patients treated with MTX[117,118]. Most studies concluded that MTX therapy is safe, and that Fibroscan is useful for monitoring liver fibrosis in patients treated with this drug. Conclusions drawn from several studies indicate that severe liver fibrosis is a rare event in patients treated with MTX, and that it is probably unrelated to the dose. A recent work also studied the accuracy and feasibility of Fibroscan and Fibrotest to detect MTX-induced liver fibrosis in 24 psoriasis patients[119]. The results obtained using Fibroscan and Fibrotest were compared with those obtained by liver histology. In this cohort, Fibrotest accurately predicted the presence of liver fibrosis, while Fibroscan accurately predicted the absence of liver fibrosis in MTX users. These findings suggest that a combination of approaches should prospectively be evaluated in monitoring and detecting significant MTX-induced liver fibrosis.

An association between MTX-induced toxicity and genetic polimorfism was suggested. Fisher *et al*[120] conducted a meta-analysis of published studies including 1400 patients for association of the C677T polymorphism of the gene encoding methylene tetrahydrofolate reductase (MTHFR), and over 660 patients for the A1298C variant. They observed that the former but not the latter *MTHFR* gene variant was significantly related to MTX toxicity, including hepatotoxicity (OR = 1.71; CI: 1.32-2.21, $P < 0.001$). Despite results for MTHFR

A1298C are not conclusive, C677T polymorphism appears to be a promising risk factor for the development of low-dose-MTX-induced hepatotoxicity. Only few studies reported variants in genes that are predictive for MTX-induced hepatotoxicity[121].

Recent results showed that the administration of metformin in rats receiving MTX normalized altered liver function tests and improved liver histopathological findings. Therefore, this result suggests that this drug confers hepatoprotection against MTX-induced hepatotoxicity[122].

Minor abnormalities of liver enzymes are relatively common when using anti-tumor necrosis factor (TNF) agents, such as infliximab, etanercept, and adalimumab, as anti-inflammatory and immunosuppressive compounds for the treatment of autoimmune diseases[123,124]. Severe hepatic reactions are much less common, and include jaundice, hepatitis, cholestasis, and acute liver failure[125-127]. AIH is a rare, but increasingly recognized adverse event linked to treatments with anti-TNF agents[122]. In addition, lupus-like syndrome and anti-TNF-α-induced SLE were the most common disorders listed in a registry of autoimmune diseases associated with anti-TNF-α agents[128,129]. Finally, rituximab is listed as able to reactivate HBV, even in patients with HBsAg negative and anti-HBsAg positive. This concept was recently reinforced by Seto *et al*[130], who reported a HBV reactivation rate of 24% in HBs Ag-negative, anti-HBc-positive patients undergoing rituximab-based chemotherapy for hematologic malignancies, with most of reactivations occurring during the first 6 mo of therapy. The Food and Drug Administration recently announced the requirement of a Boxed Warning for the anti-cancer immunosuppressive drugs Rituxan (rituximab). The Boxed Warning is specific for the risk of HBV reactivation in patients who were previously infected with the virus. Use of these drugs in patients with previous HBV infection can result in severe liver damage if the virus is reactivated[131].

Minocycline, a drug used in the treatment of rheumatoid arthritis and acne, can induce a lupus-like syndrome[132]. In addition, statins, which inhibit hydroxymethylglutaryl-coenzyme A reductase, are widely used nowadays in SLE patients due to their immunomodulator and antiatherogenic effect. Several reports have suggested that this drugs may also induce acute hepatitis and a lupus-like syndrome[133]. Finally, cyclophosphamide, an immunosuppressive and potent alkylating agent that improves the outcome of major organ disease when administered at high doses to SLE patients unresponsive to conventional therapy[134], was reported to induce hepatotoxicity associated with liver inflammation in isolated cases[135,136]. There is a report of one case in the literature showing that this effect may occur even when the drug is administered at low doses[137].

CONCLUSION

Liver abnormalities is very common among patients with SLE, especially if they are assessed from the biochemical point of view. It is generally asymptomatic, and frequently associated with steatosis, reactive unspecific changes and drug-related hepatotoxicity. Severe and progressive liver injury may occur, and even more often in the context of a coexisting primary liver disease or during pharmacotherapy.

SLE by itself is not usually associated with aggressive liver disease, but with an often asymptomatic entity referred to as "lupus hepatitis", which is characterized by a mild increase in serum transaminase levels. However, there are overlapping profiles with other autoimmune disease, such as AIH and PBC, related to chronic and aggressive damage, sometimes accompanied by changes in immunological liver tests that help to establish an accurate diagnosis. These overlap syndromes are thought to be variants of an underlying general autoimmune disease, which shows up in a variable arrangement of autoimmune disorders. An etiological role for anti-ribosomal P antibodies in triggering both lupus hepatitis and AIH has been proposed, but it remains uncertain and controversial.

SLE patients often present comorbility with non-autoimmune liver diseases. They includes HCV, thrombotic events in the splachnic vasculature, PCT, and drug-induced hepatotoxicity, among others.

Hepatic circulation disorders may lead to adaptive parenchymal regenerative processes (*e.g.*, RNH, FNH) or formation of hemangiomas. RNH must be ruled out in all lupus patients who present evidence of portal non-cirrhotic hypertension associated with hepatic pseudonodular images.

Drug-induced liver toxicity is also a common event in SLE, and may be ascribed to the chronic use, at high doses, of medicines used to control the autoimmune disorder (*e.g.*, thiopurine analogues, anti-TNF-α agents, statins, minocycline, cyclophosphamide) or to mitigate SLE symptoms (*e.g.*, NSAIDs, MTX). SLE is an oxidative-stress-prone condition, and the pro-oxidant effects of many of these drugs may be a causal factor.

Due to the relatively frequent multifaceted manifestations of liver diseases in SLE, with an often difficult differential diagnosis each others, an assessment of immunological, serological and virological markers should be systematically carried out in patients with elevated levels of liver enzymes. Testing for AMA, ASMA, and HCV may be particularly helpful. In addition, an analysis of the patient's medical history so as to have an accurate record of the drugs taken by the patient should be carefully done. Finally, histology is in some cases the only reliable method of diagnosis, and should be carried out accordingly. We hope the information provided by this review helps to systematize the knowledge of the field, so as to make the challenge of identifying liver diseases associated with SLE more approachable to the clinician.

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MINIREVIEWS

Management of autoimmune hepatitis: Focus on pharmacologic treatments beyond corticosteroids

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Abstract

In autoimmune hepatitis, patients who are intolerant or with toxicity experience, non-responders, relapsers or refractory are challenging. Non-standard drugs are being tried to preemptively avoid corticosteroid-related side effects. Prognosis and quality of life of life rely on treatment optimization. Recently, emergence of powerful immunosuppressive agents, mainly from liver transplantation, challenged the supremacy of the corticosteroid regime and promise greater immunosuppression than conventional medications, offer site-specific actions and satisfactory patient tolerance. Successes in experimental models of related diseases have primed these molecular interventions. We performed a literature review on alternative treatments. Azatioprine intolerance is the principal indication for mycophenolate use but it can be used as a front-line therapy. Cyclosporine A and tacrolimus have been tested for non-responders or relapsers. Rituximab may be used as salvage therapy. Anti-tumor necrosis factor-alpha agents may be used for incomplete responses or non-responders. Methotrexate is possibly an alternative for induction of remission and maintenance in refractory patients. Cyclophosphamide has been included in the induction regimen with corticosteroids. Ursodeoxycholic acid action is mainly immunomodulatory. Non-standard treatments are coming slowly to the attention, but its use should be cautious performed by experienced centers.

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Core tip: With our review we pretend to describe the non-standard pharmacologic treatments available for autoimmune hepatitis, the indications for its use and the main applications. Also, we pretend to enhance that those alternatives are only available guided by the experience in liver transplant patients and should be only used by experienced centers. The difficult-to-treat patients lead to the application of those therapies mainly as salvage treatments.

INTRODUCTION

Liver chronic inflammation, interface hepatitis (on histol-

ogy), hypergammaglobulinemia, and autoantibodies presence are landmarks of autoimmune hepatitis (AIH)[1,2]. AIH is an immune-mediated liver disease, its etiology is unknown[3]. A loss of tolerance seems to be the principal immunologic explanation[3,4]. Women are more affected than men and it occurs across all ages. Men are diagnosed at 40 years of age and women at 50 years of age in median[5]. Prevalence and incidence data on AIH are still limited. In Western Europe and North America Caucasian people the estimated prevalence ranges from 50 to 200 cases per million[6]; the annual incidence in Northern Europeans is 1.9 cases per 100000 persons per year[1,2,7]. An acute presentation occurs in 25% of patients, fulminant presentation is rare but AIH should be considered as etiology in the study of acute liver failure[4]. In addition, the prognosis of disease is influenced by age (young patients having an increased risk), presence of cirrhosis, treatment response (as opposed to activity) and relapses.

The clinical manifestations are heterogeneous. Unspecific symptoms like fatigue, lethargy, jaundice and right upper quadrant pain, are the most frequent clinical presentation. The complications of portal hypertension, *i.e.,* ascites, esophageal varices, hypersplenism and encephalopathy, may ensue on the natural course of the disease. About 25% of patients present extrahepatic immune-mediated symptoms and diseases, arthralgia is the most frequent[4]. Clinical criteria were developed in 1993 and they help to establish the diagnosis when there isn't a single clinical or biochemical test to affirm it[8,9]. These diagnostic criteria include hypergammaglobulinaemia; positivity for autoantibodies: anti-nuclear antibody (ANA), smooth muscle antibody (SMA) or anti-LKM1; typical histology; other causes of hepatitis (viral or toxic) should be excluded as well as other diseases with similar presentation of AIH[8,10]. The autoantibody profile helps to classify AIH: in type 1, SMA and ANA are present; on type 2 anti-LKM1 antibodies are present. Type 1 AIH affects adults and children, while type 2 AIH is mainly a disease of children and adolescents[11]. The scoring system for AIH has a sensitivity of 97% to 100%. In the presence of chronic hepatitis C, the specificity for excluding AIH relies between 66% to 92%[7,10].

Inflammatory activity at the onset of disease and cirrhosis are the main determinants of natural history and prognosis of AIH. Without treatment, mortality of 90% in 10 years is expected when a 5- to 10-times elevation of aspartate aminotransferase and a twofold increase of γ-globulins are present. Cirrhosis occurs in 17% within 5 years of the diagnosis in patients periportal hepatitis and in 82% of patients with bridging necrosis or necrosis of multiple lobules[2]. At diagnosis, 58% of mortality is expected within 5 years of diagnosis[2,4].

The diagnostic criteria may be too strict when applied to diverse ethnic groups because heterogeneous clinical phenotypes and outcomes may be present[12-14] which may be determined by antigenic exposure, variations in immune response, genetic predisposition and cultural, social and economic factors[14]. The diagnosis may be delayed as the institution of corticosteroid treatment[14].

The human leukocyte antigen (HLA) profile also determines the clinical outcome of AIH: HLA DR3 is associated with more severe disease; HLA DR4 is associated with onset at a later age and a more benign outcome of AIH[4]. The HLA-DRB1 locus, specially the alleles HLA-DR3 (DRB1*0301) and DR4 (DRB1*0401) are related to AIH type 1 susceptibility in European populations and North Americans and the strongest genetic associations contributing to the diagnosis of AIH and are included in the IAIHG revised diagnostic scoring system[15]. Genetic profile determines the response to treatment: those who do not respond to corticosteroid treatment have usually DRB1*0301 alleles[15]. Also, clinical manifestations and prognosis may be determined by genetic profile.

AIH, if left untreated, may lead to cirrhosis, liver failure and even death[16]. Survival is increased when immunosuppressive therapy is used. Initially, induction of remission is the main goal[4]. Corticosteroid regimens are effective[2,17]. Prednisolone, alone or in association to azathioprine leads to symptom improvement, laboratory and histologic manifestations of liver inflammation within 6-12 mo in the majority of patients[4,18]. Standard therapy leads to complete biochemical response in 77% in 6 mo[19], improves hepatic fibrosis[18] and 20-year life expectancy is increased in 80%[20].

Early recognition and treatment of the disease, treatment until complete resolution of inflammation, prevention of complications of treatment and early identification and treatment of problematic patients may improve the outcomes of current therapy[21]. Main prognostic determinant is the response to corticosteroid therapy: rapid disease progression is expected when the treatment is delayed or deferred[17].

Between those 23% that do not respond, 5% are intolerant or present toxicity, 7% are non-responders or have refractory disease and the remain 10% have incomplete responses[2]. The relapses after drug withdrawal are frequent (50%-86%)[2,22]. Other efficient treatments are needed. Complete biochemical remission determines the outcome[23] and, therefore, optimization of treatment has implications on prognosis and quality of life[2]. Liver transplantation supersedes empirical drug therapy in decompensated patients[21].

PHARMACOLOGIC TREATMENT OF AIH

The recognition of the response of AIH to immunosuppression changed its prognosis[24,25]. Immunosuppressive treatments should be established immediately, especially in the presence of severe disease[24].

The goal of AIH treatment includes: induction of remission; maintenance of remission; prevention of the establishment of cirrhosis and complications using the lowest possible dose of medication[11,15]. AIH has a good response to immunosuppressive treatment with 80% of remission rate[15,26].

According to the American Association for Study of Liver Diseases guidelines treatment is indicated for patients with established diagnosis of AIH, elevation

of aminotransferase activities [≥ 5 times upper normal limit-(ULN)], rises of immunoglobulin G (≥ 2 times upper normal value) and presence of interface hepatitis or necroinflammatory activity (Ishak score 4-6)[2,16]. If untreated, high mortality of 60% at 6 mo is expected when serum aspartate aminotransferase (AST) levels of 10 times the ULN or more than 5 times the ULN especially if associated with serum γ-globulin level more than twice the ULN. Also, in 82% there is progression from bridging necrosis or multilobular necrosis at presentation to cirrhosis, associated with 45% mortality within 5 years[2,16]. Corticosteroid treatment is indicated in the presence of these findings[2,16]. Treatment should also be started in the presence of incapacitating, such as fatigue and arthralgia[2].

Standard therapy may be not an option if coticosteroids, azathioprine or other immunosuppressive therapies are contraindicated by itself or by patient risk factors. The treatment doesn't alter the outcome in patients with decompensated liver cirrhosis on waiting list for liver transplantation or in those with cirrhosis without inflammatory activity[6].

The outcomes of therapy include: remission, relapse, treatment failure, and stabilization[11]. Normal inflammatory parameters and histology is necessary to assume remission. Histological remission should be differentiated from biochemical remission (complete normalization of aminotransferase levels including IgG). Treatment should definitely be considered in any patient with proven AIH, histological activity and a more than marginal elevation of aminotransferase levels, not only in patients with levels greater 5 × ULN. In 65% to 75% of patients after 24 mo on standard therapy remission is achieved[11,27]. Relapse is defined as a flare in aminotransferase levels with symptoms under treatment, following the minimum dose of maintenance therapy, or after withdrawal. Relapse occurs in about 50%; loss of remission in 42% within 6 mo of treatment withdrawal and in 80% after 3 years; progression to cirrhosis occurs in 38% and liver failure in 14%[11,17]. Retrospective analysis indicates that loss of remission or relapse occurs in virtually all patients with AIH in long-term remission when immunosuppressive therapy is discontinued[28]. Treatment failure should be assumed when there is progression of symptoms, non-improvement of histological parameters and deterioration of serologic features during standard therapy. In case of treatment failure, diagnosis should be reconsidered to exclude an overlap syndrome with primary sclerosing colangitis or primary biliary cirrhosis or different etiologies[11]. Partial remission corresponds to stabilization of the disease[11].

STANDARD PHARMACOLOGIC TREATMENT

The standard initial treatment of AIH includes the corticosteroids only or combined with azathioprine. Combination therapy is the first choice and low-dose of prednisolone (30 mg/d) with 1 mg/kg azathioprine are used in the induction phase[11]. In the United States, 50 mg is used for azathioprine, but in Europe a dose of 1-2 mg/kg bodyweight is used[2]. Alternatively, monotherapy may be used, with 60 mg of steroid and reductions of 10 mg/wk to maintenance dose of 20 mg for at least 6 mo, and further reduction until lowest dose in 2.5 mg decrements. Maybe the initial prednisolone dose in combination therapy should be considered since the percentage of response is higher. There are no differences in the remission induction. Combined treatment is preferred because it allows to decrease the dose of the prednisone dose to below 10 mg and reduces the steroid side effects[6].

Standard therapy is the best option unless contraindicated and may be especially useful by reducing corticosteroids side-effects in older patients, in patients with osteoporosis, metabolic syndrome or psychiatric lability[6]. Monotherapy with steroids is the best treatment option in patients with hematological abnormalities or a proven homozygous deficiency of thiopurine methyltransferase because azathioprine causes hematological side effects such as leukopenia or anemia[6]. Thiopurine methyltransferase (TPMT) is an enzyme responsible for the conversion in one of azathioprine to 6-mercaptopurine (active metabolite) and in 6-methyl mercaptopurine or 6-thiouric acid (inactive metabolites)[12]. In patients with azathioprine intolerance, lower TPMT activity is documented but measurements of TPMT activity cannot be used to identify those patients[29]. Pre-treatment TPMT testing provides some certain of the presence of risk for azathioprine toxicity and strengths physician confidence in the treatment regimens[12]. Allopurinol may safely and effectively optimize thiopurine therapy in patients with intolerance and/or nonresponse due to an unfavourable thiopurine metabolism and this is another option in order to maintain the standard treatment[30].

Corticosteroids are the first option of treatment in all populations, but its use should be individualized in the presence of cholestatic features[14].

However, as the combination treatment fails, other drugs have been tried although its use requires further validation[24].

In fulminant hepatic failure and in en-stage liver disease, transplantation is the treatment of choice. Post-transplantation AIH recurrence may occur[24].

ALTERNATIVE CORTICOSTEROID REGIMEN-BUDESONIDE

Budesonide is glucocorticoid from the next-generation, more than 90% has first pass hepatic clearance and metabolites don't have glucocorticoid activity[31]. These pharmacological properties seem to predict less secondary effects. In non-cirrhotic patients, treatment combination between budesonide and azathioprine may be an alternative in uncomplicated AIH with mild disease[32,33] or with conditions that may be worsened by prednisone treatment like hypertension, osteopenia, diabetes and obesity[34].

In corticosteroids refractory or dependent AIH patients may not be used as a rescue treatment. The budesonide

regimen normalized serum AST and alanine aminotransferase (ALT). Budesonide histological resolution and persistent response is unknown[34].

NON-STANDARD PHARMACOLOGIC TREATMENT

There are some difficult-to-treat patients for whom newer immunosuppressive agents, usually employed as anti-rejection drugs, have been tried with variable success. Immunosuppression with non-standard drugs is being tried to avoid corticosteroid side effects (13%) but are being used specially as superior regimens to corticosteroid treatment[21]. The use of such regimens has to be weighed and data available comes only from few small studies or case reports[15]. Other treatments considered are: mycophenolate mofetil (MMF)[35-43], cyclosporine A (CyA)[44,45], tacrolimus (FK506)[46-49], ritximab[50], anti-tumor necrosis factor-alpha (TNF-α) agents[51], methotrexate[52], cyclophosphamide and ursodeoxycholic acid (UDCA)[53] (Table 1). These non-standard treatments application is not widespread and they are not included into any standard management algorithm[15].

Only recently has the emergence of powerful immunosuppressive agents, mainly from liver transplantation, challenged the supremacy of the corticosteroid regimens[22,54]. Drugs outside of the standard repertoire now promise greater immune suppression than conventional medications, offer site-specific actions and satisfactory patient tolerance[22,54]. Site-specific molecular treatments are also possible because of improved understanding of the central pathogenic disease pathways and technological advances that now enable modulation of these pathways[22,54]. Furthermore, successes in experimental models and in other autoimmune diseases have primed these molecular interventions for study in AIH[22,54].

Importantly, publication bias may be considered since there is, probably, underreport of studies with negative results. Also, target populations, dosing schedules, safety profiles and monitoring strategies are not yet clear; adjunctive therapy with corticosteroids is still required; the standard algorithms do not include already the risks and expense of these drugs[55]. Newer agents are much more expensive than the standard treatment irrespectively of the generic use (as recently available generic MMF may attenuate this problem) and this may be a limitation to the accessibility to these treatments.

MMF

MMF, is most frequently used in patients with refractory AIH or azathioprine intolerance but it may be used as a first choice treatment[16,22,35,38,40,41]. It acts as a purine antagonist.

MMF is hydrolyzed by to mycophenolic acid by liver esterases and acts as reversible noncompetitive inhibitor of inosine monophosphate dehydrogenase: it selectively impairs the synthesis of nucleotides based on purines, inhibits the new synthesis of DNA, impairing proliferation of activated lymphocytes. The thiopurine methyltransferase pathway does not interfere on the activation or elimination of lymphocytes[16,22].

De novo synthesis of purines, in contrast with other cells, is essential for B and T cell proliferation: this is why MMF exerts its cytotoxicity specially on these cell populations[4].

According to eleven small single-centre experiences, MMF is effective in difficult-to-treat patients in doses ranging from 0.5 g/d to 3 g/d[22,35]; 2 g/d in divided doses was the most used regimen, initially with corticosteroids[16].

Recent studies[43,56-58] showed that 47% of the patients had positive response and 53% showed no response or drug intolerance[22]. From 11 studies, 40% of the patients included achieved complete corticosteroid withdrawal and 15% experimented treatment-ending side effects[22]. MMF treatment was more efficient in patients where it was used because of azathioprine intolerance than in patients who where treated for refractory liver disease (58% *vs* 12%)[57,58]. Nonresponders were mainly children with AIH and sclerosing cholangitis[56].

MMF has been used as first choice therapy in naive patients. MMF was used in 59 previously untreated AIH patients for up to 92 mo: 88% showed normal aminotransferase and gamma-globulin serum levels (within three months) and 12% showed partial response[59]. Corticosteroids withdrawn occurred within eight months in 58% and 3% presented serious side effects. MMF can be administered effectively and safely as a front-line treatment, but the reasons for preferring this treatment as a front-line strategy are unclear[22].

The most common side effects of treatment with MMF in AIH patients have been gastrointestinal discomfort (nausea, diarrhea and abdominal pain) (11%), rash (including skin cancers) (7%), fatigue (7%) and leukopenia (1%)[57]. The frequency of side effects has ranged from 3% to 33%[57,59] and the frequency of treatment-ending complications has been as high as 13%[57].

The differences between the costs of MMF and azathioprine may be important[60]; treatment ending side effects occur in 3% to 13%[57,59]; most patients require continuous corticosteroid therapy; the duration of treatment is indefinite; and is more efficient as a salvage therapy in patients with azathioprine intolerance than in patients with steroid-refractory liver disease[57,59]. MMF has a limited and evolving off-label role in AIH, and its use as a salvage therapy for azathioprine intolerance is currently its most effective application[22].

Data about histological remission are poor and further studies are needed before recommend MMF as a first-line treatment for AIH[16]. MMF is contraindicated in pregnancy[16,22].

Calcineurin inhibitors

CyA and FK506 are calcineurin inhibitors that alter phosphatase activity, interfere with lymphocyte T proliferation blunting cell-mediated immune responses. Cyclosporine and FK506 have each been used in AIH patients, primarily as salvage therapies for steroid-refractory disease[22,54].

Table 1 Non-standard immunosuppressive drugs used in autoimmune hepatitis

Non-standard pharmacologic treatments	Studies	Indications	Contra-indications	Outcomes
Mycophenolate mofetil, 0.5 to 3.0 g/d Purine antagonist (inhibits inosine monophosphate dehydrogenase, limits purine nucleotides, impairs lymphocyte proliferation)	[1]46 mo, 7 patients[35] [1]19 mo, 8 patients[39] [1]41 mo, 15 patients[40] [1]61.5 mo, 26 patients[56] [1]26 mo, 59 naïve-patients[59]	Azatioprine Intolerance Refractory AIH Front-line therapy	Pregnancy Hypersensitivity to mycophenolate mofetil, mycophenolic acid or mycophenolate sodium	Salvage[22,35-43,56]: 47% overall improvement 58% azathioprine intolerance 12% refractory disease 53% failure or side effects 40% steroid withdrawal 3%-33% Serious side effects Front-line[59]: 88% complete response 12% partial response 58% steroid withdrawal 3% serious side effects
Cyclosporin, 2 to 5 mg/kg per day Calcineurin inhibitor (impairs NF-κB, reduces IL-2 and lymphocyte proliferation)	6 mo, 19 patients[44] 3 mo, 5 patients[45]	Refractory AIH Relapsing AIH Non-responding AIH	Rheumatoid arthritis and psoriasis: abnormal renal function, uncontolled hypertension, malignancies Psoriasis: under PUVA, UVB therapy, methotrexate Hypersensitivity to cyclosporin or to polyoxyethylated castor oil Pregnancy	Composite results[22,44,45]: 93% improvement 7% failure/side effects
Tacrolimus, 0.075 to 4 mg/kg twice a day Calcineurin inhibitor (impairs NF-κB, reduces IL-2 and lymphocyte proliferation)	12 mo, 21 patients[46] 25 mo, 11 patients[48] 18 mo, 9 patients[49]	Refractory AIH Relapsing AIH Non-responding AIH	Hypersensitivity to tacrolimus Pregnancy	Composite results[22,46,49]: 98% improvement 2% failure/side effects
Rituximab, 1.0 g, two doses 15 d apart[1] Anti-CD20 (B-cell depletion, impairs type 2 cytokine pathway, interferes with antibody-dependent cell-mediated cytotoxicities)	5 mo, 6 patients[66] case reports; data from studies for hematological malignancies, rheumatoid arthritis	Refractory AIH Relapsing AIH Non-responding AIH	Type 1 hypersensitivity or anaphylatic reaction to murine proteins Progressive multifocal leukoencephalopathy	Biochemical improvement
Infliximab, dose of 5 mg/kg at weeks 0, 2 and 6, and then every 4 to 8 wk[1] Anti-TNF-α (neutralizing soluble transmembrane forms of TNF-α impairing cytotoxic type 1 cytokine pathway)	Case reports	Refractory AIH Relapsing AIH Non-responding AIH	Heart failure NYHA class Ⅲ/Ⅳ Hypersensitivity to infliximab or murine proteins	Biochemical improvement
Cyclophosphamide, 1 to 1.5 mg/kg per day Alkylating agents (covalent binding and crosslinking to deoxyribonucleic acid-DNA, ribonucleic acid-RNA and proteins)	95 mo, 94 patients with long-term auto-immune hepatitis[71]	Refractory AIH Relapsing AIH Non-responding AIH	Hypersensitivity to cyclophosphamide, urinary outflow obstructions, severe myelosuppression, severe renal or hepatic impairment, severe immunossupression Pregnancy	91% complete remission
Methotrexate, 7.5 mg/wk Purine antagonist (inhibits the binding of dihydrofolic acid)	Case reports[52]	Refractory AIH	Hypersensitivity Breast-feeding Pregnancy	Biochemical and histologic improvement
Ursodeoxycholic acid, 13 to 15 mg/kg per day Immunomodulation (epimer of chenodeoxycholic acid)	6 mo, 37 patients[53]	In addition to other immunosuppressive strategies	Hypersensitivity Unremitting acute cholecystitis, cholangitis, biliary obstruction, gallstone pancreatitis, biliary-gastrointestinal fistula, allergy to bile acids	Biochemical improvement Corticosteroid dose reduction

[1]Careful is needed in women of childbearing age since those treatments have uncertain effects on reproduction and are presumable teratogenic. AIH: Auto-immune hepatitis; NF-κB: Nuclear factor kappa B; TNF-α: Tumor necrosis factor-alpha; IL-2: Interleukin-2; UVB: Ultra-violet B; PUVA: Psoralen and ultra-violet A.

Calcineurin activates nuclear factor-κB *via* a pathway dependent on phosphatase activity. The activated nuclear factor binds to promoter regions of interleukin-2 (*IL-2*) gene increasing transcription of IL-2. In turn, IL-2 stimulates the cell cycle by binding to IL-2 receptor, and lymphocytes proliferate by a type 1 cytokine pathway[22,54]. In difficult-to-treat AIH patients calcineurin inhibitors have been used as a rescue treatment[15].

CyA: CyA is a calcineurin inhibitor extracted from the *tolypocladium inflatum* and *cylindrocarpum lucidum*[11]. It has been used, since 1985, mainly as a rescue therapy but also in relapsing or non-responsive AIH[22]. There are no long-term reports on safety but results in these situations seem promising[16]. Ten studies[22,44,61] showed that 93% of the 133 patients included within 26 years had a positive response, and 7% showed no response or drug intolerance[22].

Serum aminotransferases and histological activity index scores decreased over 6 mo in an open label trial of 19 patients[16,44].

In a multicenter study, 32 children were included and CyA was administered as monotherapy for 6 mo (200-250 ng/mL levels). Then, prednisolone and azathioprine were given in low doses for 1 mo and stopped after[62]. Alanine aminotransferase activity levels normalized in 25 patients by 6 mo and in all patients by 1 year of treatment. There was a trend to improvement of *Z*-scores for height during treatment[62].

Between 1994 and 2000, 84 children where recruited from five centers, CyA was administered during 6 mo in doses similar to that previously described; after 6 mo, patients with AST/ALT levels lower than 2-ULN started standard therapy. Aminotransferase levels were normal in 94% of patients, 72% within the first 6 mo of treatment[16].

In all studies, CyA adverse effects seem to be mild and transient and standard therapy is not related with relapse during follow-up[16,44,62].

The data are encouraging and CyA might be considered an alternative therapy to steroids in patients who do not achieve a complete remission. However, side effects are a serious problem and include: dyslipidemia, hypertension, renal failure, infection, hirsutism and malignancy[16].

FK506: FK506, macrolide lactone antibiotic, acts as a potent immunosuppressive agent on CD4+ T-helper cells[11]. FK506 and CyA have similar mechanisms of action however, FK506 binds to a different immunophilin (FK-binding protein) leading to the inhibition of lymphokine synthesis (IL-2, IL-3 and IFN-α), IL-2 receptor expression and the generation of cytotoxic T cells[6]. FK506 has been used as a rescue therapy since 1995[22]. Experience with this drug is reported in three studies, 41 patients were included within 16 years: 98% presented a positive response; 2% presented no response or treatment-ending drug intolerance[22,46,49]. There are no controlled trials on the use of FK506 in AIH[11]. In a preliminary trial, 21 patients were treated with FK506 (drug

levels of 0.6-1.0 ng/mL): biochemical improvement was documented after 3 mo[46]. Although the reported results are encouraging, more extensive studies are warranted before FK506 can be recommended as a safe and useful agent in AIH[11]. Remission can be achieved with FK506 for most patients, only or combined with corticosteroids. All series are limited by a short time of follow-up[16].

The success of the calcineurin inhibitors as a salvage therapy for AIH has been impressive, but the overall reported clinical experience with these agents has been lacking. Calcineurin inhibitors still lack a uniform dosing schedule, an acceptable safety profile and an established monitoring protocol for AIH despite their longstanding empirical use in this disease. Efforts to launch large, multicentre, clinical trials have been frustrated by low patient recruitment. Calcineurin inhibitors remain empirical, off-label treatments reserved for steroid-refractory disease and even in these cases should be used with caution and only in experienced centres[22].

Rituximab
Rituximab is an anti-CD20 chimeric monoclonal antibody, a surface marker expressed on B cells, from early pre-B to memory B lymphocytes. Treatment with rituximab leads to B cell depletion through both complement- and antibody-dependent cellular cytotoxicity[63]. Initially developed for the treatment of B-cell lymphoma, rituximab has since proven effective for the treatment of autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus or autoimmune haemolytic anemia[64], suggesting it might also be effective in patients with AIH.

Treatment with rituximab has been reported as effective in patients with Epstein Barr virus infection associated with lymphoproliferative disease secondary to azathioprine[64], in a patient with concurrent diagnoses of B cell lymphoma[65] and steroid resistant AIH/primary biliary cirrhosis overlap syndrome, in patients with concomitant idiopathic thrombocytopenic purpura, cryoglobulinemic glomerulonephritis, or Evans syndrome. Isolated AIH refractory to standard treatment in 6 patients was studied in a phase 1 study: they were treated with rituximab (1000 mg at days 1 and 15)[66]. All patients were maintained on stable doses of prednisolone plus azathioprine for at least 1 mo before and 3 mo after rituximab infusions, after which steroids were tapered. Biochemical remission was achieved by all patients by week 12, with good tolerance to treatment with no serious adverse event being reported during the 72-wk follow-up[66]. Although these results are promising and the toxicity profile is favourable, controlled clinical trials are needed before rituximab can be recommended as an alternative treatment in AIH[11].

Anti-TNF-α agents
TNF-α is a pro-inflammatory cytokine known to be implicated in the pathogenesis of AIH[67]. Additionally, genetic polymorphisms in the TNF promoter region have been identified in patients with AIH type 1, associated with a poorer response to corticosteroid therapy

and higher incidence of cirrhosis[68,69]. Infliximab, etanercept and adalimumab are anti-TNF-α agents commonly used for treatment of immunemediated diseases such as rheumatoid arthritis, psoriasis and inflammatory bowel disease[11]. Soluble and transmembrane forms of TNF-α are neutralized by anti-TNF-α agents. It also seems to have pro-apoptotic effect on activated lymphocytes. Its effect in AIH is explained by the impairment of activated lymphocytes activity[51].

Weiler-Normann *et al*[51] reported the first series of AIH patients treated with infliximab in a single centre. This retrospective study included 11 AIH patients who did not achieve remission with a standard immunosuppressive regimen upon diagnosis, and who also failed to respond to other alternative treatments, including cyclosporine, FK506 and cyclophosphamide. Patients were given infusions of infliximab at a dose of 5 mg/kg at weeks 0, 2 and 6, and then every 4 to 8 wk depending on response. After 3 infusions of infliximab, all patients showed a decrease in the levels of transaminases and of IgG; normalisation of transaminases and IgG levels was observed in 8 and 6 patients respectively. Of the 5 patients in whom a liver biopsy was performed after treatment, all showed reduction of inflammation, as expressed by a modified histological activity index. Some cautions in the use of this agents in AIH must be present since treatment with infliximab has been associated with the induction of severe de novo AIH in some patients treated for other diseases[51,70].

For all the above reasons, while more studies are warranted to evaluate the efficacy and tolerability of infliximab in AIH, this type of treatment should be considered in defined cases and administered only in specialised centres[11,70].

Cyclophosphamide

For the induction of remission in combination with steroids cyclophosphamide was used in the dose of 1-1.5 mg/kg per day[71]. Cyclophosphamide use is highly experimental because of the potential severe hematological side effects[6].

Methotrexate

Methotrexate is an antagonist of folate metabolism, it has anti-inflammatory and immunomodulating properties. Bone marrow suppression and mucosal ulceration at higher doses are the principal side-effects but it is generally well tolerated[52].

A once-weekly dose is reported as induction and maintenance regimen in two case reports. Fibrogenic effect might enable its long-term use[72].

UDCA

UDCA may have immunomodulatory functions. It is a hydrophilic bile acid that changes HLA-1 antigen expression on cellular surfaces and suppresses the production of immunoglobulin. Non-controlled studies show improvement in histology features, in clinical presentation and biochemical parameters. A reduction of fibrosis

wasn't established in four AIH type 1 patients. Its role in AIH treatment is not yet established[6,53]. UDCA monotherapy is effective for some Japanese AIH patients, may have a role during the taper of corticosteroids for prevention of early relapse but is not recommended on patients with high-grade inflammatory activity or poor residual capacity of liver[73].

CONCLUSION

In AIH, identification of efficient salvage treatment options is urgently needed for the difficult-to-treat patients: those who experience intolerance or toxicity, non-responders, relapsers or with refractory disease. Also, non-standard drugs are being tried as superior drugs to corticosteroid regimens and to minimize its side effects. Optimization of treatment plays a major role in long-term prognosis and quality of life for patients with AIH. Recently, the emergence of powerful immunosuppressive agents, mainly from liver transplantation, challenged the supremacy of the corticosteroid regime and promise greater immunosuppression than conventional medications, offer site-specific actions and satisfactory patient tolerance. Successes in experimental models and in other autoimmune diseases have pointed these molecular interventions for study in AIH. Some encouraging results were described, but the establishment of these non-standard drugs as alternative treatments has evolved slowly and they weren't already included into a standard management algorithm. Therefore, those treatments should be used with caution and only in experienced centers.

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World Journal of Hepatology

MINIREVIEWS

Management of hepatitis C virus infection in hemodialysis patients

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Abstract

The prevalence of hepatitis C virus (HCV) infection in patients on maintenance hemodialysis (MHD) is relatively higher than those without MHD. Chronic HCV infection detrimentally affects the life quality and expectancy, leads to renal transplant rejection, and increases the mortality of MHD patients. With the application of erythropoietin to improve uremic anemia and avoid blood transfusion, the new HCV infections during MHD in recent years are mainly caused by the lack of stringent universal precautions. Strict implementation of universal precautions for HCV transmission has led to markedly decreased HCV infections in many hemodialysis units, but physicians still should be alert for the anti-HCV negative HCV infection and occult HCV infection in MHD patients. Standard interferon alpha and pegylated interferon alpha monotherapies at a reduced dose are currently the main treatment strategies for MHD patients with active HCV replication, but how to increase the sustained virological response and decrease the side effects is the key problem. IFNα-free treatments with two or three direct-acting antivirals without ribavirin in MHD patients are waiting for future investigations.

Core tip: The new hepatitis C virus (HCV) infections during maintenance hemodialysis (MHD) in recent years are mainly caused by the lack of stringent universal precautions. Strict implementation of universal precautions for HCV transmission has led to markedly decreased HCV infections in many hemodialysis units, but the anti-HCV negative HCV infection and occult HCV infection in MHD patients still should be noted. How to increase the sustained virological response and decrease the side effects is the key problem for the currently recommended interferon alpha-based antiviral therapy in MHD patients. Interferon alpha-free treatments with two or three direct-acting antivirals without ribavirin in MHD patients are waiting for future investigations.

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INTRODUCTION

Hepatitis C virus (HCV) infection is a major public health problem worldwide which can lead to chronic hepatitis

C, liver cirrhosis and hepatocellular carcinoma (HCC)[1,2]. Prevalence of HCV infection is markedly higher in patients on maintenance hemodialysis (MHD)[3-7]. Chronic HCV infection detrimentally affects the life quality, decreases life expectancy, leads to renal transplant rejection, and increases the mortality of MHD patients suffering from chronic kidney failure[1,5,6]. Moreover, HCV infection has been shown to increase the prevalence of renal insufficiency, defined by serum creatinine ≥ 1.5 mg/dL; the mechanisms may include the direct HCV-related renal injury and HCV-related cirrhosis with subsequent renal impairment[7], and this will be harmful for patients who receive renal transplantation. The rates of HCV infection in MHD patients vary markedly among different countries and hospitals. Multiple factors are associated with the high risk of HCV transmission in MHD patients[3]. Standard interferon alpha (ST-IFNα) and pegylated IFNα (PEG-IFNα) are currently the main treatment strategies for HCV infection in MHD patients, and the key problems are how to increase the sustained virological response (SVR), control the side effects and minimize the dropout rates[1,2,8-10]. This review summarizes the advancement in understanding the prevalence, risk factors, monitoring strategy, and more importantly, prophylaxis and treatment of HCV infection in MHD patients.

EPIDEMIOLOGY

HCV infection in hemodialysis patients varies by patients' behavioral and cultural differences, geographic location, socioeconomic aspects, community exposure factors, number of patients per hemodialyzer and rigorous use of the strictest biosafety standards[4,11], with the reported prevalence ranging from 1.9% to 90% (Table 1)[3]. Generally, new cases of HCV infection related to hemodialysis are more frequent in regions that have a higher prevalence of serum anti-HCV, and HCV genotypes in hemodialysis patients are usually in accordance with those found in non-hemodialysis patients; but some HCV genotypes that are rare in the general population may be more prevalent in hemodialysis patients because of the nosocomial person-to-person transmission in the hemodialysis unit[4]. For instance, a higher prevalence of HCV genotype 2b has been found in hemodialysis populations in southern Brazil, a genotype rarely occurring in Brazil, where the 1a, 1b, or 3a are more common[4].

RISK FACTORS

Currently, it is still unclear how MHD patients become HCV-infected. Nevertheless, both intradialysis (number of blood transfusions, duration and mode of dialysis, prevalence of HCV in the hemodialysis unit, breakdown of standard infection control practices) and extra dialysis (high risk of lifestyle behaviour) variables have been identified.

First, many of these patients had severe uremic anemia needing blood transfusion, which is the most important route of HCV transmission[3,7]. Thus, it was

Table 1 Prevalence of anti-hepatitis C virus seropositivity in hemodialysis patients

Country/region	Prevalence	Investigators and year of publication
Slovenia	1.9%	Buturović-Ponikvar[12], 2001
Netherlands	3.4%	Schneeberger et al[13], 1999
Puerto Rico	3.5%	López-Navedo et al[14], 1999
United Kingdom	4.0%	Wreghitt[15], 1999
Germany	6.1%	Hinrichsen et al[16], 2002
Mexico	6.7%	Méndez-Sánchez et al[17], 2004
Belgium	6.8%	Jadoul et al[18], 2004
United States	7%-23.3%	Kalantar-Zadeh et al[5], 2007
		Kalantar-Zadeh et al[19], 2005
		Sivapalasingam et al[20], 2002
		Kelley et al[21], 2002
		Saab et al[22], 2001
Brazil	6%-90%	da Silva et al[4], 2013
		Mello Lde et al[23], 2007
		Lopes et al[24], 2006
		Albuquerque et al[25], 2005
		Carneiro et al[26], 2001
China Maintain	7.01%-37.34%	Ren et al[27], 2011
		Qi et al[28], 2003
Greece	10%-29%	Garinis et al[29], 1999
		Rigopoulou et al[30], 2005
		Sypsa et al[31], 2005
Sweden	11.0%	Almroth et al[32], 2002
Iran	13.2%	Alavian et al[33], 2003
France	16.3%	Salama et al[34], 2000
Tunisia	19%-41.7%	Bouzgarrou et al[35], 2005
		Ayed et al[36], 2003
Libya	20.5%	Daw et al[37], 2002
Italy	22.5%-32.1%	Petrosillo et al[38], 2001
		Lombardi et al[39], 1999
Sudan	23.7%	El-Amin et al[40], 2007
Vietnam	26.6%	Dunford et al[7], 2012
Bosnia and Herzegovina	59.0%	Ahmetagić et al[41], 2006
Peru	59.3%	Sanchez et al[42], 2000
Kuwait	71.0%	Wreghitt[15], 1999
Moldavia	75.0%	Covic et al[43], 1999
Senegal	80.0%	Diouf et al[44], 2000

highly possible that some of the hemodialysis patients got HCV infection through this way, especially in regions with poor socioeconomic conditions, where the qualified medical staff and equipments available to treat MHD patients were very limited. In the past two decades, the sensitivity and specificity of laboratory tests for detection of HCV have improved greatly, leading to the more stringent screening of blood donors and the marked decline of new HCV infections[6,45,46]. On the other hand, the availability of erythropoietin has reduced the need of blood transfusion in hemodialysis patients. Accordingly, the risk of HCV infection through blood transfusion in hemodialysis patients has decreased significantly in many countries[45].

Second, new HCV infections can occur in patients who lack the risk factors of blood transfusion, intravenous drug use, high-risk sexual activity, or exposure to known HCV-positive persons. It is believed that these patients were infected by HCV during the course of hemodialysis[47]. Phylogenetic analysis of HCV isolates implies that many HCV infections during hemodialysis

are surely the result of nosocomial patient-to-patient transmission[45,47-50]. The infection risk usually increases with the prevalence of HCV, and the number and length of hemodialysis exposure in corresponding hemodialysis units[4,5,31,40]. Recently, da Silva *et al*[4] reported that HCV-infected patients had been on hemodialysis for 91.9 mo, more prolonged than HCV-negative patients (*P* = 0.001). Another investigation showed that the prevalence of HCV infection at admission in a New York City hemodialysis unit was 18%, far higher than the 1.6% in the United States population overall. During 2001-2008, nine patients treated in this unit were found to have seroconversion from anti-HCV negative to positive. Of them the sources for four HCV infections were identified phylogenetically and epidemiologically as four other patients in the unit. The epidemiologic and site investigations showed that the hemodialysis unit had inadequate HCV infection surveillance and patient follow-up, inadequate cleaning and disinfection practices, failing to wear or change gloves or perform hand hygiene between contacted patients, lack of a separate clean area for medication storage and preparation, and short turnover periods between patient treatments[47]. Accordingly, it is suspected that the way for HCV transmission in these patients may be direct percutaneous exposure to infectious blood because of inadequate infection control[1]. On the contrary, the use of dedicated hemodialyzer specially prepared for each patient and the strict implementation of hygienic precautions against HCV transmission could markedly decrease the incidence of nosocomial HCV infection in hemodialysis patients[45].

MONITORING

Monitoring serum anti-HCV by enzyme-linked immunosorbent assay or enzyme immunoassay every three to six months is essential to identify HCV seroconversion[45,47]. Sometimes the recombinant immunoblot assay for anti-HCV should be added to confirm the positivity of anti-HCV[47]. Of note is that the anti-HCV tests may fail to detect HCV infection in 1.66%[45] to 7.2%[46] of MHD patients, because the immunocompromised status of these patients prevents them from having detectable anti-HCV antibodies[1]. So it is necessary to detect HCV core antigen by chemiluminescent assay or HCV RNA by polymerase chain reaction (PCR) in anti-HCV negative patients who are at high risk of HCV transmission[6,46]. If HCV RNA is positive, it is necessary to quantitate and genotype the HCV RNA further to provide important information for phylogenetic analysis of HCV isolates and selection of treatment strategy in MHD patients[45,50]. In addition, serum alanine aminotransferase (ALT) and other liver-associated biochemical tests, alpha fetoprotein and ultrasonic scan of the liver should also be conducted regularly.

Occult HCV infection, defined as detectable HCV RNA in the liver or peripheral blood mononuclear cells (PBMCs) in the absence of both serum HCV RNA and anti-HCV[51], is a serious fact that might be ignored in hemodialysis patients. Barril *et al*[51] reported that occult HCV infection, determined by the presence of genomic HCV RNA in PBMCs, was found in 45% of the 109 MHD patients, and 53% of these patients had ongoing HCV replication indicated by the presence of antigenomic HCV RNA. Patients with occult HCV infection had spent a significantly longer time on hemodialysis and had significantly higher mean ALT levels during the 6 mo before study entry. Accordingly, for patients with long time of hemodialysis and a relatively higher serum ALT level, the PBMCs or liver biopsy samples should be collected to detect HCV RNA to rule out occult HCV infection[51,52].

PROPHYLAXIS

There is no active vaccine to prevent MHD patients from HCV infection. It has been adopted by many medical centers to assign HCV-infected patients to dedicated hemodialysis machines in a dedicated room in order to separate HCV positive patients from the negative patients, and this has been considered to be able to decrease the risk of HCV transmission[53]. In those hemodialysis units with high HCV prevalence but without fulltime medical staff on HCV-infection control, this strategy may help decrease the risk of HCV transmission among patients[3]; but for hemodialysis units with strict universal precautions against HCV transmission, some specialists consider that the dedicated hemodialysis machine in a dedicated room for HCV-infected patients is somewhat unjustified and unnecessary[49,54].

Universal precautions, especially stringent adherence of all necessary biosafety measures during hemodialysis, are considered to be the keystones to minimize HCV transmission related to hemodialysis and have maximized ideal prophylactic effects[45,47,53]. These measures include: (1) applying a disposable hemodialyzer to avoid sharing of a hemodialyzer; (2) systematic decontamination of the equipment and circuits after each patient's treatment; (3) avoiding sharing of medications, such as multiuse vials of heparin among patients; (4) avoiding sharing of instruments such as tourniquets; (5) preparing any medications in a separate area; (6) disinfecting hemodialysis station surfaces timely; (7) cleaning hands and changing gloves before contacting different patients; (8) periodic testing of all patients for anti-HCV and HCV RNA; and (9) systematic training of health workers in hemodialysis units.

TREATMENT

HCV infection has a significant adverse effect on the health of persons with chronic kidney disease, leads to a higher mortality in MHD patients than non-infected MHD patients, and reduces the survival rates of patients who undergo kidney transplantation, as do their grafts. Moreover, HCV infection renders the patients at high risk of developing diabetes mellitus, membranous glomerulonephritis as well as fibrosing cholestatic hepatitis after

Table 2 Current recommendations for antiviral treatment of hepatitis C virus infection in maintenance hemodialysis patients with kidney failure[1,54,56,57]

Drug	Dosage	Notes
ST-IFNα-2a	3 million units, three times a week	Usually 48 wk for HCV genotypes 1 and 4, and 24 wk for HCV genotypes 2 and 3, or receiving response-guided treatment
ST-IFNα-2b	3 million units, three times a week	
PEG-IFNα-2a	135 μg, once a week	A more reduced dose, a longer interval between two injections, or temporary cessation
PEG-IFNα-2b	1 μg/kg, once a week	of IFNα should be considered in patients with severe side effects such as dangerous bone marrow suppression
Ribavirin	200 mg, once a day, every other day, or thrice weekly after hemodialysis	Ribavirin is applied in combination with interferon, and should be prohibited if severe anemia or other adverse effects occurs

HCV: Hepatitis C virus; ST-IFNα: Standard interferon alpha; PEG-IFNα: Pegylated interferon alpha.

kidney transplantation[1]. Accordingly, patients with MHD who are infected with HCV should be treated if conditions permit, no matter whether they will receive kidney transplantation or not. On the other hand, occult HCV infection is usually persistent and can not be eradicated spontaneously. Though it seems to be less aggressive than chronic hepatitis C, occult HCV infection may also lead to liver cirrhosis and even HCC[52,55]. Accordingly, if occult HCV infection could be confirmed in MHD patients, the antiviral therapy should be given too[40]. Recommendations for the treatment of HCV infection in MHD patients with kidney failure are summarized in Table 2.

ST-IFNα and PEG-IFNα monotherapies are currently the main treatment strategies for MHD patients with active HCV RNA replication. For adult patients, ST-IFNα-2a or ST-IFNα-2b should be given at a reduced dose of 3 million units three times a week, and PEG-IFNα-2a or PEG-IFNα-2b should be given at a reduced dose of 135 μg and 1 μg/kg once a week, respectively[1]. If the patients still cannot endure the side effects even in the use of erythropoietin, granulocyte-macrophage colony stimulating factor, interleukin-11 or other symptomatic and supporting treatments, a more reduced dose of IFNα should be given, and/or the intervals between two injections should be prolonged, or the IFNα should be stopped temporarily. Generally, the recommended treatment duration of IFNα is based on the HCV genotypes, *i.e.*, 48 wk for HCV genotypes 1 and 4, and 24 wk for HCV genotypes 2 and 3[54]; but the response-guided treatment strategy should also be emphasized, *e.g.*, shorter treatment duration for patients achieving rapid virological response (defined as seronegativity of HCV RNA at week 4 of treatment) than those with early virological response (EVR, defined as a seronegative or at least a 2 $\log_{10}$ decrease from baseline in the serum HCV RNA at week 12 of treatment), and early termination in those without an EVR[56]. Moreover, a shorter treatment duration of IFNα might be considered in patients with interleukin-28B (IL-28B) genotype rs12979860 CC or rs8099917 TT, but a longer treatment duration should be given in those with IL-28B genotype rs12979860 CT/TT or rs8099917 TG/GG[56].

Though PEG-IFNα can be used and may be associated with improved SVR rates in MHD patients[57], a group of experts in both kidney and liver disease recommended ST-IFNα in preference to PEG-IFNα for the treatment of MHD patients with HCV infection[1]. The rationale for this recommendation is that ST-IFNα has appeared as effective as PEG-IFNα in MHD persons because its excretion is reduced in these patients, its adverse effects may be lower, and management of adverse effects is relatively easier than PEG-IFNα[1].

Because ribavirin has the high risk of inducing or aggravating hemolytic anemia in uremic patients and can not be removed by hemodialysis, it should be prohibited or used at a markedly reduced daily dose with careful monitoring of anemia and other adverse effects in MHD patients[1,8-10]. If RBV is to be applied, it should be given at an individualized dosing of 200 mg once a day, or 200 mg every other day, or 200 mg thrice weekly after hemodialysis, and substantial hematopoietic support is essential[57].

In a meta-analysis made by Gordon *et al*[9] in 2009, which included 428 patients from 20 prospective studies from 1966 to February 2009, IFNα treatment for at least six months against chronic HCV infection in MHD patients was shown to result in a high overall SVR of 45%. Both univariate and multivariate regression analyses demonstrated that the higher SVR was related to the following factors: (1) three million units or higher dosage of IFNα, three times weekly; (2) completion of treatment for at least six months; (3) lower baseline HCV RNA; (4) female gender; and (5) early virological negativity[9]. In a later meta-analysis by Alavian *et al*[8] published in 2010, 491 MHD patients from 21 studies of ST-IFNα and 279 MHD patients from 12 studies of PEG-IFNα were meta-analyzed. The pooled SVR for ST-IFNα and ST-IFNα monotherapy in random effects model were 39.1% and 39.3%, respectively. Pooled dropout rates were 22.6% and 29.7%, respectively. Only age less than 40 years was significantly associated with SVR. HCV RNA level, HCV genotype, ALT pattern, female gender, duration of infection, liver fibrosis stage, and treatment duration were not associated with SVR[8]. These conclusions are conflicting with that of Gordon *et al*[9]. Accordingly, the factors associated with the SVR are worthy of further investigations.

Tolerance to initial IFNα monotherapy was lower in MHD than in non uremic patients with chronic HCV infection. The most frequent side effects requiring interruption of treatment were severe flu-like symptoms, bone marrow suppression, neurological and gastrointestinal

discomfort. However, about 40% of MHD patients with HCV infection have been successfully treated with IFNα monotherapy. Further studies are warranted to define whether longer duration of IFNα monotherapy will have a better SVR on IFNα for chronic hepatitis C in MHD population[10].

Telaprevir and boceprevir are HCV protease inhibitors (PIs) developed in recent years. No significant impact of renal dysfunction on telaprevir or boceprevir exposure was found in patients with end-stage renal disease[58], suggested that both drugs might be used to treat HCV infection in this setting[57]. A recent study that included 36 treatment-naive HCV genotype 1 infected MHD patients showed that telaprevir-containing triple therapy had superior efficacy than PEG-IFNα/RBV dual therapy, but was accompanied with anemia more frequently and severely[57]. Generally speaking, in consideration of added severe side effects and drug-drug interactions, triple or quadruple combinations based on IFNα/RBV therapy with one or two PIs are believed not very suitable for MHD patients with HCV infection. On the other hand, several IFNα-free clinical studies combining two or three new direct antiviral agents without RBV are now under investigation in HCV-infected patients without renal dysfunction[2]. This will bring new hopes to increase SVR with decreased side effects not only for HCV-infected patients without MHD, but also for patients with MHD.

CONCLUSION

MHD patients without initial HCV infection may be infected by HCV through blood transfusion or negligence of universal precautions during hemodialysis. The application of erythropoietin has decreased the necessity of blood transfusion for uremic anemia greatly, and the improved detection tests of anti-HCV, HCV core antigen and HCV RNA have minimized the risk of HCV transmission through blood transfusion. Accordingly, the new HCV infections during MHD in recent years are mainly caused by the lack of standard universal precautions. Construction of detailed surveillance systems and implementation of stringent universal precautions for HCV transmission have led to a markedly decreased prevalence of HCV infection in many hemodialysis units[16], and the effectiveness of different preventive strategies for HCV infection in hemodialysis units should be further investigated and clarified. The occult HCV infection in MHD patients should be paid more attention, and detection of HCV RNA by PCR from PBMCs or liver biopsy is necessary for MHD patients with unexplainable elevated serum ALT or liver cirrhosis. Currently, ST-IFNα and PEG-IFNα monotherapies at a reduced dose are the main treatment strategies for MHD patients with active HCV replication, and the SVRs are up to 40% or so. The emphases of future study for the treatment of HCV infection in MHD patients include how to increase the SVR, how the genetic factors such as polymorphisms of *IL-28B* gene will affect the SVR, how to optimize the

treatment duration, how to conquer the side effects of IFNα, and whether IFNα-free treatments with two or three DAAs without RBV are effective and practical for HCV eradication in MHD patients[2].

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ORIGINAL ARTICLE

Hepatitis E virus in patients with acute severe liver injury

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Abstract

AIM: To examine the incidence of hepatitis E (HepE) in individuals with acute liver injury severe enough to warrant treatment at a transplant unit.

METHODS: Hepatitis E virus (HEV) is an emerging pathogen in developed countries causing severe illness, particularly in immunocompromised patients or those with underlying chronic liver disease. HepE infection is often under diagnosed, as clinicians can be reluctant to test patients who have not travelled to regions traditionally considered hyperendemic for HepE. There are few data regarding the significance of HEV in patients with very severe acute liver injury in developed countries. Eighty patients with acute severe liver injury attending the Scottish Liver Transplant unit were tested for HEV and anti-HEV IgG and IgM. Severe acute liver injury was defined as a sudden deterioration in liver function confirmed by abnormal liver function tests and coagulopathy or presence of hepatic encephalopathy. Eighty percent of these patients were diagnosed with paracetomol overdose. No patients had a history of chronic or decompensated chronic liver disease at time of sampling. IgG positive samples were quantified against the World Health Organization anti-HEV IgG standard. Samples were screened for HEV viral RNA by quantitative reverse transcription polymerase chain reaction.

RESULTS: Four cases of hepatitis E were identified. Three of the four cases were only diagnosed on retrospective testing and were initially erroneously ascribed to drug-induced liver injury and decompensated chronic liver disease, with the cause of the decompensation uncertain. One case was caused by HEV genotype 1 in a traveller returning from Asia, the other three were autochthonous and diagnosed on retrospective testing. In two of these cases (where RNA was detected) HEV was found to be genotype 3, the most prevalent genotype in developed countries. Three patients survived, two of whom had been misdiagnosed as having drug induced liver injury. The fourth patient died from sepsis and liver failure precipitated as a result of hepatitis E infection and previously undiagnosed cirrhosis. Histopathology data to date is limited to mainly that seen for endemic HepE. All patients, with the exception of patient 1, demonstrated characteristics of HepE infection, as seen in previously described locally acquired cases.

CONCLUSION: In patients with acute severe liver injury, HEV testing should be part of the initial diagnostic investigation algorithm irrespective of suspected initial

diagnosis, age or travel history.

Core tip: Misdiagnosis of hepatitis E infection in drug induced liver injury has been noted in patients previously in South East England (13%) and the United States (3%). However, hepatitis E virus is still not given precedence when diagnosing these individuals. In our study, 5% of individuals tested were misdiagnosed and viraemic. It is an important clinical point that the diagnosis of drug induced liver injury is not secure without first excluding hepatitis E, irrespective of travel history, particularly in patients with elevated transaminases.

INTRODUCTION

Hepatitis E has previously been considered a disease of developing countries. In these hyperendemic settings hepatitis E often occurs in large outbreaks involving hundreds or thousands of cases, such as the recent epidemic in south Sudan[1,2]. In such geographical settings hepatitis E virus (HEV) is spread oro-faecally *via* infected water supplies. Most patients recover, but the mortality rate is high in pregnant females and patients with underlying chronic liver disease[2]. Over recent years, locally acquired hepatitis E has been reported from many developed countries, where it is considered to be a porcine zoonosis[3]. Locally acquired acute hepatitis E is more common in middle aged and elderly males, and in most patients causes a self-limiting hepatitis which last 4-6 wk[4].

In some European countries such as England, France and Germany, a large number of sporadic cases of locally acquired hepatitis E have been documented. For example, in 2011, 454 cases of laboratory-confirmed cases were documented in England and Wales[5] (http://www.hpa.org.uk/hpr/archives/2012/news3212.htm#hev), and these were mostly locally acquired. In contrast, in the United States only a handful of cases have been documented[6] despite an anti-HEV seroprevalence of 21%[7]. This suggests that sub-clinical and/or unrecognised infection is common.

In developed countries, there have been very few previous studies of HEV in patients with acute liver injury severe enough to warrant assessment and treatment at a liver transplant unit. The aim of this study was to retrospectively determine the role and contribution of HEV infection in patients presenting with acute severe liver injury to the Scottish Liver Transplantation Unit (SLTU), Edinburgh, Scotland.

MATERIALS AND METHODS

The SLTU admits patients from hospitals in Scotland with severe acute liver injury for assessment and treatment, and covers a population of 5254800 (http://www.gro-scotland.gov.uk/files2/stats/annual-review-2011/rgar 2011.pdf). The cohort studied included 80 patients with severe acute liver injury admitted to the SLTU between December 2008 and May 2012 (Tables 1 and 2). Severe acute liver injury was defined as a sudden deterioration in liver function confirmed by abnormal liver function tests and coagulopathy or presence of hepatic encephalopathy[8]. No patients had a history of chronic or decompensated chronic liver disease. The majority (80%) of cohort patients were referred to SLTU in the context of paracetamol overdose (POD). The other aetiologies are reflective of the diversity of patients referred to the unit. Despite the large proportion of POD cases within the cohort we felt HEV testing was justified given that infection with hepatitis A, hepatitis C andthere are several publications implicating HEV in the misdiagnosis of POD and DILI.[9-14]. Also, paracetamol ingestion is common in patients with viral hepatitis and may lead to confusion as to the cause liver injury[15].

Serum samples from all 80 patients, taken at presentation and stored at -80 ℃[16], were tested for the presence of anti-HEV IgM and IgG antibodies and HEV RNA. Antibody screening was carried out using commercial assays for anti-HEV IgM and IgG (Wantai, Beijing, PR China) according to manufacturer's instructions. IgG positive samples were quantified against the World Health Organization (WHO) anti-HEV IgG standard. HEV RNA screening was carried out using a HEV pan-genotype quantitative reverse transcription polymerase chain reaction assay with taqman probe and primer sequences targeting the open reading frame (ORF)2/3 region of the HEV genome described by Jothikumar *et al*[17]. RNA positive samples were quantified against the WHO HEV RNA standard and the limit of detection of the assay was determined to be 250 WHO IU/mL. Positive samples underwent conventional PCR using primer sequences targeting the ORF2 region previously described by Erker *et al*[18]. Cloning of the ORF2 amplicons was performed using the pGem-T-Easy vector (Promega, Southampton, United Kingdom) and sequenced (GATC, Konstanz, Germany). Sequence data was aligned against known HEV genotype sequences using alignment software ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The Health Protection Agency guidelines for HEV diagnosis were followed when assigning diagnoses in this study. Briefly, the criteria for diagnosing an acute HEV infection is defined as; clinical and/or biochemical findings consistent with acute viral hepatitis together with virology laboratory markers consistent with acute infection; this must include the de-

Table 1	Patient cohort									
n	**Gender**		**Age**	**ALT**	**Bilirubin**	**Creatinine**	**PT**	**WBC**	**Liver**	**Outcome**
	Male	**Female**	**mean ± SD**	**(10-50 IU/L)**	**(< 17 μmol/L)**	**(45-110 μmol/L)**	**(8-12 s)**	**(4.3-10.8 ×10⁹ cells/L)**	**failure[1]**	
80	36 (45%)	44 (55%)	38.7 ± 14.1	5112 ± 3492	118 ± 96	181 ± 136	52 ± 34	9.96 ± 6.51	47 (58.8%)	SWOTX 54 (67.5%)
										SWTX 11 (18.3%)
										Died 15 (13.8%)

[1]Defined as loss of hepatic cellular function and subsequent development of coagulopathy, jaundice and encephalopathy. Normal range indicated in brackets. ALT: Alanine aminotransferase; PT: Prothrombin time; WBC: White blood cell; SWOTX: Survived without transplant; SWTX: Survived with transplant.

Table 2	Prevalence of patient								
Diagnosis	POD	Acute viral hepatitis	Autoimmune hepatitis	Post LTX graft nonfunction	Fatty liver of pregnancy	Malignancy	DILI	Acute porphyria	Ischaemic hepatitis
Prevalence	64 (80%)	6[1] (7.5%)	3 (3.75%)	2 (2.5%)	1 (1.25%)	1 (1.25%)	1 (1.25%)	1 (1.25%)	1 (1.25%)

[1]The causes of acute viral hepatitis prior to retrospective testing were acute hepatitis B (*n* = 3), acute hepatitis C (*n* = 1), and acute hepatitis E (*n* = 1, case 4 who had travelled to India and was tested for hepatitis E virus at presentation). The diagnoses described are the diagnoses before retrospective testing for hepatitis E virus was undertaken. POD: Paracetamol overdose; LTX: Liver transplant; DILI: Drug induced liver injury.

tection of HEV RNA. Laboratory markers of probable cases must include the detection of anti-HEV IgG and IgM antibodies but allows for the absence or non-testing of HEV RNA (http://www.hpa.org.uk/webc/HPAweb-File/HPAweb_C/1287146735973).

RESULTS

From the 80 patients cohort; 72 (90%) patients tested anti-HEV IgG negative, anti-HEV IgM negative and HEV RNA negative; 4 (5%) patients tested anti-HEV IgG positive, anti-HEV IgM negative and HEV RNA negative; 3 (3.75%) patients tested anti-HEV IgG positive, ant-HEV IgM positive and HEV RNA positive; 1 (1.25%) patient tested anti-HEV IgG positive, anti-HEV IgM positive and HEV RNA negative (Table 3). No patient diagnosed with hepatitis B, hepatitis C, autoimmune hepatitis, post liver transplant graft non-function, fatty liver of pregnancy, ischaemic hepatitis, malignancy or acute porphyria tested positive for hepatitis E (Table 1). The 4 patients with corresponding anti-HEV IgM positive results, suggestive of active infection at time of testing, are described in further detail below.

Patient 1

A 58-year-old female presented with a 24 h history of jaundice and itch. She complained of generalised malaise/fatigue for 1 wk and dark tea-coloured urine for 3 d, but no abdominal pain, nausea or vomiting. The patient had travelled to Ibiza (Spain) 1 mo and Cornwall (England) 2 wk previously. After returning with a dry cough, she visited her General Practitioner, who prescribed a short course of clarithromycin. The patient had also recently begun taking simvastatin and diclofenac and was a regular user of aspirin and nifedipine. The patient had no history of liver problems and only light alcohol consumption (< 48 g/wk, maximum recommended limit for females = 112 g/wk). Liver function tests showed highly

elevated transaminases (Table 3). Tests for hepatitis A, B, C and autoantibodies were negative, alpha-1-antitrypsin and ceruloplasmin were normal, serum ferritin 1871 ug/L (normal range 14-150), iron 15 μmol/L (normal range 10-28), transferrin 2.1 g/L (normal range 2-4), transferrin saturation 27%. An ultrasound scan showed no focal abnormality, contracted gall bladder, no biliary dilatation, normal kidneys/spleen and no free intra-abdominal fluid.

After withdrawal from all her medication, the patient's liver function tests improved. The patients liver dysfunction was therefore attributed to drug induced liver injury (DILI). However, in retrospect a diagnosis of acute hepatitis E was made, as she was anti-HEV IgM and IgG positive, and her serum contained a high titre of HEV RNA (Table 3). Sequencing of the patient's viral RNA showed it to be of genotype 3 (Figure 1).

Patient 2

A 67-year-old female presented to the referring hospital with acute hepatitis after returning from Spain 4 wk earlier. She had significant comorbidity; non-insulin dependent diabetes, hypertension, chronic kidney disease and alcohol excess (224-258 g/wk). Because of developing hepatic encephalopathy, increasing fluid overload and renal injury the patient was transferred to SLTU. The patient underwent a transjugular liver biopsy to clarify the diagnosis. The biopsy showed cirrhosis with sparse steatohepatitis and a low grade cholestatic hepatitis (Figure 2). She developed sepsis complicated by multi-organ failure and died despite supportive care, including dialysis and norepinepherine. This patient was anti-HEV IgM and IgG positive. Retrospective RNA screening and sequencing showed this patient demonstrated HEV genotype 3 in her stored blood sample (Figure 1 and Table 3).

Patient 3

A 27-year-old male Polish immigrant living in London

Table 3 Hepatitis E virus immunoglobulin M positive patients

Patient ID	Gender	Age	Liver function tests (normal range)						HEV			Genotype	Travel history	Initial/ retrospective diagnosis	Outcome
			ALT (10-50 IU/mL)	Bilirubin (<17 µmol/L)	ALP (40-125 IU/L)	Gamma GTP (5-35 IU/mL)	Albumin (30-50 g/L)	PT (8-12 s)	IgG (IU/mL)	IgM	RNA (IU/mL)				
1	Female	58	3648	92	540	480	35	16	48.4	+	6.4×10^4	3	Spain, Cornwall	DILI/hepatitis E	Survived
2	Female	67	98	516	256	Icteric[1]	19	14	36.49	+	1.4×10^3	3	Spain	Alcohol, T2Diabetes, obesity, CLD/hepatitis E	Died
3	Male	27	5288	140	186	368	27	35	24.7	+	-	-	London	POD/hepatitis E	Survived
4	Male	27	4044	269	253	Icteric[1]	38	25	42.05	+	1.9×10^4	1	India	Hepatitis E	Survived

[1]GTP could not be calculated due to high serum bilirubin levels. Values obtained from serum samples taken on admission to SLTU. ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; GTP: Glutamyl transpeptidase; PT: Prothrombin time; HEV: Hepatitis E virus; Ig: Immunoglobulin; RNA: Ribonucleic acid; DILI: Drug induced liver injury; CLD: Chronic liver disease; POD: Paracetamol overdose.

travelled to Scotland to stay with a relative. Without suicidal intent, and in conjunction with excessive alcohol, he ingested 8 g of liquid paracetamol. He was transferred to SLTU because of deranged liver function tests (Table 3) and confusion. He developed hepatic encephalopathy and acute kidney injury with oliguria and peak creatinine 600 µmol/L which resolved spontaneously, without renal replacement therapy. Twelve days after admission he was discharged to the referring hospital with the following blood tests (normal range indicated in brackets); prothrombin time 35 s (8-12 s), bilirubin 343 µmol/L (<17 µmol/L), alanine aminotransferase 97 IU/L (10-50 IU/L), alkaline phosphatase 160 IU/L (40-150 IU/L), gamma glutamyl transpeptidase 190 IU/L (5-35 IU/L), Albumin 19 g/L (30-50 g/L). In retrospect, this patient tested positive for anti-HEV IgM and IgG antibodies but no viral RNA could be detected in his serum.

Patient 4

A 27-year-old male experienced a short self-limiting episode of nausea and diarrhoea just before returning to Scotland from working at a sanitation project in Northern India. He rapidly became jaundiced and fatigued. He was febrile, had no stigmata of chronic liver disease, but was deeply jaundiced with a palpable non-tender liver. Upper abdominal ultrasound was normal and other liver diseases excluded by serology, biochemistry and immunology. A transjugular liver biopsy was performed, revealing a severe acute lobular hepatitis (Figure 3). Due to the patient's travel history he was contemporaneously tested for HEV, and was IgM, IgG and PCR positive. Sequencing performed in retrospect showed the patient to be infected with HEV genotype 1 (Figure 1).

DISCUSSION

In developed countries there have been few previous studies of HEV in patients with acute liver injury. The current study shows that in four of 80 (5%) of patients with acute severe liver injury the cause was hepatitis E, thus making HEV the commonest cause of viral hepatitis in this cohort. Three of the four cases were only diagnosed on retrospective testing and were initially erroneously ascribed to drug-induced liver injury (n = 2) and decompensated chronic liver disease, with the cause of the decompensation uncertain (n = 1). These findings suggest that in patients with acute severe liver injury HEV testing should be part of the initial diagnostic investigation algorithm, irrespective of suspected initial diagnosis, age or travel history.

In the case of patient 1, we initially misdiagnosed the case as DILI because, at the time, we did not consider hepatitis E as a diagnostic possibility. A study from England showed that 6/47 (13%) of patients with criterion-referenced drug-induced liver injury had been misdiagnosed, as they had locally-acquired hepatitis E infection[12]. A similar study from the United States showed that 9/318 (3%) had been similarly misdiagnosed[13]. More recently, Chen *et al*[14] described another case of hepatitis E which had been erroneously diagnosed as "DILI". An accurate diagnosis of drug-induced liver injury depends on excluding of all other possible causes of hepatocellular injury[21]. Patient 1 case illustrates the important clinical point that the diagnosis of drug-induced liver injury is not secure without first excluding hepatitis E, irrespective of travel history, particularly in patients with highly elevated transaminases.

Figure 1 **Phylogenetic relationship between the 4 hepatitis E virus geno-type reference sequences and those isolated from a United Kingdom swine (AF503512), a United Kingdom patient with locally acquired hepatitis E virus (AY362357), genotype 3 subtypes described in**[19] **and the patients discussed in this paper. Sequences were assembled using ClustalW and the phylogenetic tree expressed in the Newick format using NJ plot**[20]. **The 121 bp sequences correspond to nucleotides 6332-6476 of hepatitis E virus genotype 3 reference strain AF060668.**

Patient 2, although presenting with severe acute liver injury, had previously undiagnosed cirrhosis, likely due to a combination of alcoholic and fatty liver disease, and died from multi-organ failure precipitated by hepatitis E infection. Studies from Europe and Southeast Asia show, that hepatitis E infection in patients with underlying chronic liver disease, have a poor prognosis, with a 12-mo mortality rate from subacute liver failure of up to 70%[22,23]. The diagnosis of acute hepatitis E infection in such patients is easily overlooked, and may commonly be ascribed to other causes such as alcoholic hepatitis[24]. Patient 2 illustrates this diagnostic difficulty, as there were no specific clinical or laboratory clues which prompted consideration of hepatitis E as a diagnostic possibility, and the liver biopsy appearances could easily have been ascribed simply to decompensated alcoholic cirrhosis in a previously undiagnosed patient. Often the only clue in such hepatitis E cases is the elevation of transaminases at presentation[25]. Within a week, the alanine aminotransfer-ase declines to the range seen in alcoholic hepatitis[24,25], as was the case in patient 2 (98I U/mL) (Table 3). Despite the difficulties in identifying cases, it is important to establish an early diagnosis of hepatitis E infection in patients with underlying chronic liver disease, as the prognosis may be improved by early anti-viral therapy with ribavirin[26,27]. Indeed, this case highlights the poor prognosis in older patients with underlying liver disease and acute hepatitis E infection.

In the case of patient 3 we were unable to detect HEV RNA, despite the patient being anti-HEV IgM positive. However, the absence of detectable HEV RNA does not exclude recent infection as peak viremia occurs during incubation and the window of detectable RNA is narrow (approximately 2-3 wk)[28,29]. Also, studies show patients with a history of alcohol abuse who are exposed to HEV are significantly more likely to develop clinically apparent hepatitis[30], as this patient did. Interestingly, this patient was originally considered to have paracetamol induced liver necrosis, developing significant coagulopa-thy and an acute kidney injury as is commonly observed in such cases. Although the stated dose of paracetamol was relatively low and more recent studies have reported the validity of patient history in the context of hepato-toxicity, the reported dose of paracetamol is not related to eventual outcome[31,32]. It is possible that HEV, like other viral infections, can augment the hepatotoxicity of paracetamol[9-11]. However, coexisting acute hepatitis E in patients with paracetamol hepatotoxicity in this cohort was an uncommon finding (1.6%) and it is not possible to make comparisons with other cases of paracetamol hepatotoxicity based on a single case.

Of the four cases of hepatitis E identified in this study, only 1 patient (patient 4) had travelled to a region considered hyperendemic for HEV (India). Phylogenetic analysis revealed this patient to be infected with HEV genotype 1 (Figure 1 and Table 2), supporting the hy-pothesis that this was an imported case of HEV infec-tion. This was the only case that was diagnosed contem-poraneously, as HEV testing was prompted by the travel history. The remaining 3 patients had travelled outside of Scotland, although only to regions previously considered non-endemic for HEV (England and Spain). Phylogo-netic analysis of patient's 1 and 2 samples revealed these patients were infected with HEV genotype 3, the most prevalent genotype in cases of autochthonous hepatitis E infection in developed countries[2]. It is not possible to determine for certain whether these patients contracted their HEV infection in Scotland or the other "non-en-demic" regions they visited prior to onset of symptoms. However, given the absence of cases of hepatitis E in patients in this series who had not recently travelled out-side of Scotland, it suggests that locally acquired hepa-titis E infection in Scotland is uncommon. This notion is supported by a low IgG seroprevalence rate of 4.5% in blood donors from southeast Scotland and, at least compared to other European countries, a modest rate of asymptomatic viraemia at the time of donation (1 in 14500)[33].

Patient 4, aligned with Genotype 1 to confirm the ac-quisition of HepE during travel. However, HEV3 infec-tion in developed countries is commonly associated with the ingestion of contaminated food products such as undercooked pork, game meat and molluscs cultivated in contaminated water, as well as occupational exposure to pigs or their effluent[2]. Patient 1 and patient 2 had geno-types with homology to genotype 3f and 3e respectively.

Figure 2 Patient 2, transjugular needle biopsy. A: Low magnification view of part of the biopsy, showing red-stained nodular fibrosis indicating cirrhosis (x 20 original magnification, picrosirius red stain); B: Ballooned hepatocyte (arrow) containing a Mallory-Denk body and with surrounding neutrophils (satellitosis), features of steatohepatitis. (× 600 original magnification, H and E stain). Small clusters of these cells were present in the biopsy, with sparse small droplet macro-steatosis; C: Low grade hepatitic infiltrate (arrows) of lymphocytes with occasional plasma cells in the portal area (× 400 original magnification, H and E stain); D: Prominent chol-angiolitis (periportal ductules with oedema and neutrophils) (region indicated by arrows), with adjacent liver parenchyma showing canalicular cholestasis (arrowheads); E: Lobule showing mild disarray with cholestasis, increased lymphocytes and Kupffer cells within sinusoids and scattered apoptotic/necrotic cells (arrowheads).

These genotypes were isolated from European swine, however, pre-infection exposure to defined environmental and dietary risk factors is unknown and so we cannot narrow their possible source of infection further. Infection *via* blood transfusion has also been documented[2] and it was confirmed that none of the patients had recently undergone a blood transfusion.

The liver biopsy findings deserve comment given the differing genotypes. There are only limited previously published data on the liver biopsy appearances of endemic and locally acquired acute hepatitis E[23,34-36], as such cases usually have a self-limiting illness and so a liver biopsy is not commonly clinically indicated. There is a lobular hepatitis of varying severity between patients 2 and 4, from mild lobular disarray with Kupffer cell hypertrophy and scattered individually necrotic hepatocytes with adjacent neutrophils or lymphocytes, through to se-

vere lesions with confluent necrosis and collapse. A cholestatic element is often present within lobules and portal tracts, including canalicular cholestasis, mild bile duct inflammation and typically quite prominent neutrophilic cholangiolitis around the portal tracts as seen in patient 2 (Figure 2D and E). Similar pathology has been reported by Malcolm *et al*[35] in their locally acquired cases and by others[36]. These features can easily be mis-attributed to a drug-related cholestatic hepatitis. When occurring in patients with chronic liver disease such as alcoholic cirrhosis the viral effects may be overshadowed by or mis-attributed to steatohepatitis-related changes or sepsis-related decompensation. A plasma cell-rich portal, interface and lobular hepatitis such as characterises flares of autoimmune hepatitis is not normally seen in acute hepatitis E, although loose lymphoid aggregates in portal tracts have been described in occasional patients, which might misdi-

Figure 3 Patient 4, transjugular needle biopsy showing severe acute lobular hepatitis. A: Low magnification view showing the diffuse nature of the liver inflammation and injury (original magnification × 40, H and E); B: Severely inflamed lobule with numerous infiltrating inflammatory cells, including occasional plasma cells (long arrow), hepatocyte cell death (short arrow) and ballooning injury (arrowhead) (original magnification × 400, H and E); C: Shows an inflamed portal tract (delineated by arrows) expanded within by mononuclear inflammatory cells without bile duct injury and with only rare plasma cells or eosinophils. There is neutrophil cholangiolitis around the portal tract (just within the arrows) but no prominent interface hepatitis (original magnification × 200, H and E).

rect when there is pre-existing liver fibrosis. In patient 4, the absence of a prominent interface hepatitis and neutrophil cholangiolitis was comparable to a recent study on acute endemic HEV[34]. Whether autochthonous and endemic hepatitis E differ qualitatively in histological characteristics, remains uncertain given the small number of case comparisons including this study[34,35,37]. The variable underlying severity of the hepatitis and other selection factors prompting biopsy could skew the comparison and more data is required[38].

In summary, in 4 of 80 (5%) of patients with acute liver injury severe enough to warrant assessment and treatment at the Scottish Liver Transplant Unit, the cause was hepatitis E. Only one of these patients had a history of travel to an area traditionally considered hyperendemic for HEV. This is in line with the sero-prevalence in the Scottish population and higher than the rate of viraemia[31]. This study shows that clinicians should have a low threshold for considering hepatitis E as a possible diagnosis in any patient with severe acute liver injury. This should include those with possible paracetamol hepatotoxicity, irrespective of their age or travel history.

COMMENTS

Background
Hepatitis E virus (HEV), the etiological agent responsible for hepatitis E infection, is now recognised as an emerging zoonotic disease in industrialized countries. HEV genotypes 3 and 4 are responsible for sporadic cases of autochthonous hepatitis E infection in countries such as the United Kingdom, United States, France, Italy and Japan. HEV can cause a mild, self-limiting infection but it can also cause more serious health problems such as cirrhosis of the liver and fulminant hepatitis. The ability of HEV genotype 3 and 4 strains to cross the species barrier has been documented and there is a growing body of evidence that HEV can be transmitted to humans *via* the consumption of infected or contaminated food products.

Research frontiers
In developed countries, there have been very few previous studies of HEV genotype 3 in patients with acute liver injury severe enough to warrant assessment and treatment at a liver transplant unit.

Innovations and breakthroughs
Recent reports have highlighted the importance of HEV diagnosis in a number of clinical situations. In this study, we confirm the need for increased diagnostic testing in patients presenting with drug induced liver injury. In addition, liver pathogenesis clearly differs with the genotype causing the infection. Finally, routes of infection need further clarification.

Applications
Misdiagnosis of hepatitis E infection in drug induced liver injury has been noted in patients previously in South East England (13%) and the United States (3%). However, hepatitis E virus is still not given precedence when diagnosing these individuals. In the authors' study, 5% of individuals tested were misdiagnosed and viraemic. It is an important clinical point that the diagnosis of drug induced liver injury is not secure without first excluding hepatitis E, irrespective of travel history, particularly in patients with elevated transaminases. This data contributes to the increasing need to screen patients for the presence of the HEV.
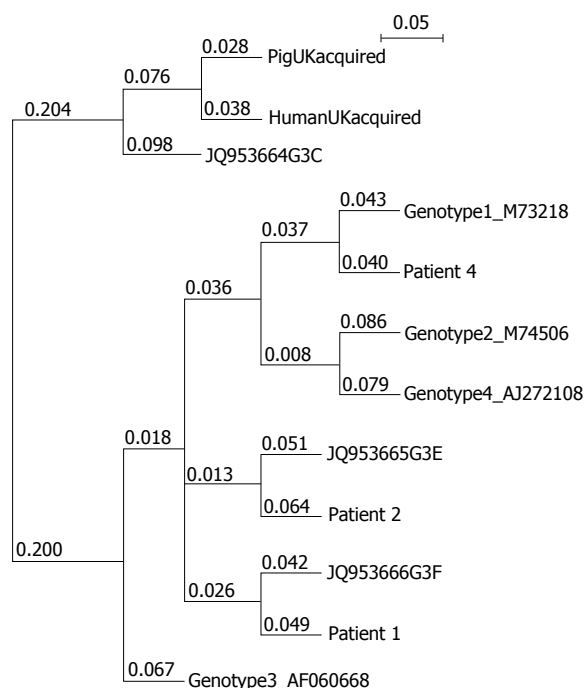
Terminology
HEV is a member of the Hepeviridae and Genotype 3 recognised as a zoonotic infection. The role of HEV in severe acute liver disease has yet to be defined.

Peer review
This is a well written manuscript, dealing with the prevalence of hepatitis E among a series of patients subjected to liver transplantation, in whom the etiology of liver failure was masked by coexisting cirrhosis and/or drug overdose. Authors correctly stress the importance of excluding HEV infection in Western countries, irrespective of the travel story.

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CASE CONTROL STUDY

Pooled genetic analysis in ultrasound measured non-alcoholic fatty liver disease in Indian subjects: A pilot study

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Abstract

AIM: To investigate genetic susceptibility in Indian subjects with non-alcoholic fatty liver disease (NAFLD) by performing a pooled genetic study.

METHODS: Study subjects (n = 306) were recruited and categorized into NAFLD and control groups based on ultrasound findings of fatty infiltration. Of the 306 individuals, 156 individuals had fatty infiltration and thus comprised the NAFLD group. One hundred and fifty (n = 150) individuals were normal, without fatty infiltration of the liver, comprising the control group. Blood samples, demographic and anthropometric data from the individuals were collected after obtaining informed consent. Anthropometric data, blood glucose, lipids and liver function tests were estimated using standard methods. Genome wide association studies done to date on NAFLD were identified, 19 single nucleotide polymorphisms (SNPs) were selected from these studies that were reported to be significantly associated with NAFLD and genotyping was performed on the Sequenom platform. Student's t test for continuous variables and χ^2 test was applied to variant carriers from both groups. Required corrections were applied as multiple testing was done.

RESULTS: The mean age of the control group was 39.78 ± 10.83 and the NAFLD group was 36.63 ± 8.20 years. The waist circumference of males and females in the control and NAFLD groups were 80.13 ± 10.35; 81.77 ± 13.65 and 94.09 ± 10.53; 92.53 ± 8.27 cms respectively. The mean triglyceride and alanine transaminase (ALT) levels in the control and NAFLD groups were 135.18 ± 7.77 mg/dL; 25.39 ± 14.73 IU/L and 184.40 ± 84.31 mg/dL; 110.20 ± 67.05 IU/L respectively. When χ^2 test was applied to the number of individuals carrying the variant risk alleles between the control and NAFLD group, a significant association was seen between rs738409 of the patatin-like phospholipase domain containing 3 (*PNPLA3*) gene (P = 0.001), rs2073080 of the *PARVB* gene (P = 0.02), rs2143571 of *SAMM50* gene (P = 0.05) and rs6487679 of the pregnancy zone protein (*PZP*) gene (P = 0.01) with the disease. Variant single nucleotide polymorphisms (SNPs) in *NCAN* and *PNPLA3* gene were associated with higher levels of ALT, whereas variant SNPs in APOC3, PNPLA3, EFCAB4B and COL13A1 were associated with high triglyceride levels. Apart from the above associations, rs2073080, rs343062 and rs6591182 were significantly associated with high BMI; rs2854117 and rs738409 with high triglyceride levels; and rs2073080, rs2143571, rs2228603, rs6487679 and rs738409 with high ALT levels.

CONCLUSION: Pooled genetic analysis revealed an association of SNPs in PNPLA3, PARVB, SAMM50 and *PZP* genes with NAFLD. SNPs in NCAN and *PNPLA3*

gene were associated with higher levels of ALT, whereas variant SNPs in APOC3, PNPLA3, EFCAB4B and COL13A1 were associated with high triglyceride levels.

Key words: Non-alcoholic fatty liver disease; Genome wide association studies; Genetic association; Hepatic steatosis; Genotyping; Single nucleotide polymorphisms; Susceptibility

Core tip: Non-alcoholic fatty liver disease (NAFLD) describes a range of conditions caused by build-up of fat within liver cells in the absence of alcohol consumption. Although obesity, diabetes, age, hypertension and hypertriglyceridemia contribute to the disease, genetics also has an important role to play. Furthermore, in 26%-35% of patients, genetic component is believed to contribute to NAFLD. By identifying significant single nucleotide polymorphisms from genome wide association studies reported from different ethnic populations for NAFLD and performing a pooled genetic association study, this study has identified important genetic risks that could help in identifying individuals with susceptibility at an early stage, thus aiding in better management of the disease.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a global epidemic, the incidence of which is reported to be as high as 25%-30% in different populations[1]. Differences in prevalence, clinical profile, histological severity and outcome of NAFLD in different ethnic groups suggest a genetic contribution; and NAFLD in 26%-35% of patients is believed to be contributed by genetic component[2,3]. In recent years, genetic heritability has been a major focus of research, although changing dietary habits and modifying life style have been demonstrated to benefit patients with hepatic steatosis[4]. Genome wide association studies (GWAS) from different ethnic populations revealed a strong association of PNPLA3 variant[3,5], apart from few other variants[6-8], and an independent study identified APOC3 variants associated with higher triglyceride levels and risk of NAFLD in migrant Indians[9].

A recent GWAS[6] of hepatic steatosis revealed loci in or near the neurocan (*NCAN*), glucokinase regulatory protein, lysophospholipase-like protein 1 and protein phosphatase 1, regulatory subunit 3B (*PPP1R3B*) genes that have associations with glycemic traits, serum lipid

levels, hepatic steatosis, hepatic inflammation/fibrosis, or a combination of these. Specific genotypic information in the form of single nucleotide polymorphisms (SNPs) which confer susceptibility for an individual have to be identified so that early preventive measures can be initiated, especially in children and adolescents. Patatin-like phospholipase domain containing 3 (PNPLA3) missense variant was studied and compared with MR spectroscopy for predicting NAFLD[4]. However, since it is now known that multiple SNPs are associated with the disease, identifying other susceptibility SNPs apart from PNPLA3 would enhance the predictive capability.

The prevalence of NAFLD in the Indian population is estimated to be around 25%-30%[10-16]. In addition, the prevalence of hepatic steatosis in non-obese (lean NAFLD) was shown to range between 11%-31.7% according to a recent study[17]. Increase in the incidence of obesity, metabolic syndrome and the presence of lean non-alcoholic steatohepatitis (NASH) in the Indian population warrants genetic susceptibility studies in Indian NAFLD subjects. In this preliminary pilot study, we selected SNPs (Table 1) from already reported GWAS across different populations and genotyped the same in Indian subjects.

MATERIALS AND METHODS

A total of 450 individuals with fatty infiltration were recruited for the study during 2011-2012 (1 year) from hepatology clinics of the hospital. As shown (Figure 1), 156 individuals were found to be eligible for the pooled genetic analysis. Statistical power analysis was not used to compute the sample size as this is a pilot study. Although liver biopsy is considered to be the gold standard for identifying NAFLD and NASH, lack of indication for asymptomatic individuals, the costs involved, risk of complications and ethical concerns limit its use in these types of studies. Therefore, subjects were recruited based on ultrasound findings of hepatic steatosis as per earlier reports[18,19]. Healthy subjects (*n* = 150) from the institute who volunteered to be part of the study were recruited as controls based on the sole criteria of the absence of fatty liver on ultrasonography and normal alanine transaminase (ALT) levels. Written informed consent was obtained from each individual. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Review Committee. Demographic and anthropometric details [height, weight, magnetic resonance imaging (BMI) and waist circumference] were collected in a structured pro forma. Whole blood (5 mL) was collected in pre coated EDTA containers from the study group and stored at -20 ℃ until further analysis. Biochemical investigations like ALT, viral markers and lipid profiles were estimated as per standard methods.

Definitions

Individuals with BMI less than 18.5 kg/m² were defined

Table 1 List of single nucleotide polymorphisms included in the study

SNP No	rsID	Risk allele	Associated gene	Associated with	Ref.
1	rs738409	G	*PNPLA3*	Hepatic steatosis	[6]
2	rs4240624	A	*PPP1R3B*	Hepatic steatosis	[6]
3	rs2228603	T	*NCAN*	Hepatic steatosis	[6]
4	rs780094	A	*GCKR*	Hepatic steatosis	[6]
5	rs12137855	C	*LYPLAL1*	Hepatic steatosis	[6]
6	rs2645424	C	*FDFT1*	NAFLD activity score	[8]
7	rs343062	T	-	Degree of fibrosis	[8]
8	rs1227756	G	*COL13A1*	Lobular inflammation	[8]
9	rs6591182	G	-	Lobular inflammation	[8]
10	rs887304	A	*EFCAB4B*	Lobular inflammation	[8]
11	rs2499604	A	Intronic *ZP4-TRNAP23P*	Serum levels of alanine aminotransferase	[8]
12	rs6487679	C	*PZP*	Serum levels of alanine aminotransferase	[8]
13	rs1421201	C	-	Serum levels of alanine aminotransferase	[8]
14	rs2710833	T	-	Serum levels of alanine aminotransferase	[8]
15	rs2854116	A	*APOC3*	Hypertriglyceridemia in Asians	[9]
16	rs2854117	G	*APOC3*	Hypertriglyceridemia in Asians	[9]
17	rs2143571	A	*SAMM50*	NAFLD	[7]
18	rs2073080	T	*PARVB*	NAFLD	[7]
19	rs1390096	A	*HS3ST1-HSP90AB2P*	NAFLD	[7]
20	rs11206226	A	*YIPF1*	NAFLD	[7]

PNPLA3: Patatin-like phospholipase domain containing 3; NCAN: Neurocan; GCKR: Glucokinase regulatory protein; LYPLAL1: Lysophospholipase-like protein 1; PPP1R3B: Protein phosphatase 1, regulatory subunit 3B; PZP: Pregnancy zone protein.

Figure 1 Flowchart showing sample recruitment.

as underweight; 18.5-22.9 kg/m^2 were defined as normal and BMI more than 23 kg/m^2 were defined as obese. Lean NAFLD was defined as hepatic steatosis in individuals with normal BMI (< 22.9 kg/m^2) according to Asian standards[20]; likewise hypertriglyceridemia (greater than 150 mg/dL), low levels of high density lipoprotein (HDL less than 40 mg/dL in males and 50 mg/dL in females), hypertension (greater than 130/85 systolic and diastolic blood pressure level in mmHg or on anti hypertensive drugs) and high fasting glucose levels (greater than 100 mg/dL of fasting blood sugar levels) were considered as cut offs. The cut off for waist circumference was > 90 cm and > 80 cm in males and females respectively, as per Asian standards[21]. A cut off of 30 IU/L was considered for ALT[22].

Genotyping

DNA was isolated from blood using standard protocols. The concentration and integrity of DNA was measured with NanoDrop 1000 spectrophotometer (Thermo Scientific, USA) and agarose gel electrophoresis respectively. The DNA with 260/280 ratios between 1.8-2.0 and agarose gel image showing a high molecular weight intact DNA band were included for further genotyping analysis. The samples were genotyped for the 19 SNPs on the Sequenom platform (Sequenom®, San Diego, CA, United States) using the manufacturer's protocol. Primers for one SNP (rs2854116) could not be designed because of proximal SNPs present very near to the target SNP and so was not included in the study. The raw data files generated by Sequenom MassARRAY were analyzed for the intensity peaks of calibrant to ascertain the quality of the data. An overall call rate of > 95% was maintained. Five percent of the samples were duplicated across the plate, their genotypes compared and they had 100% concordance. Negative controls (master mix without DNA) were also included.

Correlation of demographic and anthropometric phenotypes, like BMI, waist circumference, liver enzymes (ALT) and triglyceride levels, to the genotype was done to identify significant risk factors.

Statistical analysis

The data collected was edited for consistency and completeness and entered into MS-Excel for further analysis. Patient characteristics were compared using Student's *t* test for continuous variables and proportion test for categorical variables. χ^2 test was used on the number of variant

Table 2 Demographic and clinical characteristics of the study group			
Parameter	Controls (*n* = 150)	Patients (*n* = 156)	*P* value
Mean ± SD	39.78 ± 10.83	36.63 ± 8.20	0.004
Age, yr	18-63	19-62	
Range			
Males	110 (73.33%)	138 (88.46%)	-
Females	40 (26.66%)	18 (11.53%)	-
Waist circumference			
Males	80.13 ± 10.35	94.09 ± 10.53	0.0001
Females	81.77 ± 13.65	92.53 ± 8.27	0.01
BMI (kg/m^2)	24.04 ± 7.77	27 ± 5.86	0.001
Triglycerides (mg/dL)	135.18 ± 7.77	184.40 ± 84.31	0.0001
HDL (mg/dL)	41.86 ± 9.70	39.56 ± 13.02	0.2923
ALT (IU/L)	25.39 ± 14.73	110.20 ± 67.05	0.0001
AST (IU/L)	25.99 ± 8.46	69.14 ± 37.77	0.0001
Hypertensives	4 (2.6%)	18 (11.53%)	-
Diabetics	19 (12.66%)	27 (17.3%)	-

BMI: Body mass index; HDL: High density lipoprotein; ALT: Alanine transaminase; AST: Aspartate amino transferase.

carriers in the control and NAFLD groups for identifying SNPs associated with NAFLD. To correct for multiple comparison testing, the Benjamini and Hochberg false discovery rate correction[23] was applied to "*P* values". All SNPs were divided into risk and non-risk groups and 2X2 contingency tables were prepared to estimate odds ratio for all variables like age, gender, BMI, ALT levels *etc*. Multiple logistic regression was used to identify independent predictor variables for NAFLD. The data was analyzed using Statistical Package for Social Sciences (SPSS Version 17). In this study, a *P* value ≤ 0.05 was considered statistically significant. Haplotype analysis was carried out using software[24]. An excel sheet was prepared as per instructions with "0" representing wild type allele and "1" representing heterozygous or mutant variants.

RESULTS

The clinical characteristics, such as age, waist circumference, BMI, triglyceride and ALT levels, of the groups are presented in Table 2. Categorization of the study population yielded two groups based on ultrasonographic detection of hepatic steatosis in the liver, namely the NAFLD (*n* = 156) and control group (*n* = 150). The inclusion of individuals in the control group was based on the absence of hepatic steatosis and retrospectively it was seen that few of the individuals in the control group were obese (BMI > 23 kg/m^2). So the group was divided based on BMI and a comparison of both clinical characteristics and the genotype was made (data not shown) between the normal and obese controls. Such an analysis did not show any significant differences between the normal and obese control group with respect to the genotype. However, the waist circumference in males (*P* = 0.0001) and females (*P* = 0.02) and the triglyceride levels (*P* = 0.0057) were high in the obese controls, apart from BMI (*P* = 0.0001), and the difference in all the other characteristics

studied was statistically not significant.

Association between clinical characteristics, SNPs and risk of NAFLD

When an analysis was done between the control and NAFLD group, a significant difference in clinical characteristics was noted in BMI (*P* = 0.001), waist circumference of males (*P* = 0.0001) and females (*P* = 0.01), high triglyceride levels (*P* = 0.0001) and ALT (*P* = 0.0001) levels in the NAFLD group.

In the single allelic analysis, tests for associations between NAFLD and the SNPs revealed that variants in PARVB, SAMM50, NCAN, intronic SNP (rs2499604), *APOC3*, pregnancy zone protein (*PZP*) and *PNPLA3* genes were associated with NAFLD; however, after correction for multiple testing was applied, only variants in PARVB, SAMM50, PZP and PNPLA3 were significant (Table 3).

Significant SNPs associated with clinical traits

To identify SNPs which may be associated with clinical traits like triglyceride and ALT levels but not necessarily to the disease, the individuals in the study group were divided into two groups, namely individuals with normal and those with high levels of the mentioned clinical traits irrespective of the disease status. Such an effort identified significant SNPs which are likely to be associated with clinical traits. SNPs in NCAN (*P* = 0.04) and PNPLA3 (*P* = 0.001) were significantly associated with high ALT levels and SNPs in *APOC3* (*P* = 0.01), *PNPLA3* (*P* = 0.05), *EFCAB4B* (*P* = 0.04) and *COL13A1* (*P* = 0.02) genes were significantly associated with high triglyceride levels.

Odds of developing NAFLD

Among the various characteristics like age, BMI and the SNPs that were studied, rs2073080 in PARVB, rs343062 (intronic) and rs6591182 (intronic) were significantly associated with higher odds of obese individuals with NAFLD. Likewise, SNPs in various genes studied were associated with clinical parameters like ALT and triglyceride levels (Table 4).

Haplotype analysis

Since PNPLA3, SAMM50 and PARVB are found on the same locus on chromosome 22, haplotype analysis was done for the 3 SNPs and it was noted that heterozygous or homozygous variants in these genes were overrepresented in the NAFLD group compared to the control group (8 in controls against 63 in the NAFLD group) (Table 5).

Multivariate logistic regression analysis

Multiple logistic regression analysis was applied to the data to estimate the risk of an individual for NAFLD. The dependent variables were the NAFLD group and controls. The variables that were significant in the univariate analysis, namely age (less than 40 years), BMI, waist circumference, triglyceride levels, HDL, hypertension,

Table 3 Comparison of variant carriers between patients and controls

SNP-gene name	Allele frequency controls (n = 150)		Allele frequency patients (n = 156)		χ^2	P value	Corrected P value[1]	OR	95%CI
	Major	Minor	Major	Minor					lower-upper
rs1227756 COL13A1	0.49	0.51	0.44	0.56	0.02	0.88	0.93	1.04	0.54-2.03
rs12137855 LYPLAL1	0.77	0.23	0.73	0.27	0.41	0.51	0.62	1.20	0.68-2.12
rs1390096 HS3ST1-HSP	0.68	0.32	0.66	0.34	0.007	0.93	0.93	0.97	0.55-1.70
rs1421201 intronic	0.88	0.12	0.85	0.15	1.60	0.20	0.36	1.55	0.78-3.10
rs2073080 PARVB	0.81	0.19	0.69	0.31	8.42	0.003	0.02	2.36	1.31-4.22
rs2143571 SAMM50	0.80	0.20	0.69	0.31	6.25	0.01	0.05	2.07	1.16-3.69
rs2228603 NCAN	0.97	0.03	0.92	0.08	4.09	0.04	0.12	3.29	1.10-9.84
rs2499604 intronic	0.53	0.47	0.60	0.40	3.76	0.05	0.13	1.92	0.98-3.75
rs2645424 FDFT1	0.5	0.50	0.56	0.44	1.21	0.27	0.40	0.69	0.36-1.32
rs2710833 intronic	0.60	0.40	0.66	0.34	3.09	0.07	0.17	0.56	0.30-1.07
rs2854117 APOC3	0.58	0.42	0.55	0.45	4.24	0.03	0.12	1.83	1.02-3.28
rs343062 intronic	0.55	0.45	0.52	0.48	1.96	0.16	0.32	1.53	0.84-2.80
rs4240624 PPP1R3B	0.94	0.06	0.91	0.09	1.21	0.27	0.40	1.56	0.69-3.52
rs6487679 PZP	0.89	0.11	0.75	0.25	9.65	0.001	0.01	2.81	1.44-5.48
rs6591182 intronic	0.63	0.37	0.59	0.41	0.99	0.31	0.44	1.39	0.72-2.67
rs738409 PNPLA3	0.92	0.08	0.60	0.40	46.37	0.0001	0.001	12.66	5.45-29.38
rs780094 GCKR	0.76	0.24	0.74	0.26	0.48	0.48	0.62	1.22	0.69-2.15
rs887304 EFCAB4B	0.82	0.18	0.83	0.17	0.19	0.65	0.73	0.87	0.47-1.59
rs11206226 YIPF1	1.00	0.00	1.00	0.00	-	-	-	-	-

[1]Benjamini and Hochberg false discovery rate correction was applied to the "P value". PNPLA3: Patatin-like phospholipase domain containing 3; NCAN: Neurocan; GCKR: Glucokinase regulatory protein; LYPLAL1: Lysophospholipase-like protein 1; PPP1R3B: Protein phosphatase 1, regulatory subunit 3B; PZP: Pregnancy zone protein.

diabetes and SNPs, were included for the multivariate analysis (Table 6).

DISCUSSION

The main objective of this study was to identify susceptibility SNPs for NAFLD in Indian subjects utilizing pooled genetic SNP data from various GWAS performed in different populations to date. Variants in *SAMM50*, *PARVB*, *PZP* and *PNPLA3* genes were significantly associated with NAFLD, thus suggesting involvement of multiple loci in Indian NAFLD.

A significant association of PNPLA3 (rs738409) (P = 0.001) with NAFLD was observed in Indian subjects and is consistent with the genetic association of PNPLA3 in other populations, like Caucasians, European descent, Hispanics and Japanese[6-8]. Furthermore, this SNP was also significantly associated with higher ALT (P = 0.001)

and triglyceride levels (P = 0.05), suggesting that individuals with the variant may be at higher risk for NAFLD. In addition to this SNP, PZP rs6487679 located on the 12th chromosome, demonstrated to have a role in clearance of transforming growth factor-beta from human plasma and hepatic fibrogenesis[25], was also significantly associated with NAFLD in Indian subjects. This finding corroborates with similar earlier findings in non-Hispanic Caucasians[8].

rs2073080 of the beta-parvin (PARVB) located on chromosome 22 that codes for a protein beta-parvin in humans[26] was significantly associated with the disease (P = 0.018) in the present study. Not much is known about the polymorphism, but in general the protein is believed to play a role in cytoskeleton organization and cell adhesion apart from having a role in tumor suppression (Entrez Gene: *PARVB*). The association of rs2143571 of the SAMM50 sharing the same locus on chromosome 22

Table 4 Significant single nucleotide polymorphisms associated with higher odds of body mass index, triglycerides and alanine transaminase based on clinical characteristics

Variable	SNP-gene	OR	*P* value	95%CI lower-upper
BMI (obese and non-obese)	rs2073080-PARVB	1.81	0.0470	1.00-3.25
BMI (obese and non-obese)	rs343062-intronic	2.25	0.0100	1.21-4.21
BMI (obese and non-obese)	rs6591182-intronic	2.05	0.0340	1.05-4.00
TG (abnormal and normal)	rs2854117-APOC3	2.31	0.0040	1.29-4.13
TG (abnormal and normal)	rs738409-PNPLA3	1.94	0.0170	1.12-3.37
ALT (abnormal and normal)	rs2073080-PARVB	1.92	0.0200	1.10-3.36
ALT (abnormal and normal)	rs2143571-SAMM50	1.77	0.0200	1.10-3.36
ALT(abnormal and normal)	rs2228603-NCAN	3.23	0.0180	1.22-1.37
ALT (abnormal and normal)	rs6487679-PZP	1.92	0.0300	0.05-3.51
ALT (abnormal and normal)	rs738409-PNPLA3	5.07	0.0001	0.03-10.92

BMI: Body mass index; TG: Triglycerides; ALT: Alanine transaminase; SNP: Single nucleotide polymorphisms.

Table 5 PARVB-SAMM50-PNPLA3 haplotype data

Total counts	Haplotype	Number in controls	Number in patients
138	000	90	48
31	001	6	25
10	010	10	0
4	011	2	2
4	100	2	2
48	110	32	16
71	111	8	63

"Total Counts" column consists of the number of haplotypes generated in both controls and patient group combined. In the haplotype column, 0 stands for wild type allele and 1 for either heterozygous or homozygous variant carrier. The sequence of the genes is *PARVB, SAMM50* and *PNPLA3*. The individuals count for the haplotypes for controls and patient group is given in the Number in controls and Number in patients column.

Table 6 Results of multiple logistic regression analysis

Variable	Regression coefficient	Standard error	*P* value	OR	95%CI Lower	95%CI Upper
PZP	0.880	0.415	0.034	2.41	1.05	5.44
PNPLA3	2.289	0.480	< 0.0001	9.86	3.85	25.29
Triglyceride levels	1.502	0.391	0.000	4.48	2.08	9.67
Constant	-0.619					

PNPLA3: Patatin-like phospholipase domain containing 3; PZP: Pregnancy zone protein.

as PNPLA3 and PARVB encoding sorting and assembly machinery component 50 homolog was also significantly associated with NAFLD in the Indian subjects[27]. This protein has a function in the assembly of beta-barrel proteins into the outer mitochondrial membrane. A recent genome wide scan[7,28] also identified similar SNPs in PNPLA3, SAMM50 and PARVB in the Japanese population which was significantly associated with NAFLD. Our results corroborate with this study, indicating that these 3 variants are commonly seen in an Asian population.

Apart from the promoter polymorphism of the *APOC3* gene and variant in *PNPLA3* gene, EFCAB4B and COL13A1 polymorphisms were identified as significantly associated with higher triglyceride levels. Polymorphisms in NCAN and PNPLA3 were associated with higher ALT levels. Although previous studies[6,7] reported an association of the above mentioned SNPs with NAFLD, their associations with triglycerides and ALT levels have been identified for the first time in Indian subjects.

When lean and obese controls were compared for significant differences in clinical characteristics and genotype, BMI between the groups was significantly different, with a higher BMI in the obese group as expected. The triglyceride levels were also significantly higher in the obese control group without hepatic steatosis compared to the lean controls; however, there were no significant differences in the genotype with respect to the 19 SNPs studied. Based on this, these individuals were found to be suitable to be included in the control group for further analyses.

The incidence of lean NAFLD in the present study (19.87%) is in agreement with an earlier study from North India[17] which had more or less a similar incidence (13.2%) and there was no significant difference in the incidence between the two groups (*P* = 0.08).

When odds were computed based on obese and non-obese status in the study group, PARVB and two intronic SNPs (rs343062 and rs6591182) were significantly associated with higher odds of NAFLD in the obese, suggesting that an obese individual with these variants is at a higher risk of hepatic steatosis compared to a non-obese individual. This important finding has a clinical implication in that, if an individual with the above mentioned variants can be identified at an early age, the significant modifying risk factors like higher waist circumference and triglyceride levels can be managed, thus reducing the predisposing risk component because of the variants in SNPs and thereby delaying the onset of hepatic steatosis.

Haplotype data for the three SNPs on PNPLA3, SAMM50 and PARVB suggests that three SNPs might be linked as heterozygous or mutant variant carriers were overrepresented in the NAFLD group (63 counts) compared to the control group (8 counts) (Table 5).

A recent study[29] from North India reported a higher frequency of CG and GG genotypes of rs738409 polymorphism in the *PNPLA3* gene in North Indians and a significant association of the genotype to ALT (*P* = 0.003) and AST levels (*P* = 0.04). The values of triglycerides were slightly higher in the cases but were not significantly different in comparison to controls. This study is in agreement with the above study from the North Indian center with respect to the PNPLA3 polymorphism and

its association with NAFLD and ALT levels. Our study also found a significant association of higher triglyceride levels with rs738409 polymorphism. However, the present study has looked at an additional 18 polymorphisms, which is by far the most comprehensive pooled genetic analysis taken up in Indian subjects with NAFLD.

To estimate the strength of the relationship between several independent variables and a continuous dependent variable, multiple logistic regression analysis was done with significant SNPs and patient characteristics like triglyceride levels, BMI from the univariate analysis as the independent variables and NAFLD as the dependent variable. While high levels of BMI, triglyceride levels, waist circumference both in males and females, ALT levels and variant SNPs in PARVB, SAMM50, NCAN, intronic SNP rs2499604, PZP and PNPLA3 were significantly associated with NAFLD when univariate analysis was done, only variants in *PZP* and *PNPLA3* genes and high triglyceride levels were significantly associated with NAFLD when multivariate analysis was done, suggesting that these three are independent risk factors to predict hepatic steatosis and that the others probably interact with the modifying risk factors like BMI and waist circumference in the causation of NAFLD.

In conclusion, an analysis between the control and NAFLD groups revealed significant differences in BMI, triglyceride levels, waist circumference in both males and females and ALT levels with higher levels associated with the NAFLD group. Variant SNPs in *NCAN* and *PNPLA3* genes were significantly associated with high ALT levels, which are the clinical phenotype of hepatic necroinflammation state, and SNPs in APOC3, PNPLA3, EFCAB4B and COL13A1 were associated with higher triglyceride levels.

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COMMENTS

Background

Non-alcoholic fatty liver disease (NAFLD) encompasses a spectrum of conditions associated with lipid deposition in the hepatocytes, ranging from simple steatosis (fatty liver) to non-alcoholic steatohepatitis (fatty changes with inflammation and hepatocellular injury or fibrosis), to advanced fibrosis and cirrhosis. It is the most common cause of liver disease, with a prevalence of 25%-30% in the general population. The presence of metabolic syndrome is the most common risk factor for NAFLD and it is now believed that NAFLD is the hepatic manifestation of metabolic syndrome. The other important risk factors are obesity, type-2 diabetes, total parenteral nutrition, jejunoileal bypass operation and use of certain medications. However, genetics play an important role in NAFLD and it is believed that 26%-35% of the patients who develop NAFLD have an underlying genetic component. So, it is important to identify the genetic aspects of the disease and their environmental interactions for better management of the disease.

Innovations and breakthroughs

Studies have identified that variant single nucleotide polymorphisms (SNPs) in genes, namely PNPLA3, NCAN, glucokinase regulatory protein, lysophospholipase-like protein 1, FDFT1, COL13A1, SAMM50, PARVB and pregnancy zone protein (PZP), were associated with NAFLD. A pooled genetic study was carried out by identifying significant SNPs from genome wide association studies and this study identified SNPs which are associated with Indian NAFLD. Apart from these associations, variant SNPs which contribute to hypertriglyceridemia, and alanine transaminase levels were also identified. By genotyping for these SNPs, an individual's predisposing risk can be identified at an early age and lifestyle-based modifications would ensure delayed onset of fatty infiltration.

Applications

Susceptibility loci for Indian NAFLD have been identified for the first time. The genotype data can be used in early identification and better management of the disease.

Terminology

Genome wide association study is the examination of many common genetic variants known as SNPs (single nucleotide polymorphisms) in two sets of individuals. One set of individuals with disease and the other set without the disease are compared for a large number of SNPs (approximately 9 lakhs) and analysis is done to identify those SNPs with a higher frequency in the disease group and these SNPs are said to be associated with the disease.

Peer review

This well written and interesting pilot study of genetic susceptibility of NAFLD in an Indian population has shown that multiple SNPs and loci are involved in the development of NAFLD. Variant SNPs in *PZP* and *PNPLA3* genes were found to be independent risk factors for the development of NAFLD. PARVB, SAMM50, neurocan and intronic SNP rs2499604 were significant risk factors along with other associations. So, genetics play an important role along with metabolic factors in the development of NAFLD. These findings may add a new level to the existing knowledge about the genetic basis of NAFLD, especially in the Indian population, and be valuable for clinical interference.

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SYSTEMATIC REVIEWS

Reuse of liver grafts following the brain death of the initial recipient

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Abstract

AIM: To determine if there is a reasonable prospect of success of a re-use liver transplantation.

METHODS: We systematically searched for reports of liver graft re-use using electronic searches of PubMed and Web of Knowledge. We performed hand searches of references lists of articles reporting re-use of grafts.

RESULTS: A systematic review of the literature reveals 28 liver transplantations using previously transplanted grafts. First and second recipients ranged in age from 4 to 72 years and 29 to 62 years respectively. Liver disease in the first recipient was varied including 5 (18%) patients with fulminant liver failure who died subsequently of cerebral edema. The second transplantation was performed after a median interval of 5 d (one day-13 years). Viral hepatitis was present in 3 (11%) of the initial recipients and in 8 (29%) of final recipients. Hepatocellular carcinoma was present in 6 (21%) of the final recipients. Early survival after the final transplantation was 93%, whereas long-term survival was 78% with a mean follow-up of 23.3 (3-120) mo.

CONCLUSION: Outcomes of transplantation using previously transplanted grafts in this select population are similar to those seen with conventional grafts.

Key words: Reuse; Liver graft; Brain death; Liver transplantation

Core tip: Reuse of a previously transplanted liver graft may be considered if the first recipient suffers neurological death at some time after liver transplantation.

Tanaka H, McAlister VC, Levstik MA, Ghent CN, Marotta PJ, Quan D, Wall WJ. Reuse of liver grafts following the brain death of the initial recipient. *World J Hepatol* 2014; 6(6): 443-447 Available from: URL: http://www.wjgnet.com/1948-5182/full/v6/i6/443.htm DOI: http://dx.doi.org/10.4254/wjh.v6.i6.443

INTRODUCTION

The growing disparity between the demand for and supply of organs for transplantation has restricted the availability of grafts for patients whose indications for transplantation fall outside of conventional guidelines and it has led to new strategies to increase donor utility. On rare occasions, a donor situation is such that it is not acceptable for routine transplantation but a novel rationale is present for an expectation of success so that the graft

may be offered to candidates who would otherwise be excluded from transplantation. We encountered this situation for a patient with hepatitis C virus (HCV) and hepatocellular carcinoma (HCC) that had advanced beyond criteria for transplantation when another liver recipient unexpectedly suffered neurological death from intracerebellar bleeding, 13 d after transplantation. Organ donation for allocation to patients on the conventional liver transplantation waiting list had been declined by the organ procurement organization. To determine if there was a reasonable prospect of success of a re-use transplantation, we undertook a systematic survey of the literature.

MATERIALS AND METHODS

We systematically searched for reports of liver graft re-use using electronic searches of PubMed (1966 to January 2013) and Web of Knowledge (1981 to January 2013). The following key words were used: "liver transplantation"; "reuse"; "graft" or "liver graft". The search was limited to the English literatures and humans. We performed hand searches of references lists of articles reporting re-use of grafts. We collected the data to determine the age range of each donor and recipient, their liver disease and cause of death (if applicable), the interval between the initial and final liver transplantation and the outcome of the final transplantation.

RESULTS

Systematic review of the literature revealed 14 papers describing 27 liver recipients of previously transplanted grafts with an early survival rate of over 90% for both the patients and the re-used grafts[1-14]. No review of this aspect of liver transplantation was located. We proceeded with the re-use liver transplantation in London, ON. The initial recipient was a 55-year-old man with end-stage liver disease secondary to hepatitis C. His blood type was O and hepatitis B core antibody was positive. The initial donor for this recipient was a 69-year-old man with blood type O, who developed brain death from intracranial hemorrhage. Unfortunately, he suffered a huge intracerebellar bleed on the 4th day after transplantation and was declared brain dead on day 13. The second recipient was 54-year-old man with hepatitis C cirrhosis and a history of ruptured HCC two years earlier. He had been put on capecitabine 1000 mg/m^2 and multiple liver lesions were embolized by angiogram 7 mo earlier. Even though his HCC appeared to be stable and there was no evidence of extrahepatic disease, long-term survival without liver replacement was considered unlikely. The opportunity was discussed with the patient and his family including its known risks and uncertainties. The blood type of the second recipient was B and hepatitis B serology was negative. At retrieval surgery 14 d after the initial transplant, the liver graft was found to be larger than before with a stiff texture (Figure 1). The liver graft was perfused with Histidine Tryptophan Ketogluterate (HTK) solution *via* the portal vein. Arterial perfusion was done

Figure 1 Reuse graft after the retrieval. It was slightly enlarged with a stiff texture.

on the back-table confirming good flow of the perfusate. In the final recipient wide resection of tissue surrounding the liver was performed including areas of diaphragm, peritoneum, omentum, extrahepatic nodes and lymphatic tissue. Occlusive thrombus was removed from the native portal vein. Cold ischemic and warm ischemic times were 9 h and 1.5 h, respectively. His postoperative course was straightforward except for temporary renal impairment. His transaminases went up more than 4000 IU/L, but graft function improved significantly thereafter. His induction immunosuppressive therapy was basiliximab and steroid, and he was maintained on sirolimus and steroid thereafter. Prophylaxis for hepatitis B started according to our protocol. He was put on capecitabine again on day 4. He was discharged 15 d after transplantation. Evidence of recurrent hepatitis C virus was diagnosed 8 mo later. Although the graft continued to function well, he expired 16 mo after transplantation due to recurrence of HCC.

Data from the 28 reuse transplantations are given in Table 1. Initial donors and recipients ranged in age from 4 to 72 years and 29 to 62 years respectively. Liver disease in the first recipient was varied with the notable exception of higher than expected incidence of fulminant liver failure in 5 (18%) patients. These patients became donors when brain death from cerebral edema was diagnosed after liver transplantation. The commonest cause of death of the initial recipient was cerebrovascular accident 4 d (median, one day-13 years) after transplantation. Brain anoxia was the cause of death in one patient but is not recorded in the remaining patients. The second transplantation was performed 5 d (median, one day-13 years) after the initial transplantation. Viral hepatitis was present in 3 (11%) of the initial recipients and in 8 (29%) of final recipients. HCC was present in 6 (21%) of the final recipients. One reused graft failed to function and a second graft failed from hepatic artery thrombosis giving an initial patient and graft survival of 93%. Long-term survival is 78% with a mean follow-up of 23.3 (3-120) mo.

DISCUSSION

The outcomes described in this report of liver transplantation using previously transplanted grafts is comparable

Table 1 Reuse of liver grafts following the brain death of the initial recipient

Location (ref. NO.)	Donor			Interval (d) initial to final transplant	Recipient		Outcome	
	Age (yr)	Liver disease	Cause of death		Age (yr)	Liver disease	Early after second transplantation	Long-term
London, Canada (current report)	55	HCV	CVA	14	54	HCV/HCC	No complications	Died of recurrent HCC at 16 mo
Madrid, Spain[1,2]	57	PBC	CVA	1	29	CR post LTx for PSC and CCC	No complication	Died of recurrent CCC at 48 mo
Madrid, Spain[2]	54	PSC	CVA	2	32	CR post LTx for HCV	Sepsis	Died at 4 mo
Madrid, Spain[2]	51	CR post LTx (cause N/A)	CVA	2	56	HCV/HCC	AR	Alive at 25 mo
Creteil, France[3]	24	CR post LTx for cryptogenic cirrhosis	CVA	5	52	Alcoholic	AR	Alive at 6 mo
Essen, Germany[4]	N/A	Cryptogenic cirrhosis	CVA	1	46	Recurrent HBV post LTx	AR	Alive at 5 mo
Barcelona, Spain[5]	55	Alcoholic	CVA	5	58	HCV	No complication	Alive at 14 mo
Brussels, Belgium[6]	47	ALF (acetaminophen)	Cerebral edema	2	53	HCV/HCC	AR	Alive at 22 mo
Lille Cedex, France[7]	21	ALF (acetaminophen)	Cerebral edema	2	61	HCV	No complication	Alive at 11 mo
UNOS #1[8]	6	N/A	N/A	1	N/A	N/A	N/A	Alive at 111 mo
UNOS #2[8]	60	Cryptogenic cirrhosis	CVA	8	44	N/A	N/A	Alive at 62 mo
UNOS #3[8]	21	N/A	N/A	1	N/A	N/A	N/A	Alive at 3.5 mo
UNOS #4[8]	49	N/A	N/A	4.9 yr	N/A	N/A	failed at 0.1 mo (cause N/A)	-
UNOS #5[8]	48	N/A	N/A	5	N/A	N/A	N/A	Failed at 11 mo
UNOS #6[8]	56	HCV	Anoxic brain injury	2	N/A	HCV, alcoholic	No complication	Alive at 25.4 mo
UNOS #7[8]	49	N/A	N/A	6	N/A	N/A	N/A	Alive at 4.8 mo
UNOS #8[8]	35	ALF (acetaminophen)	Cerebral edema	3	56	PSC	No complication	Alive at 12 mo
UNOS #9[8]	46	N/A	N/A	2	N/A	N/A	N/A	Alive at 11 mo
UNOS #10[8]	25	N/A	N/A	2.8 yr	N/A	N/A	N/A	Alive at 5.9 mo
UNOS #11[8]	44	N/A	N/A	17	N/A	N/A	N/A	Alive at 3.0 mo
Barcelona, Spain[9]	55	Alcoholic	CVA	5	58	HCV	No complication	Alive at 120 mo
Barcelona, Spain[9]	58	Alcoholic	CVA	14	55	Budd Chiari synd	No complication	Alive at 13 mo
Barcelona, Spain[9]	58	Alcoholic	CVA	10	47	Ischemic cholangitis	AR	Alive at 7 mo
Creteil, France[10]	72	Alcoholic	CVA	13 yr	61	Cryptogenic cirrhosis,	No complication	Alive at 12 mo
Montreal, Canada[11]	26	ALF (acetaminophen overdose)	Cerebral edema	2	62	Hemochromatosis HCC	No complication	Alive at 30 mo
Berlin, Germany[12]	53	Cryptogenic cirrhosis	Cerebral edema	24	43	Alcoholic, HCC	Biliary obstruction by stones	Alive at 6 mo
Stuttgart, Germany[13]	38	Budd Chiari synd	CVA	5 yr	51	Polycystic liver disease	No complication	Alive at 18 mo
Malatya, Turkey[14]	4	ALF (hepatitis A)	Cerebral edema	5	31	Cryptogenic cirrhosis, HCC	HAT at one month, died at 1.3 mo	-

HCV: Hepatitis C virus; CVA: Cerebrovascular accident; HCC: Hepatocellular carcinoma; PBC: Primary biliary cirrhosis; CR: Chronic rejection; LTx: Liver transplant; PSC: Primary sclerosing cholangitis; CCC: Cholangiocarcinoma; N/A: Not available; AR: Acute rejection; HBV: Hepatitis B virus; UNOS: United Network for Organ Sharing; ALF: Acute liver failure; HAT: Hepatic artery thrombosis.

to transplantation from conventional donors. There may be a publication bias where poor outcomes have been excluded from reportage. The inclusion of patients from mandatory databases and the large number of centers reporting from several jurisdictions may mitigate this risk of publication bias.

There are several causes that lead to severe brain damage in liver transplant recipients[15,16], and some of those circumstances make re-use of liver grafts possible. In the series described here, cerebrovascular accident and cerebral edema are the commonest causes of death of the donor of the previously transplanted graft. Brain death from cerebral edema is a particular concern in candidates with fulminant liver failure as recovery from coma may unpredictably occur after a considerable interval from successful liver

replacement. Knowledge that these grafts may be available for re-use should a recovery not occur, may permit the teams to give candidates with fulminant failure the benefit of the doubt.

Moreno González *et al*[2] considered several factors to be important for successful reuse of liver grafts: all reused grafts should be obtained from young and stable initial donors, excellent graft function in the first recipient, early reuse (within 48 h), short preservation times, biopsy showing minimal preservation injury, negative donor-recipient crossmatch, ABO compatibility, absence of viral, bacterial, and fungal infection. While it is wise to be prudent, the current report suggests that criteria for donation after transplantation may be similar to conventional donation after neurological death. The age of donor here ranged from 4 to 72 years. The interval between transplantations was up to several years. Biopsy before reuse was not routinely reported but should be considered. All of the teams reported efforts to shorten cold and warm ischemic times. Extension of criteria to include donation after cardiac death has not been reported.

There is limited experience of re-use of HCV infected grafts with only two reports in this series. Both of the final recipients experienced recurrence of HCV. One died from recurrent HCC at 16 mo (our case) but the other is well at 25.4 mo after transplantation[8]. Biopsy of HCV infected grafts should be performed before re-use using the same protocols as for initial transplantation.

Clinical indications for the re-use of the liver grafts is varied in the current series but the incidence of HCC, chronic rejection and recurrent hepatitis suggest that candidates may have been offered this unconventional form of transplantation because access to the conventional list was limited. There has been no established guideline so far for the recipients' indication of reuse liver transplantation. A marginal recipient whose general condition is deteriorating or whose stage of malignancy is almost beyond the criteria for liver transplant and suitable donor is not available may take advantage of the reuse liver transplant. If so, the results presented here confirm that the courage shown by the patients was properly rewarded. Even though the results in this select group of transplantations are good, the world wide experience is so limited that we do not advocate for previously transplanted grafts to be included in the conventional donor pool. This report will hopefully guide medical teams faced with unusual circumstances where a liver recipient unexpectedly dies after transplantation in a manner that permits organ donation.

Nowadays transplant programs are increasingly accepting marginal donors such as old donors, donors with fatty liver, or other conditions such that delayed graft function or poor outcome might be anticipated after the transplant compared to the transplants from non-marginal donors. The local Ethical committee should be ideally called before accepting the reuse liver, and this paper will help the committee understand the feasibility of the rare form of transplants.

COMMENTS

Background
The growing disparity between the demand for and supply of organs for transplantation has restricted the availability of grafts for patients whose indications for transplantation fall outside of conventional guidelines and it has led to new strategies to increase donor utility.

Innovations and breakthroughs
Reuse of a previously transplanted liver graft may be considered if the first recipient suffers neurological death at some time after liver transplantation.

Applications
This report will hopefully guide medical teams faced with unusual circumstances where a liver recipient unexpectedly dies after transplantation in a manner that permits organ donation.

Peer review
This is a very novel article focused on the Reuse of liver grafts following the brain death of the initial recipient. Subject to certain restrictions, there maybe some bias. However, liver transplantation secondary use, which provide a new method to solve the liver source, and it deserves further study.

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CASE REPORT

Grade 4 febrile neutropenia and Fournier's Syndrome associated with triple therapy for hepatitis C virus: A case report

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Abstract

The use of triple therapy for hepatitis C not only increases the rate of sustained virological responses compared with the use of only interferon and ribavirin (RBV) but also leads to an increased number of side effects. The subject of this study was a 53-year-old male who was cirrhotic with hepatitis C virus genotype 1 A and was a previous null non-responder. We initially attempted retreatment with boceprevir (BOC), Peg-interferon and RBV, and a decrease in viral load was observed in the 8th week. In week 12, he presented with disorientation, flapping, fever, tachypnea, arterial hypotension and tachycardia. He also exhibited leucopenia with neutropenia. Cefepime and filgrastim were initiated, and treatment for hepatitis C was suspended. A myelogram revealed hypoplasia, cytotoxicity and maturational retardation. After 48 h, he developed bilateral inguinal erythema that evolved throughout the perineal area to the root of the thighs, with exulcerations and an outflow of seropurulent secretions. Because we hypothesized that he was suffering from Fournier's Syndrome, treatment was replaced with the antibiotics imipenem, linezolid and clindamycin. After this new treatment paradigm was initiated, his lesions regressed without requiring surgical debridement. Triple therapy requires knowledge regarding the management of adverse effects and drug interactions; it also requires an understanding of the importance of respecting the guidelines for the withdrawal of treatment. In this case report, we observed an adverse event that had not been previously reported in the literature with the use of BOC.

Core tip: Triple therapy is a recently developed strategy for the treatment of hepatitis C that requires extensive knowledge of adverse effects and drug interactions. It also requires an appreciation of the importance of respecting the guidelines for treatment withdrawal. The case report presented here describes a serious adverse event associated with this new therapy that has not previously been reported in the literature. This finding emphasizes the importance of adequately managing patients according to international clinical protocols, and our study allows for an exchange of experience among experts in the conduct of real-life cases.

Figure 1 Viral kinetics in response to clinical treatment. TW 0: 0th week of treatment.

INTRODUCTION

The hepatitis C virus (HCV) is the principle cause of chronic liver disease, cirrhosis of the liver and hepatocarcinoma (CHC) throughout the world[1-4]. It is estimated that 120 to 200 million individuals are chronically infected with HCV worldwide, with chronic infections by the HCV genotype 1 being the most prevalent globally (40% to 80%)[1]. Although the incidence of acute hepatitis C has diminished significantly since screening for HCV in the donors of blood and its derivatives began in 1990, the number of patients who present with decompensated cirrhosis and CHC is expected to increase, reaching a peak in approximately 2020[1].

Fifty to 80% of individuals with an acute HCV infection will develop the chronic form of this infection. Of the infected individuals, 2% to 20% will develop cirrhosis within the first 20 years, and most evidence suggests that the disease progression may then increase in a nonlinear fashion. From the point at which cirrhosis is established, the rate of CHC development ranges from 1% to 6% per year. Numerous factors have been associated with rapid progression to cirrhosis, such as a greater age at the time of infection, being male, alcohol consumption, co-infections with the human immunodeficiency virus or hepatitis B virus, non-alcoholic fatty liver disease and tobacco smoking[1].

Over the past 10 years, standard therapy for chronic hepatitis C has consisted of a combination of Peg-interferon alpha and Ribavirin (RBV). This treatment results in a sustained virological response (SVR) in 40% to 50% of HCV genotype 1 patients and in approximately 80% of patients with genotypes 2 or 3[1].

Two direct-acting antiviral agents, telaprevir (TVR) and boceprevir (BOC), both of which are first generation protease inhibitors (PIs), have recently been approved for the treatment of chronic hepatitis C genotype 1[1,3,5]. In Brazil, the approval of these PIs has been granted exclusively for mono-infected HCV genotype 1 patients with advanced fibrosis or compensated cirrhosis of the liver[6].

Triple therapy that comprises a PI in combination with Peg-interferon alpha and RBV increases the SVR rate to approximately 70% and shortens the required treatment duration by approximately 50% in naïve patients (*i.e.*, individuals who had not been previously treated). The SVR rates in previously treated patients depend on the response to prior treatment and the degree of liver fibrosis. Prior work has shown that this rate may vary from > 80% in previous relapse cases to approxi-

mately 15% to 30% in null responders and individuals with an advanced degree of fibrosis[1,3,7]. However, these advances occur at the expense of an increased incidence of adverse events and higher therapy costs[1,6,7].

Triple therapy for hepatitis C has been associated with a higher incidence of adverse events, a fact that can limit its tolerability. These unwanted side effects require greater monitoring of the patient compared with treatment with only Peg-interferon alpha and RBV[6,8]. The augmentation of hematological toxicity that occurs with triple therapy can also lead to a rise in the use of growth factors, which results in increased strain on the medical resources in the health system[6,7]. Furthermore, PIs carry the risk of inducing mutations that lead to HCV resistance. Extensive monitoring of patients for their virological response, attention to criteria for treatment cessation and counseling on compliance would be necessary to minimize the development of resistant variants[1].

The higher incidence of adverse events requires PI discontinuation in 10% to 21% of patients. Adverse events that occur at a higher frequency among individuals who received triple therapy include anemia, neutropenia, dysgeusia (BOC), gastrointestinal discomfort, fatigue, cutaneous eruption (TVR) and perianal discomfort (TVR)[1,6].

The present work aimed to report a case of febrile neutropenia and the development of Fournier's Syndrome in a cirrhotic patient with HCV genotype 1 A. This patient was a null responder to two prior treatments (a change in viral load was undetectable following previous treatments, with no decrease in HCV-RNA of at least 2-log after 12 wk of treatment). The patient's retreatment for HCV included triple therapy with BOC, Peg-interferon alpha and RBV.

CASE REPORT

This study describes the case of a 53-year-old male with cirrhosis induced by HCV genotype 1 A. This patient was a null non-responder to two previous treatments (Peg-interferon alpha and RBV for 48 wk), and he denied the previous use of alcohol and other drugs. He was treated again with BOC, a double dose of Peg-interferon alpha [180 micrograms (μg)] and RBV. He obtained a sharp drop in viral load in the 8th week of treatment (TW 8), and viral negativity was observed in week 12 as illustrated in Figure 1.

In the 12th week of treatment (TW 12), he presented with a fever (40 ℃), dyspnea and diarrhea. During the initial evaluation, he was confused and disoriented, with

Figure 2 Photos documenting the involvement of the right and left inguinal regions, respectively.

flapping, fever, tachypnea, arterial hypotension and tachycardia. Laboratory analyses revealed leucopenia (300 leukocytes/mm³) with neutropenia (10 neutrophils/mm³). Cefepime and filgrastim were indicated, and treatment for hepatitis C was suspended.

A myelogram demonstrated hypoplasia with cytotoxicity and maturational retardation; the chosen reposition consisted of folic acid and vitamin B12, in addition to the continuance of filgrastim.

After 48 h of antibiotic therapy, the patient started to present with bilateral erythematous lesions in the inguinal region, and these lesions evolved within 2 d with diffuse erythema throughout the perineal area extending to the root of the lower limbs, with exulcerations and an outflow of seropurulent secretions (Figure 2).

The patient underwent computed tomography (CT) of the pelvis to evaluate the depth of the lesion, and involvement of the lesion in deep planes was not observed (Figure 3). CT revealed a thickening of the skin of the inguinal region and the root of the thighs and scrotum, which was associated with a slight blurring of the adjacent fat.

Based on these symptoms, a hypothesis of Fournier's Syndrome was postulated, and the therapy was replaced with the antibiotics imipenem, linezolid and clindamycin. The blood cultures were positive for multi-sensitive *Pseudomonas,* and the urine cultures were positive for *Staphylococcus aureus* that was sensitive to oxacillin.

The patient's lesions regressed without requiring surgical debridement, and his neutrophil count normalized with the use of filgrastim. The patient was discharged from the hospital after 14 d of antibiotic therapy.

DISCUSSION

The advent of triple therapy for chronic hepatitis C with PIs, Peg-interferon alpha and RBV in HCV genotype 1 carriers has increased the rates of SVRs in naïve patients, previous relapsers and null responders to rates of 70%, > 80% and 30%, respectively. Nevertheless, the observed parallel increase in the incidence of adverse events limits the tolerability of this therapy and raises its associated costs[1,3,6].

The hemolytic anemia that has been associated with RBV use and the suppression of hematopoiesis observed with the use of Peg-interferon alpha require extensive monitoring of hemoglobin levels and absolute neutrophil numbers to achieve adequate management of anemia and neutropenia, two frequent adverse effects that have been related to double therapy. The frequency of these adverse events is increased in patients treated with BOC or TVR, and it results in greater reductions in the doses of RBV and/or Peg-interferon alpha, the use of growth factors or even the discontinuation of PI therapy[3,4,7].

The various dermatological manifestations associated with HCV are classified into three types according to their etiology. The first type involves the direct action of the virus on the skin, and it includes the involvement of lymphocytes, dendritic cells and blood vessels. The second type occurs secondary to the interruption of the immune response, and the third type involves a non-specific cutaneous response secondary to HCV involvement in other organs[9-11].

Interferon has the clinical potential to cause adverse effects on the skin, and these effects are secondary to interferon's immunomodulatory activity[9-11].

The well-described association of adverse dermatological events with the use of TVR is less evident when BOC is chosen. Light-to-moderate cutaneous eruptions can be treated with oral antihistamines and/or topical corticosteroids, but TVR therapy must be terminated immediately for severe cutaneous eruptions (> 50% of the body surface area) or any eruption associated with significant systemic symptoms, including evidence of the involvement of internal organs, facial edema, mucosal erosions or ulcers, target lesions, epidermal dislocation, vesicles or blisters. For these types of serious adverse effects, the patient must be immediately referred for dermatological medical assistance. Drug Rash with Eosinophilia and Systemic Symptoms Syndrome and Stevens Johnson Syndrome occur in < 1% of patients treated with TVR[1,3,11].

The increase in the neutropenia incidence [absolute neutrophil count (ANC) < 750 per cubic millimeter] is similar in patients treated with TVR, Peg-interferon and RBV compared with patients treated only with Peg-interferon and RBV. However, higher costs are incurred among the more severe cases when the proposed scheme includes BOC, Peg-interferon and RBV. Approximately 23% of patients treated with BOC had grade 3 neutropenia (ANC between 500 and 750 per cubic millimeter), and approximately 7% of these individuals experienced grade 4 neutropenia (ANC < 500 per cubic millimeter) in comparison with 13% and 4%, respectively, in patients who received Peg-interferon and RBV. It may be necessary to reduce the Peg-interferon doses and to use granulocyte colony-stimulating factor in patients treated with BOC[3].

There are no reports in the literature that have reported the appearance of Fournier's Syndrome with triple therapy for HCV. However, it should be taken into consideration that this rare condition is most common in patients with diabetes, alcohol abusers and in immu-

Figure 3 These images reveal a thickening of the skin in the inguinal region, as well as in the thigh roots and scrotum. These features are associated with slight blemishing of the adjacent adipose tissue.

nosuppressed individuals. Although this diagnosis would be made primarily using clinical data, imaging exams may be useful in cases of atypical presentation or when there is concern regarding the true extent of the disease. The most common sites of involvement are the genitourinary tract, the lower gastrointestinal tract and the skin. Fournier's Syndrome is a mixed infection caused by both aerobic and anaerobic bacteria; thus, the management of Fournier's Syndrome requires immediate debridement and wide-spectrum antibiotic therapy[11-15].

The present case report documents severe neutropenia associated with a serious infection, Fournier's syndrome, during triple therapy for HCV. Given the seriousness of the adverse events and the good patient outcomes observed with this treatment paradigm, this case report may have extremely important implications for patient care.

COMMENTS

Case characteristics
A 53-year-old cirrhotic patient with hepatitis C virus (HCV) genotype 1 A who had exhibited no response to prior treatment was initiated on retreatment with boceprevir (BOC), Peg-interferon (at a double dose of 180 μg) and ribavirin (RBV). However, he exhibited fever and signs of hepatic encephalopathy during the 12th week of treatment.

Clinical diagnosis
The patient exhibited fever (40 ℃), dyspnea, diarrhea, confusion and disorientation, flapping, tachypnea, hypotension, tachycardia and evolving diffuse erythema throughout the perineal area to the root of the lower limbs, with exulcerations and seropurulent secretions.

Differential diagnosis
Septic shock and an infection of the gastrointestinal tract were also considered.

Laboratory diagnosis
Leucopenia (300 leukocytes/mm³) with neutropenia (10 neutrophils/mm³) was diagnosed based on laboratory results.

Imaging diagnosis
A computed tomography scan of the abdomen showed a thickening of the skin in the inguinal region, the roots of the thighs and the scrotum, and these fea-

tures were associated with a slight blurring of the adjacent fat.

Pathological diagnosis
A myelogram demonstrated hypoplasia with cytotoxicity and maturational retardation.

Treatment
The patient was initially treated with cefepime and filgrastim after the cessation of treatment for hepatitis C. This treatment was subsequently exchanged for treatment with the antibiotics imipenem, linezolid and clindamycin.

Related reports
Triple therapy for hepatitis C has been previously shown to increase the rate of sustained virological responses (SVRs), and we observed an increase in the frequency and severity of adverse events related to this treatment.

Term explanation
Triple therapy for hepatitis C involves the use of PIs (BOC-boceprevir/telaprevir-telaprevir) in combination with Peg-interferon and RBV, and it should be considered for early treatment viral kinetics to treatment previously performed. Naïve: a patient who has not received prior treatment; Relapser: a patient characterized by undetectable levels of HCV-RNA after an initial treatment, without a SVR because of a positive viral load after the discontinuation of treatment; Partial responder: a patient whose HCV-RNA levels fell by more than 2-log after 12 wk of treatment, but HCV-RNA levels were detectable at the end of the treatment period; null responder: a patient whose HCV-RNA levels fell at least 2-log after 12 wk of treatment.

Experiences and lessons
The case report presented here describes a serious adverse event associated with this new triple therapy that has not yet reported in the literature. The authors' data emphasize the importance of adequate patient management plans that are in accordance with international clinical protocols, and these findings allow experts to gain access to and experience with the conduct of this real-life case.

Peer review
Dr. Oliveira *et al* presented an interesting case report. The design of the study is adequate. The finding of the presented case report is of interest for the general reader.

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REVIEW

Colorectal hepatic metastasis: Evolving therapies

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unresectable disease.

Abstract

The approach for colorectal hepatic metastasis has advanced tremendously over the past decade. Multidrug chemotherapy regimens have been successfully introduced with improved outcomes. Concurrently, adjunct multimodal therapies have improved survival rates, and increased the number of patients eligible for curative liver resection. Herein, we described major advancements of surgical and oncologic management of such lesions, thereby discussing modern chemotherapeutic regimens, adjunct therapies and surgical aspects of liver resection.

Key words: Colorectal cancer; Hepatic metastasis; Hepatectomy; Survival; Chemotherapy; 5-fluorouracil leucovorin and oxaliplatin

Core tip: The management of colorectal hepatic metastasis is complex, and should involve a multidisciplinary tumor board involving specialized medical and surgical oncologists. Although liver resection still remains as the key step in the management of liver metastasis, the introduction of new chemotherapeutic regimens and recent adjunct therapies, including radiofrequency ablation, cryotherapy and radioembolization improved patient care, and prolonged survival in patients with

INTRODUCTION

Colon cancer is the third most common malignancy in the United States, and comprising around 10% of all cancer-related mortality[1]. Most disease-related mortality is associated with metastatic disease. Approximately 25% of patients is diagnosed with metastases at initial presentation, and around 50% will present metastases during the clinical management of the disease[2,3]. The survival for untreated colorectal hepatic metastasis (CHM) are dismal with medial survival estimated in only 6 to 9 mo[4].

Although liver resection still remains as the most important modality in the treatment of CHM, the introduction of recent adjunct therapies, including radiofrequency ablation (RFA), cryotherapy and radioembolization improved patient care, and prolonged survival in patients with unresectable disease. Concurrently, the evolution of chemotherapy with the introduction of multidrug therapy optimized response rates, and expanded the number of surgical candidates for curative liver resection. Herein, we describe the current management of CHM, thereby discussing major advancements in chemotherapeutic regimens, adjunct therapies and surgical technique, and describe paradigm changes in resectability and outcomes.

DETERMINATION OF STRATEGY

The management of CHM is complex, and should involve a multidisciplinary tumor board including oncologists, radiologists, colorectal and hepatobiliary surgeons. Clinical and laboratory suspicion of metastasis should be routinely confirmed by radiological imaging. Options

available include computed tomography (CT), ultrasound, fluorodeoxyglucose-positron emission tomography (PET), and magnetic resonance imaging (MRI). Multi-detector CT is widely available, and is routinely used for detection of CHM[5]. MRI is being used more commonly, and provides better visualization of liver lesions as compared to CT by some experts[6]. PET scan is usually associated with CT (PET-CT), and is superior to CT or MRI for identification of equivocal lesions, metastases, and local recurrence, prior to resection of metastatic disease[7-10].

Several prognostic factors should be considered during definition of therapeutic strategy, including: staging of the primary tumor, interval diagnosis between the primary and metastatic lesions, number and size of metastases, presence of surgical margins and extrahepatic recurrence, and elevated biochemical markers such as carcinoembrionic antigen, alkaline phosphatase, and albumin[11-15]. The most important decision for definition of the therapeutic plan is defined based on resectability of metastatic disease. Patients should be stratified as suitable for resection, potentially resectable after chemotherapy and/or adjunct therapies, and those with unresectable disease.

MANAGEMENT OF RESECTABLE DISEASE

Liver resection continues to be the most crucial step in the management of CHM, potentially offering definitive treatment to a subset of patients. The use of chemotherapy is used as an adjunct therapy, thereby enhancing the 5-year survival at approximately 37%-58%[16,17]. Assessment of resectability is based on the volume of future remnant liver with adequate vascular inflow and outflow and biliary drainage[18]. For patients with normal liver function, 20% of remnant tissue is required, whereas in the presence of steatosis and cirrhosis, 30% and 40% of residual liver is necessary, respectively. Negative margins of 1-cm is associated with improved outcomes, and is currently recommended by most experts[19,20]. Contraindications to resection include uncontrollable extrahepatic disease, extensive lymph node involvement, including retroperitoneal or mediastinal nodes, bone or central nervous system metastases[21]. Local predictors of unresectability are determined by hepatic vascular involvement, and bilaterally, that would leave an inadequate functional liver remnant. Perioperative combination with chemotherapy with 5-fluorouracil, leucovorin, and oxaliplatin (FOLFOX) regimen given 3 mo prior and 3 mo following resection of metastases enhances survival by 8% at 3 years[22]. Neoadjuvant chemotherapy for patients with resectable liver metastases is still under investigation, and currently, remains controversial. Another topic of major debate is regarding the timing of the colectomy relative to the hepatectomy in cases of synchronous CHM. Typically, the primary colorectal cancer (CRC) is resected first, however in select cases where the liver disease is marginally resectable and primary CRC is small, the liver resec-

tion may be considered as initial approach to avoid progression of CHM. Combined resections are associated with shorter hospital stay and less morbidity, with similar 5-year survival and technically more challenging[23].

MANAGEMENT OF POTENTIALLY RESECTABLE DISEASE

Initially unresectable liver metastases can become resectable after being downsized by neoadjuvant chemotherapy, and, in such cases, resection may be advocated. Bismuth *et al*[24] reported the first experience with downstaging of unresectable lesions to resectable. They found similar outcomes to those patients with initially resectable lesions[25]. Nuzzo *et al*[26] found similar operative complications, and 3-year overall survival between initially resectable patients and those with initially unresectable but downstaged lesions. Subsequent reports showed conversion rates between 30%-50% with the combination of hepatic artery infusional fluoxuridine with systemic chemotherapy[27,28]. In these patients, response to initial chemotherapy appears to be a predictor of outcome[29].

Initial experience with addition of a vascular endothelial growth factor (VEGF) or epidermal growth factor receptor (EGFR) target agent (bevacizumab or cetuximab, respectively) is associated with higher resection rates in patients with initially unresectable disease. Resection is usually performed 5-8 wk after the last chemotherapy cycle with cetuximab or bevacizumab, respectively. The decision for resectability in these patients is often challenging, and involves a multidisciplinary team, depending on the experience of hepatobiliary surgeon and assessment for sufficient remnant liver. Many surgeons and oncologists would offer resection as soon as the lesion has become resectable, whereas others usually continue chemotherapy for 4 to 9 mo regardless of the response[30].

Several techniques have been recently introduced aiming at downsizing metastatic disease and improving resectability, including radioembolization, intra-arterial chemotherapy, and local ablation techniques, especially radiofrequency ablation. These adjunct modalities will be discussed separately.

MANAGEMENT OF UNRESECTABLE DISEASE

The majority of patients with CRC and concurrent metastasis has unresectable disease. However, due advances in systemic therapy, the survival of these patients is progressively improving[31]. The median survival is improved, estimated in up to 24 mo.

The approach for unresectable metastatic disease with synchronous CRC is still controversial. Resection of the bowel cancer initially is associated with precise definition of nodal and peritoneal status, prevention of local complications, the theoretical advantage of reduced total-body tumor load as well as psychological benefits for the patient[11]. However, the chemotherapy-first approach

is considered better by other experts due to the avoidance of postoperative morbidity and mortality, potential downstaging of unresectable CHM to resectability, and data showing equivalent survival benefits[11].

Monoclonal therapy against VEGF and EGFR should be considered especially in refractory cases, and will be further discussed in this review. For non-curative therapy of CHM, in addition to using the standard FOLFOX or FOLFIRI chemotherapy regimens, single agent strategies have been used with survival benefits as evidenced by the MRC FOCUS (using 5-FU-LV) and CAIRO (using capecitabine) trials[32-34].

TREATMENT MODALITIES

Resection
Surgery is the key step in the management of patients with CHM and represents the only chance for cure. Resection of CHM is considered a relatively safe operation with an operative mortality less than 5% by most recent series[30,35,36]. In high volume centers, median hospital stay ranges between 5 and 10 d for minor and major resections[36,37]. With increased outcomes, hepatectomies are now safely performed in elderly patients[38].

In cases of multiple, bilateral CHM, surgical options include: parenchyma-sparing approaches, and two-stage hepatectomy. In a two-stage operation, a portion of the liver disease is removed, and the contralateral portal vein is occluded, followed by 1 to 3 mo interval to allow for hypertrophy of the remaining liver and a curative-intent, second-stage hepatectomy. In such cases, the portal vein is occluded intraoperatively or subsequently by percutaneous embolization. Most experts perform minor segment resection first followed by resection of major liver. The minor-first approach spares the patient with progressive disease to undergo a major hepatectomy.

Within 2 years, most patients developed a recurrence[11,39]. Approximately 40% of them are eligible to undergo reoperation. The 5-year survival after first and second hepatectomies was 47% and 32%, respectively[40].

The experience with laparoscopic resection of CHM is yet minimal. Buell *et al*[41] and Mala *et al*[42] demonstrated tumor clearance, feasibility and safety of laparoscopic liver resection in 31 and 42 patients with CHM, respectively[41,42]. Long-term outcomes compared to open approach remains unknown.

CHEMOTHERAPY

Although chemotherapy plays a vital role in managing resectable and unresectable CHM, the timing of delivery is still controversial. For resectable disease, delivery of chemotherapy may be offered before colon resection (preoperative), after colon resection but before liver resection (peri-operative) or after both resections (post-operative).

Pre- and peri-operative chemotherapy for resectable disease
For patients with potentially resectable CHM, response to chemotherapy has become an important adjunct in deciding whether to proceed with surgery. Typically, most tumors either reduce in size or remain unchanged following chemotherapy[22,43-46].

The recommended approach of delivering neoadjuvant chemotherapy to patients with resectable CHM consists of a 2-3 mo course of FOLFOX in order to limit chemotherapy-induced liver injury[46]. Chemotherapy application is considered safe to be used in patients with intact colorectal tumors[47]. In order to avoid difficulties locating both colorectal tumors and CHM that respond well to systemic chemotherapy, it would be prudent to mark the lesions before initiation of therapy, typically done using India ink tattoo or metallic coils placed by interventional radiology[48]. The disadvantages of preoperative chemotherapy application include the development of new extrahepatic lesions[49] as well as a possible increased incidence of post-operative sequelae[22].

Adjuvant chemotherapy
The application of 5-FU-based chemotherapy post-CHM resection is established in most clinical practice despite prospective data limited to only two studies[50-52]. Pooled analysis of these two trials demonstrated a trend towards longer disease-free survival but no difference in median progression-free survival or overall survival. At present, there is no role for irinotecan-containing chemotherapy regimen (FOLFIRI) following hepatic resection with no benefit demonstrated when compared to 5-FU based regimens[53].

The application of systemic chemotherapy for CHM is associated with hepatotoxicity, a sequelae that has been recognized to increase the risk of peri- and post-operative mortality for CHM resection candidates. Amongst these hepatotoxic sequelae are hepatic steatosis seen in 30%-47% of patients on 5-FU[17], non-alcoholic steatohepatitis (NASH) in 12%-25% of patients on irinotecan[18] and sinusoidal dilation in 78% of patient on oxaliplatin[37]. The impact of these hepatotoxic effects is somewhat varied, although it is clear that the irinotecan-associated NASH appears to be the most significant with established evidence of increased post-operative mortality due to liver failure. Although previously the recognition of these adverse reactions was the domain of oncologists, the significant impact on post-operative outcomes has made it imperative for surgeons to be mindful of them too before considering operative intervention.

MOLECULAR TARGETED THERAPIES
Monoclonal antibodies against VEGF and EGFR have added an additional therapeutic option for treatment in select patients when used in combination with chemotherapy. Evidence of the therapeutic benefit of this treatment modality was initially found using the anti-VEGF monoclonal antibody, bevacizumab, with findings of improved survival when used in combination with therapy of IFL (irinotecan, 5-FU and leucovorin)[54]. Additional studies have demonstrated similar benefits in response

rate, disease-free progression and overall survival of using bevacizumab in combination with 5-FU/LV alone and FOLFOX in the first-line and second-line settings respectively[29]. Bevacizumab has, however, been associated with a number of complications, most notably gastrointestinal perforation, risk of bleeding and wound healing problems. As a result, the use of this modality requires careful monitoring, with treatment withheld for 6-8 wk prior to resection[55,56].

Panitumumab and cetuximab are EGFR inhibitors that have also demonstrated benefits in treating patients with metastatic CRC. Benefits have particularly been found using cetuximab in chemorefractory patients, improving survival compared to standard therapies[57]. Indeed, similar to bevacizumab, cetuximab appears to have superior effects when used in combination with[29]. It also appears that EGFR inhibitors are most effective for non-mutated (wild-type) K-ras colorectal tumors[55]. The side-effect profile for anti-EGFR antibodies is less extensive, limited to acneiform rash and hypomagnesemia and allergic reactions with cetuximab only[29] and no significant hepatotoxic effects seen thus far.

ADJUNCT THERAPIES

With the role of surgical resection for CHM widely accepted, the roles of non-operative liver directed therapies continue to evolve. With numerous new adjunctive therapies coming to the fore in recent years producing encouraging outcomes (including downstaging of CHM and increasing survival), the decision to integrate these options into current practice is challenging. Broadly speaking, there are three non-operative, liver directed therapies in use; intra-arterial therapies, ablative therapies, and radiotherapies.

INTRA-ARTERIAL THERAPIES

The role of intra-arterial therapies continues to evolve. The delivery of intra-arterial therapies uses the principal that hepatic metastases deriving their blood supply from hepatic arteries[58,59]. Therefore, intra-arterial therapy enhances drug delivery to hepatic tumors, maximizing local tumor therapy and limiting systemic therapy with its side-effects.

Hepatic arterial infusion chemotherapy

The hepatic arterial infusion (HAI) modality delivers chemotherapy directly to the liver *via* intra-abdominal catheters or infusion pumps cannulating the gastroduodenal artery[60,61]. An intimate understanding of hepatobiliary anatomy by surgeons is required to avoid placement of these catheters within aberrant anatomy leading to organ underperfusion with associated peptic ulceration, pancreatitis or biliary sclerosis[62]. The complex technical skills for correct placement of these infusion pumps requires experience often attainable at high volume centers. The delivery of HAI may be initiated as soon as the first post-operative week provided the patient has recovered well. In the United States, the chemotherapeutic agent most commonly used for HAI is fluoxuridine (FUDR) due to its high uptake by the liver limiting systemic toxic effects[63], although the low toxicity benefit may be lost by concomitant systemic chemotherapy use[64]. Dexamethasone has been delivered in conjunction with FUDR HAI-therapy, reducing biliary sclerosis, increasing tumor response rate and patient survival[65]. In Europe, 5-FU based HAI chemotherapy has also been used with some success.

HAI in unresectable disease

The role of HAI chemotherapy in unresectable disease is yet to be defined. This is largely due to inconclusive evidence from trials regarding patient outcomes[66]. On one hand, HAI has been found to produce higher tumor response rates than systemic therapy alone, but on the other no significant survival advantage has been found *via* the numerous randomized trials performed so far[67]. The application of combination therapy of HAI and systemic chemotherapy as second-line therapy following failed conventional chemotherapy[68] or to downstage initially unresectable CHM[28] have been suggested roles for HAI.

HAI as adjuvant therapy

The evidence supporting adjuvant HAI-therapy is even less established. To date, there has only been evidence from a single RCT that demonstrated a significant survival advantage applying HAI chemotherapy over systemic chemotherapy in the adjuvant setting[69]. This subject is therefore under ongoing scrutiny in current studies assessing HAI chemotherapy *vs* modern chemotherapy regimens.

Radioembolization

Radioembolization (or selective internal radiation therapy; SIRT) delivers high-energy beta-emitting radiation locally to CHM, delivering its effects specifically on tumor vasculature and minimizing collateral hepatic damage[70]. At present, this modality is delivered *via* two forms; Yttrium-90 (^{90}Y)-labeled resin microspheres (SIR-Spheres®; Sirtex Medical, Sydney, Australia) and ^{90}Y-labeled glass microspheres (Therasphere®; MDS Nordion, Ottawa, Canada). Radioembolization therapy is performed by injecting radioactive microspheres designed to embolize into small vessels around the metastases *via* branches of the hepatic artery, usually using a percutaneous femoral approach and fluoroscopic monitoring[71].

The current benefits with radioembolization using ^{90}Y microspheres have been reduced tumor load of unresectable CHM particularly if refractory to conventional chemotherapy. Indeed, combing radioembolization with chemotherapy has produced longer tumor suppression compared to chemotherapy alone[72]. The results of the recently ended SIRFLOX trial evaluating the efficacy of first-line therapy of FOLFOX6 combined with SIR-Spheres® *vs* FOLFOX6 alone will hopefully provide ad-

ditional evidence in favor of this treatment strategy in patients with unresectable CHM[73,74].

Further, trials have also demonstrated similar benefits of ^{90}Y microspheres used in combination with other treatment modalities like HAI therapy, demonstrating a superior time to progression compared to HAI alone[73,74].

The evidence supporting the use of ^{90}Y glass microspheres in CHM is less extensive with limited research demonstrating CHM tumor regression in upto 88% of patients with chemo-refractory tumors treated with ^{90}Y glass microspheres[75]. The further assessment of ^{90}Y-glass microspheres as salvage therapy continues to be evaluated with an ongoing phase III multicenter randomized trial (EPOCH trial) which will hopefully provide corroborative evidence in support of this modality[17]. The long-term toxicity effects of radioembolization techniques are yet to elucidated.

Chemoembolization

Chemoembolization [or transcatheter arterial chemoembolization (TACE)] is a form of transarterial therapy that also utilizes the principal of liver tumors' predominantly arterial supply, allowing for regional therapy to the tumors. Similar to HAI, TACE is delivered using selective angiographic techniques by injection of chemotherapeutic drug combined with embolic material resulting in selective ischemic and chemotherapeutic effects on the CHM[76].

At present, there is no standard approach to delivering TACE therapy, although the application of a newer approach, drug-eluting beads composed of irinotecan (DEBIRI®; Biocompatibles United Kingdom Ltd, Farnham, United Kingdom) is gaining wider acceptance through ongoing clinical trials[77-79]. Irinotecan is preferentially used in this modality due to its properties allowing for application to the beads. Administration of DEBIRI® occurs *via* a selective arterial catheter, depositing the beads adjacent to the CHM tumors. This allows for slow release of irinotecan locally to the tumors.

Although DEBIRI® is presently not approved by the United States Food and Drug Administration, there are promising early results on its efficacy and safety. Available clinical trials suggest that DEBIRI® treatment may be associated with a median survival time of 15-25 mo, which is broadly equivalent to the outcomes achieved for unresectable CHM with the use of best-practice systemic chemotherapy[76]. In addition, the majority of patients that had responded to TACE treatment had failed first-line chemotherapy regimens[76]. Further evidence from additional trials[78,80-82] have also found successful downstaging of unresectable CHM to resectable status with most trials describing minimal toxicity effects[76].

It must be mentioned that the majority of the available trials to date have methodological flaws, and their conclusions must be interpreted with caution. To address the lack of high-quality randomized comparative trials assessing DEBIRI® use, there is ongoing research to evaluate its benefits when used in combination with systemic chemotherapy.

ABLATION TECHNIQUES

Ablation techniques aim to induce local destruction of the CHM. At present, the exact role of ablative techniques in the treatment of CHM is unclear, although there have been suggestions that its roles may include to reduce tumor size minimizing the extent of liver resection required, adjunctive therapy for patients either unfit for surgery or with unresectable disease. Ablative approaches can be subdivided into cryoablation, RFA and microwave ablation.

Cryoablation

Cryoablation was the first thermal ablative modality attempted to treat unresectable hepatic malignancies[83]. Cryoablation (or cryosurgery) is induced by local delivery of liquid nitrogen or argon on a probe tip to the CHM, resulting in tumor destruction by intracellular ice crystals that form from the rapid cooling. The "iceball" that forms around the tip of the probe can be measured by real-time intraoperative ultrasound although there has been some suggestion that the tissue furthest away from the tip may not be cooled sufficiently to cause tissue destruction[17].

Cryotherapy applications

Cryoablation application appears to vary between institutions. In general, its primary use has been for the ablation of unresectable CHM. Despite initial thoughts that cryoablation could be used in patients with resectable CHM, high tumor recurrence following cryosurgery has tempered this enthusiasm. So far, previous research has demonstrated a modest 5 year survival of 26% but also low mortality rates of less than 5% following cryotherapy for CHM[84]. Cryoablation used in combination with surgery has also been shown to produce similar survival benefits to surgery alone in patients with initially unresectable CHM[85].

The application of cryotherapy to the remnant liver resection margins (edge cryotherapy) remains undecided. Although some authors have reported the decreased application of edge cryotherapy due to report higher complication rates than hepatic resection alone[17], other institutions have reported positive outcomes with this approach, finding potential cure of up to 13% of advanced unresectable CHM compared with resection alone.

Additional benefits of cryosurgery include its facility in treating bilobar CHM or recurrent hepatic tumors following resection in addition to evidence from animal models that shows decreased secretion of factors that stimulate growth of occult micrometastases following cryotherapy compared to post-surgical resection[86]. One of the shortcomings of cryoablation is its poor ability to destroy tumors next to larger blood vessels due to the "heat-sinking" effect[87], resulting in recurrence rates as high as 44%. Another disadvantage of this modality is that for unclear physiologic reasons, patients may suffer from a systematic

inflammatory response (cryoshock phenomenon)[82,88,89] associated with periprocedural deaths[88,89].

RFA

By far, the most extensively evaluated ablative approach is RFA. RFA is the most widely applied ablative modality due to ease and safety of application and inexpense of equipment[17]. This modality is applied by placing needles within and adjacent to CHM through which alternating electrical current is delivered at radiofrequency range generating heat to desiccate the tumors[90,91].

Application

Although RFA is in widespread use across many institutions internationally, a paucity of randomized controlled trials up to now has prevented the development of a consistent approach to its use. Indeed, to date, there are no RCTs comparing surgical resection with RFA in resectable CHM, a study that at present seems inconceivable and unethical considering established survival data from surgical resection. At present, most evidence from the retrospective studies available comparing RFA and resection has demonstrated the inferiority of RFA compared to surgical resection with increased local recurrence rates (16%-60% *vs* 0%-24%) and worse long-term survival[91,92].

At present, RFA is being used to treat unresectable CHM only, with no extrahepatic metastatic disease[93]. Tumors amenable to successful treatment with RFA have typically been solitary CHM or a few which are not close to large hepatic vessels[93]. Tumor size in particular has been limited to 3-cm due to the circumferential rim of ablation currently delivered by ablation probes being approximately 4-cm in diameter, a limitation that may be addressed with advancement of the technology. Overlapping ablations can be used to treat larger tumors although this has been associated with less successful complete ablation[94]. The presence of large blood vessels limits RFA efficacy because their high blood flow acts a "heat sink", protecting adjacent cells from thermal ablation[17].

RFA is delivered *via* open, laparoscopic or percutaneous approaches[93]. The application of ultrasound, CT and MRI are particularly important to guide the needle in the percutaneous approach while intraoperative ultrasound is an additional adjunct used to directly visualize the tumor in the operative approaches. It appears at present that RFA *via* laparotomy is associated with the lowest recurrence rate followed by laparoscopy, and finally by percutaneous approach. The trade-off of using the least invasive percutaneous approach must be weighed up against poor tumor visualization increasing the potential for recurrence. The surgical approaches are typically applied at the time of primary or hepatic metastasis tumor resection.

In addition to the aforementioned advantages of RFA, it has a relatively lower morbidity profile of < 10% independent of the approach used for delivery being surgical or percutaneous[95]. Amongst the complications that have been seen, thermal injury (bowel and biliary injury),

mechanical (biliary and vessel injury) and septic (abscess and peritonitis) have been the most widely reported. A more infrequent presentation of post-ablative syndrome where patients suffer from self-limiting constitutional upset including malaise, febrile episodes, myalgia, nausea and vomiting has also been reported[93].

Microwave ablation

Microwave ablation (MWA) is a more recently developed technique used for CHM. MWA is applied *via* a microwave probe delivered into the tumor *via* image-guided percutaneous, or ultrasound guided surgical approaches. *Via* these probes, microwave radiation between 900 MHz and 2.4 GHz is delivered that causes polarized water molecules within the tissue to oscillate generating friction that produces heat that destroys tissue by coagulative necrosis[96].

MWA application

As this modality is relatively new, the evidence of its efficacy is limited and has included too many different liver tumor types particularly hepatocellular carcinoma. The exact application of MWA for CHM is therefore still unclear. Although reported local recurrence rates have been extremely variable ranging from 3% to 50%, encouraging evidence from the largest series reported rates as low as 3% and 6%[97,98]. Further research would therefore provide the evidence to define its role as an ablative therapy in CHM management.

The purported advantages of MWA have been the more extensive nature of tissue destruction created by the heating mechanism generated by this technique. This mechanism also appears to be less prone to the "heat-sink" effect seen with RFA therapy[99]. There has also been suggestion that intra-operative hepatic inflow occlusion (Pringle maneuver) increases the size of ablated lesions[100]. Further, there appears to be reduced occurrence of charring using MWA and it creates larger ablation zones up to 6 cm away more rapidly than RFA[96]. Interestingly, there is now growing interest over a further method of cell death induced by microwaves characterized by normal-looking but non-viable cells. If indeed this is correct, this would have important implications in the post-procedure observation of the ablated tumors, requiring likely routine histopathology to differentiate seemingly viable tumor from completely ablated ones.

The complication rates from MWA range from 6% to 30%, most often associated with cases where laparotomy and additional procedures had been performed[90,97,98]. There are at present concerns of potential inadvertent injury to surrounding organs due to the higher energy generated by this modality.

STEREOTACTIC BODY RADIOTHERAPY

Stereotactic body radiotherapy (SBRT) is another newer technology that has generated growing interest for use in ablating CHM[101]. Unlike external beam radiation therapy (EBRT) which had previously been abandoned for use

in liver tumors due to the narrow therapeutic window between tumoricidial and hepatoxic effects, SBRT uses more modern technology that allows for safe treatment delivery in lung and liver with hypofractionation[101].

Application

SBRT is based on techniques used in stereotactic radiosurgery for brain tumors[101]. In this modality, the tumor location is identified using four-dimensional imaging that maps the target area accounting for patient movements during breathing. Gold seeds called fiducials are then placed within the tumor, which guide treatment. Using the predetermined tumor coordinates, high-dose radiation is delivered over a relatively shorter duration compared to conventional EBRT.

Although encouraging evidence of tumor local control rates as high as > 90% have been demonstrated in lung tumors using SBRT[102,103], its application in liver tumors specifically CHM is still under scrutiny with few well-designed studies presently available in current literature. The optimum radiation dosage is also undetermined, although it appears that a higher dose of up to 60 Gy is most effective, eliminating high local progression rates seen at lower doses[104], maximizing tumor response rate (up to 90%) and 2-year local control rate of 100%.

Although the treatment is focused, it does not eliminate surrounding toxicity. Specifically, acute gastrointestinal and liver toxicity in addition to chest wall pain have been reported side effects of the therapy. In addition, and more importantly, although there is some early evidence of local tumor control with SBRT, it is not yet been demonstrated to significantly impact survival.

However, the encouraging early results have lead to the assertion that SBRT be considered as an option in patients not offered surgery after chemotherapy to locally ablate their CHM[101].

CONCLUSION

The management of CHM is complex, and should involve a multidisciplinary tumor board involving specialized medical and surgical oncologists. Although overall survival has increased tremendously over the last 5 years with the introduction of adjunct therapies, more efficient chemotherapeutic regimens still need to be discovered. Concurrently, the criteria for resection is much more liberal and should be based on functional remnant liver volume. Even in situations where multiple, bilobar liver metastases are present, resection may be a considered option. Both basic studies and prospective trials are necessary to further understand the molecular aspects of colorectal hepatic metastasis, and therefore improve outcomes.

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Juvenile autoimmune hepatitis: Spectrum of the disease

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Abstract

Juvenile autoimmune hepatitis (JAIH) is a progressive inflammatory liver disease, affecting mainly young girls, from infancy to late adolescence, characterized by active liver damage, as shown by high serum activity of aminotransferases, by elevated immunoglobulin G levels, high titers of serum non organ-specific and organ-specific autoantibodies, and by interface hepatitis on liver biopsy. It is a multifactorial disease of unknown etiology in which environmental factors act as a trigger in genetically predisposed individuals. Two types of JAIH are identified according to the autoantibody panel detected at diagnosis: AIH-1, characterized by the presence of anti-smooth muscle antibody and/or antinuclear antibody and AIH-2, by anti-liver-kidney microsomal antibody type 1 and/or by the presence of anti-liver cytosol type 1 antibody. Epidemiological distribution, genetic markers, clinical presentation and pattern of serum cytokines differentiate the two types of AIH suggesting possible pathogenetic mechanisms. The most effective therapy for AIH is pharmacological suppression of the immune response. Treatment should be started as soon as the diagnosis is made to avoid severe liver damage and progression of fibrosis. The aim of this review is to outline the most significant and peculiar features of JAIH, based

largely on our own personal database and on a review of current literature.

Core tip: Juvenile autoimmune hepatitis is an inflammatory liver disease affecting mainly young girls from infancy to late adolescence, characterized by active liver damage, elevated immunoglobulin G levels, high titers of serum non organ-specific and organ-specific autoantibodies, and interface hepatitis on liver biopsy. Two types are identified according to the autoantibody panel, with differences in the epidemiological distribution, genetic markers and clinical presentation. The most effective therapy for autoimmune hepatitis is pharmacological suppression of the immune response. Treatment should be started as soon as the diagnosis is made to avoid severe liver damage and progression of fibrosis.

INTRODUCTION

Autoimmune hepatitis (AIH) results from an autoimmune attack of the liver parenchyma. The term "autoimmune hepatitis" was first employed by Mackay *et al*[1] in 1965 to describe "a persistent liver disease, with highly elevated levels of serum transaminase, sometimes over 1000 units, elevated serum gamma globulins, up to 6.0 g per 100 mL, piecemeal necrosis on liver biopsy with diffuse lymphoid infiltration and fibrosis, progressing to cirrhosis, various autoantibodies reactions in the serum,

improving with immunosuppressive drugs". The pattern of the serum autoantibodies that characterize patients with AIH led to a classification of this disease[2,3].

In children, after anecdotal reports of cases of chronic hepatitis and hypergammaglobulinaemia in the sixties[4], it was clearly shown that about half of the children with the histological features of chronic active hepatitis had hypergammaglobulinemia and high titers of serum autoantibodies[5] and that these patients responded in most cases to prednisone and azathioprine treatment, therefore suggesting an autoimmune mechanism[6,7]. In the same period a peculiar form of autoimmune hepatitis, now called AIH-2 was described as a distinct entity in children[8] and later confirmed in adults[9].

The aim of this review is to outline the most significant and peculiar features of juvenile AIH (JAIH), based largely from our own personal database; additionally we searched PubMed with the term of "juvenile autoimmune hepatitis", "autoimmune hepatitis", "epidemiology", "pathogenesis" and "treatment", filtered for age "birth-18 years".

DEFINITION

AIH is a liver disease of unknown origin, pathogenetically characterized by an inflammatory liver disease, as shown by high serum activity of aminotransferases, by elevated immunoglobulin G levels, high titers of serum non organ-specific and organ-specific autoantibodies, and by interface hepatitis on liver biopsy[10]. It affects mainly young girls and spontaneously progresses to severe liver damage. Immunosuppressive therapy, which should be started as soon as diagnosis is made, induces clinical and biochemical remission in most treated patients. If untreated, cirrhosis and terminal liver failure may rapidly occur[6].

EPIDEMIOLOGY

AIH can be diagnosed at any age in both sexes. Mean annual prevalence in European adults ranges from 11.6 per 100 individuals to 17 per 100[11,12] with point prevalence in homogeneous populations, such as Alaskan natives, of 42.9 per 100 individuals[13]. Epidemiological data on JAIH are largely incomplete. There is only one recent report on the incidence and prevalence of JAIH, which was conducted in Utah, United States. In this study, the incidence and prevalence of JAIH was reported to be 0.4 and 3.0 cases per 100000 children, respectively[14]. AIH-1 is the more common type of AIH, which also affects adults and often presents at puberty, while AIH-2 is typical of pediatric age, presenting at a younger age, and even during infancy[8,15].

PATHOGENESIS

AIH is a multifactorial disease of unknown etiology. Environmental factors act as a trigger with self-perpetuating liver inflammation in predisposed individuals who carry a complex genetic background. Moreover, a defective immunoregulatory function, possibly genetically related, fails to control autoreactive clones and let the disease become clinically evident. The histological picture of interface hepatitis, in which a mononuclear and plasma cell infiltrate, which originates in the portal tracts, and disrupts the parenchymal limiting plate, morphologically illustrates this process. Among the inflammatory cells, activated T lymphocytes, positive for the CD4+ helper/ inducer phenotype, predominate. These cells are believed to recognize self-antigens on the hepatocyte surface and to trigger the autoimmune liver damage[16].

Genetics

Main susceptibility HLA alleles for AIH-1 in Europe and North America were found to be *DRB1*0401* and *DRB1*0301*. The presence of these alleles confers an increased risk of developing AIH-1 and influences some features of the disease[17]. Geographic variation of the genetic predisposition to AIH-1 exists: in some countries such as Japan, Mexico and Argentina, DR3 haplotype is poorly represented in the general population, and the principal susceptibility alleles for AIH-1 are *DRB1*0404* and *DRB1*0405*[18-20]. European children display the typical pattern for AIH-1 of Caucasian patients with a significant prevalence of *DRB1*0301* and *DRB3*0101*[21].

Knowledge of the genetic background of AIH-2 is limited. In Europe, *DRB1*03* and *DQB1*02* alleles may have an important role, whereas other studies reported an increased frequency of *DRB1*07*, *DRB4*01* and *DQB1*06*. In a pediatric population from Brazil, a significant increase of *DRB1*07*, *DRB4* and *DQB1*02* was observed. Moreover, *HLA-DRB1*07* allele was found significantly associated with the presence of anti-liver-kidney microsomal antibody type 1 (LKM-1) alone and *HLA-DRB1*03* allele with anti-liver cytosol type 1 antibody (LC-1)[22-24].

A partial deficiency of HLA class III complement component C4, genetically determined, has been associated with JAIH[25].

Environmental factors

A number of drugs may cause unpredictable, dose-independent, immune-mediated liver damage. Autoimmune hepatitis related to halothane, tienilic acid, dihydralazine and minocycline are typically associated with LKM autoantibodies even though the molecular targets are different from AIH-2 (*i.e.*, CYP2E1 for halothane and CYP2C9 for tienilic acid)[26].

Several viruses have been proposed as triggering factors for AIH such as HAV, measles, EBV or HSV, based on clinical or epidemiological criteria[27]. CYP2D6, the specific target of LKM-1 antibodies, shows epitopes that cross-react with homologous region of HCV, CMV and HSV[28]. Although definite evidence supporting this mechanism is lacking, it is conceivable that, infections with otherwise common viruses might lead, within a per-

missive genetic background, to break tolerance to self-antigens like CYP2D6, which could also be expressed, under particular conditions, on the hepatocyte surface[29].

Autoimmune reaction as a defect of regulatory function

A defect in a subpopulation of T lymphocytes regulating the immune response to liver- antigens expressed on the hepatocyte membrane has been reported in patients with AIH-1[30]. This T-cell subpopulation, bearing the interleukin 2 receptor α-chain (CD25+) and known as functional regulatory T-cells (T-regs), has been extensively studied as the putative main subset of regulatory cells for immune tolerance maintenance. In AIH patients, T-regs lymphocytes were found to be defective in number[16]. Moreover, functional studies have suggested that these cells are defective in promoting secretion of regulatory cytokines by their targets and in regulating CD4+ and CD8+ T-cell proliferation and interferon-gamma production and that they are unable to restrain monocyte activation and function[16]. However, using different markers, such as FOXP3, to identify T-regs lymphocytes, their role, in AIH, as the main immunoregulatory cells was recently challenged[31,32].

In AIH-2, the principal autoantigen (CYP2D6) is known, and the dominant epitopes target of the B and T-cell immune responses are also well characterized. On this basis, generation and expansion of HLA-restricted specific T-reg lymphocytes has been attempted and their immunomodulatory properties have been described *in vitro*[33]. Targeted immunotherapy with autologous infusion of *ex-vivo* expanded T-regs was demonstrated to induce remission of experimental AIH of mice[34].

Animal models

Advances in understanding the pathogenesis of AIH has been limited by the lack of accurate animal models. Murine models have been generated through DNA immunization with a chimeric fusion protein containing human CYP2D6 and human forminotransferase cyclodeaminase, the two self antigens of type 2 AIH, together with the extracellular region of mouse, cytotoxic T-lymphocyte antigen 4, as an immunological modulator[34,35]. Another model for AIH-2 uses CYP2D6 transgenic mice and tolerance mechanisms are overrun with the use of an adenovirus-CYP2D6 vector[36]. Immunized or infected mice developed chronic histological changes in the liver close to interface hepatitis, resembling those of AIH, with the development of a specific immune response with the production of anti-LKM1 and anti-LC-1 antibodies. A third animal model was created without the use of active immunization against xenopeptides, but using a transgenic mouse expressing chicken ovalbumin on the hepatocyte surface[37].

CLINICAL FEATURES

A specific autoantibody panel identifies two types of AIH: the presence of anti-smooth muscle antibody (SMA) and/or antinuclear antibody (ANA), in AIH-1[21,38], and LKM-1 and/or LC-1, in AIH-2[8,39]. Epidemiological distribution, genetic markers, clinical presentation and pattern of serum cytokines differentiate the two types of AIH suggesting possible pathogenetic mechanisms[40]. AIH-1 presents at any age, from infancy to the elderly, and in both sexes, while AIH-2 presents almost exclusively in childhood, with a very high incidence in females[8,39]. Patients with AIH-2 present at younger age that AIH-1, and are at higher risk to develop an acute liver failure[41]. Hypergammaglobulinemia is common in AIH-1, but it can be absent in AIH-2[8,38]. Moreover, AIH-2 is almost never associated with evidence of bile duct lesions while bile duct lesion is commonly observed in AIH-1[38]. Extra hepatic diseases of autoimmune mechanism are frequently observed in patients with both types of AIH. Autoimmune thyroid diseases (Grave's and Hashimoto diseases) and autoimmune skin diseases such as vitiligo or alopecia are more frequently observed in AIH-2[8,38].

Three patterns of clinical onset characterize JAIH: (1) Acute onset with anorexia, nausea, vomiting and abdominal pain followed by jaundice, eventually suggesting an acute viral hepatitis, is the most frequent. In particular, patients, with AIH-2, are at higher risk than AIH-1 to develop acute liver failure with encephalopathy; (2) Insidious onset with progressive fatigue, anorexia, and intermittent jaundice lasting for several months/years before diagnosis, can be observed in about a third of patients. All these patients have clinical evidence of chronic liver disease and/or of cirrhosis at diagnosis; and (3) About 10% of patients may be asymptomatic when the liver disease is serendipitously discovered by the finding of clinical signs of chronic liver disease or by an increase of aminotransferase activity.

In a few patients, JAIH may reveal itself with symptomatic portal hypertension or with symptoms related to an extrahepatic autoimmune disease such as autoimmune thrombocytopenia, autoimmune haemolytic anemia, diabetes type 1, autoimmune thyroiditis, vitiligo, cutaneous vasculitis, uveitis, glomerulonephritis, juvenile chronic arthritis, systemic lupus erythematosus, Sjögren's syndrome, celiac disease and inflammatory bowel disease[8,15,38].

LABORATORY FEATURES

At diagnosis, the "activity" of the liver disease can be documented by the presence of an almost constant increase of liver enzyme, in particular of serum transaminase activity that may increase up to 50 times or more the upper normal limit, while gamma glutamyltransferase (GGT) activity may be normal or only slightly elevated. An increase of GGT should suggest bile duct damage as in the case of autoimmune hepatitis/cholangitis overlap syndrome. Serum gamma globulins and immunoglobulins G are usually elevated, sometimes markedly, up to 6-8 g/L. Serum albumin may be normal in absence of liver function impairment and ascites. Serum immunoglobulin A deficiency and/or genetically determined low levels of

C4 can be observed in AIH-2[8,25]. Prolonged prothrombin time suggests severe liver function impairment.

Autoantigens and autoantibodies

Recognition of pathogenetic autoantigens in AIH might be one of the key factors to develop an etiologic-based therapy. Unfortunately most of the antigens recognized by autoantibodies detected in AIH are either non organ-specific or intracellular molecules, unlikely involved in triggering autoimmune reaction. The most studied candidate autoantigens are the asialoglycoproteins receptor (ASGP-R) for type AIH-1 and the cytochrome P4502D6 (CYP2D6) for AIH-2.

The ASGP-R is an organ-specific antigen expressed in the hepatocyte membrane. Even if several experimental studies had been published[42], its role in pathogenesis of AIH is still controversial[42]. Both peripheral and infiltrating lymphocytes collected from adult and pediatric patients with AIH show a proliferative response to human ASGP-R[43], and a lack in T-suppressing function of CD4+ T-cells specific for ASGP-R and corrigible by immunosuppressive therapy, has been described both in patients and in their healthy relatives.

Seven isoforms of cytochrome P450 are expressed in human liver and all of these isoforms are targets of LKM reactivity in different types of autoimmune, viral or drug induced liver disease. CYP2D6 is an intracellular enzyme active in detoxification of several drugs and is the molecular target of AIH-2[44]. By effect of some cytokines, CYP2D6 can be expressed on hepatocytes surface becoming a potential target for autoreactive T-cells[29].

Detection of serum non-organ-specific autoantibodies (ANA, SMA and LKM-1) known to be associated with autoimmune liver diseases is a critical component of diagnostic criteria developed by International Autoimmune Hepatitis Group[2,3]. Their assessment should preferably be performed by indirect immunofluorescence (IIF) on frozen section of rat liver, kidney and stomach, and the presence of ANA/SMA and LKM-1 is virtually mutually exclusive. Sera screened positive for ANA/SMA should be further examined to assess the pattern of nuclear staining by the use of HEp2 cell monolayers, or to define the target of the SMA reaction[45].

The autoantibody profile does not markedly vary in the course of AIH with the exception of ANA reactivity that can be detected "*de novo*" in both subgroups of AIH. Autoantibodies titers varies during the course of the disease usually reducing in titer in case of remission, but also independently[46,47].

Autoantibody titers are not predictive of biochemical or histological remission. High titers at onset do not suggest a more aggressive disease and their disappearance from serum is not predictive of a better disease control during treatment or of a sustained remission in case of discontinuation of treatment.

Antinuclear and anti smooth muscle antibody, the serological hallmark of AIH-1, are usually present at high (≥ 1:100) titer, but they are not specific of AIH. ANA and SMA can, in fact, be detected in other liver diseases (viral or drug induced hepatitis, steatohepatitis and hepatocellular carcinoma) and also in non-hepatic disorders, however at lower titers.

Various patterns of ANA staining can be observed: homogeneous (60%) and speckled (15%-25%) are the most frequent, however they are not considered of clinical importance and they may vary in the same patient, during treatment. Several nuclear antigens have been identified, as a target of ANA reactivity: single and double-stranded DNA, histones, chromatin, ribonucleoprotein complexes, cyclin A and centromere, but no single AIH specific antigen has been detected so far. In AIH-1, ANA can be detected either alone or in conjunction with SMA. In children, ANA is considered positive when the titer is ≥ 1:40, however since ANA reactivity at low titer can be frequently found in children we suggest raising the positivity cut-off to at least to 1:100. Moreover, Anti dsDNA antibodies can be detected in 25% of ANA-reactive AIH-1 patients[46].

When using rat stomach as substrate for SMA: uniform IIF stain of the muscolaris mucosa, blood vessels walls (V) and parietal cell occurs. With rat kidney tissue, staining of the mesangial area of glomeruli (G) and of proximal renal tubular cells (T) also occurs. "VG" and "VGT" staining patterns are the most frequent IIF patterns encountered in AIH. SMA reactivity usually stains structural components of the cytoskeleton such as desmin and troponin. In AIH SMA reactivity is directed against filamentous (F) actin. Anti-F-actin can be detected using cultured human fibroblast or HEp2 cells. Anti-F-actin specificity is higher than SMA but anti-F-actin antibodies may be found also in viral infection, connective tissue disease and celiac disease.

Anti-LKM-1 serum reactivity defines the AIH-2, the most common type of JAIH occurring in infancy and childhood[8]. LKM-1 are present in 30%-70% of sera of patients with AIH-2 with anti-LC-1 antibody. Occasional patients with both ANA and LKM-1 have been defined as AIH-2.

LKM-1 stains hepatocytes and the proximal renal tubular cells (P3 portion) of liver and kidney sections in mice. Occasional staining of the distal renal tubules usually generates confusion with anti-mitochondrial autoantibody (AMA). AMA positivity in children is rare and Primary Biliary Cirrhosis is exceptional in pediatric age.

Anti-LC-1 is an organ-specific antibody, which homogeneously stains, in IIF, the cytoplasm of the hepatocytes, sparing the perilobular layer of central veins and without staining of the proximal renal tubules[48]. LC-1 can also be detected with both immunodiffusion and immunoblotting. LC-1 antibody reacts with forminotransferase cyclodeaminase, a 58-62 Kd liver specific antigen[49] and together with LKM-1, characterizes AIH-2. In fact LC1 reactivity can be found associated with LKM-1 in about 50% of AIH-2, but LC-1 may characterize on its own, as a sole autoantibody children with AIH-2[39].

The simultaneous presence of LKM-1 may obscure

Figure 1 Interface hepatitis with piecemeal necrosis and lymphocyte spillover across the limiting plate.

Figure 2 Liver biopsy of a 6 mo old infant with autoimmune hemolytic anemia showing diffuse giant cell transformation and moderate inflammatory portal infiltrate.

LC-1 IIF reactivity. In these cases it is necessary to use another method to detect the presence of LC-1 such as immunodiffusion, ELISA, Western blot or dot blot.

Anti-SLA is a non organ-specific antibody which target antigen is likely to be a 50 Kd protein identified as O-phosphoseryl-tRNA: selenocysteinyl-tRNA synthase. Anti-SLA is considered a specific marker for AIH-1 being present in 6% to 58% of adults and children with AIH-1, alone or in combination with SMA and/or ANA[50]. Its detection could be particularly useful in patients who are negative for conventional markers of the disease (ANA, SMA), but its diagnostic role in JAIH is not relevant.

Anti-human ASGPR is a species-specific, liver-specific autoantibody that can be detected in sera of patients with various inflammatory liver diseases, but predominantly in AIH. The absence of a commercialized assay restricts its use to few laboratories.

Anti-neutrophil cytoplasmic antibodies (ANCA) constitute a heterogeneous group of autoantibodies directed against various subcellular components of neutrophils or myeloid cells and their presence has been proven to be a reliable diagnostic tool in systemic vasculitis. They are routinely detected by IIF on ethanol fixed human neutrophils and commonly classified in cytoplasmic (cANCA), perinuclear (pANCA) and atypical (pANNA). Atypical pANCA are characterized by non-homogeneous labeling of the nuclear periphery together with multiple intranuclear fluorescent staining and have been reported in patients with autoimmune liver disease including sclerosing cholangitis associated with inflammatory colitis and in AIH-1.

LIVER HISTOLOGY

The International Autoimmune Hepatitis Group has affirmed the role of liver biopsy for the diagnosis of AIH[2,3]; liver biopsy is thus recommended in all patients suspected AIH unless there is a significant contraindication[51]. The histological hallmark of AIH is "interface hepatitis" (formerly called piecemeal necrosis) (Figure 1). A considerable amount of eosinophilic granulocytes can be observed within the portal infiltrate, especially in such cases associated to celiac disease[52].

In patients with AIH presenting as an acute liver disease, liver histology allows for differentiation between spontaneous exacerbation of a chronic liver disease ("acute-on-chronic") and a newly developed disease. In the latter case, centrilobular zone 3 necrosis is the most typical pattern[53]. Subsequent transition to the classic features of "interface hepatitis" usually occurs. Other possible liver biopsy findings in AIH include the presence of giant multinucleated hepatocytes[54]. Moreover, diffuse giant cell transformation characterizes a distinct form of AIH in infants, associated with autoimmune hemolytic anemia[55] (Figure 2).

Massive liver cell necrosis may be present in AIH with acute severe/fulminant onset and may be associated with bridging necrosis and/or with multilobular or panlobular necrosis. These histological findings support but do not constitute firm evidence for the diagnosis of AIH.

Biliary ducts are usually not affected in AIH and the presence of lymphocytic cholangitis, or that of a mixed inflammatory infiltrate surrounding and infiltrating the bile ducts, has received a negative diagnostic rating on the IAIHG diagnostic score[3]. However, the incidental presence of bile duct inflammatory changes has been recognized in patients with AIH responding to immunosuppressive treatment[56]. In children, bile duct inflammatory changes are common in AIH-1, but are very rare or absent in AIH-2.

Liver biopsy also provides information on prognosis, identifying the presence of cirrhosis. Cirrhosis may be present at diagnosis or rapidly develop in JAIH[57]. Cirrhosis is more frequent at diagnosis in AIH-1 than in AIH-2[58], however, concerning the diagnosis of cirrhosis, "blind" percutaneous liver biopsy has been demonstrated to be of low diagnostic sensitivity, since up to 50% of patients may not be correctly diagnosed[57].

DIAGNOSIS

JAIH has variable clinical manifestations and should be considered in the diagnostic work-up of any patient with

a cryptogenic liver disease. Diagnosis of JAIH basically relies on the exclusion of other possible known causes of the hepatic disease, such as chronic viral infections and Wilson's disease and by clinical biochemical and histological "positive" criteria. Diagnosis is not challenging when all the major clinical and biochemical elements of the disease are present, such as the occurrence of an autoimmune disease in the same patient, a biochemically "active" liver disease, an elevation of serum gamma globulins, presence in serum of autoantibodies known to characterize JAIH, and compatible histopathological features on liver biopsy. However, sometimes the diagnosis may become difficult and for this reason, in 1993, an international board of physicians published a set of criteria to identify patients as having either "definite" or "probable" autoimmune hepatitis[2]. Once used primarily for scientific and research purposes, this scoring system is now widely used in clinical practice after being reviewed in 1999[3].

A simplified scoring system has since been proposed based solely on four parameters (autoantibodies, IgG levels, liver histology and exclusion of viral hepatitis), and has been validated in adults with 88% sensitivity and 97% specificity[59]. When comparing both scoring system in adults, we see that the revised original scoring system has shown greater sensitivity for the diagnosis than the simplified scoring system (100% *vs* 95%), while the simplified score had greater specificity (90% *vs* 73%) and predictability (92% *vs* 82%) for AIH than the revised original system[60]. The original scoring system was assessed in children using the GGT/aminotransferase ratio instead of the alkaline phosphatase/aminotransferase ratio to improve its specificity[61]. When tested in children the simplified scoring system was not proved to be effective mainly because of its low sensitivity[62]. In conclusion, no validated scoring system for the diagnosis exists for JAIH[63]. In challenging cases, once Wilson's disease is excluded, and in absence of liver function failure, an immunosuppressive treatment should be attempted for at least 6 wk. A positive response to this treatment would suggest AIH. Moreover, relapse after immunosuppressive drug withdrawal is positively weighted in the IAIHG diagnostic criteria[2,3].

MANAGEMENT

The most effective therapy for JAIH is pharmacological suppression of the immune response. Treatment should be started as soon as the diagnosis is made to avoid severe liver damage and progression of fibrosis. Standard therapy includes a combination of prednisone and azathioprine[6] or occasionally prednisone as a monotherapy[6,7,21]. Prednisone or prednisolone is used at a higher dose that used in adults (2 mg/kg per day, up to a maximal daily dose of 60 mg/d in the adolescent) and azathioprine is administered starting from 1 mg/kg per day up to a maximum of 2.5 mg/kg per day. First line combination therapy including prednisone and azathioprine can be more effective than prednisone alone[64]. Moreover, the "steroid-sparing" effect of the azathioprine allows

reducing more rapidly the steroid dose, thus tapering side effects related to the prolonged use of steroids at high dose.

The goal of the treatment is to obtain clinical and biochemical remission of the liver disease clinical signs with normalization of the "activity" of the disease (transaminase, gamma globulins) and of the liver function (prothrombin activity; INR). The definition of treatment-induced remission in JAIH should be stricter than that used in adult disease: the serum activity of aminotransferase should be maintained within the upper limit of normal, serum immunoglobulin G levels within the normal range for age, and serum autoantibodies absent or at very low titer[6,15,21]. Even clinical and biochemical remission do not always reflect histological resolution of inflammation, the proof of histological remission is not required. The rapidity and degree of response to treatment depends on the disease severity at onset. In JAIH, treatment is associated, in over 90% of cases, with a measurable clinical and laboratory response within 4 to 8 wk. Complete normalization of biochemical parameters may, however, take several months. On histopathological evaluation, the immunosuppressive treatment improves the fibrosis score, with an arrest in its progression into cirrhosis. Fibrosis control is mainly associated with regression of necroinflammatory activity[65]. Once remission is obtained, it must be maintained in the long term on the lowest possible dose of medication. Different therapeutic schedules of treatment discontinuation exist and should be tailored on individual patients. Prednisone is usually first decreased; the shift to alternate-day use of steroids may be suitable because of the lower incidence of side effect[66]. In cases of severe liver function impairment at diagnosis, liver function may further deteriorate despite an appropriate therapy. In these patients immunosuppressive therapy should be modified with the introduction of a third drug such as cyclosporine. In case of further non-response, the possibility of a liver transplant should be considered.

When complete remission is achieved, the goal of the immunosuppressive treatment is to maintain remission and to prevent relapse of the disease. Prednisone should be further reduced to the lowest dose that allows a biochemical remission. Alternate-day doses of prednisone associated to azathioprine are usually effective in maintaining remission. A relapse may occur at any time, the most frequent cause of a relapse is patient's non-compliance. It is questionable that a histological remission has to be demonstrated through a liver biopsy in patients with clinical and biochemical remission, since the presence of histological remission has not been shown to be sufficiently indicative of an absence of possibility of relapse in the case of further reduction of the immunosuppression[6]. Liver fibrosis rarely progresses in patients who maintain a persistent biochemical remission and it can even diminish during treatment. Duration of the immunosuppressive treatment before attempting discontinuation is unknown; stopping treatment within the first two years is usually followed by a relapse[6]. We suggest

that sustained remission should be maintained for at least five years, thereafter, in case of combined treatment of prednisone and azathioprine, prednisone is stopped and the patient is maintained on azathioprine monotherapy. Azathioprine monotherapy had been demonstrated to maintain remission in most patients with AIH[67]. Undetectable serum autoantibodies do not exclude the risk of relapse, but an increase of the titer of autoantibodies suggests caution in modifying the dose of immunosuppressive therapy.

Particular variant forms of AIH, as celiac disease-associated AIH on gluten free diet, might have a lower risk to relapse after treatment discontinuation[68]. In such cases a discontinuation attempt after less than 5 years of treatment could be justified.

Steroids mostly cause side effects of immunosuppressive therapy, including increase of food intake leading to moderate and reversible weight increase and a reduction of height growth. Severe side effects include obesity, growth failure, severe cosmetic changes, cutaneous striae, vertebral collapse, hyperglycemia, and cataracts, causing both visual impairment and, potentially, psychosis. Azathioprine is usually a safe drug, and cytopenia necessitating a dosage reduction is a rare event. Teratogenicity and oncogenicity issues resulting from azathioprine use in humans have not been conclusively demonstrated. Pregnancy should however be excluded in adolescent girls before starting treatment with azathioprine. However, if prolonged azathioprine treatment is needed, pregnancy has been demonstrated to be safe, in the long term, in young females with AIH[69,70]. During pregnancy, higher doses of prednisone may be an alternative option for those young women who prefer azathioprine withdrawal. However, vigilance is required at all times, and patients need careful monitoring, especially in the postpartum period, because of the possibility of relapses.

In case of non-response to conventional treatment or in the presence of severe side effects of corticosteroids the use of cyclosporine A is indicated. Cyclosporine A at a median dose of 5 mg/kg per day induces remission in children and adolescents with AIH with a initial target concentration in serum of cyclosporine of 200-250 ng/mL[71,72]. Cyclosporine treatment side effects including mild gingival hyperplasia and reversible irsutism in some patients, are usually well tolerated and disappear after reduction of the dose[73]. Normality of renal function should be verified before starting this drug. In the follow-up, once remission is obtained, the dose of cyclosporine can be reduced with a target concentration of 100 ng/mL or the patient may be shifted to conventional treatment.

In children who either did not tolerate azathioprine or did not respond to conventional treatment, mycophenolate-mofetil (MFM, 20 mg/kg per day) in addition to steroids therapy has been shown to induce and maintain remission[74]. Side effects of MFM include headache, diarrhea, dizziness, hair loss and neutropenia.

Budesonide, a steroid that is rapidly metabolized with low systemic exposure, in combination with azathioprine, has been recently shown in a trial including patients with JAIH to induce and maintain remission with fewer side effects than prednisone[75]. However, the low proportion of remission observed in this study compared to that reported in others pediatric studies using prednisone and azathioprine schedules, do not support its use as first-line treatment of JAIH[76]. Recently Rituximab, a monoclonal antibody against CD20, a B-lymphocyte surface antigen, has been successfully used in selected cases as a rescue therapy[77].

Liver transplantation should be considered as a therapeutic option for children and adolescents with JAIH and chronic end stage liver disease or in patients with acute liver failure at onset not responding to rescue immunosuppression. Five-year post-transplant survival in JAIH patients is scored at 86%[78].

LONG-TERM OUTCOME

Immunosuppressive treatment has convincingly altered the outcome of most patients with AIH[6,21,79-82]. Indeed according to previous prognostic studies on adults, 40% of patients with severe disease without treatment die within six months of diagnosis and cirrhosis eventually develops in at least 40% of untreated survivors[83,84].

On the other hand the 10-year survival rates among treated adults is 60% for those with cirrhosis on the initial liver biopsy[83,85] and more than 80% for those patients without cirrhosis at presentation[86].

The long-term outcome of JAIH still remains scarcely known, however, in case of full and prompt response to immunosuppressive therapy, the prognosis is usually satisfactory and most patients survive in the long-term with excellent quality of life and, in the majority of cases, on low dose immunosuppression.

In the five largest published series of children with AIH, overall survival rate in long-term treated patients exceeded 80% with a 5-year survival with native liver ranging between 67% and 87%; follow-up ranged from 4, 8 to 10 years[21,38,87-89].

The presence of cirrhosis on initial liver biopsy did not seem to impact long-term survival in children with AIH[87,88] while elevated total bilirubin and prolonged INR are independent risk factors of death and/or need of liver transplantation[21]. Immunosuppressive treatment requires to be prolonged in the long term in the majority of patients; however, sustained remission after treatment discontinuation has been reported in 13% to 20% of patients[21,87].

End-stage liver disease leading to liver transplantation has been reported to develop up to 14 years after diagnosis in 8% to 16% of children with JAIH compliant to immunosuppressive therapy and in absence of an evident biochemical relapse[21,87].

VARIANT FORMS OF JAIH

Giant cell hepatitis with autoimmune hemolytic anemia
Giant cell hepatitis with autoimmune hemolytic anemia is a rare entity described by Bernard *et al*[55] in 1981, pre-

senting in early childhood with severe progressive liver disease in combination with Coombs positive hemolytic anemia. The clinical course is usually aggressive leading to hepatic failure and death.

The mechanism of liver disease is not known, but an autoimmune process is believed to be responsible for this component of the disease as well. A study by Whitington *et al*[90] has recently provided evidence that systemic B cell autoimmunity might play a pathogenetic role even if autoantibodies are usually absent, histological features characteristic of auto-immune hepatitis are missing, and the disease is highly refractory to therapy that would usually be effective in AIH. Conventional immunosuppressive treatments (steroids, azathioprine, cyclophosphamide, cyclosporine, mycophenolate) are associated with high toxicity and often produce only partial or short-lasting remission[91]. Liver transplantation is associated with high rate of disease recurrence. In more recent studies the use of anti-CD20 monoclonal antibody (Rituximab) has been reported to be effective in patients with refractory hepatitis[92]. Intravenous immunoglobulins have been also reported to be efficacious in case of severe liver function impairment at onset or during a relapse in patients treated by multiple immunosuppression although their efficacy seems to be only temporary[93].

Autoantibody-negative autoimmune hepatitis

Cryptogenic hepatitis with autoimmune features in absence of detectable serum autoantibodies is described as "autoantibody-negative AIH"[94]. It affects a small proportion of adult patients presenting with a cryptogenic liver disease with acute or chronic presentation with the clinical, biochemical and histopathological features of AIH and responsive to immunosuppressive treatment[94-96]. The comprehensive international scoring system can support, but never override the clinical diagnosis pre-treatment, and non-standard serological markers should be sought in order to enhance diagnostic confidence[3,60,97]. A 3-mo treatment trial with corticosteroids should be considered in all candidates for the diagnosis, regardless of the serological findings[98,99]. This entity has been reported in children only in small series or in single case report[100].

Celiac disease associated-AIH

Celiac disease (CD) is common in patients with AIH, especially in children, as it has been shown in a previous Italian multicenter survey[52] and in more recent small pediatric series[68,101-103]. These studies reported a prevalence of CD in up to 19% of children with JAIH. The pathogenetic role of gluten in triggering AIH is uncertain, however, both types of AIH have been described in association with CD as well as autoantibody-negative AIH[52,100]. Liver damage, as evidenced by elevated aminotransferase activity, has been reported as being present from the first observation of celiac patients in some cases while in other, CD was diagnosed by a serological screening in patients with a known AIH[101,102]. Therefore

all patients with AIH should be serological screened for CD and moreover all CD patients with clinical and/or biochemical signs of liver damage should be closely followed-up to exclude an AIH, especially in the case of persistent elevation of liver enzymes on a gluten free diet.

Children with co-existent CD seem to have an apparently more favorable response to treatment, suggesting a positive effect of gluten withdrawal on AIH co-existent with CD. Gluten withdrawal might potentiate the immunosuppressive effect of the immunosuppressive drugs, maintaining remission even when the treatment has been withdrawn[68,103].

Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy-associated JAIH

Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) is a rare autosomal recessive disorder caused by mutations in autoimmune regulator gene (AIRE) inducing a loss in central immune tolerance, failure to eliminate autoreactive T cells in the thymus, and their escape to the periphery. APECED is characterized by an extremely variable pattern of destructive autoimmune reaction, mainly mediated by specific autoantibodies toward different endocrine and non-endocrine organs. Virtually, all tissues and organs may represent the target of the autoimmune attacks, thus leading to a wide spectrum of clinical features. The three main components of APECED are chronic mucocutaneous candidiasis, chronic hypoparathyroidism and Addison's disease. Generally chronic mucocutaneous candidiasis develops first and it is often followed by chronic hypoparathyroidism, before the age of 10 years, and later on by adrenal insufficiency. In addition to the main components, the spectrum of minor manifestations may include ectodermal dystrophy, other endocrinopathies, such as hypergonadotropic hypogonadism, insulin-dependent diabetes, autoimmune thyroiditis, and pituitary dysfunction. Moreover, skin diseases (vitiligo and alopecia) and gastrointestinal disorders (chronic atrophic gastritis, pernicious anemia) and particularly AIH, may be present. AIH shows a clinical phenotype akin to AIH-2 and it is present in 15%-20% of cases with CYP1A2 and CYP2A6 as a specific target antigens[104]. Heterozygous mutations of *AIRE* gene have been reported in children with AIH-1 suggesting a possible predisposition role[105].

De novo autoimmune hepatitis

De novo autoimmune hepatitis, after liver transplantation, was first described in 1998 by the group of King's College Hospital in London[106]. It is a form of late graft dysfunction characterized by abnormal liver function tests, high serum concentration of immunoglobulin, presence of autoantibodies, and histological features of interface hepatitis coupled with a rich plasma cell infiltrate[107]. This recently recognized entity affects patients transplanted for disorders other than AIH and usually of non-autoimmune nature. Since its first description several authors reported the occurrence of *de novo* AIH in children and adults

transplanted for non-autoimmune conditions[108-115].

The pathogenesis of *de novo* AIH is not yet defined and there are a variety of potential mechanisms leading to autoimmune liver disease post-transplant. Possible pathogenetic mechanism include the release of autoantigens from damaged tissue, as well as molecular mimicry, whereby exposure to viruses sharing amino acid sequences with autoantigens leads to cross-reactive immunity[106]. *De novo* AIH responds to treatment with corticosteroids and azathioprine allowing excellent graft and patient survival. Early recognition and appropriate management are therefore essential to avoid graft loss[107,116].

CONCLUSION

Juvenile AIH is a severe liver disease of childhood and adolescence progressing rapidly toward cirrhosis and severe liver function impairment unless immunosuppressive treatment is promptly started. Its clinical spectrum is broad: from asymptomatic liver damage to acute symptomatic and even severe hepatitis. Early diagnosis is mandatory but no scoring system of sufficient sensitivity exists. Serum autoantibodies are a relevant tool, but not essential for diagnosis and liver histology has distinct but non-pathognomonic features. The vast majority of treated patients responds to the immunosuppressive treatment, but relapses are frequent and mostly related to defective compliance to treatment. Long-term outcome studies on JAIH concerning the possibility of safely stopping the immunosuppressive treatment are needed for appropriate counseling to families and patients.

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Focal liver lesions detection and characterization: The advantages of gadoxetic acid-enhanced liver MRI

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Abstract

Since its clinical introduction, several studies in literature have investigated gadolinium ethoxybenzhyl diethylenetriaminepentaacetic acid or gadoxetic acid (Gd-EOB-DTPA) properties. Following contrast injection, it provides dynamic vascular phases (arterial, portal and equilibrium phases) and hepatobiliary phase, the latter due to its uptake by functional hepatocytes. The main advantages of Gd-EOB-DTPA of focal liver lesion detection and characterization are discussed in this paper. Namely, we focus on the possibility of distinguishing focal nodular hyperplasia (FNH) from hepatic adenoma (HA), the identification of early hepatocellular carcinoma (HCC) and the pre-operative assessment of metastasis in liver parenchyma. Regarding the differentiation between FNH and HA, adenoma typically appears hypointense in hepatobiliary phase, whereas FNH is isointense or hyperintense to the surrounding hepatic parenchyma. As for the identification of early HCCs, many papers recently published in literature have emphasized the contribution of hepatobiliary phase in the characterization of nodules without a typical hallmark of HCC. Atypical nodules (no hypervascularizaton observed on arterial phase and/or no hypovascular appearance on portal phase) with low signal intensity in the hepatobiliary phase, have a high probability of malignancy. Finally, regarding the evaluation of focal hepatic metastases, magnetic resonance pre-operative assessment using gadoxetic acid allows for more accurate diagnosis.

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Key words: Magnetic resonance imaging; Liver; Image enhancement; Gadolinium diethylenetriaminepentaacetic acid; Carcinoma; Hepatocellular

Core tip: This study highlights the added value of gadoxetic acid-enhanced liver magnetic resonance imaging (MRI) in the detection and characterization of focal liver lesions. Three main topics are summarized: the role of gadoxetic acid in the evaluation of solid benign hepatic lesions, represented by hepatocellular adenoma and focal nodular hyperplasia; the diagnostic capability of hepatobiliary phase of gadoxetic acid-enhanced liver magnetic resonance imaging in the early identification of small hepatocellular carcinoma; the high diagnostic accuracy powered by gadoxetic enhanced-liver MRI in the detection of hepatic metastasis.

INTRODUCTION

Since the first studies were reported in literature in 1991-1992, several authors have investigated the potentialities of gadolinium ethoxybenzhyl diethylenetriaminepentaacetic acid or gadoxetic acid (Gd-EOB-DTPA) enhanced magnetic resonance imaging (MRI) liver[1-5]. In a previous article published by Mühler *et al*[5], spin-echo

Table 1 Imaging features of focal liver lesions in the dynamic vascular phases (after contrast administration) and in the hepatobiliary phase

	Phases			
	Arterial	Portal	Delayed	Hepato-biliary
FNH	Hyperintense	Isointense	Isointense	Hyperintense/isointense (hypointense[1])
Adenoma	Hyperintense	Isotense/slightly hypointense	Isotense/slightly hypointense	Hypointense (hyperintense or mixed hypo/hyperintense[1])
Typical HCC	Hyperintense	Hypointense	Hypointense	Hypointense
Pre/early HCC (decreased portal supply)	Isointense	Hypointense	Hypointense	Hypointense
Pre/early HCC (increased arterial supply)	Hyperintense	Isointense	Isointense	Hypointense
Metastasis (hypovascular)	Irregularly hypointense	Irregularly hypointense	Irregularly hypointense	Hypointense
Metastasis (hypervascular)	Irregularly hyperintense	Isointense or hypointense	Inhomogeneously hypointense	Hypointense

[1]Atypical behaviours of focal liver lesions. FNH: Focal nodular hyperplasia; HCC: Hepatocellular carcinoma.

(SE) sequences and short tau inversion recovery (STIR) sequences were compared in the detection of experimental liver metastases[5]. Relative enhancement and lesion-to-liver contrast were also analysed in the mentioned study. After contrast administration, the authors reported lesion-to-liver contrast increased by approximately 500% with both SE and STIR sequences. Therefore, we can see that the role of Gd-EOB-DTPA in focal liver lesion (FLLs) detection has been studied from the beginning.

Subsequently, the usefulness of hepatospecific contrast in liver MRI has been confirmed by other studies. In fact, detection and characterization of focal liver tumours have been compared in the same patient using Gd-EOB-DTPA and Gd-DTPA enhanced MRI[6]. In the assessment of FLLs, Gd-EOB-DTPA has also been compared with intra-operative findings in a multicenter analysis[7].

Although research on focal lesions is the most common, some authors have observed that, because of its properties, Gd-EOB-DTPA could be potentially used as a tracer of liver functionality[8-10].

The mechanisms of contrast uptake and excretion have been documented[11-14]. The uptake of Gd-Eob-DTPA is achieved by functional hepatocytes, which have the cloned organic anion transporting polypeptides (OATPs). In humans the contrast is introduced through OATP1 and OATP3 transporters, located at the apical membrane of hepatocytes[15]. Then, the contrast has urinary and biliary excretion rates (the latter up to 50%, much higher than other hepatospecific contrasts). Regarding biliary excretion, the contrast is excreted through Multidrug Resistance-associated Proteins (MRPs) to bile canaliculi (MRP2 = apical transporter) or sinusoidal spaces (MRP3, MRP4 = basolateral transporters)[11-15].

Thus, in normal liver parenchyma starting during dynamic vascular phases, hepatocytes increase the uptake of gadoxetic acid. The uptake process is gradually followed by contrast discharging through the bile canaliculi. Generally, the hepatobiliary phase, where hepatocytes reach maximum signal intensity, is obtained 20 min after contrast administration. The variable contrast uptake by FLLs represents an additional diagnostic tool in liver imaging.

The aim of this topic highlight is to discuss the advantages of gadoxetic acid-enhanced liver MRI in the study of FLLs, focusing on: (1) Evaluation of hepatic adenoma and focal nodular hyperplasia; (2) Identification of early hepatocellular carcinoma (HCC); and (3) Detection of hepatic metastases detection in oncology patients. Typical and atypical behaviours of FLLs using gadoxetic acid-enhanced MRI are summarized in Table 1, which shows imaging features observed also in the hepatobiliary phase.

EVALUATION OF HEPATIC ADENOMA AND FOCAL NODULAR HYPERPLASIA

The use of Gd-Eob-DTPA allows for characterization of hepatic adenoma (HA) and focal nodular hyperplasia (FNH). In some cases, diagnosis between these solid lesions cannot be reliably achieved using only dynamic vascular phases, and hepatobiliary contrast agents are very useful in their differentiation. In fact, in a previous study, although using gadobenate dimeglumine-a different liver specific contrast from gadoxetic acid-Grazioli et al[16] reported an overall accuracy of 98.3% in the differentiation of FNH from HA and liver adenomatosis, with positive predictive value of 100% and negative predictive value of 96.4%.

FNH was described for the first time by Edmondson in 1956[17]. The lesion is considered a non-neoplastic and hyperplastic response of the liver parenchyma to "a pre-existing local arterial spiderlike malformation"[18]. It occurs in asymptomatic women. The relationship between FNH and contraceptives is still unclear as several authors have demonstrated that contraceptives may favour FNH progression[19]. The lesion is generally represented by a solid circumscribed mass, sometimes with lobulated contour (Figure 1), with a central scar surrounded by nodules of hyperplastic hepatocytes and small bile ductuli[20]. FNHs may show a certain degree of histological heterogeneity, due to the variable degree of intra-lesional inflammation, fibrosis or fat content (the latter has been described as steatotic FNH).

Figure 1 Typical imaging features of focal nodular hyperplasia in a 29-year-old woman. Gadoxetic acid-enhanced magnetic resonance imaging; axial images (A-D) were obtained in dynamic phases and hepatobiliary phase. A shows a solid circumscribed mass (white arrow), lobulated in contour, with a central scar (black arrow); the lesion is hyperintense on the arterial phase (A) and persists slightly hyperintense in the portal and venous phases (B and C respectively). In hepatobiliary phase (D) the mass is slightly hyperintense or isointense to the surrounding liver. The presence of biliary canaliculi, even if not functioning, leads to retention of gadoxetic acid in comparison to the surrounding parenchyma.

Hepatic adenoma is a rare monoclonal benign liver tumour, predominantly found in young females and associated with the use of contraceptives[21]. It generally appears as an uncapsulated mass, formed by large plats or cord cells very similar to hepatocytes. In a work by Grazioli et al[22], they are defined as "these plats are separated by sinusoids, which consist of small capillaries perfused through the arterial pressure". This histological architecture explains the morphological behaviour of adenomas during the dynamic phases after contrast administration. In fact, lesions often appear hypervascular in the arterial phase, and are generally isointense or hypointense to the surrounding liver in the portal phase. The vascular supply in the portal phase is not observed because of the adenomas lack of a portal vascularization[22]. Adenomas have a poor number of Kupfer cells, and this histological feature could explain the absence of technetium (Tc)-99m sulfur colloid uptake. In addition, HAs do not have bile canaliculi[23,24].

The significant capability of Gd-Eob-DTPA in distinguishing FNH from adenomas depends on histological features and cellular expression of molecular transporters. Bile ductuli are present in FNHs, whereas they are missing in HAs. The molecular transporter Organic Anion Transporting Polypeptide 8 (OATP 8) is usually absent or minimally expressed in cellular adenomas. This transporter is instead expressed in FNH, explaining the uptake of Gd-Eob-DTPA[25].

Thus, typically HAs appear hypointense, whereas FNHs are isointense or hyperintense to the surrounding hepatic parenchyma (Figures 1 and 2, Table 1). Several studies have described the mentioned imaging features.

In a work published in 2001, all three adenomas studied in the hepatobiliary phase by Grazioli et al[22] showed hypointense appearance following liver contrast agent administration. Zech et al[25] reported enhancement in the hepatobiliary phase in the 90% of FNH examined in their series where only a minority of lesions showed no enhancement or peripheral enhancement. The presence of biliary canaliculi, even if not functioning, leads to a "slower excretion in comparison to the surrounding parenchyma", and this gadoxetic acid retention explains the hyperintense appearance of FNH[26] (Figures 1 and 2).

Nevertheless, atypical lesions are very difficult to diagnose, even using Gd-EOB-DTPA. In fact, the heterogeneity of FNH could also explain the atypical imaging presentation that has recently been well described in many articles[27,28]. In another case series published in literature, Grazioli et al[29] found that 62 out of 68 FNHs (91.2%) were hyperintense or isointense to the surrounding liver, with only 6 lesions showing an atypical pattern[29]. One atypical enhancement pattern explanation was the presence of a large central scar. These lesions appeared hypointense in hepatobiliary phase, showing only a little marginal enhancement. Two atypical lesions, in the series reported by Grazioli et al[29], were hypointense for

Figure 2 Magnetic resonance imaging of a small focal nodular hyperplasia. Arterial, venous and hepatobiliary phases (A, B and C), acquired in a 44-year-old woman shows the typical enhancement of a small focal nodular hyperplasia (white arrows). The lesion is located in the fourth liver segment, between medium and left sovrahepatic vein. In hepatobiliary phase (C) the lesion is slightly hyperintense to the surrounding liver parenchyma, due to uptake of hepatospecific contrast.

the presence of large fibrous components and abundant fat contents (steatotic FNH).

On the other hand, atypical HAs may not appear hypointense in the hepatospecific phase. Atypical behaviours, appearing as hyperintense lesions, have been reported in literature[15]. In fact, inflammatory adenomas could enhance in the hepatospecific phase. Hyperintense HAs in the hepatobiliary phase have been observed in the series by Denecke *et al*[15]. They reported one hepatic adenoma homogeneously hyperintense and two HAs with a mixed pattern (hypo-/hyperintense). In the subgroup of fatty hepatic adenomas, 14 adenomas were hypointense and 1 was mixed hyper-/hypointense. Also, Huppertz *et al*[30] describe in their FLLs series two out of three adenomas with hyperintense appearance in comparison to the surrounding liver. However, based on a quantitative analysis, all HAs, with hypointense signal to the surrounding liver on hepatobiliary phase, showed a certain degree of increase in signal intensity[15]. This could probably be explained by contrast retention in the interstitium or fibrotic tissue.

In addition, in the series reported by Denecke *et al*[15], the proportion between hyperintense and hypointense adenomas in hepatobiliary phase was approximately equal both in the non-steatotic group and in the steatotic of fatty adenomas[29]. The mechanism of Gd-EOB-DTPA uptake in these minority HAs is still unclear and further studies with histological correlation are needed.

IDENTIFICATION OF EARLY HCC

The progressive differentiation of a regenerative nodule to a dysplastic nodule, and then to an early-HCC has been well investigated[31-34]. In this differentiation, the nodule increases its arteriolar supply progressively and reduces the portal vascularization[32,33]. This vascular change is a crucial step in the carcinogenesis. In view of this consideration, HCC diagnosis with imaging techniques is based on a "vascular analysis" of enhancing pattern, with an increased signal intensity or "wash-in" during the arterial phase and a "wash-out" pattern in the portal or equilibrium phase[35] (Table 1).

In 2012 the European Association for the Study of the Liver (EASL) and European Organization for Research and Treatment of Cancer (EORTC) provided common guidelines for the management of the liver[36]. The joint committee established that non-invasive assessment for HCC could be made only by applying a 4-phase multidetector computed tomography (CT) scan or dynamic contrast-enhanced MRI. In addition, the guidelines postulated that diagnosis is based on a typical morphological hallmark of HCC (Figure 3), with hypervascular pattern in the arterial phase and wash-out in the portal venous or delayed phases[36]. It has to be remarked that while only one technique is required for nodules greater than 1 cm in diameter (evidence 2D, recommendation 2B), a more conservative approach using 2 techniques is recommended in suboptimal settings[36].

Similarly, in 2010 an update of The American Association for the Study of Liver Disease (AASLD) recommended that nodules greater than 1 cm should be investigated with either 4-phase multidetector CT scan or dynamic contrast enhanced MRI[37]. In case of atypical nodules, a second contrast methodical is required (level Ⅱ), or alternatively a biopsy.

Nevertheless, the characterization of a nodule, based on these approaches, is not possible if both mentioned imaging features, "wash-in" and "wash-out", are not observed. Nodules may have hypervascular appearance in arterial phase, without evident wash-out in the portal or equilibrium phase (Figure 4). They could also have the same attenuation or signal intensity to the surrounding liver parenchyma during the dynamic arterial phase on CT and MRI images respectively, and may manifest a wash-out only in the portal phase. In this case the diagnosis is difficult and so, a further analysis is usually required in order to evaluate other important features such as a change in size or a tumour marker. A more invasive approach could be also adopted by choosing a biopsy.

In addition, small nodules (< 2 cm) very often lack the typical behaviour of HCC. Arterial neovascularization or reduced portal supply cannot be identified on imaging techniques, probably because these vascular changes are not significant. Adopting only hypervascularity criteria in

Figure 3 Imaging features of a typical hepatocellular carcinoma. Axial magnetic resonance images show a hypervascular lesion in the arterial phase (A, white arrow), located in the top of the liver, with wash-out clearly in the portal venous phase (B, white arrow). This enhancement pattern represents the typical morphological hallmark of hepatocellular carcinoma. The nodule has an increased arteriolar supply and reduced portal vascularization. In hepatobiliary phase, the lesion appears hypointense to the surrounding liver parenchyma.

Figure 4 Imaging features of a small hepatocellular carcinoma. The lesion (white arrow), located in the fifth segment of right hepatic lobe, is detectable in the arterial and hepatobiliary phase. It has hypervascular appearance in arterial phase (A), without evident wash-out in the portal phase (B). The lesion is hypointense in the hepatobiliary phase (C). As reported in literature, the low or absence of gadolinium ethoxybenzhyl diethylenetriaminepentaacetic acid or gadoxetic acid uptake could precede the decrease of portal vascularization in malignant differentiation.

the diagnosis of HCC, MR sensitivity for nodules < 20 mm is about 63%[38,39].

An important diagnostic tool for the evaluation of lesions in the hepatospecific phase has now been added. In fact, papers have recently emphasized the contribution of hepatobiliary phase in the characterization of nodules without a typical hallmark of HCC. In a recent paper by Iannicelli et al[40], a total of 120 nodules were retrospectively evaluated using gadoxetic acid-enhanced liver MRI. In this study, 92 out of 120 nodules (76.6%) reported typical vascular behaviour of HCC, with hypervascularization appearance in the arterial phase. In the hepatobiliary phase, 90/92 nodules showed low signal intensity, whereas two nodules were hyperintense. The other 28 cases, with non-hypervascular behaviour in the arterial phase, were hypointense in hepatobiliary phase. Among these non-hypervascular nodules, only 15 cases had hypointense signal in the equilibrium phase. In the follow-up study, 50% of non-hypervascular nodules with low signal intensity in the hepatobiliary phase acquired the typical vascular behaviour of HCC.

The high accuracy in the identification of early HCCs will probably change the diagnostic algorithm in hepatocellular carcinoma[41]. It facilitates the diagnosis of hypervascular advanced HCC and the differentiation of early HCC and dysplastic nodules from pseudovascular lesions.

The hypointense appearance in hepatobiliary phase will probably be considered a "radiological marker of nodule differentiation". In the study by Golfieri et al[42], 62 out of 215 nodules were atypical for radiological behaviour. Their histological analysis showed 20 high-grade dysplastic nodules (HGDN)/early HCC, 21 low-grade nodules dysplasia, 17 regenerative nodules and 4 nodular regenerative hyperplasia. Nineteen out of 20 HGDN/early HCC nodules were hypointense in hepatobiliary phase. In another work, Kogita et al[43] found that low or absence of Gd-EOB-DTPA uptake precedes the decrease of portal vascularization in malignant differentiation (Figure 4).

In conclusion, gadoxetic acid-enhanced liver MRI could be very helpful in the early identification of HCC. However, differentiation between HCC and dysplastic nodule remain very difficult. Atypical nodules require better investigation, studying their behaviour in the hepatospecific phase.

LIVER METASTASES DETECTION IN ONCOLOGY PATIENTS

Detection of liver metastases in oncology patients is essential in order to choose the best possible management and treatment. In this regard, many studies have demonstrated the high diagnostic accuracy of liver MRI[44]. Nevertheless, routine liver MRI is generally not performed for the staging of extra-hepatic oncology diseases. For example, the American College of Radiology Appropriateness Criteria for pre-treatment staging of colorectal cancer recommended CT of the chest, abdomen and pelvis for the initial evaluation of disease[45]. In the majority of the cases, staging liver MRI is required to evaluate doubtful FLLs.

The identification of liver involvement by metastases disease is essential because surgical resection has improved patient survival, especially in cases of colorectal cancer[46,47].

Gadoxetic acid-enhanced liver MRI allows for a vascular dynamic study of the hepatic parenchyma and adds hepatospecific phase for characterization of FLLs[46,48-50]. Lee et al[46] evaluated Gd-EOB-DTPA liver MRI and triple-phase multidetector computed tomography (MDCT) in the detection of suspected hepatic metastases, reporting that dynamic MR images with or without hepatospecific phase show better diagnostic performance than MDCT images. The sensitivity increased significantly with the addition of hepatobiliary phase in gadoxetic acid-enhanced MRI ($P < 0.0001$). In particular, the diagnostic accuracy was greater for small lesions (< 1 cm)[46]. Gadoxetic acid-enhanced liver MRI showed higher capability than enhanced MDCT in detection liver metastases from pancreatic carcinoma. In fact, in a recent work by Motosugi et al[48], higher values of sensitivity for detection of metastases were reported, with values of 85% for MRI and 69% for MDCT.

Acquisition of hepatospecific phase takes some time in a liver MRI protocol because it is generally performed 20 min after contrast administration. Less time would be important, in order to reduce the length of a liver MRI protocol. Diagnostic accuracy for metastases detection and lesion conspicuity was evaluated in hepatospecific images obtained 10 min and 20 min after gadoxetic acid administration[51]. In the study performed by Jeong et al[51], the hepatobiliary phase images obtained at 10 and 20 min after Gd-EOB-DTPA administration improve detection of metastases in comparison with pre-contrast images and dynamic acquisitions only. It has been demonstrated that sensitivity in the detection of metastases does not differ significantly using delay images acquired at 10 min and 20 min after contrast injection. However, in our opinion, the interval time between dynamic acquisitions and 10-min hepatobiliary phase, and between the 10-min and 20-min hepatobiliary phases, could be maintained in a standard liver MRI protocol. In fact, these intervals offer the possibility to acquire other sequences, thus acquiring a more complete liver MRI protocol. Diffusion weighted imaging (DWI) using multiple b values could

require more time for its acquisition. In line with what has previously been reported in literature[52], morphological T2-weighted sequences, including axial breath-hold steady-state free-precession, axial breath-hold single shot spin-echo and axial breath-hold fast spin-echo sequences are acquired after dynamic imaging in our protocol. After these T2-weighted sequences, radiologists may acquire the first hepatospecific phase (10 min after contrast administration). Then, between 10-min and 20-min hepatobiliary phases, DWI could be placed without any considerable influence on imaging quality[52].

Recently in the field of FLL detection and characterization, it has been evaluated whether diagnostic performance of gadoxetic acid-enhanced liver MRI could be enriched by DWI. The contribution of DWI has been widely applied in different radiology fields[53-58]. In detection and characterization of FLLs, diffusion imaging reported higher scores in comparison with conventional T2-weighted sequences. In view of these results, several studies have compared the diagnostic capability of DWI and gadoxetic acid-enhanced liver MRI in detection FLLs. Donati et al[59] found that adding DWI to Gd-EOB-DTPA did not significantly increase diagnostic accuracy compared to Gd-EOB-DTPA imaging alone. Considering the detection of small metastases, Shimada et al[60] reported higher diagnostic accuracy of Gd-EOB-DTPA in comparison to DWI. Probably, both imaging modalities represent very important diagnostic tools in the evaluation of FLLs, as recently described in a study by Macera et al[61]. They found that the combination of DWI with Gd-EOB-DTPA-enhanced MRI imaging significantly increases the diagnostic accuracy sensitivity in patients with colorectal liver metastases treated with pre-operative chemotherapy[61].

CONCLUSION

The topics discussed clearly demonstrate the importance of gadoxetic acid-enhanced liver MRI in the evaluation of FLLs. In fact, it significantly increases diagnostic accuracy in the detection and characterization of FLLs. Furthermore, it allows for the diagnosis of benign solid hepatic lesions such as FNH and HA, thanks to the different contrast uptake observed in hepatobiliary phase.

Some atypical nodules in vascular behaviours could be diagnosed as HCC if they lack Gd-EOB-DTPA retention in the hepatobiliary phase. The HCC guidelines need to underline the recent use of a liver hepatospecific agent. Finally, MR pre-operative assessment using gadoxetic acid allows for higher diagnostic accuracy in the detection of hepatic metastases.

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REVIEW

Differential diagnosis and management of liver tumors in infants

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Abstract

During the first year of life, most of the liver neoplasms are benign in origin, but some of these histologically benign lesions may be challenging in their management. Although most hepatic hemangiomas can be safely observed until involution is documented, some patients will need treatment due to progressive hepatomegaly, hypothyroidism and/or cardiac failure. Large mesenchymal hamartomas may require extensive hepatic resection and an appropriate surgical plan is critical to obtain good results. For malignant neoplasms such as hepatoblastoma, complete surgical resection is the mainstay of curative therapy. The decision about whether to perform an upfront or delayed resection of a primary liver malignant tumor is based on many considerations, including the ease of resection, surgical expertise, tumor histology and stage, and the likely chemosensitivity of the tumor. This article reviews the initial management of the more common hepatic tumors of infancy, focusing on the differential diagnosis and treatment options.

Core tip: Management of liver neoplasms during the first year of life may be challenging. Some of these tumors may be observed but others require extensive surgical resection and adjuvant therapies. Differential diagnosis and treatment options are discussed in our article.

INTRODUCTION

The management of infants with liver tumors may be challenging and it may require a complete work-up because of symptoms or concern about malignancy. Initial evaluation should be focused on patient history, pregnancy evaluation, gestational age at birth, weight and findings on physical exam. Diagnostic imaging modalities may facilitate the identification of benign and malignant liver tumors, however biopsy or resection for histological diagnosis sometimes becomes necessary. Some of these infantile hepatic neoplasms are highly vascularized and surgical interventions are at high risk of bleeding. Certain tumor markers may be helpful in the initial work-up and evaluation of response to therapy. Alpha-fetoprotein (AFP) level may be elevated in children with malignant lesions such as hepatoblastoma and hepatocellular carcinoma, but cautious interpretation is warranted as AFP level is frequently elevated in infants up to 6 mo of age and may be slightly elevated with benign tumors and with hepatic insult or regeneration. Therapy must be tailored according to the nature of the lesion. Observation is recommended for asymptomatic hepatic hemangioma,

	Clinical findings	Laboratory findings	Biopsy findings	Therapy	Outcome
			Table 1 Hepatic tumors characterisctics		
Hepatic hemangioma	Cutaneous hemangiomas	Decreased T3, T4	Glut-1 positive/negative	Observation Propranolol Embolization	Favourable
Focal nodular hyperplasia	Bleeding Torsion	-	Glutamine synthetase	Observation Surgery	Favourable
Mesenchymal hamartoma	Hepatomegaly	-	Vimentin, desmin, a-1 antitrypsin, actin, cytokeratins	Surgery	Favourable
Hepatoblastoma	Hepatomegaly	Elevated AFP	Small cells Embryonal epithelial cells	Chemotherapy Surgery	EFS 30%-90%
Billiary tract rhabdomyosarcoma	Jaundice Hilum of the liver	Cholestasis	Embryonal or botryoid subtype	Chemotherapy Radiation therapy Surgery	EFS 60%-90%
Angiosarcoma	Metastatic disease	-	Glut-1 negative	Chemotherapy Radiation therapy Surgery	Unfavourable
Malignant rhabdoid tumor	Metastatic disease	-	INI1/BAF 47	Chemotherapy Surgery	Unfavourable
Undifferentiated embryonal sarcoma	Right lobe of the liver	-	SMA, a-ACT, desmin, vimentin	Chemotherapy Surgery	Unfavourable
Metastatic hepatic disease from NB	Hepatomegaly	Elevated catecholamines	MYC-N	Chemotherapy Radiation therapy Surgery	EFS 50%-90%

AFP: Alpha-fetoprotein; MYC-N: MYC-N proto-oncogene protein; EFS: Event free survival; SMA: Smooth muscle actin; ACT: Actin; INI1/BAF: INI1/BAF protein; NB: Neuroblastoma.

Figure 1 Cutaneous hemangiomatosis.

whereas complete surgical resection in the mainstay of treatment in hepatoblastoma. Benign primary liver tumors described in infants include hemangioma, focal nodular hyperplasia and mesenchymal hamartomas. Hepatic adenoma is almost exclusively a disease of older children. Malignant lesions include hepatoblastoma, biliary tract rhabdomyosarcoma, angiosarcoma, rhabdoid tumor, undifferentiated embryonal sarcoma and metastatic neuroblastoma (Table 1). The aim of this article is to review the clinical features and management of infants diagnosed with a liver tumor.

BENIGN LIVER TUMORS IN INFANTS

Hepatic hemangioma

Hepatic hemangioma (HH) is the most common benign liver tumor of infancy and it must be differentiated from misnamed hepatic hemangiomas seen in adults, which correspond actually to hepatic venous malformations[1,2]. These adult cases are histologically described as cavernous hemangiomas with large, dilated, blood-filled vessels lined by flattened endothelium, whereas HH are true vascular tumors composed of proliferating endothelial cells. A great variety of pediatric vascular lesions is incorrectly referred to as "hemangiomas" in the medical literature and a significant number of patients receive ineffective and potentially harmful treatment based on misclassification. In 2007, Christison-Lagay *et al*[3] from Vascular Anomalies Center in Boston Children's Hospital postulated three principal categories of HH (focal, multifocal, and diffuse) and a clinical practice algorithm. These lesions share the same patterns of growth, histological findings and involution as their cutaneous counterparts, the infantile hemangioma (IH) and the Rapidly Involuting Congenital Hemangioma (RICH)[4-6]. Focal hemangioma seems to correspond with a RICH, a vascular tumor completed formed at birth with no postnatal growth in which involution is normally observed in the first 12-18 mo after birth. Multifocal and diffuse HH correspond with IH, the most common vascular tumor in children that shows a rapid postnatal growth (0-12 mo) followed by slow involution (1-5 years). It is probable that most HH remain undiagnosed since they are asymptomatic self-limiting lesions, although they often come to clinical attention while screening for visceral hemangioma based on the presence of multiple cutaneous IH (Figure 1), since the liver is the most commonly involved organ[3,7,8]. Some patients may develop a congestive heart failure associated with high-volume vascular shunting and treatment is warranted. Unresponsive patients to therapy may develop a severe cardiac failure with hypothyroidism (IH express type 3

Figure 2 Abdominal computed tomography-contrast. Focal hepatic hemangioma that shows centripetal enhancement and central sparing because of thrombosis, necrosis and/or intralesional hemorrhage.

Figure 3 Abdominal magnetic resonance imaging-contrast. Diffuse hepatic hemangioma that nearly totally replaces the liver.

iodothyronine deiodinase that converts thyroid hormone to its inactive form, resulting in an acquired hypothyroidism), abdominal compartment syndrome, and death[9-12].

Differential diagnosis with malignant liver tumors should be performed and AFP should be included in the initial lab work. Focal HH (Figure 2) shows centripetal enhancement and central sparing because of thrombosis, necrosis, or intralesional hemorrhage on computed tomography (CT) or gadolinium magnetic resonance imaging (MRI). Multifocal HH shows multiple well-defined, spherical lesions with intervening areas of normal hepatic parenchyma, whereas diffuse lesions (Figure 3) nearly totally replace the liver. On CT, lesions are hypodense relative to liver without contrast but enhance centripetally with contrast. Central sparing, thrombosis, or necrosis is not seen in multifocal and diffuse HH. Radiologists who are very specialized in looking at vascular lesions feel comfortable in many cases saying that something is an hemangioma *vs* another tumor based upon its radiographic presentation. Hepatoblastomas tend to be heterogeneous on T2-weighted imaging and angiosarcomas seem to have central enhancement rather than centrifugal enhancement, but if there is any question about the diagnosis, a biopsy is recommended, although this procedure is at high risk of bleeding[13-16].

Most of the diagnosed HH may be observed closely with serial abdominal ultrasonography until involution is documented. If the lesions become symptomatic (hemodynamically significant shunting), medical therapy is firstly recommended. Recently, propranolol has been introduced as an effective treatment for cutaneous IH and several recent cases have been reported showing excellent response of diffuse HH to propranolol, even in patients with associated hypothyroidism. Corticosteroids have been first line treatment of infantile hemangioma, but the use of propranolol is emerging as the treatment of choice for high-risk infantile hemangiomas[17,18]. Other therapeutic options include arterial embolization, hepatic artery ligation, resection, or liver transplantation[3].

Focal nodular hyperplasia

Focal nodular hyperplasia (FNH) of the liver is a rare benign lesion, usually seen in older children rather than infants. Girls are more affected than boys. An asymptomatic incidental finding on a diagnostic study is commonly observed[19,20]. A cumulative incidence is reported in oncologic pediatric patients after completion of therapy and differential diagnosis to other focal hepatic lesions, such as metastasis, is often challenging. Infants with neuroblastoma and metastatic hepatic disease seem to be a specific risk-group for FNH development, especially if they underwent chemotherapy and/or radiation therapy to the liver during treatment[21-23]. Gutweiler *et al*[24] reported a hepatoblastoma case presenting with FNH after treatment of neuroblastoma. FNH should be considered in patients with persistent late imaging changes. Classical CT-contrast picture is a lesion enhanced when compared with normal liver and a central scar that becomes hyperintense owing to concentration of the contrast. Currently, liver ultrasound (US) and MRI are the recommended diagnostic imaging tools for characterizing the lesion and subsequent follow-up. Glutamine synthetase is a nitrogen metabolism enzyme with a distribution in the human liver characterized by its strict pericentrolobular localization[25]. It has emerged as a good marker for identification of resected FNH and for differentiating FNH from all other types of hepatocellular nodules developed on normal liver[26]. Acute abdominal pain may develop owing to torsion or rupture of the lesion with bleeding. Although FNH is a benign lesion that is typically managed conservatively in adults, most children with FNH undergo biopsy or resection because of increasing size, concerning symptoms or inability to rule out malignancy, especially in pediatric cancer survivors[27].

Mesenchymal hamartoma

After hemangiomas, mesenchymal hamartoma of the liver (MHL) is the second commonest benign hepatic tumor in childhood, but these tumors are relatively rare. Most MHLs are large benign multicystic masses that present in the first 2 years of life[28]. Prenatal diagnosis of MHL has been reported, most often in the last trimester of pregnancy and it may be a cause of severe hydrops. An early prenatal diagnosis and a subsequent follow-up could help to establish the best time for delivery. Fetal intervention

Figure 4 Abdominal computed tomography and magnetic resonance imaging. A: Abdominal computed tomography-contrast shows enhancement of the solid component, septate, and the peripheral rim; B: Abdominal magnetic resonance imaging-contrast shows a high signal intensity on T2-weighted magnetic resonance sequences.

may be beneficial in selected cases. If the fetus is becoming hydropic, early delivery or fetal treatment (particularly if the tumor is composed of a few large cysts) should be considered. Most affected fetuses have been successfully delivered vaginally[29].

Postnatal presentation is more common with abdominal distension and/or an upper abdominal mass. Liver function tests are usually normal. AFP is occasionally elevated though not to the degree that occurs in hepatoblastoma. About 75% of MHL occur in the right lobe of the liver. In the newborn, the tumor may expand rapidly and cause life-threatening abdominal distension with respiratory distress[30]. Diagnostic imaging studies demonstrate a multiloculated cystic tumor with a variable amount of solid tissue[31]. This may be seen in undifferentiated embryonal sarcoma of the liver (UESL), but rarely in hepatoblastoma. Intratumor calcification, which can be frequently detected in hepatoblastoma or hepatic hemangioma, has been reported very rarely for a MHL.

Ultrasound demonstrates the presence of thin mobile septate and/or round hyperechoic parietal nodules within the cysts, but rarely containing debris. The hepatic architecture is normal beyond the outer rim of compressed liver. On CT-contrast the solid component, septate, and the peripheral rim may enhance. On MRI, MHL has a low signal intensity on T1-weighted magnetic resonance sequences and a variable signal intensity on T2-weighted sequences (Figure 4)[32]. In most patients, the diagnosis of MHL is suggested by imaging and confirmed by histological examination of the resected specimen. If radiological diagnosis is not clear, a percutaneous or open tumor biopsy can be performed[33].

Although a laparoscopic or open surgical biopsy is considered by some authors, SIOPEL (International Childhood Liver Tumor Study Group of the International Society of Paediatric Oncology) currently recommends image-guided coaxial plugged needle biopsy for liver tumors (obtaining numerous cores)[34]. Fine needle aspiration cytology is of limited value because hepatoblastoma or a malignant mesenchymal tumor is difficult

to exclude. MHL has been considered a focal tumor, but small satellite lesions at the tumor margin have been described, which could explain tumor recurrence after apparent complete resection. Clinical and histological evidence suggest that UESL can develop within a preexisting MHL[28,30]. Both tumors share similar features on gross pathology (cystic and solid components, sometimes pedunculated), histology (mesenchymal elements with benign bile duct epithelial structures), and immunohistochemistry (positive staining for vimentin, desmin, a-1-antitrypsin, actin, cytokeratins). Flow cytometry studies have shown that although most MHLs are diploid, some are aneuploid and cytogenetic studies have demonstrated a balanced translocation involving the same breakpoint on chromosome 19 (band 19q13.4) and chromosome 11. These abnormalities have been found in both, UESL and MHL[28].

The management of MHL remains still controversial. MHL has the potential to involute spontaneously, especially for those tumors with a prominent angiomatous component. Nonoperative management may be appropriate in selected cases (*e.g.*, infants with a biopsy-proven MHL and a prominent vascular component). Percutaneous aspiration or drainage of larger cysts may temporarily control tumor size in life-threatening lesions and it may helpful for the definitive surgical resection. The standard of care is complete resection with the goal of achieving negative margins to avoid the risks of local recurrence and long-term malignant transformation. Enucleation may be adequate in case of very large tumors that replace most of the liver parenchyma. Liver infiltration by MHL is rarely seen and a surgical plain is normally found for resection (Figure 5). Pedunculated lesions are amenable to laparoscopic resection. Marsupialization or partial resection are suboptimal because of the risk of tumor recurrence. Liver transplantation can be considered for unresectable tumors[28,30].

MALIGNANT LIVER TUMORS IN INFANTS

Hepatoblastoma

Hepatoblastoma (HB) is the most common malignant liver tumor in infancy and early childhood, accounting for over 65% of all liver cancer diagnosed in children under 15 years of age. Recent publications indicate that the incidence rates for HB have increased in the last decades[34,35]. Maternal smoking, parental occupation and genetic susceptibility (gene *MPO*, *NQ01*, *SULT*, *IGF-2* and so on) have been associated with HB and recent studies provide support for an increased risk of HB in low (1500-2500 g) and very low (< 1500 g), birth weight infants, in which HB is diagnosed at older ages and in more advanced stages than HB cases of normal birth weight. Neonatal therapies including supplemental oxygen, phototherapy, administration of numerous drugs, total parenteral nutrition and blood transfusions may play a role in the development of HB[36]. An infant with HB usually presents with an abdominal mass often detected by a parent.

Figure 5 Surgical resection of mesenchymal hamartoma of the liver.

Figure 6 Doppler-US of the liver allows to investigate the relation between the tumor and the hepatic vessels. 1: Aorta; 2: Inferior vena cava; 3: Hepatic portal vein; 4: Left portal vein; 5: Right portal vein; 6 Tumor.

Table 2 Pre-treatment evaluation system of tumor extension staging system	
PRETEXT I	One section is involved and three adjoining sections are free
PRETEXT II	One or two sections are involved, but two adjoining sections are free
PRETEXT III	Two or three sections are involved, and no two adjoining sections are free
PRETEXT IV	All four sectors involved, any involvement of caudate lobe indicates a minimum of PRETEXT II

PRETEXT: Pre-treatment evaluation system of tumor extension.

Other frequent presenting findings include anorexia, failure to thrive, abdominal pain, and abdominal distension. Jaundice is rarely seen since the liver function is otherwise normal. The presence of jaundice in a pediatric patient with a liver mass is most commonly seen in biliary rhabdomyosarcoma and undifferentiated sarcoma of the liver. Marked thrombocytosis is a typical finding in the laboratory work of a HB patient due to a paraneoplastic effect related to the tumor production of interleukin-6, a potent growth factor for megakaryocytes. The measurement of the serum AFP level is an useful test in infants with a liver mass and is elevated in at least 70% of children with HB. Moreover, patients with low AFP level at diagnosis (< 100 ng/mL) tend to have a more aggressive biological tumor behaviour and ultimately an unfavourable clinical outcome[37]. AFP is also an extremely useful marker of the tumor response to therapy and in the early detection of tumor recurrence. Attention must be paid to the correction of residual fetal.

AFP in infants under 6 mo of age. Elevation of AFP may also be seen in infants with yolk-sac tumors, sarcomas and hamartomas.

Abdominal ultrasonography-Doppler should be the first imaging modality in an infant with suspicion of a liver tumor and provides information about the origin of the mass, the extention of the lesion, and discerns whether the lesion is solid or cystic and whether it is a solitary or a multifocal tumor. It represents a valuable tool of resectability assessment as it allows to investigate the relation between the tumor and the hepatic vessels (Figure 6). It can be also used intraoperatively. On CT, HB shows heterogenous, low attenuation mass which enhance during arterial phase and hypoattenuates during portal phase. MRI shows HB as hypointense in comparison to normal liver in T1-weighted sequences and hyperintense in T2-weighted sequences, while dynamic imaging with gadolinium shows early enhancement with rapid washout. Chest CT should be performed to investigate pulmonary metastatic disease[34].

The pre-treatment evaluation system of tumor extension was developed by the SIOPEL and aims to define tumor extension before any therapeutic intervention. This system divides the liver into four sectors, an anterior and a posterior sector on the right and a medial and a lateral sector on the left (Table 2). The Children's Oncology Group (COG) adopted a different system based mostly on surgical findings[38-40].

In SIOPEL protocol, a tumor biopsy is required to confirm diagnosis before starting chemotherapy and this does not upstage a patient if a subsequent complete resection is performed. Biopsy can be done with open or laparoscopic surgical technique, but a percutaneous approach ultrasound-guided is preferred. Tumor seeding should be prevented by advancing the needle through a short depth of normal liver tissue (a portion that will be resected at future surgery)[41,42]. COG protocol allows

a primary tumor resection without a biopsy if it seems feasible. Patients with negative margin and microscopic positive margin will receive a less intensive chemotherapy regimen compared with those patients with gross residual disease or initial biopsy only. Patients with negative margin and pure fetal histology are observed and will receive no adjuvant chemotherapy in the COG protocol[43].

For both protocols, surgical resection is the mainstay of curative therapy, but only one-third to one-half of newly diagnosed patients with HB will have resectable disease at diagnosis. The combination of cisplatin-based chemotherapy and surgery has improved survival in patients with unresectable HB by increasing the number of patients whose tumors can be resected. Patients whose tumor may not be resectable even after neoadjuvant chemotherapy should be referred to a liver transplant center[44-48].

Biliary tract rhabdomyosarcoma

Rhabdomyosarcoma (RMS) of the biliary tree is a rare mesenchymal neoplasm that arises as an intraluminal biliary mass or cluster of grape-like masses and it typically presents with features of obstructive jaundice[49]. Median age at presentation is 3 years, but it should be included in the differential diagnosis of an infant presenting with jaundice and a mass in the porta hepatis. Other diagnostic possibilities include undifferentiated sarcoma of the liver, pancreateoblastoma, papillary cystic tumor of the pancreas, metastatic lesions and more rarely, hepatoblastoma[50,51]. Additionally, a RMS in this location can mimic the radiological appearance of a choledochal cyst because of its combined cystic and solid component[52]. Once the radiological diagnosis is performed by US, CT or MRI, an endoscopic retrograde cholangio-pancreatography can be performed to relieve biliary obstruction, visualize the biliary tree and obtain a biopsy[53,54]. This procedure may be challenging in an infant and an open, laparoscopic or needle biopsy may be required to confirm the diagnosis. Outcome in RMS appears to have improved over the last several decades secondary to the tumor chemosensitivity. Multiagent neoadjuvant chemotherapy following the biopsy may avoid important complications associated to a massive primary resection. Even after chemotherapy, gross total resection is rarely possible but outcome is good despite residual disease after second-look surgery[48,51].

Angiosarcoma

Most of the vascular liver neoplasms in infants are benign and correspond to infantile hemangioma (multifocal and diffuse hepatic hemangioma) and rapidly involuting congential hemangioma (focal hepatic hemangioma). If an hepatic hemangioma shows an unusual progression, malignancy should be suspected and a tumor biopsy is warranted. Hepatic angiosarcoma is a rare and high-grade malignant neoplasm that accounts for 2% of liver tumors in children[55-57]. Early metastatic disease to the lungs is commonly seen. Diagnosis may be challenging and an open wedge biopsy may be a good choice to avoid potential bleeding and obtain an accurate histolog-

ical diagnosis. Prognosis is poor, even after multiagent chemotherapy, surgical resection, radiation, and liver transplantation[58,59].

Malignant rhabdoid tumor

Malignant rhabdoid tumor of the liver (MRTL) is a rare and aggressive neoplasm that share clinical features with HB such as male predominance, thrombocytosis, anemia, and only moderate derangement of overall liver function at presentation[60,61]. However, patients at diagnosis are younger compared with HB patients and LDH is typically elevated. Accurate diagnosis of MRTL may be challenging due to extensive tumor necrosis and immunohistochemistry studies for INI1/BAF 47 protein (which is abnormally lost in all rhabdoid tumors) has emerged as an useful tool for diagnosis[62]. For infants with liver tumors and normal AFP level at diagnosis, detailed cytogenetic, immunohistochemical and/ or molecular analysis of INI1/BAF 47 protein may be helpful in distinguishing MRTL from HB[63]. Hepatoblastomas of small cell undifferentiated histology can mimic MRTL but do not have *INI1* mutations[63,64]. Outcomes for patients with MRTL are very poor. Multiagent chemotherapy including vincristine, doxorubicin, cyclophosphamide, ifosfamide and etoposide in combination with complete surgical resection is the mainstay of treatment[65].

Undifferentiated embryonal sarcoma

Undifferentiated embryonal sarcoma of the liver (UESL) is an uncommon malignant hepatic neoplasm that occurs more frequently in older children but has also been described in infancy[66,67]. Malignant transformation from mesenchymal hamartoma or a solitary liver cyst to UESL has been reported. It is generally considered to be a highly invasive malignant tumor with lung, peritoneum and pleura as the typical sites for distant metastasis. The diagnosis may be challenging and relies on postoperative pathology and immunostaining analysis (positive expression of SMA, a-ACT, desmin, vimentin). If the tumor is not suitable to primary resection, a biopsy should be obtained followed by chemoradiation. Survival rates have significantly improved in the last decades and long-term survival cases have been reported[68,69]. In an Italian-German soft tissue sarcoma study[70], 12 of 17 children with UESL achieved remission following treatment with chemoradiation and surgery. Patients whose tumor is not able to be resected or who have postoperative local recurrence of the tumor without distant metastasis may be candidates for liver transplantation.

Metastatic neuroblastoma

Neuroblastoma (NB) is the most common extracranial solid tumor in the pediatric population, accounting for 6%-10% of all childhood cancers and 15% of all cancer related mortalities in children. Common sites for metastasis are bone marrow, bone and liver. Stage 4S or MS represents 5% of NB cases and it is defined as disease with a localized primary adrenal or extra-adrenal tumor

Figure 7 Extensive hepatic infiltration by neuroblastoma. Surgical abdominal decompression by patch placement.

and metastasis restricted to the liver, skin, and bone marrow involvement less than 10%[71,72]. Although, it is associated with survival rates of 70%-97% due to the possibility of regression and spontaneous tumor maturation, some of these young patients may present with extensive and diffuse liver involvement (Figure 7) that can cause respiratory compromise and symptoms of abdominal compartment syndrome with decreased venous return, renal impairment and coagulation disorders secondary to extensive hepatic infiltration. Chemotherapy and liver radiation have been advocated as therapeutical options for those infants who present with progressive disease and life-threatening symptoms[73,74]. Surgical management by abdominal decompression may be necessary in case an abdominal compartment syndrome is present[75,76]. However, the decision of when and how to treat remains controversial.

Our experience

Benign hepatic tumors: we have evolved from corticosteroids treatment to oral propranolol in the last 5 years for the management of symptomatic hepatic hemangiomas. We have observed a more rapid response to propranolol on ultrasound follow-up compared with steroids. Oral propranolol is discontinued until lesion involution is documented which it normally occurs after the first year of age. Patients with asymptomatic lesions have been observed with good results.

Most of our FNH patients underwent incisional biopsy to rule out malignancy. We have observed FNH as a residual lesion of primary vascular anomalies.

In our experience, a surgical plain may lead the resection of MHL with good residual liver parenchyma. All our patients have a normal liver function on follow-up.

Malignant hepatic tumors: at our institution, we follow the SIOP protocol for the management of hepatoblastoma with an initial incisional biopsy at presentation followed by cisplatin-based chemotherapy and surgical resection. Our overall survival is 70% and it does not differ from the results published by other groups.

As for RMS in other locations, our experience in the management of biliary tract RMS has evolved from pri-

mary tumor resection in the last decades to initial biopsy followed by neoadjuvant chemotherapy and non-massive second look surgery for tumor resection and evaluation of tumor response.

We have anecdotal cases of infants with tumors other than hepatoblastoma (angiosarcoma, malignant rhabdoid tumor, undifferentiated embryonal sarcoma) and conclusions are difficult to be drawn.

We have successfully managed liver infiltration by neuroblastoma with standard protocols based on chemotherapy and radiation therapy. We have only performed one surgical abdominal decompression by patch placement in a patient with an abdominal compartment syndrome (Figure 7) who finally died.

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ORIGINAL ARTICLE

CEUS and Fibroscan in non-alcoholic fatty liver disease and non-alcoholic steatohepatitis

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Abstract

AIM: To determine intra-hepatic blood flow and liver stiffness in patients with non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) using contrast-enhanced ultrasound and fibroscan.

METHODS: This prospective study included 15 patients with NAFLD, 17 patients with NASH and 16 healthy controls. In each patient, real-time ultrasound was used to locate the portal vein (PV) and the right liver lobe, and 5 mL of SonoVue® was then injected intravenous in a peripheral vein of the left arm over a 4-s span. Digital recording was performed for 3 min thereafter. The recording was subsequently retrieved to identify an area of interest in the PV area and in the right liver parenchyma (LP) to assess the blood flow by processing the data using dedicated software (Qontrast®, Bracco, Italy). The following parameters were evaluated: percentage of maximal contrast activity (Peak%), time to peak (TTP, s), regional blood volume (RBV, cm^3), regional blood flow (RBF, cm^3/s) and mean transit time (MTT, s). At 24-48 h post-injection, liver stiffness was evaluated using Fibroscan and measured in kPa. The statistical evaluation was performed using Student's *t* test.

RESULTS: In the PV, the Peak%, RBV and RBF were significantly reduced in the NAFLD and NASH patients compared with the controls (Peak%: NAFLD 26.3 ± 6.6, NASH 28.1 ± 7.3 *vs* controls 55.8 ± 9.9, *P* < 0.001; RBV: NAFLD 4202.3 ± 3519.7, NASH 3929.8 ± 1941.3 *vs* controls 7473 ± 3281, *P* < 0.01; RBF: NAFLD 32.5 ± 10.8, NASH 32.7 ± 12.1 *vs* controls 73.1 ± 13.9, *P* < 0.001). The TTP in the PV was longer in both patient groups but reached statistical significance only in the NASH patients compared with the controls (NASH 79.5 ± 37.8 *vs* controls 43.2 ± 30, *P* < 0.01). In the LP, the Peak%, RBV and RBF were significantly reduced in the NAFLD and NASH patients compared with the controls (Peak%: NAFLD 43.2 ± 7.3, NASH 41.7 ± 7.7 *vs* controls 56.6 ± 6.3, *P* < 0.001; RBV: NAFLD 4851.5 ± 2009, NASH 5069.4 ± 2292.5 *vs* controls 6922.9 ± 2461.5, *P* < 0.05; RBF: NAFLD 55.7 ± 10.1, NASH 54.5 ± 12.1 *vs* controls 75.9 ± 10.5, *P* < 0.001). The TTP was longer in both patient groups but did not reach statistical significance. The MTT in both the PV and LP in the NAFLD and NASH patients was not different from that in the controls. Liver stiffness was significantly increased relative to the controls only in the NASH patients (NASH: 6.4 ± 2.2 *vs* controls 4.6 ± 1.5, *P* < 0.05).

CONCLUSION: Blood flow derangement within the liver present not only in NASH but also in NAFLD suggests that a vascular flow alteration precedes liver fibrosis development.

Core tip: The use of contrast-enhanced ultrasound (CEUS) assisted by dedicated software (Qontrast®) in combination with Fibroscan examination could provide a non-invasive tool to evaluate the level of fatty-liver disease. In this study, we found that there were reductions in portal and intra-parenchymal blood flow in patients affected by non-alcoholic fatty liver disease and non-alcoholic steatohepatitis (NASH), whereas liver stiffness was increased only in NASH patients. Qontrast®-assisted CEUS could be used to quantify early changes in intra-parenchymal liver flow before the onset of fibrosis.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver disease worldwide[1,2]. Liver biopsy, which is the gold standard for diagnosing NAFLD is an invasive procedure with potential adverse effects and large inter- and intra-observer variability[3]. NAFLD cannot be diagnosed reliably without clear imaging or biopsy evidence of hepatic steatosis and without excluding excessive alcohol consumption, viral hepatitis and medications. NAFLD is further divided into non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH). NAFL is simple steatosis with no evidence of hepatocellular injury, whereas NASH is steatosis with inflammation, hepatocellular injury and possible fibrosis. NASH can lead to cirrhosis and hepatocellular carcinoma, whereas NAFLD has a very slow, if any, progression to NASH. NAFL and NASH, therefore, can be considered different steps in the same histological disease spectrum[3,4]. The pathogenesis of NAFLD is not completely known[5,6]. The fat accumulation occurring in NAFLD is key to the onset of vascular impairment[7]. The fat accumulation is responsible for liver structural and functional changes, leading to increased hepatic vascular resistance and finally to portal hypertension.

To study blood flow in the liver, pulsed continuous Doppler ultrasound (US) is used as the first-line imaging investigation. Doppler US can evaluate the blood flow in large and small vessels but fails to analyze the flow in the capillaries or sinusoids, where the velocity of the red blood cells is too slow to produce a Doppler signal[8]. Hence, changes in the hepatic microcirculation may be assessed using contrast-enhanced ultrasonography (CEUS) that consists of an intravenously administered suspension of gas-filled microbubbles that remain entirely within the intravascular space, thus acting as a blood pool tracer[9,10]. The obtained data can be processed using a post-processing computational tool (Qontrast®, Esaote, Florence, Italy) that includes a suite of software applica-

Table 1 Study populations

Population characteristics	Controls	NAFLD	NASH
Number	16	15	17
Male/female	8/8	12/3	16/1
Mean age (range)	37 yr	48 yr	45 yr
	(26-69 yr)	(26-75 yr)	(20-74 yr)
AST (mean ± SD)	20.6 ± 4.5	19.3 ± 5[b]	45.2 ± 22.1[d]
ALT (mean ± SD)	24.4 ± 7.0	27.4 ± 8.1[b]	86.4 ± 55.7[d]
GGT (mean ± SD)	18.3 ± 10.1	25.6 ± 20[b]	73.1 ± 43[d]
ALP (mean ± SD)	144.4 ± 45.4	154.1 ± 38.3	176.5 ± 57.4

[b]$P < 0.001$, NAFLD *vs* NASH; [d]$P < 0.001$, NASH *vs* controls. AST: Aspartate aminotransferase: reference range 0-37 (IU/L); ALT: Alanine aminotransferase: reference range 0-40 (IU/L); GGT: Gamma-glutamyl transferase: reference range 7-50 (IU/L); ALP: Alkaline phosphatase: reference range 98-279 (IU/L); NASH: Non-alcoholic steatohepatitis; NAFLD: Non-alcoholic fatty liver disease.

tions for image analysis designed to use alternative representations to extract and present brightness information that is already present in the image.

Liver fibrosis directly affects the mechanical properties of the liver parenchyma, such as stiffness, which indicates tissue resistance to deformation under mechanical stress. A greater stiffness corresponds to a higher tissue resistance to deformation. Liver stiffness can be studied using three physical measurements: two measures based on sonographic techniques, such as Fibroscan[11] and acoustic radiation force impulse[12,13], and one that is MR-based, such as magnetic resonance elastography[14]. Regardless of the specific technique, the measured parameter is correlated with the histological fibrosis stage, and the results can be used to accurately predict moderate to severe fibrosis[10,11,15].

In NASH and NAFLD, it remains unclear whether early changes in intrahepatic blood flow are associated with an early production of fibrous tissue. Therefore, the aim of this study was to evaluate the liver blood flow in the large and small intra-parenchymal vessels and fibrosis using CEUS and Fibroscan in patients with NAFLD and NASH compared with healthy controls.

MATERIALS AND METHODS

Populations

The study population was enrolled from August 2010 to December 2013. All of the participants were Caucasian and underwent physical examinations, laboratory tests for liver function, upper and lower abdominal real-time ultrasonography (RUS) and computed tomography (CT) scan when necessary.

Sixteen healthy controls and 32 patients with US-documented steatosis were recruited (Table 1). Fifteen patients affected by NAFLD as defined according the latest guidelines established by the American Association for the study of liver diseases[3] and 17 patients with NASH defined as having fatty liver on abdominal ultrasound examination and either aspartate aminotransferase or alanine aminotransferase more than 1.5 times the upper normal limit on two occasions during the six months before en-

Figure 1 Example in a healthy control of the split-screen display during the contrast-enhanced ultrasound procedure upon the injection of SonoVue® using a low mechanical index. Left: B-mode frame; Right: Contrast-enhanced ultrasound frame. PV: Portal vein; LP: Liver parenchyma. The B-mode frame shows more detail because of the higher gain.

rollment were included. Exclusion criteria were laboratory data and image studies as assessed with ultrasound or CT scan when necessary, compatible with hepatitis B and C, autoimmune hepatitis, sclerosing cholangitis, Wilson's disease, alpha-1 anti-trypsin deficiency, hemochromatosis and hepatic cirrhosis[16]. Additional exclusion criteria were patients with medical histories of malignancy, previous abdominal or thoracic surgery and history of heart and pulmonary disease that may impair the flow of the contrast bubbles to the liver as well as severe concomitant diseases. Finally, patients with pregnancy and breastfeeding as well as pediatric patients were also excluded.

Contrast-enhanced ultrasound and dedicated software

The patients were examined after fasting for 8 h and after having obtained written informed consent. The CEUS examination was always performed by the same expert operator using RUS with a 3.5-MHz convex probe (MyLab70 XVision, Esaote, Ansaldo, Italy) through a longitudinal intercostal scan, in which the portal vein (PV) and right liver parenchyma (LP) could be easily identified while keeping the patient or subject in the supine position. The US contrast medium (SonoVue®, Bracco Spa, Milan, Italy) consisted of 2.5 μm sulfur hexafluoride-filled microbubbles (hence, they are smaller than red blood cells, which have a diameter of 7 μm) stabilized by a lipid monolayer membrane[9]. The microbubbles can generate a nonlinear harmonic response to a low mechanical index (MI), thus permitting continuous real-time imaging. In our study, we used a signal-processing algorithm installed on the ultrasonographic machine (Contrast-Tuned Imaging™, CnTI™, Esaote, Genoa, Italy) that automatically sets a low MI of 0.06 and holds this value constant during the entire CEUS procedure. These features allow the contrast medium microbubbles to travel through the smallest blood vessels without bursting. SonoVue, a blood-pool contrast agent, has no cellular uptake; thus, it enhances only the US image generated by the blood vessels[9]. In CEUS studies, there are 3 overlapping vascular phases: the arterial phase, which

starts within 20 s after the injection and lasts 30-45 s; the portal venous phase, which usually lasts until 2 min after the injection; and the late phase, which corresponds to the clearance of the US contrast agent from the circulation. The CEUS screen, because of the low gain, shows signals only from intensely reflective structures, which limits the ability to identify the proper scan area. To overcome this problem, a split-screen display was used on the ultrasound machine to show the conventional B-mode image beside the CEUS image (Figure 1). Using contrast-processed data, the blood flow through the small capillaries of the liver interstitial tissue could be measured in terms of the volume and flow.

The procedure started with a 5-mL contrast medium injection (always performed by the same expert nurse) using a 20-gauge (G) needle cannula over a 4-s span into the antecubital vein of the left arm with the patient in the supine position. The line was then flushed with a 5-mL bolus of saline solution, also injected over a 4-s span. During the contrast medium injection, digital recording was started and performed for 3 min; during this operation, the patients were asked to breathe slowly to minimize respiration-related movements. The video recordings were then analyzed by the same trained operator using Qontrast® software (Esaote, Florence, Italy), which performs a parametric analysis of perfusion within a selected set of higher signal intensity frames in the region of interest (ROI). In each patient, we evaluated two ROIs: one in the PV and one in the right LP. To correct for translational movements in the ROI, a Gamma variate (bolus)-corrected parametric curve model was selected. The Qontrast® software was then allowed to process the perfusion in each of the previously determined ROIs, calculate the parameters automatically and plot the measured and calculated curves. The following parameters were generated (Figure 2): Peak%, the maximum signal intensity (SI) reached during SonoVue® bolus transit at time T, where T was the time to peak (TTP, s), the time to reach the maximum SI; regional blood volume (RBV, cm^3), the blood volume in the ROI, proportional to the area under the time intensity curve; mean transit time (MTT, s), the contrast medium mean transit time in the ROI; and regional blood flow (RBF, cm^3/s), the RBV to MTT ratio. The reproducibility of the data obtained by Qontrast® analysis of CEUS was tested according to the method of Ridolfi *et al*[17].

Transient elastography

Transient elastography was performed 24-48 h after CEUS using a Fibroscan device (Echosens, Paris, France). Fibroscan consists of a 5-MHz US transducer probe installed on the axis of a vibrator that generates a 50-Hz vibration (completely painless to the patient) that causes an elastic shear wave to propagate through the skin and subcutaneous tissue and finally to the LP, the stiffness of which is directly related to the velocity of the wave. Fibroscan measures the stiffness of a cylindrical volume 1 cm in diameter, 4 cm in length and 25 to 45 cm from

Figure 2 Example of region of interest selection in the portal vein and Qontrast®-assisted contrast-enhanced ultrasound analysis of portal vein parameters in healthy control. A: Region of interest (ROI) drawn in a region of the portal vein (PV) in a selected set of higher signal intensity frames 1 min and 10 s after SonoVue® injection; B: A gamma variate (bolus)-corrected parametric curve for the translational movement caused by breathing activity. SI (%): Signal intensity; PEAK (%): Maximum signal intensity reached during SonoVue® bolus injection; TTP (s): Time to peak; RBV (cm³): Regional blood volume; RBF (cm³/s): Regional blood flow; MTT (s): Mean transit time.

the skin. Acquisition was performed by the same expert operator through an intercostal scan, in which the probe was placed perpendicular to an area free of large vascular structures. During acquisition, the patient lay in the supine position with the right arm in abduction. Liver stiffness was determined by computing the median value of 10 successful acquisitions in kPa.

Statistical analysis

Continuous variables (laboratory values, SonoVue® data processed by Qontrast® software and elastosonography data) are expressed as group means ± SDs. Age was analyzed as a mean. Comparisons of all gathered data among the groups were tested by a Welch-corrected unpaired *t* test. *P* values were two-tailed, and all *P* values less than 0.05 were considered statistically significant. All statistical relationships were assessed using correlation analysis. The statistical analyses were performed using the GraphPad Prism software, version 3.00 (GraphPad Software, San Diego, California, Unites States).

RESULTS

Qontrast

We could not analyze two PV ROIs in NAFLD patients and three in NASH patients because of poor video recording due to liver steatosis that interfered with the returning echoes to the US probe.

The PV analysis showed a significantly shorter Peak% (Figure 3A) and decreased RBV and RBF (Figure 3C and D) in both the NASH and NAFLD patients compared with the controls. The TTP in the PV was longer in both patient groups but reached significance only in the NASH patients (Figure 3B).

The LP analysis yielded similar results, with Peak% (Figure 4A), RBV and RBF (Figure 4C and D) significantly reduced in both the NASH and NAFLD groups compared with the normal controls. The TTP was longer in both NASH and NAFLD patients compared with the controls but did not reach significance (Figure 4B). The

MTT in both the PV and LP in the NAFLD and NASH patients was similar to that in the controls (Figure 5).

Fibroscan

The values of liver stiffness measured in kPa were found to be significantly greater in the NASH patients compared with the control group (Figure 6).

Adverse effects

CEUS studies were performed successfully in all of the patients and were well tolerated, with no side or adverse effects reported.

DISCUSSION

Our study showed that blood flow, as assessed by Qontrast®-assisted CEUS analysis of the PV and LP, was decreased in patients affected by NAFLD and NASH. We also found that liver stiffness, as assessed by Fibroscan, was increased only in NASH patients.

Based on the data obtained by the Qontrast® analysis of ROIs in the PV and LP, significant reductions in the Peak%, RBV and RBF were found in both groups of patients, whereas a delayed TTP was found only in the PV of the NASH group. Our results suggest the hypothesis that in patients with NAFLD, there is a reduced vascular compliance in the liver due to augmented hepatic vascular resistance to portal blood flow and an increased hepatic vascular tone that starts before the onset of fibrosis. This change was previously demonstrated by Francque *et al*[18] in an experimental animal model; in their study, Wistar rats fed with a methionine- and choline-deficient diet for four weeks developed severe steatosis associated with a significant increase in intrahepatic resistance before the onset of fibrosis and inflammation. These changes involved functional (liver endothelial dysfunction and vasoconstrictor overproduction) and structural (sinusoidal altered microvascular architecture) factors. Another study by Pasarín *et al*[19], performed on rats fed with a cafeteria diet for one month, showed that the impaired response

Figure 3 Contrast-enhanced ultrasound of the portal vein in controls, non-alcoholic fatty liver disease and non-alcoholic steatohepatitis. A: Peak (%); B: TTP (s); C: RBV (cm³); D: RBF (cm³/s); Peak (%): Maximum signal intensity (SI) reached during SonoVue® bolus injection; TTP (s): Time to peak; RBV (cm³): Regional blood volume; RBF (cm³/s): Regional blood flow; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis.

Figure 4 Contrast-enhanced ultrasound of liver parenchyma in controls, non-alcoholic fatty liver disease and non-alcoholic steatohepatitis. A: Peak (%); B: TTP (s); C: RBV (cm³); D: RBF (cm³/s); Peak (%): Maximum signal intensity (SI) reached during SonoVue® bolus injection; TTP (s): Time to peak; RBV (cm³): Regional blood volume; RBF (cm³/s): Regional blood flow; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis.

to endothelial-dependent vasodilation caused endothelial dysfunction, leading to augmented intrahepatic resistance and reduced portal flow. Even in this study, the functional features of intrahepatic vascular changes preceded the

onset of fibrosis and inflammation[19].

Another interesting observation is that our data obtained by Qontrast®-assisted CEUS were similar to those from other studies that analyzed liver blood flow with

Figure 5 Contrast-enhanced ultrasound of the portal vein (A) and liver parenchyma (B) in controls, non-alcoholic fatty liver disease and non-alcoholic steatohepatitis: Mean transit time (s). NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis.

Figure 6 Fibroscan in controls, non-alcoholic fatty liver disease and non-alcoholic steatohepatitis. NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis.

CEUS using SonoVue® as the contrast medium in cirrhotic patients. Lin *et al*[20] studied the flow in the right PV by means of color Doppler and CEUS, and they found that the arrival time of SonoVue® in the right PV was prolonged, whereas the velocity and flow volume were decreased. Similar results were found in another study by Ridolfi *et al*[17], who evaluated liver blood flow in the PV and in the parenchyma by means of CEUS and subsequent analysis by Qontrast® in cirrhotic patients and healthy subjects. They found a reduced Peak% and prolonged TTP and MTT in cirrhotic patients compared with controls. These data suggest that NASH and, more interestingly, NAFLD might be considered precursors of liver cirrhosis due to the presence of similar hemodynamic changes in liver blood flow.

In our patients affected by NASH, the delay in reaching the maximum signal intensity (TTP), only present in the PV, together with the reduction in blood flow in either the PV or LP, could be the consequence of not only intraparenchymal microcirculation variations but also increased liver stiffness. Liver stiffness data in our patients with NAFLD and NASH could be included among those with no fibrosis (NAFLD) or mild fibrosis (NASH) as classified by Wong *et al*[21]. These authors studied a large cohort of patients with hepatic steatosis using Fibroscan and liver histology. They found that patients with no fibrosis or mild fibrosis showed liver stiffness values (kPa) that were consistent with those of our patients with NAFLD (no

fibrosis) and NASH (mild fibrosis), respectively. They also identified some patients with steatosis with liver stiffness values that were much higher than those found in this present study. These patients were classified at histology as having a fibrosis pattern compatible with early cirrhosis, which was an exclusion criterion in our study.

The major limitation of this present study was the small number of patients that were examined; further studies in a much larger population are required to draw definitive conclusions regarding the value of the digital data generated by Qontrast®. However, the differences between the control, NAFLD and NASH groups for the main measure of the analysis (Peak% in the PV and LP) were so large that the statistical power of the study could be considered satisfactory. The absence of liver biopsy data in our study was another limitation, although other authors have found a significant correlation between US and histopathologic data in the evaluation of steatosis[22-24]. The CEUS procedure may be incorrectly applied when the US machine does not meet the criteria of good sensitivity, good tissue suppression and good temporal and spatial resolution as reported in the Guidelines of European Federation of Societies for Ultrasound in Medicine and Biology[25]. In our study, we obtained a good tissue suppression by means of CnTI™, which maintains a low MI throughout the study and avoids microbubble bursting as well as bioeffects in the target organs.

In conclusion, CEUS evaluated by Qontrast® might be able to quantify functional vascular liver changes not otherwise detectable with any other non-invasive procedure and before the development of fibrosis. The combined use of Fibroscan and Qontrast®-assisted CEUS could be helpful in assessing the level of disease and could be potentially useful for monitoring the effects of therapeutic interventions.

COMMENTS

Background

Non-alcoholic fatty liver disease (NAFLD) is among the most common cause of chronic liver disease worldwide. NAFLD is further subdivided into non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH). Whereas NASH is represented by steatosis with inflammation, hepatocellular injury and possible fibrosis, NAFL is a simple steatosis with no evidence of hepatocellular injury.

Fat accumulation within the hepatocytes leads to narrowing and distortion of the sinusoidal lumen, leading to increased hepatic vascular resistance and finally to portal hypertension and fibrosis that are among the stigmata of hepatic cirrhosis. Whether these vascular hemodynamic changes are present in NAFLD and NASH remains unclear. Pulsed continuous Doppler ultrasound (US) is the first-line imaging tool for studying blood flow in the liver and allows for the evaluation of flow in the great hepatic vessels but fails to analyze the flow in the capillaries or sinusoids, where the velocity of the red blood cells is too slow to produce a Doppler signal. Hence, to assess changes in hepatic microcirculation, the authors used US to analyze a Doppler signal generated by an intravenously administered suspension of gas-filled microbubbles (each bubble is one-third the diameter of a red blood cell) stabilized by a lipid monolayer membrane; these features allow these bubbles to remain entirely within the intravascular space, thus acting as a blood pool tracer. The obtained data can be processed with a post-processing computational tool (Qontrast®, Esaote, Firenze, Italy), which allowed them to extrapolate objective and quantitative parameters of microvascular damage in the liver. Liver fibrosis directly affects the mechanical properties of the liver parenchyma and may also contribute to portal hypertension. Liver stiffness can be studied with Fibroscan that consists of measuring the resistance of the liver tissue to the propagation of a US beam within the tissue.

Research frontiers
Considering the increasing prevalence of NAFLD with potentially severe outcomes and the limitations of the actual gold standard (liver biopsy) as a diagnostic procedure, the development of a non-invasive technique that allows for an early assessment of liver damage in terms of the derangement of intrahepatic microcirculation and the development of fibrosis appears to be a stimulating research field. This approach also has therapeutic implications in terms of the development of new drugs and monitoring of their therapeutic effects.

Innovations and breakthroughs
The US contrast medium (SonoVue®, Bracco Spa, Milan, Italy) consisted of 2.5 μm sulfur hexafluoride-filled microbubbles, which are smaller than red blood cells. The microbubbles have no cellular uptake, unlike the contrast media used for computed tomography scan or magnetic resonance, and can travel through the smallest liver blood vessels without bursting. A Doppler signal not otherwise detectable with the standard US machine is therefore generated, and the flow in microvessels can be measured. In addition, the use of a computer program that analyzes the signal intensity within the US image allows for the standardization of data in term of the blood flow and volume. By means of contrast-enhanced ultrasound (CEUS) and computer-assisted determination of flow and volume, it has been possible for the first time to detect a derangement in the microcirculation within the liver parenchyma not only in NASH but also in NAFLD. Fibrosis otherwise appears to be limited only to NASH.

Applications
To non-invasively monitor the development of liver disease and to study the effect of drugs on hepatic micro-circulation and fibrosis.

Terminology
CEUS: contrast-enhanced ultrasound, SonoVue®: microbubbles of 2.5 μm in diameter filled with sulfur hexafluoride that are stabilized by a lipid monolayer membrane. QONTRAST® is a suite of software applications for image analysis designed to extract and present, in alternative representation, brightness information that is already contained within the images. FibroScan® is a sonography-based non-invasive and rapid bedside method for the diagnosis and quantification of hepatic fibrosis (by measuring liver stiffness).

Peer review
This is a clinical study which evaluated the findings of contrast-enhanced US and Fibroscan in patients with NAFLD and control. The authors found some differences of the hepatic hemodynamics and liver stiffness among control NAFL and NASH.

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RETROSPECTIVE STUDY

Clinical outcomes of compensated and decompensated cirrhosis: A long term study

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We analyzed differences in cirrhosis aetiology, time to and mode of decompensation, hepatocellular carcinoma (HCC) occurrence and ultimately patient survival.

RESULTS: Five hundreds and twenty-two patients with median age 67 (range, 29-91) years and average follow up 9 years-10 mo (range, 1-206 mo) were studied. Commonest aetiology was hepatitis C virus (HCV, 41%) followed by alcohol (31%). The median survival time in compensated cirrhotics was 115 mo (95%CI: 95-133), whereas in decompensated patients was 55 mo (95%CI: 36-75). HCV patients survived longer while HBV patients had over twice the risk of death of HCV patients. The median time to decompensation was 65 mo (95%CI: 51-79), with alcoholics having the highest risk (RR = 2.1 *vs* HCV patients). Hepatitis B virus (HBV) patients had the highest risk of HCC, alcoholics the lowest. Leading causes of death: liver failure, hepatorenal syndrome, sepsis and HCC progression.

CONCLUSION: Cirrhosis aetiology and decompensation at presentation were predictors of survival. Alcoholics had the highest decompensation risk, HBV cirrhotics the highest risk of HCC and HCV cirrhotics the highest decompensation-free time.

Core tip: Hepatitis C was the most common cause in our cirrhotics and many hepatitis C virus patients were aged and demonstrated a long, mild course. Alcoholic and non alcoholic steatohepatitis cirrhosis is becoming a significant problem. Ascites was the commonest type of decompensation. Survival in compensated cirrhotics was at least double that of decompensated patients. Variceal bleeding was more frequent in alcoholics; nevertheless it was unexpectedly related to better survival

Abstract

AIM: To study these characteristics and prognostic patterns in a Greek patient population.

METHODS: We analyzed a large cohort of cirrhotic patients referred to the department of Gastroenterology and Hepatology and the outpatient clinics of this tertiary hospital, between 1991 and 2008. We included patients with established cirrhosis, either compensated or decompensated, and further decompensation episodes were registered. A data base was maintained and updated prospectively throughout the study period.

than decompensation with ascites or encephalopathy. This was attributed to the improvements in the management of variceal bleeding together with the importance of abstinence from alcohol after the episode was successfully treated. Hepatocellular carcinoma patients with a history of hepatitis B virus had the highest risk of mortality.

INTRODUCTION

Cirrhosis and its complications represent the end in the spectrum of chronic liver diseases, irrespective of aetiology. The natural history of cirrhosis is classically characterised by an asymptomatic phase termed compensated cirrhosis, followed by the development of complications from portal hypertension and/or liver dysfunction, termed decompensated cirrhosis. The transition has been estimated to occur at a rate of 5%-7% per year. In recent years this process has been proposed as a series of critical steps that if unchecked, culminate in hepatic decompensation[1].

Chronic infection with hepatitis B virus (HBV) and hepatitis C virus (HCV), represents the commonest cause of cirrhosis worldwide[2]. Nevertheless, hepatitis C often has indolent course for a long period of time[3,4] with a median time from infection to cirrhosis of 30 years; several confounding factors have been associated with disease progression[4]. Despite higher risk for decompensation in HCV infection, cirrhosis presents earlier in HBV patients[5]. Longitudinal studies of patients with chronic hepatitis B have shown a 5 year cumulative incidence of developing cirrhosis 8%-20% with a 5 year survival in compensated cirrhosis 85% and 15%-35% in decompensated cirrhosis[6]. Various factors are related with progression in HBV infection, but clearly its course is modified by antiviral therapy and HBV DNA suppression[7,8]. Sustained response to anti-HCV treatment also significantly determines patients' outcome regarding decompensation, liver failure, death or orthotopic liver transplantation and decreases does not completely eliminate the HCC risk[9].

Hepatocellular carcinoma (HCC) is a major complication of viral cirrhosis, both compensated and decompensated, and a major cause of death[2]. HCC incidence appears to be increasing worldwide[10] and several clinico-pathological variables have been identified as predictors for outcome[11,12]. The annual incidence of HBV related HCC ranges from 2%-5%[6]. Two other very common causes of chronic liver disease and subsequent complications are non alcoholic steatohepatitis (NASH) and alco-

holic liver disease, which especially in western countries are becoming significant public health issues[13,14].

The purpose of this study was to evaluate-in a region of southern Greece-a cohort of patients with either compensated or decompensated cirrhosis at presentation; to identify the time to and mode of decompensation, investigate the occurrence of HCC and assess the impact of all the aforementioned on patient survival.

MATERIALS AND METHODS

Study design

The study was performed in a tertiary hospital which is the reference centre for the island of Crete, in the south of Greece. The population of 800000 is largely homogenous. The few non-Greek patients are mostly from Eastern Europe and the Balkans. The study conformed to the principles of declaration of Helsinki and was approved by the ethics committee of the University Hospital of Heraklion. The participants in the study provided verbal consent; for those patients with hepatic encephalopathy, consent was given by relatives. Written informed consent was provided from those undergoing interventional procedures (*i.e.*, liver biopsies, endoscopies and abdominal paracentesis).

We included patients with a diagnosis of cirrhosis who were seen as outpatients in the liver clinic or were hospitalized, mostly for chronic liver disease complications. Starting date of the study was January 1991 and their data were registered in a data base until June 2008. During the long period of this study, many patients that fulfilled the criteria were included and were therefore followed up prospectively. In that sense the study is both prospective and retrospective.

All patients with established cirrhosis were included. Diagnosis was based on liver biopsy (all patients with compensated cirrhosis) and/or clinical evidence of decompensation combined with endoscopic and radiological findings. We excluded from the study (1) paediatric liver disease; (2) patients with primary biliary cirrhosis (PBC); (3) autoimmune hepatitis (AIH) cirrhosis; and (4) 12 patients who did not wish to participate in the study. PBC patients have a discrete clinical course and our experience has been reported elsewhere[15]. AIH patients are few in Crete (less than 20 patients have been diagnosed during the study period) and all but one run a good course under treatment. Thus AIH as a separate group for cirrhosis aetiology was excluded due to small numbers.

The diagnosis of decompensated cirrhosis was based on the presence of any of the following: ascites, variceal bleeding or encephalopathy. The classification as compensated cirrhosis precluded any past history of the above criteria. The diagnosis of liver failure was made when one or more of the following were observed in decompensated cirrhotics: Hepato-renal syndrome type 2/type 1, progressively worsening liver biochemistry with prolongation of international normalized ratio and/or deepening jaundice (frequently due to sepsis), severely worsening encephalopathy, or liver failure in the context

of massive infiltration from tumour.

All patients with HBV related cirrhosis had received standard antivirals, initially lamivudine/adefovir and later either entecavir or tenofovir. Antiviral treatment started at the time of initial diagnosis of chronic HBV infection and continued after the diagnosis of cirrhosis until death or end of follow up.

No patient with decompensated hepatitis C related cirrhosis received antiviral treatment with the standard regimen. The number of compensated HCV cirrhotics in this population on treatment with interferon and ribavirin was too small to draw conclusions. HCV decompensated cirrhotics received only supportive treatment as indicated (diuretics, antibiotics, varices ligation, repeated paracentesis and terlipressin). Approximately half of HCV cirrhotics had no antiviral treatment prior to their cirrhosis diagnosis due to either old age, side effects or unavailability of treatment. In any case antivirals were discontinued on diagnosis of cirrhosis according to the guidelines at a certain time period. A 30% of alcoholics discontinued alcohol consumption.

A careful evaluation was performed to document any episode of decompensation at scheduled outpatient Hepatology clinic visits or at hospitalization for any reason. For patients who had not attended the outpatient clinic for three months after their previous visit, information regarding the outcome was obtained by telephone interviews with patients or relatives.

Liver biopsies were taken using ultrasound guidance and were initially performed with Menghini needles, later substituted by Tru-cut needles; few biopsies were done intraoperatively or transjugularly. All patients with bleeding were scoped within 24 h to diagnose and treat portal hypertensive bleeding. Ascites and encephalopathy were diagnosed according to standard criteria; all patients with ascites on presentation and according to clinical suspicion underwent abdominal paracentesis to check for spontaneous bacterial peritonitis ever since this was internationally accepted practice. Screening for HCC was performed every 6 mo with ultrasound (US) and a-fetoprotein, and during the last 3 years of the study contrast-enhanced US was used. HCC was diagnosed either histologically or according to European association of the study of the liver/American association of the study if the liver criteria ever since these were published[16,17].

Viral hepatitis markers were detected by Abbott Elisa immunoassays and viral load was measured quantitatively using polymerase chain reaction test wherever appropriate since the method was available. Alcohol misuse (defined as exceeding 40 g of ethanol daily in male-20 g daily in female patients) was identified after interviewing the patient during hospitalisation or in the outpatient alcoholic clinic, as well from information provided by social services. The study included patients with a diagnosis of alcoholic cirrhosis who were either active drinkers or were abstainers at evaluation. Three distinct end points were considered: decompensation, death (or liver transplantation) and HCC. Few patients received a transplant due to the late development of liver transplantation services in the country. NASH related cirrhosis and cryptogenic cirrhosis were evaluated as a single group.

Statistical analysis

Univariate comparisons of patient characteristics between the aetiological groups were undertaken using the chi-squared test and one-way ANOVA according to the type of characteristic. Bonferroni post-hoc comparisons were made when the ANOVA comparison was found to be statistically significant.

The median follow-up time was calculated using the reverse Kaplan Meier estimator[18]. Kaplan-Meier estimates of survival curves were constructed for both overall survival and decompensation-free survival. Median survival times were compared using the log-rank test. Both univariate and multivariate Cox's proportional hazards models were used to estimate hazard ratios (relative risks). A significance level of 5% was chosen for all hypothesis tests. SPSS version 17 was used throughout.

RESULTS

A total of 522 patients were included in the study. The majority of these patients had compensated cirrhosis on presentation ($n = 360$, 69%). One hundred and eighty five patients developed decompensation during follow up (35.4% of the entire cohort and 51.3% of the initially compensated cirrhotics) and there were 231 deaths (44%) over the follow-up period. Median follow-up was 9 years 10 mo, and ranged from 1 mo to just over 17 years. There were 183 patients with a minimum follow up of 5 years in the entire cohort.

Seventy eight patients (15%) were lost to follow up. The distribution of cirrhosis causes in those lost to follow up was found to be similar to those remaining in the study ($n = 444$). Leading causes of death were: liver failure which resulted in 55 deaths (23.8%) hepatorenal syndrome ($n = 50$, 21.6%), sepsis ($n = 25$, 10.8%), massive portal hypertensive bleeding ($n = 15$, 6.5%). These were followed by HCC progression, extrahepatic cancer, cardiovascular events, and other causes. In 21 patients (9%) who died the cause was not verified.

Characteristics of the patient cohort are presented in Table 1. Mean patient age was 67 (range 29 to 91) years. The most common cause of cirrhosis was hepatitis C (41%, 215 patients), followed by alcoholic liver disease (31%, 162 patients). The age distribution within each etiologic group is summarized in Figure 1.

The mean age of the alcoholic liver disease (ALD) patients was 62 (SD ± 12) years, of HBV patients 67 (SD ± 10) years, of HBV + ALD patients 56 (SD ± 15) years, of HCV patients 71 (SD ± 9) years, HCV + ALD patients 65 (SD ± 11) years, of NASH/cryptogenic patients 70 (SD ± 13) years. Average patient age differed to a statistically significant extent between groups ($P < 0.0001$). Post-hoc pairwise comparisons indicated that HCV and NASH/cryptogenic patients were older on average than ALD or

Table 1 Patients' cohort characteristics

	n (%)
Number of patients	522
Male	342 (66)
Female	180 (34)
Cirrhosis aetiology	
HCV	180 (34)
HCV/ALD	35 (7)
HBV	67 (13)
HBV/ALD	15 (3)
ALD	162 (31)
NASH/other	63 (12)
HCC	88 (17)
Patients alive	213 (41)
Patients died	231 (44)
Lost to follow up	78 (15)
Initially compensated	358 (69)
Initially decompensated	164 (31)
Decompensated during Follow Up	185 (35)
Initial episode of decompensation	
Ascites	256 (73)
Variceal bleed	37 (11)
Encephalopathy	10 (3)
More than 1	22 (6)
Not known	24 (7)

HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HBV: Hepatitis B virus; ALD: Alcoholic liver disease; NASH: Non alcoholic steatohepatitis.

HBV/ALD patients ($P < 0.0001$ in all cases). HBV/ALD patients were also younger, on average, than patients with HBV alone ($P = 0.035$). There have been only 5 patients with HDV co-infection, three of them with HBV/HDV/HCV with a history of intravenous drug use. Six patients had co infection HBV/HCV. Due to the small number no separate analysis for the viral co infections was performed.

The median survival time of those presenting with compensated cirrhosis was 115 (95%CI: 95-135) mo whereas decompensated patients had a median survival of 55 (95%CI: 36-75) mo. Kaplan-Meier survival curves also indicated a worse overall prognosis for patients presenting with decompensated cirrhosis (Figure 2) ($P < 0.0001$).

Survival was also strongly influenced by cirrhosis aetiology: Kaplan-Meier survival curves are presented according to etiologic group in Figure 3, in which HBV patients (90% e-antigen negative) appear to have the worst overall survival ($P = 0.004$). Using univariate Cox regression analysis, HBV patients were found to have just over twice the risk of death of HCV patients (RR = 2.1, $P < 0.0001$) whilst the NASH/cryptogenic group had a RR of 1.6 ($P = 0.042$) compared to the HCV group.

At presentation, both cirrhosis aetiology and decompensation remained significant predictors of survival (P values 0.007 and < 0.0001 respectively) after adjusting for age ($P = 0.633$) and sex ($P = 0.505$) in a multivariable model. The RR was 2.6 for patients that were decompensated at diagnosis (95%CI: 1.9-3.6) compared to compensated patients. Patients with HBV had RR = 1.8 (95%CI: 1.2-2.7, $P = 0.005$) compared to HCV patients but none of the other groups had a statistically significantly elevated risk compared to HCV patients (all P values > 0.1).

Figure 1 Patients' age in the different etiologies of cirrhosis. HBV: Hepatitis B virus; HCV: Hepatitis C virus.

Figure 2 Survival curves in compensated and decompensated cirrhosis.

Figure 3 Survival curves according to the etiology of cirrhosis. HBV: Hepatitis B virus; HCV: Hepatitis C virus.

The median time to decompensation was 65 mo (95%CI: 51-79 mo) and varied according to aetiology. Kaplan-Meier curves are presented by etiologic group in Figure 4 ($P = 0.003$). The highest median decompensation-free time was seen in the HCV patient group (median 105, 95%CI: 60-150 mo), the lowest in

Figure 4 Risk for decompensation according to the etiology of cirrhosis. HBV: Hepatitis B virus; HCV: Hepatitis C virus.

Figure 5 Survival in relation to the type of decompensation.

the NASH/cryptogenic (median 58, 95%CI: 48-68) and HBV groups (median 57, 95%CI: 35-79 mo). Aetiology remained a statistically significant predictor of risk of decompensation in a multivariable Cox model ($P = 0.026$), adjusting for age ($P = 0.611$) and sex ($P = 0.878$). Patients with alcoholic aetiology had the highest risk of decompensation compared to those with HCV (RR = 2.1, 95%CI: 1.3-3.2).

The most frequent type of decompensation was presentation of ascites (73%, 256 patients) while 6% (22 patients) had more than one complication on the same date, 15 having both ascites and encephalopathy. The latter group of patient had high mortality (64%, 14 patients out of 22, died during follow-up). Variceal bleeding was diagnosed in 37 patients. The leading aetiology in patients with variceal bleeding was ALD (51%, 19 patients), followed by NASH/crypto (27%, 10 patients) and then HCV (19%, 7 patients).

From the Kaplan-Meier curve it appears that patients who decompensated with variceal bleeding had the best overall survival, followed by those decompensating with ascites whilst the worst outcomes were evident in the group presenting with more than one complication (Figure 5). The corresponding log rank test indicated, however that the differences in survival were not statistically significant ($P = 0.354$).

HCC was diagnosed in 10 patients at the time of first presentation, whilst 78 patients developed HCC over the follow-up period. The mean time to the development of HCC in the entire cohort was 164 mo (95%CI: 156-172 mo). The incidence of HCC during the follow-up period was associated with cirrhosis aetiology ($P = 0.003$), even after adjusting for age and sex in a multivariable model ($P = 0.027$); the only pairwise statistically significant comparison was ALD compared to HBV, with ALD patients having an HCC risk of 0.3 times that of those with HBV aetiology (95%CI: 0.15-0.60, $P = 0.001$). In addition, female cirrhotics had an HCC risk 0.38 times that of men (95%CI: 0.20-0.71). Age was not statistically significant (P

$= 0.205$). Cirrhosis aetiology was a borderline statistically significant predictor of survival after the diagnosis of HCC ($P = 0.064$) after adjusting for age ($P = 0.494$) and sex ($P = 0.159$).

DISCUSSION

In this homogenous cohort of patients with extended follow up from a single centre we studied the clinical course of cirrhosis and analyzed it according to the aetiology. The most common aetiology in this southern region of Greece was hepatitis C, in keeping with previous publications from the island and mainland[19,20]. In our cohort alcoholic was the second most common cause of cirrhosis and this cause has displayed an increasing trend over recent years.

Hepatitis C patients were, on average, older followed by the NASH/cryptogenic group, with HBV cirrhotics tending to be diagnosed at a younger age in this study. The older age in the HCV cohort could be expected as HCV infection is asymptomatic in the majority of patients with slow progression over decades, until cirrhosis is established[5]. Alcohol abuse in HCV infected patients is a well recognised negative prognostic factor and explains the younger average age of this group compared to the HCV group. Even in the younger HBV cirrhotics, however, alcohol abuse significantly lowered the age of cirrhosis diagnosis. In our study, age appeared to influence the survival after decompensation while comorbidities like cardiovascular diseases or diabetes had significant effect in those with NASH related cirrhosis.

NASH is an increasingly recognised cause of cirrhosis, delineating a significant percentage of cryptogenic cases[13]. Although the adult obesity epidemic has not been yet evident in Greece, most of the NASH cirrhotics were identified recently and many of these cases were linked to diabetes mellitus. Their relatively small number in our study is obviously due to the usually long period until cirrhosis develops. This picture is expected to change over the next decade. In our department in a sur-

vey of 2000 liver biopsies, NAFLD/NASH comprised 22.5% of the biopsies between the years 2003-2006, as compared to 5% between the years 1990-1995[21].

The majority of patients were diagnosed with compensated cirrhosis, but a considerable number decompensated during follow up. Patients who presented with compensated cirrhosis had a significantly better survival than those presenting with decompensated cirrhosis. A recent interesting study from the United Kingdom showed that survival of cirrhosis is significantly higher in patients diagnosed and followed in an ambulatory setting than those with first diagnosis in the occasion of a hospital admission[22]. In this study aetiology affected prognosis in young patients to a greater extent than in older ones.

In our cohort, those with HCV aetiology remained compensated for a longer period of time on average, with alcoholics having the highest risk for decompensation. These data are similar to those of other studies of Greek cirrhotic patients (as reported by Giannousis *et al*[20]) as well as to those from a cohort of 4537 cirrhotics from a general practice data base in the United Kingdom. In the later study, alcoholic aetiology had higher rate of decompensation compared to others during the first year after diagnosis; nevertheless this difference was not evident following the first year[23].

Ascites was the most common type of presentation in decompensated cirrhosis while patients with multiple presentations (*i.e.*, combination of ascites, variceal bleeding, and encephalopathy) had the worst prognosis. In a study on acute-on-chronic liver failure (ACLF) by Moreau *et al*[24], ascites was a risk factor for development ACLF because it is an independent predictor of kidney failure following bacterial infections. Benvegnù *et al*[2] reported (using a large cohort of viral cirrhosis, mainly HCV related, cirrhosis patients) that the most frequent complication was HCC, followed by ascites which is also the experience published by Sangiovanni *et al*[25], in an elegant natural history study of 214 HCV patients. A recent paper[26] showed that the HCC incidence was significantly higher among HCV patients with varices compared to those without.

In the present study, alcoholics had significantly more episodes of variceal bleeding. Unexpectedly patients who decompensated with variceal bleeding displayed a better survival compared to other presentations of decompensation. This may be attributed to the large number of alcoholics in this group of decompensated patients, in whom abstinence may have effectively influenced the prognosis. Moreover the established approach in variceal bleeding which includes a combination of pharmacologic and early endoscopic therapy may also be responsible for improved survival displayed in these patients[27]. Primary and secondary prophylaxis might also account for the decreased incidence of variceal bleeding observed in the recent years as compared to episodes seen in the first years of the study.

Our cohort's average survival was almost similar in compensated cirrhotics (10 years) and slightly better in decompensated (4.5 years) to the survival reported in the seminal natural history paper by D'Amico *et al*[28]. The somewhat better survival in our decompensated group could be due to our study being more recent (with documented improvements in the medical and endoscopic management of these patients), and also due to the development of alcohol services in our department and the course of HCV patients with the longest survival. Fattovich *et al*[3] in a previous classical study also reported a long survival in a cohort of 384 HCV cirrhotics[25,29]. It should be stressed that survival in HCV cirrhosis was better compared to HBV cirrhotics despite the fact that HCV cirrhotics received only symptomatic and supportive treatment while practically the vast majority HBV patients received antiviral treatment.

The lowest survival rates were found in the HBV group. This might be related to the increased incidence of HCC in this group and to the fact that more than 90% of our HBV patients had HBeAg negative chronic hepatitis. Indeed the incidence of cirrhosis and its subsequent complications are much more frequent in HBeAg negative than in HBeAg positive HBV infected patients, both in Europe and in Asia[7]. Moreover, we have included patients from the first era of the antiviral therapy when treatment for HBV aetiology was not as effective as treatments now available. This, together with the development of lamivudine resistance in a percentage of the HBV patients (data under preparation) as well as with the correlation with HCC all contributed to the uneven outcome of these patients. The poor outcomes for the combined aetiology, HBV plus alcohol group, is no surprise as alcohol can worsen the natural course of viral hepatitis at any time[4,7].

Alcoholic cirrhotics despite their higher decompensation risk had a relatively high overall survival rates and this can again be explained by the fact that a proportion of these patients successfully discontinued or reduced their alcohol intake. Thirty percent of the whole cohort of patients with alcoholic aetiology became abstinent, mostly by attending the alcohol services at the hospital. Similar to our findings, the study by Toshikuni *et al*[30] reported that survival of HCV cirrhotics was similar to survival of alcoholic cirrhotics, with the same risk for decompensation and mortality. A study in Danish patients[31] showed that alcoholic cirrhotics had high prevalence of complications at the time of diagnosis and these were predictors of 1-year mortality. In this series ascites was also the most frequent type of decompensation, while there was also high risk of variceal bleeding or encephalopathy. As in our series, more than one complication was associated with worse prognosis.

HCC development was observed mostly in HCV and HBV cirrhosis, and NASH had the smallest incidence. The risk was highest in HBV cirrhosis and lowest in those with alcoholic aetiology. Similarly, Fattovich *et al*[11] reported that in the absence of HBV or HCV infection, HCC incidence is lower in alcoholic cirrhotics and these data were confirmed by a retrospective study from Japan.

However recent data confirm that heavy alcohol consumption significantly increases the risk of HCC in HBV-related cirrhotics[32].

Survival after HCC development was marginally related to the aetiology in our group of patients in keeping with the data by Trevisani *et al*[12]. However, the development of HCC was a catastrophic event in the natural course of the disease[2,25]. The poor survival of the HCC group was also influenced by the fact that many of these patients were referred from district hospitals after the diagnosis of large tumours, not amenable to radical treatments (resection or transplantation). This, together with a heterogeneous approach to HCC screening amongst the referring hospitals obviously affected both the actual incidence and the outcome. Treatment of these patients has been reported in the randomized trial with sc octreotide[33] or im long acting somatostatin analogues[34]. The remaining few patients underwent chemoembolization and have been reported elsewhere (Samonakis *et al*[34] submitted). Only 3 patients were transplanted due to the recent development of transplant services in the country, where even today there is only one liver transplant centre with a rather limited activity.

The causes of death in this cohort of cirrhotic patients were mostly related to complications of liver disease and/or HCC rather than the presence of comorbidities. This in keeping with most published experience in natural history studies[35]. An exception to this was the NASH-cryptogenic group where death from cardiovascular complications was frequent (data not shown). It is increasingly recognised that cardiovascular diseases may seriously contribute to the mortality of cirrhosis, contrary to previously thoughts that liver cirrhosis is protective for coronary artery disease[36].

This study has several limitations. Due to the original design it has a retrospective and a prospective arm. Moreover some patients were lost to follow up after a successful management of an acute episode, so data on survival or cause of death are missing for this population. We could not provide an analysis in relation to the model end-stage liver disease (MELD) score as it was introduced after 2002. A recent publication[37] showed that aetiology of cirrhosis has an impact on 1-year survival predicted by the MELD score. The study findings are further limited by a long accrual period. Standard survival analysis methods, such as those applied in the present study, are valid under the assumption that the probabilities of death are stable with respect to absolute time.

In conclusion, in this cohort of patients with a long follow up we found that cirrhosis aetiology and decompensation were predictors of survival at presentation. Alcoholics had the highest risk of decompensation and HBV cirrhotics were at the highest risk of developing HCC. On average HCV cirrhotics had the highest decompensation-free time. The improvement in the management of cirrhosis complications, recent advances in the treatment of viral hepatitis and the development of specialized services for alcoholic liver disease could affect the development of complications and ultimately patient survival.

COMMENTS

Background
Cirrhosis and its complications represent the end in the spectrum of chronic liver diseases, irrespective of aetiology. Its natural history is typically characterised by an asymptomatic phase termed compensated cirrhosis, followed by the development of complications from portal hypertension and/or liver dysfunction, termed decompensated cirrhosis. Cirrhotics have diverse presentation and prognosis according to stage, aetiology and geographic region.

Research frontiers
The research objective was to study disease progression in the authors' cohort of compensated and decompensated cirrhotics; to identify the time to and mode of decompensation, to assess the occurrence of hepatocellular carcinoma and the ultimate impact of cirrhosis on patient survival.

Innovations and breakthroughs
Previous studies from various parts of the world have presented local experience. This is the largest study in their country, both with respect to the study population and the follow up period. It reflects various critical issues on the epidemiology, natural history and survival of this large cohort of cirrhotics.

Applications
The study provides insight on the natural course of common causes of liver cirrhosis, denotes the increasing problem of alcoholic liver disease, whereas provides useful information on the importance of aetiology in prognosis.

Terminology
Cirrhosis arises as a result of different mechanisms of liver injury and represents a common denominator to various aetiologies; it represents an increasing cause of liver morbidity and mortality. Chronic infection with hepatitis B and C virus, represent the commonest cause of cirrhosis. Hepatocellular carcinoma is a primary neoplasm that frequently develops on a cirrhotic liver.

Peer review
The article of Samonakis *et al* entitled "Natural history study of compensated and decompensated cirrhosis: a long term single centre study" is a retrospective study aimed to reporting the characteristics and evolution of a wide cohort of cirrhotic patients in Greece. The article is of interest in clinical practice, the methodology is appropriate and the size of the population is big enough to support the conclusions that authors have drawn in this comprehensive work.

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CLINICAL TRIALS STUDY

Patients with multiple synchronous colonic cancer hepatic metastases benefit from enrolment in a "liver first" approach protocol

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Abstract

AIM: To assess a protocol for treating patients with multiple synchronous colonic cancer liver metastases, which are unresectable in one stage.

METHODS: Patients enrolled in the "liver first" protocol presented with colon-only (not rectal) cancer and multiple synchronous hepatic metastases (type II or III). All patients showed good performance status (ECOG PS 0-1) and were treated with curative intent. Complete oncologic staging including positron emission tomography-computed tomography was performed in order to rule out extrahepatic disease. If bowel obstruction was imminent, an intraluminal colonic stent was placed endoscopically. Subsequently, all patients received standardised neo-adjuvant chemotherapy, that is, FOLFOX or XELOX regimens combined with an antiangiogenic agent (bevacizumab or cetuximab). Provided that a response to chemotherapy was observed, patients underwent either one or two hepatectomies with or without portal vein embolization followed by the indicated colectomy. Further chemotherapy was administered after each procedure. Re-staging was performed after each chemotherapeutic treatment. Disease progression at any stage resulted in discontinuation of the protocol and conversion to palliative disease management.

RESULTS: Prospectively recorded data from 11 consecutive patients (8 men) were analysed for this study. Their mean age at the time of their first assessment was 65.7 (SD ± 15.3) years. Six (54.6%) patients presented with type III metastatic disease. The minimum and maximum follow-up periods were 7.3 and 39.6 mo, respectively. The mean overall survival of all patients was 16.5 (95%CI: 10.0-23.2) mo. A colonic stent had to be placed in 5 (45.5%) patients due to the onset of an intraluminal obstruction. Four (36.4%) patients succeeded in completing all planned surgical operations. Their mean overall survival was 27.2 (95%CI: 15.1-39.3) mo and the mean disease-free survival was 7.7 (95%CI: 3.0-12.5) mo. Patients, who were obliged to shift to palliative treatment due to disease progression, had a mean overall survival of 10.5 (95%CI: 8.6-12.4) mo. None of these patients underwent palliative colectomy. No postoperative mortality was recorded.

CONCLUSION: The implementation of a structured "liver first" approach protocol for the treatment of pa-

tients with extensive, liver-limited colon cancer metastatic disease may be beneficial.

Core tip: Complete tumour burden resection remains the only possible curative therapy for liver-limited colon cancer metastatic disease. However, there are different approaches regarding treatment of the primary tumour and its hepatic metastases, if the latter are synchronous and unresectable with one surgical procedure. For this subgroup of patients, a "liver first" approach protocol is introduced in order to assess standardised treatment as well as to prevent overtreatment in cases of undetected extra-hepatic metastatic dissemination or disease progression.

INTRODUCTION

Approximately every second patient who suffers from colorectal cancer (CRC) will at some point be diagnosed with either synchronous or metachronous metastatic disease[1,2]. Liver is the most frequently affected organ. Resection of the complete tumour load has long been accepted as the only therapeutic option that results in improved long-term survival or even cure[3]. During the past decade a significant prolongation of overall survival and an increase in survival rates has been reported. This development is based on the improvement of systemic chemotherapy and introduction of antiangiogenic agents, but also on the utilisation of advanced surgical strategies and equipment[4-6].

Whereas in metachronous resectable disease, the timing of necessary operative procedures seems obvious, various approaches are currently being implemented if resectable (or potentially resectable) hepatic metastases, with no evidence of extrahepatic disease, are detected at the time of the primary tumour diagnosis[7]. The "classic" approach consists of targeting the primary tumour first, followed by chemotherapy and resection of the hepatic metastases[8]. This strategy remains essential, if diagnosis of the disease coincides with an existing acute lower gastrointestinal bleeding or significant bowel obstruction. The "simultaneous" approach includes resection of the primary tumour as well as any hepatic metastases in one stage. This option is often preferred, especially in experienced centres, when a minor hepatectomy is sufficient in clearing the existing tumour load[9]. Finally, the "reverse" strategy has been introduced in recent years[10,11]. In this approach, liver specific procedures such as portal vein embolization and hepatectomies come first, followed by colectomy. All operative procedures take place either after chemotherapy alone or after combination with radiotherapy, when the diagnosis is rectal cancer. The rationale behind this strategy is that patients with multiple hepatic metastases are more likely to become incurable by not timely confronting the extensive liver metastatic disease.

Important criteria for choosing the appropriate therapeutic plan are patient's performance status, primary tumour location, disease extent, available diagnostic and therapeutic tools and methods, as well as the centre's medical and surgical team experience. Due to the complexity of the disease, the patient population is heterogenic. In addition, conclusions regarding best possible management are based on retrospective series of patients suffering from CRC and liver metastases[12]. Therefore, treatment of those patients is routinely based on patient and centre specific ("individually tailored") approaches rather than generally accepted guidelines.

For this study, a subgroup of CRC patients was defined, that is, patients who had been diagnosed with stage IV colonic (not rectal) cancer and presented with multiple, bilobar, synchronous, liver-only metastases, that were either potentially resectable after more than one procedure (type II) or initially unresectable, but possibly resectable after tumour downsizing (type III)[13,14]. These patients were enrolled in a prospective "liver first" approach protocol which included staging, certain oncologic therapy and surgical therapeutic steps. The aim of the study was to assess the implementation of this algorithm, especially in terms of applicability and safety.

MATERIALS AND METHODS

Ethics
This study was conducted in a tertiary care private hospital according to the guidelines of the Declaration of Helsinki of the World Medical Association[15]. The hospital's ethics committee approved the study protocol. Written informed consent was obtained from all patients. Their enrolment was discussed during and approved by the hospital's weekly tumour board. All patients were treated with curative intent.

Definitions
Nomenclature regarding the extent of hepatic resections is that endorsed by the International Hepato-Pancreato-Biliary Association[16]. Decisions on resectability were taken by the hepato-pancreato-biliary surgeons of our centre based on the recommendations made on the Consensus Conferences on the Multidisciplinary Treatment of Colorectal Cancer Metastases[17,18]. Postoperative complications are reported according to the Dindo-Clavien

Figure 1 Algorithm of the "liver first" protocol. PET-CT: Positron emission tomography–computed tomography; PVE: Portal vein embolization; RFA: Radiofrequency ablation; MWA: Microwave ablation.

classification[19].

Patients

Inclusion criteria for patients enrolled in the "liver first" protocol included the diagnosis of colon-only (not rectal) cancer and synchronous, multiple, bilobar, liver metastases (type II or III), age ≥ 18 years, no previous disease-specific therapeutic management and Eastern Cooperative Oncology Group (ECOG) performance status grade 0 or 1. Patients who were diagnosed with extrahepatic disease were excluded.

Study protocol

The protocol was performed within the scope of an intent-to-treat study. Initially, a complete oncologic staging, that is clinical examination, blood tests, liver function tests, tumour marker determination, coloscopy, primary tumour histology, abdominal and thoracic cross-sectional imaging, positron emission tomography-computed tomography (PET-CT), was performed. In the case of an imminent bowel obstruction, an intraluminal colonic stent was placed by endoscopy (Figure 1). All patients then received standardised neo-adjuvant chemotherapy including an antiangiogenic agent. In the case of post-chemotherapy disease response, patients underwent either portal vein embolization, in order to achieve an increase in the future liver remnant, or/and one or two hepatectomies. If indicated, radiofrequency ablation or microwave ablation was performed intraoperatively. In between, (sandwich) chemotherapy was administered. This particular protocol phase was called "liver molding". If the disease remained stable, a PET-CT scan was performed in order to assess the neoplasm's response to chemotherapy. Following the "liver molding" phase, chemotherapy and re-staging was repeated. Only in the case of absence of extrahepatic disease at this stage, patients underwent the indicated colectomy. Adjuvant chemo-

therapy regimens were administered. On the other hand, disease progression at any stage of the protocol resulted in its discontinuation and conversion to palliative disease management.

Chemotherapy

First-line chemotherapy comprised of 5-fluorouracil, leucovorin, and oxaliplatin (FOLFOX4), or capecitabine and oxaliplatin (XELOX) combined with a vascular endothelial growth factor inhibitor (bevacizumab). In second-line chemotherapy, oxaliplatin was replaced by irinotecan and/or bevacizumab was replaced by an epidermal growth factor receptor inhibitor (panitumumab), the latter was administered if patients had non-mutated disease (KRAS wild-type).

Statistical analysis

Continuous and categorical variables were recorded and analysed with descriptive statistics. Survival analysis was performed by the use of Kaplan-Meier curves. Statistical analysis was performed by means of the IBM SPSS Statistics Package, version 19.9 (SPSS Inc., Chicago, IL, United States).

RESULTS

For this study, prospectively collected data were analysed. Between July, 2010 and October, 2011 eleven consecutive patients (eight men) who met the inclusion criteria were enrolled in the "liver first" protocol. Demographic and clinical characteristics at the time of their first assessment are displayed in Table 1. Patients' mean age was 65.7 (SD ± 15.3) years. Seven patients (63.6%) presented with the primary tumour located in the sigmoid colon. Five patients (45.5%) presented with type II metastatic disease. Six patients (54.6%) presented with type III metastatic disease. The number of hepatic metastases

Table 1 Patients' first assessment demographic and clinical characteristics

Patients	Gender	Age (yr)	Primary colonic tumour location	Metastatic type (liver-limited)	Colonic obstruction > stent placement
1	Male	67	Sigmoid	II	-
2	Male	75	Sigmoid	II	-
3	Female	37	Sigmoid	III	-
4	Male	79	Sigmoid	III	√
5	Male	79	Descending	III	√
6	Male	40	Sigmoid	II	√
7	Female	75	Sigmoid	II	-
8	Male	59	Descending	III	-
9	Female	78	Descending	III	√
10	Male	59	Sigmoid	III	√
11	Male	75	Ascending	II	-

ranged between seven and more than thirty, while their size ranged between 2 cm and 16 cm. A colonic stent was placed in five patients (45.5%) before the start of neo-adjuvant therapy due to an imminent intraluminal obstruction. Four patients (36.4%), all presenting with type II metastatic disease at the time of first assessment, completed all scheduled surgical procedures and correspondingly the entire protocol. They underwent two or three operations (mean: 2.75), including the indicated colectomy as the last operative step. Pathology confirmed negative margins (R0) of all resected specimens. One out of five "type II" patients (20.0%) suffered disease progression before reaching the time point of the planned hepatectomy. In only one out of six "type III" patients (16.7%) the neoplasm was able to be converted to "type II" following neo-adjuvant chemotherapy. No palliative colectomy was necessary for the seven patients who had to be allocated to palliative therapy due to disease progression (Table 2).

The minimum and maximum follow-up periods were 7.3 mo and 39.6 mo, respectively. The mean overall survival of all patients was 16.5 (95%CI: 10.0-23.2) mo. Patients who were able to complete the "liver first" protocol had a mean disease-free survival of 7.7 (95%CI: 3.0-12.5) mo and a mean overall survival of 27.2 (95%CI: 15.1-39.3) mo. On the contrary, patients, who were obliged to shift to palliative treatment due to disease progression during the period of their enrolment did not became free of disease at any time point and had a mean overall survival of 10.5 (95%CI: 8.6-12.4) mo (Table 2).

With regard to severe complications associated with chemotherapy, one patient suffered from upper gastrointestinal bleeding after receiving the FOLFOX and bevacizumab regimen. Two severe postoperative complications (Grade III) were documented. One patient suffered an anastomotic site bleeding following sigmoidectomy, which was confirmed and treated by endoscopy and blood transfusions, and one patient suffered a bile leakage following hepatectomy, requiring percutaneous drainage. Furthermore, no postoperative (90-d) mortality was recorded.

DISCUSSION

Patients presenting with metastatic CRC represent a large, but significantly heterogeneous population as distinctions can be made based on primary tumour location, extension of metastatic spread and diagnosis time point of metastases (synchronous *vs* metachronous). Currently, complete neoplasm resection is regarded as the only curative therapeutic option for those patients[20]. Despite broadening resectability criteria in recent years, only a selected group (20%-30%) will be candidates for curative resection[21]. Historically, the first step of implementing therapeutic treatment was to resect the primary colorectal tumour and subsequently target hepatic metastases ("classic" approach). Due to improvements in both chemotherapy and surgical techniques, simultaneous resection of primary and liver-limited secondary disease ("combined" approach) or the prioritised resection of liver metastases ("reverse" approach) are being performed in experienced centres[22,23].

For this study, we selected a patient cohort as homogenous as possible. To be more specific, we included patients with synchronous liver-only metastatic disease that was diagnosed at the same time as the primary tumour and was either resectable in more than one stage or potentially resectable after successful downsizing. We excluded patients with rectal cancer because of the "interference" of radiotherapy treatment phases with the specific protocol steps. We also excluded patients who had to be treated with the "classic" approach, for example patients with ileus secondary to complete bowel obstruction. In addition, patients who could be treated with the "combined" approach, for example due to the presence of a solitary liver metastasis, were also excluded. Finally, we excluded patients with potentially resectable extrahepatic neoplasm dissemination.

In theory, the proposed "liver first" protocol may take advantage of the fact that neo-adjuvant chemotherapy in CRC patients provides an assessment of tumour biology[24]. Its effectiveness influences future therapeutic strategies because it may downsize the existing tumour load, so that initially unresectable metastases may become resectable[25]. Adding biological agents reportedly increases oncologic response and resectability rate[26]. On the other hand, this approach helps to avoid unnecessary operative procedures, and thus potential complications and delay in chemotherapy administration in patients whose neoplasm's biology is not favourable.

Upfront colectomy in the treatment of CRC with synchronous hepatic metastases in the context of the curative or even palliative setting became controversial the last few years. Even though some authors conclude that upfront colectomy is beneficial in terms of overall survival, this standpoint has been challenged because the rate of primary-related complications seems low, even when using modern antiangiogenic therapy[27-30]. In our small cohort of patients, we did not encounter any primary-related complications. Whenever a bowel obstruction

Patients	Metastatic type (liver-limited)	Hepatectomy 1	Hepatectomy 2	Colectomy	Disease-free Survival period (mo)	Overall Survival period (mo)
1	II	RE and wedge and RFA-MWA	-	√	8.23	39.57
2	II	Right	Left lateral and RFA-MWA	√	2.20	14.17
3	III	-	-	-	-	13.97
4	III	-	-	-	-	7.33
5	III	-	-	-	-	13.37
6	II	Laparoscopic left lateral	Right	√	15.27	39.17
7	II	Left lateral	Right	√	5.27	15.57
8	III	-	-	-	-	9.43
9	III	Laparoscopic left lateral	-	-	-	7.80
10	III	-	-	-	-	11.5
11	II	-	-	-	-	10.1

Table 2 Patients' operative treatment and oncologic characteristics

RE: Right extended; RFA: Radiofrequency ablation; MWA: Microwave ablation.

was imminent, a stent placement prevented acute surgery and enabled the protocol enrolment for each patient. In fact, one of five patients who received a colonic stent completed all planned operations and thus, the stent was resected with the colectomy specimen.

In spite of meticulous and repeated staging, three out of four patients (75.0%), who completed the "liver first" protocol and became disease-free, were finally diagnosed with recurrence (mean disease-free survival of 7.7 mo). This trend coincides with several large retrospective series[31,32]. A recent study suggests that pathologic characteristics of the primary colorectal tumour are more prognostic than relevant metastatic features[33].

A significant limitation of this study is the absence of a control group with matched diagnosis for comparing the "reverse" with the "classic" approach. Another important limitation is that the number of patients enrolled in the applied protocol is small.

The main goal of this work was to examine the feasibility and safety of realising a prospective "liver first" approach protocol-to our knowledge, it is the first one - for patients with liver-limited metastatic colon cancer. It focuses on a specific subgroup, namely patients with synchronous, multiple, bilobar hepatic metastases that are resectable after several interventions or disease downsizing. Treatment for these patients is usually "individually tailored" since the criterion of metastatic load resectability and the availability of therapeutic options may differ significantly among medical teams. Even though the number of patients is low, a noticeable trend can be observed, that is, patients who showed disease progression during the various steps of this algorithm had a worse outcome than those patients who succeeded in completing the protocol and became disease free, even for a short period of time. Furthermore, patients with disease progression avoided at least one operation (colectomy) without developing primary-related complications that needed surgical intervention.

In conclusion, the implementation of a structured "liver first" approach protocol for the treatment of patients with extensive, liver-limited colon cancer metastatic disease is feasible, safe, and may be beneficial. The appli- cation of such a protocol requires strict multidisciplinary decision-making process and therapeutic management.

COMMENTS

Background
Liver-limited colon cancer metastatic disease is a common entity in oncological and surgical practice. Complete tumour burden resection combined with systemic chemotherapy currently constitutes the only possible curative therapy.

Research frontiers
No consensus has yet been reached concerning both the timing and the sequence of primary tumour and synchronous, multiple hepatic metastases resection in case this cannot be achieved in one stage ("simultaneous" approach). Depending on the patient's clinical situation and the existing medical expertise, the primary tumour is either targeted upfront ("classic" approach) or subsequent to one or more liver resections ("reverse" or "liver first" approach).

Research frontiers
For this subgroup of patients, a structured "liver first" approach protocol has been introduced and implemented in order to assess standardised treatment as well as to prevent overtreatment in cases of undetected extra-hepatic metastatic dissemination or disease progression.

Applications
This study suggests that, regarding the treatment of patients with multiple synchronous colonic cancer liver metastases, which are unresectable in one stage, the application of a "liver first" approach protocol, which is based on a strict multidisciplinary decision-making process and therapeutic management is feasible, safe and potentially beneficial.

Terminology
A synchronous colorectal cancer metastasis is usually defined as metastatic neoplasmatic tissue that is detected either concurrently with diagnosis of the primary tumour or three to twelve months after the diagnosis. With respect to the described treatment protocol, a synchronous colorectal cancer metastasis was defined as metastatic neoplasmatic tissue which was diagnosed at the same time as the primary tumour. In contrast, metachronous metastases were identified at a later stage.

Peer review
The present manuscript deals with a novel and very interesting approach protocol to treat patients with colon cancer and hepatic metastasis.

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CLINICAL TRIALS STUDY

Pegylated interferon alfa-2b plus ribavirin for treatment of chronic hepatitis C

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Abstract

AIM: To study the safety and efficacy of pegylated interferon alfa-2b, indigenously developed in India, plus ribavirin in treatment of hepatitis C virus (HCV).

METHODS: One-hundred HCV patients were enrolled in an open-label, multicenter trial. Patients were treated with pegylated interferon alfa-2b 1.5 μg/kg per week subcutaneously plus oral ribavirin 800 mg/d for patients with genotypes 2 and 3 for 24 wk. The same dose of peginterferon plus weight-based ribavirin (800 mg/d for ≤ 65 kg; 1000 mg/d for > 65-85 kg; 1200 mg/d for > 85-105 kg; 1400 mg/d for > 105 kg body weight) was administered for 48 wk for patients with genotypes 1 and 4. Serological and biochemical responses of patients were assessed.

RESULTS: Eighty-two patients (35 in genotypes 1 and 4 and 47 in 2 and 3), completed the study. In genotype 1, 25.9% of patients achieved rapid virologic response (RVR): while the figures were 74.1% for early virologic response (EVR) and 44.4% for sustained virologic response (SVR). For genotypes 2 and 3, all patients bar one belonged to genotype 3, and of those, 71.4%, 87.5%, and 64.3% achieved RVR, EVR, and SVR, respectively. In genotype 4, 58.8%, 88.2%, and 52.9% of patients achieved RVR, EVR, and SVR, respectively. The majority of patients attained normal levels of alanine aminotransferase by 4-12 wk of therapy. Most patients showed a good tolerance for the treatment, although mild-to-moderate adverse events were exhibited; only two patients discontinued the study medication due to serious adverse events (SAEs). Eleven SAEs were observed in nine patients; however, only four SAEs were related to study medication.

CONCLUSION: Peginterferon alfa-2b, which was developed in India, in combination with ribavirin, is a safe and effective drug in the treatment of HCV.

Key words: Hepatitis C virus; Genotype; Peginterferon alfa-2b; Ribavirin; Treatment

Core tip: In a multicenter study, the safety and efficacy of pegylated interferon alfa-2b, indigenously developed

in India, plus ribavirin was evaluated on 100 hepatitis C virus (HCV) patients with genotypes 1, 2, 3, and 4. Eighty-two patients completed the study. Most patients had mild-to-moderate adverse events, although 11 serious adverse events were reported in 9 patients. However, only 4 of these were related to study medication. The percentage of serologic response (rapid virologic response, early virologic response, and sustained virologic response rates) of patients was similar to that reported in published studies. In conclusion, peginterferon alfa-2b, developed in India, is a safe and cost-effective drug in the treatment of Indian patients with HCV infection.

INTRODUCTION

According to the World Health Organization's estimates, over 170 million people (3% of the world's population) are infected with chronic hepatitis C virus (HCV) worldwide[1]. Each year, about five million people are newly infected, and more than 350000 people, despite availability of treatment, die from HCV-related complications[2]. Hepatitis is an emerging infection in India, with a paucity of large scale prevalence studies on hepatitis C in the general population. The reported prevalence rates also vary widely (range 0.09% to 7.89%)[3]. However, regardless of prevalence rates, the burden of HCV infection in India is expected to be high with a population over 1.2 billion; as a result, its treatment modalities, as well as success rates, demand attention.

The HCV genotype plays a significant role in therapeutic guidelines, since HCV genotypes 1 and 4 are more resistant to treatment compared to HCV genotypes 2 and 3. Yet, irrespective of genotype, pegylated interferon, in combination with ribavirin, is considered the gold standard in the treatment of chronic HCV infection[4-7]. Currently, both pegylated interferon alfa-2a and pegylated alfa-2b are available in India. These drugs are exorbitantly priced and are not easily accessible to the majority of Indian patients. In view of this, Virchow Biotech developed pegylated interferon alfa-2b from *Escherichia coli* by using recombinant DNA technology, and priced it competitively. The aim of the present study is to evaluate the safety and efficacy of pegylated interferon alfa-2b in chronic hepatitis C patients.

MATERIALS AND METHODS

Patient selection

Male and female patients aged 18-65 years-old (both

years inclusive) that attended the outpatient department of 12 hospitals were screened. 100 consecutive patients were enrolled if they had chronic hepatitis C infection as per the following criteria: presence of HCV RNA and persistent elevation of serum alanine aminotransferase (ALT) levels 1.5 times greater than normal (N < 40 IU/L); compensated liver disease at the time of baseline visit as defined by Child-Pugh class A; hemoglobin $\geq$ 9 g/dL (females), $\geq$ 10 g/dL (males); platelet count $\geq$ 75 × 10^9/ L; neutrophil count $\geq$ 1.5 × 10^9/L; and thyroid stimulating hormone within normal limits (0.35-5.50 mIU/mL). Only treatment näive patients were included in the study. Patients were excluded if they had evidence of other liver diseases such as hepatitis A virus, hepatitis B virus, alfa-2 antitrypsin deficiency, Wilson's disease, primary biliary cirrhosis, autoimmune liver disease, or hemochromatosis. Other criteria for exclusion were: chronic alcoholism; history of drug abuse; immune suppression associated with organ transplantation; history of hypersensitivity to interferon or its diluents; significant psychiatric disease, especially depression; severe cardiovascular disease; patients with co-infection of human immunodeficiency virus infection; and pregnant and lactating women. Study procedures were explained to each participant and written informed consent was obtained before enrolment into the study.

Study design

This is an open-label, multicenter study that was conducted, with the approval of the Drugs Controller General of India, at 12 centers across eight Indian cities between March 2010 and March 2013. The study, conducted in accordance with principles under the 1964 Declaration of Helsinki and later revisions, was initiated after obtaining approval of the study protocol from the institutional ethical committee at respective centers. This trial was registered in Clinical Trial Registry India (CTRI/2011/000028).

Treatment regimen

Treatment consisted of the administration of peginterferon alfa-2b (manufactured by Virchow Biotech Private Ltd, Hyderabad, India) 1.5 μg/kg per week subcutaneously, in combination with ribavirin 800 mg/d orally, for patients with genotypes 2 and 3 for 24 wk. The same dose of peginterferon was administered in combination with weight-based ribavirin (800 mg/d for $\leq$ 65 kg; 1000 mg/d for > 65-85 kg; 1200 mg/d for > 85-105 kg; 1400 mg/d for > 105 kg body weight) for 48 wk for patients with genotypes 1 and 4.

Dose modification/discontinuation

Ribavirin dose was reduced to half if hemoglobin level was < 10 g/dL; treatment was discontinued if hemoglobin level was < 8.5 g/dL. Peginterferon dose was reduced to half in patients with white blood cells (WBC) < 1.5 × 10^9/L, neutrophils < 0.75 × 10^9/L, or platelet count < 50 × 10^9/L. Peginterferon treatment was discontinued in

Table 1 Baseline characteristics of patients with genotypes 1, 3 and 4			
Parameter	Genotype 1 (*n* = 27)	Genotype 3 (*n* = 56)[1]	Genotype 4 (*n* = 17)
Age (yr)	41.9 ± 13.2	41.7 ± 10.9	46.3 ± 9.3
Weight (kg)	60.5 ± 12.0	63.3 ± 11.5	63.7 ± 10.8
Male number (%)[2]	19 (70.3%)	11 (19.7%)	11 (64.7%)
Hemoglobin (g/dL)	14.1 ± 1.6	13.8 ± 1.9	14.2 ± 1.2
White blood cell count (10^9/L)	6682 ± 1682	7086 ± 1886	7201 ± 1886
Neutrophils (%)	58.4 ± 8.4	56.0 ± 11.8	53.3 ± 8.0
Platelet count (10^3/L)	200 ± 80	199 ± 78	170 ± 50
Alanine Aminotransferase (U/L)	88.1 ± 41	127.7 ± 87.4	104.9 ± 61.1
HCV RNA $\log_{10}$ IU/mL	5.5 ± 1.2	5.4 ± 1.1	5.5 ± 0.9

[1]Includes one patient with genotype 2; [2]Value in percentage. HCV: Hepatitis C virus.

patients with WBC $< 1.0 \times 10^9$/L, neutrophils $< 0.5 \times 10^9$/L, or platelet count $< 25 \times 10^9$/L.

Assessment of efficacy
The primary efficacy endpoint was the percentage of patients with sustained virologic response (SVR), defined as undetectable serum HCV RNA 24 wk after cessation of therapy. Secondary efficacy endpoints were: rapid virologic response (RVR), defined as undetectable serum HCV RNA at week 4; early virologic response (EVR), defined as undetectable serum HCV RNA or 2-$\log_{10}$ reduction in HCV RNA from the baseline at week 12; end of treatment virologic response (ETVR), defined as undetectable serum HCV RNA at weeks 24 and 48[1]; with normalization of ALT at weeks 12, 24, 48[1], and 24 after cessation of therapy ([1]only for patients with genotypes 1 and 4). Data on non-responders, relapse, and breakthrough were also collected[4]. Non-responders were defined as those who failed to clear HCV RNA from serum after 24 wk of therapy. Relapse was defined as undetectable HCV RNA at the end of treatment, followed by the reappearance of HCV RNA during follow-up. Breakthrough was defined as undetectable HCV RNA during treatment, followed by the appearance of HCV RNA, despite continued treatment.

Blood samples were obtained for serologic tests for quantitative HCV RNA by polymerase chain reaction (PCR) at baseline and at weeks 4, 12, 24, and 48 for genotypes 2 and 3; while for genotypes 1 and 4 this was at baseline and at weeks 4, 12, 24, 48, and 72. Cobas Taqman HCV test (Roche), using the real-time PCR method with a lower detection limit of < 25 IU/mL, was employed for quantification of HCV RNA in serum. A linear array detection kit from Roche was used in HCV genotyping.

Assessment of safety
Vitals (respiratory rate, pulse rate, body temperature, and blood pressure), hematology (complete blood picture, hemoglobin, and platelet count), and ALT levels were measured at each visit. Biochemical parameters (serum lactate dehydrogenase, creatinine, potassium, and phosphorus) were also measured at specified screening visits; weeks 4, 12, 24, and 48 for genotypes 2 and 3, and weeks 4, 12, 24, 48, and 72 for genotypes 1 and 4. Patients were monitored for adverse events (AE) and medication compliance throughout the duration of study. Adverse events were graded as mild, moderate, or severe. Treatment was suspended or modified according to the severity of adverse events. The dosage of peginterferon alfa-2b, ribavirin, or combination of the two was again increased to the original level after the resolution of adverse events. Serious adverse events (SAEs) were documented and communicated to the institutional ethics committee and Drugs Controller General of India.

Sample size
Various trials conducted on patients with genotypes 1 and 4 or 2 and 3 have reported around 40%-80% SVR, which reflects the efficacy of peginterferon alfa-2b in the treatment of HCV[6,7]. In our earlier pilot study conducted on 25 patients with HCV infection, a SVR of 60% was observed. Considering the 60% efficacy, 95%CI, 80% power, and 15% error with a 15% dropout rate with two tailed *t*-test, the calculated sample size was 100 patients.

Statistical analysis
Values were expressed as mean (SD). Since an open-label study design was adopted, efficacy assessment basically relied upon descriptive statistics rather than inferential analysis. Intention-to-treat (ITT) analysis was carried out on the population that included all patients who met the eligibility criteria and had received at least one dose of the investigational drug during the study period. Per protocol analysis was also carried out, which included patients who completed the stipulated study period.

Safety parameters, such as vital signs and laboratory findings including hematology and biochemical parameters, were analyzed by repeated measure analysis of variance. Two-sided *P*-values were reported, with those less than 0.05 being considered statistically significant. All analyses were performed using IBM SPSS version 19.0 for Windows.

RESULTS

Patients' characteristics
A total of 100 consecutive patients with chronic hepatitis C who met the inclusion/exclusion criteria were enrolled into the study. Among them, 27 pertained to genotype 1, 17 for genotype 4, only one for genotype 2, and 55 for genotype 3. Since there was only one patient with genotype 2, the results presented on genotypes 2 and 3 basically represent only those of genotype 3. The demographic and baseline characteristics of the 100 enrolled patients are presented in Table 1. At baseline, values of hematological and biochemical investigations were within normal limits except for liver function tests such as serum ALT, aspartate aminotransferase, and alkaline

Figure 1 Disposition of patients. [1]Includes one patient with genotype 2.

Figure 2 Mean alanine aminotransferase levels during the study period in patients with different genotypes. [1]Includes one patient with genotype 2. ALT: Alanine aminotransferase.

Table 2 Percentage of patients who responded in terms of rapid virologic response, early virologic response, end of the treatment virologic response, and sustained virologic response in genotypes 1, 3 and 4 by intention-to-treat analysis

Parameter	Genotype 1 (n = 27)	Genotype 3[1] (n = 56)	Genotype 4 (n = 17)
RVR	25.9%	71.4%	58.8%
EVR	74.1%	87.5%	88.2%
ETVR	59.2%	78.6%	70.5%
SVR	44.4%	64.3%	52.9%

[1]Includes one patient with genotype 2. RVR: Rapid virologic response; EVR: Early virologic response; SVR: Sustained virologic response; ETVR: End of treatment virologic response.

Table 3 Percentage of patients who responded in terms of rapid virologic response, early virologic response, end of treatment virologic response, and sustained virologic response in genotypes 1, 3 and 4 by per protocol analysis

Parameter	Genotype 1 (n/N)	Genotype 3[1] (n/N)	Genotype 4 (n/N)
RVR	25.9% (7/27)	74.1% (40/54)	58.8% (10/17)
EVR	74.1% (20/27)	100% (49/49)	88.2% (15/17)
ETVR	84.2% (16/19)	89.8% (44/49)	75.0% (12/16)
SVR	60.0% (12/20)	76.6% (36/47)	60.0% (9/15)

[1]Includes one patient with genotype 2; *n*: Number of responding patients; N: Total number of patients studied; RVR: Rapid virologic response; EVR: Early virologic response; SVR: Sustained virologic response; ETVR: End of treatment virologic response.

Table 4 Percentage of patients with normalization of alanine aminotransferase levels during different study periods *n* (%)

Weeks	Genotype 1 (n = 27)	Genotype 3[1] (n = 56)	Genotype 4 (n = 17)
4	16 (59.2)	27 (48.2)	8 (47.0)
12	17 (62.9)	29 (51.7)	8 (47.0)
24	17 (62.9)	35 (62.5)	9 (52.9)
48	17 (62.9)	40 (71.4)	11 (64.7)
72	17 (62.9)	-	12 (70.6)

[1]Includes one patient with genotype 2.

phosphatase. Barring serum ALT levels, other demographic, hematological, and biochemical parameters, including HCV RNA levels, were not significantly different between genotypes 1, 3, and 4. The mean ALT levels in genotype 3 patients were significantly higher ($P < 0.02$) than those in genotype 1; but these were similar to those of genotype 4.

Figure 1 shows the flow of patients through the study. Among the 100 patients, 82 completed the study. Eighteen patients did not complete the study for the following reasons: lost to follow-up (8), withdrew (6), discontinued due to SAE (2), and discontinued therapy due to non-response by the investigator (2). Treatment compliance was monitored by maintaining a patient dairy. During the study period, the mean daily intake of ribavirin was 14.3 ± 1.84 mg/kg body weight in genotypes 1 and 4 and 12.84 ± 2.29 mg/kg body weight in genotype 3.

Treatment response
Overall, 57%, 84%, 72%, and 57% of enrolled patients achieved RVR, EVR, ETVR, and SVR, respectively. Results on virologic response of genotypes 1, 3, and 4, evaluated by ITT and per protocol analysis, are presented in Tables 2 and 3, respectively.

Data on the percentage of patients with normalization of ALT at weeks 4, 12, and 24 of treatment, at the end of treatment (week 48 in genotypes 1 and 4, and week 24 in genotypes 2 and 3), and at 24 wk after cessation of therapy are presented in Table 4. In general, the majority of patients, irrespective of their genotype, attained normal levels of ALT by 4 to 12 wk of therapy and the effect was sustained even during follow-up. Mean ALT levels during different study periods are presented in Figure 2.

Side-effects
The majority of patients tolerated the scheduled treatment with peginterferon and ribavirin, though with the usual known adverse events with these drugs. Adverse events were analyzed for safety of peginterferon alfa-2b and presented in Table 5. Ninety-one patients reported 328 adverse events; 95 events by genotype 1 patients, 68 events by genotype 4 patients, and 165 events in geno-

Table 5 Patients with adverse events

Adverse event	*n* (%) of patients		
	Genotype 1 (*n* = 27)	Genotype 3[1] (*n* = 56)	Genotype 4 (*n* = 17)
Injection-site reactions	9 (33.3)	16 (28.6)	7 (41.2)
Flu-like symptoms	24 (88.8)	49 (87.5)	14 (82.3)
Tiredness	4 (14.8)	5 (8.9)	2 (11.7)
Weight loss	1 (3.7)	3 (5.4)	1 (5.8)
Chest discomfort	1 (3.7)	2 (3.6)	2 (11.7)
Arthralgia	3 (11.1)	0 (0)	1 (5.8)
Alopecia	2 (7.4)	10 (17.9)	3 (17.6)
Anorexia	2 (7.4)	7 (12.5)	3 (17.6)
Nausea	3 (11.1)	8 (14.3)	3 (17.6)
Vomiting	2 (7.4)	3 (5.4)	0
Dyspepsia	1 (3.7)	3 (5.4)	2 (11.7)
Gastritis	1 (3.7)	0 (0)	0
Mucous stool	1 (3.7)	0 (0)	0
Diarrhea	1 (3.7)	4 (7.1)	1 (5.8)
Melena	1 (3.7)	6 (10.7)	0
Ascites	1 (3.7)	0 (0)	0
Thrombocytopenia	2 (7.4)	5 (8.9)	1 (5.8)
Anemia	9 (33.3)	13 (23.2)	6 (35.3)
Neutropenia	12 (44.4)	15 (26.8)	8 (47.0)
Anxiety	1 (3.7)	3 (5.4)	1 (5.8)
Depression	2 (7.4)	4 (7.1)	3 (17.6)
Insomnia	1 (3.7)	1 (1.8)	2 (11.7)
Hypothyroidism	2 (7.4)	2 (3.6)	2 (11.7)
Giddiness	1 (3.7)	1 (1.8)	2 (11.7)
Dry throat	1 (3.7)	1 (1.8)	0 (0)
Cough	2 (7.4)	2 (3.6)	2 (11.7)
Sinusitis	1 (3.7)	0 (0)	0 (0)
Bleeding gums	2 (7.4)	1 (1.8)	0 (0)
Palpitation	1 (3.7)	0 (0)	0 (0)
Pruritus	0 (0)	0 (0)	1 (5.8)
Yellow-colored sputum	0 (0)	0 (0)	1 (5.8)
Urinary tract infection	1 (3.7)	0 (0)	0 (0)
Death	0 (0)	1 (1.8)	0 (0)
No. of patients reporting AEs	24 (88.8)	49 (87.5)	14 (82.3)
Discontinued due to SAEs	0 (0)	2 (3.6)	0 (0)
Temporary discontinuation of therapy	4 (14.8)	3 (5.4)	3 (17.6)
Temporary dose reduction	11 (40.7)	16 (29.6)	5 (29.4)

[1]Includes one patient with genotype 2. SAE: Serious adverse event.

type 3 patients. Administration of peginterferon alfa-2b resulted in common mild-to-moderate AEs, which included flu-like symptoms, nausea, and loss of appetite. None of the patients permanently stopped treatment due to adverse events, with the exception of two patients who discontinued due to SAEs. Ribavirin was temporarily discontinued due to anemia in ten patients. Twenty-four patients required ribavirin dose reduction, four needed peginterferon alfa-2b dose reduction, and four required both ribavirin and peginterferon alfa-2b dose reduction for management of anemia and thrombocytopenia. Nine patients reported 11 SAEs, which were all relieved with relevant therapy aside from one patient who died. Among the 11 SAEs, four were related to the study medication and the remaining seven, including the case of death, were unrelated to it.

DISCUSSION

Infection with HCV is one of the most important medi-cal and public health problems worldwide in view of its life-threatening complications, including hepatocellular carcinoma, cirrhosis, and liver failure[8-10]. The goal of therapy in chronic HCV infection is to achieve SVR and thereby prevent long-term complications. Despite the promising role of new antiviral therapies[11], the use of pegylated-interferon alfa combined with ribavirin continues, to date, to be the standard care of treatment in HCV infection.

Since genotype constitutes one of the important determinants of the course and outcome of therapy, 24 or 48 wk combination therapy with peginterferon alfa and ribavirin has been recommended for genotypes 2 and 3 and genotypes 1 and 4 patients, respectively[4-7]. The present open-label, multicenter study using standard-of-care therapy was undertaken to establish that the safety and efficacy of peginterferon alfa-2b is comparable to the results of historical controls in the treatment of chronic HCV infection.

One-hundred eligible patients with chronic HCV infection were enrolled, with the majority (55%) having HCV genotype 3 which is in accordance with the published prevalence studies conducted in India[12,13]. There was only one patient with genotype 2, which is rare among Indians, and thus it should be noted that the reported combined results of patients with genotypes 2 and 3, in fact, only reflects those of genotype 3. Anticipating 15% attrition, 100 patients were enrolled. However, there was instead an 18% dropout, and as a result, 82 patients completed the specified study period of therapy.

Since the dose and duration of therapy were different, the data on outcome measurements were analyzed separately for genotypes 1 and 4 and for genotypes 2 and 3. The SVR (44.4%) observed in the present study for genotype 1 is comparable with those of reported studies[14-16]. In genotypes 2 and 3, 64.3% of patients achieved SVR, which fits with the conformity figure results reported by Manns *et al*[17]. The rates of SVR in treatment naïve genotype 2 patients were reported to be 86.5%[18], which is higher than that of genotype 3. Since our genotype 2 and 3 patients, except for one, belonged to genotype 3, a lower SVR (64.3%) was observed in the present study. In genotype 4, 52.9% patients achieved SVR, which is comparable with values from published studies[19,20]. Apart from genotype, baseline viral load has been shown to be one of the determinants of SVR[21]. However, perhaps due to the small number of patients covered in the present study, our stratified statistical analysis showed that baseline viral load had no impact on SVR.

In view of the cost factor and incidence of adverse events with peginterferon use during long-duration treatment, individualized treatment, based on the results of RVR and EVR, has been emphasized. In this respect, the presence of RVR is highly predictive of ultimate SVR with a full treatment course of 48 wk in genotype 1 patients[22]. In the current study, all genotype 1 patients (*n* = 7) who achieved RVR also attained SVR; while a study reported a SVR rate of 86.8% in patients with RVR[15]. In genotype 4 patients, 80% with RVR attained SVR,

whereas the published study reported 86%[23]. Similarly, among the genotype 3 patients who had RVR, 83.3% attained SVR, which is similar (83.7%) to that reported in the literature[24]. This further confirms the utility of RVR in predicting SVR.

Among the patients who attained EVR, 10 (76.9%) in genotype 1 and 9 (75%) in genotype 4 achieved SVR. In patients with genotypes 2 and 3, the percentage with EVR attaining SVR was 100%. This is in line with the literature[25], which shows that patients with genotype 3 who fail to achieve EVR also fail to achieve SVR. Since the duration of treatment for genotypes 2 and 3 is only 24 wk, it has been reported that EVR testing is not cost-effective in these patients[25]. This indicates that utility of RVR is higher than EVR in the prediction of SVR.

Overall, 16 patients had relapse; 5 (31.2%) patients in genotype 1, 8 (18.2%) patients in genotypes 2 and 3, and 3 (25%) patients in genotype 4. Among the 100 patients, 5 were non-responders to the study treatment; 1 (3.7%) patient in genotype 1; 2 (3.6%) patients in genotypes 2 and 3, and 2 (11.7%) in genotype 4. In addition 4 patients had breakthrough during the treatment; 2 (7.4%) patients in genotype 1; 1 (1.8%) patient in genotype 3, and 1 (5.8%) patient in genotype 4.

Biochemical response of peginterferon alfa-2b was assessed by the percentage of patients attaining normalization of ALT levels. Overall, the majority of patients (51%) had normalization of ALT levels as early as week 4. This denotes that peginterferon is very effective in producing a biochemical response in patients with chronic hepatitis C.

The treatment was well-tolerated in the majority of patients, though with the common side-effects usually attributed with interferon or ribavirin. In 32% of patients, temporary dose modifications in peginterferon (4%), ribavirin (24%), or both (4%), and temporary discontinuation of therapy in 10% of patients, were required. Though 11 SAEs were observed in 9 patients, only 4 were related to study medication, with such SAEs also being reported in earlier studies[15,17,26,27].

The limitations of the study are that it is a single arm study and the results on the outcome measures were compared with those of historical controls. Earlier studies on Indian patients with HCV infection were conducted using peginterferon alfa-2b in two studies-one study was carried out on 103 patients, but only on genotype 3 patients[25]; the other study, despite covering all four genotypes, had only 16 patients[28]. We are not aware of any study conducted with an adequately powered sample of Indian patients with HCV infection following the global guidelines on peginterferon plus ribavirin[4-7,29].

Therefore, despite the limitation of a lack of comparator, our results on serological responses such as RVR, EVR, ETVR, and SVR provide valuable information on the safety and efficacy of peginterferon alfa-2b, in combination with ribavirin, in the treatment of Indian patients with chronic HCV infection. Currently, Virchow Biotech-developed peginterferon alfa-2b is marketed in India and other emerging countries at a very competitive rate. In view of the relatively low incidence of the adverse events and improved virologic and biochemical response, the results of the study show that peginterferon alfa-2b, in combination with ribavirin, is a safe and cost-effective drug in the treatment of chronic hepatitis C.

COMMENTS

Background
Hepatitis C virus (HCV) infection is a major cause of chronic liver disease in India, with a high morbidity and mortality due to its complications. Pegylated interferon, in combination with ribavirin, is the standard recommended treatment for chronic hepatitis C. One of the reasons for this could be due to its cost factor, with another being that studies evaluating the safety and efficacy of these drugs in India are limited. Therefore, an attempt is being made to evaluate the efficacy of peginterferon alfa-2b, a drug locally developed in India, in combination with ribavirin.

Research frontiers
This prospective study presents results on the efficacy, in terms of virologic response, of indigenously-developed peginterferon alfa-2b plus ribavirin in Indian patients with different genotypes of chronic hepatitis C. Adverse events observed with this combination are also reported.

Innovations and breakthroughs
There have been a few prior studies on Indian patients with HCV infection using peginterferon alfa-2b. However, these were limited to a small number of patients or confined to one genotype.

Applications
This study demonstrates that virologic response of peginterferon alfa-2b and ribavirin, when given as per global guidelines in Indian patients with different types of chronic hepatitis C, is similar to that of historical controls.

Terminology
Success rate of treatment is assessed based on sustained virologic response, which is defined as undetectable HCV RNA in blood 24 wk after cessation of therapy.

Peer review
This is a straightforward clinical control study.

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Acute fatty liver of pregnancy associated with severe acute pancreatitis: A case report

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Abstract

Acute fatty liver of pregnancy is a rare disease that affects women in the third trimester of pregnancy. Although infrequent, the disease can cause maternal mortality. The diagnosis is not always clear until the pregnancy is terminated, and significant complications, such as acute pancreatitis, can occur. Pancreatic involvement typically only occurs in severe cases after the development of hepatic and renal impairment. To date, little knowledge is available regarding how the disease causes pancreatitis. Treatment involves supportive measures and pregnancy interruption. In this report, we describe a case of a previously healthy 26-year-old woman at a gestational age of 27 wk and 6 d who was admitted with severe abdominal pain and vomiting. This case illustrates the clinical and laboratory overlap between acute fatty liver of pregnancy and pancreatitis, highlighting the difficulties in differentiating each disease. Furthermore, the hypothesis for this overlapping is presented, and the therapeutic options are discussed.

Core tip: A previously healthy 26-year-old woman at 27 wk and 6 d of pregnancy was referred for investigation of abdominal pain. She presented with complaints of diffuse abdominal pain with nausea and vomiting associated with hepatic and renal dysfunction. Acute fatty liver of pregnancy and severe acute pancreatitis were diagnosed. Acute fatty liver of pregnancy is rarely associated with severe acute pancreatitis, which can complicate the diagnosis. The possible mechanisms involved in this association and the current therapies are discussed, focusing on the relevant aspects to improve the management of similar cases.

de Oliveira CV, Moreira A, Baima JP, Franzoni LC, Lima TB, Yamashiro FS, Coelho KYR, Sassaki LY, Caramori CA, Romeiro FG, Silva GF. Acute fatty liver of pregnancy associated with severe acute pancreatitis: A case report. *World J Hepatol* 2014; 6(7): 527-531 Available from: URL: http://www.wjgnet.com/1948-5182/full/v6/i7/527.htm DOI: http://dx.doi.org/10.4254/wjh.v6.i7.527

INTRODUCTION

Acute fatty liver of pregnancy (AFLP) is a disorder unique to pregnancy that is characterized by microvesicular fatty infiltration of hepatocytes[1]. AFLP was first described in 1940 and was initially considered fatal[2]. However, early diagnosis has dramatically improved the prognosis and maternal mortality; therefore, maternal mortality is currently the exception rather than the rule[1]. AFLP typically occurs in the third quarter of pregnancy, but it is not always diagnosed prior to delivery, as was the case described herein.

The most common initial symptoms are anorexia, nausea, vomiting, abdominal pain, and jaundice. A condition that must be excluded is hemolytic anemia elevated liver function and low platelet count syndrome (HELLP) syndrome, which is characterized by hemolysis, elevated liver enzymes, and low platelet count. AFLP and HELLP syndrome can occur together in some overlapping cases, making the diagnosis more difficult. However, the signs of liver failure, such as hypoglycemia and hepatic encephalopathy, are suggestive of AFLP. Additionally, HELLP syndrome is likely to occur in patients with hypertension, whereas AFLP often occurs in the absence of hypertension. The differential diagnosis of these two diseases was evaluated in a recent study, which indicated that the incorporation of antithrombin activity less than 65% into the diagnostic criteria for AFLP may facilitate prompt diagnosis of this disease[3].

CASE REPORT

A previously healthy 26-year-old woman at a gestational age of 27 wk and 6 d was referred to our hospital due to a diagnostic hypothesis of acute appendicitis. She was complaining of diffuse abdominal pain, nausea, and vomiting during the week. During her physical exam, she was pale and prostrated with mild tachycardia (108 beats/min) and normal blood pressure (110/70 mmHg). No signs of acute appendicitis were noted, but she displayed a potent and diffuse abdominal pain. Cardiotocography revealed signs of fetal distress, so an emergency cesarean section was performed. During the surgery, the possibility of appendicitis was eliminated. Because the newborn displayed bradycardia and an absence of heartbeats at delivery, he was submitted to initial resuscitation protocols and sent to the intensive care unit (ICU). Laboratory tests on the mother revealed leukocytosis, anemia, and hepatic and renal impairment, but no significant proteinuria was found (Table 1).

Abdominal ultrasonography revealed only pancreatic edema without signs of biliary obstruction. After the delivery, abdominal computed tomography (CT), upper gastrointestinal endoscopy, and biochemical tests were performed. The endoscopy was performed exclusively to investigate the possibility of peptic ulcer or other gastroduodenal diseases, but no pathological findings were found. The CT showed only pancreatic edema without peripancreatic collections (Figure 1). Given that the amy-

Table 1 Main laboratory tests demonstrating the development of liver and pancreatic

Blood tests	Admission	48 h after admission	Hospital discharge	1 yr after discharge
Hemoglobin (g/dL)	9.8	11.2	-	-
Leukocyte count (mm³)	24000	21500	-	-
Glucose (mg/dL)	586	71	91	76
Alkaline phosphatase (U/L)	532	392	248	-
g-GTP (U/L)	282	325	234	-
Calcium (mg/dL)	8.4	7.7	-	-
Amylase (U/L)	460	642	-	59
LDH (U/L)	938	977	-	-
ALT (U/L)	202	112	34	15
AST (U/L)	343	179	38	17
TB (mg/dL)	4.0	4.9	0.6	0.5
Creatinine (mg/dL)	2.6	3.3	0.7	0.8
Urea (mg/dL)	78	85	20	28
INR	2.13	2.78	1.16	0.98
Proteinuria	0.06 g/24 h	0.03 g/24 h	-	-

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TB: Total bilirubin; LDH: Lactate dehydrogenase; INR: International normalized ratio; g-GTP: Gamma-glutamyl transpeptidase.

Figure 1 Abdominal computed tomography scan showing diffuse pancreatic edema.

lase increase was greater than sixfold higher than the normal upper limit and that pancreatic edema was confirmed by ultrasonography and CT exams, the presence of pancreatitis was conclusive. According to the Ranson criteria, the patient had a severe disease that achieved 4 points at admission based on the leukocyte count, aspartate aminotransferase, glycemia, and lactate dehydrogenase values (Table 1). Additionally, she had acute renal failure and achieved 14 points according to the APACHE II criteria, which corresponds to an estimated 18.6% risk of hospital death.

The patient developed somnolence and exhibited a progressive decrease in her level of consciousness. Tracheal intubation and mechanical ventilation were needed, so she was transferred to the ICU. At this time, the blood glucose remained normal, but she had abdominal distension and decreased bowel sounds. Then, the diagnostic hypotheses changed to acute liver failure, severe acute pancreatitis, and renal failure. Suddenly, she presented recurrent episodes of hypoglycemia, even with continuous

Figure 2 Histopathological analysis of the liver biopsy according to three stains. A: Hematoxylin-eosin staining reveals canalicular cholestasis, hepatocellular ballooning, and microgoticular steatosis; B: Masson staining demonstrates microgoticular steatosis and perivenular and pericellular fibrosis; C: Oil red staining shows the microdroplets of fat clearly stained in red.

dextrose infusion and parenteral nutrition. In response to these new symptoms, AFLP became the major diagnostic hypothesis. Serum factor V was normal, so a percutaneous liver biopsy was performed.

The liver biopsy analysis showed centrilobular microgoticular steatosis, ballooning degeneration, and reticular collapse. The Masson staining showed areas of reticular thickening and intralobular collapse. The "red oil" stain was positive in focal areas, yielding the diagnosis of AFLP (Figure 2).

Seven days following the delivery, she exhibited a clear improvement in consciousness level and liver function tests. She was discharged on postoperative day 24 and returned to the hospital 4 mo later without neurological sequelae. Additionally, laboratory tests and abdominal CT were normal. Despite the problems during the birth, her child exhibited normal development.

DISCUSSION

AFLP is a rare condition that affects approximately 1 in 7000 to 1 in 20000 births[4-8]. AFLP is more common in women with multiple pregnancies and, possibly, in underweight women. However, this case of AFLP occurred in a primiparous, normal-weight woman but not delivering twins.

Approximately half of AFLP patients display signs of preeclampsia at the beginning of or at some time during the course of the disease[9]. Extrahepatic complications may occur, which can be life-threatening[10,11]. The patients rarely develop pancreatitis, which can be severe. Similar to the case described herein, pancreatitis is typically noticed only after the development of hepatic and renal dysfunction[12]. In this case, the patient had AFLP with severe acute pancreatitis, an association that is rarely documented in the literature. The acute renal failure was a complication of the pancreatitis, so it was treated only by supportive measurements and the delivery, thereby confirming that it was a consequence of the underlying disease. No renal replacement therapy was needed. The

liver function tests demonstrated severe hepatic impairment, which was the cause of the jaundice. Therefore, even in the presence of severe pancreatitis, liver disease remained the major disease.

Women with AFLP have impaired liver function with increased bilirubin and transaminase levels and leukocyte counts, which are typically higher than those observed in a normal pregnancy. The platelet count can be reduced with or without additional signs of disseminated intravascular coagulation in association with a significant reduction of antithrombin Ⅲ[13]. Severely affected patients also have elevated serum ammonia, prolonged prothrombin times, and hypoglycemia caused by liver failure. Acute renal failure and hyperuricemia are often present[14]. However, in the case presented, the recurrent episodes of hypoglycemia, even with appropriate correction, were the main diagnostic clue.

The association between AFLP and inherited defects in the mitochondrial beta-oxidation of fatty acids, especially the impairment of long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD), suggests that some affected women and fetuses have an inherited enzyme deficiency in beta-oxidation that predisposes the mother to this disorder[15-17]. LCHAD catalyzes the third step of the beta-oxidation of fatty acids in the mitochondrion (the formation of 3-ketoacyl-CoA from 3-hydroxyacyl-CoA). The accumulation of long-chain metabolites of 3-hydroxyacyl produced by the fetus or placenta is toxic to the liver and can serve as the cause of the liver disease. The role of the pathogenesis of LCHAD in AFLP has been illustrated in various studies[18-20].

The mechanism by which pancreatitis may develop as a complication of fatty liver of pregnancy is not well understood because this association is rare. Our hypothesis is that the accumulation of long-chain metabolites of 3-hydroxyacyl is toxic to the liver and the pancreatic tissue. Thus, the pancreas could be affected when an increased concentration of these metabolites is present, as occurs in cases of severe hepatic disease. This hypothesis serves as a reasonable explanation for the pancreatic impairment displayed in this case of hepatic failure.

The diagnosis of LCHAD deficiency in newborns can save lives; therefore, all women with AFLP and their children should be administered a molecular test for LCHAD, which should at least evaluate the most common mutation, namely, G1528C[21,22]. In the present case, it was not possible to perform this type of test because it was not available.

The clinical diagnosis of AFLP is typically performed according to the definition, presentation, and laboratory-compatible image results. The liver imaging is primarily used to exclude other diagnoses, such as hepatic infarction and hematoma[23]. Various authors reported steatosis on ultrasound or CT, but these tests are only useful for performing comparative analyses[24,25]. The AFLP diagnosis can only be made through a liver biopsy showing microvesicular fatty infiltration in hepatocytes. The fat droplets are centrally distributed around the cellular nuclei,

giving the cytoplasm a foamy aspect and typically sparing the cells around the portal tract[26]. A special staining (oil red) must be used to confirm the diagnosis in patients without obvious vacuolation[27,28]. As the liver biopsy is invasive, it is not always performed. Liver biopsy should be performed with caution during pregnancy and be reserved for cases where the diagnosis remains unclear.

A specific treatment is not available for AFLP. The primary treatment is delivery, which typically occurs on an emergency basis after maternal stabilization *via* the infusion of glucose and reversal of the coagulation disturbances. Because hypoglycemia is common and dangerous, glucose levels should be monitored until liver function normalizes[7]. It is typically necessary to treat hypoglycemia with the continuous infusion of a 10% glucose solution. Some patients with severe hypoglycemia may require additional bolus administrations of 50% glucose[7].

The liver function tests typically begin to normalize after delivery; however, after the first few days, a transient worsening of renal and hepatic function may be observed, followed by a definite improvement. In severe cases, particularly when the diagnosis is delayed, more days of illness can be observed, requiring supportive treatment in an ICU. Mechanical ventilation, dialysis, parenteral nutrition, or surgery to treat bleeding during cesarean delivery may be needed. Even the most severely ill patients can recover without liver disease sequelae[7]. However, substantial morbidity and even maternal mortality may occur[8]. Some reports have also described patients who required liver transplantation, which is rarely needed when a diagnosis and pregnancy termination were achieved in sufficient time[29,30]. A case series of five patients indicated good recovery after plasma exchange and renal replacement therapy, showing that the accumulation of long-chain metabolites of 3-hydroxyacyl is most likely the major cause of the liver disease. The authors suggested that these therapies are safe and effective; thus, they should be used immediately at the onset of hepatic encephalopathy and/or renal failure in AFLP patients[31]. None of the patients treated by plasma exchange and renal replacement therapy developed pancreatitis, indicating that our pancreatic toxicity hypothesis may be correct.

Of note, AFLP can recur in subsequent pregnancies despite negative LCHAD mutation screening results[6,15,18,32-35]. However, the exact risk of recurrence is unknown. Affected women should be advised of this possibility, and a maternal-fetal medicine specialist should closely monitor subsequent pregnancies. Given that the initial symptoms of AFLP can be atypical, the diagnosis can be easily misidentified, leading to multiple organ dysfunctions. Once the diagnosis is confirmed, it is important to be vigilant to avoid serious complications, such as pancreatitis, renal failure, and hypoglycemia, as described here.

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COMMENTS

Case characteristics
A previously healthy 26-year-old woman with a gestational age of 27 wk and 6 d presented with diffuse abdominal pain associated with hepatic and renal failure.

Clinical diagnosis
Diffuse abdominal pain and vomiting.

Differential diagnosis
Hemolytic anemia elevated liver function and low platelet count syndrome syndrome.

Laboratory diagnosis
Amylase: 642 U/L; glucose: 586 mg/dL; alanine aminotransferase: 202 U/L; aspartate aminotransferase: 343 U/L; total bilirubin: 4.0 mg/dL; creatinine: 2.6 mg/dL; urea: 78 mg/dL; international normalized ratio: 2.13.

Imaging diagnosis
Pancreatic edema was confirmed by ultrasonography and computed tomography.

Pathological diagnosis
The liver biopsy results were compatible with acute fatty liver of pregnancy (AFLP).

Treatment
Pregnancy interruption and supportive measures.

Related reports
AFLP rarely presents with severe acute pancreatitis.

Experiences and lessons
Because the early symptoms of AFLP can be uncharacteristic, the disease can be misdiagnosed. It is important to be vigilant to make the correct diagnosis of AFLP and identify complications, such as pancreatitis.

Peer review
This article describes a rare case with acute fatty liver disease complicated with acute pancreatitis. This is an interesting case report.

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CASE REPORT

Portal vein thrombosis with protein C-S deficiency in a non-cirrhotic patient

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Abstract

There are several conditions that can lead to portal vein thrombosis (PVT), including including infection, malignancies, and coagulation disorders. A new condition of interest is protein C and S deficiencies, associated with hypercoagulation and recurrent venous thromboembolism. We report the case of a non-cirrhotic 63-year-old male diagnosed with acute superior mesenteric vein thrombosis and PVT and combined deficiencies in proteins C and S, recanalized by short-term low molecular heparin plus oral warfarin therapy.

Key words: Portal vein thrombosis; Mesenteric venous thrombosis; Protein C and S deficiency; Anticoagulant therapy; Transient elastography

Core tip: Abdominal pain, diarrhea, rectal bleeding, abdominal distention, ascites, anorexia, fever, lactacidosis, sepsis, and splenomegaly are common features of acute portal vein thrombosis (PVT). Etiological factors in non-cirrhotic PVT patients are prothrombotic states and local factors, although more than one factor is often identified. Our patient, a 63-year-old man, without personal or familial history of venous thromboembolism developed portal and mesenteric vein thrombosis after an acute gastrointestinal infection by *Escherichia coli*. Clinicians need to be aware of this potential complication in patients with persistent abdominal pain and ascites after abdominal infections.

INTRODUCTION

Portal vein thrombosis (PVT) is defined as complete or partial obstruction of blood flow in the portal vein, associated with a thrombus in the vasal lumen[1]. The first case of PVT was reported in 1868 by Balfour and Stewart, in a patient showing splenomegaly, ascites, and variceal dilatation[2]. PVT is rare in the general population having been reported with mean age-standarized incidence and prevalence rates of 0.7 and 3.7 per 100000 inhabitants, respectively[3]. However among patients with cirrhosis, these rates jump to between 4.4%-15%, and cause about 5%-10% of overall cases of portal hypertension[4]. Some 22%-70% of patients without cirrhosis

demonstrate prothrombotic states and local factors are present in 10%-50%[3-5], although more than one factor is often identified[6]. PVT also shows different clinical presentations in acute *vs* chronic onset patients and collateral circulation, both its development and extent. Intestinal congestion and ischemia, with abdominal pain, diarrhea, rectal bleeding, abdominal distention, nausea, vomiting, anorexia, fever, lactacidosis, sepsis, and splenomegaly are common in acute PVT. More difficult to diagnose, chronic PVT can be completely asymptomatic, or present splenomegaly, pancytopenia, varices, and, on rare occasion, ascites[2].

PVT is classified into four categories: (1) thrombosis confined to the portal vein beyond the confluence of the splenic and superior mesenteric vein (SMV); (2) extension of thrombus into the SMV, but with patent mesenteric vessels; (3) diffuse thrombosis of splanchnic venous system, but with large collaterals; and (4) extensive splanchnic venous thrombosis, but with only fine collaterals. Currently this anatomical classification is mainly used to determine operability, but it may also have etiological and prognostic relevance, since patients with thrombus interference with mesenteric vasculature risk bowel infarction and have a lower risk of variceal bleeding than those with isolated PVT. In all cases, patients with PVT should be tested for an underlying thrombophilic condition[6]. Hereditary thrombophilias known to predispose for PVT include mutations of the prothrombin, or factor V, genes, and deficiency of one of the natural anticoagulant proteins C, S, or antithrombin. Fisher *et al*[7] in a study with twenty-nine adult patients with portal hypertension caused by PVT, found that 18 patients (62%) had deficiencies in one or more of the natural anticoagulant proteins, and six had combined deficiency of all three proteins. Of these, eight cases (28%) had combined C and S protein deficiency, nine (31%) had C protein and antithrombin deficiency, seven (24%) showed protein S and antithrombin deficiency, and six cases (21%),as mentioned, had combined deficiency of all three proteins. Due to increased use and improvement of non-invasive imaging techniques in diagnostic evaluation of abdominal pain, acute portomesenteric venous obstruction is an increasingly recognized disorder[1,2,4,5].

CASE REPORT

The patient was a 63-year-old man with glaucoma treated with timolol and latanoprost. He had undergone a resection of thyrogloid cyst 50 years previously. There was no personal history of venous thromboembolism and familial history was unrevealed. No abdominal trauma was reported. The patient had developed an acute gastrointestinal infection by *Escherichia coli* three months before admission, and received treatment with ciprofloxacin. Since that infection, he had felt intermittent mesogastric abdominal pain after meals, nausea and diarrhea, that increased in frequency 2 wk before admission, when he also noted increased abdominal girdle and peripheral ede-

ma. He did not note mucus or blood in feces. On admission, the patient had a fever 39 ℃ and blood pressure of 100/70 mmHg. He was alert and oriented without signs of encephalopathy. His bowel sounds were hypoactive and minimal epimesogastric tenderness was present with no rebound tenderness. He had non-tense ascites and edema in the lower extremities. Heart, lungs, throat and skin were unremarkable. Laboratory studies showed a hematocrit of 42.2%, mean corpuscular volume of 87 fL, and a sedimentation rate of 51%, white cell count of 6.8/mm^3, neutrophils 65.6%, lymphocytes 19.0%, monocytes 15.1%, eosinophils 0.3%, platelet count 271/mm^3, prothrombin time 10.6 s, 97.6%, international normalized ratio (INR) 0.96. Serum chemistry values and urine test were normal. Liver function test showed: albumin 3.3 g/dL; total bilirubin 1.94 mg/dL; alanine aminotransferase 51 U/L; aspartate aminotransferase 40 U/L; alkaline phosphatase was 96 (32-91 U/L); lactic dehydrogenase was 251 U/L (98-192 U/L); g-glutamyl transpeptidase was 139 U/L (7-50 U/L). Amylase 44 U/L, Lipase 23 U/L. Viral B and C antibodies were negative. Tumoral markers CA-19-9, ACE, alkaline phosphatae (AFP) were negative. His antiphospholipid antibodies and cardiolipin antibodies were negative. A thrombophilia workup, not including screening for JAK2V617F mutation, revealed normal homocysteine blood levels; C-reactive protein levels was 216.5 (0-7.4 mg/L); D-dimer was 5770 (0-199 ng/mL); fibrinogen levels was 443 (177-410 mg/dL); low levels and little activity of the protein C antigen [protein C antigen level, 39%; protein C activity, 54% (normal 70%-140%)] and protein S antigen [protein S antigen level, 59%; protein C activity, 30% (normal 65%-140%)] were found; antithrombin Ⅲ levels were 89% (normal 75%-125%). Factor V Leiden mutation was homozygote. His father was dead and his mother and sister neglected screening. Hematological, urine, ascites fluid and pharyngeal cultures were negative. Upper endoscopy revealed mild portal hypertensive gastropathy without gastric and esophageal varices. Ultrasonography of the abdomen showed that the portal vein could not be identified in the porta hepatis, which was occupied by several abnormal tubular structures suggestive of cavernous transformation (Figure 1A). The computed tomography scan of the abdomen showed cavernous transformation following PVT. The portal venous thrombus extended from the superior mesenteric vein (Figure 2). A transient elastography (TE) (Fibroscan) was abnormal with stiffness 7.4 kPa. We treated the patient with low molecular weight heparin (enoxaparine, 1 mg/kg) during the first week and chronic anticoagulation therapy (warfarin 2.5 mg/d, INR 2-3) to date. A new Doppler ultrasound, five months after admission, improved his portal flow with complete recanalization and without ascites (Figure 1B). The patient is asymptomatic three years after hospital discharge.

DISCUSSION

In recent years, PVT has increasingly been diagnosed by

Table 1 Hypercoagulable etiologies			
Thrombophilic disorders		**Local factors**	
Inherited disorders	Acquired disorders	Inflammatory	Related to surgery
Factor V Leyden mutation	Myeloproliferative disorders	Cirrhosis	Post liver transplant
Prothrombin mutation	Malignancy	Sepsis	Splenectomy
Antithrombin III	Antiphospholipid syndrome	Pancreatitis/cholecystitis	Colectomy
Protein C deficiency	Anticardiolipin antibody	Diverticulitis	Umbilical vein catheterization
Protein S deficiency	Paroxysmal nocturnal hemoglobinuria	Appendicitis	Portocaval shunting
	Hyperhomocystein-emia	Peptic ulcer disease	
	Oral contraception pills	Inflammatory bowel disease	
	Pregnancy/post-partum	Blunt abdominal trauma	

Figure 1 Doppler ultrasound. A: Liver Doppler ultrasound. The image shows the thrombus in the portal vein; B: Doppler ultrasound, performed 4 mo after discharge, revealed that the portal vein thrombi had disappeared and a smooth bloodstream was observed in the portal vein.

Figure 2 Coronal reconstruction of contrast-enhanced computed tomography image with arrows indicating portal venous thrombosis and evidence of cavernous transformation.

wide use of ultrasound-Doppler equipment. When cirrhosis is not present, the lifetime risk of getting PVT in the general population is reported to be 1%[8,9]. Currently recognized etiologies can be divided into 2 categories: thrombophilic disorders and thromboses thought to be caused from local factors (Table 1).

Protein C is a thrombin-dependent anticoagulant enzyme known to deactivate coagulation cofactors V and VIa and to stimulate fibrinolysis[10]. Protein C deficiency, often inherited as an autosomal dominant trait, is a risk factor for venous thrombosis.

The prevalence of protein C deficiency, as indicated solely by plasma level, is 1 in 200-500 persons in the general population. However, this number is unreliable as many affected individuals remain asymptomatic throughout their lives. However, protein C deficiency is present in approximately 2%-5% patients presenting VTE. Severe homozygous or compound heterozygous protein C deficiency is found in 1 in 500000-750000 live births. Protein S deficiency occurs in 1.35% of the patients with venous thrombosis.

There is evidence to suggest that thrombosis in unusual sites, such as cerebral sinus venous thrombosis, mesenteric vein thrombosis, PVT, and suprahepatic vein thrombosis (Budd-Chiari syndrome), in young individuals is associated with inherited thrombophilia.

Liver function impairment, which can be a result of PVT, cannot account for the low C and S protein levels in our patient, as the levels of other function tests and indirect markers of liver fibrosis (TE) were abnormal.

It is not known whether the unexplained bout of abdominal pain and diarrhea which occurred three months before our patient, was due to thrombosis, to a resolutive episode of intestinal ischaemia secondary to mesenteric vein thrombosis, or to an unrelated illness, although abdominal pain, diarrhea, abdominal distention, nausea, anorexia, and fever are common in acute PVT[4].

In Mexico, Majluf-Cruz *et al*[11], studied 36 patients who had thrombosis-related portal hypertension and found an incidence of 30% of protein C deficiency, whereas 9% had protein S deficiency in patients with primary thrombophilia[12]. Similarly in Mexican patients with non-cirrhotic PVT, 31% had protein C deficiency[13].

Table 2 Proposed mechanism for reduction in concentrations of procoagulant and anticoagulant proteins in patients with portal vein thrombosis

Hereditary or acquired thrombophilia
Reduced hepatic blood flow
Reduced synthesis
Portal hypertension
Portosystemic shunting
Clearance or consumption
Portal pyaemia or other local inflammatory disease
Portal vein thrombosis
Reduced levels of procoagulant and anticoagulant proteins

However, a French study has found a high number of patients with non-cirrhotic PVT showed Protein S deficiency[14] and in a study from United Kingdom, protein S deficiency was found in 38% of patients with PVT[15]. Other cases have also reported C and S protein deficiencies in patients with idiopathic portal hypertension accompanied by PVT[16,17]. Valla *et al*[14], argue that C and S protein deficiencies do not explain the majority of idiopathic portal thrombosis. Nevertheless, we agree with others that measurements of C and S proteins should be performed in patients with portal thrombosis when no overt cause is located. However, since a low number of cases of PVT may be due to underlying hereditary anticoagulant protein deficiency, this can only be confirmed by careful investigation of background of family members, preferably including both parents. When studies of the parents is not feasible, another possibility might be screening siblings, which could be used for both diagnostic and counseling purposes. Lastly, the recent use of gene sequencing in the elucidation of anticoagulant protein gene mutations may now allow determination of whether such anticoagulant deficiencies in PVT are truly primary or not[18]. Some possible mechanisms for reduction in concentrations of procoagulant and anticoagulant proteins in patients with PVT are shown in Table 2.

Visualization of abnormalities associated with PVT is crucial to diagnosis and appropriate intervention. Cavernous transformation of the portal vein occurs in one-third of patients after PVT. An ultrasonographically diagnostic triad would consist of: (1) failure of visualization of the extra-hepatic portal vein; (2) demonstration of high-level echoes in the region of the porta hepatis (the "diamond sign"); and (3) visualization of multiple serpiginous vascular channels around the portal vein[19]. Dynamic contrast-enhanced computed tomography (CT) is the best means of diagnosing PVT and evaluating possible causative diseases. The findings of PVT in a dynamic CT include: filling defect partially or totally occluding the vessel lumen and rim enhancement of the vessel wall[20]. Signs and symptoms of PVT may be subtle or nonspecific and are secondary to the underlying illness. On the other hand, presence of a well-developed cavernoma usually indicates an old thrombosis. A previous PVT, however, can be associated with a recently superimposed thrombus, which is then responsible for the acute manifestations which lead

to imaging studies. An abdominal magnetic resonance imaging may prove more useful than Doppler ultrasound in identifying venous collateral development and cavernoma[21]. An important step in PVT is to disclose malignancy. We only performed some tumoral markers (CA-19-9, ACE, AFP), but screening for JAK2V617F in order to discard myeloproliferative neoplasms and positron emission tomography-scan were not performed. TE is a non-invasive technique to assess liver fibrosis, which assesses liver fibrosis by calculating the velocity of a low-frequency transient shear wave produced by a mechanical probe that is placed directly on the skin of the patient. Liver stiffness is expressed in kPa. The method is easy to learn (the procedure can be performed by a technical assistant), and results are immediately available. One meta-analysis evaluating the predictive performance of TE in patients with chronic liver disease suggests the optimal cut-off value for the diagnosis of significant fibrosis is 7.65 kPa and for cirrhosis 13.01 kPa[22]. In our patient, stiffness of 7.4 kPa was highly predictive for significant fibrosis ($F \geq 2$). There is no data on the use of TE in PVT, but this method may be useful to determine liver fibrosis in these patients. Complications during follow-up frequently include: esophageal and gastric varices, portal hypertensive gastropathy and bleeding. Portal hypertensive gastropathy is reported to be 44% in patients without cancer and cirrhosis, as was the case with our patient[23]. Therefore, it would be wise to screen all PVT patients endoscopically. Although spontaneous resolution of PVT has been reported in the literature, a specific therapeutic management strategy is necessary. The goal of treatment is similar in acute and chronic PVT, and includes correction of causal factors, prevention of thrombosis extension and achievement of portal vein patency. Currently, anticoagulant therapy is the best way to obtain portal vein recanalization; however, its application is not universally accepted. No controlled trial has been performed on the use of anticoagulants in acute PVT[24]. After 6 mo of therapy, complete recanalization has been reported in about 50% of patients, with good outcomes in mesenteric vein involvement, and very few complications. What is certain is that, in acute PVT onset, the sooner the treatment is given, the better the prognosis; the rate of recanalization is about 69% if anticoagulation is begun within the first week after diagnosis, while it falls to 25% when begun in the second week[25]. Thrombolytic therapy may also be effective, but efficacy is significantly lower and mortality increases compared to conservative treatment[26]. Surgical thrombectomy is usually not recommended. Other approaches, such as transyugular intrahepatic portosystemic shunt, should be reserved for liver transplant patients developing acute PVT or as an alternative when anticoagulation fails[4]. In non-cirrhotic and non-neoplastic patients, PVT has shown promising results with overall survival at 1 year and 5 years of 92% and 76% respectively[3,23,27,28].

In conclusion, our case shows that PVT can be provoked by C and S protein deficiency and that the PVT

can be recanalized by short-term low molecular heparin plus oral warfarin therapy. Although the evidence is not definitive, existing literature supports the idea that the risk-benefit ratio favors anticoagulation in chronic non-cirrhotic PVT.

COMMENTS

Case characteristics
Upon admission the patient felt intermittent colicky abdominal pain and non-bloody diarrhea after meals with increased abdominal girdle and peripheral edema at physical examination.

Clinical diagnosis
The patient presented with non-tense ascites and imaging evidence of portal vein thrombosis (PVT) on a background of non-liver disease.

Differential diagnosis
Differential diagnosis was performed between inherited *vs* acquired disorders of coagulation in PVT using ultrasound Doppler, dynamic computed tomography (CT) and specific laboratory tests.

Laboratory diagnosis
A thrombophilia workup, not including screening for JAK2V617F mutation, revealed normal homocysteine blood levels; C-reactive protein levels were 216.5 (0-7.4 mg/L); D-dimer was 5770 (0-199 ng/mL); fibrinogen levels was 443 (177-410 mg/dL); low levels and little activity of the protein C antigen [protein C antigen level, 39%; protein C activity, 54% (normal 70%-140%)] and protein S antigen [protein S antigen level, 59%; protein C activity, 30% (normal 65%-140%)] and homozygote factor V Leiden mutation was found; abnormal liver function tests (albumin 3.3 g/dL; total bilirubin 1.94 mg/dL; alanine aminotransferase 51 U/L (31-45 U/L); alkaline phosphatase 96 (32-91 U/L); lactic dehydrogenase 251 U/L (98-192 U/L); g-glutamyl transpeptidase 139 U/L (7-50 U/L) were found; antithrombin Ⅲ levels, viral B and C antibodies ,CA-19-9, ACE, alkaline phosphatae, antiphospholipid antibodies and cardiolipin antibodies were normal or negative.

Imaging diagnosis
Liver Doppler ultrasound showed a thrombus in the portal vein that was corroborated by CT image indicating portal venous thrombosis and evidence of cavernous transformation.

Pathologic diagnosis
Histologic examination was not indicated.

Treatment
The patient was treated with low molecular weight heparin (enoxaparine, 1 mg/kg) during the first week and chronic anticoagulation therapy (warfarin 2.5 mg/d, INR 2-3) to date.

Experiences and lessons
Even if Doppler ultrasound or abdominal CT play a key role in the diagnosis of PVT, the protocol to find the etiology of the thrombosis may be complex.

Peer review
This manuscript is interesting and presents a remarkable presentation about diagnosis and management of PVT associated with C and S protein deficiency in a non-cirrhotic patient.

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REVIEW

Vertical hepatitis C virus transmission: Main questions and answers

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Author contributions: Tosone G and Orlando R designed the review and made the overview of the manuscript; Maraolo AE and Palmiero G collected and analyzed the data on hepatitis C virus infection in pregnancy and vertical transmission; Mascolo S and Tambaro O collected and analyzed the data on antiviral drugs, including animal model data.

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Abstract

Hepatitis C virus (HCV) affects about 3% of the world's population and peaks in subjects aged over 40 years. Its prevalence in pregnant women is low (1%-2%) in most western countries but drastically increases in women in developing countries or with high risk behaviors for blood-transmitted infections. Here we review clinical, prognostic and therapeutic aspects of HCV infection in pregnant women and their offspring infected through vertical transmission. Pregnancy-related immune weakness does not seem to affect the course of acute hepatitis C but can affect the progression of chronic hepatitis C. In fact, postpartum immune restoration can exacerbate hepatic inflammation, thereby worsening the liver disease, particularly in patients with liver cirrhosis. HCV infection increases the risk of gestational diabetes in patients with excessive weight gain, premature rupture of membrane and caesarean delivery. Only 3%-5% of infants born to HCV-positive mothers have been infected by intrauterine or perinatal transmission. Maternal viral load, human immunodeficiency virus coinfection, prolonged rupture of membranes, fetal exposure to maternal infected blood consequent to vaginal or perineal lacerations and invasive monitoring of fetus increase the risk of viral transmission. Cesarean delivery and breastfeeding increases the transmission risk in HCV/human immunodeficiency virus coinfected women. The consensus is not to offer antiviral therapy to HCV-infected pregnant women because it is based on ribavirin (pregnancy category X) because of its embryocidal and teratogenic effects in animal species. In vertically infected children, chronic C hepatitis is often associated with minimal or mild liver disease and progression to liver cirrhosis and hepatocarcinoma is lower than in adults. Infected children may be treated after the second year of life, given the adverse effects of current antiviral agents.

Core tip: Hepatitis C virus (HCV) infection during pregnancy is an emerging problem. While not negatively affecting acute hepatitis, it may exacerbate chronic hepatitis and worsen liver function in woman with liver cirrhosis. HCV does not affect delivery outcome apart from an increased risk of premature membrane rupture and cesarean delivery. The mother-to-child HCV transmission rate is low (3%-5%) and is related to high maternal viremia, human immunodeficiency virus (HIV) coinfection, prolonged rupture of membranes, vaginal lacerations and invasive fetal monitoring. Cesarean delivery and no breastfeeding are indicated for HIV/HCV coinfected women. Antiviral therapy is not routinely offered to pregnant women and infants because of its side effects.

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INTRODUCTION

Since its discovery in 1989, hepatitis C virus (HCV) has been recognized as a global public health problem that affects about 3% of the world's population (150-200 million people)[1,2]. In the United States, almost 4 million people have been infected by the virus and more than half of these are estimated to have chronic hepatitis C[3,4]. In most European countries, the prevalence of HCV in the general population ranges between 0.5% and 2% (*i.e.*, 5 to 10 million people)[5]; the prevalence rate peaks in subjects between 40 and 59 years old[1,3].

HCV infection can cause chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC). Acute HCV infection, which is asymptomatic in 50% to 90% of cases, can progress to chronic hepatitis in more than half of patients and can be associated with variable rates of fibrosis progression[1,3]. About 10% to 20% of patients with chronic C hepatitis develop cirrhosis 20-30 years after contracting the infection. Patients with liver cirrhosis have a risk of about 1% to 5% of developing HCC[1,3]. In addition, HCV can be associated with extra-hepatic complications such as lymphoma[4,6]. Notably, HCV-related liver cirrhosis is the major cause of liver transplantation in developed countries[1,3]. Unfortunately, the burden of liver cirrhosis, HCC and death related to HCV is expected to increase in the next few years[6], although the incidence of acute infection is declining[1,5].

HCV infection occurs after exposure to infected blood, through the parenteral and inapparent parenteral route[7]. Injection drug use, unsafe medical practices, high-risk sexual practices and birth to an infected mother are the most frequent routes of infection[3]. In most countries, only people with high risk behaviors are currently tested for HCV infection. However, the United States Center for Disease Control recommends HCV screening for all individuals born between 1945 and 1965 (irrespective of risk factors) because of the high prevalence of HCV infection in that birth cohort[8].

Despite research conducted in the last 20 years, an effective vaccine against HCV has not yet been developed. On the contrary, antiviral therapies are now available that guarantee viral clearance (also called "sustained virological response") in a remarkable percentage of patients affected by chronic hepatitis C.

An emerging problem is HCV infection during pregnancy. In fact, the incidence of pregnancies and deliveries has increased (four-fold and between 5% and 10%, respectively) in women over the age of 40 years in most western countries, including the United States[9,10]. Since these women are at a higher risk of HCV infection than younger women, physicians might have to treat an increasing number of HCV-infected pregnant women in the near future. The true size of the problem has not yet been defined; in fact, data related to the prevalence of HCV infection in pregnant women are largely discordant. Between 1% to 2% of pregnant women in the United States and Europe have been estimated to be anti-HCV positive[11-17] and more than 70% of them have HCV viremia[12,13]. The prevalence is reported to be higher in pregnant women with high risk behaviors for blood-transmitted infections (*i.e.*, intravenous drug use, multiple sexual partners, co-infection with human immunodeficiency virus (HIV), or who live in developing countries)[16-20]. Because HCV screening is recommended only for high risk subjects, a large number of infected women in the general population without classical risk behaviors or history of blood exposure eludes the screening strategy. Unfortunately, even in Italy, where a free-of-charge test for HCV, hepatitis B virus (HBV) and HIV is offered to all women from the 33th to the 37th week of pregnancy[21], many women remain untested until delivery.

Here we review the clinical, prognostic and therapeutic aspects of HCV infection in pregnant women, as well as aspects of HCV vertical infection. Specifically, we address the following topics: (1) Can pregnancy worsen HCV-related disease? (2) Can HCV increase obstetrical complications? (3) What is the risk of transmitting HCV infection to the newborn and how is it prevented? (4) What is the course of HCV infection in the newborn? and (5) What are the benefits and risks of antiviral therapy for the mother and her child?

HCV INFECTION AND PREGNANCY: RECIPROCAL EFFECTS

During pregnancy, the maternal immune system undergoes various modifications that enable tolerance of the paternal alloantigens, therefore preventing anti-fetal immune aggression[17,22,23]. In fact, consequent to these modifications, pregnant women experience a condition of immunological weakness that results in increased immunoglobulin production, a decreased T-cell mediated response (due to a shift in the Th1/Th2 balance toward the Th2 response) and expansion of regulatory T-cells[22]. Also, sex hormones and immunosuppressive cytokines produced by pregnant women may concur to modulate the immune response to HCV[22,23]. Pregnancy-associated immune modulation can also influence the immune response to HCV, thereby affecting both the maternal viral disease and mother-to-child transmission of the virus.

The innate immune system, through natural killer (NK) cells, also plays a role in modulating immune response to the virus. This process involves the interaction between the inhibitory NK cell receptor KIR2DL3, which belongs to the family of cell immunoglobulin-like receptors (KIR), and its human leukocyte antigen C group 1 (HLA-C1), which is an inhibitory receptor for self-MHC class I ligand. The effector functions of NK cells occur only when activating signals overcome inhibitory signals. Therefore, individuals with two copies of HLA-C1 alleles (HLA-C1C1) and homozygous for KIR2DL3 (which binds HLA-C1 with less affinity than

other inhibitory receptors) tend to resolve HCV infection. In these subjects, the weaker inhibitory receptor-ligand interaction is easily overridden by activating signals and results in a stronger activity of NK cells. This effect was demonstrated in Caucasians and African Americans with expected low infectious doses of HCV but not in those with high-dose exposure, in whom the innate immune response is likely to be overwhelmed[24].

Question 1: Impact of pregnancy on maternal HCV-related disease

Acute hepatitis C has been rarely reported during pregnancy[17,23,25]. Consequently, the data available are not sufficient to draw any conclusion about its course. The few reports available indicate that pregnant women with acute hepatitis C may have the same course and outcome as non-pregnant women, except for an increased risk of developing jaundice[17,23,25].

Various studies have been carried out on women affected by chronic hepatitis C who become pregnant. The results showed that serum aminotransferase levels (ALT) decrease and reach normal range during the second and third trimester of pregnancy[17,22,26,27]. The HCV viral load increases concomitant with the decrease in serum ALT and reaches a peak during the third trimester. These fluctuations, which are similar to those described in HBV-infected women during pregnancy[28], were not found in another study[23]. Only one study reported sustained clearance of HCV RNA during the second trimester of pregnancy[29]. After delivery, restoration of the maternal immune system results in a better control of HCV replication. In fact, exacerbation of chronic hepatitis C, including rebound of ALT levels and worsening liver histopathology (Knodell score, portal necrosis, lobular degeneration and inflammation) were reported in the postpartum period, together with a reduction in the plasma HCV load. It is feasible that the decrease in ALT levels and the increased HCV viral load observed in the third trimester of pregnancy in women chronically infected with HCV could be due to a pregnancy-associated decline in immune-mediated hepatocellular destruction. Indeed, expansion of CD4+ CD25+ Treg cells begins early in gestation and reaches a peak in the second trimester. CD4+ CD25+ T regulatory cells may affect the clinical presentations of chronic HCV infection by suppressing CD4+ T cell responses. Le Campion *et al*[22] and Bolacchi *et al*[30] reported that the HCV-specific TGF-β response induced by CD4+ CD25+ (high) T cells was significantly greater in patients with a normal ALT level than in patients with abnormal ALT levels. This phenomenon is the hallmark of exacerbation of hepatic inflammation[17,22] which, in some patients, can worsen the course of chronic hepatitis C[31-35] but in a few cases it can be associated with viral clearance[36], suggesting that postpartum may be an optimal time to start antiviral therapy in the attempt to achieve a sustained response.

HCV-infected pregnant women seem to develop cholestasis earlier and more frequently than anti-HCV-negative women. This phenomenon has been attributed to altered transport of sulfated hormones in the liver, a failure in the transport of toxic substances, and a defect of the bile salt export pump[16,23,37-41], but its pathogenesis is still being debated.

Lastly, HCV-infected women with advanced liver disease seem to be at a high risk of developing liver decompensation, which results in worsening of the portal hypertension and coagulopathy[42-44]. Hence, pregnancy should be strongly discouraged in these women.

Question 2: Effect of HCV infection on pregnancy and delivery

Very few studies have investigated the impact of maternal hepatitis C infection on pregnancy outcome. Although it can be difficult to separate the role of HCV from other risk factors (*i.e.*, alcohol intake, tobacco smoking and drug abuse), the data available indicate an increased risk of gestational diabetes (reported in patients with excessive weight gain), premature membrane rupture and an increased rate of caesarean delivery in HCV-infected pregnant women than in anti-HCV-negative pregnant women[16,17,22,45-47]. In addition, various obstetrical complications have been reported in HCV-infected women, namely, higher rates of preterm delivery, placental abruption, low birth weight, prematurity, low Apgar scores at 1 min, increased neonatal jaundice, congenital malformations and newborn perinatal mortality[22,45,48]. However, these findings were not confirmed in other studies[23,49,50].

Question 3: Risk of mother-to-child HCV transmission and preventive measures

Numerous studies have evaluated the risk of mother-to-child HCV transmission (vertical transmission) with conflicting results. In fact, the rates of transmission varied from 0% to 30%[11-20,22]. These large fluctuations are probably due to differences in study size (*e.g.*, the number of HCV-infected mothers enrolled), the study methodology (prospective or retrospective study; detection of maternal infection based on anti-HCV antibody positivity or on HCV RNA positivity) and the diagnostic criteria of neonatal HCV infection (*e.g.*, number of polymerase chain reactions performed and duration and timing of follow-up in the neonates)[14,15]. The rate of HCV transmission is estimated to be lower[14,15,17,23,51-57] than the rate of HBV and HIV transmission. However, unlike HIV-infected or HBV-infected pregnant women, no drugs or vaccines are available for HCV-infected pregnant women to reduce and/or prevent vertical transmission, which is the main cause of HCV infection in the pediatric setting[15,22,51]. Thus, when a HCV-infected pregnant woman asks "How can I avoid infecting my child?", the answer is unfortunately, "we do not know".

The pathogenesis of vertical transmission, specifically the timing and route of transmission of the virus, and the host's defense mechanisms are unknown. The timing of vertical transmission is based on the appearance of HCV RNA positivity in the newborn: if a neonate tests HCV-RNA-positive at delivery or within the first 3 d of life, he/she was probably infected *in utero* (intrauterine

Table 1 Clinical factors and risk of vertical transmission

Associated with vertical transmission

  Pregnant woman:
    High HCV viral load
    Elevated ALT levels before pregnancy
    HIV-HCV co-infection iv drug abuse[1]
  Obstetric procedures:
    Prolonged rupture of membranes vaginal and/or perineal lacerations
    invasive monitoring of fetus intrauterine pressure catheter
    amniocentesis (debated)
  Father HCV infection[1]
  Fetus gender[1]
Not associated with vertical transmission
  Maternal HCV genotype
  Mode of delivery[2]
  Breastfeeding[2]

[1]To be confirmed; [2]Except in the presence of HIV-HCV coinfection. HCV: Hepatitis C virus; HIV: Human immunodeficiency virus.

transmission); if a neonate who tests HCV-RNA-negative in the first 3 d of life becomes positive, he/she was probably infected in the *peripartum* or *postpartum* period (perinatal transmission)[22,23]. The data available support both intrauterine[23,58,59] and perinatal transmission[22,23,53,60,61], the former accounting for 30% of cases and the latter for 40-50% of cases. Many sites of human placenta could act as HCV-receptors and/or entry cofactors (*e.g.*, claudin-1, occludin, SR-B1, LDLr or DC-SIGN)[22,23]. Consequently, they could be directly implicated in HCV infection of placental cells. However, the rate of vertical transmission (3%-5%) seems to be lower than the potential biological exposure of fetus/neonate to maternal HCV. In fact, in the case of intrauterine transmission, although maternal HCV RNA has not been detected in amniotic fluid[11], a very large amount of virus (1×10^{13} to 1×10^{14} virions) has been estimated to reach the placental bed during gestation[11]. Therefore, the fetus could be exposed to free virions or to the cell-associated virus that crosses the placenta[22] in a percentage higher than the reported transmission rate (30%)[22,62]. Also, in the case of perinatal transmission, leakage of maternal HCV-infected blood into the fetal bloodstream and/or labor trauma (which exposes the offspring to maternal HCV-infected blood)[15] occurs more frequently than vertical transmission of the virus (40%-50%)[15,22].

The reason for the low rate of vertical transmission is not known. Various biological and immunological factors could protect the fetus against HCV infection, *i.e.*, placental immune cells, fetal cellular adaptive immunity, fetal plasma inflammatory markers, maternal HLA class II alleles and IL-28B genotype[22,60,63-65], but their role is poorly understood. Also, the suggested association with gender (girls seem to be infected twice as often as boys) could reflect biological differences in susceptibility or in the response to infection[66], although this has yet to be confirmed.

Many factors related to the status of pregnant women and delivery/obstetrical practices have been associated with an increased risk of transmission (Table 1)[11,16,17,22,23].

The first factor is the mother's viral load, especially at the time of delivery. In fact, there is evidence that HCV-RNA-negative mothers have a low risk of infecting their infant, whereas this risk increases in HCV-RNA-positive mothers parallel to increases in levels of viral load above 10^5 IU/mL[60,67] and reaches a maximum in women who have viremia levels above 10^7 IU/mL[68]. Moreover, a high maternal serum ALT level in the 12 mo before conception and/or at the time of delivery has recently been associated with a higher rate of vertical transmission. In fact, a high ALT level is a hallmark of high viral replication in both the maternal bloodstream and in mononucleated blood cells[22,54,55]. However, the effects of maternal HCV disease activity on vertical transmission are not completely understood. Lastly, HCV genotype is not considered a significant risk factor in terms of vertical HCV transmission[22].

The second factor is HIV co-infection, which can cause a three-four fold increase of the risk of mother-to-child-transmission[16,22,69]. How HIV-1 infection enhances the rate of HCV transmission is unclear. It is conceivable that HIV-1 infection facilitates HCV entry and replication in peripheral mononucleated blood cells[16,22,69].

Moreover, HIV induces immune suppression, which can result in a less effective HCV-specific innate or cell-mediated maternal immune response at the maternal/fetal interface[16,22]. On the other hand, HIV can infect trophoblasts, thereby compromising the integrity of the placenta and enhancing the passage of HCV through this barrier. HIV-associated chorioamnionitis could also induce placental microtransfusions through which HCV infection can be transmitted to the fetus[22,69]. Lastly, antiretroviral treatment of HIV/HCV co-infected pregnant women can dramatically lower the risk of HCV transmission from 19% to 8%[16]. Additional risk factors, namely, intravenous drug abuse by the mother and concomitant HCV infection of the father, have been proposed but have yet to be confirmed[16].

The main obstetrical factors associated with the risk of vertical transmission are prolonged rupture of membranes (more than 6 h before delivery), exposure of the fetus to maternal infected blood during vaginal delivery (consequent to vaginal and/or perineal lacerations) and invasive monitoring of the fetus with scalp electrodes or intrauterine pressure catheter placement[11,16,22,60]. Amniocentesis may contribute to the risk of maternal-to-fetus transmission[17,22], although its impact is still being debated[17]. On the contrary, neither the delivery mode nor breastfeeding (two main concerns for the pregnant woman) appear to influence the risk of transmission[11,16,17,22,66,70,71] in HCV-infected women. In fact, a cohort study of 1787 mother-child pairs showed that the rate of vertical HCV transmission was 6.2% and was not influenced by caesarean section[66]. The failed protective effects of cesarean delivery was confirmed in a meta-analysis study[72]. The issue of breastfeeding is more complex and needs to be discussed with the mother. In fact, although the amount of HCV in maternal milk and colostrum is very low and probably inactivated in the infant'

s digestive tract, the presence of cracked or bleeding nipples can be a contraindication to breastfeeding because it can expose the infant to contaminated milk. On the other hand, cesarean delivery must be recommended for HIV/HCV co-infected patients, associated with antiretroviral therapy to prevent or to reduce the risk of transmission of both viral agents[54]. Vaginal delivery and breastfeeding are contraindicated in HIV/HCV co-infected mothers[16,54,71,73].

Question 4: Outcome of HCV infection in the newborn

HCV infection is the most common cause of chronic hepatitis in childhood. The prevalence of pediatric infection seems to be very low in the United States and Europe (0.05%-0.36%), while it increases (1.8%-5.8%) in some developing countries and reaches its highest prevalence in Egypt, Sub-Saharan Africa, the Amazon Basin and Mongolia; the highest prevalence worldwide has been reported in Egypt (9% and up to 50% in certain rural areas)[22,74]. Vertical or perinatal transmission is the most common route of pediatric HCV infection[11] and can lead to an estimated 10000-60000 cases per year[11].

At the time of delivery and during the first year of life, the anti-HCV positivity detected in the newborn can be due to the passive transfer of maternal antibodies. Therefore, the diagnosis of HCV infection based on antibody assays in children of HCV-infected mothers before the age of 12 mo is not reliable[75]. The diagnosis can be made by testing neonates for HCV RNA, preferably 1 or 2 mo after birth[76]. Indeed, the sensitivity of PCR for HCV RNA is about 22% at birth and increases to 70%-85% 1 mo after birth. Similarly, the predictive positive value of PCR testing is 33% at birth and reaches 78% when the child is 9 mo old[77]. These findings could reflect the very low viral loads in the first month of age and/or the incubation period of HCV that ranges from 2 wk to 6 mo[11]. Notably, a negative PCR test at birth/first month of age cannot exclude HCV infection and must be confirmed by further testing.

Spontaneous clearance of HCV has been reported in up to 25%-30% of HCV-infected children[78,79] irrespective of the route of infection (vertical or parenteral transmission). However, the rate of chronicity seems to be higher in infants with perinatally acquired HCV infection than in infants infected by parenteral transmission[80-83]. Various factors are associated with HCV clearance, namely, a younger age of the child, normal ALT levels[84], the IL-28B genotype[74] and IFN-γ responses against structural and non-structural recombinant HCV antigens[85]. The clinical course of chronic HCV infection in childhood seems to differ from that in adulthood. Pediatric HCV infection is associated with minimal or mild liver disease. In fact, advanced liver damage is uncommon[86-88], although another study suggested that fibrosis can be severe in children despite the relatively short duration of infection[89]. Progression of liver damage in children depends on viral load, serum ALT levels, gender, ethnicity, obesity, toxins, environmental factors and co-morbid risk factors (hemolytic anemias, chemotherapy for malignancy, immu-

nosuppression and concomitant HIV or HBV infection) and genetic factors such as the IL-28B genotype[90].

Differently from adults, data about the rate of progression from cirrhosis to HCC in childhood and early adolescence are scarce but it seems that HCC is rare in children with HCV infection[91] and the number of HCV-infected children requiring liver transplantation is low in developed countries[91]. Long-term studies are required to quantify the incidence of cirrhosis and HCC in adults who acquired hepatitis C infection by vertical transmission.

In childhood, membranoproliferative glomerulonephritis is one of the most frequent extra-hepatic manifestations of chronic HCV infection but, unlike adults, neither cryoglobulinemia nor lymphoma have been reported in children[91]. The involvement of the central nervous system in HCV-infected children could explain the developmental delay, learning disorders and cognitive deficits that have been reported in some cases[92,93].

Question 5: Antiviral therapy of hepatitis C in pregnant women and infants

The last, but not least, question regards the treatment of HCV infection in both the pregnant woman and the newborn. While some anti-HBV and anti-HIV drugs can be safely used to prevent or reduce the risk of vertical transmission, the two cornerstones of the standard-of-care treatment for HCV infection, namely, pegylated interferon (PEG IFN) and ribavirin (RBV), have several side effects or contraindications that limit their use during pregnancy and childhood[11,16].

The problem of therapy in HCV-infected pregnant women is not negligible. In fact, in a United States study of 45690 HCV-infected patients, pregnancy was the third most common contraindication (1.9%) to treatment, after bipolar disorders (6.5%) and anemia (5.9%). In addition, about 1.3% of women became pregnant during a median follow up of 33 mo[94]. Consequently, the concern is not only about the indication of antiviral therapy for pregnant women but also how to manage a woman who becomes pregnant during antiviral therapy. The answers to these issues are mainly based on limited clinical data. Recombinant interferon alpha is classified by the United States Food and Drug Administration in pregnancy category C. In fact, given its abortifacient effect in animals[1], it could have the same effect in humans[95,96] as PEG INF[97,98]. In fact, abortifacient effects have been observed in Macaca mulatta (rhesus monkeys) treated with interferon alpha-2b or alpha 2a during the early to middle fetal period of organogenesis (gestation day 22 to 70). Abortifacient activity was also observed in pregnant rhesus monkeys treated (500 times the human dosage) during late fetal development (days 79 to 100 of gestation). These drugs may impair fertility. In fact, in nonhuman primates, menstrual cycle irregularities, *i.e.*, prolonged or shortened menstrual periods and erratic bleeding (anovulatory cycle) have been observed and the females returned to a normal menstrual rhythm after discontinuation of therapy. Decreased serum estradiol and progesterone concentrations have been reported in

women treated with human leukocyte interferon. No mutagenic effects or toxicity has been reported. However, due to the species specificity of interferon, the effects in animals are unlikely to be predictive of those in man. Lastly, the injectable solution contains benzyl alcohol that can be transmitted *via* the placenta and could be toxic in premature infants. No effect on male fertility has been reported[95,96]. No studies on the teratogenic effect of PEG-IFN are available. Since non-pegylated interferon alpha resulted in a statistically significant increase in abortions of Macaca, PEG-IFN should also be assumed to have abortifacient potential. There are no well-controlled studies in pregnant women[97,98].

Nevertheless, in clinical practice, IFN-alpha is used to treat essential thrombocythemia in pregnant women to prevent or lower the risk of thrombocythemia-related fetal loss[99]. A systematic review of data about pregnancies exposed to IFN-alpha (60% of women had received IFN throughout pregnancy) showed that IFN did not significantly increase the risk of major malformation, miscarriage, stillbirth or preterm delivery above the rates observed in the general population[99]. Therefore, the treatment of HCV-infected pregnant women with IFN does not seem to entail a risk for the offspring. Data on PEG-IFN treatment during pregnancy are lacking. It is not known whether IFN is excreted in human milk. Given the potential for serious adverse reactions in nursing infants, a decision should be made whether to discontinue nursing or to discontinue the drug based on the importance of the drug for the mother. The main concern of IFN therapy is not only the risk for the fetus, but also the risk of serious psychiatric side effects, namely exacerbation of postpartum depression[11]. Therefore, all pregnant women who are candidates for PEG-IFN must be carefully selected, also considering their psychological and psychiatric conditions.

The other drug available for HCV infection is RBV, which is classified by the USA Food and Drug Administration in pregnancy category X[100,101]. Ribavirin is absolutely contraindicated, not only for HCV-infected pregnant and childbearing women, but also for HCV-infected men whose partners may become pregnant. These subjects are recommended to take contraceptive measures during RBV therapy because of its significant embryocidal and teratogenic effects[1] in animals[100-102]. Malformations of the skull, palate, eye, jaw, limbs, skeleton and gastrointestinal tract have been described in the offspring of female animals that have been directly exposed to the drug[102]. In addition, RBV caused cell toxicity, mutagenicity and a decrease in the epididymal sperm count in all the animal species studied; these sperm cell mutations are believed to cause cell death or to be associated with infertility[102].

In HCV-infected treated men, the RBV concentration was two-fold higher in seminal fluid than in serum[103]. In addition, the round cell/spermatozoa ratio (suggestive of spermatogenic abnormality) and the sperm DNA fragmentation index were significantly higher in a HCV-infected man during RBV therapy and returned to base-line levels only four and eight months, respectively, after treatment withdrawal[104]. All these data indicate the need to avoid pregnancy for longer than the recommended 6 mo after discontinuing RBV treatment in men[102].

Only a few cases of direct or indirect exposure to RBV have been reported in pregnant women and these resulted in healthy infants and no miscarriages or elective terminations[102,105-110]. However, it is difficult to quantify the true risk of direct or indirect exposure to RBV. Consequently, a Ribavirin Pregnancy Registry was established in 2003 to monitor pregnancy exposure to RBV. Between 2003 and 2009, 118 live births from mothers exposed to RBV (49 direct and 69 indirect exposures) were recorded. Birth defects were reported only in 6 cases (3 direct and 3 indirect exposures): torticollis (2 cases), hypospadias (1 case), polydactyly and a neonatal tooth (1 case), glucose-6-phosphate dehydrogenase deficiency (1 case), ventricular septal defect, and cyst of the 4th ventricle of the brain (1 case). Although these preliminary results did not indicate that RBV exerts a teratogenic effect, it is not possible to draw conclusions about the risk of direct or indirect prenatal exposure to the drug in humans[102].

More recently, new therapeutic approaches targeting essential components of the HCV life cycle have been developed, including the protease inhibitors (boceprevir, telaprevir) and polymerase inhibitor (sofosbuvir), indicated mainly for the treatment of chronic hepatitis C patients infected by genotype 1 virus.

Boceprevir and telaprevir are classified by the United States Food and Drug Administration in the Pregnancy Category B[111,112]. In fact, although no adequate and well-controlled studies are available in humans, the absence of negative effects on fetal development in animals (mice, rats and rabbits) seems to indicate *"no evidence of risk in humans"*[2], although the chance of fetal harm still remains possible. Boceprevir did not cause genotoxicity in *in vitro* or *in vivo* assays, including bacterial mutagenicity, chromosomal aberration in human peripheral blood lymphocytes and mouse micronucleus assays[111]. Nevertheless, reversible effects on fertility and early embryonic development in female rats have been reported, as well as decreased fertility in male rats, most likely due to testicular degeneration. No effects on fetal development have been observed in rats or rabbits exposed to boceprevir at doses higher than the recommended dosage in humans[111]. A clinical study showed the absence of testicular toxicity in humans[107] at the recommended clinical dose, while a decrease in percent motile sperm and an increase in non-motile sperm count occurred in rats at exposures 0.3-fold the human recommended clinical dose[111]. Telaprevir did not result in fetal harm in mice or rats. The effects on fertility parameters in rats (*e.g.*, decreased percent motile sperm and increased non-motile sperm count) may be associated with testicular toxicity in male animals. Telaprevir did not affect the birth body weight of rat offspring[112].

Since these drugs cannot be used as monotherapy but have to be associated with PEG-IFN and RBV (triple therapy), their use is contraindicated during pregnancy

and childbearing females have to take adequate contraceptive measures. Similarly, the excretion of protease inhibitors into human breast milk is not known yet; in lactating rats, the levels of both boceprevir (or its metabolites) and telaprevir in the milk were slightly higher than levels observed in maternal blood[111,112]. Because of the potential adverse reactions in infants, nursing must be discontinued prior to starting the treatment[111,112].

The last licensed polymerase inhibitor (sofosbuvir[1]) is classified by the United States Food and Drug Administration in the pregnancy category B. In this case also, adequate and well-controlled studies with the drug in pregnant women are missing but no effects on fetal development have been observed in rats and rabbits at the highest doses tested[113]. Similarly, no data are available on the excretion of sofosbuvir and/or its metabolites in human breast milk; no data are available for the pediatric setting[113]. When used in triple therapy (associated with PEG IFN and RBV), sofosbuvir is contraindicated as well as the protease inhibitors; when used in regimen IFN and RBV-free, it could be a promising option in the treatment for the pregnant women.

The therapy of HCV-infected infants is still being debated in the absence of a consensus on when or how to optimally treat. Because of the low rates of vertical transmission (overall between 3% and 5%) and the favorable course of hepatitis C (relatively high rate of spontaneous resolution of HCV infection, the lack of symptoms, *etc.*) in the first class of age, the rationale is to only treat the child with chronic hepatitis C at high risk of progression[11]. A previous meta-analysis showed that children had a higher SVR and tolerated IFN alpha monotherapy better than adults[114]. In contrast, there are few pediatric trials on the standard of care therapy; a systematic review of 4 randomized controlled trials and 31 non-randomized studies showed that children had an SVR similar to adults[115]. The standard of care therapy seemed to be well tolerated in the large majority of children; the main adverse effects (*i.e.*, flu-like symptoms and neutropenia) were mild or moderate. The rate of treatment discontinuation was low but half of the children required a reduction of PEG-IFN dosage[115].

The decision of when to start antiviral therapy in the early ages must be based on several factors: the estimated/known duration of infection, HCV genotype, presence/degree of fibrosis, co-morbidities, predicted parents' compliance with the therapy, expected adverse events and possible interference with home life or school activities and the IL-28 genotype[111]. Injectable solutions of IFN contain benzyl alcohol and are not indicated for use in neonates or infants because of reports of death in neonates and infants exposed to excessive doses of benzyl alcohol[91-94].

It has been suggested that treatment with weight-adjusted doses of PEG-IFN and RBV should be offered to HCV-infected children over 2 years old and with significant hepatic fibrosis (detected by liver biopsy or transient elastography), irrespective of HCV genotype[116]. Moreover, such treatment should be avoided in children under

2 years of age because of the risk of PEG-IFN-related neurotoxicity[115] and growth suppression described in older children[11,116]. In fact, PEG IFN-a-2a has an inhibitory effect[1] on children's growth. A study of 31 Japanese children showed that the Z-scores of height and body weight decreased during treatment and, although they improved after withdrawal, they were significantly lower than pre-treatment scores. This growth inhibitory effect was smaller in children aged 10 years and older[116].

Antiviral therapy for hepatitis C can be routinely offered to all HCV-infected newborns only when new drugs with a well demonstrated long-term safety profile become available[11], but at the moment both the safety and effectiveness of protease or polymerase inhibitors in pediatric patients have not been established.

CONCLUSION

The problem of HCV infection in pregnancy is still a matter of concern. The first concern is the possible impact of HCV infection on the mother's health during pregnancy and in the postpartum period due to the intense physiological changes and the virus/host interaction that characterize this period. Pregnancy-associated immune modulation affects the immune response against HCV because it leads to immune tolerance during pregnancy and immune restoration immediately after delivery. This phenomenon does not seem to impact negatively on liver disease in most pregnant women but may worsen liver function in some cases. Differently, childbearing women with HCV-related liver cirrhosis are at high risk of liver decompensation during pregnancy.

The second concern is the impact of HCV on delivery outcome. HCV-infected women may have an increased risk for premature membrane rupture and for cesarean delivery but there is no evident risk for complications for offspring.

The main concern is that HCV-infected women may transmit the infection to their offspring during pregnancy, upon delivery or during breastfeeding. The overall rate of vertical transmission is low (3%-5%) but the risk is higher for women with high viremia or HIV co-infection and in the case of exposure of the neonate to infected blood (*i.e.*, during prolonged rupture of membranes or vaginal lacerations and consequent to invasive monitoring of the fetus during pregnancy). Cesarean delivery, which limits the exposure to vaginal/perineal lacerations, was formerly suggested to avoid this risk of transmission.

However, it is currently recommended only for HIV/HCV coinfected women. The problem of breastfeeding is complex and must be discussed with the woman. In fact, the risk is not due to milk or colostrum (which contain a very low amount of virus and can be inactivated in the infant's digestive tract) but to contamination by infected blood through damaged or cracked nipples. HIV/HCV co-infected pregnant women are recommended to avoid vaginal delivery and breastfeeding because of the high risk of infecting their offspring.

The last concern is antiviral therapy. Currently, the

consensus is not to routinely offer antiviral therapy to all HCV-infected pregnant women and HCV-infected offspring. Given the side effects of the drugs available in these settings, candidates for therapy must be carefully selected based on the benefits of therapy and the severity of the disease. The ideal solution would be to encourage young women infected with HCV to start and complete therapy before pregnancy in order to lower or clear the virus and so reduce the risk of vertical transmission. Another strategy would be to start treatment postpartum and to avoid breastfeeding.

In the childhood setting, the standard of care therapy should be started only after the second year of life, except in cases that require immediate treatment to avoid rapid progression of liver disease.

The recently approved new generation drugs (protease and polymerase inhibitors) for the treatment of HCV infection have opened a new perspective in HCV therapy for pregnant women and infected infants since one of these agents, *i.e.*, sofosbuvir, has been reported to also be effective in IFN-free and RBV-free regimens.

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REVIEW

From portal to splanchnic venous thrombosis: What surgeons should bear in mind

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Abstract

The present study aims to review the evolution of surgical management of portal (PVT) and splanchnic venous thrombosis (SVT) in the context of liver transplantation over the last 5 decades. PVT is more commonly managed by endovenous thrombectomy, while SVT requires more complex technical expedients. Several surgical techniques have been proposed, such as extensive eversion thrombectomy, anastomosis to collateral veins, reno-portal anastomosis, cavo-portal hemi-transposition, portal arterialization and combined liver-intestinal transplantation. In order to achieve satisfactory outcomes, careful planning of the surgical strategy is mandatory. The excellent results that are obtained nowadays confirm that, even extended, splanchnic thrombosis is no longer an absolute contraindication for liver transplantation. Patients with advanced portal thrombosis may preferentially be referred to specialized centres, in which complex vascular approaches and even multivisceral transplantation are performed.

Core tip: The present study aims to review the evolution of surgical management of portal and splanchnic venous thrombosis in the context of liver transplantation.

INTRODUCTION

Portal vein thrombosis (PVT) has been described as a multi-factorial condition resulting from the combination of both inherited and acquired factors[1]. Cirrhosis represents the most common etiologic factor, accounting for up to 24%-32% of cases[2]. Other common causes include cancer, infection, inflammation and thrombophilic disorders.

The incidence of PVT also correlates with the severity of cirrhosis[3], thus being a common problem during liver transplantation (LT). PVT usually arises within the liver and extends downwards into the extra-hepatic portion of the portal vein (PV). In some cases the thrombosis further extends to the mesenteric branches resulting in

Venous involvement	Grade 1	Grade 2	Grade 3	Grade 4
PV	< 50%	> 50%	Complete	Complete
"Proximal" SMV	± Minimal	± Minimal	Complete	Complete
"Distal" SMV	None	None	None	Complete

Figure 1 Portal vein thrombosis classification according to Yerdel et al[3]. PV: Portal vein; SMV: Superior mesenteric vein.

a splanchnic venous thrombosis (SVT).

Until the late 1980's, PVT and SVT were considered contra-indications for LT due to concerns about compromised portal allograft inflow.

The first successful LT in a patient with PVT was reported by the Pittsburgh group in 1985 using a free iliac vein allograft[4]. Two years later, the same group presented the first large series of LT in patients with PVT (*n* = 22), representing a landmark paper in this field[5]. Since that seminal experience, several new techniques have been proposed to overcome this problem. The present study reviews the surgical evolution in this field of LT over the last five decades.

DIAGNOSIS AND CLASSIFICATION

Despite progress in preoperative and cross-sectional imaging, a substantial number of cases of PVT or SVT are still discovered at the time of LT[6,7]. Doppler-ultrasound examination remains the most common initial diagnostic tool. However, it has limitations in detecting thrombosis due to (spontaneous or medical) recanalization and because of thrombus extension to the mesenteric veins, which cannot always be visualised clearly. Therefore, computed tomography and magnetic resonance angiography have an important role in diagnosing this condition[8]. The presence of arterial enhancement in contrast-enhanced ultrasound may help differentiate between malignant and benign thromboses[9].

The sensitivity in detecting complete venous thrombosis ranges from 92% to 100%, decreasing to 14%-50% in partial thrombosis[10]. The preoperative identification of PVT enables surgical planning and the exclusion of patients with malignant thrombosis from listing for LT. Several classifications have been proposed so far; the Yerdel classification gained the greatest acceptance and widespread clinical application[3] (Figure 1). Grade I and II PVT can almost always be managed by portal vein resection with or without thrombectomy; grade III and IV PVT require a more complex technique (Figure 2).

Management of grade I - II PVT

The initial strategy for grades I - II PVT is the removal of the thrombus. This is best done by removing it together with the innermost layer of the vessel (thrombo-endovenectomy)[3]. If the thrombosis involves a short segment of the PV, this can be resected; the residual part of the thrombus can also be fixed to the vessel wall[3]. The thrombus is separated from the PV wall using an endarterectomy spatula and the thrombus is freed under direct vision whilst everting the vessel wall[11-13]. Thrombi extending up to the mesenteric vein can be extracted successfully with this technique. Blind extraction using

Figure 2 Algorithm for the management of portal and splanchnic vein thrombosis during liver transplantation. CPHT: Cavo-portal hemitransposition; CPHT E-E: End-to-end cavo-portal hemitransposition; CPHT S-E: Side-to-end cavo-portal hemitransposition; ETEV: Eversion thromboendovenectomy; IVC: Inferior vena cava; MC: Mesocaval shunt (spontaneous or surgical); PV: Portal vein; PVT: Portal vein thrombosis; RPA: Reno-portal anastomosis; SMV: Superior mesenteric vein; SR: Splenorenal shunt (spontaneous or surgical); ST: Splanchnic tributary (coronary, gastroepiploic vein). From Paskonis *et al*[43], with modifications.

vascular clamps should be avoided as it can rip the vessel, which may result in uncontrollable bleeding, especially at the level of the pancreatic head. The completeness of the thrombectomy can be verified by restoration of an adequate portal blood flow (Figure 3).

Eversion thromboendovenectomy (ETEV) is another surgical technique applicable to type I-III thromboses. Type IV thrombosis can only be occasionally treated with this technique, but typically requires more complex procedures. With ETEV, the clot is progressively and circumferentially freed with the aid of a tonsil clamp by everting the venous wall, and clamping the free edge of the clot with a tonsil. Some authors consider ETEV a risky technique, as a piece of diseased venous wall with thrombogenic potential is left in place[14].

Pan *et al*[15] described a modification of ETEV, called improved eversion thrombectomy, in which 1 cm of the anterior wall of the PV is cut, with the final removal of the smooth wall of PT after clot removal. This technique was reported in 23 type I-III cases, with no PVT recurrences or post-operative deaths.

Several single-centre series have been reported in relation to the treatment of grade I-II PVT[14-19]. A large review of 1957 LT recipients with PVT[10] showed that thrombectomy and/or thromboendovenectomy with end-to-end portal anastomosis was the most frequently used technique (75% of cases) with a very low risk of

PVT recurrence and complications.

Management of grade III PVT

In the case of type III PVT, ETEV alone can be insufficient, due to involvement of the distal portion of the SMV[15]. If portal flow is insufficient, different options can be considered in order to establish an adequate portal flow (> 600 mL/min). Porto-systemic shunt collaterals can be suture-ligated; in the case of spontaneous or surgical spleno-renal shunt, the left renal vein can be divided[20]. Sometimes a reno-portal anastomosis using a free iliac vein graft between the left renal vein and the PV (end-to-side or end-to-end anastomosis) can provide adequate portal inflow[21]. Another technique in grade III PVT may consist of anastomosing (eventually with a venous graft) the PV to recipient collaterals (coronary or choledochal veins). All these techniques can be considered when the PV is found to be a small fibrotic vessel.

A jump graft can be used in cases in which a low dissection of the retro-pancreatic PV or distal SMV part is required. This method avoids hazardous dissection with potential fatal bleeding and risk of pancreatitis[5,22].

Rodríguez-Castro *et al*[10], adopted venous interposition grafting between donor and recipient PV in 158 cases (8.4%), which represents the second most commonly used surgical technique after thrombectomy/thromboendovenectomy.

Figure 3 Eversion venous thrombectomy technique. A and B: Schematic representation of the manoeuvre; C: Intraoperative image of thrombectomy procedure; D: The thrombus removed from the portal vein. Modified from Lerut *et al*[13]. Figures from the experience of Prof. Jan Lerut.

Figure 4 Intraoperative image of a large coronary vein and a thrombosed portal vein. Figure from the experience of Prof. Jan Lerut.

Kim reported 50 cases of living donor LT with PVT: in one case (2.4%) of partial PVT, the PV was reconstructed using a cryopreserved interposition graft after resection of a thrombosed segment; in 3/7 cases of total PVT, the distal SMV or coronary vein were used for the inflow using a jump graft; two patients with SVT, both needed jump grafts[17].

Management of grade IV PVT

Up to 15 years ago, patients with diffuse SVT were not considered for LT. More recently, 5 different surgical techniques to restore portal inflow have been suggested: (1) anastomosis to a patent splanchnic tributary (APST); (2) PV arterialization (PVA); (3) reno-portal anastomosis (RPA); (4) cavo-portal hemitransposition (CPHT); (5) hepato-intestinal or (6) multi-visceral transplantation (MVT).

APST represents, when feasible, the preferred approach in the case of SVT as it is the "easiest" to perform. This technique was initially described by Lerut *et al*[5] and Hiatt *et al*[23]. In the review by Rodríguez-Castro *et al*[10], 49 (2.4%) cases of APST were described; the reported series rarely contain more than 5 cases[3,24]. Virtually any large collateral (2 cm of diameter or more) can suffice to supply the graft; these are mostly a bile duct varix or a middle colic or coronary (left gastric) veins (Figure 4). The venous flow must be tested before implanting the graft to ensure adequate inflow. An interposition graft is sometimes necessary[25]. Particular care must be taken when suturing these variceal structures to the donor portal vein.

PVA is a simple method to restore the portal blood flow into the graft, anastomosing the PV of the graft to the hepatic or gastro-duodenal artery or aorta using an iliac interposition graft. This revascularisation procedure is well documented in surgery for portal hypertension[26] and post-LT arterial thrombosis[27] or, more commonly in the setting of PVT during LT[28-35]. It is occasionally used to deal with early PVT complicating LT. Here, PVA is usually associated with PV thrombectomy. PVA has been reported once in a case of auxiliary heterotopic LT[36].

The PV can be directly anastomosed to the recipient hepatic artery[28,29,32,33], or anastomosed to the supra- or infra-renal aorta with an interposition graft from a segment of donor iliac artery[28-31]. In one case, PV was anastomosed to the accessory right hepatic artery originating from the superior mesenteric artery[34]. However, this is associated with significant mortality due to haemorrhage, right heart failure, acute[28,32] and secondary PVT[28,30-34]. Some patients developed graft fibrosis due to modified hepatic microcirculation[28,32], right-sided heart decompensation[29] as well as persistent portal hypertension due to "over-arterialization"[28,32]. Experimental syngenic rat

Figure 5 Examples of different techniques of portal flow reconstruction during cavo-portal hemitransposition. A: End-to-end cavoportal anastomosis; B: Side-to-end cavoportal anastomosis with retro-hepatic caval vein constriction; C: End-to-side cavoportal anastomosis using vein interposition graft distal to the conventional portal vein anastomosis. Modified from Paskonis *et al*[43]. Figures from the experience of Prof. Jan Lerut.

models confirmed that PV "over-arterialization" during LT can lead to liver fibrogenesis[37]. A possible solution for this problem is the surgical modulation of the arterialized portal inflow. There is, however, no agreement regarding the "ideal" flow to aim at in this setting; some authors propose 0.6-0.8 L/min[33], others 1 L/min[30] or even 1.5-1.8 L/min[32]. The calibration of arterio-portal anastomosis can also be done either surgically or radiologically using coil embolization of the artery anastomosed to the PV[29,36]. In the 3 cases in which calibration of PVA was performed either surgically or radiologically favourable outcome were observed, with a follow-up up to 36 mo[29,36]. However, the progressive aneurysmal dilatation of intrahepatic portal branches and the possible risk of liver fibrosis suggest that PVA should be used only exceptionally.

RPA was originally reported by Sheil *et al*[38], and subsequently modified with a venous interposition graft by Bhangui *et al*[39] and Kato *et al*[40]. This approach represents a good option in grade IV PVT when engorged collateral vessels are unavailable or when their blood flow is inadequate. However, the liver pathophysiological consequences of this reconstruction are not yet clearly elucidated[41,42]. This method, when possible to adopt, is safe[43]. Until now, RPA has been reported in about 50 cases worldwide; however, only a handful of series contain more than five cases[39,40].

After identification and control of the renal vein, a free iliac vein can be anastomosed either end-to-end or side-to-end to the renal vein. Next, the renal vein itself or

the interposition graft will be anastomosed to the donor PV. Kocherisation of the duodenum can be useful when the renal vein has a lower location.

This technique also has some complications such as ascites, renal dysfunction, GI-bleeding, deep venous thrombosis and oedema of lower limbs. These events are all due to a persistent portal hypertension that is resolved only partially by this technique[44].

CPHT represents an exceptional technique to overpass an extensive splanchnic venous thrombosis. The inflow from IVC is used to perfuse the PV of the allograft. CPHT was developed in animal models for the treatment of some metabolic diseases[45-47]. The first human series were performed by Starzl *et al*[48] and Riddell *et al*[49] for glycogen storage disease. In 1998, Tzakis *et al*[50] reported a series of nine cases of CPHT performed during LT due to diffuse PVT. To date, 107 cases have been reported worldwide (Table 1) with the largest series described by Tzakis ($n = 23$)[14,15,24,50-80].

CPHT can be performed either as an end-to-end or an end-to-side anastomosis between IVC and PV; with the latter carried out in the case of IVC sparing hepatectomy (Figure 5). Both connections may require the use of an interposition graft. In the end-to-side anastomosis, the IVC is best ligated in order to redirect the systemic venous blood flow to the allograft. This procedure carries a high mortality [36 (33.6%) patients] mainly due to sepsis and multiple organ failure. The longest reported survival is 139 mo.

Postoperative complications are mainly related to

Table 1 Cavoportal hemitransposition: Experiences worldwide

Ref.	Year	Country	Pts	Post-LT			Mortality	Survival (mo)[1]
				Variceal bleeding	Cavo-portal thrombosis	Severe renal failure		
Weeks *et al*[52]	2000	United States	1	0	1	0	0	20
Varma *et al*[53]	2000	United States	1	0	0	0	0	12
Olausson *et al*[55]	2001	Sweden	6 (1[2])	1	2	2	1	13
Santaniello *et al*[56]	2001	Italy	1	1	0	1	0	9
Bakthavatsalam *et al*[58]	2001	United States	1[2]	0	0	0	0	12
Urbani *et al*[59]	2002	Italy	6 (2[2])	1	1	1	1	23
Gerunda *et al*[60]	2002	Italy	2	2	0	2	1	12
Azoulay *et al*[61]	2002	France	8	2	0	1	3	37
Shrotri *et al*[62]	2003	United Kingdom	1	0	1	0	0	12
Kumar *et al*[63]	2003	United Kingdom	1	0	0	0	0	24
Verran *et al*[64]	2004	Australia	1[2]	0	0	0	0	6
Bertelli *et al*[66]	2005	Italy	1	1	0	0	0	84
Wang *et al*[67]	2005	China	1	0	0	0	0	6
Ozden *et al*[68]	2006	Turkey	1[2]	0	0	0	0	13
Lipshutz *et al*[69]	2006	United States	7 (1[2])	0	0	0	2	96
Egawa *et al*[70]	2006	Japan	1	0	0	1	1	0
Lladó *et al*[24]	2007	Spain	1	0	0	0	1	1
Selvaggi *et al*[71]	2007	United States	23	7	6	3	13	112
Li *et al*[72]	2008	China	1	0	1	0	0	18
Yan *et al*[73]	2008	China	3	1	0	0	1	48
Pan *et al*[15]	2009	China	1	0	0	0	1	-
Tao *et al*[16]	2009	China	2	0	1	0	0	-
Gao *et al*[74]	2009	China	2	1	0	0	2	6
Campsen *et al*[75]	2010	United States	10	0	0	0	0	-
Suarez *et al*[76]	2010	Spain	4	-	-	-	2	-
Ravaioli *et al*[77]	2011	Italy	6	0	0	0	1	-
Shi *et al*[78]	2011	China	1	0	0	0	0	-
Lai *et al*[79]	2012	Belgium	8	3	4	2	5	139
Chmurowicz *et al*[80]	2013	Poland	1[2]	0	0	0	0	-
Total			103 (7[2])	20	17	13	35	

[1]Maximum survival in the series; [2]Retransplantation. LT: Liver transplantation.

anastomotic thrombosis or stenosis, congestion of the inferior vena cava (IVC) and incompletely resolved portal hypertension. Complications related to IVC congestion are mild to severe oedema of the lower torso and limbs and renal dysfunction. Mild renal dysfunction, observed in almost all patients, usually resolves spontaneously without the need for haemodialysis; haemodialysis was required in 13 (12.1%) patients. Within the second group of complications, the most commonly observed were ascites and (early or delayed) variceal bleeding. In 21 (19.6%) patients, bleeding occurred post-operatively due to persistent portal hypertension. Varma reported a case in which a venous graft was interposed between a retroperitoneal varix and the PV in order to improve the drainage of the portal venous system[53]. The majority of variceal bleedings were controlled with sclerotherapy, splenic artery embolization or splenectomy with or without gastric devascularization[79].

Thrombosis at the level of the anastomosis was seen in 17 (15.9%) patients. Such complication can sometimes be treated by endovascular stenting.

Hepato-intestinal or MVT represents the very last surgical option in grade Ⅳ PVT, allowing replacement of the entire splanchnic venous system of the recipient[81]. Such a radical expedient still represents a major technical and immunological challenge, and carries a high risk of rejection, infection and surgical complications. In recent years, surgical technique and peri-operative management have evolved substantially, achieving one- and three-year survival rates up to 80% and 70%[65,82,83]. Particular attention should be given to the graft procurement and to ensure a low cold ischemia time ($\leqslant$ 6 h) in order to avoid irreversible intestinal mucosal injury[84,85]. The MVT surgical technique consists of complete replacement of the abdominal viscera after exenteration[86]. Arterial inflow is established with a unique anastomosis between the donor aortic patch encompassing the coeliac trunk and superior mesenteric artery and recipient aorta. Venous anastomosis is routinely performed with a piggy-back technique. A terminal ileostomy is created in order to allow endoscopic monitoring of the bowel graft. The decision to opt for a MVT may be undertaken during the transplant procedure. If adequate portal flow can be restored, the Indiana group led by Vianna proposed that only the liver should be implanted and the multivisceral graft split on the back table; the remaining organs are next directed towards a backup recipient needing an isolated intestinal transplantation. If portal flow cannot be established, then a MVT is required[87].

SVT used to represent a common indication for MVT in adults. The Pittsburgh experience consisting of five hundred transplants included MVT for SVT in 10% of

cases[88]. The Indiana group obtained excellent one- and three-year survival rates of 80% and 72%. However, the procedure carried a complication rate of 56% with half the patients requiring a surgical re-exploration[83]. Nowadays, MVT is less frequently offered in cases of SVT due to the introduction of more sophisticated surgical techniques when dealing with extended SVT.

CONCLUSION

PVT and SVT are no longer a contraindication for LT. However, in order to achieve satisfactory outcomes, the surgical strategy needs to be carefully planned before the transplant procedure. Patients with extended splanchnic thrombosis may need complex vascular interventions; others may even require a multivisceral transplantation.

ACKNOWLEDGMENTS

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Palliation: Hilar cholangiocarcinoma

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Abstract

Hilar cholangiocarcinomas are common tumors of the bile duct that are often unresectable at presentation. Palliation, therefore, remains the goal in the majority of these patients. Palliative treatment is particularly indicated in the presence of cholangitis and pruritus but is often also offered for high-grade jaundice and abdominal pain. Endoscopic drainage by placing stents at endoscopic retrograde cholangio-pancreatography (ERCP) is usually the preferred modality of palliation. However, for advanced disease, percutaneous stenting has been shown to be superior to endoscopic stenting. Endosonography-guided biliary drainage is emerging as an alternative technique, particularly when ERCP is not possible or fails. Metal stents are usually preferred over plastic stents, both for ERCP and for percutaneous biliary drainage. There is no consensus as to whether it is necessary to place multiple stents within advanced hilar blocks or whether unilateral stenting would suffice. However, recent data have suggested that, contrary to previous belief, it is useful to drain more than 50% of the liver volume for favorable long-term results. In the presence of cholangitis, it is beneficial to drain all of the obstructed biliary segments. Surgical bypass plays a limited role in palliation and is offered primarily as a segment Ⅲ bypass if, during a laparotomy for resection, the tumor is found to be unresectable. Photodynamic therapy and, more recently, radiofrequency ablation have been used as adjuvant therapies to improve the results of biliary stenting. The exact technique to be used for palliation is guided by the extent of the biliary involvement (Bismuth class) and the availability of local expertise.

Core tip: The majority of patients with hilar cholangiocarcinoma present in advanced stages and are candidates for palliation only. The techniques of palliation, primarily at endoscopy or by percutaneous techniques, are evolving as better stents become available. Alternate techniques, such as endosonography-guided procedures, are also becoming popular. Photodynamic therapy and radio-frequency ablation are also used to improve the results of biliary stents. This review article provides a consolidated picture of the present knowledge in this field based on recent data.

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INTRODUCTION

Bile duct cancers at or around the confluence of the right and left hepatic ducts are termed as hilar cholangiocarcinomas (HC) or Klatskin's tumors. HC is the most common type of bile duct cancer throughout the world and constitutes 46%-97% of all bile duct cancers[1,2]. It has a particularly high prevalence in certain Asian countries, such as Thailand and China[3]. This could be related to

Figure 1 Hilar Cholangicarcinoma-Bismuth classification. Type Ⅰ: Confluence of right and left hepatic duct is intact; Type Ⅱ: Right and left hepatic ducts are separated; Type Ⅲ: Tumor extends into second degree branches of right (ⅢA) or left (ⅢB) hepatic duct; Type Ⅳ: Involvement of both side secondary branches.

Table 1　Criteria for non-resectability of hilar cholangiocarcinoma
Bilateral hepatic duct involvement up to the secondary biliary radicles (Bismuth type Ⅳ)
Encasement or occlusion of the main portal vein (relative)
Unilateral tumor extension to secondary biliary radicles (Bismuth type Ⅲ) with contralateral portal vein or hepatic artery involvement or encasement
Hepatic lobar atrophy with contralateral portal vein or hepatic artery involvement or encasement
Hepatic lobar atrophy with contralateral tumor extension to the secondary biliary radicles

Available from Aljiffry *et al*[6].

the liver fluke infestation in these areas. Due to the critical nature and site of the disease, patients with HC suffer greatly from progressive jaundice, anorexia, pruritus, cholangitis and liver failure. Unfortunately, a majority of HC cases manifest late and are diagnosed at a stage when curative resection is not possible[4,5]. Palliation, therefore, is the goal for this subset of patients. This review addresses the indications, techniques and results of various palliative methods. There are a number of "grey zones" in the palliative treatment of HC; these have also been addressed.

INDICATIONS FOR PALLIATION

Only approximately 20%-30% of patients with HC are diagnosed at a stage when surgical resection is possible. Table 1 gives the criteria for surgical non-resectability[6]. Moreover, the associated co-morbidity often precludes any form of surgery. While the median survival for resected patients (R0) can be up to 4 years, for those without the feasible option of resection, survival is almost always less than 1 year[4,7]. Multi-detector CT scan (MDCT) and magnetic resonance imaging (MRI) with MR Cholangiography continue to be the most two accurate modalities in the evaluation of the stages and resectability of HC[8,9]. The accuracy of predicting hepatic artery invasion, portal vein involvement, lymph nodal metastasis and the extent of biliary ductal spread is approximately

85%-95% in both of these approaches[8,9]. Endoscopic ultrasonography (EUS), positron emission tomography (PET) and diagnostic laparoscopy are additional means of evaluating the resectability of HC, but their role is not yet fully established[10-12].

Not every patient with unresectable HC needs palliative intervention. Patients with complications of cholangitis and intractable pruritus are definite candidates for palliation. Palliation is also often performed in patients with abdominal pain and high bilirubin with the hope of ameliorating their pain and sense of well-being, respectively. There are three established methods for the palliation of HC: endoscopic insertion of a stent, percutaneous placement of biliary drainage and surgical bypass. Endosonography-guided procedures have been evolving as alternatives to these standard techniques.

ENDOSCOPIC PALLIATION BY STENTING

Before planning any palliative drainage, either by endoscopy or percutaneously, it is mandatory to obtain a cholangiogram to define the extent of biliary ductal involvement. Magnetic resonance cholangio-pancreatography (MRCP) continues to be the most preferred investigation for this purpose, and HC has been classified as Bismuth type Ⅰ to type Ⅳ at cholangiogram (Figure 1).

Metal vs plastic stents

Both plastic stents (PS) and self-expanding metal stents (SEMS) have been used for biliary drainage (Figure 2). PS have smaller diameters (10-12 Fr), resulting in faster occlusions and a median patency time of only 1.4 to 3 mo[13]. SEMS have a wider diameter (8-10 mm), resulting in longer patency of 6-10 mo[13]. SEMS, which are used for HC, are uncovered with an open mesh, allowing the drainage of side branches. Perdue *et al*[14], in a multicenter study involving 62 patients, compared metal stents with plastic stents in HC for 30 d outcomes. Adverse effects, including cholangitis, stent occlusion, migration, perforation and the need for reinterventions, occurred in a

Figure 2 Stenting for hilar cholangiocarcinoma. A: Unilateral plastic stent; B: Bilateral plastic stents; C: Unilateral metal stent; D: Bilateral metal stents (previously placed percutaneous drains are still in place).

Figure 3 Hilar stricture: Unilateral metal stent placed in dilated left lobe duct. A: Left duct selectively cannulated and showing dilated system; B: Metal stent being deployed in the left ductal system.

Table 2 Unilateral *vs* bilateral drainage for hilar cholangiocarcinoma

	Unilateral	Bilateral	*P*
No. of pts	79	78	-
Stent insertion (%)	88.6	76.9	0.041
Successful drainage (%)	81.0	73.0	0.049
Early complication (%)	18.9	26.9	0.026
Survival (d)	140	142	0.482

Available from De Palma *et al*[16].

significantly higher proportion of patients in the plastic stent group (39.3%), compared to metal stents (11.8%). These results were revalidated in a recent study from Thailand[15]. In this randomized study involving 108 patients, Sangchan *et al*[15] demonstrated that metal stents were better in terms of success rate (70% *vs* 46%) and patient survival (126 d *vs* 49 d). In spite of its high initial cost, SEMS are considered more cost-effective than PS. This result could be due to the greater success rate, shorter hospital stays, fewer blockages, fewer re-interventions and lower antibiotic needs associated with SEMS. The superior cost-effectiveness of SEMS compared to PS is particularly apparent if patient survival is expected to be more than 4-6 mo. However, in situations where a decision of palliation has not been made, plastic stents may be preferred because uncovered SEMS used for hilar blocks are not easily removable.

Volume of drainage and number of stents (unilateral vs bilateral)

For Bismuth type I HC, it is obvious that one stent is sufficient to drain both lobes of the liver because the confluence is patent. However, the placement of single stent for Bismuth type II to type IV HC would drain only a proportion of liver; instead, multiple stents are often placed in these advanced HC patients. However, no consensus has been reached in terms of the need for multiple stents.

De Palma *et al*[16] reported better results with unilateral stenting (*vs* bilateral stenting) in terms of successful stent placement, effective drainage and complication rates (Table 2). However, approximately one-third of the patients in this series had Bismuth type I HC, leading to a bias in the study result. Contrary to this study, Naitoh *et al*[17] demonstrated a trend towards longer survival and lower cholangitis with bilateral, compared to unilateral, drainage. An important conclusion was drawn in a study by Chang *et al*[18], which showed that patients with HC faired very poorly, with high cholangitis rates (32%), high 30 d mortality (30%) and poor survival (45 d) if only one stent was placed after opacifying both sides of the liver. This was in contrast to the other two groups, in which the opacified lobes were drained by one or two stents (Table 3). It therefore seems that any contrast placed in an obstructed system must be drained. An experienced endoscopist dealing with hilar blocks is always reluctant to inject any contrast until a guide wire has been negotiat-

Table 3 Malignant hilar obstruction-1 stent or 2			
	Group A	**Group B**	**Group C**
n	32	29	37
Early cholangitis	6%	0%	32%
30-d mortality	0%	3%	30%
Survival (d)	145	225	45

Group A: One lobe opacified and the same lobe drained; Group B: Both lobes opacified and both lobes drained; Group C: Both lobes opacified with just one lobe drained. Available from Chang *et al*[18].

Table 4 Computed tomography/magnetic resonance cholangio-pancreatography -Guided Selective Unilateral Stenting			
Ref.	**Hintze *et al*[25]**	**Freeman *et al*[26]**	**De Palma *et al*[16]**
No. of pts	35	35	61
Stent	Plastic	Metal	Metal
Tech. success (%)	100	100	97
Effective drain (%)	86	77	97
Early cholangitis (%)	6	0	5
Median patency (d)	-	165	169
Median survival (d)	300	150	140

ed well beyond the site of the biliary obstruction. A study in India[19] demonstrated the importance of contrast-free unilateral endoscopic palliation in Bismuth type II HC. In 18 patients submitting to this technique, successful drainage was achieved in all, with no cholangitis or 30 d mortality.

Conventional teaching suggests that, in the absence of cholangitis, it is only necessary to drain 25% of the liver volume for the adequate palliation of jaundice[20]. However, a recent study by Vienne *et al*[21] showed better results with the drainage of more than half of the liver volume. More than a 50% drainage of the liver volume was associated with a greater decrease in bilirubin levels, a lower incidence of early cholangitis and longer patient survival compared to patients with less than a 50% drainage of the liver volume. It is known that the right lobe, left lobe and caudate lobes of liver constitute 55%-60%, 30%-35% and approximately 10% of the liver volume, respectively[22]. Incorporating this information in the results of the study by Vienne *et al*[21], a significant proportion of patients should undergo the draining of at least two segments and the placement of more than one stent to achieve good long-term palliation. However, more data are needed in this respect. It is important to note that, in the presence of cholangitis, all infected ductal systems need to be drained.

It is important to select the appropriate segments of the liver that need to be drained when unilateral or incomplete drainage is planned in patients with advanced HC (Bismuth type Ⅱ to type Ⅳ). It is advisable to select segments with dilated ductal systems and to avoid atrophic segments (Figure 3). MDCT or MRI can provide this useful information prior to palliative stenting[23,24]. A number of studies with both PS and SEMS have shown the usefulness of CT/MRCP-guided, selective biliary drainage (Table 4)[16,25,26]. This CT/MRCP approach can reduce the need for further intervention and has been found to be cost-effective compared to routine bilateral stenting[27]. A recent study[28] with a small number of patients used air cholangiography rather than MRCP to act as a lower cost road map and found air cholangiography to be safe and effective with no cholangitis and no 30 d mortality or morbidity.

Endoscopic stenting technique
Stents in HC are mostly introduced at endoscopy[23]. In terms of the complexity of endoscopic procedures,

stenting a hilar block is considered one of the most difficult. In a recent consensus, the American Society of Gastrointestinal Endoscopy graded endoscopic hilar stenting as a level 3 ERCP procedure in terms of complexity, with level 1 being the simplest and level 4 being most complex[29]. In general, a higher level of complexity is associated with a lower success rate and a higher complication rate[30]. Therefore, hilar stenting should be practiced only be experienced therapeutic endoscopists.

A variety of plastic and SEMS are available and have been used for the stenting in HC. In general, 10 Fr plastic stent and uncovered SEMS are preferred. The distal end of stents may be left in the duodenum or in distal bile duct, but the later situation may make reintervention more difficult. When more than one stent (plastic or SEMS) is to be placed, the stents are usually placed side by side (Figure 4). However, in the last few years, a new dual stent design called "stent-in-stent" has been developed for metal stenting[31,32] (Figure 5). In this device, the first stent has an open-cell design, allowing the second stent to pass easily through the first stent. In a recent study by Lee *et al*[33] from South Korea, stent-in-stent technology for bilateral stenting was evaluated in 84 patients with inoperable, high-grade HC. Technical and clinical success was achieved in 95.2% and 92.9% of patients, respectively. The median survival and patency were noted to be 256 d and 239 d, respectively. Still, this new stent design can be problematic if the first stent becomes occluded. In the study by Lee *et al*[33], 30.8% patients had an obstruction of the primary biliary stents. For revision stenting, bilateral metal stents could be placed in 55%, while plastic stents were placed in the remaining patients[33].

It is important to institute antibiotic prophylaxis in patients with anticipated incomplete biliary drainage by any technique[34]. Antibiotics should be continued in cases of incomplete biliary drainage. The choice of antibiotics should be based on local hospital data.

The occlusion of metal stents, while more common and earlier with plastic stents, can still occur[35]. Blocked plastic stents should always be removed, and the bile ducts should be cleaned of all sludge by a balloon. Then, a new plastic stent can be positioned. Metal stents, however, may be the better choice, particularly in the presence of thick bile or purulent material. It may also be a good idea to temporarily place a nasobiliary drain for repeated flushing prior to restenting[35]. Uncovered SEMS

Figure 4 Two metal stents placed side by side in a Type II Bismuth hilar cholangiocarcinoma. A: Magnetic resonance cholangiopancreaticography showing the stricture; B: Endoscopic view showing two metal stents placed side by side; C: X-ray showing two parallel stents with proximal ends in the right or left ductal systems.

Figure 5 Y stent (stent-in-stent) from Tae Woong Medical (South Korea) for Hilar Cholangiocarcinoma. A: Two stents one with open mesh in the middle (arrow) and other with normal mesh; B: Y stent system in which the normal mesh stent passes through the open mesh of the second stent.

cannot be removed and therefore should be cleaned if they become clogged. However, it is advisable to place another stent through the blocked metal stent. The new stent could be either plastic or SEMS, depending upon the patient's life expectancy.

PERCUTANEOUS BILIARY DRAINAGE

With technical expertise, it is also possible to perform palliative intervention in HC by percutaneous techniques (Figure 6). Percutaneous procedures have the theoretical advantage of precise lobar selection, which can result in less cholangitis. Moreover, the percutaneous approach can be performed with minimal sedation, which may be beneficial in an unstable patient. Alternatively, the percutaneous approach may be a two-step procedure with external drainage (percutaneous transhepatic biliary drainage) during first session, followed by internalization and subsequent stenting. Pain at the puncture site and the risk of biliary peritonitis are additional concerns with the use of percutaneous approach.

Just as with the endoscopic data, the advantages of metal stents over plastic stents have been demonstrated in percutaneous approaches as well. Prolonged survival and lower morbidity have been shown with metal stents compared to plastic stents[36]. While using multiple SEMS percutaneously, stents may be placed side by side. However, a T or Y configuration (Figure 7), in which one stent crosses the block and the second stent only reaches the first stent or crosses from the left to the right ducts, have also been used successfully[37]. T or Y configuration stents have been shown to have a median patency of 279 and 218 d and a median survival of 334 and 375 d, respectively[38,39].

Few studies have compared percutaneous procedures with endoscopic techniques in HC, and no randomized controlled trials are available addressing this issue. A multicenter retrospective study[40] from South Korea has compared the results of endoscopy *vs* percutaneous techniques for advanced HC (Bismuth type III and IV). In their 85 patients (endoscopy 44, percutaneous 41), they noted better results for percutaneous procedure, (compared to endoscopy) with a significantly higher success rate (93% *vs* 77%, P = 0.049) and a trend towards a lesser cholangitis risk (22% *vs* 29.5%). Similar conclusions were drawn from two other studies from Asia[41,42]. Non-infective complications, such as bleeding and pancreatitis, were more frequent in the percutaneous group. This result suggests that, if infective complication can be avoided by performing contrast-free cannulation, ERCP may have an advantage, even in advanced HC.

In contrast to these results, endoscopy in general is the preferred approach for less advanced Bismuth type (Type I and II) HCs, mainly because endoscopy is a less invasive and faster procedure. However, no comparative data are available, and local expertise usually guides the treatment approach in these patients. Occasionally, palliation after endoscopic stenting may be suboptimal due to the presence of pus flakes, mucus or blood clots in the biliary ductal system. A temporary placement of a percutaneous catheter can allow repeated saline irrigations and may enable effective biliary drainage. It must be recognized that HC requires a multidisciplinary approach, with close co-ordination between the endoscopist and radiologist. For example, a failed endoscopic drainage after ductal opacification may result in suppurative cholangitis,

Figure 6 Percutaneous stenting in Bismuth Type I hilar cholangiocarcinoma. A: Stent assembly; B: Cholangiogram showing the block; C: Guide wire being negotiated across the stricture; D: Balloon dilatation being performed; E: Stent (arrow) after deployment.

Figure 7 Percutaneous double stenting. A: X-ray showing the Y configuration of the stent (Boston Scientific MA, United States); B: Transverse limb of a T stent (Taewoong Medial, South Korea) showing the open mesh in the center; C: Assembly of a T stent showing the vertical stent passing through the open mesh of the transverse stent.

and an emergency percutaneous drainage may became mandatory.

ENDOSONOGRAPHY GUIDED BILIARY DRAINAGE

Endosonography (EUS)-guided biliary drainage is emerging as an alternative to ERCP and percutaneous biliary stenting[43-46]. In the present scenario, an EUS-guided procedure is performed only if the ERCP fails in the presence of a tight hilar block or distorted duodenal anatomy. It remains unclear if EUS-guided drainage should be the

second preferred modality or if it should be performed only when the percutaneous procedure fails or is contraindicated. Prospective randomized trials are needed to solve this issue.

The EUS technique involves the puncture of the left hepatic ducts, usually through the gastric wall, *i.e.*, hepaticogastrostomy. A guide wire is then passed through this tract. Stenting can then be achieved in one of the three ways: (1) negotiating the wire across the hilar stricture and then passing it through the ampulla into the duodenum; the rendezvous procedure is then performed by catching the wire at the papilla, positioning the stent as in the ERCP

Figure 8 A simplified algorithm suggested for the palliation of Hilar Cholangiocarcinoma, based on Bismuth type. Additional percutaneous procedures may be required if the endoscopic stenting results are suboptimal. PDT: Photodynamic therapy; RFA: Radiofrequency ablation.

Table 5 Photodynamic therapy as an adjunct to biliary stenting: Improved survival

	Year	No. of pts	Median survival (mo)		P value
			Stenting alone	**Stenting with PDT**	
Ortner *et al*[56]	2003	39	3	16	< 0.0001
Cheon *et al*[57]	2004	47	10	18	0.0143
Zoepf *et al*[58]	2005	32	7	21	0.0109

PDT: Photodynamic therapy.

LOCAL ABLATIVE THERAPY

A few exciting techniques have been used to improve the results of biliary stenting with the aim of delaying stent blockage and prolonging patient survival. These techniques include photodynamic therapy, radiofrequency ablation and chemo/radiotherapy.

Photodynamic therapy

Photodynamic therapy (PDT) is a relatively new technique that has evolved over the last 10 years[55]. PDT uses a photosensitive agent that concentrates preferentially in malignant tumors. Subsequent photoactivation with red laser lights of a specific wavelength[56] creates reactive oxygen, leading to selective tumor cell death. Table 5 summarizes some of the studies[56-58] that have shown improved patient survival by the addition of PDT to biliary stenting. Studies have also shown improved quality of life after PDT[56,58]. In HC patients who have been treated with plastic stents, the stents should be removed temporarily for light delivery during PDT. In cases of SEMS, PDT can be performed through the stent by adjusted the required light dose to compensate for the decreased transmission of light[59]. A further moot point that should be addressed is whether these results can be reproduced in certain Asian countries, in which the sun does not affect skin photosensitivity to the same extent as in the Western white population[57,60].

In general, PDT is preferred through the percutaneous route [compared to the endoscopic] because of the lower chance of laser tip fracture, the easy repeatability of the procedure and the feasibility of obtaining cholangiogram. Shim *et al*[61] showed that PDT using percutaneous cholangioscopy is safe and effective, improving quality of life with a good median survival time (*i.e.*, 558 ± 178.8 d). Cholangitis, hemobilia and photosensitivity are known complications of PDT.

A recent study by Wagner *et al*[62] used temoporfin rather than conventional porfimer for PDT in 10 patients with HC. Temoporfin PDT was highly tumoricidal and had double the depth of local tumor ablative effect, compared to porfimer PDT. Infectious complications and skin photosensitivity were similar with both agents[62].

Radiofrequency ablation

Radiofrequency ablation (RFA) is an ablative procedure that is well established for treating small liver cancers. With the introduction of an endoscopic probe for RFA

procedure[43,44]; (2) negotiating the wire across the hilar stricture and then placing the stent across the stricture[45]; or (3) placing the stent across the hepatico-jejunostomy itself without negotiating the wire across the stricture[46].

In experienced hands, EUS-guided biliary drainage has a technical success rate of 70%-98%. However, up to 20% of patients can have complications, such as bile leak, biliary peritonitis, pneumoperitoneum, bleeding, pain or stent migration[43-50]. This complication rate is higher than that reported historically with ERCP. All types of stents, including plastic, uncovered SEMS and covered (partial or total) SEMS, have been used. However, covered SEMS are usually preferred. Between the three techniques described above, the rendezvous technique is more natural and is preferred; however, there are no comparative data with other techniques, and the procedure is more demanding[43,44].

EUS-guided procedures are exciting but still evolving. Because most of the data are in the form of a case series, prospective randomized studies are required to position the modality correctly in the management algorithm of hilar blocks.

SURGICAL BYPASS

Due to its invasiveness, surgical bypass has a very limited role to play in palliating hilar tumors. It is only accepted when all of the other techniques described above have failed or are not available, as well as occasionally when laparotomy has been performed for curative intent but an unresectable tumor is revealed[51,52]. The various surgical drainage procedures that can be carried out include segment III hepaticojejunostomy, stent placement across the tumor and sectoral duct (*i.e.*, right anterior, right posterior or left hepatic duct) bypass. However, segment III bypass is the preferred choice because it resolves jaundice in approximately two-thirds of HC patients and has a median survival of approximately 6.3 mo[53,54].

and the use of a lower power setting with the existing generators, it is now possible to treat pancreatico-biliary cancers with this modality[63]. Within the bile duct, it has the potential of improving stent patency by decreasing tumor ingrowth and benign epithelial hyperplasias. Steel *et al*[64] were the first to demonstrate its safety and efficacy in 22 patients with biliary malignancy (pancreatic/cholangiocarcinoma). A recent study involving 20 patients with pancreatico-biliary malignancy (11 with cholangiocarcinoma) showed a significant increase in bile duct diameter after RFA[65]. Further studies are required to revalidate these results, but the preliminary data are interesting.

Chemo/radiotherapy

Radiotherapy has a very limited role in HC. Intraluminal brachytherapy has been delivered to cholangiocarcinoma using Ir-S192 seeds, with a total dose of 30-50 Gy through the percutaneous route. Good short-term effects were demonstrated, showing prolonged stent patency and improved survival[66]. A number of studies from Japan have shown better SEMS patency (10-18 mo *vs* 4-12 mo) after external beam radiotherapy[67,68].

Chemotherapy has been used in HC as a palliative therapy for both locally advanced cancers and metastatic disease[69]. However, most of these studies involved case series with a heterogeneous mixture of patients with HC or distal bile duct cancers, as well as pancreatic or gall bladder cancers. A retrospective study comparing a gemcitabine-based regimen with a cisplatin-based therapy and a fluoropyrimidine-based therapy clearly showed the gemcitabine-based regimen to be superior in terms of the lowest fatality[70]. Gemcitabine has been used in combination with cisplatin, oxaliplatin or capecitabine to improve its efficacy[69]. Valle *et al*[71,72] compared gemcitabine with a combination of gemcitabine with cisplatine in their two ABC trials, which were published sequentially. ABC-02 trial by Valle *et al*[72], which involved 410 patients with advanced biliary cancers in a multicenter randomized phase Ⅲ study, clearly showed the superiority of the combination over gemcitabine alone. The combination improved progression-free survival and overall survival by 30% over gemcitabine alone. Another multicenter trial from Japan[73] also demonstrated the superiority of gemcitabine combined with cisplatin over gemcitabine alone in terms of one year survival, median survival time, median progression-free survival time and overall response rate. These data have resulted in this combination being used as standard of care chemotherapy for advanced cholangiocarcinoma, including HC. Gemcitabine-free combinations, such as capecitabine with oxaliplatin, capecitabine with cisplatin and 5 fluorouracil with cisplatin, have also been used for bile duct cancers with modest results[69]. However, newer drugs are being investigated for bile duct cancers; these include erlotinib, cetuximab and bevacizumab[69].

CONCLUSION

HC is the most common type of malignancy of the bile duct and is highly prevalent in the Eastern Hemisphere. Unfortunately, the majority of patients with HC manifest too late for resection. Therefore, palliation is the goal in the majority of patients. Palliation is usually performed with ERCP or through a percutaneous route. Figure 8 shows our approach and suggested guidelines based on Bismuth types. For advanced HC (Bismuth type Ⅲ and Ⅳ), we prefer the percutaneous route because this has been shown to be superior to the endoscopic approach. For Bismuth type Ⅰ and Ⅱ, ERCP is usually preferred in view of its being less invasive and faster. Generally, we prefer metal stents over plastic stents because of the former's documented better median patency and patient survival. Plastic stents are only offered when the expected survival is very short. Controversy continues as to whether single or multiple stents should be preferred for advanced HC. Recent data suggesting that better outcomes will be obtained if > 50% of the liver is drained have made us change our policy such that we offer two stents in the majority of patients. EUS-guided biliary drainage is also gradually emerging as an alternative modality. Percutaneous or EUS-guided biliary drainage is at present offered for all patients who fail ERCP or for patients who cannot undergo ERCP due to their altered anatomy or duodenal obstructions from tumors. A few reports showed that photodynamic therapy and radiofrequency ablation could improve the patency of biliary stenting and patient survival, suggesting that this procedure should be considered in advanced tumors (Bismuth type III and IV). Local expertise is often the deciding factor for choosing one modality over the other.

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Role of adipokines and peroxisome proliferator-activated receptors in nonalcoholic fatty liver disease

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Abstract

Intrahepatic fat deposition has been demonstrated in patients with nonalcoholic fatty liver disease (NAFLD). Genetic and environmental factors are important for the development of NAFLD. Diseases such as obesity, diabetes, and hypertension have been found to be closely associated with the incidence of NAFLD. Evidence suggests that obesity and insulin resistance are the major factors that contribute to the development of NAFLD. In comparing the factors that contribute to the buildup of excess calories in obesity, an imbalance of energy homeostasis can be considered as the basis. Among the peripheral signals that are generated to regulate the uptake of food, signals from adipose tissue are of major relevance and involve the maintenance of energy homeostasis through processes such as lipogenesis, lipolysis, and oxidation of fatty acids. Advances in research on adipose tissue suggest an integral role played by adipokines in NAFLD. Cytokines secreted by adipocytes, such as tumor necrosis factor-α, transforming growth factor-β, and interleukin-6, are implicated in NAFLD. Other adipokines, such as leptin and adiponectin and, to a lesser extent, resistin and retinol binding protein-4 are also involved. Leptin and adiponectin can augment the oxidation of fatty acid in liver by activating the nuclear receptor super-family of transcription factors, namely peroxisome proliferator-activated receptor (PPAR)-α. Recent studies have proposed downregulation of PPAR-α in cases of hepatic steatosis. This review discusses the role of adipokines and PPARs with regard to hepatic energy metabolism and progression of NAFLD.

Core tip: Nonalcoholic fatty liver disease (NAFLD) is one of the principal causes for chronic liver disease. Recent reports suggested a positive association between cytokines secreted by the adipocytes, such as tumor necrosis factor-α, transforming growth factor-β, and interleukin (IL)-6 in NAFLD. Furthermore, hepatic natural killer T-cells produce IL-13 and IL-4; IL-13 may then activate hepatic stellate cells to produce pro-inflammatory cytokines and initiate oxidative stress, iron overload and fibrosis. Downregulation of peroxisome proliferator-activated receptors (PPAR), particularly PPAR-α in cases of hepatic steatosis, may facilitate the activity of hepatic proinflammatory cytokines. Hence, PPAR-γ and PPAR-α ligands have been considered for administration to prevent the initial inflammatory reactions and render protection to the liver cells.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a disease spectrum encompassing simple steatosis, steatohepatitis, fibrosis and, ultimately, liver cirrhosis[1]. It is reported to be one of the principal causes of chronic liver disease, and is predominant in developed countries. Results of several studies proposed NAFLD as the hepatic component of metabolic syndrome (MS), which is characterized by hyperinsulinemia, insulin resistance (IR), obesity, type Ⅱ diabetes mellitus (TDM), dyslipidemia, and hypertension[2]. NAFLD has a wide histological spectrum ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), which may progress to cirrhosis. In simple NAFLD, the presence of macrovesicular fat droplets in hepatocytes, foci of lobular inflammation, mild portal inflammation, and lipogranulomas may be seen, while increased portal inflammation has been reported in untreated NAFLD patients[3]. In approximately 25% of cases, histological signs of fibrosis and necroinflammatory injury that define NASH are present[4]. Patients with NASH are at higher risk of developing fibrosis, cirrhosis and hepatocellular carcinoma[4]. The exact cause of NAFLD is still unknown. However, incidences of NAFLD have been associated with cases of IR in both diabetes and obesity, and with hypertension. It has been proposed that an imbalance in energy homeostasis may be the basis for obesity and thus NAFLD.

Complex physiologic processes, constant communication within and among tissues such as adipose tissue, liver, skeletal muscle, pancreas, and the central nervous system are required for energy homeostasis. The brain is referred to as the central regulator of energy homeostasis and thus the primary controller of body weight. Peripherally, the major organs participating in the regulation of food intake are the stomach, intestines, pancreas, and adipose tissue[5]. The imbalance in energy homeostasis stems etiologically from either excess food intake or insufficient energy expenditure. This may also be secondarily related to endocrinopathies such as hypothyroidism, Cushing's syndrome, etc., that evolve into MS, which, in turn, is related to NAFLD[6,7].

Among various peripheral organs, the role played by adipose tissue in energy homeostasis remains central. Adipose tissue, far from being an inert depot of storage fat, is an active endocrine organ, as evidenced by the variety of hormones or adipokines it synthesizes, including leptin and adiponectin, among others[8]. Signals controlling energy intake originate from adipose tissue, mediated by leptin. The afferent and efferent signals fluctuate in a coordinated manner to maintain energy homeostasis. It is necessary to determine the role played by various adipokines and nuclear receptors such as peroxisome proliferators-activated receptors (PPARs), in the initiation and progression of NAFLD. Research in this field is evolving to explain the exact role of the adipokines in NAFLD. This review discusses the role of adipokines and PPARs in NAFLD.

ADIPOSE TISSUE IN ENERGY HOMEOSTASIS

Energy homeostasis is maintained by the integration of major metabolic functions such as lipogenesis, lipolysis, and fatty acid oxidation, and is mediated through adipose tissue[9]. Of the two types of adipose tissues, white (WAT) and brown (BAT), WAT is concerned with energy balance in adults and has been found to be increased in obesity[10,11]. Cells constituting WAT include preadipocytes, fibroblasts, endothelial cells, macrophages, and monocytes[12]. The functional adipocyte secretes several factors known as adipokines to communicate actively with the liver, muscle, and hypothalamus[13,14]. Such factors include pro-inflammatory cytokines produced by resident macrophages such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, and monocyte chemoattractant protein (MCP)-1. The majority of adipokines, however, maintain equilibrium in the utilization of energy substrates between adipose and non adipose tissues in the intestine, liver, brain, and skeletal muscle[14]. Among such adipokines, leptin and adiponectin and, to a lesser extent, resistin and retinol binding protein-4 have a role in energy homeostasis.

ROLE OF LEPTIN, ADIPONECTIN, RESISTIN, AND RETINOL BINDING PROTEIN-4 IN LIPID METABOLISM

Leptin (16 kDa) is a peptide hormone synthesized by adipocytes and in inconsequential amounts by the liver and skeletal muscle[15]. Its gene expression (*Lep/Ob* gene) is regulated by food intake, energy status, hormones, and the overall inflammatory state[16]. Leptin signals are mediated through a membrane receptor and signaling pathways involve Janus activating kinase/signal transducer and activator of transcription, mitogen-activated protein kinase, phosphatidyl-inositol 3' kinase, and AMP-dependent protein kinase (AMPK)[17,18]. Through such pathways, leptin acts on the hypothalamus to reduce appetite and thus function as an adipostat[16]. The stimulating pathways, in general, favor fatty acid oxidation and decrease lipogenesis. Leptin also tends to decrease the ectopic deposition of fat in liver and muscle. Furthermore, leptin can also act on the pancreas, inhibiting both insulin and glucagon secretion *via* short-term/non-genomic and long-term/genomic mechanisms, thereby promoting glucose homeostasis[19]. In excess calorie states, such as obesity, hyperleptinemia associated with leptin receptor inactivation/downregulation has been observed in the hypothalamus and liver of obese rats[20].

Adiponectin (30 kDa) is a protein hormone, which exists in several globular, trimeric, and high molecular weight forms in circulation. Adiponectin stimulates insulin sensitivity, decreases hepatic glucose production, increases glucose utilization in muscle and oxidation of fatty acid in muscle, liver, and peripheral tissues, thus

Figure 1 Mechanism of action of adenosine monophosphate-dependent protein kinase. AMP: Adenosine monophosphate; AMPK: AMP-dependent protein kinase.

down-regulating the secretion of pro-inflammatory cytokines IL-6, IL-8 and MCP-1[21,22]. Adiponectin receptors activate AMPK, p38 mitogen-activated protein kinase, and PPAR-α, which in turn regulate fatty acid metabolism[23]. Low levels of adiponectin are associated with low-grade inflammation, oxidative stress, and endothelial dysfunction[24]. Its receptors are largely decreased in obese animals and humans.

Both leptin and adiponectin stimulate AMPK in skeletal muscle, liver, and adipose tissue[25]. In skeletal muscle, AMPK activation promotes glucose transport, glycolysis, and fatty acid oxidation, and inhibits fatty acid synthesis (Figure 1). AMPK directly inhibits acetyl CoA carboxylase, preventing lipogenesis; consequentially, carnitine palmitoyl transferase-1 is stimulated leading to fatty acid oxidation[22]. AMPK is activated during exercise and is involved in glucose transport and fatty acid oxidation in skeletal muscle. Hepatic lipid turnover is regulated by transcription factors carbohydrate-responsive element-binding protein, sterol regulatory element binding protein 1c (SREBP-1c), CCAAT-enhancer-binding protein α, and PPARs. In liver, AMPK is a cellular metabolic sensor, inhibiting lipogenesis and cholesterol synthesis; its activation suppresses the SREBP1c[9]. Disequilibrium in lipid homeostasis causes triglycerides to accumulate in the liver.

Resistin (12.5 kDa) is a 108 amino acid protein; its circulatory form consists of a dimer united by a single disulfide bridge. Although its function in humans is still unclear, in mice it has been shown to increase blood glucose and insulin concentrations by means of promoting hepatic IR and gluconeogenesis[26]. Its exact role in IR and obesity has not yet been determined; however, studies have demonstrated that obese individuals display higher serum resistin values than lean subjects and a positive correlation may exist between BMI and resistin, although the latter remains under debate[15].

Correspondingly, retinol binding protein-4 (RBP4) (21 kDa), the transport protein for retinol in blood, has also been linked to cases of IR[27]. Although primarily hepatic in origin, high levels of RBP4 were detected in the adipose tissue of glucose transport protein 4 knock-out mice[28]. The blood concentration of RBP4 was also found to be increased in obese and diabetic human individuals. Alternatively, RBP4 knockout mice displayed increased insulin sensitivity[27]. A more positive association, however, was distinguished between adipocyte inflammatory cytokine production in cases of IR and RBP4 levels[28].

ROLE OF LEPTIN, ADIPONECTIN, RESISTIN, AND RETINOL BINDING PROTEIN-4 IN NAFLD

NAFLD usually occurs concomitantly with obesity, TDM, and/or hyperlipidemia. The current explanation for the pathogenesis of NAFLD is two-fold; according to the "two-hit" theory, IR develops first. Excess free fatty acid flux occurs from adipose stores, and discrepancies in liver function lead to excessive fatty acid synthesis, insufficient fatty acid oxidation, and/or inadequate incorporation of fatty acids into very low density lipoproteins, eventually result in steatosis[12,26,29]. Secondly, oxidative stress paves the way for portal inflammation, lipid peroxidation, and ultimately fibrosis[12]. Assuming defective lipid metabolism as one of the underlying causes for intrahepatic fat deposition in NAFLD, serum adipokine levels are of certain interest. As such, leptin and adiponectin are of foremost importance in the formation of NAFLD.

Although the exact role of adipokines in the development and progression of NAFLD is unknown, Huang *et al*[29] proposed a positive association between blood leptin levels and incidences of NAFLD, while soluble leptin receptors were found to be significantly reduced in such cases. Other studies expounded on this claim and found that the elevated leptin levels cannot be tied definitively to IR in adults, but rather may be linked to the fibrogenesis noted in NAFLD[30]. It was found that hepatic stellate cells (HSCs) are responsible for the fibrotic changes that characterize NAFLD; HSCs produce leptin, suggesting that they may be implicated in fibrogenesis. According to Canbakan *et al*[30], leptin may increase the level of reactive oxygen species (ROS) in the liver, provoking Kupffer cells to produce TNF-α and other cytokines, thus enhancing collagen production and the progression

Figure 2 Role of adipokines in inflammation and fibrosis.
NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis. TNF-α: Tumor necrosis factor alpha; HSCs: Hepatic stellate cells.

of fibrosis (Figure 2). In children, hyperleptinemia was observed and was found to be correlated to the degree of liver impairment[31]. However, such studies performed in adults yielded inconclusive results, implying that hyperleptinemia may emerge in cases of early NAFLD, leading ultimately to IR and obesity. It is thought that the hyperleptin state only aggravates the inflammatory changes and fibrogenesis initiated by HCSs[31].

Adiponectin enhances glucose utilization and hepatic fatty acid oxidation *via* its receptors, AdipoR1 and AdipoR2. NAFLD patients displayed low adiponectin levels along with IR[12]. It was observed in mice that the hyperinsulinemic state in most liver disorders led to the downregulation of adiponectin receptors, leading to adiponectin resistance; studies have demonstrated, however, that the characteristically low adiponectin level observed in NAFLD is linked more to intrahepatic fat deposition rather than to the degree of liver damage[32]. High adiponectin levels in mice have succeeded in preventing intrahepatic fat deposition *via* inhibition of fatty acid synthesis. Bugianesi *et al*[32] identified increased serum activity of alanine transaminase (ALT) and γ-glutamyl transpeptidase (GGT) associated with hypoadiponectinemia in healthy individuals, highlighting the importance of adiponectin in preventing liver damage. Adiponectin plays a role in the inflammation observed in NAFLD; unlike leptin, however, high levels of adiponectin have been shown to inhibit the secretion of TNF-α by HSCs. One may surmise that there exists interplay between leptin-adiponectin levels and the inflammatory and fibrotic changes seen in NAFLD[12]. Adiponectin was reported to have antifibrogenic and antisteatogenic effects in the liver as opposed to leptin, which is involved in fibrogenesis. A retrospective study of serum adipokine levels in patients with NAFLD, who underwent liver biopsy due to elevated transaminase activities, found that a lower serum adiponectin/leptin ratio was useful as a non-invasive approach to discriminate NASH from simple steatosis[33]. The hepatic expression of AdipoR2 was found to be significantly higher in patients with NASH compared with controls and was related with necroinflammatory

injury[33].

Adult NAFLD patients displayed increased serum resistin values. Although there is debate surrounding the exact relation between resistin and obesity and IR, recent studies seem to support the notion that increased resistin levels may be correlated with IR, BMI, and the severity of NAFLD[33]. Women were found to display a higher level of serum resistin than men, attributable to the disparity in body fat content. Murad *et al*[34] discovered that patients with moderate to severe steatosis displayed higher serum resistin values than those with mild steatosis. Such a finding may be related to the link between resistin and IR and later inflammation. Resistin may exacerbate the inflammation brought on by TNF-α and IL-6 secreted from HSCs[34]. Specifically, it is proposed that resistin can induce the secretion of TNF-α and IL-12 from macrophages *via* a nuclear factor-kappa B-dependent cascade to control the release of IL-6 and IL-1β[35-37]. There exists the likelihood that HSCs themselves may produce resistin, as they do leptin, contributing to the hyper-resistin state observed in NAFLD[34].

Serum RBP4 was found to be increased in cases with IR, including obesity, TDM, and impaired glucose tolerance, suggesting that RBP4 may play a similar role in NAFLD[38]. Earlier research indicated that RBP4 is raised in non-diabetic NAFLD patients, and selected studies claimed that there exists a positive association between RBP4 and liver enzymes, specifically ALT and GGT[38]. However, Alkhouri *et al*[39] reported an inverse relationship between the degree of fibrosis and RBP4 levels, such that patients with late fibrosis and cirrhosis displayed lower RBP4 values.

Additionally, there have been several studies investigating the association between polymorphisms in the genes for adipokines and the susceptibility to NAFLD within populations sharing common environmental and metabolic predisposing factors[40]. Of the many leptin polymorphisms reported, the *LEPR* G3057A variant was shown to be expressed by many NAFLD patients, implying that this polymorphism may be an independent risk factor for developing NAFLD in patients with TDM[41].

Figure 3 Activation of peroxisome proliferator-activated receptors α and associated biological response in adipose tissue. TNF-α: Tumor necrosis factor α; COX-2: Cyclooxygenase-2; IL-1: Interleukin-1; FFA: Free fatty acids; RXR: Retinoid receptor; SMRT: Silencing mediator of retinoid and thyroid hormone receptors; NCoR1: Nuclear receptor co-repressor 1; PPAR: Peroxisome proliferator-activated receptors.

Adiponectin gene short nucleotide polymorphisms (SNPs) 45TG and 276GT were demonstrated in non-obese and non-diabetic NAFLD patients. Furthermore, these reported SNPs indicated the extent of liver injury in NAFLD[42]. A recent finding showed that a SNP in the Patatin-like phospholipase domain-containing 3 (*PNPLA3*), *i.e.*, I148M *PNPLA3* variant predicts the extent of steatosis in NAFLD[43]. The I148M *PNPLA3* genotype may represent a genetic determinant of serum adiponectin levels. Therefore, in carriers of the 1148M *PNPLA3* variant, modulation of serum adiponectin might be involved in mediating the susceptibility to steatosis[44].

FUNCTION OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS IN LIPID METABOLISM

The key element in the process of lipogenesis and lipolysis in adipose and non adipose tissues is mediated by PPARs. They are members of the steroid/retinoid nuclear receptor superfamily, and they act namely in two ways: transactivation and transrepression[45]. Three types of PPARs have been identified: alpha, gamma, and delta (beta)[46]. PPAR-α is expressed in the liver, kidney, heart, muscle, adipose tissue, and other organs. PPAR-β is expressed in many tissues, but markedly in the brain, adipose tissue, and skin[47]. Finally, PPAR-γ (γ2 and γ3) is expressed namely in adipose tissue.

PPAR-γ is activated by fatty acids and their derivatives. It plays a role in insulin sensitivity, adipogenesis, placental function, and transcription activation in concert with coactivators like steroid receptor coactivator-1. Coordination is required between PPAR-α and PPAR-γ activity for the maintenance of equilibrium between oxidation and synthesis of fatty acids. PPAR-α regulates the expression of genes involved in peroxisomal and mitochondrial beta-oxidation in liver and skeletal muscle; such genes encode for proteins such as fatty acid transport protein, fatty acid translocase, liver cytosolic fatty acid-binding protein, and uncoupling proteins-2 and 3. PPAR-γ2, on the other hand, is the central regulator of adipogenesis, favoring the deposition of excess calories in adipocytes. PPAR-γ2 is involved in growth arrest, clonal expansion, early and terminal differentiation, and anti-inflammatory effects in adipose tissue macrophages[48]. It modulates differentiation and cytokine production. Endogenous ligands of PPARs include FFA and eicosanoids[49].

Activation of PPAR-γ can promote secretion of anti-hyperglycemic adipokines like adiponectin, and shift the deposition of non-esterified fatty acids (NEFAs) to adipose tissue and away from liver and skeletal muscle, as it serves as an activator of lipoprotein lipase[50]. Moreover, adiponectin can increase PPAR-γ in adipose tissue. This process enhances its anti-inflammatory effects and thus insulin sensitivity in adipose tissue. The mechanism of activation of PPAR-γ is shown in Figure 3. It functions as a heterodimer with retinoic acid receptor (RXR), which binds direct repeat sequences AGGTCA in the promoter

region of its target genes, enhancing their expression[51]. Histone deacetylase and RNA pol Ⅱ activation are observed in response to PPAR-γ action in BAT and WAT[52]. Other ligands have been found to activate PPAR-γ and may be suitable as insulin-sensitizing agents to treat DM2.

ROLE OF PPARS IN NAFLD

Recent studies have proposed a downregulation of PPAR-α in cases of hepatic steatosis and obesity-related IR[53], thereby favoring lipogenesis over oxidation. This effect may be further aggravated by PPAR-γ upregulation, promoting overall lipogenesis. Adiponectin is responsible for the expression of PPAR-α in liver cells[54]. It has also been suggested that PPAR-α downregulation may facilitate the activity of hepatic pro-inflammatory cytokines, expediting the transition from steatosis to steatohepatitis; however, further research must be conducted to confirm such proposals[55]. Accordingly, PPAR-γ has been implicated in preventing pro-inflammatory cytokine gene expression *via* transrepression. As such, PPAR-γ ligands have been considered for administration in cases of inflammation, including NAFLD. In mice, PPAR-γ ligands successfully reversed the effects of cytokines produced by HSCs and managed to restore HSC quiescence. The thiazolidinedione class of antidiabetic drugs, which includes rosiglitazone and pioglitazone, acts as a PPAR-γ agonist in adipose tissue, reducing the release of NEFAs and enhancing hepatic insulin sensitivity. Although reversal of fibrosis was not observed in the 6-mo treatment with pioglitazone, improvement was noted in the 12-mo treatment of non-diabetic NASH subjects[45]. Treatment of NAFLD patients with n-3 polyunsaturated fatty acid, a known PPAR-α ligand, slightly alleviated steatosis and decreased transaminase activity[45].

Of the PPAR polymorphisms reported, a Leu162Val SNP for PPAR-α has been observed and severity of fibrosis in NAFLD[56]. Similarly, a Pro12Ala SNP has been documented for PPAR-γ. Though earlier studies have documented many NAFLD patients with the alanine variant of PPAR-γ, recent meta-analyses have failed to establish such an association between Pro12Ala SNP and NAFLD risk[50]. Further investigations are required to corroborate such findings.

ROLE OF PROINFLAMMATORY CYTOKINES IN NAFLD

Cytokines are soluble chemical mediators credited with a number of functions. They are renowned for their role in inflammatory diseases, including NAFLD. They are not normally secreted by the liver. Gradual hepatic lipid accumulation provokes HSCs and Kupffer cells to synthesize various cytokines, leading to portal inflammation, slow necrosis or apoptosis, and eventual fibrosis[57]. Of the cytokines implicated in NAFLD, TNF-α, TGF-β, and IL-6 will be discussed.

TNF-α is secreted by a number of body cells, but in particular by HSCs, Kupffer cells, and adipocytes. Selected studies have demonstrated a link between TNF-α expression and IR in steatohepatitis associated with NAFLD, indicating that adipocyte TNF-α has a key role in inflammation and IR by binding to the tyrosine kinase insulin receptor[57-59]. It has also been demonstrated that TNF-α induces swelling of hepatic mitochondria, resulting in eventual rupture and disruption of respiratory chain complexes, principally complexes I and Ⅲ[60]. Reduced activity of mitochondrial complexes were reported in experimental animals followed by hepatotoxicity[61,62]. Although the results from rodent studies supported an association between enhanced TNF-α expression in inflammatory diseases, such as NAFLD and NASH, and IR, in human subjects, such an association is under debate. In both species, however, TNF-α upregulation was observed in obese subjects, and a positive association was observed between the severity of hepatic fibrosis and serum TNF-α levels. Additionally, in both mice and humans, treatment with either anti-TNF-α antibodies or a TNF-α inhibitor (pentoxifylline), respectively, served to alleviate inflammatory and fibrotic changes. In humans, the drug also succeeded in reducing serum aminotransferase activity. Excess TNF-α is also thought to enhance the expression of SREBP-1c, and promote lipogenesis[58].

Of the many isoforms of TGF-β, TGF-β1 is most predominant within the inflamed liver and has been suggested to induce the transformation of HSCs to myofibroblasts through the production of proteins like collagen 1[57]. The production of TGF-β1 leads to a cascade of irreversible events, including further synthesis of TGF-β1, connective tissue growth factors, and type I collagen; a reduction in cell turnover as a result of impeded DNA synthesis; inhibition of metalloprotease expression; and initiation of apoptosis *via* the phosphatidylinositol 3-kinase (PI3K)-AKT pathway and of fibrosis *via* increased production of fibronectin[63]. It was also suggested that elevated serum TGF-β1 may be considered an independent predictor of fibrosis[63].

IL-6 possesses a contradictory role; a study in a mouse model claimed that in addition to activating hepatocytes, stem cells, and osteoclasts, IL-6 acts as a protective shield for the liver, hindering mitochondrial dysfunction in cases of hepatic steatosis[57]. Recent reports suggested a positive association between IL-6 and IR through its inhibition of hepatic cytokine signaling[59]. Administration of anti-IL-6 antibodies in obese mice was observed to enhance insulin sensitivity, and NAFLD mice and human subjects have demonstrated elevated serum IL-6 and IL-6 receptor levels. Mahmoud *et al*[63] established IL-6 to be the most important biomarker for NAFLD in their study. In contrast, IL-10 has been suggested to inhibit inflammation, and prevent steatosis, while some research has also shown IL-10 serum levels to be inversely related to incidence of MS[57]. There is preliminary evidence that hepatic natural killer T-cells accumulate in liver diseases and produce IL-13 and IL-4; IL-13 may then activate HSCs to produce further pro-inflammatory cytokines, recruiting TGF-β and initiating fibrosis[58].

Figure 4 Cytotoxic effects of free fatty acids in nonalcoholic fatty liver disease. FFA: Free fatty acid; JNK: c-Jun NH(2)-terminal kinase; ROS: Reactive oxygen species; TRAIL-R2: Tumor necrosis factor-related apoptosis inducing ligand; ATP: Adenosine triphosphate.

Figure 5 Changes of adipokines and peroxisome proliferator-activated receptors in nonalcoholic fatty liver disease. TGFβ1: Transforming growth factor beta 1; TNF-α: Tumor necrosis factor alpha; RBP4: Retinol binding protein 4. NAFLD: Nonalcoholic fatty liver disease; PPAR: Peroxisome proliferator-activated receptors.

Mild overload of iron is frequently observed in NAFLD. Significantly increased local as well as systemic TNF-α may favor the accumulation of iron in the liver. The progressive retention of iron can be ascribed to the impaired iron export from liver cells *via* cytokine-mediated downregulation of the iron exporter, ferroportin-1 (FP-1) in sinusoidal Kupffer cells. Iron accumulation may also result from an ineffective sensing of hepatic iron due to low hemojuvelin (HJV) expression. The increased hepatic and serum concentrations of TNF-α in NAFLD patients were inversely correlated with liver FP-1 and HJV mRNA levels whereas, it was positively associated with body mass index and hepatic hepcidin mRNA. Hepatic iron accumulation as well as increased level of TNF-α stimulates hepcidin formation, which decreases the duodenal and hepatic FP-1 expression and results in the blockage of duodenal iron uptake to compensate for liver iron overload. Nevertheless, decreased intestinal absorption of iron, and hepatic iron overload is found in NAFLD patients. Iron reduction therapy was found to be beneficial with regard to NAFLD disease activity and insulin sensitivity[64].

Mechanisms of NAFLD are closely linked to chronic inflammatory and oxidative stress responses. Increased intrahepatic levels of fatty acids as well as iron load provide a source of oxidative stress. Fatty liver is injured by ROS generated from microsomal, mitochondrial, and/or other hepatocellular pro-oxidant pathways when the antioxidant defenses are critically lowered. In the diminished antioxidant response, cells are susceptible to mitochondrial damage and cellular apoptotic injury. Ajith *et al*[65-67] reported reduced activity of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase during acute and chronic liver injury in experimental animals. In NASH, FFAs induce lipoapoptosis in hepatocytes[68]. Cytotoxic products of lipid peroxidation (*e.g.*, malondialdehyde, and 4-hydroxynonenal) may impair cellular functions including nucleotide and protein synthesis and may play a role in hepatic fibrogenesis. Increased ROS can release more TNF-α from hepatocytes, Kupffer

cells, and adipose tissue[69], and can further upregulate pro-inflammatory pathways. Increased levels of nitric oxide and superoxide radical interact to form peroxynitrite, which is an important mediator of free radical toxicity. The role played by the toxic FFAs is depicted in Figure 4. TNF-α can induce mitochondrial ROS and thus exacerbate NAFLD by attenuating the anti-inflammatory effects of adiponectin and PPAR-γ[70], and results in secondary inflammation and fibrosis in NAFLD. Animal models of NAFLD suggest that an increased translocation of bacterial endotoxins lead to an activation of toll-like receptor-dependent signaling cascades and increased formation of ROS[71].

CONCLUSION

NAFLD is one of the principal causes of chronic liver disease. Cytokines secreted by the adipocytes, such as TNF-α, TGF-β, and IL-6, are implicated in NAFLD. TNF-α, IL-6 and leptin have been shown to exert pro-inflammatory effects and adiponectin has been shown to exert anti-inflammatory effects at the liver level. Furthermore, preliminary evidence suggests that hepatic natural killer T-cells accumulate in liver diseases and produce IL-13 and IL-4; IL-13 may then activate HSCs to produce further pro-inflammatory cytokines, increase TGF-β and initiate fibrosis. Downregulation of PPAR-α in cases of hepatic steatosis favors lipogenesis over oxidation (Figure 5). PPAR-α downregulation may facilitate the activity of hepatic proinflammatory cytokines, expediting the transition from steatosis to steatohepatitis; however, further research must be conducted to confirm such proposals. PPAR-γ ligands have been considered for administration in cases of inflammation, including NAFLD; PPAR-γ ligands successfully reversed the effects of cytokines produced by HSCs and managed to restore HSC quiescence. Treatment of NAFLD patients with n-3 polyunsaturated fatty acid, a known PPAR-α ligand, slightly alleviated steatosis and decreased transaminase activity. Hence, ligands that activate these receptors may prevent the initial inflammatory reactions and render protection to the liver cells. Mechanisms of NAFLD are closely linked to chronic inflammatory and the oxidative stress response. However, there are insufficient data to support the use of antioxidant supplements for patients with NAFLD. Iron reduction therapy and lipid lowering drugs were found to be beneficial in NAFLD. Future research in these fields is required to design specific compounds to prevent fat deposition in liver cells.

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MINIREVIEWS

Pictures of focal nodular hyperplasia and hepatocellular adenomas

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Abstract

This practical atlas aims to help liver and non liver pathologists to recognize benign hepatocellular nodules on resected specimen. Macroscopic and microscopic views together with immunohistochemical stains illustrate typical and atypical aspects of focal nodular hyperplasia and of hepatocellular adenoma, including hepatocellular adenomas subtypes with references to clinical and imaging data. Each step is important to make a correct diagnosis. The specimen including the nodule and the non-tumoral liver should be sliced, photographed and all different looking areas adequately sampled for paraffin inclusion. Routine histology includes HE, trichrome and cytokeratin 7. Immunohistochemistry includes glutamine synthase and according to the above results additional markers such as liver fatty acid binding protein, C reactive protein and beta catenin may be realized to differentiate focal nodular hyperplasia from hepatocellular adenoma subtypes. Clues for differential diagnosis and pitfalls are explained and illustrated.

Key words: Focal nodular hyperplasia; Hepatocellular adenoma; Inflammatory hepatocellular adenoma; Beta catenin; Hepatocyte nuclear factor 1 alpha

Core tip: In this paper are illustrated macroscopic and microscopic aspects of focal nodular hyperplasia and hepatocellular adenoma. These illustrations represent typical as well as less usual aspects of these two benign hepatocellular tumors. Microscopic pictures are performed using classical routine stains such as HE, trichrome or cytokeratin 7 or less usual markers proven of great interest to identify focal nodular hyperplasia (FNH) such as glutamine synthase or liver fatty acid briding protein, C reactive protein or b catenin to identify hepatocyte nuclear factor 1 alpha mutated hepatocellular adenoma (HCA), inflammatory HCA, β-catenin mutated HCA respectively. These illustrations combined with brief clinical information should be helpful for pathologists for their practice. Indeed if FNH are rather frequent tumor easy to recognize, there are difficulties when key features are lacking or when features such as steatosis or sinusoidal dilatation, features of HCA, are present. The great message of this paper is the possibility to identify HCA subtypes, a key feature for the coming years to better manage patients.

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INTRODUCTION

In the most recent liver pathology textbooks[1-4], the description of focal nodular hyperplasia (FNH) and hepatocellular adenoma (HCA) taking into account the new classification of HCA allows to better differentiate FNH from HCA and to identify HCA subtypes, but not surprisingly there are few pictures of these 2 entities by lack

of space.

The aim of this atlas is to illustrate more extensively than in textbooks[1-4] and journals[5-7] the most frequent pathological aspects of FNH and HCA both macroscopically and microscopically, using traditional routine stainings and more recently described immunohistochemical approach.

It is important to mention that if these new immunohistochemical techniques have allowed the classification of HCA into subtypes by the pathologists, the definite criteria to classify HCA relies on molecular analysis[8]. The strict correlation between the interpretation of the HCA classification based on the phenotype (macroscopy, routine techniques and immunohistochemistry) versus the genotype defined by the molecular analysis has to be strictly performed. In addition, the molecular classification is still in progress; therefore some data presented here should be interpreted cautiously in particular regarding the subgroups of β-catenin mutated HCA (β-HCA) and unclassified HCA (UHCA).

FOCAL NODULAR HYPERPLASIA

FNH are frequent hepatocellular nodules. Until recently the diagnosis was not always easy[9]. If the diagnosis is certain, resection is not recommended[10]; however, resection is occasionally performed upon pain, organ compression, doubt in differential diagnosis with HCA or with hepatocellular carcinoma. Using routine histopathological techniques [HE, trichrome, cytokeratin 7 (CK7)] the diagnosis is certain in more than 80% of cases. Glutamine synthase (GS) staining has the great advantage to confirm rapidly and with high confidence the diagnosis[11]. When combining all the markers, the accuracy of the diagnosis is close to 100%.

HEPATOCELLULAR ADENOMA

HCA are rare hepatocellular nodules frequently resected when their size exceed 5 cm. HCA are divided into subtypes using molecular markers and immunohistochemistry[12]. The most common HCA: HNF1A mutated HCA (H-HCA, 35%) and inflammatory HCA (IHCA, 50%) can be recognized with some degree of confidence by imaging techniques and by HE[13-15]. GS is a surrogate marker to identify β-catenin mutated HCA (β-HCA, 10% and β-catenin activated inflammatory HCA, β-IHCA, 10% of IHCA)[15,16]. Identification of β-catenin mutated HCA is of major clinical relevance because of the highest risk of malignant transformation[17,18]. The mutation can be confirmed using β-catenin staining. The absence of aberrant nuclear staining is not however an argument to refute the diagnosis, because it is often focal[15,16]. When GS is difficult to interpret, molecular biology is necessary to demonstrate β-catenin mutation in order to confirm the diagnosis[8]. When the diagnosis of H-HCA or IHCA is not self-evident on routine stainings, immunostainings included liver-fatty acid binding protein (L-FABP), C-re-

active protein (CRP), markers which are useful to identify H-HCA (absence of LFABP) and IHCA (diffuse positivity of CRP). The absence of the above markers identifies unclassified HCA (less than 10%). The differential diagnosis between FNH and HCA is important as well as the identification of HCA subtypes thought to be of great importance for the present and future management[19-22]. Indeed, in the past there has been a great confusion between FNH and HCA[23,24].

Below are figures of FNH and HCA. For each macroscopic or microscopic pictures, we have given in the legend a minimum of clinical information. In this practical atlas we have not illustrated HCA with malignant transformation.

DIAGNOSIS

To increase the chance to make a correct diagnosis, some rules must be respected: the specimen must be carefully sliced; photographs should be taken to illustrate necrosis, hemorrhage and fibrotic bands; all areas of interest must be sampled as well as the non tumoral liver and the junction between the nodule and the non tuomral-liver; routine histology includes HE, trichrome, and CK7; these markers may be sufficient to make a diagnosis of FNH, HCA and eventually HCA subtypes. When needed to differentiate an FNH from an HCA or to identify HCA subtypes, additional immunomarkers may be useful. In some occasions all of them may be useful when there is no indication for a specific diagnosis or HCA subtypes: it includes LFABP, C reactive protein (CRP), GS and β-catenin. Even when all the rules are followed, difficulties in interpretation may occur. First of all to have to check the quality of the technique; it is not necessary to overinterpret doubtful immunohistochemical data. When there is no way to interpret satisfactorily the data, one is forced to rely on the molecular data. This is why it is highly recommended for referral center to freeze material (tumor and non tumoral tissue) when their counterparts are analysed by routine histology.

RESULTS

FNH

The macroscopic aspects are presented in Figure 1. Microscopic typical features on Masson's Trichrome, GS and CK7 immunostainings are presented in Figure 2. Microscopic atypical features on Masson's trichrome and GS immunostaining are presented in Figure 3. The other microscopic aspects dealing with steatotic, pre-FNH and regressing FNH are presented in Figure 4.

HCA

The macroscopic aspects of different subtypes are presented in Figure 5.

H-HCA

The microscopic typical and atypical features are present-

Figure 1 Focal nodular hyperplasia the macroscopic aspects. A: Man born in 1988; 100 kg/1.95 m; abdominal pain; abnormal liver function tests; magnetic resonance imaging (MRI): liver mass 9.5 cm: Focal nodular hyperplasia (FNH)/hepatocellular adenoma (HCA) (bisegmentectomy in 2009). Fresh specimen: typical aspect of FNH: Tan, vaguely plurinodular tumor, non encapsulated, with central stellate scar. This macroscopic aspect is typical and the diagnosis of FNH is evident. The diagnosis was confirmed on HE and CK7. B: Woman born in 1951; acute abdominal pain followed by discomfort and pain on abdominal palpation; ultrasound (US): 2 nodules, largest one 3.5 cm. Magnetic resonance imaging (MRI): FNH (left hepatectomy in 2008). Fixed specimen: plurinodular tumor with thin fibrous bands, non encapsulated but well demarcated from surrounding liver parenchyma, with large portal tract at the interface (arrow). The diagnosis of FNH is evident. The diagnosis was confirmed on HE and CK7. C: Woman born in 1986; abdominal pain; MRI 2007: FNH 6.4 cm close to the biliary convergence; US in 2000: hemangioma 15 mm; 2004: 4.5 cm (tumorectomy in 2007). Fresh specimen: pedunculated irregular nodule with eccentric fibrous scar, well demarcated from the surrounding liver. The diagnosis of FNH is likely. D: Woman born in 1965; oral contraceptives for 18 years; abdominal pain; imaging (6 cm): FNH [surgery in 2005 (tumorectomy)]. Fresh specimen: clear-tan, vaguely plurinodular tumor, without clear-cut fibrous scar. The diagnosis of FNH is not self-evident. The diagnosis was confirmed on HE, CK7 and glutamine synthase (GS). E: Woman born in 1961; abnormal liver function tests; liver US: nodule 5.5 cm, MRI and US favors HCA over FNH (segmentectomy VII in 2009). Fresh specimen: irregular cut surface with tan nodules separated by congestive/reddish, atrophic areas. The diagnosis of FNH is unlikely. F: Woman born in 1956; check-up for arterial hypertension; liver imaging (2 cm nodule): probable HCA (tumorectomy in 2003) (other nodules were found). Fresh specimen: well-limited, non encapsulated, smooth nodule, with small reddish areas, without any fibrous bands or scar visible. The diagnosis of FNH is unlikely. The diagnosis was confirmed by HE, CK7 and GS.

ed in Figures 6 and 7 respectively. The different aspects of LFABP and GS stainings are presented in Figures 8 and 9 respectively.

Inflammatory HCA

The typical microscopic aspects are presented in Figures 10 and 11. The immunohistochemistry is presented in Figure 12.

β-IHCA

Microphotographs are presented in Figures 13-15.

β-catenin

Micrographs are presented in Figures 16 and 17 showing

Figure 2 Focal nodular hyperplasia microscopic typical features on Masson's trichrome, glutamine synthase and CK7 immunostainings. A: Same patient as Figure 1C. Glutamine synthase (GS) immunostaining: typical aspect of focal nodular hyperplasia (FNH) with large anastomosed positive areas in a "map-like" pattern[11]. B-D: Woman born in 1969; abnormal liver function tests (GGT); ultrasound (US): nodule interpreted as a hemangioma, computed tomography scan and magnetic resonance imaging: FNH 8 cm, segment VIII with minor dilatation of the right biliary tree (compression by the tumor). Left lobectomy + segmentectomy VIII in 2002 (other nodule segment II : 0.5 cm). B, C: GS immunostaining: typical aspect of FNH with anastomosed positive areas often centered by veins (arrowhead) and at distance of fibrous bands (arrows). C and D are from 2 serial sections. D: CK7 immunostaining: ductular reaction at the periphery of fibrous bands. E, F: Same patient as Figure 1C. E: Masson's trichrome - fibrous bands surrounding benign hepatocytic nodules of different sizes. F: CK7 immunostaining - prominent ductular reaction at the junction between parenchymal nodules and fibrous bands. Typical aspect of FNH.

Figure 3 Focal nodular hyperplasia microscopic atypical features on Masson's trichrome and glutamine synthase immunostaining. A, B: Same patient as Figure 1E. A: Masson's trichrome - large areas of sinusoidal dilatation (arrowhead), nearby solid hepatocytic areas (right) with thin, short fibrotic bands. B: Glutamine synthase (GS) immunostaining - typical aspect of focal nodular hyperplasia (FNH) with anastomosed positive areas in a "map-like pattern" in the nodular area (right); no staining in the sinusoidal dilation area (left). This aspect is very unusual. This nodule should not be interpreted as a mixed tumor (part FNH and part hepatocellular adenoma) and should not be called "telangiectatic FNH"[23,24]. A better term could be FNH with major sinusoidal dilatation. C, D: Woman born in 1969, 2003; 2 FNH: first hepatic resection (tumorectomy in 2003 for a 7-cm FNH). In 2004, persistence of abdominal discomfort (no change in size of the 7 cm FNH). Right hepatectomy. C: Typical GS staining (left); no GS staining in the area of sinusoidal dilatation (right). D: Obvious ductular reaction on the CK7 immunostaining. Although the 2 above cases are very rare, FNH with areas of sinusoidal dilatation are seen occasionally.

Figure 4 Other aspects (steatotic, pre-focal nodular hyperplasia, regressing focal nodular hyperplasia). A, B: Woman born in 1959; heavy alcohol consumption, tobacco; hepatomegaly, no evidence for cirrhosis on liver function tests, virus negative. Computed tomography scan: nodule 3.5 cm and others (1.5, 0.3 and 0.8 cm). No firm radiological diagnosis: ?focal nodular hyperplasia (FNH)/hepatocellular adenoma (HCA)/HCC, left hepatectomy in 2001. A: Masson's trichrome - the nodule is composed of benign steatotic hepatocytes separated by fibrous bands. Steatosis is not a rare event in FNH. B: Glutamine synthase (GS) immunostaining - anastomosed GS positive areas, favoring the diagnosis of steatotic FNH. The diagnosis of FNH remains doubtful, however. In this case, the diagnoses of HNF1A mutated HCA, inflammatory HCA, β-HCA and β-IHCA were ruled out (specific IHC markers were negative). The diagnosis of unclassified HCA cannot, however, be ruled out. Molecular analysis is necessary to confirm the diagnosis. C, D: Woman born in 1950; abdominal pain: gallbladder lithiasis; Magnetic resonance imaging (MRI): 2.5 cm nodule, probably an HCA. Left hepatectomy plus cholecystectomy in 2000. C: Fixed specimen - subcapsular, not well defined nodule barely visible, clearer than the surrounding liver. D: GS immunostaining - the positive perivenular areas are slightly but significantly larger than in the surrounding normal liver; this aspect is interpreted as a "pre-FNH" without fibrosis and nodulation. Pre FNH is probably not a rare entity but to-day there is no consensus concerning its denomination[9]. This type of lesion has been named in congenital vascular abnormalities such as Rendu-Osler disease. E, F: Woman born in 1948; intrahepatic hemorrhagic rupture of a large nodule of segment Ⅶ in 2009. MRI favors HCC, additional 4 nodules known since 2005 and interpreted as FNH. Size of the FNH nodule resected with the HCC has decreased significantly. Right hepatectomy (HCC + 1 additional not identified nodule, previously known as FNH). E: Fresh specimen: close to the hemorrhagic HCC, a 4 cm hard white/brown nodule, under the capsule (arrow), with irregular surface. F: GS immunostaining: limited positive areas (map-like pattern) surrounded by large areas of dense fibrous tissue (arrowhead). All these features are interpreted as a regressing FNH. This interpretation needs to be confirmed by additional cases.

Figure 5 Hepatocellular adenoma macroscopic aspects of different subtypes: A-C: Examples of HNF1A mutated hepatocellular adenoma (H-HCA). A: Woman born in 1972; oral contraceptives for 8 years. Discovery of a liver nodule (3.5 cm). No regression after stopping contraceptives. Fear of complications by the patient. Left hepatectomy in 2008; fresh specimen: yellowish tumor, clearer than the surrounding liver. The diagnosis of H-HCA is likely but not self-evident. The diagnosis was confirmed by immunohistochemistry. B: Woman born in 1974; oral contraceptives for 12 years. Liver nodules discovered by chance on imaging and diagnosis of adenomatosis. Largest nodule 5 cm. Segmentectomy IVb 2006. Other small nodules. One nodule was an focal nodular hyperplasia (FNH). Fixed specimen of one nodule: yellowish, clear tumor, non encapsulated, contrasting with the surrounding liver. The diagnosis of H-HCA is likely. The diagnosis was confirmed by immunohistochemistry. C: Woman born in 1978. Massive right hepatomegaly discovered in the obstetric department (miscarriage at 6 wk). No oral contraception. Right hepatectomy in 2004. Fresh specimen: large irregular, mammillated tumor occupying the whole right liver. This is an exceptional case. The diagnosis was confirmed by immunohistochemistry. D-F: Examples of IHCA (D, E) and β-IHCA (F). D: Woman born in 1968; overweight, BMI > 40 kg/m^2. Oral contraceptives for 18 years. Several nodules detected on imaging. Doubtful diagnosis. Surgical biopsies: HCA. Right hepatectomy in 2008 (largest nodule 10 cm). In 2009, another known HCA removed in the left liver (11 cm). Fresh specimen: reddish tumor with congestive areas. The diagnosis of inflammatory HCA (IHCA) is likely. The diagnosis was confirmed by immunohistochemistry. E: Woman born in 1956; oral contraceptives for 31 years. Biological abnormalities (inflammatory syndrome). CT scan and MRI multiple liver nodules (largest nodule 7 cm) in favor of IHCA. Surgery in 2007: bisegmentectomy Ⅵ-Ⅶ plus 2 tumorectomies. Fresh specimen: ill defined tumor with congestive strands. The diagnosis of IHCA is very likely. The diagnosis was confirmed by immunohistochemistry. F: Woman born in 1971; liver hemorrhage. Imaging: 5 nodules, largest 8 cm. No oral contraceptives; BMI 20.4 kg/m^2. Segmentectomy Ⅲ, Ⅵ, Ⅷ 2007. Fixed specimen: large hematoma and a narrow viable tissue at the periphery. No obvious diagnosis. The diagnosis of HCC cannot be ruled out. The diagnosis was confirmed by immunohistochemistry. G: Example of β-catenin HCA. Woman born in 1981; one nodule 8 cm discovered by chance. Imaging HCA. Oral contraceptives for 8 years; BMI 21.1 kg/m^2. Right hepatectomy 2005. Fresh specimen: well limited clear nodule. The diagnosis of HCA is likely. H-HCA and IHCA are unlikely. H: Example of HCC developed on β-IHCA. Woman born in 1934; intramuscular injection of hormones as contraceptive. Liver nodule interpreted as hemangioma, known for several years. Growth of the nodule. Segmentectomy in 2000. Fresh specimen: irregular, multinodular tumor, with a large necrotic and hemorrhagic area (arrowhead) surrounded by a fibrous rim. The diagnosis of HCC is likely. I: Example of unclassified HCA. Woman born in 1983; abdominal pain. Imaging: one nodule 8 cm; no final diagnosis. Oral contraceptives for 8 years. BMI 20.2 kg/m^2. Right hepatectomy 2007. Fresh specimen: well limited clear nodule with a pale reddish area. The diagnosis of HCA is likely. H-HCA and IHCA are unlikely.

Figure 6 HNF1A mutated hepatocellular adenoma microscopic typical features. A: Woman born in 1955; surgery in 2010 for an HCC (6 cm) developed on a non fibrotic liver. Discovery of another small nodule on the surgical specimen. No oral contraceptives; BMI 24.1 kg/m^2. HE: typical aspect of HNF1A mutated hepatocellular adenoma (H-HCA): nodule with lobulated contours, made of benign hepatocytes with diffuse steatosis; some congestive areas (arrowhead); sharp contrast with non steatotic surrounding liver. The diagnosis was confirmed by immunohistochemistry. B, C: Woman born in 1952; abdominal pain; imaging: nodule 1.8 cm, no firm diagnosis by magnetic resonance imaging. Oral contraceptives, 27 years; BMI 22.0 kg/m^2. Tumorectomy in 2008. B: Nodule composed of benign, clear hepatocytes, sometimes steatotic, separated by thin strands of atrophic hepatocytes (arrow). C: Same nodule seen at higher magnification. Although clear hepatocytes are not the hallmark of H-HCA, the lobular pattern is very characteristic. It consists of steatotic or clear hepatocytes arranged in a lobular pattern separated by tumoral, atrophic, not steatotic hepatocytes. Commonly, arterioles/small arteries are seen in this space (not shown on this micrograph). The diagnosis was confirmed by immunohistochemistry.

Figure 7 HNF1a inactivated hepatocellular adenoma microscopic atypical feature. A, B: Woman born in 1952; abnormal liver function tests; oral contraceptives 13 years; BMI 25.4; magnetic resonance imaging (MRI): adenomatosis, largest nodule 6 cm. Segmentectomy Ⅳ/Ⅵ in 2001. A: ill defined nodule composed of clear hepatocytes, with mild steatosis. B: Same nodule seen at a higher magnification. The normal portal tract from the periphery of non tumoral parenchyma is entrapped in the nodule. The diagnosis of HNF1A mutated hepatocellular adenoma (H-HCA) is less evident than in Figure 6B, C. The diagnosis was confirmed by immunohistochemistry. C, D: Woman born in 1950; abnormal liver function tests; imaging: probable FNH 12 cm. Right hepatectomy 2002. HE: proliferation of benign hepatocytes, no steatosis, mild sinusoidal dilatation. This is a rare case. The diagnosis of H-HCA cannot be suspected without performing immunohistochemistry. E, F: Woman born in 1965; past history of cancer. Abdominal pain. Oral contraceptives 10 years, BMI 21 kg/m^2. MRI: Adenomatosis, largest nodule 3.9 cm. Segmentectomy and tumorectomy in 2011. HE: Proliferation of benign hepatocytes, no steatosis, mild sinusoidal dilatation, numerous ectatic vessels. As above, in this rare case the diagnosis of H-HCA cannot be suspected without immunohistochemistry, and malignant transformation has to be ruled out.

Figure 8 HNF1a inactivated hepatocellular adenoma different aspects of liver-fatty acid binding protein staining. A: Same patient as Figure 7C, D. Typical aspect: absence of liver-fatty acid binding protein (LFABP) staining in tumor contrasting with normal expression in non tumoral liver (NT). B: Same patient as Figure 7E, F. Bands of non tumoral (NT) parenchyma normally expressing LFABP are penetrating within the unstained tumor. Indeed, the growth of HNF1A mutated hepatocellular adenoma (H-HCA) is due to the coalescence of several adenomatous liver nodules and it is not rare to find non adenomatous liver lobules squeezed in between. C: Woman born in 1962; abdominal pain. Oral contraceptives 25 years; BMI 25 kg/m². Two liver nodules, largest 3.7 cm. Segmentectomy Ⅴ/Ⅵ (2005). Bands of hepatocytes expressing LFABP within the unstained typical H-HCA. Same comment as above. D: Same patient as Figure 7E, F. LFABP expression in a few perivenular hepatocytes, particularly at the periphery of H-HCA, is a frequent observation, as well as a rim of positive hepatocytes at the border. E, F: Woman born in 1967; adenomatosis suspected during coelioscopy for extrauterine pregnancy. Segmentectomy for a 6 cm liver nodule. On HE (E), at distance from the main lesion, the presence of several steatotic small nodules well identified on LFABP staining (F). This is a very specific and characteristic aspect of H-HCA adenomatosis.

Figure 9 HNF1a inactivated hepatocellular adenoma different aspects of glutamine synthase immunostaining. A: Same patient as Figure 7E, F. There is no staining within the lesion, except around some veins, mainly at the borders where liver-fatty acid binding protein staining (not shown) demonstrates intermingled HN-F1A mutated hepatocellular adenoma (H-HCA) and normal parenchyma areas. It was impossible on HE staining to clearly identify the border of the tumor. B: Woman born in 1964; one nodule discovered by chance, 5.5 cm; oral contraceptives 21 years; BMI 18.4 kg/m². Biopsy: β-HCA. Segmentectomy Ⅷ 2007. Diffuse, moderate glutamine synthase (GS) staining, contrasting with normal staining in non tumoral liver in which the GS staining is limited to 1-3 rows of centrilobular hepatocytes. Today, in the absence of β-catenin nuclear staining, the significance of this abnormal GS staining in HCA remains unknown and molecular analysis is mandatory to search for β-catenin mutations. C, D: Same patient as Figure 7C, D. Heterogeneous, mild staining in tumoral hepatocytes, sometimes arranged in rosettes. The presence of rosettes is often considered as a criterion suggesting a possible malignant transformation; it may also reflect cholestatic features. Same comment as above: in the absence of molecular analysis, it is not possible to conclude and a search for β-catenin mutations is mandatory.

Figure 10 Inflammatory hepatocellular adenoma typical microscopic aspects. A: Woman born in 1959; adenomatosis discovered by chance. Size of the largest nodule: 11 cm. Oral contraceptives for 23 years; BMI 27.0 kg/m^2. Left hepatectomy 2008. HE: typical aspect of an inflammatory hepatocellular adenoma (IHCA) with many small inflammatory foci (arrows) dispersed within the tumor, associated with areas of moderate sinusoidal dilation (arrowhead). In this area, sinusoids are dilated, another hallmark of this subgroup. B: Woman born in 1969; abnormal liver function tests. One liver nodule, 12 cm. Oral contraceptives for 16 years; BMI 26.0 kg/m^2. Right hepatectomy 2004. HE: areas of sinusoidal dilatation and pseudo portal tracts with thick walled arteries and inflammatory cells (arrows), hallmarks of this subgroup. The diagnosis was confirmed by immunohistochemistry. C: Man born in 1968; abnormal liver function tests. One nodule 12 cm. BMI 30.0 kg/m^2. Right hepatectomy 2011. HE: prominent inflammatory foci dispersed in the tumor; thick vessels at the border of the HCA (arrow). The diagnosis was confirmed by immunohistochemistry. D: Woman born in 1966; liver nodule, 3.5 cm discovered by chance. No oral contraceptives, BMI 24.5 kg/m^2. Right hepatectomy 2004. HE: inflammatory foci, areas of sinusoidal dilatation; in this area, tumoral hepatocytes are steatotic. E: Woman born in 1973; overweight. Adenomatosis discovered by chance. Largest nodule 7 cm. Biopsy HCA. Tumorectomy IV, VI, VII 2003. HE: ill defined benign hepatocellular tumor (arrow); limited areas of sinusoidal dilatation (arrowhead) predominating at the periphery of the tumor. The non tumoral liver is steatotic, a frequent finding in this group of patients. F: Woman born in 1966; abnormal liver function tests. Several liver nodules. Biopsy HCA; oral contraceptives for 10 years, BMI 29.6 kg/m^2. Left hepatectomy and tumorectomy IV and VI, 2007. Masson's trichrome: pseudo portal tracts (arrows) with arteries in fibrous tissue; large areas of steatosis, within the tumor. The tumor is limited by thick arteries and veins from the non tumoral liver (NT).

Figure 11 Inflammatory hepatocellular adenoma typical microscopic aspects. A: Same patient as Figure 10 D. B: Same patient as Figure 10F. HE: thick-walled arteries surrounded by inflammatory cells. These pseudo portal tracts are very characteristic of inflammatory hepatocellular adenoma (IHCA). C, D: Woman born in 1972; oral contraceptives for 19 years. BMI 19.6 kg/m^2. One nodule 10 cm discovered by chance. Magnetic resonance imaging: IHCA. Right hepatectomy 2009. HE: prominent sinusoidal dilatation. E, F: Same patient as Figure 10F, different tumors. E: HE - tumor ill-defined from the surrounding liver without any inflammation or sinusoidal dilatation. F: HE - the histological aspect is different with a more typical aspect of IHCA. Here, thick arteries are surrounded by inflammatory cells and fibrous tissue within the hepatocellular proliferation.

Figure 12 Inflammatory hepatocellular adenoma immunohistochemistry. A, B: Same patient as Figure 10 F, different tumors. C-reactive protein (CRP): typical aspect of inflammatory hepatocellular adenoma (IHCA) with strong and diffuse expression in tumoral hepatocytes, with sharp demarcation from the surrounding non tumoral liver (A); more irregular CRP staining with limited areas remaining negative (B); C: Same patient as Figure 10D. CRP is expressed only in hepatocytes. D, E: Same patient as Figure 10F. D: Glutamine synthase: no abnormal staining; positivity only in some perivenous hepatocytes at the periphery of the nodule. E: CK7: faint staining around pseudo portal tracts, underlining ductular reaction, a common finding in IHCA. F: Same patient as Figure 10A. CK7 highlights the major ductular reaction at the periphery of pseudo portal tracts.

Figure 13 β-catenin activated, inflammatory hepatocellular adenoma. A-F: Man born in 1971. BMI 21.6 kg/m². By chance, discovery of one nodule 6 cm. Imaging: focal nodular hyperplasia. Right hepatectomy 2006. A: Fresh specimen: pigmented, irregular tumor. Non tumoral liver is normal. B: HE: Hepatocellular adenoma with sinusoidal dilatation and inflammatory infiltrate (on the left); large vessels at the junction with non tumoral liver. C: Diffuse expression of C-reactive protein by tumoral hepatocytes, with sharp demarcation from the non tumoral liver. D: Strong and diffuse glutamine synthase (GS) expression contrasting with normal staining of GS in adjacent non tumoral liver (in a few pericentrolobular hepatocytes). E: Large areas are positive for CD34, but not diffuse diffusely; F: Aberrant nuclear and cytoplasmic expression of β-catenin in quite numerous hepatocytes.

Figure 14 β-catenin activated, inflammatory hepatocellular adenoma. A, B: Woman born in 1967. Oral contraceptives 20 years, BMI 20.0 kg/m². By chance, discovery of one nodule 18 cm. Imaging hepatocellular adenoma (HCA). Segmentectomy IV and V 2005. A: HE: features of inflammatory HCA (IHCA): sinusoidal dilatation, thick vessels, mild inflammation. B: Glutamine synthase immunostaining is abnormal, but faint and heterogeneous with reinforcement around veins. C, D: Woman born in 1974; oral contraceptives 13 years. BMI 21.0 kg/m². By chance, discovery of one nodule 6.5 cm. Imaging IHCA. Segmentectomy Ⅵ and Ⅶ 2009. C: Marked but not diffuse CD34 immunostaining. D: Very few tumoral hepatocytes expressed aberrant nuclear β-catenin.

Figure 15 β-catenin activated, inflammatory hepatocellular adenoma. A-C: Woman born in 1971; liver hemorrhage. No oral contraceptives. BMI 20.4 kg/m². Imaging 5 nodules, largest 8 cm. Segmentectomy Ⅲ, Ⅵ, Ⅷ 2007. A: HE - large arteries at the periphery of the nodule. Mild sinusoidal dilatation in the non tumoral liver (NT). B: Abnormal patchy GS staining in one nodule. C: Abnormal homogeneous glutamine synthase (GS) staining in another nodule. D-F: Woman born in 1959; oral contraceptives for 21 years. BMI 21.8 kg/m². Abnormal liver function tests. Imaging: 3 nodules, largest 10 cm. Right hepatectomy 2005. In the three nodules, GS staining is different but definitely abnormal. D: Homogeneous. E: Extremely faint. F: Patchy GS staining. The difficulty in interpreting GS often comes from the positivity that can be found around veins. This perivenular staining is normal when it is strictly limited to 1 or 2 rows of perivenular hepatocytes; the interpretation of a GS staining larger than 2 or 3 rows of hepatocytes, even if faint or patchy, remains poorly understood in the absence of molecular analysis.

Figure 16 β-hepatocellular adenoma.. A, B: Man, 16 years old under androgen treatment for a hematological disorder. Imaging: several nodules; tumorectomy of one nodule 3 cm. HE: Hepatocellular adenoma (HCA) with some glandular arrangements (arrowhead) and a few larger, irregular nuclei (arrow). According to the clinical context, the diagnosis of β-catenin is very likely. C, D: Woman born in 1953; oral contraceptives for 4 years. Danazol one year (endometriosis). BMI 19.5 kg/m². Asthenia. Imaging: one nodule 3 cm. Right hepatectomy in 1989. C: HE: Well limited HCA with no features of H-HCA, or of IHCA. By default, the diagnosis of β-catenin is therefore a possibility. D: Aberrant cytoplasmic and nuclear expression of β-catenin in numerous hepatocytes confirms the diagnosis of β-HCA; E, F: Woman born in 1980; oral contraceptives for 12 years. BMI 20.4 kg/m². Abdominal pain. Imaging: one nodule 15 cm: HCA. Right hepatectomy 2009. E: HE - numerous vessels within the HCA. This aspect seems to be quite characteristic of β-HCA and of some unclassified HCA; F: Aberrant expression of β-HCA in very few hepatocytes confirms the diagnosis of β-HCA.

Figure 17 β-hepatocellular adenoma different types of glutamine synthase immunostaining. A: Same patient as Figure 16C, D. Strong and diffuse expression of glutamine synthase (GS) in hepatocellular adenoma (HCA) (left), contrasting with non tumoral liver (positivity in only few pericentrolobular hepatocytes). B-D: Same patient as Figure 16 E, F. Strong, heterogeneous (patchy) positivity of GS seen at different magnification, with a reinforcement rim at the periphery of the HCA (the rim positivity has no pathological significance). E, F: Woman born in 1986; abnormal liver function tests. Imaging: one nodule 9 cm, HCA. Left hepatectomy 2007. E: HE - no specific abnormalities. The presence of numerous vessels is, however, intriguing in this young patient. F: Patchy positivity of GS from mild to strong.

Figure 18 β-catenin hepatocellular adenoma. A, F: Same patient as Figure 5G. A: HE: Numerous vessels dispersed within the hepatocellular proliferation. B: Quite numerous CK7+ cells dispersed within the tumor; some are small, looking like progenitor cells; others are larger as intermediate cells. C, D: Diffuse positivity of CD 34 within the tumor. E, F: Patchy positivity of glutamine synthase.

Figure 19 Unclassified hepatocellular adenoma. A, B: Woman born in 1988; oral contraceptives for 4 years. BMI 16.8. Abdominal pain. Imaging: one nodule 11 cm. No final diagnosis. Left hepatectomy 2009. A: Diffuse expression of CD34 in hepatocellular adenoma (HCA) (top) contrasting with adjacent non tumoral liver (below). B: No expression of glutamine synthase (GS) except at the periphery of the HCA. Here the nodule is divided in 2 parts at its periphery by a thin band of normal tissue containing vessels. C, D: Woman born in 1975; oral contraceptives for 12 years. BMI 24.2 kg/m^2. Hemorrhage. Imaging: one nodule 5 cm, HCA. Right hepatectomy 2003. C: Widespread but not diffuse expression of CD34 within the HCA. D: Numerous cells overexpressing CK7: small cells looking like progenitor cells and intermediate cells. GS was normal. E, F: Same patient as Figure 5I. E: HE: thick fibrous rim around a necrotic area (arrowhead); peliotic areas within the viable HCA. F: Some small CK7 positive cells dispersed within the HCA. GS (not shown) was normal. CD34 staining was more or less diffuse (not shown).

Figure 20 Unclassified hepatocellular adenoma. Woman born in 1980; oral contraceptives for 6 years. BMI 22.2 kg/m^2. Abdominal pain. Imaging: one nodule 3 cm, hepatocellular adenoma (HCA). Segmentectomy VI 2004. A, B: On CD34, three zones (1-3) are seen in this nodule. Zone 1 is the external limit of the nodule; zone 2 is intermediate and zone 3 represents the quasi totality of the nodule. Only zone 3 is diffusely positive. In zone 2, CD34 positivity is seen along vascular axis. C, D: Glutamine synthase (GS) staining: zone 3 is negative. Zone 1 is negative except around veins. In zone 2, GS staining is patchy. E, F: CK 7 - in zone 3, few cells, possibly progenitor cells are positive. In zone 2, positive cells are seen along vascular axis. This nodule has been classified as UHCA because all specific markers were negative. It is not rare to observe a thin peripheral rim which is CD34 negative /GS positive in unclassified HCA or β-HCA. In this case, the presence of 2 zones different from the bulk of the tumor remains unexplained but should not change the diagnosis.

different types of GS staining and Figure 18.

UHCA

UHCA are still poorly understood entities. However, their histological characteristics are worrisome and in many aspects in at least 50% of cases are reminiscent of b catenin HCA. Micrographs are presented in Figures 19 and 20.

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MINIREVIEWS

Pancreatic neuroendocrine tumor accompanied with multiple liver metastases

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Abstract

Pancreatic neuroendocrine tumor (P-NET) is rare and slow-growing. Current classifications predict its prognosis and postoperative recurrence. Curative resection is ideal, although often difficult, because over 80% of patients have unresectable multiple liver metastases and extrahepatic metastasis. Aggressive surgery for liver metastases is important to improve survival. Aggressive or cytoreductive surgery for liver metastases is indicated to reduce hormone levels and improve symptoms and prognosis. Liver transplantation was originally conceived as an ideal therapy for unresectable liver metastases. Unfortunately, there is no clear consensus on the role and timing of surgery for primary tumor and liver metastases. Surgeons still face questions in deciding the best surgical scenario in patients with P-NET with unresectable liver metastases.

Key words: Gastroenteropancreatic neuroendocrine tumor; Pancreas; Liver metastasis; Liver surgery; Liver transplantation

Core tip: Pancreatic neuroendocrine tumor is rare. Current classifications predict its prognosis and postopera-

tive recurrence. Curative resection is often difficult, because over 80% of patients have unresectable multiple liver metastases and extrahepatic metastasis. Aggressive or cytoreductive surgery for liver metastases is indicated to reduce hormone levels and improve symptoms and prognosis. Liver transplantation was originally conceived as an ideal therapy for unresectable liver metastases. However, there is no clear consensus on the role and timing of surgery for primary tumor and liver metastases.

INTRODUCTION

Pancreatic neuroendocrine tumor (P-NET) is a rare and slow-growing tumor[1]. The American Joint Committee on Cancer stated a new TNM classification in 2009, based on tumor size, including direct invasion and lymphoid and distant metastases[2]. In 2010, the World Health Organization categorized gastroenteropancreatic neuroendocrine tumor (GEP-NET) into three categories (G1, G2 and G3) based on histopathological differentiation, proliferation index (Ki-67), neuroendocrine biomarkers (such as chromogranin A and synaptophysin), hormonal behavior, tumor size, direct invasion, and distant metastasis[3]. These classifications are useful for predicting the prognosis and postoperative recurrence[1]. Curative resection is ideal for this slow-growing tumor[1,4-6], and postoperative surveillance of at least 10 years is required, because long-term recurrence can occur after surgery[1].

Curative surgery is often difficult, because over 80% of P-NET patients already have unresectable multiple liver metastases and extrahepatic metastasis[1]. Some cur-

rent opinions suggest an expanded surgical indication for P-NET patients with liver metastases, because survival is improved[1,6-9]. Aggressive surgery for liver metastases or cytoreductive surgery for over 90% of the visible tumors are important to improve survival[6,9]. Cytoreductive surgery for liver metastases is indicated to reduce hormone levels and improve clinical symptoms and prognosis[1,6,9]. Liver transplantation (LT) was originally conceived as an ideal therapy for unresectable liver metastases[1,10].

Unfortunately, there is no clear consensus on the role and timing of surgery for primary tumor and liver metastases, although current reports refer to liver surgery including LT for unresectable liver metastases. Surgeons still face questions in deciding the best surgical scenario in patients with P-NET with unresectable liver metastases. Here, we reviewed previous studies about therapeutic strategies for P-NET, with our regretful case.

RESECTION OF PRIMARY TUMOR

Approximately half of P-NETs are nonfunctioning[11], and tumors < 10-30 mm are not indications for surgery[1,6]. Functional P-NET should be removed even if the tumor is < 10 mm[1,6], because functional P-NET has malignant potential despite a small tumor size[1]. Some factors, such as young age, hormonal function, and surgical resection, are important for overall survival[6,12]. Seventy to ninety percent of enlarging P-NETs have malignant potential[1], and the aim of surgery for primary nonfunctioning tumor is to avoid malignant change and subsequent distant metastasis[6]. Although endoscopic ultrasonography with fine-needle aspiration biopsy is useful for determining the malignant potential and predicting prognosis[13-15], there are no definitive criteria regarding whether P-NET should be removed or observed based on tumor size[1,6]. Curative resection is considered as standard therapy in well-differentiated GEP-NET G1/G2 with a Ki-67 index of < 10%[1,4]. Cytoreductive surgery for primary tumor is indicated to reduce hormone levels and improve clinical symptoms[1,6,16], although the effects on prognosis are still controversial[1,5]. Overall, surgery for primary tumor should be curative resection[1,4-6], although palliative therapy may be indicated if there is a possibility of improvement of clinical symptoms, such as endocrine symptoms, oppression on surrounding organs by primary tumor, jaundice and oral passage disturbance[6,17].

RESECTION OF LIVER METASTASES

Curative surgery is often difficult, because over 80% of P-NET patients already have unresectable multiple liver metastases and extrahepatic metastasis[1]. Current opinions suggest extended surgical indications for P-NET patients with liver metastases, because survival is improved and P-NET is a slow-growing tumor[1,6-9]. For liver metastasis without extrahepatic metastasis, standard/aggressive surgery is the first choice for well-differentiated P-NET categorized as GEP-NET G1/G2[1,7,8]. Aggressive surgery for liver metastases and cytoreductive surgery for > 90% of the visible tumors are important to improve survival[6,9]. Cytoreductive surgery for liver metastases is indicated to reduce hormone levels and improve clinical symptoms and prognosis[1,6,9].

LT FOR UNRESECTABLE LIVER METASTASES

LT was originally conceived as an ideal therapy for advanced hepatic malignancy, because it eliminates the liver tumors and the potential for recurrence in the liver remnant[1,10]. LT for unresectable metastases has essentially been abandoned[10]. Several attempts to implement this strategy between 1960 and the 1980s showed poor results, although LT for early hepatocellular carcinoma has been established[18]. It is well known that highly selected P-NET patients with liver metastases may be candidates for LT[10,19-21]. The only prospective study recommended strict selection criteria for LT with curative intent (*i.e.*, low grade, removal of primary tumor, liver involvement < 50%, age < 55 years, and stable disease for ≥ 6 mo before LT)[21], and a study reported 96% overall survival and 80% disease-free survival[22]. However, it was also reported that P-NET patients with liver metastases who received LT had a follow-up term of no longer than 5.8 years, and the longest tumor-free survival was 5.1 years[23], and a high rate of tumor recurrence was reported at almost 60%[20].

Use of LT for extended indications always presents an ethical dilemma[10]. The United Network for Organ Sharing has generally held that LT for malignancy should be considered only when results are essentially equivalent to results with standard indications, generally requiring a 5-year survival rate of 60%-70%[10]. LT in selected GEP-NET patients has shown a 5-year recurrence-rate as low as 30%[21]. Previous results that indicate LT for P-NET[20-22] must be interpreted cautiously[10], especially given the global scarcity of liver grafts available[10]. These results should not justify LT at this time[10]. The Milan Criteria is maybe a better definition of selection criteria for LT[21]. In the last decade, selection criteria based on clinical presentation have been integrated with a proper histopathologic classification and diagnostic techniques[21]. In particular, Ki67 expression has been considered as a prognostic factor of risk of recurrence[21,24-28]. A Ki67 proliferation index of < 10% is a characteristic of well-differentiated tumor, which we have adopted as a cut-off value to consider GEP-NET patients for LT candidates[21,24]. Current studies suggest a growing consensus concerning LT for liver metastases of P-NET as follows[20,24-28]: (1) liver metastases of symptomatic or asymptomatic P-NET are unresectable; (2) disease is confined to the liver, and extrahepatic metastases are ruled out; (3) LT is indicated for well-differentiated P-NET categorized as GEP-NET G1/G2. Poorly differentiated P-NET categorized as GEP-NET G3 is considered as a contraindication for LT. Ki67 index < 10% is recommended; and (4) LT should not be associated with major extrahepatic

resection. Primary tumor should be removed before LT.

As described above, primary tumor should be removed before LT. However, optimal timing for LT in patients with stable versus progressive disease remains unclear[20]. In previous report, 83% of patients had undergone surgical treatment for primary tumor, and a 5-year overall survival has increased to 59% in relation with fewer patients presenting poor prognostic factors[20]. Favorable outcomes in cases of unknown primary tumor might suggest that a failure to detect the primary tumor before LT should not be considered as an absolute contraindication[20].

MANAGEMENT OF UNRESECTABLE LIVER METASTASES

For metastatic poorly-differentiated P-NET categorized as GEP-NET G3, cisplatin-based combination therapy is considered as the first-line therapy. Radiofrequency ablation, transarterial chemoembolization (TACE), transcatheter arterial infusion (TAI) and selective inhibitor of mammalian target of rapamycin are available as optional treatments[1]. Systemic biotherapy, such as somatostatin analog and interferon-α, is indicated for functional P-NET and postoperative recurrence[1].

Peptide receptor radionuclide therapy (PRRT) with radiolabeled somatostatin analogs is a novel treatment in patients with somatostatin receptor-expressing, well-differentiated and metastatic neuroendocrine tumors[29-31], and the PRRT with yttrium and/or lutetiumis a potent therapeutic approach. On the other hand, transarterial radioembolisation [*i.e.*, selective internal radiotherapy (SIRT)] is an innovative therapy in liver-limited unresectable, neuroendocrine liver metastases[32-34]. SIRT is an effective treatment option for patients with metastatic liver disease in a salvage setting with acceptable toxicity.

OUR REGRETFUL CASE

A 39-year-old man was diagnosed with nonfunctioning P-NET in the pancreatic head, with multiple liver metastases. The tumor was 2.5 cm in diameter, and was histopathologically well-differentiated with a Ki-67 expression of < 10%. He was asymptomatic. Small but multiple metastases were detected in the liver, and no extrahepatic metastases were observed. We initially intended to control the liver metastases before resection of the primary tumor. To begin with, TACE/TAI were repeated. Thereafter, TACE/TAI, systemic chemotherapies and biotherapies were repeated. Although liver metastases seemed to be stable for a while, the primary tumor was enlarged even after therapy. At 3.5 years after initial diagnosis, the primary tumor became symptomatic. Liver metastases enlarged and massive swelling of the para-aortic lymph nodes was observed. Thereafter, palliative therapy was the main course of action. He died at 4.3 years after initial diagnosis. We understand that P-NET patients often have unresectable liver metastases at initial diagnosis[1],

and that surgical indications for P-NET with liver metastases should be determined individually in each case[6]. Resection of the primary tumor in metastatic nonfunctioning P-NET patients with unresectable liver metastases does not significantly improve survival[4]. Presence of liver metastases is a major prognostic factor for P-NET patients[1,20], and surgical management of liver metastases remains controversial[9]. In our case, we initially intended to control the liver metastases before resection of the primary tumor, because we considered liver metastases as the most important prognostic factor. Our decision at that time may have been consistent with previous opinions[1,4,6,9,20]. However, in our case, aggressive surgery for liver metastases seemed to be difficult even during a period of stable liver metastases, and resection of primary tumor is required before LT. We retrospectively regret that aggressive surgery for primary tumor and subsequent LT for unresectable liver metastases may have provided a better course in our case.

DISCUSSION

Currently, classification of GEP-NET is useful for evaluating malignancy, predicting prognosis, and determining therapeutic strategies[1,2]. Though this report focused surgical options for P-NET with liver metastases, novel managements (*i.e.*, PRRT and SIRT) are currently available for unresectable liver metastases, with acceptable side effects[29-34]. Effective and beneficial treatment options for P-NET patients with liver metastases should be carefully considered. From the viewpoint of surgical option, surgical indications for primary tumor[1,4-6,16] and hepatic surgery, including LT for liver metastases[1,10,20,24-28] have already been stated. However, it seems to be not easy to decide optimal timing of surgery for primary tumor and liver metastases. Currently, surgical procedures and devices are well developed, and the question is whether pancreatoduodenectomy or distal pancreatectomy is risky. We believe that pancreatic surgery is safe and beneficial for patients, if indicated.

In LT for P-NET patients, previous excellent reports focused on a prognostic factors for overall survival, a post-transplant risk of recurrence, a better selection criteria, a difference between P-NET and others, and an importance of the post-transplant surveillance[21,24,28]. There is a difference in behaviors between P-NET and the other tumors, the indication for LT for unresectable liver metastases is unique for P-NET[21,24]. Also, an importance of careful surveillance after LT due to the risk of recurrence was documented[21,24]. Tumor re-staging should be scheduled at least 4 times per year for the first two years and continued thereafter with progressively longer follow-up intervals[21].

Though we understand that any decisions cannot be made based on a single patient experience, we retrospectively speculate that a negative approach to aggressive surgery for primary tumor may have resulted in poor quality of life and deprived patient of the opportunity of LT for unresectable liver metastases. P-NET patient with

liver metastases could have been a candidate for initial surgery for primary tumor and might have had a chance of subsequent LT for unresectable metastases. Surgeons still face questions in deciding the best surgical scenario in patients with P-NET with liver metastases.

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ORIGINAL ARTICLE

Assessing liver injury associated with antimycotics: Concise literature review and clues from data mining of the FAERS database

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Abstract

AIM: To inform clinicians on the level of hepatotoxic risk among antimycotics in the post-marketing setting, following the marketing suspension of oral ketoconazole for drug-induced liver injury (DILI).

METHODS: The publicly available international FAERS database (2004-2011) was used to extract DILI cases (including acute liver failure events), where antimycotics with systemic use or potential systemic absorption were reported as suspect or interacting agents. The reporting pattern was analyzed by calculating the reporting odds ratio and corresponding 95%CI, a measure of disproportionality, with time-trend analysis where appropriate.

RESULTS: From 1687284 reports submitted over the 8-year period, 68115 regarded liver injury. Of these, 2.9% are related to antimycotics (1964 cases, of which 112 of acute liver failure). Eleven systemic antimycotics (including ketoconazole and the newer triazole derivatives voriconazole and posaconazole) and terbinafine (used systemically to treat onychomicosis) generated a significant disproportionality, indicating a post-marketing signal of risk.

CONCLUSION: Virtually all antimycotics with systemic action or absorption are commonly reported in clinically significant cases of DILI. Clinicians must be aware of this aspect and monitor patients in case switch is considered, especially in critical poly-treated patients under chronic treatment.

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Key words: Drug-induced hepatotoxicity; Antimycotics; Drug safety; Pharmacovigilance; Spontaneous reporting systems

Core tip: The recent regulatory interventions (United States restriction and Europe suspension) concerning ketoconazole for drug-induced liver injury (DILI) poses a prescribing challenge to clinicians, who should now carefully consider safer therapeutic alternatives. Data mining of FAERS database (2004-2011) highlighted that: (1) antimycotics are involved in approximately 3% of DILI cases (including acute liver failure events); (2) virtually all systemic antimycotics (*e.g.*, azole derivatives), are associated with disproportionality signals; careful monitoring is therefore recommended, especially in critical poly-treated patients with multiple co-morbidities; and (3) topical antimycotics, as expected, do not generate a post-marketing signal of DILI, thus indicating the accuracy of our approach.

Raschi E, Poluzzi E, Koci A, Caraceni P, De Ponti F. Assessing liver injury associated with antimycotics: Concise literature re-

INTRODUCTION

Following the recent interest and regulatory measures on ketoconazole-related liver injury, the present manuscript provides: (1) a concise overview of the literature on drug-induced liver injury (DILI), namely a practical guide for clinicians to realize the need for active post-marketing vigilance; and (2) a case study on antimycotics, based on key results obtained from our original analysis of the international publicly available Food and Drug Administration (FDA) database, to advance the reporting pattern of DILI in the real-world practice. This approach can provide physicians with practical clues to assign the level of DILI risk among antimycotics.

Overview of DILI

Definition of DILI: Hepatotoxicity and DILI are interchangeable terms adopted by clinicians, researchers as well as drug developers to describe a broad spectrum of liver manifestations. Indeed, DILI can be defined as a liver injury induced by a drug or herbal medicinal products leading to liver test abnormalities or liver dysfunction with reasonable exclusion of other competing etiologies. More than 1100 medicines, natural products, vitamins, dietary supplements, recreational and illicit compounds have been reported to cause DILI, and the list is constantly growing. Antibiotics, anticonvulsants, and antidepressant therapy remain the commonest causes of DILI in the Western Hemisphere[1]. Among others, tumor necrosis factor-alpha antagonists, fluoroquinolones, tyrosine kinase inhibitors, statins and food supplements are gaining appreciation[2].

Issues in clinical practice: A number of classifications have been proposed to facilitate diagnoses and research in the field. From a pharmacological standpoint, DILI can be divided into "intrinsic" (*i.e.*, dose-related) and "idiosyncratic" (*i.e.*, dose-independent)[3]; however, the validity of this classification remains controversial and debated[4]. The term idiosyncratic was first used to identify a reaction occurring in susceptible individuals, usually associated with variable or prolonged latency (several weeks to 1 year) and generally unexpected on the basis of the pharmacological action of the drug. Idiosyncratic DILI is linked to the vast majority of compounds, although a common misconception of idiosyncratic DILI regards its non relation with the dose. As a matter of fact, recent data suggested that a daily dose > 50 mg (and especially when the dose exceeds 100 mg daily) for drugs with high lipophilicity undergoing extensive hepatic metabolism is associated with a higher risk of idiosyncratic DILI[5-7]. The latest evidence revealed that (1) drugs which are sub-

strates of CYP enzymes would have the higher likelihood of causing DILI (which is dose-independent); (2) drugs which are cytochrome P450 (CYP) inhibitors would have the higher likelihood of generating DILI only when they are administered at a high daily dose; and (3) drugs which are CYP inducers are not observed to be associated with DILI[8].

From a clinical standpoint, DILI can be classified into acute and chronic persistent (*i.e.*, evidence of continue liver injury after discontinuation of causative agent beyond 12 mo of follow-up). Paracetamol (acetaminophen in the United States) represents the leading cause of acute dose-dependent DILI. More precisely, an international DILI working group of clinicians and scientists developed uniform consensus criteria to standardize the phenotypes of DILI[9]. Although liver enzymes such as transaminases lack specificity, liver injury in the context of DILI has been defined decades ago as an elevation in the serum concentration of alanine aminotransferase (ALT), conjugated bilirubin or alkaline phosphatase exceeding 2 × the upper limit of normal (ULN)[10]. The expert working group proposed a cut-off level of 5 × ULN for ALT as better threshold to exclude clinically unimportant and self-limited drug-related hepatic events as well as nonalcoholic steatohepatitis not related to DILI.

The most common clinical presentations of DILI are hepatocellular, cholestatic and mixed, which should be defined on the basis of biochemical criteria. The R value is use for this purpose [R = (ALT/ULN)/(ALP/ULN)]; when R ⩾ 5 the pattern is considered hepatocellular, whereas if R < 2 is cholestatic. Usually, the damage induced by amoxicillin-clavulanate is considered to be cholestatic, as compared to the hepatocellular pattern caused by methotrexate.

Diagnosis is a major challenge for clinicians and is based on a combination of factors, which are influenced by the expertise: (1) exclusion of other causes that may elevate hepatic biochemical tests (*in primis* hepatitis viruses); (2) causality assessment methods, which may be based on expert opinion or on standardized liver-specific scoring instruments such as the roussel uclaf causality assessment method (RUCAM), endorsed by the Council of International Organization of Medical Sciences[11]; and (3) the presence of a signature pattern indicative of a specific causative agent (*e.g.*, typical features of DILI by telithromycin include short latency and abrupt onset of fever, abdominal pain, and jaundice, sometimes with the presence of ascites even in cases that resolved)[12]. In this context, a clinical research workshop took place to review and attempt to standardize the current nomenclature and terminology used in DILI research. Because DILI is a diagnosis of exclusion, selected elements of the medical history, laboratory tests, and previous reports were proposed to improve causality assessment[13]. The role of liver biopsy is still controversial, especially histological features and their relationship with biochemical parameters, although a systematic approach has been recently proposed as a foundation to correlate DILI pathology with its causality and outcome[14]. Efforts have been also

directed to identify a list of essential elements (minimum requirements) when submitting case reports for publication[15]. Recently, a dedicated website was finally created as an aid for clinicians and researchers to quickly extract updated information on DILI (http://livertox.nih.gov/).

Issues in drug development: The regulatory impact of DILI is appreciably expanding, as demonstrated by the fact that specific Consortia have been established, especially in the United States, where the DILI Network was created in 2003 to prospectively identify a large number of patients with *bona fide* DILI that will allow for collection of epidemiological data and biological samples for future mechanistic studies[16].

DILI has been a major cause of drug withdrawals, non approval and variegate regulatory actions in the past 50 years. In one study of 38 drugs withdrawn from the market between 1990 and 2006, 14 (37%) were related to hepatotoxicity[17]. A more recent review highlighted that of the 25 safety-based withdrawals in Europe and United States, ten (40%) were for cardiovascular events and seven (28%) for gastrointestinal, primarily hepatic, adverse events. It is clear that the majority of these regulatory measures concerned rare events with delayed onset and were not predicted based on known pharmacological action[18]. It is also remarkable that spontaneous reporting systems have been a primary source of information that mainly contributed to regulatory actions, especially for newly marketed drugs and events that are likely to be drug- induced.

From a historical perspective, in several circumstances, hepatotoxic agents were identified after being approved by regulators and marketed for some time; this was the case, for instance, of bromfenac (withdrawn 11 mo after its approval) and more recently troglitazone, which was marketed in 1997 and withdrawn worldwide in March 2000. In other cases, DILI led to non approval or interruption of late phases of drug development (*e.g.*, in 2006, the manufacturer of the oral anticoagulant ximelagatran withdrew a pending application to the FDA).

In this context, potential safety issues on DILI should be recognized as early as possible during drug development in order to accurately predict the actual risk in the post-marketing phase[19]. There are currently a number of challenges and controversies in exploiting predictive preclinical studies, especially for animal models, where ethical issues pose important limitations[20].

In line with the clinical situation, there are no specific biomarkers that may be used for optimal prediction of the risk. In fact, the frequency of asymptomatic rise in serum ALT in the pre-approval phase does not correlate *per se* with the frequency of symptomatic liver injury in the post-marketing period. The quest for highly predictive biomarkers has been underway for years and remains an ongoing challenge[21]. Although some genetic associations (*e.g.*, flucloxacillin and HLA-B[1]5701) have been identified, the clinical utility of genetic polymorphisms associated with drug-specific DILI appears still limited[1]. In addition, there are at least 3 groups of individuals

showing different pattern of hepatic response: tolerates (the vast majority of patients without significant changes in liver biochemical tests), susceptibles (showing progressive increase in ALT level that continues to increase and evolves into clinically significant liver damage with signs and symptoms) and adaptors (showing transient increase in enzyme levels, which eventually return to baseline despite continuation of the drug)[19].

So far, regulatory authorities and drug developers have mainly relied on the so-called Hy's law or rule[22], coined following Zimmerman's observations, to predict post-marketing risk of serious hepatic events and for making recommendations on whether treatment should be continued, stopped or its dose reduced, following biochemical abnormalities with the suspect drug. To define a clinical trial subject as a Hy's law case, the following components must be present: (1) ALT increase to a level ≥ 3 times ULN; (2) total bilirubin increase ≥ 2 times ULN; (3) no significant increase in ALP (initial Alp values does not reach 2 times ULN); and (4) no other cause is found to explain liver injury.

When the above criteria are met, Hy's rule predicts a mortality rate that can exceed 10%. In the last decade, two population-based studies further support the validity of this rule by demonstrating a mortality rate of 9%-12%[23,24]. Based on the 2009 FDA guidance, finding two cases is considered highly predictive that the drug has the potential to cause severe DILI when given to a larger population[25]. Thus, Hy's law calls for enhanced vigilance on the patient so that the drug is discontinued before the patient crosses the threshold of hepatotoxic irreversibility. The correct timing of discontinuation and the exact level of ALT elevation is still a matter of debate; an eight-fold increase from baseline is generally considered a standard cut-off, although even levels greater that 3 time the ULN may be sufficient if accompanied by additional symptoms. Given the importance of monitoring thousands of laboratory parameters during clinical phases, the eDISH method was developed to visualize, assess and summarize hepatic safety data during clinical trials[26]. As regards causality assessment, pharmaceutical companies prefer to rely on a 3-category scale instead of using the 5-category scale adopted by the DILIN consortium or the RUCAM score[19].

Antimycotics and DILI: clinical and regulatory aspects: The true incidence of DILI associated with antimycotics in the post-marketing phase challenges precise estimation, especially as compared to other antimicrobials[27]. Previous studies, mostly based on data derived from clinical trials, reported a 2%-23% range, depending on the drug of interest and especially the severity of the disease[28]. Clinical evidence on ketoconazole emerged in early 80', when 54 reports of alleged ketoconazole-induced liver injury submitted to the FDA from the time of initial marketing in 1980, of which 33 were labeled as likely. The incidence of symptomatic, potentially serious hepatic injury appeared to be very low, perhaps 1 in 15000 exposed individuals, whereas the incidence

of mild, asymptomatic, reversible elevations in serum transaminases has been estimated to be 5%-10%[29]. Also García Rodríguez *et al*[30] found that ketoconazole was associated with significant risk. Very recently, a systematic review and meta-analysis documented an overall incidence of 3.6%-4.2%, which may be considered as common[31]. Data on other antifungals such as terbinafine are more scant and mostly based on individual case reports[32-34]. The latest population-based study in Taiwanese concluded that oral antifungal agents are associated with a low incidence if DILI, which may be fatal (especially in the elderly) and increased for longer treatment[35].

On July 26th, 2013, the European Medicines Agency (EMA) and the FDA issued important safety announcements: the EMA's Committee on Medicinal Products for Human Use (CHMP) has recommended that the marketing authorisations of oral ketoconazole-containing medicines should be suspended throughout the European Union (the CHMP concluded that the risk of liver injury is greater than the benefits in treating fungal infections) (http://www.ema.europa.eu/ema/index.jsp?curl=pages/news_and_events/news/2013/07/news_detail_001855.jsp&mid=WC0b01ac058004d5c1), whereas the FDA limits usage of Nizoral® (ketoconazole) oral tablets due to potentially fatal liver injury and risk of drug interactions and adrenal gland problems (http://www.fda.gov/drugs/drugsafety/ucm362415.htm). The FDA has revised the Boxed Warning, added a strong recommendation against its use (contraindication) in patients with liver disease, and included new recommendations for assessing and monitoring patients for liver toxicity. The FDA has also approved a new patient Medication Guide containing information on the potential risks associated with Nizoral® tablets, which must be dispensed with every prescription for the drug. In the revised US drug label, indications for dermatophyte and Candida infections have been removed and the indications for treatment of blastomycosis, coccidioidomycosis, histoplasmosis, chromomycosis, and paracoccidioidomycosis have been retained only for patients in whom other antifungal treatments have failed or are not tolerated.

These regulatory measures were driven by the following reasons: (1) suspension of the drug in France (June 2011), which asked the EMA to carry out a full assessment of the benefit-risk balance of oral ketoconazole-containing medicines; (2) the incidence and severity of liver injury with oral ketoconazole were higher than with other antifungals; (3) reports of liver injury occurred early after starting treatment with recommended doses, and it was not possible to identify measures to adequately reduce this risk; and (4) the widespread off-label use of oral ketoconazole for treating patients with Cushing's syndrome; and (5) currently available alternative treatments are deemed to be safer.

Possible contribution of pharmacovigilance in DILI research: Pharmacovigilance, namely post-marketing phase of drug development, represents the mainstay to evaluate the safety of recently marketed drugs or to monitor high-priority adverse events[36]. On one hand,

spontaneous reports and data mining of large spontaneous reporting systems still represent the traditional source for safety surveillance; on the other hand, exploiting electronic healthcare records. Both approaches are important and can be viewed to complement each other. However, it is a common opinion (corroborated by original research) that spontaneous reporting systems are more suited for newly approved drugs (as they aim at early signal detection) and rare events with suggestive drug-related component[18]. It is noteworthy that the diagnostic potential of commonly applied signal-detection algorithms is reasonably efficient and accurate to discriminate true drug-event associations from those that are likely to be spurious[37].

In this context, the aim of this study is to show that spontaneous reporting systems can contribute in characterizing the hepatotoxic potential of systemic antifungals. As a matter of fact, a recent systematic review highlighted that regulatory measures on drug risk (*e.g.*, dear doctor letters, safety warnings) may differently but substantially impact prescribers' attitudes, thus causing a switch towards therapeutic alternatives, perceived as safe[38]. This could be the case for some systemic antifungals, especially following marketing suspension of oral ketoconazole, both in US and Europe, for risk of DILI.

MATERIALS AND METHODS

Data source

The publicly available international FDA Adverse Event Reporting System, now termed FAERS database (2004-2011), was used for data mining of DILI associated with antimycotics. FAERS serves as repository of potential adverse events and medication errors associated with chemical and biological agents spontaneously submitted by healthcare professionals, patients and manufacturers. Since 2003, more than 7 million reports have been accumulated into FAERS, with more than 700000 records entered in 2011. In the light of its large catchment area (including also European reports potentially related to serious events) and public availability (since 2004), FAERS plays a leading role in signal detection and characterization, especially for rare events with high drug-attributable risk such as torsade de pointes (TdP)[39-41]. DILI shares different clinical, pharmacological and regulatory issues with TdP and, remarkably, the accuracy of FAERS to investigate DILI cases has been very recently demonstrated, especially as an important aid to systematically track emerging signals of DILI for newly marketed drug[42].

Data selection and analysis

Data processing and management (*e.g.*, duplicate detection and removal, handling of missing data) were performed according to previous methodology, extensively described in a dedicated book chapter[43]. In this study, main selection criteria regards the role code assigned to the drug (*i.e.*, only "Primary Suspect", "Secondary Suspect" or "Interacting", whereas cases where antimycotics were reported as "Concomitant" were not included) and information on age and gender (records with missing

PT	Liver injury	Acute hepatic failure
Acute hepatic failure		X
Alanine aminotransferase abnormal	X	
Alanine aminotransferase increased	X	
Ammonia increased	X	
Aspartate aminotransferase abnormal	X	
Aspartate aminotransferase increased	X	
Bilirubin conjugated increased	X	
Bilirubin urine	X	
Blood bilirubin abnormal	X	
Blood bilirubin increased	X	
Blood bilirubin unconjugated increased	X	
Cholestasis	X	
Coma hepatic		X
Cytolytic hepatitis	X	
Hepatic encephalopathy		X
Hepatic enzyme abnormal	X	
Hepatic enzyme increased	X	
Hepatic failure		X
Hepatic function abnormal	X	
Hepatic necrosis	X	
Hepatitis	X	
Hepatitis acute	X	
Hepatitis cholestatic	X	
Hepatitis fulminant		X
Hepatitis toxic	X	
Hepatocellular damage	X	
Hepatotoxicity	X	
Hyperammonaemia	X	
Hyperbilirubinaemia	X	
Jaundice	X	
Jaundice cholestatic	X	
Jaundice hepatocellular	X	
Liver function test abnormal	X	
Liver injury	X	
Liver transplant		X
Mixed hepatocellular-cholestatic injury	X	
Subacute hepatic failure	X	
Transaminases abnormal	X	
Transaminases increased	X	
Urine bilirubin increased	X	

PT: Preferred term of the MedDRA terminology. "X" indicates that the PT was used to define the liver event; MedDRA: Medical Dictionary for Regulatory Activities.

data in these fields were not considered). Drugs of interest were represented by antimycotics with systemic use, including those agents used for topical indications that may have systemic absorption (*e.g.*, terbinafine and griseofulvine). These compounds were mapped through an *ad hoc* created archive of drug names and converted into relevant active substances. Events of interest were selected according to a pre-specified search strategy (see below) and based on the standard MedDRA (Medical Dictionary for Regulatory Activities) terminology for codification.

Search strategy

In line with a previous multidisciplinary collaborative work[44], a two-fold approach was carried out to identify any type of liver damage, namely acute and chronic injuries as well as acute liver failure events. To this aim, dif-

ferent MedDRA Preferred Terms (PTs) were identified to define two patterns of liver event with different severity and clinical implication: "liver injury" (LI, including both chronic and acute liver events) and "acute liver failure" (ALF, a severe liver injury potentially reversible in nature and with onset of encephalopathy[45]) (Table 1). In order to assign a certain report to a precise group, at least one pre-specified PT should be present. A mutually exclusive approach was performed; a single case report of interest was classified only in one group based on the following priority: ALF>LI. Therefore, LI does not include cases with ALF; this allowed us the identification of a so-called "overall liver injury" (OLI).

Statistical analysis

Description of the overall number of cases was first provided, both in terms of ALF, LI and OLI; demographic information (age and gender) were also analysed. Then, a case/non case disproportionality approach was carried out by calculating the Reporting Odds Ratio (ROR) with relevant Confidential Interval (CI). Statistically significant disproportionality was formally defined when the lower limit of the 95%CI was > 1, with at least 3 cases[46]. Cases were represented by reports of DILI according the aforementioned pre-specified MedDRA PTs, whereas non cases were defined as all other reports (*i.e.*, those without such PTs). The ROR was first calculated by comparing a given antimycotic against all remaining drugs recorded in the entire FAERS database (*i.e.*, comprising all spontaneous reports from all drugs), by considering ALF and LI both separately and cumulatively (*i.e.*, by providing the ROR for OLI). Finally, where appropriate, the ROR was calculated within antimycotics by using a subset of data (*i.e.*, reports associated only with antimycotics). Restricting the analysis among agents belonging to the same pharmaco-therapeutic class may be useful as a sensitivity approach to test whether any potential intraclass differences exist in terms of risk. Cumulative time-trend disproportionality analysis was also performed, when appropriate[47].

RESULTS

Over the 8-year period, 2612807 reports with at least one mapped active substance were processed; in 3.4% of these, at least one pre-selected PT of interest was recorded. The precise allocation of reports is provided in Figure 1, according to case definition and drugs of interest. Based on our selection criteria, 68,115 DILI cases (OLI) were identified, corresponding to 4% of overall FAERS reports; the majority (91.7%) regarded LI. Antimycotics were reported in 3% of LI and 2% of ALF cases.

In the entire FAERS database, 52% of cases of DILI occurred in females, whereas only in 44% of cases associated with antimycotics female gender was recorded. The analysis on age distribution showed a peak occurring in patients with 50-60 years of age (all FAERS database, no matter the drug under scrutiny), both for ALF and LI; only 26% and 27% of DILI cases occurred in patients

Figure 1 Flowchart describing data-mining approach to allocate cases of interest according to selection criteria and case definition.

ATC code	Active substance	Cases LI	Cases ALF	Cases OLI	ROR (95%CI) LI	ROR (95%CI) ALF	ROR (95%CI) OLI
N02BE01	Paracetamol	2780	645	3425	3.69 (3.54-3.84)[1]	9.58 (8.82-10.41)[1]	4.31 (4.16-4.48)[1]
C10AA05	Atorvastatin	1763	131	1894	3.46 (3.29-3.64)[1]	2.62 (2.20-3.12)[1]	3.43 (3.27-3.60)[1]
C10AA01	Simvastatin	1516	80	1596	2.62 (2.49-2.77)[1]	1.44 (1.15-1.80)[1]	2.54 (2.41-2.67)[1]
N06AX21	Duloxetine	1456	59	1515	3.34 (3.15-3.53)[1]	1.42 (1.10-1.83)[1]	3.32 (3.14-3.50)[1]
L03AB07	Interferon beta-1	1377	46	1423	0.73 (0.70-0.78)	0.27 (0.20-0.36)	0.69 (0.66-0.73)
J05AF05	Lamivudine	1162	130	1292	4.68 (4.40-4.98)[1]	5.22 (4.38-6.22)[1]	4.86 (4.57-5.16)[1]
L01BA01	Methotrexate	1200	86	1286	2.89 (2.72-3.07)[1]	2.15 (1.74-2.66)[1]	2.85 (2.69-3.02)[1]
J01CR02	Amoxicillin/clavulanic acid	1164	94	1258	5.86 (5.49-11.36)[1]	4.50 (3.67-5.53)[1]	5.89 (5.54-6.27)[1]
C02KX01	Bosentan	1177	49	1226	9.53 (8.91-10.19)[1]	3.35 (3.53-4.45)[1]	9.20 (8.61-9.83)[1]
L01XE05	Sorafenib	920	271	1191	4.98 (4.64-5.35)[1]	15.39 (13.58-17.44)[1]	6.22 (5.83-6.63)[1]

Table 2 Top-10 drugs ranked in FAERS according to their reporting frequency for overall liver injury

[1]Statistically significant disproportionality (Lower limit 95%CI > 1). ATC: Anatomical therapeutic chemical classification; ALF: Acute liver failure; LI: Liver injury; OLI: Overall liver injury; ROR: Reporting odds ratio.

aged > 65 years (ALF and LI, respectively). Concerning antimycotics, a peak was found for patients aged 60 (number of OLI cases = 48), with only 30% of OLI cases occurring in subjects aged > 65 years. The analysis on the outcome of the events revealed that: (1) in the entire FAERS database, death was recorded in 37% and 17% of cases, whereas hospitalization or life-threatening conditions were reported in 52% and 49% of cases (ALF and LI, respectively); and (2) as regards antimycotics, death occurred in 51% and 24% of events, hospitalization or life-threatening conditions in 39% and 47% of events (ALF and LI, respectively).

As a first screening to test the sensitivity of the approach in detecting drug-event associations, we generated a list of top-10 drugs based on their reporting frequency for DILI (Table 2). Paracetamol ranked first both for ALF and LI and also generated statistically significant

disproportionality; it was followed by a number of drugs with known hepatotoxic potential such as statins, duloxetine, amoxicillin clavulanate, bosentan. Except for interferon, all these agents showed statistically significant ROR. Sorafenib ranked second in terms of ALF.

The analysis on antimycotics revealed a total of 1964 DILI cases; 35% were submitted by European Countries, 21% by Japan and 18% by United States. Terbinafine ranked first in terms of number of cases (422), followed by fluconazole and voriconazole (Table 3). Except griseofulvin, and miconazole, all remaining compounds generated a significant disproportion for ALF or LI or OLI. The intraclass analysis on OLI among azole-derivate agents showed the following ranking: ketoconazole (ROR = 1.70; 95%CI: 1.34-2.15) > voriconazole (1.47; 1.29-1.68) > posaconazole (1.36; 1.04-1.77) > fluconazole> itraconazole> miconazole (no statistical significance obtained).

Table 3 Key results of drug-induced liver injury associated with antimycotics: Number of cases with relevant disproportionality analyses

Pharmacological class	Active substance	Cases LI	Cases ALF	Cases OLI	ROR (95%CI) LI	ROR (95%CI) ALF	ROR (95%CI) OLI
Antibiotics	Amphotericin B	251	14	265	5.33 (4.65-6.10)[1]	2.86 (1.69-4.84)[1]	5.20 (4.55-5.94)[1]
Imidazole derivatives	Miconazole[2]	16	-	16	0.33 (0.20-0.54)	-	0.30 (0.18-0.50)
	Ketoconazole[2]	88	6	94	6.68 (5.28-8.44)[1]	4.22 (1.88-9.45)[1]	6.64 (5.28-8.34)[1]
Triazole derivatives	Fluconazole	381	31	412	4.25 (3.81-4.74)[1]	3.46 (2.42-4.93)[1]	4.26 (3.83-4.73)[1]
	Itraconazole	178	4	182	3.73 (3.19-4.37)[1]	0.84 (0.32-2.25)	3.50 (2.99-4.09)[1]
	Voriconazole	342	19	361	5.61 (4.99-6.31)[1]	2.97 (1.89-4.67)[1]	5.48 (4.89-6.14)[1]
	Posaconazole	65	5	70	5.39 (4.12-7.04)[1]	4.00 (1.65-9.66)[1]	5.39 (4.16-6.99)[1]
Other antimycotics for systemic use	Flucytosine	6	-	6	3.06 (1.31-7.13)[1]	-	2.80 (1.20-6.52)[1]
	Caspofungin	161	7	168	7.03 (5.90-7.37)[1]	2.79 (1.32-5.87)[1]	6.78 (5.71-8.05)[1]
	Micafungin	48	2	50	6.90 (5.02-9.49)[1]	-	6.64 (4.86-9.09)[1]
	Anidulafungin	13	1	14	4.97 (2.75-9.00)[1]	-	4.97 (2.79-8.84)[1]
Antimycotics used for topical indications (dermatological use) with systemic absorption	Griseofulvin[3]	3	-	3	2.00 (0.62-6.47)	-	1.83 (0.57-5.92)
	Terbinafine[3]	395	27	422	5.11 (4.58-5.69)[1]	3.39 (2.32-4.96)[1]	5.06 (4.55-5.62)[1]
Antimycotics for topical use with potential systemic absorption (*e.g.*, gynecological and intestinal use)	Nystatin	12	-	12	2.01 (1.12-3.62)[1]	-	1.84 (1.02-3.31)[1]
	Econazole	6	-	6	3.25 (1.39-7.60)[1]	-	2.97 (1.27-6.94)[1]
	Ciclopirox	3	-	3	3.39 (1.02-11.30)[1]	-	3.10 (0.93-10.33)

Only agents with at least three cases for the event of interest are shown (*i.e.*, those for which disproportionality can be calculated). [1]Statistically significant disproportionality (Lower limit 95%CI > 1); [2]Frequent topical use; [3]Also topical use. ALF: Acute liver failure; LI: Liver injury; OLI: Overall liver injury; ROR: Reporting odds ratio.

The time-trend cumulative analysis of the ROR (performed on the two most recently marketed compounds and ketoconazole, the only antimycotic receiving recent regulatory intervention on DILI) showed that: (1) ketoconazole reached significant ROR in the first quarter of 2005 (*i.e.*, well before the recent safety measure); (2) posaconazole (marketed in October 2005 by EMA and in September 2006 by the FDA) showed significant disproportion in the second quarter of 2005 (*i.e.*, before its actual marketing approval), which persisted throughout the period of analysis, although fluctuations were recorded during the first 2-year period; and (3) voriconazole (marketed in March 2002 by EMA and May 2002 by FDA) showed significant disproportion in the second quarter of 2002, a striking correspondence with its regulatory approval (Figure 2).

DISCUSSION

This study shows that virtually all antimycotics for systemic use are associated with clinically significant risk of DILI in the post-marketing setting, where comorbidities and poly-pharmacotherapy exist. Although the summary of product characteristics of all highlighted compounds and the livertox database already addressed this safety issue (*e.g.*, by mentioning liver monitoring in the warning section), clinicians should be aware of this aspect, especially following the marketing suspension of oral ketoconazole. This means that switching prescription towards therapeutic alternatives of ketoconazole does not sufficiently protect patients from risk and active vigilance is still needed.

Although actual incidence cannot be inferred from spontaneous reporting systems, the fact that antimycotics are reported in approx 3% of the total cases in FAERS suggests that the risk could be common. Our study also confirmed that miconazole and griseofulvin are not associated with clinically significant risk of DILI, a finding in line with recent data from EU-ADR and OMOP Consortia (Europe and United States projects, respectively), which labeled these drugs as "negative controls" in drug safety studies investigating DILI, based on the existing scientific literature and expert opinion[48,49]. As a matter of fact, miconazole is mainly used as topical preparation, whereas griseofulvin has specific tropism for keratin tissues with only low plasma concentrations. This kinetic features could explain the lack of a signal of liver injury. On the other hand, one recent population-based study in Taiwanese detected 8 cases of DILI associated with griseofulvin, although the cumulative exposure dose and the concomitant co-prescription of antibiotics and antinflammatory drugs (which have hepatotoxic potential) may have contributed to the occurrence of liver events[35].

Clinicians should interpret our data in the light of limitations affecting pharmacovigilance data. In particular, apart from well-described bias inherent to all spontaneous reporting systems (*e.g.*, underreporting, causality assessment, influence of media attention/safety alerts[50]), it should be kept in mind that these analyses, although formally quantitative, do not allow firm quantification of risk, especially because the role of concomitant drugs as culprit agents in the occurrence of the event cannot be ruled out with certainty. However, they allow early identification of alert signals, which require further confirmation through pharmacoepidemiological approaches. In our case, the evaluation of "old" drugs may be useful to

Figure 2 Cumulative time trend analysis of disproportionality (*i.e.*, lower limit of 95%CI) on overall liver injury for ketoconazole (recently restricted/suspended), voriconazole and posaconazole (the most recent antimycotics receiving marketing authorization in United States and Europe). Relevant regulatory milestones (*i.e.*, marketing approvals) are indicated by arrows. The total number of cases is also indicated when disproportionality was calculated for the first time (the first point of the lower limit of the 95%CI is provided when at least 3 cases of interest were recorded); EU: Europe; US: United states.

understand the reporting pattern over time and contribute to the general picture of perceived risk in a consolidated clinical setting. In any case, it should be recognized that DILI cases cannot be fully validated and precisely classified because laboratory values and liver biopsy are not available.

Nonetheless, our analysis has some strengths which can be summarized as follows. First, the validity of FAERS in early detection of DILI risk has been recently shown in a study allowing discrimination of a broad range of hepatotoxic drugs with clinically significant serious DILI, including those with only clinically apparent risk (*e.g.*, amlodipine)[42]. In this context, our top-10 ranking drugs have already known hepatotoxic risk (*e.g.*, paracetamol), a finding in line with similar pharmacovigilance study recently performed on Vigibase[44], and can be considered our positive controls. Second, it should be noticed that: (1) sorafenib (a novel tyrosine kinase inhibitor for which liver toxicity is under scrutiny and requires diligent surveillance[51]) is ranked second in terms of ALF (after paracetamol); and (2) in our analysis, voriconazole and posaconazole are associated with significant disproportion already in their first year of marketing life (Figure 2). Although the indication for unresectable hepatocellular carcinoma complicates the causality assessment for sorafenib, EMA and FDA amended the prescribing information to add the hepatotoxic potential in the label (2010 and 2012, respectively)[52,53].

An incidental observation of this study is that female gender does not seem to be associated with a reporting pattern of DILI for antimycotics, which differs from current data showing that females predominated in liver cases associated with a number of drug class[54].

The large number of cases associated with triazole antifungals and terbinafine can be partially explained by the underlying clinical conditions of patients (critically ill patients, usually with systemic infections, cancer and other diseases that may increase the likelihood of adverse drug reactions). Moreover, when discussing and interpreting pharmacovigilance results, drug consumption data should be also taken into account can provide an additional perspective to regulators and clinicians in assessing the possible consequences of side effects of drugs (*e.g.*, by mapping the level of risk in a population standpoint)[55,56]. Notably, the first ESAC survey on 2007 consumption data found that terbinafine, ketoconazole, itraconazole and fluconazole represented > 94% of the total outpatient antimycotic and antifungal use in 20 European Countries. Terbinafine use represented > 50% of the total systemic antimycotic and antifungal use in 16 out of 20 Countries[57]. The updated analysis on 2009 data showed an increased outpatient use and confirms terbinafine and fluconazole as the most used compounds[58]. Two point-prevalence ESAC surveys in European hospitals revealed that the most used antifungal was fluconazole (60.5%) followed by caspofungin (10.5%).

Table 4 Clinical, pharmacological and regulatory aspects of antimycotics for systemic use currently on the market (ketoconazole not shown)

Drug	Approval (EMA-FDA)	Indication(s)	Main hepatic issues	Drug interactions
Amphotericin B	1995 (FDA)[1]	Empirical therapy for presumed fungal infection in febrile, neutropenic patients Cryptococcal Meningitis in HIV infected patients Visceral leishmaniasis	No detailed information provided (general statement in the section on side effects)	Unclear from the label (metabolic pathway unknown)
Miconazole	No centralized approval	Onychomycosis (topical) Local candida infections (topical and systemic)	Not reported	CYP3A4 and CYP2C9 inhibitor (when administered systemically)
Fluconazole	1990 (FDA)	Acute vaginal candidiasis when local therapy is not appropriate Candidal balanitis when local therapy is not appropriate Mucosal and invasive candidiasis, genital candidiasis (trush), cryptococcal meningitis, dermatomycosis, coccidiodomycosis and onychomycosis (EMA revision in 2011)	Hepatic injury (warning FDA and EMA)	Potent CYP2C9 inhibitor; moderate CYP3A4 inhibitor; CYP2C19 inhibitor
Itraconazole	1992 (FDA)	Onychomycosis of the toenail caused by Trichophyton rubrum or T. mentagrophytes (FDA)	Hepatic injury (FDA)	Potent CYP3A4 inhibitor (drug interactions in warnings)
Voriconazole	2002 (EMA and FDA)	Invasive aspergillosis Candidemia in non-neutropenic patients Esophageal candidiasis (FDA) Fluconazole-resistant serious invasive Candida infections (including *C. krusei*) (EMA) Serious fungal infections caused by *Scedosporium spp.* and *Fusarium spp.*	Hepatic toxicity and monitoring of hepatic function (EMA and FDA)	CYP2C9, 2C19 and 3A4 inhibitor (several contraindicated drugs)
Posaconazole	2006 (FDA) 2005 (EMA)	Refractory IFI/Patients with IFI intolerant to first line therapy Oropharyngeal candidiasis Prophylaxis of IFI	Hepatic toxicity and monitoring of hepatic function (EMA and FDA)	Potent CYP3A4 inhibitor (drug interactions in contraindications)
Caspofungin	2001(EMA and FDA)	Empirical therapy for presumed fungal infections in febrile, neutropenic patients Invasive candidiasis Invasive aspergillosis (patients refractory or intolerant)	Hepatic effects	No CYP3A4 inhibition; No P-gp induction and poor substrate
Micafungin	2005 (only FDA)	Treatment of invasive candidiasis Treatment of esophageal candidiasis Prophylaxis of Candida infection	Hepatic effects	No P-gp induction or substrate
Anidulafungin	2006 (FDA) 2007 (EMA)	Invasive candidiasis in adult non-neutropenic patients (EMA) Esophageal candidiasis (FDA)	Hepatic effects (warning FDA and EMA)	No CYP substrate, inhibitor or inducer
Terbinafine	1995[2]	Fingernail onychomycosis Toenail onychomycosis	Hepatotoxicity	CYP2D6 inhibitor
Griseofulvin	1975 (FDA)	Various forms of tineas (corporis, pedis, cruris, barbae, capitis, unguium)	Hepatotoxicity	Unclear from the label

Information are derived from relevant summary of product characteristic (EMA) or product information (FDA), as of March 26th, 2013. When no information could be obtained from EMA and FDA documents, the Italian label was used. [1]On 28 August 2006, orphan designation (EU/3/06/391) was granted by the European Commission to Nektar Therapeutics United Kingdom Ltd, United Kingdom, for amphotericin B (for inhalation use) for the prevention of pulmonary fungal infections in patients deemed at risk; [2]Pediatric investigation plan agreed with EMA. EMA: European Medicines Agency; FDA: Food and Drug Administration; IFI: Invasive fungal infections.

Notably, antifungal-antibacterial combinations were frequently used (77.5%)[59]. As clearly emerged from these drug utilization studies, widely used antimycotics are also frequently reported in DILI cases extracted from FAERS. Therefore, not only active monitoring is warranted, but also appropriateness of prescriptions should be carefully considered by clinicians.

Another important aspect regards the issue of potential drug interactions. As a matter of fact, several antimycotics are metabolized or even inhibit crucial liver cytochromes for drug metabolism (*e.g.*, CYP3A4) (Table 4). Although the clinical relevance of drug interactions is still

a matter of debate, concomitant administration of drugs and/or herb (which are recognized to have hepatotoxic potential) should be assessed for potential interactions or interference with hepatic metabolism. Remarkably, the clinical implications of our data are also influenced by the different therapeutic indications of antimycotics (Table 4). In this context, griseofulvin, terbinafine, fluconazole and itraconazole share onicomycosis as main indication, whereas micafungin, voriconazole, posaconazole, caspofungin and amphotericin are used for systemic invasive infections, implying different baseline patients' conditions that may contribute to increase their hepatotoxic poten-

tial. In particular, voriconazole and posaconazole are the most recently marketed compound and are indicated as second-line treatment for invasive fungal infections (*e.g.*, following treatment failure with fluconazole); therefore, a potential channeling bias should be considered (*i.e.*, the possibility that drugs may be differently prescribed in relation to the severity of disease).

In a conclusion, we have used the recent regulatory case of ketoconazole to provide clues to clinicians needing practical guidance to assign the level of DILI risk among antimycotics.

From our analysis, it clearly emerges that it is not possible to identify a safe systemic antimycotic because all agents show a disproportionality signal for DILI. Although this safety issue was already formally mentioned in the label, it is still possible that clinicians do not fully appreciate this aspect.

The recent marketing restrictions of ketoconazole by regulatory Agencies should not lead physicians to overlook hepatotoxicity due to other systemic antifungals. Careful monitoring is therefore recommended, especially in critical poly-treated patients, keeping in mind that treatment usually requires drug exposure for a significant period of time.

It is clear that drug developers and clinicians face a challenging task in early recognition of DILI, a rare but potentially serious event for which drug discontinuation remains the key aspect of management, provided that diagnosis is correct. Therefore, the creation of novel multidisciplinary projects and the implementation of existing consortia (*e.g.*, the DILI network) is desirable to achieve the best risk prediction in the preclinical phase and make pharmacovigilance a reliable indicator of the post-marketing risk. This is also in line with new European pharmacovigilance legislation (in force since July 2012), which advocates the need for global risk-benefit assessment.

COMMENTS

Background
The recent regulatory interventions (United States restriction and Europe suspension) concerning ketoconazole for drug-induced liver injury (DILI) poses a prescribing challenge to clinicians, who should now carefully consider safer therapeutic alternatives.

Research frontiers
Eleven systemic antimycotics (including ketoconazole and the newer triazole derivatives voriconazole and posaconazole) and terbinafine (used systemically to treat onychomicosis) generated a significant disproportionality, indicating a post-marketing signal of risk.

Innovations and breakthroughs
Authors have used the recent regulatory case of ketoconazole to provide clues to clinicians needing practical guidance to assign the level of DILI risk among antimycotics.

Peer review
This is a well-written manuscript that provides valuable information about drug hepatotoxicity, particularly those induced by antimycotics.

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Circulating microRNAs in patients with non-alcoholic fatty liver disease

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Abstract

AIM: To identify novel non-invasive biomarkers for non-alcoholic fatty liver disease (NAFLD).

METHODS: Twenty patients with histologically proven NAFLD and 20 controls were included. All NAFLD cases were scored using the NAFLD activity score. The relative expressions of miR-197, miR-146b, miR-10b, miR-181d, miR-34a, miR-122, miR-99a and miR-29a were analyzed using real-time polymerase chain reaction.

RESULTS: Serum levels of miR-181d, miR-99a, miR-197 and miR-146b were significantly lower in biopsy-proven NAFLD patients than in the healthy controls. Serum levels of miR-197 and miR-10b were inversely correlated with degree of inflammation and miR-181d and miR-99a were inversely correlated with serum gamma glutamyl transferase levels in non-alcoholic steatohepatitis patients.

CONCLUSION: NAFLD is associated with altered serum miRNA expression pattern. This study provides clues for defining the non-invasive diagnosis of NAFLD.

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Key words: Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; MicroRNA; Noninvasive; Serum markers

Core tip: Due to the limitations of liver biopsy, the use of non-invasive markers has emerged in recent years. MicroRNAs (miRNAs) are a class of naturally occurring small noncoding RNAs that regulate gene expression. Altered miRNA expression has been reported in animal and human liver samples in non-alcoholic fatty liver disease (NAFLD). There is, however, only limited information on their detection in blood and their correlation with histological disease severity in patients with NAFLD. For this reason, we measured the serum levels of some miRNAs in non-alcoholic steatohepatitis (NASH) patients. Of these microRNAs, miR-181d, miR-99a, miR-197 and miR-146b were expressed at lower levels in NASH patients than in controls. Serum levels of miR-197 and miR-10b were inversely correlated with degree of inflammation and miR-181d and miR-99a were inversely correlated with serum gamma glutamyl transferase levels in NASH patients.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in Western populations. It comprises a disease spectrum which includes variable degrees of simple steatosis (fatty liver), non-alcoholic steatohepatitis (NASH) and cirrhosis which are likely to be characterized by different pathogenesis and clinical history[1]. Liver biopsy is used in daily practice for the diagnosis of NASH and histologic severity. The identification of novel non-invasive biomarkers for NAFLD is needed due to the invasive nature of liver biopsy.

MicroRNAs (miRNAs) are a class of naturally occurring small noncoding RNAs of approximately 22 nucleotides in length that regulate gene expression either by promoting mRNA degradation or by attenuating protein translation[2,3]. miRNAs can influence NAFLD through pathways involving inflammation, fibrosis, insulin resistance, lipid metabolism and the metabolic syndrome. Recently, altered miRNA expression has been reported in animal and human liver samples in NAFLD[4-6]. Serum levels of miR-29a were significantly down-regulated in fibrotic/cirrhotic livers compared with nonfibrotic livers[7]. miRNA-10b was proven to regulate the level of steatosis in human hepatocyte cell culture[8]. miR-122 was significantly underexpressed in liver samples of NASH subjects compared to controls and miR-34a was significantly overexpressed[5]. miR-181d may play a role in regulating the lipid content of hepatocytes[9]. miR-197 and miR-99, in the visceral adipose tissue, were significantly associated with pericellular fibrosis in NAFLD[10]. Serum free fatty acid (FFA) levels negatively correlated with adipose tissue level of miR-99a[11]. miR-146b was up-regulated in adipose tissue after long-term high-fat diet-induced obesity in mice[12].

There is insufficient data in the literature regarding serum miRNA expression patterns in NAFLD. In the present study, we analyzed the serum expression profile of these eight miRNAs (miR-29a, miR-10b, miR-122, miR-34a, miR-181d, miR-197, miR-99a, miR-146b) which are related to NAFLD progression and pathogenesis, and are related to serum FFAs, insulin resistance, and adipose tissue.

MATERIALS AND METHODS

Study population

A total of 40 patients were enrolled in this study between September 2010 and January 2012 at Erciyes University Department of Gastroenterology. Twenty patients with histologically proven NAFLD were included. The inclusion criteria were as follows: (1) 18 years or older; (2) persistently elevated (for at least 6 mo) aminotransferases; (3) ultrasonographic presence of hyperechogenic liver and (4) liver histology with a diagnosis of non-alcoholic steatohepatitis (NASH) without cirrhosis obtained no more than 6 mo before the study. The exclusion criteria were as follows: (1) a history of any level of alcohol consumption; (2) hypertension ($\geq$ 135 systolic, $\geq$ 85 diastolic or antihypertensive use); (3) any other form of chronic liver disease; (4) use of any medications thought to cause or affect NAFLD; (5) abnormal thyroid function tests; (6) plasma fasting glucose $\geq$ 126 mg/dL or antidiabetic drug use; (7) chronic obstructive pulmonary disease; (8) peripheral and cerebral vascular disease; (9) hematologic disorders; (10) acute or chronic infection; (11) history of cancer; (12) chronic kidney diseases and (13) documented coronary artery disease.

The control group consisted of 20 healthy age-matched subjects from our institution staff with normal liver enzymes and abdominal ultrasonography findings. All subjects underwent a clinical examination and were questioned regarding their medical history. BMI was calculated as body weight/height^2.

The study was performed in accordance with the principles of the Helsinki Declaration of 1975, as revised in 2008. This study was approved by the ethics committee of the Medical School of Erciyes University and informed consent was obtained from all participants.

Biochemical measurements

Blood samples were drawn after an overnight fast from an antecubital vein; fasting plasma glucose, serum basal insulin level, high density lipoprotein cholesterol, triglycerides, low density lipoprotein cholesterol, creatinine, alanine aminotransferase, aspartate aminotransferase, serum total bilirubin, serum indirect bilirubin, alkaline phosphatase, and gamma glutamyl transferase (GGT) were determined by standard methods.

The estimate of IR by the homeostasis model assessment insulin resistance index (HOMA-IR) was calculated using the formula: fasting serum insulin (mIU/L) × fasting plasma glucose (mmol/L)/22.5.

Histopathologic analysis

The liver tissue was stained with hematoxylin-eosin and Masson's trichrome stains. The review of the specimens was carried out by a single experienced liver pathologist. NASH was diagnosed according to the consensus arrived at a meeting of the American Association for the Study of Liver Diseases[13]. All cases were scored using NAS to compare miRNA expression and laboratory parame-

ters[14]. A 4-point scale for steatosis [(0) < 5%, (1) 5%-33%, (2) > 33%-66%, and (3) > 66%], lobular inflammation [(0) no foci, (1) < 2 foci, (2) 2-4 foci, and (3) > 4 foci] and a 3-point scale for ballooning [(0) none, (1) mild, and (2) moderate-marked] resulted in a maximal sum score of 8. A NAS score of 5 and over correlated with salient NASH, patients with scores < 3 were diagnosed as not having NASH, and patients with scores of 3 and 4 were diagnosed as having borderline NASH. Fibrosis was scored by Masson's trichrome stain using the NASH scoring system [(0): none, (1) perisinusoidal or periportal fibrosis, (2) perisinusoidal and periportal fibrosis, (3) bridging fibrosis, and (4) cirrhosis]. Steatosis was coded as 0 = mild (steatosis grade 1) or 1 = moderate to severe (steatosis grade 2-3). Ballooning was coded as 0 = mild (ballooning grade 1), or 1 = moderate-severe (ballooning grade 2). Lobular inflammation was coded as 0 = absent-mild (lobular inflammation 0-1) or 1 = moderate-severe (lobular inflammation 2-3). Fibrosis was coded as 0 = no significant fibrosis (F0-F1) or 1 = significant fibrosis (F2-F4).

miRNA quantitation

miRNA was isolated from serum using a miRNeasy Mini Kit (Qiagen, Cat; 217004) according to the manufacturer's protocol. The yield and purity of RNA were measured using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies). cDNA was isolated from obtained miRNA samples using a miScript Reverse Transcription Kit (Qiagen, Cat; 218060). We performed quantitative Real-Time PCR (qPCR) according to the manufacturer's instructions (miScript SYBR Green PCR Kit Qiagen Cat. No: 218073) to profile the distribution of eight miRNAs in serum samples and RUN1A1 was used as an internal control. The qPCR assays were performed in duplicate. The relative expressions of miR-197, miR-146b, miR-10b, miR-181d, miR-34a, miR-122, miR-99a and miR-29a were analyzed using the standard 2-ΔΔCT method[15]. These miRNAs were selected due to their pathophysiological relation with NAFLD.

Statistical analysis

For statistical analysis of clinical data, the Shapiro-Wilk's test was used, and histograms and q-q plots were constructed to check data normality. Either a two-sided independent samples *t*- test or Mann-Whitney *U* test was used for the comparison of continuous variables. χ^2 analysis was used for the comparison of categorical variables. Values are expressed as frequencies and percentages, mean ± SD deviation or median and 25th-75th percentiles.

miRNA expression data analysis was performed according to the 2-ΔΔCT method. For additional pre-processing we applied a logarithmic transformation to the data, then centered genes as follows: [(value)-mean(gene)/ standard deviation(gene)]. A *t* test was used to find the differentially expressed (DE) miRNAs between the NAFLD and control groups. Significant analysis of microarrays was also performed using 100 permutations to assess if the significance of the differential expression was by chance. The agglomerative hierarchical clustering

method with average linkage and the Pearson correlation distance metric were used for DE miRNAs and a cluster heat-map was constructed to visualize the data values of the samples and DE miRNAs simultaneously in a hierarchical cluster structure. Receiver operating characteristic (ROC) curves were plotted for DE miRNAs, and the area under the ROC curve values were calculated with 95% intervals and compared with each other. A series of cut-off values were applied, sensitivity and specificity statistical measures were computed for each cut-off value. Next, a ROC curve was generated and the area under this curve was calculated for each miRNA using the trapezoidal rule. Also, Pearson and point-biserial correlation coefficients were calculated to identify the relationship between laboratory parameters, NAS-scale parameters and miRNAs.

All *P* values obtained from clinical data and miRNA expression data were adjusted with the false discovery rate to control the multiple testing problem. Analyses were performed using several packages of R 2.14.0 software.

RESULTS

The clinical and laboratory data of age- and sex-matched patients and controls are summarized in Table 1. To determine whether serum levels of miR-181d, miR-10b, miR-122, miR-34a, miR-146b, miR-197, miR-99a and miR-29a change in patients with NAFLD, we measured these eight miRNAs in sera collected from 20 patients diagnosed with definite steatohepatitis according to Sanyal *et al*[13] and compared them with those of 20 controls.

miR-181d expression was significantly lower in the serum of NASH patients compared to healthy controls (*P* < 0.0001). miR-99a, miR-197 and miR-146b serum levels were also lower in NASH patients (*P* < 0.05). Serum levels of miR-10b, miR-122, miR-34a and miR-29a did not differ between the control and patient groups (Figure 1). miR-181d levels were decreased by 2.49-fold in NASH patients compared to healthy controls (q < 5%). miR-99a levels were decreased by 1.92-fold, miR-197 levels were decreased by 1.61-fold and miR-146b levels were decreased by 1.52-fold in NASH patients compared to healthy controls (q < 5%) (Table 2). A cluster heat-map which displays the data values of samples and differentially expressed miRNAs simultaneously in a hierarchical cluster structure was produced and is shown in Figure 2.

In our study, serum levels of miR-197 and miR-10b were negatively correlated with degree of inflammation (*P* < 0.05) (Table 3). Serum levels of these two miRNAs were decreased, while the degree of inflammation increased from absent-mild to moderate-severe. miRNA levels were similar in both groups according to NAS, steatosis, ballooning and fibrosis. Because both miR-197 and miR-10b levels correlated with degree of inflammation, we investigated the relationship between their serum levels and laboratory parameters in NASH patients (Table 4). Based on the correlation results between laboratory parameters and miRNAs (Table 4), a negative moderate and significant correlation was detected between GGT and miR-181d (r = -0.464, *P* < 0.05), and between GGT

Figure 1 Box plots showing the expression value of 8 genes between control and non-alcoholic fatty liver disease groups. The middle line indicates the median statistic, the bottom and top of the box show the 25th and 75th percentiles, the lower and upper whiskers show the minimum and maximum values of the data after detecting outliers (circles). *P* values were obtained from a two-sided *t* test and adjusted with false discovery rate. NAFLD: Non-alcoholic fatty liver disease.

Figure 2 A cluster heatmap displaying the data values of samples and differentially expressed miRNAs simultaneously in a hierarchical cluster structure. Bright red: Under-expression; black: No change; bright green: Over-expression.

Table 1 Clinical and demographic characteristics of non-alcoholic fatty liver disease patients and controls

Variable	NAFLD (n = 20)	Control (n = 20)	P
Age (yr)	42.75 ± 8.17	44.50 ± 9.31	0.761
Sex (male/female)	9 (45.0)/11(55.0)	9 (45.0)/11 (55.0)	0.761
BMI (kg/m^2)	31.86 ± 4.76	27.26 ± 3.07	0.006
Waist circumference (cm)	103.25 ± 13.65	90.75 ± 11.02	0.014
Systolic blood pressure (mmHg)	120.25 ± 8.66	120.25 ± 7.69	0.999
Diastolic blood pressure (mmHg)	76.50 ± 6.09	77.50 ± 8.96	0.761
Fasting glucose (mg/dL)	97.85 ± 13.88	86.80 ± 11.92	0.034
Triglycerides (mg/dL)	151.10 ± 65.06	167.70 ± 51.46	0.645
HDL-C (mg/dL)	43.00 (36.50-48.50)	45.00 (38.45-49.95)	0.761
LDL-C (mg/dL)	125.50 (100.00-139.00)	108.00 (89.25-121.25)	0.511
Insulin (mIU/mL)	14.81 (11.88-22.75)	11.76 (8.61-15.70)	0.229
HOMA-IR	4.05 (2.71-5.51)	2.21 (1.77-3.12)	0.034
Total biluribin (mg/dL)	0.65 (0.50-0.80)	0.60 (0.55-0.80)	0.761
Direct biluribin (mg/dL)	0.20 (0.12-0.25)	0.20 (0.10-0.20)	0.761
AST (IU/L)	45.00 (34.50-59.00)	21.00 (18.00-27.00)	< 0.001
ALT (IU/L)	68.50 (52.50-85.50)	18.50 (14.00-29.00)	< 0.001
AP (IU/L)	76.00 (55.50-96.00)	69.00 (57.00-78.50)	0.761
GGT (IU/L)	48.00 (33.00-73.00)	20.00 (14.50-33.00)	< 0.001
Creatinine (mg/dL)	0.87 ± 0.15	0.80 ± 0.18	0.401
WBC (μL)	6709.50 ± 1709.47	6618.00 ± 1425.37	0.892
Trombocyte (10^3/μL)	252.10 ± 64.85	275.90 ± 69.82	0.511
Hct	43.37 ± 3.53	44.25 ± 4.19	0.761

Values are expressed as n (%), mean ± SD or median (25th-75th percentiles). P values were adjusted with FDR to control multiple testing problem. NAFLD: Non-alcoholic fatty liver disease; BMI: Body mass index; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol; HOMA-IR: Homeostasis model assessment insulin resistance index; AST: Aspartate aminotransferase ; ALT: Alanine aminotransferase; AP: Alkaline phosphatase; GGT: Gamma glutamyl transferase; WBC: White blood cell.

Table 2 Log₂ (Fold change) ratios of differentially expressed miRNAs which were statistically significant by t test (P < 0.05) and further confirmed by SAM test (q < 5%)

miRNAs	log$_2$ (Fold change)		q value (%)
	Mean	SEM	
Over-expressed			
-	-	-	-
Under-expressed			
miR-197	-1.61	0.54	0
miR-146b	-1.52	0.55	0
miR-181d	-2.49	0.50	0
miR-99a	-1.92	0.53	0

A q value less than 5% by SAM corresponds to a P value less than 0.05 by t test. SEM: Standard error of mean.

and miR-99a (r = -0.479, P < 0.05). Other correlation coefficients between laboratory parameters and miRNAs were not statistically significant (P > 0.05).

To determine the presence of NASH, we performed ROC curve analyses of miR-197, miR-146b, miR-181d and miR-99a (Figure 3). AUROC values were 0.77 (0.60-0.88), 0.75 (0.59-0.87), 0.86 (0.72-0.95), and 0.76 (0.60-0.88), respectively. mir-181d seemed to be the best marker for NASH, and there was no statistically significant difference between any two miRNA AUROC values (P > 0.05). Using the Youden index, best cut-off values and the related diagnostic measures are given in Table 5.

DISCUSSION

The microRNA expression pattern changes in NAFLD

have been demonstrated in animal and human studies[4,5]. There is, however, only limited information regarding their detection in blood and their correlation with histological disease severity in patients with NAFLD. For this reason, we measured the serum levels of miR-181d, miR-10b, miR-122, miR-34a, miR-146b, miR-197, miR-99a and miR-29a in NAFLD patients. Of these microRNAs, miR-181d, miR-99a, miR-197 and miR-146b were expressed at lower levels in NASH patients than in controls.

miR-29a was significantly down-regulated in fibrotic/cirrhotic livers compared with nonfibrotic livers[7]. Roderburg et al[7] also showed that significantly lower serum levels of miR-29a were observed in fibrosis patients compared with healthy controls. In our study, miR-29a serum levels were not different from controls. miRNA-10b significantly increased the triglyceride level and lipid content in human hepatocyte cell line L02 cells. miRNA-10b was proven to regulate the steatosis level in human hepatocyte cell culture via the peroxisome proliferator-activated receptor-α pathway[8]. miR-122 was significantly underexpressed in liver samples of NASH subjects compared to controls[5]. Overexpression of miR-122 in cell culture resulted in a significant decrease in lipogenic genes[5]. Although miR-10b and miR-122 were found to regulate lipid content in hepatocyte cell cultures, serum expression patterns did not differ in patients with NASH in our study. The miR-34a expression pattern was not changed in our study, which was significantly overexpressed in NASH in human liver tissue[5].

There is insufficient data on miR-181d in NAFLD. miR-181d may play a role in regulating the lipid content

Table 3 Point-biserial correlation coefficients between NAS-scale parameters and miRNAs in non-alcoholic fatty liver disease patients

	miR-197	miR-146b	miR-10b	miR-181d	miR-34a	miR-122	miR-99a	miR-29a
Steatosis	0.229	0.191	0.179	0.179	0.245	0.067	0.311	0.256
Inflammation	-0.457[1]	-0.396	-0.492[1]	-0.402	-0.302	-0.23	-0.431	-0.315
Ballooning	0.154	0.114	0.175	0.254	0.165	-0.037	-0.029	0.096
Fibrosis	-0.112	-0.171	-0.213	-0.098	0.033	-0.137	-0.197	-0.103
NAS	0.051	0.015	0.035	0.069	0.124	-0.027	0.074	0.041

[1]Correlation is significant at the 0.05 level (2-tailed).

Table 4 Correlation analysis between laboratory parameters and miRNAs in non-alcoholic fatty liver disease patients

	miR-197	miR-146b	miR-10b	miR-181d	miR-34a	miR-122	miR-99a	miR-29a
AST	-0.257	-0.331	-0.225	-0.252	-0.084	-0.225	-0.138	-0.322
ALT	-0.131	-0.147	-0.080	-0.215	0.122	0.103	0.077	-0.143
GGT	-0.327	-0.225	-0.303	-0.464[1]	-0.29	-0.231	-0.479[1]	-0.221
Triglyceride	0.008	-0.039	0.009	-0.035	-0.075	0.087	-0.239	-0.058
HDL-C	0.027	0.051	-0.031	0.042	0.052	0.111	0.16	0.034
LDL-C	0.078	0.042	0.067	0.015	0.139	0.102	0.213	0.155
HOMA-IR	-0.055	0.117	0.011	-0.015	0.195	0.205	0.221	0.177

[1]Correlation is significant at the 0.05 level (2-tailed). ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HOMA-IR: Homeostasis model assessment insulin resistance index; GGT: Gamma glutamyl transferase; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol.

Table 5 Statistical diagnostic measures for miR-197, miR-146b, miR-181d and miR-99a in identifying non-alcoholic fatty liver disease

miRNA	Cut-off value	SEN (95%CI)	SPE (95%CI)	PPV (95%CI)	NPV (95%CI)
miR-197	≤ -1.0144	60.0 (36.1-80.9)	95.0 (75.1-99.9)	92.3 (64.0-99.8)	70.4 (49.8-86.2)
miR-146b	≤ -0.7689	55.0 (31.6-76.9)	100.0 (83.0-100.0)	100.0 (71.3-100.0)	69.0 (49.2-84.7)
miR-181d	≤ -0.2861	70.0 (45.7-88.0)	85.0 (62.1-96.6)	82.4 (56.6-96.0)	73.9 (51.6-89.7)
miR-99a	≤ -0.1562	65.0 (40.8-84.5)	95.0 (75.1-99.2)	92.9 (66.1-98.8)	73.1 (52.2-88.4)

SEN: Sensitivity; SPE: Specifity; PPV: Positive predictive value; NPV: Negative predictive value.

Figure 3 Comparison of area under receiver operating characteristic curve values of miR-197, mir-146b, miR-181d and mir-99a miRNA's in the detection of non-alcoholic fatty liver disease. Area under receiver operating characteristic values were 0.77 (0.60-0.88), 0.75 (0.59-0.87), 0.86 (0.72-0.95), and 0.76 (0.60-0.88), respectively, and there was no statistically significant difference between any two miRNAs (*P* > 0.05).

of hepatocytes. miR-181d has been shown to decrease lipid droplets, and to reduce cellular triglycerides and cholesterol ester in cell culture[9]. In our study, serum levels of miR-181d were decreased in NAFLD by 2.49-fold compared to controls. Estep *et al*[10] studied miRNA expression in the visceral adipose tissue of patients with NAFLD. They found that significant changes in miRNA expression were characterized by overall down-regulation in the visceral adipose tissue. They also found that down-regulation of miR-197 and miR-99 in the visceral adipose tissue was significantly associated with pericellular fibrosis in NAFLD patients. We also found that miR-99 and miR-197 were down-regulated in the serum of NASH patients, but we did not find any relationship between fibrosis stages. A study conducted by Klöting *et al*[11] showed that the human adipose tissue level of miR-99a negatively correlated with FFA levels. NAFLD is significantly associated with obesity, type II diabetes mellitus, and the metabolic syndrome. Serum FFA levels are elevated in all of these disorders[16]. It was reported that the main source for TG in hepatocytes was derived from circulating FFAs in NAFLD[17]. Although we did not analyze serum fatty acid levels in our study, down-regulation of miR-99a in NASH was concordant with this evidence. Chartoumpekis *et al*[12] examined miRNA profiling after long-term high-fat diet-induced obesity in mice and found that miR-146a and miR-146b were up-regulated in adipose tissue.

In one study, the miR-146a expression levels were significantly decreased in peripheral blood mononuclear cells from patients with Type 2 diabetes compared to control subjects. They also reported that reduced miR-146a levels are associated with insulin resistance and poor glycemic control[18]. Although miR-146a and miR-146b are encoded on different chromosomes, their mRNA targets are predicted to overlap significantly[19]. The data in this study showed decreased miR-146b levels which seems reasonable in light of these findings.

During our research, the study by Cermelli *et al*[20] showed that serum levels of miR-122, miR-34a and miR-16 were significantly higher in NAFLD patients. They also found that miR-122 and miR-34a levels were correlated with liver enzyme levels, fibrosis stage and inflammation activity in NAFLD. These findings regarding miR-122 and miR-34a were not confirmed by our study results. This may be attributed to patient selection. We did not include hypertensive patients due to their impaired miRNA profile[21,22].

The current study provides evidence that biopsy proven NAFLD is associated with altered serum miRNA expression. The potential targets of differentially expressed miRNAs are known to play a role in metabolism, immune function, cell proliferation, apoptosis, tissue development and differentiation; all these are key processes involved in the development and progression of NASH[5]. While our findings do not provide direct proof of the involvement of these differentially expressed miRNAs in the development, progression and systemic effect of NASH, they serve as a resource for future studies on the role of miRNAs in the non-invasive assessment of NASH. This was a principal aim of this study.

The pattern of miRNA expression can be affected by hypertension[21,22]. This possibility was minimized by excluding patients with hypertension. The miRNA expression pattern is also possibly affected by the development of cirrhosis. We excluded patients with cirrhosis from this study. We want to point out that the aim of this study was to determine whether biopsy proven NAFLD was associated with altered serum miRNA expression without hypertension. Therefore, only subjects without hypertension were studied. At the same time, this study does not provide any information on the serum miRNA expression pattern in those with isolated hepatic steatosis due to insufficient funding. Differential expression of microRNA with two distinct clinical entities, in simple steatosis and NASH, may exist. It will be very interesting to investigate the serum expression pattern of miRNA in simple steatosis and NASH. In addition, this study had a cross-sectional design. Due to the financial constraints we selected these eight miRNAs from earlier studies which were related to NAFLD progression and pathogenesis and were also related to serum FFA, insulin resistance and adipose tissue.

This study showed the following: (1) miR-181d, miR-99a, miR-197 and miR-146b expression was significantly lower in the serum of biopsy proven NAFLD patients than in healthy controls; (2) The serum levels of miR-197 and miR-10b were inversely correlated with degree of inflammation; and (3) miR-181d and miR-99a were inversely correlated with serum GGT levels in NASH patients. In conclusion, biopsy proven NAFLD is associated with an altered serum miRNA expression pattern. This study provides clues for defining the non-invasive diagnosis of NAFLD.

COMMENTS

Background
Due to the limitations of liver biopsy, the use of non-invasive markers has emerged in recent years. MicroRNAs (miRNAs) are a class of naturally occurring small noncoding RNAs that regulate gene expression. Altered miRNA expression has been reported in animal and human liver samples in non-alcoholic fatty liver disease (NAFLD). There is, however, only limited information regarding their detection in blood and their correlation with histological disease severity in patients with NAFLD.

Research frontiers
miRNAs are a class of naturally occurring small noncoding RNAs of approximately 22 nucleotides in length that regulate gene expression either by promoting mRNA degradation or by attenuating protein translation. miRNAs can influence NAFLD through pathways involving inflammation, fibrosis, insulin resistance, lipid metabolism and the metabolic syndrome. A research hotspot is the evaluation of miRNAs to determine hepatic damage and fibrosis in patients with NAFLD.

Innovations and breakthroughs
miR-181d, miR-99a, miR-197 and miR-146b were expressed at lower levels in non-alcoholic steatohepatitis (NASH) patients than in controls. Serum levels of miR-197 and miR-10b were inversely correlated with degree of inflammation and miR-181d and miR-99a were inversely correlated with serum gamma glutamyl transferase levels in NASH patients.

Applications
Biopsy proven NAFLD is associated with an altered serum miRNA expression pattern. This study provides clues for defining the non-invasive diagnosis of NAFLD.

Terminology
NAFLD is the most common cause of chronic liver disease in Western populations. It comprises a disease spectrum which includes variable degrees of simple steatosis (fatty liver), NASH and cirrhosis which are likely to be characterized by different pathogenesis and clinical history. The identification of novel non-invasive biomarkers for NAFLD is needed. miRNAs are a class of naturally occurring small noncoding RNAs of approximately 22 nucleotides in length that regulate gene expression either by promoting mRNA degradation or by attenuating protein translation. miRNAs can influence NAFLD through pathways involving inflammation, fibrosis, insulin resistance, lipid metabolism and the metabolic syndrome.

Peer review
This is a good descriptive study in which authors evaluate the serum miRNA levels to determine hepatic damage and fibrosis in patients with NAFLD. The results are interesting and suggest that miRNAs may be useful to determine hepatic inflammation with a non-invasive tool in NAFLD patients. The data analysis and presentation were appropriate, and the manuscript was well prepared.

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OBSERVATIONAL STUDY

Early termination of immune tolerance state of hepatitis B virus infection explains liver damage

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Abstract

AIM: To assess an early termination of immune tolerance state of chronic hepatitis B virus infection in Bangladesh and its clinical significance.

METHODS: From a series of 167 treatment-naive chronic hepatitis B patients aged between 12 to 20 years (mean ± SD; 17.5 ± 2.8 years), percutaneous liver biopsies of 89 patients who were all hepatitis B e antigen negative at presentation were done. Of them, 81 were included in the study. They had persistently normal or raised serum alanine aminotransferase (ALT) values. A precore mutation (PCM) study was accomplished in 8 patients who were randomly selected.

RESULTS: Forty-four (53.7%) patients had significant necroinflammation (HAI-NI > 7), while significant fibrosis (HAI-F ≥ 3) was seen in 15 (18.5%) patients. Serum ALT (cut off 42 U/L) was raised in 29 (35.8%) patients, while low HBV DNA load (< 10^5 copies/mL) was observed in 57 (70.4%) patients. PCM was negative in all 8 patients.

CONCLUSION: This study indicates that the current concept of age-related immune tolerance state of HBV infection deserves further analyses in different population groups.

Key words: Chronic hepatitis B; Immune tolerance; Early termination

Core tip: Immune tolerance phase usually prevails for up to 20-25 years in subjects with chronic hepatitis B virus (HBV) infection. However, the present study showed that considerable numbers of chronic HBV-infected patients of Bangladesh lost hepatitis B e antigen and developed anti-HBe. Early termination of immune tolerance phase of these young patients was also associated with elevated alanine aminotransferase, hepatic necroinflammation and considerable hepatic fibrosis in some patients. Treatment guidelines are warranted for these patients as there is a paucity of information about their entity.

INTRODUCTION

Maybe nearly 2 billion people have been infected with hepatitis B virus (HBV) worldwide. The clinical manifestations vary widely with asymptomatic acute viral B hepatitis at one end and hepatocellular carcinoma (HCC) at

the other. There are about 400 million chronic HBV carriers worldwide. Of them, 75%-80% resides in Asia and the Western Pacific region. HBV is responsible for over one million deaths per year globally. It is a major cause of cirrhosis of liver and HCC worldwide[1].

Although there is a paucity of information about a nation-wide survey regarding HBV prevalence in Bangladesh, published data show that about 5%-6% of apparently healthy individuals are HBV carriers in Bangladesh[2-4]. There may be about 6-8 million chronic HBV carriers in Bangladesh and most of them are unaware of their disease. In intermediate prevalence countries like Bangladesh, lifetime risk of acquiring HBV infection is above 40%[1].

The precore/core region of the HBV genome encodes the nucleocapsid protein (HBcAg) and hepatitis B e antigen (HBeAg)[5,6]. The biological role of HBeAg in HBV replication cycle is uncertain. Expression of HBeAg is nonessential for virus replication in animal models[7] and in humans[8]. In utero exposure to HBeAg can induce immune tolerance in newborn mice[9]. Mutations in the precore region of the HBV genome have been described[10-12] resulting in HBeAg negative HBV infection. The core promoter region (nucleotides 1742 to 1849) has an important role in HBV replication as well as HBeAg production[13] and mutations in this region commonly lead to HBeAg negative HBV infection[14].

Chronic HBV infection can be divided into different phases, which may not be sequential. Patients may present: (1) in a state of immune tolerance; (2) with hepatitis B e antigen (HBeAg)-positive chronic HBV; (3) with HBeAg-negative chronic HBV; or (4) as an inactive hepatitis B surface antigen (HBsAg) carrier. A state of immune tolerance with minimum liver damage is usually seen in chronic HBV carriers until 25 years of age.

The present study was accomplished to evaluate the biochemical, virological and immunological statuses of young chronic HBV carriers in Bangladesh. It seems that there may be an early termination of immune tolerance state of HBV in Bangladesh. However, there is no therapeutic recommendation for these young HBV-infected patients. Here, we provide evidence suggesting that considerable numbers of these patients should be treated as otherwise they may develop complications of chronic HBV infection.

MATERIALS AND METHODS

Patients
One hundred and sixty-seven treatment naive young chronic HBV-infected patients, aged between 12 and 20 years (17.5 ± 2.8 years, *n* = 167), were enrolled in this study. At presentation they were asymptomatic with no features of chronic liver disease. They were all HBsAg positive either at vaccination, school health screening or family screening. All of them had at least two HBsAg positive results at a minimum of 6 mo apart.

Of these patients, 89 were HBeAg negative, while the others tested positive for HBeAg. They were all negative

for serological markers of hepatitis C virus, IgM antibodies to hepatitis A virus and hepatitis E viruses. Also, they had no history of alcohol consumption and no evidence of pregnancy. None of the patients had received an antiviral drug for treatment of HBV infection. The Ethical Committee of Farabi General Hospital, Dhaka, Bangladesh gave ethical approval for the study. The nature and purpose of the study were explained to all patients or their guardians in the case of minors. Informed written consent for undergoing percutaneous liver biopsy was obtained. Patients were excluded from further analyses if an adequate amount of liver tissue (*i.e.*, 1.0 cm) was not available at liver biopsy[15]. Eight patients were excluded from final analyses as adequate amounts of liver tissue were not available from them. Thus, a total of 81 HBeAg negative chronic hepatitis B (CHB) patients were included for final analyses.

Biochemical and serological tests
The level of ALT in serum was measured by auto-analyzer (Microlab 300, Vitalab Micro Series, Vital Scientific NV, The Netherlands). The cut off for the upper limit of normal (ULN) was ALT 42 U/L. HBsAg was assessed using an ELISA kit (Diasorin, Fallugia, Italy). HBeAg was checked in the sera using an ELISA kit (Abbott Labs, Chicago, IL, USA).

Quantification of serum HBV DNA level
First, HBV DNA was extracted from the patient's serum. It was then amplified by polymerase chain reaction (PCR) and detected using fluorescent reporter dye probes specific for HBV (Amplicon HBV Monitor Assay, Roche Molecular Systems, CA, United States). The lower limit of detection was 250 copies of HBV DNA/mL.

Amplification of the pre-core region by the PCR
Oligonucleotide primers were synthesized in a 380B DNA synthesizer (Applied Biosystems Japan, Tokyo, Japan). PCR was performed by a modification of the procedure originally described by Saiki *et al*[16]. Briefly, 10 uL of the DNA sample was heated at 95 ℃ for 7 min to denature proteases, spun in a microcentrifuge for 5 seconds and allowed to cool at room temperature. Target sequences were amplified in a 100-pd reaction volume with the use of the Gene Amp DNA amplification reagent kit (Perkin-Elmer Cetus, Norwalk, Conn., United States), as recommended by the manufacturer. The amplification was carried on for 30 cycles in a programmable DNA thermal cycler (Perkin-Elmer Cetus). The reaction was allowed to proceed at 94 ℃ for 1 min, at 55 ℃ for 1.5 min, and at 72 ℃ for 3 min in each cycle. In the last cycle, the reaction at 72 ℃ was continued for 10 min to ensure complete DNA extension.

Liver biopsy
Percutaneous liver biopsy was performed under local anesthesia using a 16G Tru-cut biopsy needle (Cardinal Health, McGaw Park, IL, United States). A biopsy specimen of more than 1.0 cm in length with five to six portal

Table 1 Baseline characteristics of study population	
Parameters	
Total number of patients	81
Male	60[a] (74%)
Female	21 (26%)
Age (yr)	17.5 ± 2.8 (12-20)
ALT ≤ 42 (U/L)	52[a] (65.2%) (13-42)
ALT > 42 (U/L)	29 (34.8%) (44-281)
DNA ≤ 100000 (copies/mL)	57[a] (70.4%)
DNA > 100000 (copies/mL)	24 (29.6%)
Non-significant hepatic necroinflammation (HAI-NI ≤ 7)	44 (53.8%)
Significant hepatic necroinflammation (HAI-NI > 7)	37 (45.7%)
Non-significant hepatic fibrosis (HAI-F < 3)	66[a] (81%)
Significant hepatic fibrosis (HAI-F ≥ 3)	15 (18.5%)
Cirrhosis	2/15

Figure in the round bracket indicates the percentage and the square bracket indicates range. [a]$P < 0.05$ *vs* same parameter. HAI-NI: Histologic activity index-necroinflamation; ALT: Alaninaminotransferase; HAI-F: Histologic activity index-fibrosis.

tracts was evaluated. Histology was graded according to histological activity index (HAI) using the criteria of Knodell *et al*[17]. The total HAI score comprises necroinflammation (HAI-NI) and fibrosis (HAI-F) scores. The HAI-NI scale includes three components (0-10, piecemeal necrosis; 0-4, lobular necrosis and inflammation; 0-4, portal inflammation). HAI-F was graded according to severity: 0, absence of fibrosis; 1, fibrous portal expansion; 3, bridging fibrosis; 4, cirrhosis.

Statistical analysis
Data are shown as mean ± SD. Means were compared using the Student's *t* test. For differences determined by the *F* test, the t test was adjusted for unequal variances (Mann-Whitney's *U* test). $P < 0.05$ was considered statistically significant.

RESULTS
A total of 81 patients with HBeAg-negative chronic HBV infection were enrolled in this study as a sufficient amount of liver biopsy specimens could be collected from these patients. The baseline data of these patients is given in Table 1. Sixty of them (74%) were male and the remaining 21 were female (26%). The numbers of male patients were significantly higher than females (60 *vs* 21, $P < 0.05$). The age of the patients was 17.5 ± 2.8 years (*n* = 81). The levels of ALT were below ULN in 52 patients (65.2%) (28.7 ± 8.6 IU/L, range, 13-42 IU/L) and ALT levels were above ULN in the remaining 29 patients (34.8%) (79.7 ± 47.4 IU/L; range, 44-281 IU/L, $P < 0.05$). The levels of HBV DNA varied considerably among patients, ranging from 779 copies/mL to 1.4×10^{12} copies/mL. In 57 patients (70.4%), the levels of HBV DNA were less than 100000 copies/mL whereas these were more than 100000 copies/mL in 24 patients (29.6%). Considering 100000 copies HBV DNA as a cut-off point

of "high level" of HBV DNA, significantly higher levels of patients had low levels of HBV DNA (HBV DNA < 100000 copies/mL) compared to patients with high levels of HBV DNA (HBV DNA > 100000 copies/mL) ($P < 0.05$).

Significant levels of hepatic necroinflammation (HAI-N1 > 7) were detected in 37 of 81 patients (46%) (Table 1) and this was not statistically different with patients with low hepatic necroinflammation (44 patients, $P > 0.05$). Significant levels of hepatic fibrosis (HAI-F ≥ 3) were detected in the liver biopsy specimens of 15 patients (19%). Among these, cirrhosis of liver was detected in two patients (Table 1).

DISCUSSION
Our study reveals that young HBeAg negative CHB patients can have significant necroinflammation and/or fibrosis in the liver. This is in contrast to our existing understanding of clinical course of chronic HBV infection that patients in the immunotolerance age group tend to have no significant hepatic pathology.

Although the study shows that a significant proportion of our patients were at risk of developing more severe liver diseases, they were not aware of this scenario. More importantly, no major guideline recommends treatment of this group of patients[18]. Similar studies have been conducted in different parts of the world to assess the extent of a similar scenario. Kumar *et al*[19] from India showed that more than 50% of their 1387 incidentally-detected chronic HBV carriers had evidence of progressive liver diseases for which treatment was indicated. A similar outcome has also been reported from Pakistan, Egypt and other countries[20-25].

There are studies from Bangladesh, India, South Korea and Turkey suggesting that HBeAg negative CHB patients as a whole tend to develop more significant liver fibrosis than those who are HBeAg positive[26-30].

An exact explanation for such a high incidence of HBeAg negative infection in our young chronic HBV infected population of the immunotolerant age group is difficult to reach. All 8 patients in our series, who were randomly picked up, tested negative for precore mutation. However, in Bangladesh most HBV infections are acquired early in life, either soon after birth or at a preschool age[1]. This possibly leads to early termination of an immune tolerance state in our population.

Non-alcoholic fatty liver disease (NAFLD) is now regarded as a leading cause of chronic liver disease in Bangladesh, perhaps second only to HBV infection. The incidence of non-alcoholic steatohepatitis (NASH) in our NAFLD patients is 88.5%[31]. Co-existence of NASH and CHB may also be responsible for significant hepatic injury in many of the apparently healthy chronic HBV infected population; however, this is an area that needs much more exploration. Finally, viral genotype may also be responsible[32].

Although many patients included in this present study were suitable candidates for anti-viral treatment, they are

usually not considered for treatment owing to complex socio-economic problems, social taboos and lack of scientific information. However, we recommend that all HBV-infected patients, irrespective of their age, should be properly evaluated for anti-HBV therapy.

Our study has a few limitations. One is that HBV DNA, ALT and liver histology were assessed only once. Serial assessment of virological, biochemical and histological parameters would provide more insight into the natural disease course in these patients. Our main aim was to gain an insight into the pathogenesis of these patients to initiate a strategy for their management. We found that a considerable number of young Bangladeshi HBV infected individuals have significant liver damage. This is important evidence to convince physicians and policy makers in developing countries to develop a management strategy for such patients.

In conclusion, chronic HBV infection is a complex disease entity and here we describe a group of such patients whose clinical course is not well studied and is difficult to explain. Although considered to be apparently healthy, a proportion of them are eligible for treatment. They not only pose a threat to themselves. In fact, in the fragile health economics of the developing world, they simply give rise to more questions than answers. As clinical hepatologists of the developing world, it remains our responsibility to look into these issues in further detail and develop a strategy for their appropriate management.

COMMENTS

Background
The immune tolerance phase usually persists for 20-25 years in chronic hepatitis B virus (HBV) infected subjects. However, early termination of the immune tolerance phase is seen in clinics.

Research frontiers
The clinical, biochemical, virological and histological aspects of young chronic HBV-infected patients of Bangladeshi origin were analyzed.

Innovations and breakthroughs
Early termination of the immune tolerance phase was detected in considerable numbers of chronic HBV-infected patients in this cohort. Many of them also developed progressive liver damage and increased fibrosis.

Applications
The management of these patients remains a major challenge as they express anti-HBe, a marker usually considered to have a better prognosis in the context of chronic HBV infection.

Terminology
Immune tolerance phase: HBV infected patients expressing hepatitis B e antigen, high HBV DNA but no liver damage.

Peer review
The article is properly written, endeavored and well constructed. Although there are an inadequate number of patients, it is an interesting article in terms of having insight on regional data.

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REVIEW

Living-donor *vs* deceased-donor liver transplantation for patients with hepatocellular carcinoma

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Abstract

With the increasing prevalence of living-donor liver transplantation (LDLT) for patients with hepatocellular carcinoma (HCC), some authors have reported a potential increase in the HCC recurrence rates among LDLT recipients compared to deceased-donor liver transplantation (DDLT) recipients. The aim of this review is to encompass current opinions and clinical reports regarding differences in the outcome, especially the recurrence of HCC, between LDLT and DDLT. While some studies report impaired recurrence - free survival and increased recurrence rates among LDLT recipients, others, including large database studies, report comparable recurrence - free survival and recurrence rates between LDLT and DDLT. Studies supporting the increased recurrence in LDLT have linked graft regeneration to tumor progression, but we found no association between graft regeneration/initial graft volume and tumor recurrence among our 125 consecutive LDLTs for HCC cases. In the absence of a prospective study regarding the use of LDLT *vs* DDLT for HCC patients, there is no evidence to support the higher HCC recurrence after LDLT than DDLT, and LDLT remains a reasonable treatment option for HCC patients with cirrhosis.

Key words: Deceased donor liver transplantation; Hepatocellular carcinoma; Living donors; Living-donor liver transplantation; Recurrence

Core tip: The current opinions and clinical reports regarding differences in the recurrence of hepatocellular carcinoma (HCC) between living donor liver transplantation (LDLT) and deceased donor liver transplantation (DDLT) were reviewed. In the absence of a prospective study regarding the use of LDLT *vs* DDLT for HCC patients, only with some retrospective studies with conflicting results, there is no evidence to support the higher HCC recurrence after LDLT than DDLT, and LDLT remains a reasonable treatment option for HCC patients with cirrhosis.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the 7th most common cancer overall and the 3rd most common cause of cancer-related death worldwide[1,2]. Since the landmark report of the Milan criteria by Mazzaferro *et al*[3], which demonstrated comparable outcomes of patients with HCC having a single tumor smaller than 5 cm in diameter or up to 3 tumors smaller than 3 cm in diameter with no vascular invasion or extra-hepatic disease determined by preoperative imaging studies, deceased - donor liver transplantation (DDLT) has become an established treatment for cirrhotic patients with HCC[4,5]. Similarly, in Asian countries where living-donor liver transplantation (LDLT) comprises the majority of liver transplantation procedures, LDLT has become an established treatment

Table 1 Studies comparing living - donor liver transplantation and deceased - donor liver transplantation for hepatocellular carcinoma														
Ref.	Country	Year	Study period	Type of LT	Case number	Recurrence - free survival			*P*	% Recurrence rate	*P*	Criteria used	% Outside Milan	Difference in tumor charact- eristics	Median follow- up period (mo)
						1-yr	3-yr	5-yr							
Impaired results in LDLT															
Park *et al*[10]	South Korea	2014	1999-2010	LDLT	166	89		81	0.045	19	0.045	UCSF	NA	none	35
				DDLT	50	96		94		6					
Vakili *et al*[13]	United States	2009	1999-2007	LDLT	28					29	< 0.05	UNOS	25	none	41
				DDLT	65					12					
Kulik *et al*[12]	United States	2012	1998-2010	LDLT	100	80	66	56	0.05	38	0.0004	UNOS	59	More aggressive in LDLT	60
	Multi- center			DDLT	97	90	81	73		11			30		
Lo *et al*[14]	Hong Kong	2007	1995-2004	LDLT	43	93	71	71	0.029	29	0.029	UCSF	26	More aggressive in LDLT	33
				DDLT	17	100	100	100		0			29		
Comparable results															
Sandhu *et al*[15]	Canada	2013	1996-2009	LDLT	58	88	75	70	NS	17	NS	Toronto criteria	28	none	38
				DDLT	287	86	75	70		15			32		31
Bhangui *et al*[16]	France	2011	2000-2009	LDLT	36	100	89	88	NS	13	NS	UCSF	27	none	58
				DDLT	120	93	89	86		13			21		50
Li *et al*[36]	China	2010	2005-2009	LDLT	38	71	42		NS	50	NS	UCSF	79	none	25
				DDLT	101	76	41			55			68		
Di Sandro *et al*[35]	Italy	2009	2000-2007	LDLT	25		96	96	NS	4	NS	Milan	20	none	NA
				DDLT	154		91	89		11			31		
Sotiropoulos *et al*[20]	Germany	2007	1998-2006	LDLT	45	88	75		NS	12	NS	UCSF	44	none	NA
				DDLT	55		81			14					
Hwang *et al*[8]	South Korea	2005	1992-2002	LDLT	237	83	80		NS	18	NS		27	none	26
	Multi- center			DDLT	75	88	82			16			29		45
Gondolesi *et al*[17]	United States	2004	1988-2002	LDLT	36	82	74		NS	19	NS	UNOS	53	none	15
				DDLT	165	90	83			19					

DDLT: Deceased - donor liver transplantation; HCC: Hepatocellular carcinoma; LDLT: Living - donor liver transplantation; LT: Liver transplantation; UCSF: University of California, San Francisco; UNOS: United Network for Organ Sharing; NA: Not applicable; NS: Not significant.

for HCC patients with end-stage liver disease[6,7]. LDLT is now considered a promising treatment for HCC patients in Western countries, not only to compensate for the shortage of donor organs but also to reduce the dropout rate on the waiting list[8].

With the accumulation of LDLTs for HCC patients, the impact of LDLT on recipient outcome compared with DDLT, especially the recurrence of HCC after liver transplantation, has become an important topic of debate[9]. The aim of this review was to encompass the current opinions and clinical reports regarding the differences in outcome, especially the recurrence of HCC, between LDLT and whole liver DDLT.

STUDIES COMPARING LDLT AND DDLT FOR HCC PATIENTS

Studies comparing LDLT and DDLT for HCC patients are summarized in Table 1. All DDLTs reviewed here were done with the whole liver graft.

Studies reporting a poorer outcome in the LDLT setting

Park *et al*[10] recently reported poorer recurrence-free survival among 166 LDLT recipients (81% at 5 years) com-pared to 50 DDLT recipients (94% at 5 years; *P* = 0.045). The noteworthy finding of this study was that the smaller the LDLT graft, the poorer the recurrence - free survival. Based on this finding, Park *et al*[10] suggested that the physiology of the small graft may stimulate tumor recurrence.

The results of the A2ALL cohort in United States also demonstrated an impaired outcome in LDLT recipients. In their initial report[11], they found a higher rate of recurrence within 3 years in LDLT than in DDLT (29% *vs* 0%, *P* = 0.002), but there was a clear tendency toward more aggressive tumor characteristics in the LDLT group. The same group recently published an updated report[12], in which HCC recurrence remained significantly different between LDLT and DDLT after adjustment for tumor characteristics. They concluded that the higher recurrence observed after LDLT was likely due to differences in the tumor characteristics, pretransplant HCC management, and waiting time.

Vakili *et al*[13] reporting the Lahey Clinic experience, demonstrated that the HCC recurrence rate of LDLT (29%) was significantly higher than that of DDLT (12%) (*P* < 0.05), but survival after LDLT was significantly better than that following DDLT for HCC during the same

period (P = 0.02).

Lo *et al*[14] from Hong Kong also reported a significantly higher incidence of HCC recurrence, 29% in LDLT and 0% in DDLT (P = 0.029). While the tumor characteristics were comparable between groups, the authors speculated that LDLT as a salvage transplantation, microscopic vascular invasion, and liver regeneration led to the difference in the recurrence rate.

Studies reporting a comparable outcome

Sandhu and colleagues of the Toronto group[15] reported that LDLT and DDLT both provide similarly low recurrence rates and high survival rates. They compared the results of 58 LDLT cases with those of 287 DDLT cases having comparable tumor characteristics, in which the 1-, 3-, and 5-year recurrence-free survival rates were 88%, 75%, and 70%, and 86%, 75%, and 70%, respectively.

In a well-designed study by Bhangui *et al*[16], an intention-to-treat analysis was conducted with recurrence rate representing the primary endpoint, comparing 36 LDLT cases and 147 DDLT cases. The authors demonstrated that both LDLT and DDLT provided similar recurrence-free survival rates (88% *vs* 86% at 5 years) for patients with HCC. The dropout rate and waiting time were significantly lower in the LDLT group than in the DDLT group, and there was also a trend toward a longer time to recurrence in the LDLT group, which may guarantee additional advantages with LDLT.

The Mount Sinai group[17,18] reported comparable recurrence-free survival between LDLT (*n* = 36) and DDLT (*n* = 165; 74% *vs* 83% at 2 years, P = 0.3). When stratified by tumor size (5 cm diameter) and the existence of microvascular invasion, there was still no difference between groups.

Sotiropoulos and colleagues of Essen, Germany[19,20], also supported the comparable recurrence-free survival rates between LDLT and DDLT for HCC (75% *vs* 81% at 3 years).

Hwang *et al*[21] of South Korea performed a nationwide survey regarding this issue. Among 237 LDLTs and 75 DDLTs for HCC, the 1- and 3-year recurrence-free survival rates were 83% and 80%, and 88% and 82%, respectively, with no significant difference between them.

A comparison of outcomes after liver transplantation obtained from database studies revealed comparable patient survival rates between LDLT and DDLT. According to a report from the Japanese Liver Transplantation Society Registry[22], a total of 6097 LDLTs were performed in Japan by the end of 2010, and 1225 (32%) were indicated for HCC, which was the most common indication in adult patients. The 1-, 3-, 5-, and 10-year cumulative survival rates of LDLT for HCC were 85%, 74%, 69%, and 60%, respectively. Todo and colleagues[23] performed a detailed survey using the same database (up to the end of 2005), comprising 653 patients who had undergone LDLT for HCC in Japan. At 1, 3, and 5 years, overall patient survival was 83%, 73%, and 69%, and disease-free survival was 77%, 65%, and 61%, respectively. Based on

preoperative imaging studies, 62% were within the Milan criteria and 38% were beyond the Milan criteria, with 5-year recurrence-free survival rates of 90% and 61%, respectively (P < 0.001). These findings do not differ much from those obtained in the DDLT database of the United States and Europe[24-27], and may validate the use of LDLT for HCC patients.

CURRENT OPINIONS REGARDING THE DIFFERENCE BETWEEN LDLT AND DDLT

A randomized clinical study would be best to settle the controversy regarding the use of LDLT *vs* DDLT for HCC patients, but this is indeed difficult, if not impossible, to realize given the complicated decision-making process involved in LDLT. No prospective study has been conducted to date.

The Toronto group[28] recently performed a meta-analysis on 12 retrospective studies comparing the recurrence rates and recurrence-free survival between LDLT and DDLT recipients. A total of 633 LDLTs and 1232 DDLTs were enrolled, and the study provided evidence of lower disease-free survival after LDLT compared with DDLT for HCC (HR = 1.59, 95%CI: 1.02-2.49; P = 0.041). In contrast, there was no difference in overall survival between LDLT and DDLT (HR = 0.97, 95%CI: 0.73-1.27; P = 0.808). As mentioned by the authors of the paper, however, all involved studies were retrospective, had a low data quality score with poor reporting of baseline patient characteristics and an inadequate statistical approach, and were heterogeneous in critical aspects such as indication criteria and basal tumor characteristics, which warrant further well-designed studies to determine whether differences in HCC recurrence are due to study biases or biologic differences.

A recent review article by experts[29] concluded as follows: Although there is no strong evidence to support the higher HCC recurrence rates in LDLT than DDLT, the higher recurrence rates in LDLT recipients reported by several authors cannot be ignored. Actually, there are critical differences among societies such as: (1) differences in the allocation system for DDLT and LDLT; (2) differences in the availability of deceased donors; (3) differences in the potential waiting time; and (4) the differences in regional and national organ transplant law. In addition to taking into account these differences, liver transplant candidates with HCC and their potential live donors should be informed following risks and benefits; the waiting time for DDLT may lead to the dropout due to HCC progression which could be avoided by the prompt LDLT, however, the prompt LDLT may mask the aggressive tumor characteristics which may lead to a higher HCC recurrence rates. Although the currently available literatures can provide a low evidence for the difference of HCC recurrence between DDLT and LDLT, the tumor characteristics and biology seem to significantly influence on the recurrence, while the graft type and waiting time are less likely important as a possible risk factor.

Table 2 Graft characteristics and hepatocellular carcinoma recurrence			
	Patients with recurrence (*n* = 11)	**Patients without recurrence (*n* = 114)**	***P***
Regeneration rate at 3 mo (%)	90 ± 24	93 ± 34	0.732
Graft type: right/left	4/7	36/78	0.702
Initial graft volume ratio to standard liver volume (%)	46 ± 9	47 ± 9	0.842

POSTULATED THEORIES FOR DIFFERENCES BETWEEN LDLT AND DDLT

LDLT provides several advantages compared with DDLT, such as a shorter waiting time, good quality graft with normal liver function and shorter ischemic time, and pretransplant treatment optimization, which might contribute to improved survival in LDLT recipients. Some of these characteristics, on the other hand, may lead to a favorable milieu for tumor progression[9].

There are several hypotheses other than tumor characteristics to explain the inferior outcome of LDLT. One explanation for the higher recurrence rates in LDLT is fast-tracking patients into liver transplantation, the so - called fast-track effect[11,30]. Some patients with more biologically aggressive HCC might drop off the waiting list due to tumor progression beyond the criteria during the wait-time in the DDLT setting. In contrast, due to the shortened wait time for LDLT candidates, progression of HCC with an aggressive tumor biology might not be recognized during such a short wait-time. This scenario might account for the higher HCC recurrence in the LDLT setting.

Another hypothesized mechanism for the higher recurrence rates in LDLT is that growth factors and cytokines released during rapid regeneration of the partial grafts from living donors might contribute to tumor progression and recurrence[31-34]. A rapidly regenerating liver parenchyma and ischemic-reperfusion injury facilitated by a small-for-size graft in LDLT setting might be a more favorable environment for tumor progression and HCC recurrence.

Additionally, some authors[11,35,36] insist that the technique of LDLT per se foregoes the principles of oncologic surgery. During LDLT, the meticulous dissection and mobilization of the liver might increase the possibility of tumor capsule violation or tumor embolization through the hepatic veins, thus promoting tumor dissemination. Preserving the native vena cava and the bile duct/hepatic artery/portal vein in the hepatic hilum might increase the risk of leaving the residual tumors.

As opposed with the above-mentioned anecdotal explanations, the advanced tumor characteristics of LDLT recipients can reasonably explain the higher recurrence rate in the LDLT setting. Grafts from living donors are not limited by restrictions imposed by the organ allocation system, meaning that the relation of the graft and recipient is usually one-on-one. Consequently, selection criteria based on the tumor burden, such as the tumor size and number, can be considered relative on a case-by-case basis, taking into account the presence of risk factors for recurrence and the chance of survival, as well as the wishes of the donor[37]. Consequently, the majority of Asian transplant centers have adopted extended criteria beyond those of Milan or the University of California, San Francisco (UCSF)[38]. Based on some studies, differences in patient tumor characteristics between LDLT and DDLT remain a main reason for the higher recurrence rate in LDLT. Additionally, in the majority of the aforementioned studies comparing LDLT and DDLT for HCC patients, tumor burdens such as the size, number, vascular invasion, and poor differentiation have proved to be independent risk factors for HCC recurrence after liver transplantation, all of which may lead to a rational explanation for the impaired recurrence - free survival of LDLT compared to DDLT.

OUR EXPERIENCE

At our institution, the University of Tokyo Hospital, a total of 423 adult recipients underwent LDLT by the end of 2012. Among them, 125 (30%) patients had HCC. The principle criterion for LDLT for HCC at our center is "up to 5 nodules with a maximum tumor diameter within 5 cm", which we call the "5-5 rule"[39]. Of the 125 patients, 118 (94%) were within the 5-5 rule criteria and 109 (87%) were within the Milan criteria. Overall survival of the 125 recipients at 1, 3, and 5 years was 88%, 82%, and 76%, respectively, with a median follow-up period of 8 years. A total of 11 (9%) patients developed HCC recurrence with a cumulative recurrence rate at 1, 3, and 5 years of 6%, 9%, and 11%, respectively.

We compared the graft regeneration rate between patients with HCC recurrence (*n* = 11) and those without recurrence (*n* = 114) to confirm the association of liver regeneration with HCC recurrence. The regeneration rate was calculated as follows: (graft volume at 3 mo after LDLT- initial graft volume)/initial graft volume × 100 (%). As shown in Table 2, there was no difference in the regeneration rate between those with HCC recurrence and those without recurrence. At the same time, the graft type (right *vs* left) and the initial graft volume ratio to the recipient's standard liver volume were also compared between groups, revealing no difference. A similar result was reported by the Asan group of South Korea[40], in which the graft-recipient weight ratio had no impact on HCC recurrence after LDLT among 181 LDLT recipients with HCC. Our result as well as the report of the Asan group clearly demonstrated that graft regeneration of the partial liver graft has no impact on HCC recurrence, at least in a clinical setting. The independent predictors for HCC recurrence in our series were tumors not within the 5-5 rule (Tokyo criteria), AFP level over 400 ng/mL, and des-

gamma-carboxy prothrombin levels over 200 mAU/mL.

CONCLUSION

In conclusion, there is no strong evidence to support higher HCC recurrence after LDLT than DDLT, and it may be reasonable to use different indication criteria for LDLT and DDLT, while there could be a potential bias in choosing the articles in the present study. LDLT should always be considered as a treatment option for HCC patients with advanced cirrhosis in areas where deceased donors are scarce or for patients whose tumor status interrupts access to DDLT.

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REVIEW

Liver involvement in systemic infection

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Abstract

The liver is often involved in systemic infections, resulting in various types of abnormal liver function test results. In particular, hyperbilirubinemia in the range of 2-10 mg/dL is often seen in patients with sepsis, and several mechanisms for this phenomenon have been proposed. In this review, we summarize how the liver is involved in various systemic infections that are not considered to be primarily hepatotropic. In most patients with systemic infections, treatment for the invading microbes is enough to normalize the liver function tests. However, some patients may show severe liver injury or fulminant hepatic failure, requiring intensive treatment of the liver.

Core tip: The liver is frequently involved in systemic infections, resulting in various types of abnormal liver function test results. It is very important to know the frequency and the patterns of abnormal liver function test results in each infection for the appropriate man-agement of the patients. However, there have been few reports focusing on this issue. Here, we gather information from previous reports on this topic to provide a comprehensive summary that will help clinicians interpret abnormal liver function test results according to the associated infection.

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INTRODUCTION

It is well known that the classical hepatotropic viruses, hepatitis A through E, can infect the liver and cause hepatic injury. Other systemic infections by non-hepatotropic viruses or bacteria can also cause hepatic injury, either by direct invasion or indirectly through toxins and cytokines, but there are few reports of the correlations between liver function abnormalities and these infections. This review will describe features of liver injury caused by various systemic infections. It will discuss, in order, bacterial infections, infection by specific pathogens, non-hepatitis viral infections, fungal infections, and liver involvement of parasitic diseases.

BACTERIAL INFECTIONS

Sepsis

Sepsis is a clinical syndrome that complicates severe infection and accompanies systemic abnormalities such as tachycardia, tachypnea and/or hypotension. It is thought to be associated with vasodilation and increased microvascular permeability caused by bacterial products and cytokines. Liver function test abnormalities and jaundice frequently accompany a variety of bacterial infections, especially sepsis[1]. Various sites of infection can cause jaundice, which include intra-abdominal infection, urinary

Table 1 Mechanism of Jaundice in Sepsis

Increased bilirubin load
  Hemolysis
    Red blood cells lysed by bacterial products (*e.g.*, exotoxin)
    Red blood cells lysed by immunological mechanisms
Hepatic dysfunction
  Hepatocellular injury (hepatitis and/or necrosis)
  Hepatic ischemia
  Decreased bilirubin uptake; dysfunction of basolateral transport (*e.g.*, NTCP)
  Decreased transport of conjugated bilirubin; dysfunction of canalicular transport (*e.g.*, BSEP, MRP2)
Decreased bile flow

NTCP: Na$^+$/taurocholate cotransporting polypeptide; BSEP: Bile salt export pump; MRP2: Multidrug-resistance-associated protein 2.

tract infection, pneumonia, endocarditis, and meningitis. Although several retrospective studies have reported incidences of jaundice ranging from 0.6% to 54% in adults with sepsis[2,3], the precise incidence remains unclear because of the absence of a large-scale prospective study. In patients with sepsis, jaundice can be caused by several organisms including aerobic and anaerobic gram-negative (*Escherichia coli* and *Klebsiella*) and gram-positive bacteria (*Staphylococcus aureus*). Kanai *et al*[4] isolated microorganism species in patients with bacteremia and reported *S. aureus*, *E. coli*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa* as comprising 29.3%, 14.4%, 6.0%, and 6.0% of all isolates, respectively. Uslan *et al*[5] also reported that the most common organisms identified in the blood of patients with bacteremia were *E. coli* (25.1%) and *S. aureus* (16.6%). Of the bloodstream infections, 44.5% were community acquired, 36.5% were health care associated, and 19.1% were nosocomial[5].

Hyperbilirubinemia in the range of 2-10 mg/dL is often seen in patients with sepsis, but on rare occasions, much higher levels (30 to 50 mg/dL) have been reported[6]. Serum alkaline phosphatase (ALP) is usually elevated in range of 1 to 3 times the upper limit of normal (ULN), but serum ALT is only modestly elevated. Infected patients with bacteremia had significantly higher serum levels of gamma-glutamyl transpeptidase (γ-GTP) and ALP and significantly lower serum concentrations of albumin, cholesterol and cholinesterase as compared with those without bacteremia. If septic shock and hypoperfusion complicate, a striking elevation of aminotransferases may occur.

The pathogenesis of jaundice in systemic infections is multifactorial. Jaundice is mainly associated with cholestasis in patients with sepsis[1], but isolated jaundice without cholestasis can occur through increased bilirubin load from hemolysis in some cases[7,8]. Several bacterial infections, especially *Clostridium perfringens*, may cause hemolysis. Phospholipase C produced by *C. perfringens* may be associated with the release of lysolecithin and the lysing of red blood cell membranes. It also can produce proteolytic exotoxins, which may lead to hemolysis[9,10].

Cholestasis is mainly thought to be caused through the inhibition of the canalicular excretion of conjugated bilirubin by proinflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-1,6 (IL-1,6), which are mainly released by macrophages in response to endotoxins[11]. Interestingly the serum concentrations of ALP and bilirubin are often discordant, because jaundice in sepsis is associated with various factors including increased bilirubin load, decreased bilirubin uptake, intrahepatic processing, and canalicular excretion (Table 1)[12,13].

The major histological finding in sepsis is bland intrahepatic cholestasis with bile in the bile canaliculi and hepatocytes. Minimal degenerated changes of hepatocytes with Kupffer cell hyperplasia and lymphocyte infiltration may also be seen.

Pneumonia

Lobar pneumonia is a common disease usually caused by any one of a variety of bacteria (*e.g.*, *S. pneumonia*, *Haemophilus influenza*, *S. aureus*, or *P. aeruginosa*). Patients with pneumococcal pneumonia sometimes show elevated concentrations of serum aminotransferases and bilirubin. Jaundice was reportedly observed in 3%-25% of such patients[14]. Pneumonia-associated jaundice is mostly thought to be a result of hepatocellular damage, because hepatic necrosis is often seen in liver biopsies of patients with pneumonia[15]. In *Mycoplasma pneumonia* infection, an adult case with acute hepatitis without pulmonary manifestations was also reported by Lee *et al*[16]. They also summarized five other cases (5 to 22 years of age) with similar clinical characteristics to those of *M. pneumonia* infection. *Legionella* is an important species of bacteria which causes pneumonia, often accompanied by laboratory abnormalities indicating hepatic dysfunction, renal dysfunction, thrombocytopenia, and hyponatremia[17].

Microbial foodborne disease

Microbial foodborne illness is very common and mainly causes gastrointestinal symptoms including nausea, vomiting, abdominal pain, diarrhea, and fever. These patients may have other complications such as hepatitis, renal failure, and neurogenic symptoms (Table 2).

***Salmonella typhi* infection:** *Salmonella typhi* can cause an acute systemic illness known as typhoid fever, while being nontyphoidal *Salmonella* (most commonly *S. enteritidis* and *S. typhimurium*) primarily induces gastroenteritis. The majority of patients with typhoid fever present with fever, malaise, abdominal discomfort, and hepatosplenomegaly. Typhoid fever may also cause liver injury with elevated aminotransferases and jaundice[18]. Hepatomegaly and jaundice were reportedly observed in 44% and in 33% of patients with typhoid fever, respectively. Although severe elevation of aminotransferases is rare in patients with typhoid fever, typhoid fever and viral hepatitis A sometimes need to be discriminated because clinical features of typhoid fever are similar to those of acute viral hepatitis A infection (Table 3). The ALT/LDH ratio may be useful to distinguish these diseases; the ALT/LDH ratio has been shown to be significantly lower (< 4.0) in typhoid fever compared with the ratio (> 5.0) in acute viral hepatitis A[19]. The hepatic histology shows

Table 2 Foodbrone pathogens and manifestations

Pathogens	Manifestations
Bacteria	
Staphylococcus aureus	Vomiting (exotoxin), toxic shock syndrome
Clostridium spp	
C. botulinum	Neurogenic finding (paralysis)
C. perfringens	Diarrhea, gas gangrene, intravascular hemolysis, jaundice, liver abscess, gas in the portal vein
Campylobacter spp	
C. jejuni	Inflammatory diarrhea, liver injury (possible)
C. fetus	Systemic, liver injury (possible)
Escherichia coli	
Enterotoxigenic E. coli	Inflammatory diarrhea
Enterohemorrhagic E coli	Inflammatory diarrhea, hemolytic uremic syndrome
Listeria monocytogens	Systemic (Listeriosis), elevated aminotransferases
Salmonella spp	
Non-typhoidal	Inflammatory diarrhea
S.typhi	Systemic (Typhoid fever), acute
S.paratyphi	hepatitis (Salmonella hepatitis)
Shigella spp	Inflammatory diarrhea, cholestatic hepatitis
Vibrios spp	
V. cholera	Watery diarrhea
V. parahemolyticus	Inflammatory diarrhea
V. vulnificus	Systemic (sepsis, DIC)
Yersina entrrocolitica	Inflammatory diarrhea, multiple liver abscesses
Virus	
Hepatitis A	Acute hepatitis, jaundice
Hepatitis E	Acute hepatitis, jaundice
Norovirus	Vomiting, watery diarrhea
Rotavirus	Vomiting, watery diarrhea

minimal parenchymal changes with focal infiltration of mononuclear cells or focal hepatocyte necrosis known as "typhoid nodules"[20,21].

Campylobacter infection: *Campylobacter* enteritis is an important cause of acute diarrhea, and several complications are known in patients with *Campylobacter* infection, which include cholecystitis, reactive arthritis and Guillain-Barré syndrome. Mild to severe hepatocellular dysfunction is rarely observed in these patients, and liver biopsy shows nonspecific reactive hepatitis[22]. The symptoms and liver dysfunction are commonly improved after antimicrobial therapy.

Clostridum perfringens infection: *Clostridum perfringens* is an important cause of watery diarrhea and also a toxin-mediated disease including hemolysis, jaundice, hypotension, and renal failure. *C. perfringens* is well known to cause clostridium myonecrosis (gas gangrene), which is a life-threatening muscle infection spreading directly from the area of trauma or hematogenously from gastrointestinal tract infection[23]. Jaundice may develop in up to 20% of patients with gas gangrene. On rare occasions, it can cause necrotizing massive gas gangrene in the liver leading to fulminant hepatic failure[24].

Pelvic inflammatory disease
Pelvic inflammatory disease (PID) refers to infection of the uterus, fallopian tubes, and adjacent pelvic structures, and the most important causative organisms are *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. Occasionally, patients with these infections develop perihepatitis (Fitz-Hugh-Curtis syndrome), an inflammation of the liver capsule and adjacent peritoneal surfaces[25,26]. The clinical presentations include right-upper quadrant (RUQ) pain or pleuritic pain, and it may be confused with acute cholecystitis or pleurisy. The levels of aminotransferases are usually normal because of the minimal stromal hepatic involvement.

It has been reported the use of intrauterine devices (IUDs) causes a slight increase in the risk for PID[27-29]. Gelfand *et al*[30] reported a case of *Streptococcus milleri* bacteremia and multiple hepatic abscesses secondary to a tuboovarian abscess associated with IUD. Long-term indwelling IUDs was also reported to cause pelvic actinomycosis, which is a slowly progressive infection of *Actinomyces* species[31]. Moreover, a case of hepatic actinomycotic abscess associated with IUD was reported[32].

Toxic shock syndrome
Toxic shock syndrome is caused by the staphylococcal toxic shock syndrome toxin (TSST-1) and is commonly associated with *S. aureus* infections[33]. The clinical findings include fever, a scarlatiniform rash, mucosal hyperemia, vomiting, and diarrhea. It may cause liver dysfunction with severe jaundice and high levels of aminotransferases by hypoperfusion and circulating toxins.

Moreover, it should be noted that *Aeromonas* bacteremia, mostly *Aeromonas hydrophila*, causes significantly severe soft tissue infection such as necrotizing fasciitis with high mortality in patients with liver cirrhosis in contrast to self-limiting recovery in healthy subjects[34].

Hepatic encephalopathy and systemic infection
Systemic infection such as sepsis has been associated with the development of hepatic encephalopathy (HE). In patients with acute liver failure, rapidly progressing and severe HE is found more frequently in those with infection and inflammation[35,36]. It has also been reported that infection and inflammation exacerbate HE in patients with cirrhosis[37]. Systemic inflammation might have synergistic effects with HE. In a bile duct-ligated rat model, lipopolysaccharide (LPS)-treated rats showed severe HE with cytotoxic brain edema and increased nitrotyrosine in the frontal cortex despite preservation of the blood-brain barrier, whereas those without LPS developed precoma status only[38]. In systemic inflammation, increased cerebral blood flow, activated neutrophils or produced cytokines such as TNF-α, IL-1β or IL-6 contribute to the pathogenesis of HE[39,40].

INFECTION BY SPECIFIC PATHOGENS
Mycobacterium infection
Although the lungs are the major site for *Mycobacterium*

Table 3 Frequency of symptoms and signs in salmonella and acute viral hepatitis A

	Nausea/vomiting	Abdominal discomfort	Jaundice[b]	Diarrhea	Relative bradycardia[d]	Fever > 104° F[b]	Hepatomegaly	Splenomegaly
Salmonella hepatitis	70%	33%	33%	48%	37%	41%	44%	7%
Acute viral hepatitis A	89%	63%	89%	30%	4%	0%	66%	11%

Modified from El-Newihi et al[19]. Salmonella hepatitis vs acute viral hepatitis A: [b]P < 0.0001; [d]P < 0.002.

tuberculosis infection, liver involvement has been also reported in patients with mycobacterial infection[41]. Miliary tuberculosis is defined as hematogenous dissemination of *Mycobacterium tuberculosis*, and the liver is frequently involved. Hepatic tuberculosis can be classified into various types such as military, granulomatous, and localized hepatic tuberculosis. The clinical presentations include fever, abdominal pain, and hepatomegaly. Liver function abnormalities have been observed in patients with hepatic tuberculosis, including elevated ALP and aminotransferases in 83% and 42% of these patients, respectively[42]. Cholestatic jaundice has also been reported in miliary tuberculosis, and fulminant hepatic failure can occur, if only rarely[43]. Importantly, hepatic tuberculosis can occur in the absence of apparent pulmonary tuberculosis, and tuberculous liver abscess without lung involvement has also been reported[44]. Histologically, the presence of caseating granulomas is suggestive of hepatic tuberculosis, but the yields of acid-fast bacillus smears and cultures are low. Detection using tissue PCR for *Mycobacterium tuberculosis* has a higher sensitivity and specificity.

Atypical mycobacteremia caused by *M. avium intracellulare* or *M. genavense*, can also cause granulomatous hepatitis with an elevation of ALP and low-grade fever in immunocompromised hosts such as those with AIDS syndrome[45].

Syphilis
Hepatic involvement in patients with syphilis is not uncommon. Schlossberg et al[46] reported that 39% of early syphilis patients had liver enzyme abnormalities at the time of diagnosis and that 2.7% of syphilis patients were diagnosed with syphilitic hepatitis. Other reports also show that liver enzyme abnormalities have been observed in about 10% to 50% of patients with secondary syphilis[46,47]. Syphilitic hepatitis is characterized by a high serum ALP level and normal to mild elevation of aminotransferases. Clinical hepatitis is rare, but acute cholestatic syphilitic hepatitis has been reported[48]. Hepatic gummas consisting of a caseous mass with a fibrous capsule may present in patients with tertiary syphilis. After starting therapy using penicillin, jaundice may occur with chills, fever, and a rash (erythema of Milan), as part of the Jarisch-Herxheimer reaction.

It is well known that syphilis continues to occur at high rates among human immunodeficiency virus (HIV)-infected patients. Crum-Cianflone et al[49] reported that syphilitic hepatitis is common, occurring in 38% of HIV-positive patients with early stages of syphilis infection, and

that syphilis should be included in the differential diagnosis of HIV patients with liver dysfunction.

Leptospirosis
Leptospirosis caused by *Leptospira interrogans* is one of the most common zoonoses, and it may occur as one of two different clinical courses: anicteric leptospirosis (> 90% of cases) or icterohemorrhagic (Weil's) disease (5%-10% of cases)[50]. The former is characterized by self-limited viral infection-like symptoms with fever and conjunctival suffusion. The latter presents severe jaundice (approaching 30 mg/dL of total bilirubin) and several complications such as renal failure. Mild elevation of serum aminotransferases and thrombocytopenia can be seen[51]. Importantly, it is difficult to distinguish leptospirosis from other febrile infectious diseases such as *Salmonella typhi* or influenza because of similar clinical manifestations in the early phase.

In spite of severe functional impairment of the liver and kidneys, histopathological changes are usually slight, consisting of minimal focal hepatocyte necrosis. In severely jaundiced cases, leukocyte infiltration and bile thrombi can be observed.

Lyme disease
Lyme disease is a spirochetal infection by *Borrelia burgdorferi*, and it can involve multiple organs including skin, muscle, liver, heart, and nervous system. Hepatic involvement can be seen in 20% of patients with Lyme disease; elevations of aminotransferases and γ-GTP are commonly observed[52,53].

Q fever
Q fever is one of the zoonotic infections caused by *Coxiella burnetii*; it is characterized by relapsing fevers, headache, and myalgias, and can involve several organs including the lungs, heart and liver. Nearly 50% of patients with Q fever may have liver function abnormalities, and the clinical features may mimic anicteric viral hepatitis[54].

Rocky Mountain spotted fever
Rocky Mountain spotted fever (RMSF) is the most common manifestation of *Rickettsia rickettsii* infection in the United States. The clinical spectrum of human infection ranges from mild to fulminant, and hepatic involvement is frequent, predominantly in the form of jaundice[55,56]. RMSF is commonly mistaken for other viral or bacterial infection, because the symptoms are commonly nonspecific during the first few days of illness.

Table 4 Comparison of clinical features of hepatitis caused by various viruses

	n	Median age (range)	ALT (U/L)	ALP (U/L)	Bilirubin (μmol/L)	Lymphocyte count ($\times$ 10^9/L)	Lymphocytosis *n* (%)
EBV	17	40 (18-68)	395 (87-1362)	345 (160-756)	74 (13-165)	6.91 (3.77-24.82)	17 (100)
HAV	11	44 (20-61)	1056 (595-4122)	231 (91-342)	154 (42-214)	2.16 (1.23-4.1)	1 (9)
HBV	16	39 (20-60)	1858 (499-3856)	230 (93-406)	122 (36-355)	2.00 (1.26-3.52)	2 (12.5)
HEV	20	63 (54-81)	1387 (318-6357)	192 (139-464)	61 (8-297)	1.89 (0.96-10.25)	5 (25)

Modified from Vine *et al*[60]. EBV: Epstein-Barr virus; HAV: Hepatitis A virus; HBV: Hepatitis B virus; HEV: Hepatitis E virus.

Hepatic actinomycosis

Actinomycosis is a chronic granulomatous disease caused by filamentous, gram-positive, anaerobic bacteria, mainly *Actinomyces Israelii*. Hepatic involvement may occur through intestinal actinomycosis in the appendix and ileocecal region. The clinical presentation includes fever, anemia, body weight loss, and hepatosplenomegaly, which is not characteristic as actinomycosis; therefore, it is difficult to diagnose as actinomycosis preoperatively[57]. Percutaneous liver biopsy is useful, as sulfur granules can typically be observed.

NON-HEPATITIS VIRAL INFECTION

Although the hepatotropic viruses, hepatitis A though E, are the most common viral cause of acute liver injury (acute hepatitis), other viruses such as Epstein-Barr virus (EBV) or cytomegalovirus (CMV) can also cause acute liver injury. Serological tests and direct-detection by PCR/ISH/IHC are useful to diagnose these viruses, but it is not easy to distinguish between hepatitis viral infections and these non-hepatotropic viral infections at the time of the first medical examination.

EBV

EBV is a member of the herpes virus family. Infection commonly occurs in childhood and is asymptomatic. On the other hand, symptomatic disease develops mostly in young adults with high fever, sore throat and lymphadenopathy, known as infectious mononucleosis. Liver injury with a mild elevation of aminotransferases often occurs in patients with infectious mononucleosis, but acute hepatitis and jaundice has been observed in some patients with EBV infection without clinical features of infectious mononucleosis[58]. Manifestations of liver involvement range from mild hepatitis to hepatosplenomegaly with jaundice, and on rare occasion, acute fulminant hepatitis[59]. Vine *et al*[60] reported the clinical features of EBV hepatitis compared with those of acute viral hepatitis caused by hepatitis A, B, and E viruses (Table 4). Patients with EBV hepatitis rarely present with more than 1000 IU/L of serum ALT, and usually have lymphocytosis (> 5 $\times$ 10^9/L). Splenomegaly has been shown to be present in 88% of these patients. In this context, EBV hepatitis may be suggested by the presence of lymphocytosis and splenomegaly. Interestingly, the median age of patients with EBV hepatitis is older than that of patients with typical infectious mononucleosis, with nearly half the patients more than 60 years old[60].

It is well known that EBV primarily replicates nasopharyngeal epithelial cells and B lymphocytes, and expression of EBV-encoded small RNA is also observed in liver specimens from transplant recipients. Histologically, various findings can be seen, including sinusoidal infiltration of mononuclear cells and mildly ballooning hepatocytes with vacuolization.

Chronic active EBV infection (CAEBV) is well known as a rare disorder occurring in immunocompetent as well as immunocompromised hosts, and may cause EBV-associated lymphoproliferative diseases (LPDs). It has been reported that a third of the patients with EBV-driven LPDs have liver involvement[61]. The incidence of post-transplant lymphoproliferative diseases has been reported to range from 0.5% to 30% depending on the EBV status and the transplanted organs[62,63].

There are also several reports on chronic hepatitis by EBV infection in immunocompetent adults, which might be caused by the reactivation of EBV and increased viral-specific CTL responses[64,65]. The criteria for establishing this diagnosis have been proposed by Drebber *et al*[66]: namely, the presence of suggestive histopathological features, a specific serological profile, and detection of EBV genome in the liver tissue. When chronic hepatitis with unknown etiology is diagnosed, EBV infection could be the cause.

Human cytomegalovirus

Human cytomegalovirus (CMV) infection is commonly subclinical in immunocompetent adults, but it sometimes can cause a disease such as infectious mononucleosis or hepatic injury[67]. Liver dysfunction associated with CMV infection usually presents with mild to moderate elevation of serum aminotransferases and ALP, but jaundice is not common. CMV hepatitis rarely includes granulomatous hepatitis, cholestatic hepatitis, or acute hepatic failure with massive necrosis.

Watanabe *et al*[68] retrospectively analyzed the clinical features and laboratory data of patients with CMV hepatitis compared with EBV hepatitis. Although common signs and symptoms were similar, epigastralgia was more common in CMV hepatitis than EBV hepatitis (*P* < 0.05), and cervical lymphadenopathy was more frequently observed in EBV hepatitis than CMV hepatitis (*P* < 0.01). Also, the ratio of peripheral blood monocytes in the white blood cells was greater in CMV hepatitis (*P* < 0.01). On the other hand, CMV is one of the most important opportunistic pathogens and can cause severe pulmonary, retinal, gastrointestinal, and hepatic disease in im-

munocompromised hosts. About 10% of recipients of liver transplantation suffer from hepatitis associated with CMV, and the hepatitis may be caused by reactivation rather than primary infection[69].

The typical histological finding of CMV hepatitis is thought to be vital inclusion bodies in hepatocytes in recipients after liver transplantation, but this is not absolute. Microabscesses, lymphocytic infiltration, and parenchymal alterations are also common.

Other human herpes viruses

Other human herpes viruses besides EBV or CMV, including herpes simplex virus-1 and -2 (HSV-1, HSV-2), Varicella-Zoster virus (VZV), and human herpesvirus-6, -7 and -8 (HHV-6, -7, and -8), can occasionally cause liver injury[70]. Although HSV hepatitis is uncommon in immunocompetent patients, mild elevations of aminotransferases can be observed in 14% of patients with acute HSV infection. Severe hepatitis associated with HSV was reported in neonatal infection, pregnancy, and immunocompromised hosts. Rakela *et al*[71] reported that 6% of fulminant hepatitis cases at the Mayo Clinic were associated with HSV infection.

In primary infection by VZV, a typical manifestation is generalized exanthematous rash, which is known as varicella, and mild elevations of aminotransferases can be observed in up to 25% of children with varicella. After organ transplantations, the primary infection and reactivation of varicella can occasionally cause severe hepatitis including fatal fulminant hepatitis[72].

HHV-6 infection, known as Sixth Disease, typically occurs in infants under the age of 2. Härmä *et al*[73] reported HHV-6 was found in 80% of patients with acute liver failure (ALF) of unknown cause, which suggests that HHV-6 might be one of the causes of ALF. Reactivation of HHV-6 is also known to relate to the pathogenesis of drug-induced hypersensitivity syndrome (DIHS), which is characterized by fever, rash, lymphadenopathy, hepatitis, and leukocytosis with eosinophilia after administration of specific drugs. Shiohara *et al*[74] reported that an altered immune response, including changes of functional regulatory T cells, may influence the pathogenesis of DIHS. There are a few reports of hepatitis associated with HHV-7 infection[75]. Liver involvement of HHV-8 may occur in patients with HIV infection.

Other viruses

Parvovirus B19 infection commonly causes the erythema infectiosum in children, and causes liver dysfunction and hematologic disorder in adults. There are several case reports of parvovirus B19-infected patients with fulminant hepatitis and aplastic anemia[76].

It is well known that rubella infection during pregnancy may cause hepatocellular injury in the newborn as a part of the congenital rubella syndrome. Acquired rubella infection also may cause acute hepatitis with mild elevation of aminotransferases in adults[77,78]. In these cases, the most characteristic finding of the laboratory data

is a marked increase of LDH level, whereas cholestasis is rarely observed. It has been reported that the increase of LDH consisted of both LDH isozyme-3 derived from lymphocytes/platelets and LDH isozyme-5 derived from the liver[79,80]. The reported hepatic histological findings were compatible with acute hepatitis, including the ballooning of hepatocytes, spotty necrosis, and infiltration of inflammatory cells[79,80].

Hepatic involvement in measles has also been reported[81], with the prevalence of hepatitis in measles patients ranging from 71% to 89%[82-85]. Therefore, hepatitis should be considered as a common finding in patients with measles. Although the liver enzymes are elevated to more than 5 times ULN in 22% of patients with measles, clinical jaundice is rare[86]. It should be noted that rubella or measles could be a cause of liver dysfunction in patients with skin rash or fever, which may be misdiagnosed as drug-induced liver injury under medication.

Moreover, adenovirus commonly causes acute infections of the respiratory system and gastrointestinal tracts, and a few cases of ALF associated with adenovirus have been reported[87].

The mechanism of liver injury associated with non-hepatitis viral infection

Although the mechanism of liver injury caused by non-hepatitis viral infection remains unclear, several factors may be concerned. Hepatocellular injury may be caused by both host immune responses with activated CD8+ T cells and direct viral cytopathy. On the other hand, proinflammatory cytokines induced by virus infection may influence the function of sinusoidal and canalicular transporting systems, which may lead to cholestasis[88,89].

EBV-infected T or NK cells could cause chronic active EBV infection (CAEBV) in some cases; therefore, the possibility exists that EBV-infected T cells affect the pathogenesis of hepatitis.

An animal model study showed that activated CD8+ T cells are recruited to and trapped in the liver through interaction with intracellular adhesion molecule 1, which is expressed on sinusoidal endothelial cells and Kupffer cells[90]. A number of experiments have shown that soluble factors of the immune responses, especially interferon-γ, the Fas ligand and TNF-α, induce hepatitis[91-93].

FUNGAL INFECTION

Deep fungal infections can usually occur in immunocompromised hosts, including patients with HIV infection, neutropenia after chemotherapy, and organ-transplanted recipients. The liver is often involved in deep fungal infections, together with other organs.

Hepatosplenic candidiasis

Hepatosplenic candidiasis may be caused by *Candida* species, including *C. albicans* and *C. tropicans*, through candidemia or the portal vasculature from the gut with degenerated barriers of gastrointestinal mucosa[94]. Dis-

Table 5 Parasitic infection of the liver

Disease (organism)	Organs/status	Clinical presentation
Malaria	Pre-erythrocytic phase	Asymptomatic
(*P. falciparum, malariae, vivax, ovale*)	Erythrocytic phase	Anemia, jaundice, mild elevation of aminotransferases, tender hepatomegaly, splenomegaly
Amebiasis	Intestine	Right upper quadrant pain, fever,
(*Entameba histolytica*)	Amebic liver abscess	hepatomegaly (50%), jaundice (< 10%)
Cystic echinococcosis	Single cyst (> 70%), < 10 cm	Asymptomatic
(*Echinococcus granulosus*)	and no complication	
	Size up (1-5 cm/year), > 10 cm	Abdominal pain, mass effect (possible)
	Rupture	Peritonitis, hypersensitivity reactions
Alveolar echinococcus		Malaise, tender hepatomegaly, eosinophilia,
(*E. multilocularis*)		obstructive jaundice, portal hypertension
Schitosomiasis	Acute phase	Eosinophilic infiltrate
(*S. mansoni, japonicum*)	Chronic phase	Presinusoidal portal hypertension, splenomegaly, gastroesophageal varices
Fascioliasis (*F. hepatica*)	Acute phase	Abdominal pain, fever, hemobilia, hepatomegaly
	Chronic phase	Biliary colic, cholangitis, cholelithiasis, obstructive jaundice
Ascariasis (*A.lumbricoides*)		Abdominal pain, fever, obstructive jaundice

seminated candidemia is usually seen among patients with hepatologic malignancies with prolonged severe neutropenia. The incidence of hepatosplenic candidiasis has been reported to be 3% to 7% in these high-risk patients, but it may have decreased recently due to the use of antifungal prophylaxis.

The clinical presentation of hepatosplenic candidiasis consists of persistent fever with spikes and right upper quadrant discomfort, nausea, and anorexia. Laboratory testing commonly shows elevated serum concentrations of ALP and γ-GTP, associated with small liver abscesses or granulomas[95]. Multiple hypoechoic lesions and non-enhanced low-attenuation lesions can be detected by ultrasound and CT scan, respectively[96].

Other fungal infections

Liver involvement in other fungal infections, including disseminated aspergillosis, cryptococcosis, mucormycosis, trichosporonosis, and histoplasma capsulanum occurs on rare occasion in immunocompromised hosts[97]. The incidence of liver involvement with other fungal infections is very low, because these are acquired exogenously. Park[97] reported that up to 90% of these patients could have liver involvement in spite of the very low incidence of disseminated histoplasmosis. Hepatic histological findings could be variable, including granulomatous changes and sinusoidal Kupffer cell hyperplasia.

LIVER INVOLVEMENT OF PARASITIC DISEASES

Parasitic liver involvement is common in highly endemic areas, and it should be considered in an individual who has visited such areas. Parasitic involvement is dominated by *Plasmodium spp.*, but *Entameba histolytica*, *Schistosoma spp.* and *Echinococcus spp.* infections are also important in clinical practice[98]. Hepatobiliary involvement is also caused by *Ascaris lumbricoides*, *Fasciola hepatica* and *Liver flukes* (Table 5).

Malaria

Malaria is one of the most important public health problems worldwide, as an estimated 300 to 500 million persons suffer from malaria annually. The malarial life cycle consists of the pre-eythrocytic and the erythrocytic phases. Usually malarial schizogony takes place in the liver without obvious liver injury in the pre-erythrocytic phase. In the erythrocytic phase, symptoms such as cyclical fever, malaise, nausea, vomiting, diarrhea, tender hepatomegaly and splenomegaly develop[99]. Jaundice associated with hemolysis can be observed in severe malarial infection, and hepatic failure can occasionally be seen in patients with severe *P. falciparum* infection[100]. Jaundice in malaria consists of both unconjugated and conjugated bilirubin, which could be caused by intravascular hemolysis of parasitized red blood cells, and hepatocellular dysfunction. Hepatic histological findings may show Kupffer cell hyperplasia with pigment deposition, hepatocyte necrosis, and cholestasis.

Schistosomiasis

Schistosomiasis is caused by trematodes of the genus Schistosoma, including *S. mansoni and S. japonicum*. Mesenteric infection may cause deposition of the eggs in the liver, which may lead to presinusoidal occlusion and periportal fibrosis associated with granulomatous response[101]. Chronic hepatic schistosomiasis presents with portal hypertension with splenomegaly and gastroesophageal varices. Laboratory test abnormalities include eosinophilia and increased serum IgE levels. Because hepatic schistosomiasis is one of the most common causes of noncirrhotic portal hypertension, it should be considered in differential diagnosis for that symptom.

Amebiasis

Amebiasis is caused by the *Entamoeba histolytica*, and about 40 to 50 million persons are infected annually. Amebiasis includes amebic dysentery and extraintestinal disease such as amebic liver abscess. Patients with amebic liver abscess

usually present with RUQ pain and fever. Although hepatomegaly can be seen in about 50% of cases, jaundice can be seen in less than 10%[102]. In the liver, *E. histolytica* lyses host's tissue and infiltrating neutrophils with its proteolytic enzymes[103]. Amebic liver abscess grows inexorably, and the retardation of making diagnosis leads to perforation in about 2% to 7% of these patients[104-106] with the mortality rate being 23% to 42% in the perforated patients[105,107]. Therefore, prompt diagnosis and treatment are very important for successful treatment in patients with hepatic amebiasis[108].

Hydatid disease

Hydatid disease is caused by infection with the metacestode stage of the tapeworm *Echinococcus*. Liver involvement may occur in about two-third of patients with *Echinococcus granulosu* infection, and commonly can form single cyst. Although a patient has no symptom when the cyst is small (< 10 cm in diameter) and without complication, intra-peritoneal rupture may be frequent and cause abdominal pain. Rupture into the biliary tract may cause biliary colic, obstructive jaundice, or cholangitis.

Echinococcus multilocularis can cause alveolar echinococcus, which commonly presents obvious complaints such as tender hepatomegaly, malaise, and weight loss. Because *E. multilocularis* can invade the biliary tract, hepatic vein, inferior vena cava, and/or diaphragm, a high mortality rate has been reported in untreated patients[109].

Liver flukes (Fascioliasis)

Fascioliasis is a trematode infection caused by *Fasciola hepatica* or *Fasciola gigantic*. Fascioliasis commonly consists of two phases, the acute/invasive and chronic obstructive phase. In the acute phase, symptoms usually include fever, RUQ pain, hepatomegaly and eosinophilia. The chronic phase usually begins about six months after infection and is characterized by bile duct obstruction associated with bile duct inflammation and hyperplasia due to the presence of adult flukes. Clinical presentations include recurrent biliary colic, cholangitis, cholelithiasis, and obstructive jaundice[110].

Ascariasis

Ascaris lumbricoides is an intestinal nematode, and arrives in the liver through the bile duct by a retrograde manner. Migration of adult worms into the biliary tree can cause biliary colic, cholecystitis, cholangitis, obstructive jaundice, and secondary liver abscess[111].

CONCLUSION

The liver is exposed to many systemic infectious pathogens including not only hepatotropic but also non-hepatotropic microorganisms through both the systemic and portal circulation. These pathogens may directly or indirectly cause liver injury presenting with various manifestations described in this review, but the mechanisms

of these liver injuries have not been completely clarified. When making correct diagnosis of liver dysfunction in systemic infections, knowledge about non-hepatotropic pathogens and appropriate microbiological examinations are very important.

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Vertical transmission of hepatitis C virus: Current knowledge and perspectives

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Abstract

Hepatitis C virus (HCV) infection is a major global health issue. Infection by the HCV can cause acute and chronic liver diseases and may lead to cirrhosis, hepatocellular carcinoma or liver failure. The World Health Organization estimates that approximately 3% of the world population have been infected with HCV and the worldwide prevalence is between 1% and 8% in pregnant women and between 0.05% and 5% in children. Following the introduction of blood product screening, vertical transmission becomes the leading cause of childhood HCV infection. The prevalence of pediatric HCV infection varies from 0.05% to 0.36% in developed countries and between 1.8% and 5% in the developing world. All children born to women with anti-HCV antibodies should be checked for HCV infection. Though universal screening is controversial, selective antenatal HCV screening on high-risk populations is highly recommended and should be tested probably. Multiple risk factors were shown to increase the possibility of HCV vertical transmission, including coinfections with human immunodeficiency virus, intravenous drug use and elevated maternal HCV viral load, while breastfeeding and HCV genotypes have been studied to have little impact. At present, no clinical intervention has been clearly studied and proved to reduce the HCV vertical transmission risk. Cesarean section should not be recommended as a procedure to prevent vertical transmission, however, breastfeeding is generally not forbidden. The high prevalence of global HCV infection necessitates renewed efforts in primary prevention, including vaccine development, as well as new approaches to reduce the burden of chronic liver disease. Future researches should focus on the interruption of vertical transmission, developments of HCV vaccine and direct-acting antivirals in infancy and early childhood.

Key words: Hepatitis C virus; Vertical transmission; Perinatal infection; Chronic liver disease

Core tip: Hepatitis C virus (HCV) infection is a major global health issue. World Health Organization estimates that the worldwide prevalence is 1%-8% in pregnant women and 0.05%-5% in children. Vertical transmission becomes the leading cause of childhood

HCV infection. Current understanding of the epidemiology of mother-to-child transmission of HCV is limited. At present, no clinical intervention has been clearly studied and proved to reduce the vertical transmission risk. Though universal screening is controversial, selective antenatal HCV screening on high-risk populations is highly recommended and should be tested probably. This review provides the current knowledge and perspectives of HCV vertical transmission and summarizes the updated follow up guidelines for clinical practice.

GLOBAL EPIDEMIOLOGY OF HEPATITIS C INFECTION

Hepatitis C virus (HCV) infection is a major global health issue[1]. Infection by the HCV can cause acute and chronic liver diseases and may lead to cirrhosis, hepatocellular carcinoma or liver failure[2]. The World Health Organization estimates that approximately 3% of the world population have been infected with HCV[3]. There are approximately 170 million HCV patients worldwide, and three to four million cases are newly diagnosed every year[4,5]. It is estimated that about 0.2% to 26% of the general population in different countries are chronically infected by HCV[6]. The prevalence of HCV infection in the United States between 1999 and 2002 was found to be 1.6%[7]. In China, approximately 40 million people are infected with HCV, and 50% to 85% of them may develop chronic hepatitis; of these patients, 20% to 30% progress to liver cirrhosis and/or hepatocellular carcinoma[8,9].

Before blood product screening for HCV was introduced, transfusion represented an important route of HCV transmission for infants and children[10]. Following the introduction of blood product screening, vertical transmission becomes the leading cause of childhood HCV infection and approximately 4000 new cases are diagnosed each year in the United States[11]. It is estimated that the prevalence varies from 0.05% to 0.36% in developed countries and 1.8% to 5% in the developing world[12,13].

PREVALENCE OF HCV INFECTION IN PREGNANT WOMEN

The worldwide prevalence of HCV infection is between 1% and 8% in pregnant women and between 0.05% and 5% in children[14]. Antenatal HCV infection rates vary worldwide, from 1% to 2.5% in the United States and Europe to more than 10% in some sub-Saharan countries[15-17]. Studies have shown the prevalence to be as high as 40% in some parts of Egypt[18]. According to the

result of the maternal HCV screening project conducted in Tottori Prefecture, Japan, the prevalent rate of HCV carrier mothers who were both anti-HCV and HCV RNA positive was 0.39%, while the rate of vertical transmission was found to be 8%[19]. However, the above maternal HCV prevalent rates may be underestimated since the current practice of HCV screening among high-risk pregnant women might miss a large number of HCV-infected patients, and besides there are no large scale HCV serosurvey studies available at present[20].

In a recent study performed in Taiwan, a total of 7355 healthy asymptomatic pregnant women were screened for anti-HCV during a 6-year study period, 44 (0.6%) were found to be HCV-infected and 22 mothers were enrolled[21]. Half of the anti-HCV positive mothers were found to be positive for HCV RNA. All the mothers were negative for anti-HIV, 9 had invasive obstetric procedures such as amniocentesis. Of the 22 mother and baby pairs who were successfully followed up, two (9.1%) had eventually confirmed infected with HCV. Both of them were born to mothers with high viral load (HCV RNA > 10^5 copies/mL).

However, a methadone program in Australia showed that more than 70% of the pregnant women in this program are HCV positive, but less than 20% of their offsprings are examined for HCV status[22]. As a result of the lack of awareness of HCV in this high-risk population, many of these children are lost to follow-up and not diagnosed[23,24].

PATHOGENESIS OF HCV INFECTION DURING PREGNANCY

The pathogenesis of HCV infection during pregnancy remains poorly understood[14]. Recent studies have demonstrated there is a decrease of levels of serum alanine aminotransferase (ALT) during the second and third trimesters of pregnancy. However, the HCV viral load increases and reaches a peak during the third trimester[25-26]. Postpartum exacerbation of clinical HCV manifestations were found[27]. Conversely, seroconversion in pregnancy has been demonstrated and pregnancy may improve the natural course of HCV infection in some studies[26,28].

Besides, recent researches suggests that HCV infection during pregnancy may increase the risks for preterm delivery, low Apgar scores, low birth weight, gestational diabetes, congenital malformations and overall perinatal mortality[29-31]. Other risk factors, such as limited prenatal care and intravenous drug use, are also found to be more prevalent in HCV patients[32] which could influence maternal and fetal morbidities and outcomes[26]. Conversely, increased risks for these obstetric complications were not shown in other studies[27,33].

INCIDENCE OF VERTICAL TRANSMISSION

As mentioned previously, vertical transmission becomes

a leading cause of pediatric HCV infection after blood product screening for hepatitis C was introduced, and it is also the leading cause of pediatric chronic liver disease in developed countries[34]. Although vertical transmission leading to chronic infection is reported in 4%-8%, transient HCV perinatal infection also occurs, with an incidence of about 14%-17%[35,36]. Incidence of HCV vertical transmission has been documented to be 3%-10%[14,33,37,38] and are higher in infants born to mothers coinfected with human immunodeficiency virus (HIV).

RISK FACTORS OF VERTICAL TRANSMISSION

Multiple risk factors were studied to increase the risk of HCV vertical transmission, including coinfections with HIV, intravenous drug use, high maternal HCV viral load, mode of delivery, preterm labor, prolonged rupture of membranes and amniocentesis, while breastfeeding and HCV genotypes have little impact on vertical transmission[14,30,39-41]. However, most of the reports are still controversial.

HIV
Multiple researches have demonstrated that HCV vertical transmission rate increases 2-4-fold if coinfected with HIV[10,42,43]. Vertical transmission in the group of infants in which the mother was HIV coinfected antenatally was 5.9%, and thus supports the current recommendations for cesarean delivery in HIV and HCV coinfected mothers[13]. It has been demonstrated that coinfections with HCV and HIV during pregnancy increase the vertical transmission odds by 90% according to a meta analysis of 10 studies[43].

Viral load
Another main risk factor identified for vertical transmission was maternal hepatitis C viremia. For mothers who tested positive for HCV RNA, vertical transmission was significantly higher at 7.1% when compared with 0% transmission for those who tested HCV RNA negative antenatally[10]. This has been reported previously in the literature and reflects that viremia holds a higher risk of vertical transmission[44,45]. Many studies have demonstrated that the risk of HCV vertical transmission increases if the maternal serum HCV viral load is above 10^6 copies HCV-RNA/mL, however there are many uninfected infants even though their mothers have a higher HCV viral load[46-48]. Since the maternal serum HCV-RNA viral load may fluctuate during pregnancy, it is recommended to repeat the HCV-RNA load in the third trimester[38].

Although there are a few reports of vertical transmission in which the mother did not have viremia detected antenatally[49,50]. Maternal HCV RNA status can be of benefit in the patients counseling, patients can be reassured and advised that the risk of vertical transmission is minimal if hepatitis C RNA is not detected antenatally.

Mode of delivery
More controversial is the effect of mode of delivery on vertical transmission. Whereas some studies have shown a protective benefit from cesarean section (CS) delivery[51,52], many have not[25,53-55]. Few studies in the past recommend the use of elective CS to prevent the possible obstetric risks in order to lower the incidence of HCV vertical transmission[56]. Okamoto *et al*[19] previously reported that children born to mothers with high viral loads had a significantly higher incidence of vertical transmission when delivered transvaginally. However, other studies, including a large-scale multicenter research project conducted in Europe, have failed to show significant evidence to prove its protective effect[25,47,57]. Some questioned the results since probably because most of the studies did not analyze high viral loads incidence along with elective CS.

Several conditions must be elucidated before the recommendation of elective cesarean section to prevent HCV vertical transmission[58]. Though studies show that the HCV vertical transmission rate is low in the infants born to mothers with high viral load[38], however, taking into consideration the risks involved in CS and the natural course of HCV in infants, most research studies do not recommend elective CS for vertical transmission prevention at present[38,50,59,60]. Thus, the majority of the published literature would suggest that mode of delivery is not a key factor influencing HCV vertical transmission.

In 2008, the Japanese Society of Obstetrics and Gynecology's guideline recommended informing high viral loads women that the risk of vertical transmission might be significantly reduced by elective CS[61]. However, emergency CS should be considered separately from elective CS because emergency CS may allow conditions such as maternal blood contamination of the fetus and other complications[58,62]. However, cesarean delivery has been recommended for HCV-positive women coinfected with HIV as mentioned before[63].

Breast-feeding
Breast-feeding does not increase the vertical transmission rate[38,60,64]. Avoidance of breast feeding is not an effective way for preventing HCV vertical transmission[65]. It is true that HCV RNA has been detected in breast milk and colostrum[66], however breast-feeding does not shown to be a route of maternal to infant transmission[45,67,68]. HCV infected mothers are encouraged to breast-feed if there are no other contraindications, such as HIV co-infection[69]. The Centers for Disease Control and Prevention (CDC) suggests mothers should interrupt breast-feeding temporarily if there are bleeding or traumatized nipples, which could increase infants' HCV exposure[70].

Premature rupture of the membranes
Premature rupture of the membranes is considered a risk factor for HCV vertical transmission by exposing the fetus to maternal HCV in the birth canal[45,54]. The duration of rupture has been found to be significantly longer in

infected children[45,54,60]. These parameters are potentially related to contamination of the fetus with infected maternal blood in the birth canal.

Other factors

Besides, the European Paediatric HCV Network described the significance of infantile sex as a risk factor for HCV vertical transmission, girls are twice as likely as boys to be infected[60], however, no significant difference is found in other study[46]. In addition, some experts recommend avoiding invasive procedures that promote fetal exposure to maternal blood, such as fetal scalp monitoring[45,68]. As described previously, other parameters, such as birthweight, Apgar score, gestational age and bleeding volume during delivery were not significant risks for HCV vertical transmission[45,46,55,71,72]. Besides, HCV genotype was not associated with vertical transmission of HCV[73]. Despite an increased understanding of the risk factors involved, its transmission mechanisms and timing are still unknown and recommendations regarding prevention are limited[41,53,74].

NATURAL COURSE OF HCV-INFECTED INFANTS

HCV infection may lead to chronic hepatitis, cirrhosis and hepatocellular carcinoma in adult populations. However researches about the natural history of hepatitis C in children is little. Studies showed that perinatally-acquired HCV infection becomes chronic in approximately 80% of cases[45,75,76], similar to that observed in adults[77], but higher than that reported in children who were infected through contaminated blood products[78]. Most studies show that HCV infected children are mostly asymptomatic[75,79]. Spontaneous clearance have reported rates ranging from 21% to 75%[76,78,80,81]. The European Paediatric HCV Network evaluated 266 children with vertically-acquired HCV infection and found clearance occurred in 21%-25%. Among cases of neonatal infection, 25% demonstrated spontaneous clearance by 7.3 years[80].

However it has not been studied clearly whether the virus is completely eliminated, and there is possibility the infants will become HCV-RNA positive again later in their life. In a study performed in United Kingdom, the overall rate of spontaneous viral clearance was 17.5% with higher clearance (27%) in the transfusion group compared to the vertically acquired group (9%). Most children are asymptomatic with mildly abnormal hepatic transaminases[82]. An infected infant becomes HCV-RNA positive between 0 and 3 mo after birth[38]. Fortunately there is no case of fulminant hepatitis reported among infected infants to date.

Long-term outcomes for young HCV infected children in general are good[83-85]. Studies following patients for 10 to 20 years after perinatal acquisition of HCV show that 5% to 12% of them has significant fibrosis and 5% has cirrhosis[79,86]. No studies have yet studied the incidence of cirrhosis and hepatocellular carcinoma in

adults who acquired vertical HCV infection.

DIAGNOSIS OF PERINATAL TRANSMISSION

A practical and widely acceptable recommendation by most studies is to consider children born to anti-HCV positive mothers infected with HCV when: (1) HCV RNA is detected in at least two serum samples and at least three months apart during the first year of life; and (2) HCV antibody is positive after 18 mo of age[73]. There is agreement on delaying PCR testing until 3 mo of age and to repeat it, if positive, at 6 mo of age. Testing of HCV antibody is of limited value before 18 mo of age due to passive transfer of maternal antibodies[60,73,87].

TREATMENT AND PREVENTION

Interferon and other treatments for women with high viral load who are of child-bearing age are useful for decreasing HCV levels, both for women as carriers and to decrease the risk of possible vertical transmission in future deliveries[58]. However, the available pharmacological therapies are contraindicated in pregnancy: ribavirin for its teratogenic effects and pegylated interferon alfa for its possible effects on fetal growth[88]. Thus, these treatments of HCV are contraindicated during pregnancy and there are no antiviral treatment recommendations for HCV-infected women at present[89]. Finally, whether CS is effective in preventing vertical transmission of HCV is still unclear as stated previously[42,60,90,91].

Generally, children who are younger than 3 years should not be treated, and treatment is not approved in this age group. There are no published studies or reports of treatment in children who are younger than 3 years[92]. At present, treatment modalities that were initially restricted to adult subjects are now recommended for the treatment of HCV in children 3-17 years of age[93,94]. Treatment should consider several aspects including age, severity of disease, its adverse effects and compliance to treatment[92].

PRENATAL SCREENING

According to the recent recommendations published by the American College of Obstetricians and Gynecologists and CDC, routine prenatal HCV screening is not recommended in the general population[20,95,96]. However, women with significant risk factors should be offered screening. Generally, selective antenatal HCV screening is used on the basis of risk factors for exposure to the virus, such as a history of intravenous drug abuse[10]. However, there are currently no official recommendations addressing how often high-risk populations should be tested probably due to a lack of available data[37].

In clinical practice, HCV screening in pregnancy has proven difficult, and it is likely that most HCV infected pregnant women are not identified[97-99]. Forty to 70% of

HCV-infected pregnant women do not initially report major risk factors[25,100]. In fact, a study in the United Kingdom showed that only one-third were identified through selective antenatal screening, suggesting that there may be many unidentified perinatally infected children in the absence of routine maternal antenatal screening[81]. A recent report by Delgado-Borrego *et al*[101] estimated that about 85% to 95% of HCV-infected children in the United States have not been identified. Given the inherent inadequacies of risk factor-based screening, researches have investigated whether universal HCV screening in pregnant women would be a worthy approach.

In 2012, the CDC added recommendations for universal screening of all United States "baby boomers" regardless of reported risk factors[102]. This new recommendation was prompted by a recognition of the increasing rate of HCV complications in the United States and the failure of risk factor-based screening to identify most infected infants. Universal screening would ensure that infants born to HCV-infected women are properly identified and evaluated. However, when Plunkett and Grobman modeled universal screening in a pregnant population with 1% HCV seroprevalence, they found that it was not cost-effective, even when benefits of HCV diagnosis and treatment were considered for both mothers and infants and assuming that CS eliminated perinatal transmission[103].

Prenatal screening itself is expensive, even in developed countries. An effective screening strategy utilizes an inexpensive and sensitive test to identify asymptomatic individuals at risk of a disease that has reasonably high prevalence, serious consequences if left untreated, and an effective treatment available[58,104].

FOLLOW UP GUIDELINES

Chronic pediatric HCV infection is usually associated with minimal or mild liver disease, however some cases may progress to advanced liver damage[80,105,106]. A broad range of ALT levels have been observed during the first year of life, with some infants exhibiting acute hepatitis pictures and others showing normal or mild elevated levels[75,105,107].

In 2008, Shiraki *et al*[38] presented guidelines for doctors in consulting and treating HCV-carrying pregnant women and their infants basing on current knowledge of vertical transmission. For those infants born to mother who is positive for anti-HCV and negative for HCV-RNA, an anti-HCV test should be performed later than 18 mo after birth to confirm that the infant is negative for anti-HCV. If the infant is still anti-HCV positive, the infant is considered to have been infected with HCV, HCV-RNA viral load and ALT level should be examined to determine whether the infection is a past one or whether it has continued up to the present time.

For those infants born to HCV-RNA-positive mother, tests for AST and ALT levels and HCV-RNA load should be performed 3 or 4 mo after birth. When HCV-RNA is positive, tests for AST, ALT, HCV-RNA and anti-HCV should be performed every 6 mo starting from the 6 mo of birth to determine the persistence of infection. If the infant is negative for HCV-RNA 3 or 4 mo after birth, an HCV-RNA test should be performed at the ages of 6 mo and 12 mo to confirm the infant's negativity[38].

CDC guidelines recommend testing for anti-HCV in children born to HCV infected mothers after 12 mo of age. However, if earlier testing is required, nucleic acid-based testing for HCV RNA is recommended 1 to 2 mo after birth[96,102]. If positive for either anti-HCV or HCV RNA, children should be evaluated for liver disease, and those with persistently elevated ALT levels should be referred to a specialist for medical management[96,108,109]. To further confirm HCV-RNA negativity, anti-HCV is tested at 18 mo of age if possible, and follow-up tests are no longer required when anti-HCV is also negative.

CONCLUSION

HCV infection affects a large number of women of reproductive age worldwide, and vertical transmission remains a serious public health problem. The high prevalence of global HCV infection necessitates renewed efforts in primary prevention, including vaccine development, as well as new approaches to reduce the burden of chronic liver diseases.

Based on present knowledge of perinatal transmission of HCV, all children born to women with anti-HCV antibodies need to be tested for HCV infection. Though universal screening is controversial, selective antenatal HCV screening on high-risk populations is highly recommended and should be tested probably. At present, no intervention has been clearly demonstrated to reduce the risk for HCV vertical transmission. Cesarean section should not be recommended as a method to prevent vertical transmission of HCV, however, breastfeeding is generally not forbidden.

Awareness of HCV infection status in those high-risk population is mandatory. Novel approaches need to be considered to improve the knowledge of HCV transmission and hopefully improve HCV-associated health outcomes in high-risk populations. Future researches should focus on the interruption of vertical transmission, developments of HCV vaccine and direct-acting antivirals in infancy and early childhood. To prepare a more comprehensive and concrete standard for the prevention of HCV vertical transmission, a large scale and long-term follow-up study of children should be organized, as this may establish the need for more aggressive measures for prevention and treatment. Eventually, we believe that the number of new patients with HCV vertical transmission can be further decreased in the future.

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MINIREVIEWS

Nucleos(t)ide analogues to treat hepatitis B virus-related hepatocellular carcinoma after radical resection

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Abstract

Significant advances have been made in nucleos(t)ide analogue (NA) therapy to treat chronic hepatitis B, and this therapy reduces the risk of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC) in some patients. However, whether NAs can also prevent recurrence after radical resection of HBV-related HCC remains controversial and is an important question, given that most patients will experience recurrence within a few years of curative surgery. Here we systematically reviewed the literature since 2004 on outcomes after administering NAs to patients with HBV-related HCC following radical resection. We focused on treatment indications, duration, effects on recurrence-free survival and overall survival, and the management of NA resistance. We find that patients with HCC should strongly consider NA therapy if they are positive for HBV-DNA, and that the available evidence suggests that postoperative NA therapy can increase both recurrence-free and overall survival. To minimize drug resistance, clinicians should opt for potent analogues with higher resistance

barriers, and they should monitor the patient carefully for emergence of NA-resistant HBV.

Core tip: Significant advances have been made in nucleos(t)ide analogue (NA) therapy to treat chronic hepatitis B. However, for patients undergoing radical resection for hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC), a number of important questions remain undefined, including when NA therapy should be initiated, how long the treatment should continue, and whether NAs can prevent recurrence after radical resection. Here we review the available evidence on these questions in the Medline database. We focus on NA treatment indications, duration, effects on recurrence-free survival and overall survival, and management of NA resistance in patients with HBV-related HCC.

Ke Y, Wang L, Li LQ, Zhong JH. Nucleos(t)ide analogues to treat hepatitis B virus-related hepatocellular carcinoma after radical resection. *World J Hepatol* 2014; 6(9): 652-659 Available from: URL: http://www.wjgnet.com/1948-5182/full/v6/i9/652.htm DOI: http://dx.doi.org/10.4254/wjh.v6.i9.652

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third most frequent cause of cancer-related death in the world[1]. Hepatic resection, percutaneous ethanol injection, radiofrequency ablation are recognized as radical treatment options for HCC and are highly effective at removing tumors; however, patients' prognosis after radical resection remains poor, due to the high recurrence rate[1,2]. HCC recurrence occurs in up to

41%-50% of patients within 2 years after resection (early recurrence) and in up to 20% of patients more than 2 years later (late recurrence)[3,4]. Most early recurrence appears to reflect diffusion of primary tumors, while most late recurrence stems from *de novo* tumors spontaneously arising in the remnant diseased liver[3-5].

In China and Sub-Saharan Africa, the major risk factor for HCC is hepatitis B virus (HBV) infection. Therefore investigators reasoned that the same nucleos(t)ide analogues (NAs) that have been proven so effective against chronic HBV infection may also benefit patients with HBV-related HCC. Indeed, randomized controlled trials (RCTs)[6] and large retrospective studies[7-9] have shown that NAs can dramatically reduce the risk of HCC in patients with chronic HBV infection or cirrhosis. While this suggests that NAs are effective against primary HCC, the question of whether they can also prevent HCC recurrence after radical resection remains controversial[10].

Here we systematically reviewed the literature on this question by searching the Medline database for articles published since 2004 on outcomes of NA therapy in patients with HBV-related HCC. We used the following search terms: "nucleoside analogue", "nucleoside analog", "nucleotide analogue", "nucleotide analog", "antiviral therapy", "hepatitis B virus", "hepatocellular carcinoma", "liver resection", and "survival rate". We focused on treatment indications, duration, effects on recurrence-free survival and overall survival, and the development of NA resistance.

TYPES OF NAS

Five types of oral NAs have been used in clinical practice: lamivudine (LAM), adefovir dipivoxil (ADV), telbivudine (LdT), entecavir (ETV) and tenofovir disoproxil fumarate (TDF). LAM and LdT are L-nucleoside analogues, ADV and TDF are acyclic adenine nucleotide analogues, and ETV is a cyclopentyl guanosine analogue[11]. All 5 of these NA types can be phosphorylated in cells, and subsequently compete with natural nucleotides to be incorporated into viral DNA by HBV polymerase/reverse transcriptase. Since the analogues cannot be extended by HBV polymerase, they cause premature termination of genome replication. Studies suggest that ETV, TDF, and LdT are similarly effective at suppressing HBV-DNA synthesis and are more potent than LAM and ADV[11], although none can completely eradicate HBV due to the persistence of covalently closed circular DNA in the nuclei of infected hepatocytes[12].

INDICATIONS AND DURATION OF NA THERAPY AFTER HCC SURGERY

Nowadays there are Asian-Pacific consensus[11], Chinese Medical Association guideline[13], American Association for the Study of Liver Disease (AASLD) guideline[14], European Association for the Study of Liver (EASL)

guideline[15], Treatment Algorithm in the United States[16] and Asian-American guideline[17] related to the treatment of chronic hepatitis B infection. In these guidelines[11,13-17], the criteria for initiating treatment such as ALT level and HBV-DNA amount are different. Current Asian guidelines[11,13] recommend that NA therapy be considered if the ALT level is > 2-fold greater than the upper limit of the normal range, and the HBV-DNA level is either > 20000 IU/mL if the patient is HBeAg-positive or > 2000 IU/mL if the patient is HBeAg-negative. In America, with the same criteria about ALT level, NA therapy is recommended to patients if their HBV-DNA level is > 20000 IU/mL[14]. While a panel of Asian-American physicians with expertise in hepatitis B treatment has suggested[17] that Asia Americans should be considered for treatment when they have HBV-DNA levels above 2000 IU/mL, and serum ALT levels above the upper limit of the normal range, and so did EASL guidelines[15] in the criteria of ALT level and HBV-DNA amount, which are stricter than AASLD guideline[14].

Recommended treatment duration also varies depending on these guidelines[11,13-15]. In HBeAg-positive patients who show HBeAg seroconversion and undetectable levels of HBV-DNA, Asian-Pacific guideline[11] recommends that NA treatment can be discontinued after 12 mo of consolidation therapy, while AASLD guideline[14] recommends the duration of consolidation therapy be at least 6 mo. In HBeAg-negative patients, both Asian-Pacific and AASLD guidelines recommend NA treatment should ideally be stopped when HBsAg is no longer detectable[11,14], while Asian-Pacific guideline[11] advises if the patient remains HBsAg-positive, NA treatment can be discontinued after at least 2 years of therapy when test results show undetectable HBV-DNA levels on 3 separate occasions 6 mo apart. EASL guideline[15] suggests that in both HBeAg-positive and HBeAg-negative patients sustained off-treatment HBsAg loss is the ideal end point. Sustained off-treatment virological and biochemical response in HBeAg-negative patients (including HBeaAg-positive patients at baseline with durable anti-HBe seroconversion) is the second, and a maintained undectable HBV-DNA under long-term antiviral therapy in HBeAg-positive patients without anti-HBe seroconversion and in HBeAg-negative patients is the next most desirable end point.

Since these guidelines[11,13-17] were different from each other and were developed for patients whose major disease was chronic HBV infection, it is unclear whether they are optimal for patients with HBV-related HCC. Given the need to reduce HBV replication as much as possible in these patients, particularly before drug resistance emerges, the Chinese Medical Association[18] recommends that the threshold of viremia to initiate NA therapy for patients with HBV-related HCC should be lower than the threshold for patients without HCC, and that patients with HBV-related HCC should take NA therapy as long as they show detectable levels of HBV-DNA, regardless of ALT levels. Going even further, some investigators[19] have suggested routine prophylactic

Figure 1 Bubble plot of recurrence-free survival in patients receiving nucleos(t)ide analogue therapy or not after radical resection to treat hepatitis B virus-related hepatocellular carcinoma. Bubble size reflects relative cohort size. [a]$P < 0.05$: NA group *vs* Control group. NA: Nucleos(t)ide analogue.

NA therapy for HCC patients with HBV-DNA levels < 2000 IU/mL before liver resection. The aim is to prevent HBV reactivation after liver resection, which occurs in as many as 19% of patients within the first 1 year and which can severely reduce liver function and survival[19].

Since NA therapy cannot completely eradicate HBV, some investigators have advocated lifelong treatment, regardless of undetectable levels of HBV-DNA and HBeAg seroconversion in HBeAg-positive patients or HBsAg loss in HBeAg-negative patients. Those authors argue that long-term therapy may help prevent hepatitis flare-ups and inhibit hepatocarcinogenesis to the greatest extent[20], although there is not sufficient evidence nowadays.

POSTOPERATIVE NA THERAPY AND RECURRENCE-FREE SURVIVAL

Our extensive online search in the Medline database identified 19 studies published since 2004 that investigated outcomes of postoperative NA therapy in patients with HBV-related HCC. These references comprise 17 retrospective studies[21-37] and 2 RCTs[38,39]. Most of studies come from Asia, including Chinese mainland, Japan, Hong Kong and Tai Wan, which reflects HBV epidemiology and the high incidence of HBV-related HCC in this region. One study from the United States has a small number of patients appeared first in 2011[36] and further follow up published in 2014 with more cases and a longer follow up over 12 years[37]. Of the 19 included studies, besides patients who underwent hepatic resection (6705, 96.7%), NA therapy were also applied for patients with ablative procedures as follows: radiofrequency ablation (176, 2.5%), percutaneous ethanol injection (7, 0.1%), and transarterial chemoembolization (49, 0.7%). Patients' characteristics in these studies are shown in Table 1. The outcomes data are shown in the Table 2.

All 19 studies reported data on recurrence-free survival after radical surgery. Several retrospective studies[21-23,26-29,33,35] showed that NA treatment did not lead to significantly higher recurrence-free survival than non-NA

treatment, while other retrospective studies[24,25,30-32,34,36,37] and the RCTs[38,39] showed that NA therapy was associated with significantly higher recurrence-free survival than non-NA treatment.

To synthesize these findings quantitatively, we generated bubble plots of 1-, 3-, and 5-year recurrence-free survival, with bubble size proportional to the size of the study cohort (Figure 1). We also compared median recurrence-free survival between NA and non-NA groups using the Mann-Whitney U test. The NA group (1468 patients) showed a median recurrence-free survival of 85.0% (range 19.7%-90.0%) at 1 year, 57.0% (range 11.4%-90.0%) at 3 years, and 54.0% (range 42.6%-81.3%) at 5 years. These median survival rates were significantly higher than the corresponding values in the non-NA group (5541 patients): 78.0% (range 4.5%-86.6%) at 1 year, 56.0% (range 0%-56.0%) at 3 years, and 47.0% (range 0%-47.0%) at 5 years (all $P < 0.001$).

Next we examined whether, based on the available evidence, NA therapy prevents early recurrence, late recurrence, or both. Studies have shown that tumor factors are associated with early HCC recurrence, while high viral loads and hepatic inflammatory activity are associated with late HCC recurrence[3,4]. NAs can suppress HBV-DNA replication and promote ALT normalization but cannot affect tumor factors directly, so in theory NAs may prevent late HCC recurrence but have minimal effect on early HCC recurrence. Several retrospective studies and a RCT[27,33,35,39] support this idea. However, the other RCT[38] in our review found that NA therapy significantly decreased early HCC recurrence, while it did not report outcomes on late HCC recurrence. NA therapy may inhibit early HCC recurrence, which usually arises due to diffusion of the primary tumor, by reducing high HBV load and HBV mutations, all of which are associated with HCC metastasis and growth[40-42], as well as by inhibiting HBxAg, which promotes HCC invasiveness and metastatic potential[43,44]. Further studies are urgently needed to clarify whether and how NA therapy affects risk of HCC recurrence, since the results of RCT[38] in our review may overestimate the NA efficacy because the control group at baseline had significantly higher rates of cirrhosis, lower rates of tumor encapsulation, and higher rates of HBeAg positivity than the NA group, as well as poorer tumor differentiation and higher AFP levels.

POSTOPERATIVE NA THERAPY AND OVERALL SURVIVAL

A total of 15 studies reported data on overall survival after radical surgery. Twelve of them, including the RCTs[22,27,29-34,36-39], concluded that NA treatment leads to significantly higher overall survival than non-NA treatment, but 3 studies[21,23,26] concluded that NA therapy does not lead to significantly higher overall survival.

The 1-, 3-, and 5-year overall survival rates were summarized using bubble plots (Figure 2), and median rates were compared between NA and non-NA groups using

Table 1 Characteristics of patients with hepatitis B virus-related hepatocellular carcinoma treated with nucleos(t)ide analogues or not after radical resection

Ref.	Country or region	No. of patient[1]	Mean age (yr)[1]	TNM stage (I/II/III/IV) (n)	Multiple tumor (%)[1]	Mean tumor size (cm)[1]	Portal vein invasion (%)[1]	Mean HBV-DNA level (log10 copies/mL)[1]	Mean ALT (U/L)[1]	Cirrhosis (%)[1]	Initial treatment for HCC, (Ope/RFA/PEI/TACE)	NA therapy	Mean antiviral treatment duration (mo)	Mean follow-up duration (mo)[1]
Piao et al[21]	Japan	30 vs 40	59 vs 58	31/25/11/3	N/A	2.3 vs 2.5[2]	N/A	6.1 vs 6.5[2]	88 vs 62	N/A	22/16/0/32	LAM	N/A	24
Shuqun et al[22]	Chinese mainland	16 vs 17	48.3 vs 48.5	N/A	N/A	≥ 5 cm: 56.2% vs 70.6%	37.5 vs 23.5	N/A	N/A	100 vs 94.1	33/0/0/0	LAM	12	12-36
Kuzuya et al[23]	Japan	16 vs 33	59.8 vs 61.1	25/19/5/0	N/A	N/A	N/A	6.2 vs 4.1[2]	56.6 vs 54.2	N/A	31/18/0/0	LAM	22.7	38.0 vs 32.6
Kubo et al[24]	Japan	14 vs 10	55 vs 55	5/9/10/0	N/A	2.4 vs 2.8	28.6 vs 40.0	6.0 vs 6.0	53 vs 56[2]	42.9 vs 40.0	24/0/0/0	LAM	32	36.7 vs 7.3[2]
Hung et al[25]	Hong Kong	10 vs 62	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	72/0/0/0	LAM	N/A	18.9[2]
Yoshida et al[26]	Japan	33 vs 71	57 vs 59	I + II: 57.6% vs 73.2%	N/A	2.6 vs 2.8	N/A	≥ 3.7: 100% vs 63%	54 vs 36[2]	N/A	0/104/0/0	LAM	N/A	33 vs 47
Koda et al[27]	Japan	30 vs 20	59 vs 60	19/20/11/0	N/A	N/A	N/A	5.7 vs 5.2	78 vs 54	N/A	12/24/5/9	28LAM + 2ETV	28.6	28.6 vs 36.3
Chuma et al[28]	Japan	20 vs 30	55.7 vs 55.6	19/27/4/0	25.0 vs 23.3	1.7 vs 2.1	N/A	6.0 vs 5.9[2]	43.1 vs 37.7	55.0 vs 53.3	10/10/0/0	15LMA + 5ETV	N/A	35.5 vs 49.2[2]
Li et al[29]	Chinese mainland	43 vs 36	46 vs 45	13/27/39/0	N/A	7.1 vs 8.5	30.2 vs 27.8	6.5 vs 7.3	60.8 vs 56.5	55.8 vs 69.4	79/0/0/0	LAM	N/A	12 vs 12
Chan et al[30]	Hong Kong	42 vs 94	57 vs 55[2]	39/32/64/0	N/A	9.3 vs 9.0[2]	11.9 vs 18.1	N/A	58.0 vs 42.5[2]	73.8 vs 56.4	136/0/0/0	38LAM + 4ETV	N/A	N/A
Wu et al[31]	Tai Wan	518 vs 4051	54.4 vs 54.6	N/A	N/A	N/A	N/A	N/A	N/A	48.6 vs 38.7	4569/0/0/0	159LAM + 292ETV + 36LdT + 31Combined	17.4	31.7 vs 26.2
Urata et al[32]	Japan	46 vs 13	57 vs 58	N/A	28.3 vs 61.5	2.8 vs 3.4	34.8 vs 46.2	4.7 vs 6.1	46.8 vs 58.0	45.7 vs 30.8	59/0/0/0	22LAM + 24ETV	N/A	36.2[2]
Ke et al[33]	Chinese mainland	141 vs 141	48.9 vs 49.7	N/A	27.7 vs 24.1	4.5 vs 5.0[2]	7.8 vs 7.1	4.9 vs 4.7	39 vs 42	81.6 vs 81.6	282/0/0/0	LAM	12	24 vs 23
Yin et al[38]	**Chinese mainland**	**81 vs 82**	**47.9 vs 49.3**	**N/A**	**12.3 vs 22.0**	**≥ 3 cm: 86.4% vs 93.9%**	**3.7 vs 7.3**	**4.9 vs 4.6**	**47.3 vs 37.5**	**24.7 vs 28.0**	**163/0/0/0**	**LAM**	**N/A**	**39.9[2]**
		215 vs 402	50.1 vs 50.2	N/A	14.4 vs 12.7	≥ 3 cm: 89.3% vs 92.3%	14.0 vs 15.4	4.5 vs 3.8	> 42: 48.8% vs 36.8%	47.0 vs 35.8	617/0/0/0	LAM	N/A	23.8[2]
Su et al[34]	Tai Wan	62 vs 271	52 vs 58[2]	N/A	22.6 vs 46.9	2.7 vs 4.2[2]	11.3 vs 20.0	5.9 vs 5.5[2]	45 vs 42[2]	33.7 vs 45.8	333/0/0/0	40LAM + 19ETV + 3PEG-IFN	N/A	45.9[2]
Yan et al[35]	Chinese mainland	35 vs 25	45 vs 47	22/29/9/0	N/A	4.7 vs 5.0	65.7 vs 68.0	> 5: 54.3% vs 72.0%	41.5 vs 35.8	N/A	60/0/0/0	LAM	N/A	N/A
Hann et al[37]	The United States	16 vs 9	57 vs 53[2]	N/A	0 vs 0	2.7 vs 3.0[2]	0 vs 0	5.4 vs 6.9[2]	N/A	N/A	3/4/2/8/others[3]	8(LAM + TDF) + 3(LAM + ADV) + 2(TLV + TDF) + 2TDF + 1LAM	N/A	60.2
Huang et al[39]	Chinese mainland	100 vs 100	50.6 vs 50.5	N/A	17 vs 16	4.9 vs 5.1	0 vs 0	> 3.3: 100% vs 100%	52.6 vs 51.4	N/A	200/0/0/0	ADV	N/A	60[2]

[1]Patients who received postoperative NA treatment vs patients who received no postoperative NA treatment; [2]Median values; [3]Two patients received RFA, PEI and TACE; Three patients received RFA and TACE; One patient received cryoablation. Boldfaced data come from randomized controlled trials in our review[38,39]. ADV: Adefovir dipivoxil; ETV: Entecavir; LAM: Lamivudine; LdT: Telbivudine; N/A: Not available; Ope: Operation; PEI: Percutaneous ethanol injection; RFA: Radiofrequency ablation; TACE: Transarterial chemoembolization; NA: Nucleos(t)ide analogue.

Table 2 Survival outcomes of patients with hepatitis B virus-related hepatocellular carcinoma treated with nucleos(t)ide analogues or not after radical resection

Year of publication	Ref.	Group	n	Overall survival rate (%)				Recurrence-free survival rate (%)			
				1 yr	3 yr	5 yr	P	1 yr	3 yr	5 yr	P
2005	Piao *et al*[21]	NAs	30	100	91.3	N/A	0.12	75	46	N/A	> 0.05
		Control	40	92.4	66	N/A		58	22	N/A	
2006	Shuqun *et al*[22]	NAs	16	24	N/A	N/A	0.0053	19.7	N/A	N/A	> 0.05
		Control	17	0	N/A	N/A		4.5	N/A	N/A	
2007	Kuzuya *et al*[23]	NAs	16	100	100	N/A	0.063	86.5	64.9	N/A	0.622
		Control	33	86.6	46.8	N/A		86.6	46.8	N/A	
2007	Kubo *et al*[24]	NAs	14	N/A	N/A	N/A	N/A	90	90	78	0.0086
		Control	10	N/A	N/A	N/A		55	28	28	
2008	Hung *et al*[25]	NAs	10	N/A	N/A	N/A	N/A	90	N/A	N/A	0.03
		Control	62	N/A	N/A	N/A		75	N/A	N/A	
2008	Yoshida *et al*[26]	NAs	33	100	80	59	> 0.05	N/A	N/A	N/A	> 0.05
		Control	71	100	85	70		N/A	N/A	N/A	
2009	Koda *et al*[27]	NAs	30	96	76	76	0.02	65	15	N/A	> 0.05
		Control	20	86	48	32		72	30	N/A	
2009	Chuma *et al*[28]	NAs	20	N/A	N/A	N/A	N/A	90	55	45	> 0.05
		Control	64	N/A	N/A	N/A		85.9	50	43.7	
2010	Li *et al*[29]	NAs	43	41.9	N/A	N/A	0.0094	23.3	N/A	N/A	0.072
		Control	36	33.3	N/A	N/A		8.3	N/A	N/A	
2011	Chan *et al*[30]	NAs	42	88.1	79.1	71.2	0.005	66.5	51.4	51.4	0.05
		Control	94	76.5	47.5	43.5		48.9	33.8	33.8	
2012	Wu *et al*[31]	NAs	518	94	81	73	0.002	87	66	54	< 0.001
		Control	4051	91	74	62		78	56	47	
2012	Urata *et al*[32]	NAs	46	100	97.1	89.7	0.0025	71.6	56.8	42.6	0.0478
		Control	13	84.6	68.4	59.8		61.5	19.2	19.2	
2013	Ke *et al*[33]	NAs	141	92.1	84.4	79.1	0.009	73.1	54.7	44.5	0.503
		Control	141	89.6	66.3	52.1		68.8	47.8	43	
2013	**Yin *et al*[38]**	**NAs**	**81**	**98**	**88**	**N/A**	**< 0.001**	**81**	**46**	**N/A**	**< 0.001**
		Control	**82**	**86**	**51**	**N/A**		**50**	**20**	**N/A**	
		NAs	215	84	60	N/A	0.04	52	37.5	N/A	< 0.001
		Control	402	75	50	N/A		43	21	N/A	
2013	Su *et al*[34]	NAs	62	99	96	89	< 0.001	90	64	58	< 0.001
		Control	271	84	64	49		64	44	34	
2013	Yan *et al*[35]	NAs	35	N/A	N/A	N/A	N/A	74.3	11.4	N/A	0.283
		Control	25	N/A	N/A	N/A		80	0	N/A	
2014	Hann *et al*[37]	NAs	16	100	93.8	86.5	< 0.001	81.3	81.3	81.3	< 0.001
		Control	9	55.6	0	0		11.1	0	0	
2014	**Huang *et al*[39]**	**NAs**	**100**	**96**	**77.6**	**63.1**	**0.001**	**85**	**50.3**	**46.1**	**0.026**
		Control	**100**	**94**	**67.4**	**41.5**		**84**	**37.9**	**27.1**	

Boldfaced data come from randomized controlled trials in our review[38,39]. N/A: Not applicable; NA: Nucleos(t)ide analogue.

Table 3 Mutations of the hepatitis B virus polymerase gene arising after initial therapy with one nucleos(t)ide analogue and resulting in cross-resistance to other nucleos(t)ide analogues

Initial NA therapy	Mutational sites after initial NA therapy	Cross-resistance data				
		LAM	LdT	ETV	ADV	TDF
	Wild-type	S	S	S	S	S
LAM or LdT	M204I/V	R	R	I	S	S
ADV	N236T	S	S	S	R	I
LAM or LdT or ADV	A181T/V	R	R	S	R	I
ADV or TDF	A181T/V + N236T[1]	R	R	S	R	R
ETV	L181M + M204V/I ± I169 ± T184 ± S202 ± M250V[2]	R	R	R	S	S

[1]Resistance to ADV or TDF is associated with the substitution A181T/V and/or N235T in HBV polymerase gene; [2]Resistance to ETV is associated with substitutions at I169, T184, S202 or M250V, and with the simultaneous substitutions at L181M plus M204V/I in HBV polymerase gene. Data come from ref. [11]. ADV: Adefovir dipivoxil; ETV: Entecavir; I: Intermediate; LAM: Lamivudine; LdT: Telbivudine; NA: Nucleos(t)ide analogue; R: Resistant; S: Sensitive; TDF: Tenofovir disoproxil fumarate.

the Mann-Whitney *U* test. Median survival in the NA group (1468 patients) was 94.0% (range 24.0%-100.0%) at 1 year, 81.0% (range 60.0%-100.0%) at 3 years, and 73.0% (range 59.0%-89.7%) at 5 years. These values were significantly higher than the corresponding ones for the non-NA group (5200 patients): 91.0% (range 0-100.0%) at 1 year, 74.0% (range 0-85.0%) at 3 years, and 62.0% (range 0%-70.0%) at 5 years (all *P* < 0.001).

Figure 2 Bubble plot of overall survival in patients receiving nucleos(t)ide analogue therapy or not after radical resection to treat hepatitis B virus-related hepatocellular carcinoma. Bubble size reflects relative cohort size. [a]*P* < 0.05: NA group *vs* Control group. NA: Nucleos(t)ide analogue.

Investigators have attributed this survival benefit to 3 factors. First, NA therapy can efficiently suppress HBV replication and reactivation, ease liver inflammation and fibrosis, impede progression of liver disease, and prevent liver failure[21-23,27,29,33,38,45]. Second, liver function improvement after NA therapy increases the possibility of curative re-treatment and allows surgeons to remove a larger liver region after recurrence, which means lower risk of residual tumors[23,29,33,45]. Third, NA therapy can reduce recurrence, helping to increase overall survival[24,25,30-32,34,36-38].

To define more precisely which patients with HBV-related HCC may benefit from NA therapy, we retrospectively studied its efficacy in patients with HCC in different stages of the Barcelona Clinic Liver Cancer (BCLC) system[33]. We found that NA therapy provided significant survival benefit to patients with BCLC stage A or B disease, but not to patients with BCLC-C disease. These results are similar to those reported in 2 larger retrospective studies[30,34]. This may reflect the poor prognosis of BCLC-C patients, whose short survival provides insufficient time for NA therapy to be effective.

MANAGEMENT OF NA RESISTANCE IN HBV-RELARED HCC PATIENTS

One of the major problems associated with long-term NA therapy is the emergence of NA-resistant HBV strains[21,23,27]. Such resistance increases not only the risk of breakthrough hepatitis and liver failure, but also the difficulty and cost of subsequent treatment. LAM has the worst antiviral resistance profile among NAs, and LAM resistance is caused by mutations of the YMDD region in the active site of the HBV polymerase/reverse transcriptase gene[11]. One study[27] reported YMDD mutations in 11 of 28 patients after 28.6 ± 16.7 mo of LAM administration. Of those 11 patients, 6 exhibited breakthrough hepatitis; fortunately none of them experienced fatal liver failure because they were immediately given ADV or ETV.

To prevent NA resistance and manage its clinical ef-

fects in patients with HBV-related HCC, clinicians should obtain a thorough medical history for NA candidates. Patients who previously received NA therapy and developed resistance should receive potent NA not associated with cross-resistance (Table 3) in order to reduce the risk of eliciting multiple drug-resistant viral strains[12]. For patients who have never received any NA therapy, potent drugs with high resistance barriers, such as ETV and TDV, may be the best choice[12]. Clinicians should also not rush to incorrect conclusions about NA resistance, since about 40% of cases of HBV-related breakthrough hepatitis occur simply because of poor patient adherence to NA therapy rather than NA resistance[46]. On the other hand, drug resistance should be considered if regular follow-up tests of HBV-DNA levels and liver function every 2-3 mo give abnormal results and other possible causes can be excluded. In such cases, an appropriate rescue therapy using potent NAs without cross-resistance should be given as soon as genotypic drug resistance is confirmed[11].

CONCLUSION

Given the serious clinical consequences of uncontrolled HBV replication, patients with HBV-related HCC should consider taking NA if they are positive for HBV-DNA. Because NA therapy cannot completely eradicate HBV, patients should prepare for the possibility that they may require lifelong treatment. With the currently advanced techniques of the loco-regional ablations such as radio-frequency ablation, microwave ablation and others, NA therapy also applies for HCC patients who underwent such procedures in addition to surgical resection, and a significant body of evidence suggests that postoperative NA therapy in patients with HBV-related HCC improves both recurrence-free survival and overall survival.

Every coin has two sides. Emergence of NA-resistant HBV strains is a significant concern, highlighting the importance of regular monitoring of HBV-DNA levels and liver function during NA therapy. The most potent NAs with high resistance barriers, such as EVT and TDF, may be the best choice for NA-naïve patients. In case of drug resistance, rescue therapy should be carried out using potent NAs not associated with cross-resistance.

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ORIGINAL ARTICLE

Telaprevir- and boceprevir-based tritherapies in real practice for F3-F4 pretreated hepatitis C virus patients

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Abstract

AIM: To assess, in a routine practice setting, the sustained virologic response (SVR) to telaprevir (TPV) or boceprevir (BOC) in hepatitis C virus (HCV) null-responders or relapsers with severe liver fibrosis.

METHODS: One hundred twenty-five patients were treated prospectively for 48 wk with TPV or BOC + pegylated-interferon (peg-INF) α2a + ribavirin (PR) according to standard treatment schedules without randomization. These patients were treated in routine practice settings in 10 public or private health care centers, and the data were prospectively collected. Only patients with severe liver fibrosis (Metavir scores of F3 or F4 upon liver biopsy or liver stiffness assessed by elastography), genotype 1 HCV and who were null-responders or relapsers to prior PR combination therapy were included in this study.

RESULTS: The Metavir fibrosis scores were F3 in 35 (28%) and F4 in 90 (72%) of the patients. In total, 62.9% of the patients were null-responders and 37.1% relapsers to the previous PR therapy. The overall SVR rate at 24 wk post-treatment withdrawal was 59.8%. The SVR was 65.9% in the TPV group and 44.1% in the BOC group. Independent predictive factors of an SVR included a response to previous treatment, relapsers *vs* null-responders [OR = 3.9; (1.4, 10.6), *P* = 0.0084], a rapid virological response (RVR) [OR 6.9 (2.6, 18.2), *P* = 0.001] and liver stiffness lower than 21.3 kPa [OR = 8.2 (2.3, 29.5), *P* = 0.001]. During treatment, 63 patients (50.8%) had at least one severe adverse event

(SAE) of grade 3 or 4. A multivariate analysis identified two factors associated with SAEs: female gender [OR = 2.4 (1.1, 5.6), P = 0.037] and a platelet count below $150 \times 10^3/ mm^3$ [OR = 5.3 (2.3, 12.4), $P \leqslant 0.001$].

CONCLUSION: More than half of these difficult-to-treat patients achieved an SVR and had SAEs in an actual practice setting. The SVR rate was influenced by the response to previous PR treatment, the RVR and liver stiffness.

Core tip: To the best of our knowledge, this study marks the first time that a significant link has been shown between a sustained virological response to triple therapy and the liver stiffness measured by elastography at baseline. We also demonstrate that triple therapy is poorly tolerated. Two factors predict the development of serious adverse events: female gender and an initial platelet count of less than 150000/mm³; these factors facilitate the identification of at-risk patients.

INTRODUCTION

Approximately 80% of patients infected with the hepatitis C virus (HCV) develop chronic infections that could lead to cirrhosis and hepatocellular carcinoma[1]. Combination therapy with pegylated-interferon (peg-INF) α2a + ribavirin (PR) was the first demonstrably effective treatment[2,3]. The current combination of PR with protease inhibitors (PIs) such as telaprevir (TPV) or boceprevir (BOC) is clearly more beneficial for HCV genotype 1 patients[4-7]. The majority of HCV genotype 1 patients demonstrate a sustained virological response (SVR) to TPV (69% to 75%) and BOC triple therapy (68% to 75%)[4-7]. However, only 65% of genotype 1 HCV patients who were previously unresponsive to PR therapy produced an SVR to TPV triple therapy; only 66% of these unresponsive patients produce an SVR to BOC triple therapy[8-10]. Some predictive factors, such as high baseline viral load, HCV genotype 1a, IL-28B T/T polymorphism and severe liver fibrosis, have been associated with a poor response to antiviral treatment with PI[6,10]. Moreover, the data on the benefit of retreating

HCV genotype 1 cirrhotic patients who did not respond to a standard PR regimen with triple therapy are inconclusive[8-10]. A study on small subgroups of null-responder patients with severe liver fibrosis given TPV triple therapy determined that 39% of Metavir F3 patients and only 14% of patients with Metavir F4 produced an SVR[8]. Therefore, guidelines and new treatment strategies are required that consider the cost and adverse effects of TPV and BOC combined with PR for these difficult-to-treat patients. In these pivotal studies performed exclusively in academic centers, many patients experienced adverse effects despite restricting inclusion criteria and strict observance of treatment rules[5-10]. In addition, because very few non-responder patients with severe liver fibrosis were included in these studies, the occurrence of severe adverse effects (SAEs) in this specific population remains unclear. Therefore, we need to evaluate (in actual practice settings) the efficacy and safety profile of PI triple therapy in pretreated HCV genotype 1 patients with severe liver fibrosis.

This study assesses the SVR and safety profiles of triple therapy with TPV or BOC combined with PR in HCV genotype 1 patients with severe liver fibrosis (Metavir F3 or F4) who had previously failed to adequately respond to the standard PR treatment. This observational non-randomized prospective cohort study was performed in actual practice settings.

MATERIALS AND METHODS

Study design and patients

This study used a non-randomized multicenter prospective observational cohort from a Midi-Pyrénées network (HEPATOMIP) of hepatogastroenterology practitioners working in the Toulouse University hospital and in 9 general hospitals or private clinics. The cohort included 125 consecutive HCV genotype 1 null-responder or relapser patients with severe liver fibrosis who were seen between February 2011 and January 2012. Only those patients with severe liver fibrosis having a Metavir fibrosis score of F3 or F4 were included.

All of the patients were infected with HCV genotype 1 and did not achieve an SVR with previous standard treatments with peg-IFN α2a or 2b + ribavirin, described as follows[11]: (1) relapsers were defined as patients who achieved undetectable HCV RNA levels at the end of 48 wk of PR treatment and then subsequently relapsed; and (2) null-responders failed to achieve a decrease of at least 2 log HCV RNA IU/mL during PR treatment given for at least 24 wk.

Partial responders to previous therapy were not included in the study. After an interval of at least 6 mo, patients were given either 12 wk of TPV (750 mg every 8 h, Janssen-Cilag, Issy les Moulineaux, France) combined with PR (Roche, Meylan, France) followed by 36 wk of PR or 4 wk (lead-in phase) of PR followed by 44 wk of PR and BOC (800 mg every 8 h (MSD, Courbevoie, France) according to French label guidelines[12] (French National Agency of drugs and health products security,

ANSM cohort temporary use authorization n° 324 and n° 330). In this observational cohort, TPV or BOC triple therapy was selected by each physician; however, all of the patients received the same schedule of PR, as follows: peg-IFN α2a (180 g/wk) + ribavirin (1000 to 1200 mg/d, depending on body weight). The Toulouse University review board approved this cohort, and all of the patients provided written informed consent.

A quantification of the HCV RNA level was performed at baseline, then every 4 wk during triple therapy and at 12 and 24 wk following treatment withdrawal using real-time polymerase chain reaction (COBAS Amplicor/TaqMan, Roche Diagnostics, Basel, Switzerland) with a lower detection limit of 15 IU/mL. Fibrosis was evaluated by a liver biopsy or by measuring the liver stiffness (LS) according to the manufacturer's instructions (Fibroscan, Echosens). The results were expressed in kilopascals (kPa). Metavir F3 was defined by a liver stiffness of 9.5-12.4 kPa and Metavir F4 cirrhotic patients were defined by values of up to 12.5 kPa.

Efficacy

The response to triple therapy could be summarized as: (1) A rapid virological response (RVR), *i.e.*, negative for HCV RNA after 4 weeks of triple therapy (defined as week 4 for the TPV group and week 8 for the BOC group); (2) A virological response (VR), *i.e.*, negative for HCV RNA at the end of triple therapy; and (3) or a sustained virological response (SVR), *i.e.*, negative for HCV RNA 24 wk after the end of treatment.

Safety and adverse events

All of the patients were seen by their physicians at baseline, every 2 wk during the first 2 mo, every 4 wk during the following phase of therapy and then every 4, 12, and 24 wk after treatment withdrawal. Adverse events were graded by investigators according to a modified World Health Organization grading system. Non-life-threatening adverse events and hematological disorders were managed according to the French association of the study of the liver (AFEF) by reducing the ribavirin dose and/or giving erythropoietin (EPO) at the discretion of the physician[11]. EPO was recommended when the patient's hemoglobin (Hb) level dropped to less than 10 g/dL, despite a previous reduction in the ribavirin dose by 200 mg/d.

Statistical analysis

Statistical analyses were performed using STATA software, release 11.2 (STATA Corporation, College Station, TX, United States). Numbers and frequencies were used for the described qualitative data, and means ± SD or medians (inter-quartile range: IQR) were used when the normality assumption was not met for quantitative data. The qualitative variables were compared between groups (TPV and BOC groups; SVR and no-SVR groups; SAEs and no-SAEs groups) using the χ^2 test (or Fisher's exact test for small expected numbers). Student's t test was used to compare the distribution of quantitative data.

Alternatively, the Mann-Whitney test was used when the distribution was not normal or when homoscedasticity was rejected. We assessed the accuracy of liver stiffness to predict an SVR according to receiver operating characteristic (ROC) curves (plotting sensitivity *vs* 1-specificity at various cut-off settings), and we defined the optimal liver stiffness cut-off value of 21.3 kPa according to the best rate of correctly classified subjects {[(true positives + true negatives)/total]; 69.2%}. Odds ratios (ORs) for SVR or SAE and 95%CIs were assessed using a logistic regression model. The variables initially included in the model were those associated with SVR or SAE in the univariate analysis (*P* value < 0.20). A backward procedure was applied to assess variables that were significantly and independently associated with SVR (or SAE) (*P* value < 0.05). Because the linearity hypothesis was not fully respected, the following continuous variables were transformed into ordered data: liver stiffness (< 21.3 kPa *vs* ≥ 21.3 kPa) for the SVR model, platelet count (< 150×10^3/mm³ *vs* ≥ 150×10^3/mm³) for the SAE model. Interactions between independent covariates were tested in the final regression models, and none of these interactions was significant. All of the reported *P* values are two-sided, and the significance threshold was set at < 0.05.

RESULTS

Characteristics of patients at baseline

This prospective cohort included 125 HCV genotype 1 patients (Table 1). None of the patients had responded to previous treatment with standard PR combination therapy. There were 46/124 (37.1%) previous relapsers and 78/124 (62.49%) null-responders, and there were more men (64.8%) than women (35.2%). HCV subtype 1b (56.8%) infections were more frequent than HCV subtype 1a (31.2%) infections, although the HCV genotype was not defined as 1b or 1a in 12% of cases. As expected in this population of relapsers and null-responders to prior antiviral therapy, only 15.4% of the patients had an IL-28B genotype C/C. All of the patients had severe liver fibrosis: 28% were Metavir F3 and 72% were cirrhotic, with a Metavir F4 score. All except 2 of the cirrhotic patients were classified as Child-Pugh class A. Triple therapy was not randomized; TPV or BOC was selected by the patient's physician, with 72% of the patients treated with TPV and 28% treated with BOC. We observed no difference in the subsequent parameters for the two groups.

Virologic response to triple therapy

The overall SVR rate (Table 2) was 59.8% (73/122 patients). From the overall population, 92 patients (75.4%) had undetectable HCV RNA levels at the end of triple therapy, and 19 patients (20.6%) relapsed during the post-treatment follow-up. Three of the 57 patients (5.2%) with negative HCV RNA levels at 12 wk after the end of triple therapy suffered a late relapse after the twelfth week. These three patients were null-responders to previous PR treatment, and one of these patients had a RVR during

Table 1 Demographic and baseline characteristics *n* (%)	
Triple therapy: Peg-IFNα2a + Ribavirin + Protease inhibitor,	125
Telaprevir	90 (72)
Boceprevir	35 (28)
Ribavirin dosage mg/kg, mean (SD)	14.4 (2.1)
Age, yr, mean (SD)	56.2 (9.7)
Gender	
Male	81 (64.8)
Female	44 (35.2)
HCV genotype	
1a	39 (31.2)
1b	71 (56.8)
Undetermined subtype 1	15 (12)
IL28B genotype (rs12979860)	
C/T or T/T	88 (84.6)
Viral load, mean (log10 IU/ mL)	6.3 (0.7)
Prior response to anti-viral therapy	
Relapsers	46 (37.1)
Null-responders	78 (62.9)
Liver fibrosis grade	
Metavir F3	35 (28)
Metavir F4	90 (72)
Child-Pugh score	
A	123 (98.4)
B	1 (0.8)
C	1 (0.8)
Liver stiffness values (kPa)	
Mean (SD)	17.5 (10.3)
Median (IQR)	14.3 (10.4-20.6)
Oesophageal varices	
None	92 (75.4)
Grade 1	16 (13.1)
Grade 2 or 3	14 (11.5)
Mean hemoglobin level (g/dL, mean (SD)	15.1 (1.6)
Mean platelet count × 10^3/mm³, mean (SD)	165.69 (64.9)
Mean neutrophil count × 10^3/mm³, mean (SD)	3.4 (1.2)

Relapsers were defined as patients who had undetectable levels of HCV RNA at the end of prior treatment and subsequently relapsed. Null-responders failed to achieve a decline of at least 2 log HCV RNA IU/mL during peg-INF α2a + ribavirin treatment after a minimum duration of 24 wk. Peg-INF: Pegylated-interferon; HCV: Hepatitis C virus; IQR: Inter-quartile range.

triple therapy. The remaining 73 patients maintained their SVR until the end of the follow-up period, 24 wk after triple therapy withdrawal. The SVR rate was higher (Table 2) in the TPV group (65.9%) than in the BOC group [44.1%; $P = 0.0276$, OR = 2.49 (1.1, 5.5), univariate analysis]. The SVR rate was not significantly influenced by the HCV subtype (1a or 1b), IL-28B genotype or viral load at baseline. Non-cirrhotic patients tended to have a better SVR than the patients with cirrhosis (Table 2); however, this difference did not reach statistical significance (68.6% for Metavir F3 and 56.3% for Metavir F4, $P = 0.212$). Only 23 of the 52 patients (44%) in the subgroup of very difficult-to-treat patients (those with cirrhosis and a null-response to prior therapy) achieved an SVR. Among these SVR patients, 20/23 (87%) were given TPV triple therapy and 3/23 (13%) were given BOC triple therapy. Neither decreasing the ribavirin dosage nor anemia was associated with a loss of SVR (Table 2).

Patients who exhibited a RVR (Figure 1A), defined as a negative viral load 4 wk after the initiation of PI thera-

Table 2 Factors associated with sustained virological response *n* (%)				
	No sustained virological response *n* = 49	Sustained virological response *n* = 73	*P* value univariate analysis	*P* value multivariate analysis OR (95%CI)
Protease inhibitor				
Telaprevir	30 (34.1)	58 (65.9)	0.0276	NS[1]
Boceprevir	19 (55.9)	15 (44.1)		
Gender				
Male	27 (34.2)	52 (65.8)	0.0675	NS[1]
Female	22 (51.2)	21 (48.8)		
HCV genotype 1 subtype				
1a	17 (45.9)	20 (54.1)	0.8051	-
1b	26 (37.1)	44 (62.9)		
IL28B genotype, rs12979860, *n* (%)				
C/C	4 (25)	12 (75)	0.4420	-
C/T or T/T	40 (45.5)	48 (54.5)		
Response to prior therapy				0.008
Null-responders	40 (52.6)	36 (47.4)	0.0004	1.0
Relapsers	9 (20)	36 (80)		3.9 (1.4-10.6)
Grade of liver fibrosis				
Metavir F3	11 (31.4)	24 (68.6)	0.2118	-
Metavir F4	38 (43.7)	49 (56.3)		
Liver stiffness value (kPa)				
Median, kPa (IQR)	17.3 (11.5-28.8)	13.9 (9.4-19.7)	0.0296	0.001
< 21.3 kPa	21 (30)	49 (70)	0.002	8.2 (2.3-29.5)
⩾ 21.3 kPa	14 (66.7)	7 (33.3)		1
Rapid virological response	18 (23.7)	58 (76.3)	⩽ 0.0001	⩽ 0.001
No rapid virological response	26 (68.4)	12 (31.6)		6.9 (2.6-18.2) 1.0
Decrease of ribavirin dosage	18 (37.5)	30 (62.5)	0.6287	-
No decrease of ribavirin dosage	31 (41.9)	43 (58.1)		

Sustained virological response (SVR) was analyzed 24 wk after triple therapy withdrawal for 122 patients. Rapid virological response (RVR) under triple therapy was defined as undetectable HCV RNA levels following 4 wk of antiviral triple therapy (*i.e.*, week 4 in the TPV group and week 8 in the BOC treatment group)1. These factors were initially included in the multivariate model (*P* value < 0.2 in the univariate analysis) and were not independently associated with SVR in the final multivariate model.

py, had a better SVR than the patients who did not have this rapid drop in the HCV RNA load (76.3% *vs* 36.4%, respectively, $P < 0.0001$). In the overall population (Figure 1B), the relapsers to prior PR therapy had a better SVR than the null-responders (80% *vs* 47.4%, $P = 0.0004$). This higher rate of SVR observed in the prior PR relapsers compared with the null-responders remained significant in the TPV or BOC subgroups (80.6% *vs* 54.9%, 77.8% *vs* 32%, respectively, $P < 0.05$).

Overall, in the triple therapy population (Table 2), the liver stiffness (LS) values were significantly lower ($P = 0.0296$) in the patients with an SVR [median: 13.9 kPa, IQR (9.4-19.7)] than in the patients who failed to have an SVR [median: 17.3 kPa, IQR (11.5-28.8)]. The corresponding area under the ROC curve (Figure 2) predicting SVR was 0.64 (0.52-0.76). The optimal LS cut-off value associated with an SVR was 21.3 kPa, which had a predic-

Figure 1 Sustained virological response. A: Influence of rapid virological response on sustained virological response. Patients with rapid virological response (RVR) *vs* patients without RVR, [b]*P* < 0.001; B: Influence of response to previous treatment on sustained virological response. Prior relapsers *vs* prior null-responders, [a]*P* < 0.05. Sustained virological response was analyzed 24 wk after treatment withdrawal for 122 patients (overall numbers of patients: 88 in the telaprevir group, 34 in the boceprevir group).

Figure 2 Diagnostic value of liver stiffness measurement to predict sustained virological response. The area under the receiver operating characteristic curve was 0.64 (0.52-0.76).

tive positive value of 66.7% (43, 85) and a negative predictive value of 70% (57, 80). An SVR occurred in 70% of the patients with a LS below 21.3 kPa and in 33.3% of the patients with a LS of up to 21.3 kPa (*P* = 0.002).

The logistic regression analysis (Table 2) showed that only three factors were independently associated with SVR. These factors were a relapse after PR treatment, the LS value, and a RVR to triple therapy. The SVR rate was greater in the prior relapsers than in the prior null-responders to PR therapy [OR = 3.9 (1.4, 10.6), *P* = 0.004]. A LS of less than 21.3 kPa was associated with an improved response to triple therapy [OR = 8.2 (2.3, 29.5), *P* = 0.001]. An SVR was associated with a RVR under triple therapy [OR = 6.9 (2.48, 18.2), *P* = 0.001], defined as HCV RNA-negative after 4 wk of antiviral treatment (week 4 in the TPV group and week 8 in the BOC group). The multivariate analysis revealed no difference in the SVRs of the TPV and BOC groups.

Safety

Adverse events ≥ grade 1 occurred in 102/124 patients (82.2%) and were significantly more frequent in the pa-

tients receiving TPV (*n* = 79/89, 88.8%) compared with the patients receiving BOC (*n* = 23/35, 65.7%) [OR = 4.12 (1.4; 12), *P* = 0.0059]. Approximately half of the patients (63: 50.8%) suffered a SAE ≥ grade 3 during treatment (Table 3). These grade 3 or 4 SAEs were as follows: thrombocytopenia (*n* = 42, 66%), neutropenia (*n* = 21, 33%), anemia (*n* = 18, 28.5%), severe infection (*n* = 4, 6.3%), fatigue (*n* = 3, 4.7%), skin rash (*n* = 2, 3.2%), and hepatic failure (*n* = 2, 3.2 %). The total percentage exceeds 100% because some subjects had several grade 3 or 4 SAEs. None of the patients died during treatment. Neither the fibrosis stage (F3 or F4) nor the protease inhibitor used (TPV or BOC) influenced the occurrence of SAEs. EPO use and blood transfusions were analyzed among the 125 patients. A total of 17 patients (13.6%) were given blood transfusions, and 65 patients (52%) received EPO. The frequencies of EPO use and blood transfusions in the TPV and BOC groups were not significantly different. Treatment was discontinued because of SAEs in 11 patients (8.9%).

The univariate analysis (Table 4) showed four factors associated with an SAE during triple therapy. Women had

Table 3 Safety profile of triple therapy: Severe adverse events grade 3 or 4 *n* (%)

	Overall patients (*n* = 124)	Telaprevir (*n* = 89)	Boceprevir (*n* = 35)	*P* value univariate analysis
Premature discontinuation due to SAE	11 (8)	10 (11.2)	1 (2.9)	0.178
Death	0	0	0	-
Severe adverse events grade 3/4	63 (50.8)	46 (51.7)	17 (48.6)	0.75
Infection	4 (3.2)	3 (3.4)	1 (2.9)	1
Liver decompensation	2 (1.6)	2 (2.2)	0	1
Fatigue	3 (2.4)	3 (3.4)	0	0.558
Skin rash	2 (1.6)	2 (2.2)	0	1
Kidney failure	1 (0.8)	1 (1.1)	0	1
Digestive adverse events	1 (0.8)	1 (1.1)	0	1
Thromboembolic events	1 (0.8)	1 (1.1)	0	1
Anemia	18 (14.5)	16 (18)	2 (5.7)	0.081
Neutropenia	21 (16.8)	14 (15.7)	7 (20)	0.568
Thrombocytopenia	42 (33.6)	32 (36)	10 (28.6)	0.434
Erythropoietin use[1]	65 (52)	45 (90)	20 (57.1)	0.473
Blood transfusion[1]	17 (13.6)	15 (16.7)	2 (5.7)	0.149

Only severe adverse events (SAEs) of grade 3 or 4 were reported in the table. SAEs were known for 124 patients. [1]Erythropoietin use and blood transfusions were analyzed among the 125 patients. A single subject might have several severe adverse effects.

Table 4 Factors associated with the occurrence of severe adverse events of grade 3 or 4 *n* (%)

	Severe adverse events *n* = 63	No severe adverse events *n* = 61	*P* value univariate analysis	*P* value multivariate Analysis OR (95%CI)
Protease inhibitor				
Telaprevir	47 (51.7)	43 (48.3)	0.7548	-
Boceprevir	17 (48.6)	18 (51.4)		
Genre				0.037
Male	36 (44.4)	45 (55.6)		1.0
Female	27 (62.8)	16 (37.2)	0.0518	2.4 (1.1-5.6)
Liver fibrosis				
Metavir F3	15 (42.9)	20 (57.1)	0.2667	-
MetavirF4	48 (53.9)	41 (46.1)		
Platelets				
Mean × 10^3/mm³ (SD)	143.5 (65.43)	191.1 (54.9)	≤ 0.0001	≤ 0.001
< 150 × 10^3/mm³	34 (75.6)	11 (24.4)	0.0001	1.0
≥ 150 × 10^3/mm³	27 (37.5)	45 (62.5)		5.3 (2.3-12.4)
Albumin, mean, g/L, (SD)	39.4 (4.9)	42 (54.9)	0.0196	-
Bilirubin, median μM/L, (IQR)	13.1 (9.1-19.1)	10.8 (8-13.5)	0.0359	-

The variables initially included in the logistic regression model were those associated with SAEs in the univariate analysis (*P* value < 0.20). SAEs: Severe adverse events; IQR: Inter-quartile range.

SAEs more frequently (62.8%) than men (44.4%, *P* = 0.05). The platelet counts (mean ± SD) were lower in the patients who had a SAE (143.5 ± 65.4 × 10^3/mm³) than in the patients with no SAE 191.1 ± 54.9 × 10^3/mm³, *P* ≤ 0.0001). SAEs were more frequent in patients with low levels of serum albumin (median: 39.4 ± 4.9 and 42 ± 4.9 g/L, *P* = 0.02) or with a high bilirubin concentration [median: 13.1, IQR (9.1-19.1) and 10.8 (8-13.5) M/L, *P* = 0.036]. The two factors that remained independently associated with SAE occurrence (Table 4) were being female [OR = 2.4 (1.1, 5.6), *P* = 0.037] and a platelet count lower than 150 × 10^3/mm³ [OR 5.3 (2.3, 12.4), *P* ≤ 0.001]. A greater number of the patients with platelet counts lower than 150 × 10^3/mm³ (75.6%) experienced an SAE than did those with platelet counts higher than this cutoff (37.5%; *P* = 0.0001).

DISCUSSION

PI therapy has rarely been used to treat patients with severe liver fibrosis who failed to respond to prior treatment with PR. The few Metavir F3 or F4 patients treated were selected from within larger studies and do not always reflect the population seen in routine clinical practice[5-10]. The main objective of this study was to assess the effectiveness of triple therapy for difficult-to-treat patients with severe liver fibrosis who were null-responders or relapsers to prior PR treatment. It is necessary to understand how patients tolerate these treatments to identify the patients most at risk of suffering severe adverse side effects.

The overall SVR rate of 59.8% at 24 wk post-treat-

ment was satisfactory in this difficult-to-treat population. This high rate shows that PI could be used successfully in routine clinical practice, including for patients with severe liver fibrosis, with an efficacy equivalent to results obtained in controlled trials. The SVR rate for patients in the RESPOND-2 trial[10] with severe liver fibrosis (Metavir F3 or F4) who failed to respond to previous treatment and were retreated with BOC was 55.5%. This study, however, was carried out on a limited number of patients. The SVR rate for our patients treated with BOC was 44.1%. The lower response rate for our BOC-treated patients might be due to the type of response of these patients to the prior treatment. The majority of patients in the RESPOND-2 study[10] were relapsers (64%), whereas the majority (73.5%) of our patients were null-responders. The SVR rate for the patients in the REALIZE trial[8] who had severe liver fibrosis (Metavir F3 and F4) and who were given TPV triple therapy was 56%. The SVR for our patients given TPV triple therapy was 65.9%, which was better than that of the REALIZE patients. However, the SVR rate for those REALIZE patients[8] classified as Metavir F3 was similar (66.4%) to the rate for our patients.

We identified elements predicting an SVR. One of the main predictive factors of the virological response to triple therapy was the type of response to the previous PR treatment. Relapsers on the previous treatment had very high SVR rates (80%), whereas the SVR of null-responders was only 47.4%. In the overall population, the type of response to a previous treatment was independently associated with the SVR to triple therapy. This influence

of the type of virological response to PR treatment on the SVR to triple therapy was also described in phase III studies for patients treated with both TPV and BOC[8-10]. We observed an unexpectedly high SVR (54.9%) with TPV triple therapy in our previous null-responders (28 of 51 patients). This rate is approximately twofold higher than the SVR rate observed in the REALIZE study in the same population of null-responders with severe liver fibrosis[8]. A more detailed comparison of the characteristics of cirrhotic patients in phase III studies and our patients in the current study should provide a better understanding of this difference in the SVR rate. We confirmed that the patients who relapse after a previous double-therapy benefit more from PI treatment than null-responders. However, we also determined that an encouraging SVR could be obtained for greater than one-third of previous null-responder cirrhotic patients; therefore, triple therapy with TPV or BOC should be offered to these patients, especially to the patients who could not be included in new drug trials[13]. The rationale for treating null-responders to PR therapy with severe liver fibrosis is that the second generation of direct-acting antiviral drugs (DAAs) would be used in the near future to obtain a higher SVR[14].

We have shown an overall difference in the SVR of patients treated with TPV (65.9%) and BOC (44.1%). This difference was significant in the univariate analysis; however, it does not appear to be an independent value in the multivariate analysis. There were more null-responders to PR treatment in our BOC subgroup (73.5%) than in the TPV subgroup (58.6%). An analysis of the response to triple therapy (in terms of the previous response) indicated that the SVRs of relapsers in the TPV (80.6%) and BOC (77.8%) subgroups were similar. BOC tended to be less effective for the null-responders to prior PR therapy, although this difference was not significant after the multivariate analysis. Our study was not a randomized study; each clinician could choose to use TPV or BOC, although all of the patients were given the same treatment with the same doses of peg-IFN α2a + ribavirin, unlike those in the CUPIC trial[15], which used peg-IFN α2a or α b. More of our patients were treated with TPV than BOC because TPV was approved for use in France in January 2011, and BOC was approved at a later date. The possible difference between the SVR of the TPV and BOC groups should be confirmed in a randomized trial that includes more patients. However, taking into account the development of new DAAs, such a randomized study is unlikely to be completed. Two meta-analyses[16,17] compared the efficacy of TPV and BOC. Both studies determined that the SVR for TPV was superior. These meta-analyses included only a few trials on heterogeneous populations. The results could not be extrapolated to routine clinical practice, although they are compatible with our findings. In the overall population, a RVR was observed in 66.7% of individuals. A RVR was observed for 76.3% of the patients treated with TPV and for 31.6% of the patients treated with BOC. A Spanish study found that the RVR was significantly higher in the TPV patients, suggesting that the drug acted more

rapidly[18]. Close monitoring of the viral load, especially at the start of treatment, could lead to the selection of a sub-group of patients very likely to have an SVR[19-21]. This information could strongly motivate these difficult-to-treat patients on prolonged treatment for 48 wk to adhere more closely to the treatment protocol.

We found no link between the degree of liver fibrosis, Metavir F3 or Metavir F4, and the SVR. However, we demonstrated a statistically significant link between the SVR and the LS measured by Fibroscan® at inclusion. The SVR rate was significantly higher in the patients with an LS under 21.3 kPa. This LS value of 21.3 kPa is well above the value of 12.5 kPa that is typically used to diagnose cirrhosis[22], suggesting that the population of patients with Child-Pugh class A cirrhosis is heterogeneous. The prognosis for the patients with a high LS value is likely different from the prognosis for the patients with lower LS values. A previous study[23] of patients treated with PR showed that the liver LS values were significantly lower in patients having an SVR than in non-responders. The LS value could be used to identify portal hypertension[24-27]. Various studies have given thresholds from 13.6 kPa to 48 kPa for portal hypertension. One study on patients with various liver diseases observed that a threshold of 21 kPa is useful for predicting significant portal hypertension[25]. Other studies specifically included patients with hepatitis C for diagnosing non-invasive portal hypertension. A LS threshold of 19.8 kPa[26] in one study and of 21.5 kPa in two others[22,27] enabled to accurately predict the presence of esophageal varices in this population. These threshold values assessed in studies on heterogeneous populations are similar to the thresholds that we identified as predictive of an SVR. Our finding of a link between the LS value and the SVR suggests that there is a subgroup of patients with severe liver fibrosis who have high LS values and a diminished response to triple therapy with PI. The threshold identified in this study should be confirmed by studies on larger populations of patients.

One of our secondary objectives was to assess how well PI treatment was tolerated by difficult-to-treat patients in the context of routine clinical practice. Because the great majority of patients suffered from at least one side effect, we focused on the development of severe side effects and the factors predicting their development. We determined that 50.8% of patients treated with PI developed these severe adverse reactions, causing 8.9% of the patients to abandon their treatment early. The preliminary results of the CUPIC trial[15] demonstrated that 40% of patients suffered an SAE after 16 wk, and 14.7% of these patients abandoned treatment prematurely. Published studies indicate that 57% of patients on TPV[8,9] suffered an SAE, as did 40.4% of those on BOC[28]. In phase III trials[28], SAEs were observed in only 16% of patients with severe liver fibrosis, which was a much lower percentage than in our findings. This type of difference between initial studies and routine clinical practice is not uncommon; 24% of our patients had at least one criterion that would have excluded them from a phase III trial. Our patients were also older than the patients included in

phase Ⅲ trials and were likely more fragile, making them more susceptible to an SAE. None of our patients died during follow-up, unlike the patients in the CUPIC trial.

We had to administer EPO to 52% of the patients in this study and had to reduce the ribavirin dose for 38.7% of the patients. In addition, 13.6% of the patients required a blood transfusion. Reducing the ribavirin dose had no effect on the SVR for our patients. Anemia was treated in the SPRINT-2 trial[7], similarly to our study, with a reduction in the ribavirin dose for only 8% of patients; EPO was used for 38% of patients, and a combination of the reducing the ribavirin dose and EPO was used for 44% of patients. TPV appeared to cause anemia more frequently than BOC among our patients; however, this difference was not significant. TPV treatment was not an independent factor suggesting the development of SAEs in our study. We have confirmed that patients with severe liver fibrosis, whether Metavir F3 or F4, do not readily tolerate triple therapy with PI and suffer from a high incidence of SAEs. We determined that two factors were independently associated with the development of an SAE. One factor was the platelet count at inclusion, which was significantly lower in the patients who developed an SAE. The threshold for development of an SAE was 150000 platelets/mm³. Data from the CUPIC cohort[15] indicate that a threshold of 100000/mm³ was predictive of death and severe complications. Some of our patients had Metavir F3 or F4 scores, whereas the CUPIC cohort[15] included only patients with cirrhosis; this difference could account for the lower SVR rate in the CUPIC study. Female gender is the second factor independently associated with the development of an SAE. The PI dose should not be scaled to the patient's body weight; however, further studies on men and women are needed to define any differences in body mass index in men and women to assess whether this factor influences tolerance.

In conclusion, we studied a large cohort of patients with genotype 1 HCV infection and severe liver fibrosis (Metavir F3 or F4) who had failed to respond to an earlier PR treatment. Our data obtained in routine clinical practice confirm the satisfactory efficacy of PI triple therapy. We have also demonstrated that a threshold value of 21.3 kPa of LS is associated with an SVR. However, triple therapy with PI is rather poorly tolerated. We should use better methods to select patients for PI treatment and should be able to offer the patients at the greatest risk of treatment failure (as well as those with an intolerance for other therapies) a second generation of new DAAs.

ACKNOWLEDGMENTS

COMMENTS

Background

The addition of telaprevir (TPV) or boceprevir (BOC) in the treatment of geno-type 1 hepatitis C virus (HCV) patients has significantly increased the sustained virologic response rate of pegylated-interferon (peg-INF) α2a + ribavirin (PR). Data on these new therapeutic options are limited in the setting of very difficult-to-treat patients, although these patients are in the highest priority for achieving viral clearance.

Research frontiers

The research goal is to assess the efficacy and safety of telaprevir- and boceprevir-based triple therapies in a multicentric cohort of previously treated HCV-genotype 1 patients with severe liver fibrosis.

Innovations and breakthroughs

In the clinical setting, the efficacy of TPV and BOC-based triple therapies in previously treated HCV-genotype 1 patients with severe liver fibrosis is similar or even better than the results obtained in controlled trials [overall sustained virological response (SVR) 59.8%]. The SVR is inversely correlated to liver stiffness, as assessed by elastography with a cut-off of 21.3 kPa, which is predictive of a poor response rate. These treatments are poorly tolerated, and half of all patients experience at least one grade 3-4 adverse event.

Applications

This study suggests that telaprevir and boceprevir-based triple therapies could be used in clinical practice in the subset of very difficult-to-treat patients; these triple therapies resulted in a viral clearance rate similar to or even better than the rates obtained in controlled trials. An SVR was achieved even in cirrhosis patients. However, patients with the most advanced stage of fibrosis should be considered for other treatments because these treatments are significantly less efficient when the liver stiffness is higher than 21.3 kPa and are significantly less tolerated in the presence of biological markers of advanced liver fibrosis (platelet count < 150 × 10³/mm³).

Terminology

SVR: undetectable levels of viral RNA at 24 wk following treatment completion; Rapid virological response (RVR): undetectable levels of viral RNA at week 4 or week 8 after initiation of telaprevir- or boceprevir-based triple therapies, respectively.

Peer review

This manuscript by Bonnet *et al* described the efficacy and safety of Telaprevir or Boceprevir therapy for treatment experienced patients with advanced fibrosis. The majority of patients were cirrhosis (F4 72%) and null-responder to prior therapy (63%), thus reflecting most difficult-to-treat patients. The results are encouraging showing high SVR rate in prior relapsers (80%) and even in null-responders (47%) with low rate of premature discontinuation due to SAE (8%) and no death. This information in real-life setting may be of value for physicians treating hepatitis C.

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RETROSPECTIVE STUDY

Skin toxicity predicts efficacy to sorafenib in patients with advanced hepatocellular carcinoma

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Abstract

AIM: To study the relationship between adverse events (AEs), efficacy, and nursing intervention for sorafenib therapy in patients with hepatocellular carcinoma (HCC).

METHODS: We enrolled 37 consecutive patients with advanced HCC who received sorafenib therapy. Relationships among baseline characteristics as well as AE occurrence and tumor response, overall survival (OS), and treatment duration were analyzed. The nursing intervention program consisted of education regarding self-monitoring and AEs management, and telephone follow-up was provided once in 1-2 wk.

RESULTS: A total of 37 patients were enrolled in the study, comprising 30 males (81%) with a median age of 71 years. The disease control rate at 3 mo was 41%, and the median OS and treatment duration were 259 and 108 d, respectively. Nursing intervention was given to 24 patients (65%). Every patient exhibited some kinds of AEs, but no patients experienced G4 AEs. Frequently observed AEs > G2 included anorexia (57%), skin toxicity (57%), and fatigue (54%). Factors significantly associated with longer OS in multivariate analysis demonstrated that age ⩽ 70 years, presence of > G2 skin toxicity, and absence of > G2 hypoalbuminemia. The disease control rate in patients with > G2 skin toxicity was 13/20 (65%), which was significantly higher compared with that in patients with no or G1 skin toxicity. Multivariate analysis revealed that nursing intervention and > G2 skin toxicity were independent significant predictors for longer treatment duration.

CONCLUSION: Skin toxicity was associated with favorable outcomes with sorafenib therapy for advanced HCC. Nursing intervention contributed to better adherence, which may improve the efficacy of sorafenib.

Key words: Hepatocellular carcinoma; Molecular targeted therapy; Drug toxicity; Surrogate marker; Nursing intervention

Core tip: Sorafenib therapy for advanced hepatocellular carcinoma (HCC) often causes adverse events (AEs), subsequently leading to dose reduction or discontinuation. Conversely, few studies have associated serious AEs with a favorable response to sorafenib. We aimed to elucidate the relationship between AEs occurrence, therapeutic efficacy, and the impact of nursing intervention on adherence to therapy. We observed that

skin toxicity was associated with favorable outcomes in sorafenib therapy for advanced HCC. Furthermore nursing intervention contributed to better adherence, which may improve the efficacy of sorafenib therapy.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide[1,2]; in addition, it is one of the intractable cancers, considering its high rate of recurrence even after curative therapies[3]. In particular, vascular invasion and extrahepatic metastasis greatly decrease survival rates[4-8].

Sorafenib, an oral inhibitor, is currently used as a standard therapeutic option for advanced HCC[9-11]. This drug occasionally causes severe adverse events (AEs), which include hand-foot skin reaction (HFSR), hypertension, diarrhea, anorexia, fatigue, weight loss, and so on. Although most AEs are reversible, they can significantly impact a patient's quality of life and occasionally result in dose reduction or discontinuation of therapy[12]. On the other hand, recent studies of sorafenib therapy for HCC have reported that the occurrence of any grade (G) hypertension[13] or > G2 diarrhea[14] was associated with longer overall survival (OS); in addition, skin toxicity resulted in preferable outcomes as well[15,16]. However, studies investigating the relationship between AEs occurrence and efficacy of this drug remain insufficient.

The increase of available oral anticancer drugs has introduced a shift in responsibility from clinicians to patients and their families for self-administration of these drugs and AEs management. Reduced adherence leads to poor clinical outcomes and subsequent increase in healthcare costs[17,18]. Several studies have suggested that an adequate intervention by nurses and pharmacists may improve treatment adherence[19-22]. However, the contribution of nursing intervention to treatment adherence remains elusive.

This study aimed to elucidate the relationship between AEs occurrence and the efficacy of sorafenib therapy for patients with advanced HCC. In addition, we evaluated the impact of nursing intervention on the adherence to this drug therapy.

MATERIALS AND METHODS

Ethics

This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. This study was approved ethically by the Institutional Review Board of Tokai University (NO.10R-046). All patients provided informed written consents.

Sorafenib therapy

We enrolled consecutive patients with advanced HCC who received sorafenib therapy from August 2009 to December 2012 at Tokai University Hospital. Eligibility criteria were as follows: (1) unresectable advanced HCC; (2) resistance to or no indication of transcatheter arterial chemoembolization (TACE); (3) Child-Pugh class A or B; and (4) Eastern Cooperative Oncology Group Performance Status of 0 or 1. Patients received 800 mg sorafenib as an initial daily dose. However, lower doses were occasionally selected by doctors, particularly when patients were aged > 70 years or had liver function of Child-Pugh class B. The HCC stage was classified according to the tumor-node-metastasis criteria of the Liver Cancer Study Group of Japan[23].

Nursing intervention

The nursing intervention program consisted of education regarding self-monitoring and AEs management, and telephone follow-up was provided once in 1-2 wk[24,25]. One nurse who experienced and trained specialized care with liver cancer patients provided the nursing intervention.

Clinical evaluation

Efficacy was evaluated according to the modified Response Evaluation Criteria in Solid Tumors[26] 3 mo after the initiation of therapy. Thereafter, dynamic computed tomography (CT) scan or magnetic resonance imaging (MRI) was performed every 3 mo. The disease control rate was defined as the percentage of patients with complete response (CR), partial response (PR), and stable disease (SD). AEs were assessed according to the National Cancer Institute Common Terminology Criteria for Adverse Effects, version 4.0[27]. Skin toxicity included HFSR and any kind of rash. Patients were followed up until January 7, 2013 or death.

Statistical analysis

Relationships of efficacy to baseline patient characteristics and AEs occurrence were evaluated using Fisher's exact probability test or multiple logistic regression analysis. Further, OS and treatment duration were analyzed using the log-rank test or Cox proportional hazards regression model. Multivariate analyses were performed using the stepwise (step-up) procedure (likelihood ratio). All variables with P values < 0.15 in univariate analysis were included for multivariate analysis. P values < 0.05 were considered to indicate statistical significance. Statistical analysis was performed using the statistical package IBM® SPSS® Statistics version 21 for Windows (1989, Somers, NY).

RESULTS

Baseline patient characteristics (Table 1)

A total of 37 patients were enrolled in the study, com-

Table 1 Baseline patient characteristics *n* (%)

Variables		Number of patients
Gender	Male	30 (81)
	Female	7 (19)
Age (yr)	> 70	19 (51)
	≤ 70	18 (49)
Child-pugh class	A	33 (89)
	B	4 (11)
Etiology	HCV	20 (54)
	HBV	11 (30)
	Others	6 (16)
TNM stage	III	16 (43)
	IVa	8 (22)
	IVb	13 (35)
Previous therapies	Yes	31 (84)
	No	6 (16)
AFP (ng/mL)	> 100	20 (54)
	≤ 100	17 (46)
DCP (mAU/mL)[a]	> 1000	20 (59)
	≤ 1000	14 (41)
Initial dose of sorafenib (mg/d)	800	21 (57)
	< 800	16 (43)
Nursing intervention	Yes	24 (65)
	No	13 (35)

[a]DCP values were not available for 3 cases; TNM: Tumor-Node-Metastasis staging system; AFP: α-fetoprotein; DCP: Des-gamma-carboxy prothrombin; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

prising 30 males (81%) with a median age of 71 years (range, 36-83 years). More than half of the patients (54%) were infected with hepatitis C virus. Most patients (84%) had received other treatment for HCC before sorafenib therapy, including surgical resection, TACE, and radiofrequency ablation. Sixteen patients (43%) received < 800 mg sorafenib as an initial daily dose. Nursing intervention was given to 24 patients (65%).

Treatment efficacy
Disease control was obtained in 15 patients (41%) comprising 1 (3%) with CR, 3 (8%) with PR, and 11 (30%) with SD at 3 mo after the initiation of sorafenib therapy. The patient who achieved CR was a 69-year-old male patient, a noteworthy case that has also been reported elsewhere[28]. He received sorafenib at a dose of 800 mg for HCC metastasis to a portal lymph node metastasis that appeared 3 years after surgical resection for primary HCC. However, he discontinued sorafenib administration after 11 d because of G3 HFSR. Despite treatment termination, the portal lymph node metastasis disappeared along with the normalization of serum (des-gamma-carboxy prothrombin) DCP (and α-fetoprotein) AFP levels. A total of 27 patients (73%) died. One patient was lost to follow-up. The median OS period was 259 d (range, 41-664 d).

AEs (Table 2)
Every patient exhibited some kind of AEs, but no patient experienced G4 AEs. Frequently observed > G2 AEs included anorexia (57%), skin toxicity (57%), fatigue (54%), hypoalbuminemia (41%), and hypertension (30%).

Figure 1 Overall survival and skin toxicity. Kaplan-Meier curve shows that patients with over grade 2 skin toxicity could live longer. Thus more severe skin toxicity is associated with longer survival.

Sorafenib therapy was discontinued in 5 patients (14%) because of skin toxicity (*n* = 4) and anorexia (*n* = 1). Fifteen patients (41%) required dose reduction because of skin toxicity (*n* = 8), anorexia (*n* = 4), hyperbilirubinemia (*n* = 2), and hypertension (*n* = 1).

Factors associated with OS (Table 3)
Factors significantly associated with longer OS in univariate analysis included the presence of previous therapy, serum DCP levels ≤ 1000 mAU/mL, 800 mg initial sorafenib dose, absence of > G2 anorexia, fatigue or hypoalbuminemia, and presence of > G2 skin toxicity. Multivariate analysis demonstrated that age ≤ 70 years (HR = 0.354, 95%CI: 0.135-0.933; P = 0.036), presence of > G2 skin toxicity (Figure 1, HR = 0.267, 95%CI: 0.102-0.701; P = 0.007), and absence of > G2 hypoalbuminemia (HR = 0.221, 95%CI: 0.085-0.575; P = 0.002) were significant predictors for longer OS. The disease control rate in patients with > G2 skin toxicity was 13/20 (65%), which was significantly higher compared with that in patients with no or G1 skin toxicity [2/17 (12%); P = 0.002].

Nursing intervention and treatment duration (Table 4)
The median duration of medication was 108 d (range, 4-462 d). A total of 33 patients (89%) discontinued sorafenib: 18 (55%) for deterioration of general status, 10 (30%) for the lack of beneficial effects of sorafenib, and 5 (15%) for G3 AEs.

We provided face-to-face counseling 1-2 times per mo and telephone follow-up once in 1-2 wk to manage AEs by supporting patient's self-monitoring and self-care[24,25]. Multivariate analysis revealed that nursing intervention (HR = 0.398, 95%CI: 0.181-0.874; P = 0.022) and > G2 skin toxicity (HR = 0.225, 95%CI: 0.095-0.534; P =

Table 2 Sorafenib-related adverse events *n* (%)

Adverse events	Any Grade	Grade 1	Grade 2	Grade 3
Anorexia	29 (78)	8 (22)	10 (27)	11 (30)
Skin toxicity[a]	27 (73)	6 (16)	8 (22)	13 (35)
Fatigue	23 (62)	3 (8)	11 (30)	9 (24)
Diarrhea	20 (54)	9 (24)	8 (21)	3 (8)
Hypoalbuminemia	19 (51)	4 (11)	14 (38)	1 (3)
Weight loss	17 (46)	7 (19)	9 (24)	1 (3)
Hyperbilirubinemia	16 (43)	10 (27)	5 (14)	1 (3)
Decreased platelet count	14 (38)	3 (8)	9 (24)	2 (5)
Hypertension	13 (35)	2 (5)	6 (16)	5 (14)
Alopecia	13 (35)	5 (14)	8 (22)	0 (0)
Anemia	9 (24)	4 (11)	4 (11)	1 (3)

[a]Skin toxicity includes hand–foot skin reaction and any kind of rash.

Table 3 Variables associated with overall survival

Variables	Univariate		Multivariate	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Gender, male (*vs* female)	0.384 (0.147-1.005)	0.051		
Age, ≤ 70 yr (*vs* > 70 yr)	0.491 (0.225-1.071)	0.074	0.354 (0.135-0.933)	0.036
Previous therapy yes (*vs* no)	0.035 (0.128-0.961)	0.042		
DCP, ≤ 1000 mAU/mL (*vs* > 1000 mAU/mL)	0.416 (0.178-0.974)	0.043		
Initial dose of sorafenib, 800 mg (*vs* < 800 mg)	0.405 (0.185-0.888)	0.024		
Adverse events > grade 2				
Anorexia - (*vs* +)	0.374 (0.158-0.888)	0.026		
Skin toxicity[a] + (*vs* -)	0.278 (0.122-0.635)	0.002	0.267 (0.102-0.701)	0.007
Fatigue - (*vs* +)	0.404 (0.176-0.924)	0.032		
Hypoalbuminemia - (*vs* +)	0.379 (0.170-0.842)	0.017	0.221 (0.085-0.575)	0.002

[a]Skin toxicity includes hand–foot skin reaction and any kind of rash. HR: Hazard ratio; DCP: Des-gamma-carboxy prothrombin. This table only includes variables with *P* values < 0.15 in univariate analyses.

0.001) were independent significant predictors for longer treatment duration. Median treatment durations were 122 and 36 d in patients with and without nursing intervention, respectively. However, nursing intervention was not associated with OS, with the median OS being 258 and 274 d for patients with and without nursing intervention, respectively.

DISCUSSION

In this study, the median OS was 8.6 mo, which is comparable to previous studies: 10.7, 6.5, and 9.3 mo in the SHARP trial[9], the Asia-Pacific study[10], and the Global Infectious Diseases and Epidemiology Network study[29], respectively. The disease control rate was 41%, which was lower compared with that in the abovementioned studies, ranging from 57%[10] to 73%[9]. This difference may be attributable to the timing of tumor evaluation; CT or

Table 4 Variables associated with treatment duration

Variables	Univariate		Multivariate	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age ≤ 70 yr (*vs* > 70 yr)	0.543 (0.257-1.147)	0.110		
Other etiologies (*vs* HCV infection)	0.411 (0.191-0.886)	0.023		
DCP ≤ 1000 mAU/mL (*vs* > 1000 mAU/mL)	0.402 (0.190-0.851)	0.017		
Nursing intervention yes (*vs* no)	0.577 (0.278-1.198)	0.140	0.398 (0.181-0.874)	0.022
Efficacy, disease control (*vs* PD)	0.431 (0.206-0.903)	0.026		
Adverse events > grade 2 + (*vs* -)				
Skin toxicity[a]	0.306 (0.139-0.675)	0.003	0.225 (0.095-0.534)	0.001
Diarrhea	0.352 (0.156-0.796)	0.012		
Weight loss	0.555 (0.254-1.213)	0.140		
Alopecia	0.236 (0.081-0.686)	0.008		

[a]Skin toxicity includes hand-oot skin reaction and any kind of rash. HR: Hazard ratio; HCV: Hepatitis C virus; DCP: Des-gamma-carboxy prothrombin.This table only includes variables with *P* values < 0.15 in univariate analyses.

MRI was performed 3 and 1.5 mo after the initiation of sorafenib in the present and abovementioned studies, respectively. AEs were observed in all patients. Similar to previous studies, frequently observed AEs included anorexia, skin toxicity, fatigue, and diarrhea[9-11,29,30].

Multivariate analysis indicated that age ≤ 70 years, presence of > G2 skin toxicity, and absence of > G2 hypoalbuminemia were significant predictors of longer OS. HR of patients aged ≤ 70 years against older patients was 0.35. The Asia-Pacific study reported that a beneficial effect of sorafenib was obtained only in patients aged < 65 years[10]. Therefore, an elderly patient aged > 70 years with advanced HCC may not be a good candidate for this therapy.

We demonstrated that the occurrence of skin toxicity was associated with a higher disease control rate (65% *vs* 12%; *P* = 0.002) and longer OS (HR = 0.267). These results are in concordance with those of previous studies[15,16]. Sorafenib exerts anticancer effects by inhibiting the serine–threonine kinases Raf-1 (c-Raf) and B-Raf, and the receptor tyrosine kinase activity of the vascular endothelial growth factor receptors (VEGFRs) 1, 2, and 3 and platelet-derived growth factor receptor α[31]. Nevertheless, mechanisms underlying skin toxicity induced by this drug remain largely unknown. Recent studies demonstrated that genetic polymorphisms of VEGF[32] and VEGFR2[33] were related to the occurrence of HFSR, suggesting the involvement of VEGF signaling. Patients with a genetic predisposition to HFSR may be more sensitive to the antitumor effects of sorafenib. Further research regarding the contribution of genetic variation to skin toxicity and efficacy in this therapy is clearly required.

Other studies reported that the occurrence of hypertension[13] or diarrhea[14] was related to favorable clinical outcomes. However, we could not confirm these results in our study.

In our study, hypoalbuminemia was related to poor prognosis; this can be interpreted as a sign of progression of liver disease. In previous sorafenib studies, patients with lower pretreatment serum albumin levels had a greater risk of treatment discontinuation[28] and poor prognosis[29,34].

Because skin toxicity was associated with better prognosis, controlling this AE potentially offers benefit to patients. Moisturizers, sunscreen creams, steroid ointments, and oral antibiotics such as doxycycline can effectively prevent skin toxicity[35,36]. In particular, applying moisturizers before initiation of sorafenib therapy and avoiding stimulation to palms and soles are important[36].

In this study, we observed that nursing intervention significantly extended the treatment duration. Our nursing intervention program consisted of education on self-monitoring and AEs management and telephone follow-up. Improved management of AEs or removal of anxiety by telephone follow-up may contribute to these results. The importance of patient and family education and continuity of care along with the increasing use of oral anticancer drugs has been reaffirmed in previous studies[25]. However, the impact of nursing intervention on adherence or AEs management remains elusive. Nurse-led telephone follow-up for patients receiving oral capecitabine resulted in decreased occurrence of AEs compared with historical data[37]. On the other hand, a randomized controlled trial evaluating the role of nursing intervention in symptom management and treatment adherence for patients, who were prescribed oral chemotherapy agents, could not verify its efficacy[22]. Therefore, further studies in this regard are warranted. This study has limitations. This was a retrospective study, with a relatively small number of patients from a single institution.

In conclusion, our study revealed that skin toxicity may be a surrogate marker for preferable effects of sorafenib in the management of advanced HCC. Moreover, nursing intervention significantly contributed to treatment adherence. Establishment of better nursing intervention programs that can maintain adherence by controlling serious AEs is important in maximizing the efficacy of this oral anticancer drug.

ACKNOWLEDGMENTS

COMMENTS

Background

Sorafenib therapy for advanced hepatocellular carcinoma (HCC) often causes adverse events (AEs), subsequently leading to dose reduction or discontinu-

ation. Conversely, a few studies have associated serious AEs with a favorable response. Contributes of nursing intervention on treatment adherence to therapy were unclear.

Research frontiers

In this study, the authors revealed that skin toxicity may be a surrogate marker for preferable effects of sorafenib in the management of advanced HCC. Moreover, nursing intervention significantly contributed to treatment adherence. Establishment of better nursing intervention programs that can maintain adherence by controlling serious AEs is important in maximizing the efficacy of this oral anticancer drug.

Innovations and breakthroughs

The authors demonstrated that the occurrence of skin toxicity was associated with a higher disease control rate (65% *vs* 12%; *P* = 0.002) and longer overall survival [hazard ratio: 0.267]. These results are in concordance with those of previous studies. In this study, hypoalbuminemia was related to poor prognosis; this can be interpreted as a sign of progression of liver disease. In this study, hypoalbuminemia was related to poor prognosis; this can be interpreted as a sign of progression of liver disease. In previous sorafenib studies, patients with lower pretreatment serum albumin levels had a greater risk of treatment discontinuation and poor prognosis. This nursing intervention program consisted of education on self-monitoring and AEs management and telephone follow-up. Improved management of AEs or removal of anxiety by telephone follow-up may contribute to these results.

Applications

This study revealed that skin toxicity may be a surrogate marker for preferable effects of sorafenib in the management of advanced HCC. Moreover, nursing intervention significantly contributed to treatment adherence. Establishment of better nursing intervention programs that can maintain adherence by controlling serious AEs is important in maximizing the efficacy of this oral anticancer drug.

Terminology

Sorafenib, an oral multikinase inhibitor, is currently used as a standard therapeutic option for advanced HCC. Sorafenib exerts anticancer effects by inhibiting the serine-threonine kinases Raf-1 (c-Raf) and B-Raf, and the receptor tyrosine kinase activity of the vascular endothelial growth factor receptors 1, 2, and 3 and platelet-derived growth factor receptor â.

Peer review

In this study, the authors reported that significant skin toxicity(> grade 2), younger age (< 70 years), and absence of hypoalbuminemia were associated with better overall survival. Significant skin toxicity and nursing intervention were associated with longer treatment duration. This paper refers to skin toxicity as predictor of efficacy to sorafenib in patients with advanced HCC. The paper is of interest since it gives important clues for better selection of patients who may benefit at best from treatment with sorafenib.

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OBSERVATIONAL STUDY

Insulin resistance and steatosis in HBV-HCV co-infected patients: Role of PNPLA3 polymorphisms and impact on liver fibrosis progression

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Abstract

AIM: To evaluate steatosis, insulin resistance (IR) and patatin-like phospholipase domain-containing 3 (PNPLA3) and their relation to disease progression in hepatitis B and C viruses (HCV-HBV) co-infected patients.

METHODS: Three hundred and thirty patients with biopsy proven chronic hepatitis were enrolled: 66 had HBV-HCV, 66 HBV and 198 HCV infection. Prevalence of steatosis, IR and PNPLA3 polymorphisms and their relation to anthropometric, biochemical, virological and histological parameters were evaluated.

RESULTS: Prevalence of steatosis in group HBV-HCV was similar to that in HCV (47.0% *vs* 49.5%, respectively); group HBV showed the lowest steatosis (33.3%). Group HBV-HCV had a lesser degree of steatosis than HCV ($P = 0.016$), lower HCV RNA levels ($P = 0.025$) and lower prevalence and degree of IR ($P = 0.01$). PNPLA3 polymorphisms were associated with steatosis. Group HBV-HCV showed higher levels of liver fibrosis than group HCV ($P = 0.001$), but similar to that observed in HBV group. In HBV-HCV group, liver fibrosis was not associated with steatosis, IR or PNPLA3. HBV infection was the independent predictor of advanced liver fibrosis.

CONCLUSION: HBV-HCV co-infected patients have lower degree of hepatic steatosis, IR and HCV RNA than HCV mono-infected; co-infected patients showed a more rapid liver fibrosis progression that seems to be due to the double infection and/or HBV dominance.

Key words: Steatosis; Insulin resistance; Hepatitis B and C viruses co-infection; Patatin-like phospholipase domain-containing 3; Liver fibrosis

Core tip: We evaluated the prevalence and role of steatosis, insulin resistance and patatin-like phospholipase domain-containing 3 (PNPLA3) polymorphisms on disease progression in hepatitis B and C viruses (HCV-HBV) co-infected patients. The data showed that HBV-HCV patients have lower levels of liver steatosis and insulin resistance than HCV mono-infected patients. HBV seems to interact with HCV reducing HCV replication and HCV-related metabolic features. Thus, the influence of HCV-related steatosis and insulin resistance as well as PNPLA3 polymorphism do not significantly impact liver fibrosis progression in HBV-HCV patients. The more rapid progression of liver fibrosis observed in HBV-HCV co-infected patients seems to be mostly associated with HBV infection.

INTRODUCTION

Liver steatosis is a feature of chronic hepatitis C virus (HCV) infection[1-3]. HCV genotype 3 directly induces the highest degree and prevalence of steatosis (up to 80%), whereas HCV-related steatosis in non-3 genotypes is mainly associated with metabolic conditions[4]. A close association between steatosis and insulin resistance (IR) has been reported in HCV non-3 genotype-infected patients, but, normally, insulin resistance is not a feature of genotype-3 infection[2]. In HCV infection, IR precedes the development of steatosis and modulates fatty liver deposition through several, non-mutually exclusive, mechanisms; the appearance of steatosis, in turn, worsens IR[2,5]. Furthermore, it has been reported that in genotype-1 infection, IR correlates with the serum level of HCV RNA[6-7]. Both steatosis and IR are associated with a more rapid progression of liver fibrosis[1,8]. In chronic hepatitis B virus (HBV) infection, hepatic steatosis has been reported with a lower prevalence[9,10] than that observed in HCV infection, although one report[11] showed a high prevalence of steatosis in HBV-infected patients. Furthermore, in HBV infection, steatosis seems to be related to metabolic factors and does not seem to correlate with histological hepatic damage[9,12-14]. Recently, the single nucleotide polymorphism (SNP) of the patatin-like phospholipase domain-containing 3 (*PNPLA3*) gene, involved in the lipid metabolism, has been associated with liver steatosis in chronic hepatitis[15-20].

Chronic HBV-HCV co-infection is infrequent, but it is associated with a more severe clinical presentation[21-25] and with a more rapid progression to liver cirrhosis and hepatocellular carcinoma[26-28]. There are no direct data on liver steatosis and IR in patients with HBV-HCV co-infection, nor on their impact on the progression of the liver disease. During HBV-HCV co-infection, a reciprocal inhibition of the viral genomes has been reported[29-31] and this condition, especially in HCV-genotype-1 infection, could influence the development of IR and steatosis. Thus, at present, it remains unclear whether HBV infection affects the prevalence and level of steatosis and IR in HBV-HCV co-infected patients and their impact on liver disease progression.

Accordingly, the aim of this study was to evaluate the prevalence and degree of liver steatosis and IR and their role in the progression of liver disease in a cohort of HBV-HCV co-infected patients as compared with a cohort of HBV and HCV mono-infected patients. The role of the viral and host metabolic and genetic factors, such as PNPLA3 polymorphisms, was also evaluated.

MATERIALS AND METHODS

Patients

Three hundred and thirty Caucasian patients with histology proven chronic hepatitis were enrolled in the study. Sixty-six were HBV-HCV co-infected patients, 66 HBV mono-infected (ratio 1:1) and 198 HCV mono-infected (ratio 1:3). HBV-HCV co-infected patients were age-, gender-, and HCV genotype-matched with control mono-infected groups. The study was conducted from 2009 to 2013. However, considering the low prevalence of HBV-HCV co-infected patients, all HBV-HCV co-infected patients recorded in the data base from 2006 were enrolled if there was a serum sample stored at -30 ℃ at the time of the liver biopsy and if there was a sample available for genetic purposes.

Patients were recruited from four Liver Units (see author's affiliations) of the Second University of Naples, Italy. The patients were considered co-infected and enrolled in the study if they were HBs Ag positive/HCV-Ab positive/HBV-DNA and HCV-RNA positive; all patients HBV and HCV mono-infected were HBV-DNA positive and HCV-RNA positive, respectively. All patients included were anti-HIV- and anti-HDV-negative, naive for antiviral therapy and reported no active intravenous drug addiction or daily alcohol intake over 30 g. The possible source of infection was identified only in the minority of the enrolled patients; in fact, anamnestic blood transfusion was present in 8%, previous surgery in 4%, a family history of hepatitis infection in 4%, and a past history of drug abuse in 1.8%. All patients underwent complete physical examination, full liver function tests, fasting glucose, triglycerides, cholesterol, blood cell counts, viral markers (HBV, HCV, HDV, HIV) and liver ultrasound scan. The body mass index (BMI: kg/m^2) and waist circumference were recorded for all patients. Visceral obesity was defined as waist circumference > 102 cm in male and > 88 in female. An anamnestic estimation of possible duration of infection was made. All laboratory data presented in this study refer to the values at the time

of the liver biopsy. All blood samples were withdrawn at the time of liver biopsy and serum were stored at -30 ℃ within two hours from collection.

Serum insulin and homeostasis model assessement-insulin resistance

Serum insulin was evaluated using human insulin immunoassay (Insulin Cobas, Roche Diagnostics, Indianapolis, IN, United States). IR was determined by homeostasis model assessement-insulin resistance (HOMA-IR) using the following formula: fasting plasma glucose (mmol/dL) x fasting serum insulin (IU/mL)]/22.5. To establish the cut-off level of IR in our population, HOMA-IR was evaluated in 130 healthy subjects and the cut-off value was set at the 75th percentile of the HOMA-IR value in our mono-infected control groups[32] that was 2.60. In the three groups evaluated the determination of levels of insulinemia and glycaemia were done at the same time using the stored serum and samples from the three groups were analysed in parallel.

Liver histology

All patients gave their informed consent for liver biopsy. Liver specimens were fixed in formalin, embedded in paraffin and stained with hematoxylin-eosin and Masson's trichrome stain and evaluated in a blinded way by the pathologist. The Ishak scores to grade necro-inflammation- and fibrosis were used[33]. Steatosis was scored as follows: 0, if less than 5% of hepatocytes had fatty deposition; (1) from 5% to 29%; (2) from 30% to 59%; and (3) > 60%.

All the evaluations were conducted in accordance with good clinical practice and with the Helsinki Declaration. The local Ethics Committee approved the study.

Serological determinations

Serum markers for HBV, HCV, HDV and HIV infection were sought in serum using commercially available immune-enzymatic assays (Abbott Laboratories, North Chicago, IL and Ortho Diagnostic Systems, Raritan, NJ).

HBV and HCV genotypes and viral load

Hepatitis B virus genotypes were determined by phylogenetic analysis of sequences of 400 nt of the S region, as previously described[34]. HCV genotypes were determined using immunoblotting HCV genotype assay Lipa (VER-SANT HCV Genotype 2.0 Assay (LIPA), Siemens, Erlangen, Germany) following the manufacturer's instructions. HBV DNA and HCV RNA viral load were assessed by real-time PCR using commercial kits (COBAS® AmpliPrep/COBAS® TaqMan® HBV Test, v2.0, COBAS® TaqMan® HCV Test v2.0; Roche diagnostics, S.p.A. Monza, Italy).

PNPLA3 polymorphism study

Genomic DNA was extracted from whole blood by the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and analyzed for the PNPLA3 polymorphism. All patients were genotyped for the PNPLA3 rs738409 C to G variant underlying the I148M substitution. The following prim-

ers were used, F: 5'-GCCCTGCTCACTTGGAGAAA-3' and R: 5'-TGAAAGGCAGTGAGGCATGG-3'. The FokI restriction enzyme, as previously described, was used to identify the variant, since the G allele eliminates a FokI restriction site. Random samples were confirmed by direct genotyping, which provided concordant results in all cases[35].

Statistical analysis

The data are expressed as mean or median. Differences between the groups were evaluated by Student's *t* test for parametric data and by the Mann-Whitney *U* test for non-parametric data. Spearman's correlation test was used to identify factors significantly associated. The Chi-square test was used to evaluate differences in the prevalence. The analysis of variance was used to evaluate the different distribution of steatosis in the genetic polymorphisms. Logistic regression analysis was used to evaluate the independent factors associated with advanced liver fibrosis. The data were analysed using SPSS 13.5, and *P* < 0.05 was assumed to denote significance.

RESULTS

General characteristics of patients

The general characteristics of the study population are shown in Table 1. The three groups were comparable for demographic and anthropometric parameters. The approximate duration of the disease and the lipid profile were similar in the three groups. Waist circumference was similar in groups HCV-HBV and HCV (91.7 ± 9.8 and 90.2 ± 10, respectively). The serum glucose levels were lower, albeit not significantly, in group HBV-HCV than in group HCV (*P* = 0.09). The AST values were significantly lower in the co-infected patients than in the HBV mono-infected (*P* = 0.02), while the ALT values were significantly lower in the co-infected than in the HBV and HCV mono-infected (*P* = 0.001).

Virological characteristics of patients

The median values of the HBV DNA and HCV RNA levels are shown in Table 1. HBV-HCV co-infected patients showed lower levels of HBV DNA and HCV RNA than those observed in the HBV and HCV mono-infected patients (*P* = 0.0001 and *P* = 0.025, respectively). The majority (93%) of the HCV patients were infected by genotype non-3 (Table 1) and 98% of HBV patients had genotype D.

Steatosis and IR

Table 2 shows the prevalence and degree of steatosis in the three groups. There was no difference in the prevalence of liver steatosis between group HBV-HCV and group HCV (47.0% *vs* 49.5%), but a lower prevalence of steatosis was observed in group HBV (34%). An analysis of the degree of steatosis showed higher levels (scores 2-3) in group HCV than in group HBV-HCV (*P* = 0.016, Table 2). The above results did not change when the

Table 1 General characteristics of the 330 patients include in the study

	HBV-HCV group	HBV group	HCV group	*P*
No. of patients	66	66	198	
Medianage (range)	48.5 (25-67)	47 (23-65)	50 (22-65)	NS
Males	60.60%	63.60%	55.50%	NS
Disease duration (yr ± SD)	22.3 ± 9.7	21 ± 7.6	23.2 ± 8.4	NS
BMI (mean ± SD)	25.7 ± 3	26 ± 4.5	26.7 ± 4	NS
Glycaemia (mean ± SD) mg/dL	90 ± 13.4	85.8 ± 14.4[a]	95 ± 20[b]	0.09 (a *vs* b)
HOMA	2.48 ± 2.65[c]	2.0 ± 1.17	3.63 ± 4.5[d]	0.042 (c *vs* d)
Cholesterol (mean ± SD) mg/dL	182 ± 34	182 ± 31	182 ± 41	NS
Triglycerides (mean ± SD) mg/dL	109 ± 55	85 ± 29	103 ± 53	NS
AST (mean ± SD) IU/L	55 ± 39[e]	83 ± 84[f]	65 ± 52	0.02 (e *vs* f)
ALT (mean ± SD), IU/L	44 ± 62.5[g]	124.95 ± 92[h]	90 ± 74[i]	0.001 (g *vs* h) 0.001 (g *vs* i)
				0.01 (h *vs* i)
Median HBV DNA (range) IU/mL	1.9×10^3 (1500-10 × 10⁷)	2×10^5 (3000-1× 108)		0.0001
Median HCV RNA (range) IU/mL	1.15×10^5 (120- 6.4 × 10⁵)		6.98×10^5 (2818-8 × 10⁶)	0.025
HCV genotype:				
3	7%		8.7%	NS
Non-3	93%		91.3%	
HAI score (mean ± SD)	5.9 ± 2.9	6.2 ± 3.4	6.3 ± 3.6	NS
Fibrosis score (mean ± SD)	3.32 ± 0.45[l]	3.46 ± 0.48[m]	2.9 ± 0.30[n]	0.001 (l *vs* n)
				0.001 (m *vs* n)

HBV: Hepatitis B virus; HCV: Hepatitis C virus; HOMA: Homeostasis model assessement; AST: Aspartate transferase; ALT: Alanine transferase; NS: No significant.

Table 2 Steatosis prevalence and distribution in the different groups

	Steatosis prevalence	Steatosis grade 1	Steatosis grade 2-3
HBV-HCV group (*n* = 66)	47.0%	43.6%	3.4%
HBV group (*n* = 66)	33.3%	21.2%	12.1%
HCV group (*n* = 198)	49.5%	23.2%	26.3%[a]

[a]*P* = 0.016 *vs* group HBV-HCV. HBV: Hepatitis B virus; HCV: Hepatitis C virus.

Table 3 Distribution of patatin-like phospholipase domain-containing 3 polymorphisms and their relation to steatosis *n* (%)

PNPLA3	Steatosis no	Steatosis yes	Steatosis score 1	Steatosis score 2-3
Overall				
p.148I/I	83 (55)	65 (36)	44%	23%
p.148I/M	62 (41)	82 (46)	49%	40%
p.148M/M	6 (4)	32 (18)[b]	7%	37%
HBV/HBV-HCV groups				
p.148I/I	32 (61)	36 (45.6)	55%	12.5%
p.148I/M	20 (38)	33 (41.8)	42%	37.5%
p.148M/M	1 (1)	10 (12.6)[d]	3%	50.0%
HCV group				
p.148I/I	53 (54)	44 (44)	60%	17%
p.148I/M,	43 (44)	31 (31)	30%	33%
p.148M/M	2 (2)	25 (25)[f]	10%	50%

[b]*P* = 0.0001; [d]*P* = 0.0001; [f]*P* = 0.0001 *vs* Steatosis no group. PNPLA3: Patatin-like phospholipase domain-containing 3; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

analysis was done excluding patients with HCV genotype 3, but considering the low number of patients with genotype 3 the results deserve further evaluation. In group HBV, a higher degree of steatosis (score 2-3) was closely associated with obesity (BMI > 30).

Figure 1 shows the mean serum levels of HOMA-IR in the three groups studied. HBV-HCV co-infected patients showed an intermediate value of HOMA-IR, *i.e.*, between the highest level in group HCV and the lowest in group HBV, however, such value was significantly lower than that observed in HCV but not significantly higher than that observed in group HBV. Similarly, the prevalence of IR (HOMA-IR cut-off > 2.60) in the HBV-HCV co-infected patients was lower than that observed in group HCV (21% *vs* 54%, *P* = 0.005), but not significantly different from that observed in group HBV (23%).

The relation between IR and steatosis was evaluated and, as expected, in group HCV a correlation between the levels of IR and steatosis was observed (*r* = 0.27; *P* = 0.006), whereas, such a correlation was not seen in the HBV-HCV co-infected group (data not shown).

PNPLA3 polymorphisms and steatosis
Table 3 shows the distribution of the PNPLA3 polymor-

phisms. In accordance with our recently published data[19], the results of the present study showed that the PNPLA3 I148M polymorphism was associated with a more severe degree of steatosis both in groups HCV and HBV-HCV (*P* = 0.003). An analysis of the overall study population confirmed that the PNPLA3 I148M polymorphism caused a predisposition to liver steatosis (*P* = 0.001). In Table 3, the data have been showed aggregate (HBV and HBV-HCV groups) considering that similar results have been obtained.

Liver fibrosis progression
The data given in Table 1 show that HBV-HCV co-infected patients had similar levels of liver fibrosis to those observed in group HBV, but significantly higher than those observed in group HCV (*P* = 0.001). This higher

Figure 1 Homeostasis model assessement-insulin resistance in the three groups of patients. [b]*P* < 0.001, HCV *vs* HBV-HCV and HBV groups. HOMA-IR: Homeostasis model assessement-insulin resistance; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

degree of fibrosis in HBV-HCV group was independent of necro-inflammatory activity, because the HAI was similar in the three groups (Table 1), and, in addition, was not independently associated with liver steatosis, IR or PNPLA3 polymorphisms.

Factors associated with liver fibrosis

An overall evaluation, including all groups, on the factors associated with advanced liver fibrosis showed that the presence of HBV (*P* = 0.0001), age (*P* = 0.031), liver necro-inflammation (*P* = 0.02), and liver steatosis (*P* = 0.047) were the factors associated at univariate analysis with liver fibrosis. Regression analysis showed that HBV was the only independent factor associated with advanced fibrosis (coefficient B, 0.214; standard error of B, 0.055; 95%CI, lower: 0.104 - higher: 0.323; *P* = 0.0001).

DISCUSSION

In the present study, we explored the prevalence and possible role of liver steatosis and IR on liver disease progression in patients with chronic HBV-HCV co-infection. The data show that HBV-HCV co-infection does not influence the well-known capacity of HCV to induce steatosis; HBV-HCV co-infected patients showed a lesser amount of liver fat accumulation in comparison with HCV-infected patients. In addition, the results of this study demonstrate that HBV-HCV co-infected patients had lower serum levels of HCV RNA, a lower prevalence and degree of IR, and despite a similar duration of the disease, HBV-HCV patients showed higher levels of liver fibrosis than those observed in HCV mono-infected patients. The data suggest that HBV may interact with HCV and change some HCV metabolic characteristics. The mechanisms implicated in such interaction are not known, but some hypotheses can be made based on the results of this study.

Hepatic steatosis in chronic HCV infection is associated with alterations in the lipid and glucose metabolism[36,37]. IR in HCV infection has been reported in up to 80% of cases[38]. A close association between steatosis

and IR has been observed in HCV genotype non-3-infected patients, but IR is not generally a feature of genotype-3 infection[39,40]. HCV genotype-1-infected patients have higher prevalence of impaired glucose metabolism, and IR is correlated with the level of viral replication[6,7]. In HCV infection, IR precedes the development of steatosis and modulates fatty liver deposition[41,42]. The data of the present study show that HBV-HCV co-infected patients had lower levels of HCV RNA, IR and glucose. A fluctuating virological profile related to mutual HBV-HCV interference and the effect of this biological process on the clinical presentation and treatment strategy have been described[29,43]. It is possible that viral interference between HBV and HCV in hepatocytes might control or modulate the interaction between HCV and the lipid and glucose metabolism. However, a recent *in vitro* study[44] supports the hypothesis that HBV and HCV can replicate in the same cell without evidence of direct interference and that the *in vivo* effects may depend on the host immune response. However, the extensive virological and molecular interactions between the two viruses in co-infected patients are not well understood. Evidence seem to indicate that an in verse relationship occurs in the replication levels of the two viruses, suggesting direct or indirect viral interference[44,45]. Studies *in vitro* showed that the HCV core protein suppresses HBV replication[29,46,47]. On the other hand, an inhibition of HCV replication in patients with chronic hepatitis C who were super-infected with HBV have also been demonstrated[21,48]. Thus, the type of interaction between these two viruses in patients who are co-infected may be influenced by which virus infection is experienced first[24]. On these bases, our results seem to confirm that HBV "interference" induces lower levels of HCV replication, which may not support a significant development of IR and, in turn, not favor high amounts of liver fat deposition. Future experimental studies analyzing the effects of HBV replication on the development of IR and steatosis in HBV-HCV co-infected cells could produce interesting results.

It is well known that metabolic factors, in particular high levels of steatosis and IR are associated with a decreased likelihood of achieving a sustained virological response with interferon-based treatment[49-51], but little information is available for protease-inhibitor regimens[52]. Thus, determining the metabolic profile in HBV-HCV patients could prove useful to predict the outcome of treatment for these patients, but specific studies are necessary.

In accordance with the data available on the correlation between the PNPLA3 I148M variant and liver steatosis in NAFLD and in chronic HCV and HBV infection[15-20], the data of this study confirm the independent role of the PNPLA3 polymorphisms in inducing high degree of steatosis.

It has been well established that in chronic HCV infection, IR, a high degree of steatosis (greater than 20%-30%) and higher levels of glucose are associated with a more rapid progression of liver fibrosis[1,39]. Although the data from this study showed that HBV-HCV

co-infected patients had a more "favorable" anti-fibrotic metabolic profile, these patients had higher levels of liver fibrosis than those observed in HCV-infected patients. These data seem to indicate a prominent "direct" viral effect of the two viruses, rather than HCV-related metabolic factors, in the progression of liver fibrosis. Alternatively, considering that the levels of fibrosis in HBV-HCV co-infected patients are similar to those observed in the HBV mono-infected, and that HBV is the independent factor associated with advanced fibrosis, it is possible that HBV infection plays a dominant role in the progression of liver fibrosis.

It is necessary to underline that this study has some limitations; first, it is a cross-sectional study conducted in one geographic area; second, the very low number of HCV genotype 3 enrolled do not permit to draw conclusion about the role of genotype; third, due to the very low frequency of occurrence of double infection, a relative low number of patients have been included in the HBV-HCV co-infected group. However, despite these limitations, this study represents the essential basis for a future larger multicenter study evaluating the interaction between HBV and HCV infection.

In conclusion, the results of this study demonstrate that in HBV-HCV co-infected patients a high degree of liver steatosis is uncommon, possibly due to reciprocal viral interference causing lower levels of HCV replication and subsequently lower levels of IR. However, despite the "anti-fibrotic" metabolic profile observed, HBV-HCV co-infected patients had a higher degree of fibrosis, probably due to the dual infection and/or HBV dominance. Thus, in the unstandardized complex therapeutic managements of HBV-HCV co-infected patients an early control of HBV infection could be of importance to avoid the rapid progression of liver fibrosis.

COMMENTS

Background
Liver steatosis and insulin resistance (IR) are closely associated with chronic hepatitis C infection. The pathogenic link between steatosis, IR and chronic hepatitis C virus (HCV) infection is complex and it is associated with both viral and host factors. A host genetic factor, such as the polymorphism of the patatin-like phospholipase domain-containing 3 (*PNPLA3*) gene, involved in the lipid metabolism, is associated with liver steatosis in chronic hepatitis of different etiology. Both liver steatosis and IR are associated with a more rapid progression to liver cirrhosis. In chronic hepatitis B virus (HBV) infection, hepatic steatosis and IR have been reported with a lower prevalence than that observed in HCV infection. Chronic HBV-HCV co-infection is associated with a more rapid progression to liver cirrhosis. During HBV-HCV co-infection, a reciprocal inhibition of the viral genomes has been reported that could influence both steatosis and IR. There are no direct data on prevalence and pathogenic role of liver steatosis and IR in patients with HBV-HCV co-infection.

Research frontiers
At present, it remains unclear whether HBV infection affects the prevalence and level of steatosis and IR as well as the role of PNPLA3 in HBV-HCV co-infected patients and their impact on liver disease progression. The role of insulin resistance as promoting factor for liver steatosis and of this latter in promoting liver fibrosis has been extensively demonstrated in non-alcoholic fatty liver disease and in HCV related chronic hepatitis.

Innovations and breakthroughs
The study explores the unknown area of interaction between HBV with HCV on development of IR and liver steatosis, the role of *PNPLA3* gene polymorphisms, and their impact on the progression of liver disease. The results seem to indicate that HBV interacts with HCV reducing HCV replication and HCV-related metabolic features. Thus, steatosis and IR as well as PNPLA3 polymorphism do not significantly impact liver fibrosis progression in HBV-HCV patients. The more rapid progression of liver fibrosis observed in HBV-HCV co-infected patients seems to be mostly associated with HBV infection.

Applications
The knowledge of factors that influence the liver disease progression can improve therapeutic strategy in HBV-HCV co-infected patients.

Terminology
Liver steatosis is considered as a burden greater than 5% of triglycerides and other fats inside liver cells; it is the hepatic manifestation of the metabolic syndrome and contributes to progression of liver disease. Insulin resistance is a reduced ability of body tissues to respond to insulin, thus larger quantities of insulin are needed to maintain normal blood levels of glucose. It contributes to serious health problems including type 2 diabete and metabolic syndrome. The *PNPLA3* is a gene, involved in the lipid metabolism and has been associated with liver steatosis.

Peer review
The authors reported that a close association between steatosis and IR has been reported in HCV non-3 genotype-infected patients. The pathogenetic link between IR and chronic HCV infection is complex and is associated with HCV genotype.

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CASE REPORT

Liver fibrosis in primary intestinal lymphangiectasia: An undervalued topic

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Abstract

The relationship between primary intestinal lymphangiectasia (PIL) and liver fibrosis is an emerging topic with many obscure aspects due to the rarity of the disorder. A recent paper reported that a six-month low-fat diet improved liver fibrosis. We report the case of a 17-year-old girl affected by PIL whose hepatic fibrosis progressively worsened within one year, despite dietetic support. This and the previous case report describe extraordinary events, which do not allow clear-cut clinical aspects to be established. Nevertheless, both cases suggest that in patients with PIL, it is necessary to closely monitor liver morphology with in-depth investigations including not only ultrasonography, but also elastography.

Core tip: The relationship between primary intestinal lymphangiectasia and liver fibrosis is an emerging topic with many obscure aspects due to the disorder. The fibrosis outcome after a low-fat diet in the patient described in this report is in contrast with other literature reports. We emphasize the need for systematic monitoring of liver fibrosis in primary intestinal lymphangiectasia.

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INTRODUCTION

Primary intestinal lymphangiectasia (PIL), featuring a dilatation of intestinal lymphatic vessels and malabsorption, is a rare condition often requiring nutritional enteral/parenteral support[1]. Enteral nutrition is based on a hyperproteic, low-fat diet with vitamin and medium-chain triglyceride supplementation[2]. The association between PIL and primary liver fibrosis is uncommon[3], however, Milazzo et al[4] recently reported a case of associated PIL and liver fibrosis characterized by high stiffness at elastography. The authors reported that a six-month low-fat diet combined with medium-chain triglyceride supplementation improved liver alterations by reducing fibrosis. The authors attributed the fibrosis onset to lymphatic stasis, as occurs in cardiac congestive liver. However, since fibrosis reversibility has not been previously described in this condition, the hypothesis may be purely speculative. In this scenario, we believe our case of PIL featuring progressively worsening hepatic fibrosis, despite dietetic support, may be of interest.

CASE REPORT

A 17-year-old female patient was admitted to our unit for peripheral and facial edema, ascites and intestinal malab-

sorption (hypovitaminosis, low serum magnesium, severe hypoproteinemia with hypoalbuminemia, lymphocytopenia). The symptoms had developed four years before and progressively worsened. Her body mass index was 16.4.

Upper endoscopy and colonoscopy were performed, with biopsy samples showing only a microscopic dilatation of lymphatic vessels. Video-capsule endoscopy showed hyperemia, edema and several mucosal elevations, which was suggestive of PIL. Therefore, we evaluated intestinal protein loss by fecal alpha-1-antitrypsin clearance, which was found to be > 24 mL/d, confirming our clinical suspicion. The final diagnosis was made with technetium-labeled human serum albumin scintigraphy, which highlighted patchily distributed areas of protein dispersion in the small intestine at the level of the jejunum and ileum. During her hospital stay, ultrasonography revealed splenomegaly and hepatomegaly with inhomogeneous echogenicity, whilst transient elastography (FibroScan; Echosens, Paris, France) demonstrated hepatic fibrosis (10 kPa, interquartile range: 1.5 kPa; success rate, 100%; F3). Laboratory examinations displayed slightly increased amino transferase and gamma-glutamyl transferase (twice the normal upper limit), leading us to exclude all known causes of chronic liver disease: negative hepatitis B virus-DNA, hepatitis C virus-RNA (excluding chronic viral hepatitis); normal cupremia and ceruloplasmin (excluding Wilson's disease); normal serum iron, ferritin and transferrin saturation (excluding hemochromatosis and hemosiderosis); negative anti-nuclear, anti-smooth muscle, anti-mitochondria and anti-liver-kidney microsome antibodies (excluding autoimmune hepatitis, primary biliary cirrhosis). There was no history of alcohol or potential hepatotoxic drug use. Cardiac failure was ruled out by echocardiography.

At discharge, the patient began a hyperproteic diet (2.1 g/kg per day of amino acids), with low-fat intake and medium-chain triglycerides and vitamin supplementation[2]. Six months later, peripheral edema and ascites had improved, as well as nutritional parameters, with normalization of amino transferase and gamma-glutamyl transferase values. The decreased values were presumably due to improved nutritional conditions, and reducing the hepatic cytolysis and cholestasis that characterize malnutrition-induced liver steatosis. Indeed, these cannot be considered as markers of liver fibrosis. Paradoxically, this condition may decrease amino transferase values by reducing the hepatocyte mass.

Despite the clinical improvement, the liver stiffness value had doubled by one year later (20 kPa, interquartile range: 2.9 kPa; success rate, 100%; F4). Liver biopsy showed pericellular and periportal fibrosis. The framework was interpreted as "congenital liver fibrosis", excluding other possible causes of chronic liver diseases such as primary biliary cirrhosis and Caroli's disease.

DISCUSSION

The rarity of PIL and the extraordinary events surrounding its uncommon association with liver fibrosis are

exhibited by the present case, thus preventing the establishment of clear-cut clinical characteristics. Indeed, this report demonstrates that liver fibrosis may not improve after nutritional therapy. Nevertheless, this and a previous case[4] suggest that in patients with PIL, it is necessary to closely monitor liver function, with in-depth investigations including not only ultrasonography, but also elastography[5]. Early detection of liver involvement in PIL is important in order to promote regression and prevent progression towards portal hypertension and recurrent cholangitis.

COMMENTS

Case characteristics
Main symptoms: facial edema, abdominal swelling, weight loss.

Clinical diagnosis
Physical examination: edema, ascites, reduced body mass index (16.4), hepatomegaly.

Differential diagnosis
Malabsorption syndrome causes and chronic liver disorders were investigated.

Laboratory diagnosis
Main findings: hypovitaminosis, low serum magnesium, severe hypoproteinemia with hypoalbuminemia, lymphocytopenia, increased amino transferase and gamma-glutamyl transferase (twice the normal upper limit), negative hepatitis B-DNA and hepatitis C-RNA, normal cupremia and ceruloplasmin, normal serum iron, ferritin and transferrin, negative anti-nuclear, anti-smooth muscle, anti-mitochondria and anti-liver-kidney microsome antibodies; alpha-1-antitrypsin clearance > 24 mL/d.

Imaging diagnosis
Video-capsule endoscopy showed hyperemia, edema and several mucosal elevations, suggestive of primary intestinal lymphangiectasia. Technetium-labeled human serum albumin scintigraphy highlighted patchily distributed areas of protein dispersion in the small intestine at the level of the jejunum and ileum. Ultrasonography revealed splenomegaly and hepatomegaly with inhomogeneous echogenicity, whilst transient elastography demonstrated hepatic fibrosis (10 kPa, interquartile range: 1.5 kPa; success rate 100%; F3).

Pathological diagnosis
Microscopic dilatation of lymphatic vessels in duodenal biopsy specimens.

Treatment
Hyper-proteic diet (2.1 g/kg per day of amino acids), with low-fat intake and medium-chain triglycerides and vitamin supplementation.

Related reports
A recent case report shows an association between primary intestinal lymphangiectasia and liver fibrosis, which was improved by a 6-mo low-fat diet combined with medium-chain triglyceride supplementation.

Experiences and lessons
In patients with primary intestinal lymphangiectasia, it is necessary to closely monitor liver function with in-depth investigations including not only ultrasonography, but also elastography.

Peer review
This is a case report written in the format of a Letter to the Editor. The case report is on a 17-year old female who was diagnosed to suffer from primary intestinal lymphangiectasia. In spite of enteral nutrition which was based on hyper-proteic, vitamin, low fat and medium-chain triglyceride supplementation, the patient's liver fibrosis doubled on elastography.

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TOPIC HIGHLIGHT

Severe alcoholic hepatitis-current concepts, diagnosis and treatment options

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Abstract

Alcoholic hepatitis (AH) is an acute hepatic manifestation occurring from heavy alcohol ingestion. Alcoholic steatohepatitis (ASH) is histologically characterized by steatosis, inflammation, and fibrosis in the liver. Despite the wide range of severity at presentation, those with severe ASH (Maddrey's discriminant function $\geq$ 32) typically present with fever, jaundice, and abdominal tenderness. Alcohol abstinence is the cornerstone of therapy for AH and, in the milder forms, is sufficient for clinical recovery. Severe ASH may progress to multi-organ failure including acute kidney injury and infection. Thus, infection and renal failure have a major impact on survival and should be closely monitored in patients with severe ASH. Patients with severe ASH have a reported short-term mortality of up to 40%-50%. Severe ASH at risk of early death should be identified by one of the available prognostic scoring systems before considering specific therapies. Corticosteroids are the mainstay of treatment for severe ASH. When corticosteroids are contraindicated, pentoxifylline may be alternatively used. Responsiveness to steroids should be assessed at day 7 and stopping rules based on Lille score should

come into action. Strategically, future studies for patients with severe ASH should focus on suppressing inflammation based on cytokine profiles, balancing hepatocellular death and regeneration, limiting activation of the innate immune response, and maintaining gut mucosal integrity.

Core tip: We should further explore novel molecular targets to restore altered gut mucosal integrity, suppress inflammation based on cytokine profiles, promote hepatic regeneration, and limit innate immune responses in severe alcoholic steatohepatitis.

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INTRODUCTION

Alcoholic liver disease (ALD) is one of the main causes of end-stage liver disease worldwide[1]. ALD has a broad disease spectrum, encompassing simple steatosis, steatohepatitis, and cirrhosis. In particular, the short-term mortality in patients with severe alcoholic steatohepatitis (ASH) has been extremely high up to 40%-50%[2,3]. Although several therapeutic measures are now available to improve survival in those with severe alcoholic hepatitis (AH), overall prognoses remain gloomy.

Recently, severe AH with a significant morbidity and mortality ranks among the most costly diseases during hospitalization in the United States[4]. Thus, the early

detection of high-risk patients and prompt intervention may assist in the alleviation of healthcare cost associated with severe AH. Accordingly, the accurate prognostic stratification is crucial for individualized therapeutic decisions in patients with AH. Several prognostic scoring systems, to date, have been developed and validated for use in those with AH[5-10].

The clinical syndrome of jaundice and liver function abnormalities in alcohol abusers is generally called AH, which has often been referred to as "acute alcoholic hepatitis" historically. However, despite the sudden onset of the clinical presentation, this term seems to fade into the mists of history now that AH is usually associated with extensive fibrosis or cirrhosis and often follows a protracted natural course.

ASH is a pathologic disease entity, defined as the coexistence of steatosis, hepatocellular ballooning, neutrophilic infiltration, and perisinusoidal fibrosis[11]. ASH is not exclusively accompanied by AH but can be superimposed on any different stages of ALD comprising steatosis, steatohepatitis, fibrosis, and cirrhosis[12,13]. However, it is not much well-known which patients with ASH will progress to clinically evident AH. In addition, the true incidence and prevalence of ASH or AH among alcohol abusers remain unclear due to the uncertainties behind a clinical diagnosis of AH and the limited number of studies with liver biopsy to ascertain a histologic diagnosis of ASH.

Recently, the updated practice guidelines for management of ALD have been released from the European Association for the Study of the Liver[14] as well as the American Association for the Study of Liver Diseases[15]. Herein, we attempt to address some issues regarding different types of alcohol-induced liver failure, new prognostic scoring systems, general therapeutic measures, and potential specific therapies in patients with severe ASH from a clinical perspective.

DIAGNOSIS

Different types of alcohol-induced liver failure

Traditionally, there are two different types of liver failure, which have different prognoses and call for different therapeutic approaches. One is acute liver failure (ALF), which occurs suddenly in patients without previous any liver disease. The other is chronic liver failure (CLF) due to chronic hepatic decompensation (CHD), which is found in those with end-stage liver cirrhosis as a result of slow progression of underlying liver disease. Since the advent of albumin dialysis, a new subtype of CLF, that is, acute-on-chronic liver failure (ACLF) has been widely recognized and highlighted in the field of clinical practice[16-18]. This new entity is characterized by an acute and rapid deterioration within several weeks on the top of underlying compensated liver disease, mostly cirrhosis, leading to deep jaundice, renal impairment, hepatic encephalopathy, and multi-organ failure in the early stage[19]. Indeed, among the patients hospitalized for alcoholic cirrhosis, ACLF showed a 3-mo mortality rate of 60% *vs*

that of 20% in case of CHD, illustrating the severity of this new clinical syndrome[20]. Currently, an excessive pro-inflammatory response to bacterial components such as gut microbiota and lipopolysaccharide (LPS) seems to play an important role in ACLF, linking the gut, liver, and portal to systemic circulation[21].

In the same manner, alcohol can also instigate two different types of liver injury including ACLF and CHD. The most critically ill patients with alcohol-induced liver failure are some people suffering from severe ASH, mostly superimposed on alcoholic cirrhosis, and secondly those with decompensated cirrhosis. Precipitating events such as variceal hemorrhage, infection, and hepatitis B viral reactivation are usually crucial for the onset of ACLF, given that the rapid and aggressive control of these triggers can allow a complete reversal of ACLF. In this regard, an early use of transjugular intrahepatic portosystemic shunt effectively prevented the development of ACLF in patients with high-risk varices[22]. Similarly, early suppression of hepatitis B viremia by tenofovir prevented those with spontaneous reactivation of hepatitis B presenting as ACLF from progressing to multi-organ failure[23]. CHD is the most frequent subtype of alcohol-induced liver failure and is characterized by the complications of portal hypertension and mild to moderate jaundice in the early stage. The 1-year mortality rate was 29% in case of the appearance of ascites; however, it was 64% if hepatic encephalopathy occurred as a complication of portal hypertension in patients with alcoholic cirrhosis[24]. ACLF is the less frequent subtype of alcohol-induced liver failure but accounts for more than 40% of emergency hospitalization due to alcoholic cirrhosis in tertiary referral hospitals[20]. ACLF can be induced by several precipitating events in patients with alcoholic cirrhosis; however, one of the most common triggers is severe ASH, which occurs in roughly 25% of the patients with ACLF[20].

Prognostic scoring systems

The best way to reverse alcohol-induced ACLF is to detect and control severe ASH as early as possible, which is less likely to recover spontaneously. In this regard, a variety of prognostic scores have been developed primarily to select patients with severe ASH at high risk of early (1, 2, or 3 mo) death[5-8,10]. There are several disease-specific prognostic models (MDF: Maddrey's Discriminant Function; GAHS: Glasgow Alcoholic Hepatitis Score; ABIC: Age-Bilirubin-INR-Creatinine Score; Lille model; MAGIC: Model for Alcoholic hepatitis to Grade the severity In an Asian patient Cohort,) and a non-disease-specific model (MELD: Model for End-Stage Liver Disease) (Table 1)[5-8,10].

MDF is still one of the most commonly used prognostic models to predict survival outcomes in patients with ASH with 32 of a cutoff value[10,25]. Severe ASH (MDF ≥ 32) mostly progress to the systemic inflammatory response syndrome (SIRS) and multi-organ failure, which are often seen in other types of ACLF. MAGIC is a recently developed, new model to predict liver-related death in Asian patients hospitalized for AH[8]. The unique

Table 1 Components of clinical scoring systems to assess prognosis in alcoholic hepatitis

	Bilirubin	PT/INR	Cr/BUN	WBC	Age	Albumin	Potassium	Change in bilirubin from day 0 to day 7
MDF[10]	+	+	-	-	-	-	-	-
MELD[6]	+	+	+	-	-	-	-	-
GAHS[7]	+	+	+	+	+	-	-	-
ABIC[5]	+	+	+	-	+	+	-	-
Lille[9]	+	+	+	-	+	+	-	+
MAGIC[8]	+	+	+	-	-	-	+	+

PT/INR: Prothrombin time/international normalized ratio; Cr/BUN: Creatinine/blood urea nitrogen; WBC: White blood cell; MDF: Maddrey's discriminant function; MELD: Model for end-stage liver disease; GAHS: Glasgow alcoholic hepatitis score; ABIC: Age, serum bilirubin, INR, and serum creatinine; MAGIC: Model for alcoholic hepatitis to grade severity in an Asian patient cohort.

findings of this model are as follows: (1) the MAGIC is the first prognostic model derived from an Asian population with AH; (2) it mainly focused on the prediction of natural outcomes of untreated patients with AH; (3) it firstly brought the prognostic role of hyperkalemia in AH to light, and most importantly; and (4) the spontaneous evolution in bilirubin levels is incorporated into this new model, emphasizing the importance of early amelioration of liver function in relation to the improvement of survival. However, this model should be further validated in other ethnic populations with severe ASH.

Corticosteroids seem to improve survival outcomes in patients with severe ASH without specific contraindications such as gastrointestinal bleeding, hepatorenal syndrome (HRS), uncontrolled infection, hepatitis B virus infection, and pancreatitis[14,15]. A recent meta-analysis of individual patient data from 5 randomized controlled trials demonstrated that a 28-d survival rate was higher in corticosteroid-treated patients than in placebo-treated ones (80% *vs* 66%)[26]. MDF, GAHS, and MELD at baseline assist in defining severe ASH and guiding when to initiate steroid treatment. On the other hand, early change in bilirubin level, *in vitro* resistance to steroid, and the Lille score at day 7 allow us to decide on the responsiveness to corticosteroids and whether to stop corticosteroids during steroid treatment[9,27-29].

Recently, an alcoholic hepatitis histologic score (AHHS) has been suggested to predict survival outcomes accurately in patients with biopsy-proven, ASH[30]. The AHHS is calculated by grading the extent of fibrosis, the degree of neutrophilic infiltration, bilirubinostasis patterns, and megamitochondria[30]. In particular, the pattern of bilirubinostasis was closely associated with the development of bacterial infections during hospitalization[30,31].

TREATMENT

General therapeutic measures

Alcohol abstinence is the linchpin of therapy for AH, since abstinence failure increases mortality rates among those with AH[32]. However, anti-craving drugs such as disulfiram, naltrexone, and acamprosate are not routinely recommended to patients with severe AH due to the risk of potential hepatotoxicity. Although an anti-craving medication is not promptly given to patients hospitalized for severe AH, an abstinence treatment should be consid-

ered to reduce the recurrence of alcohol use disorders after recovery of liver function. Baclofen could effectively suppress a craving for alcohol and keep an abstinence from alcohol in patients with alcoholic cirrhosis without incurring hepatotoxicity; however, additional research is needed to prove an anti-craving efficacy in those with severe AH[33].

Patients with AH often suffer from serious malnutrition resulting from promiscuous eating habits, alcohol-related diarrhea, decreased small bowel absorption capacity, anorexia, and an excessive catabolic state, which is directly related to increased mortality[34]. Accordingly, most of them require nutritional support including the adequate calorie and protein supply as well as vitamin B and mineral repletion along with dextrose water infusion. In addition, when oral feeding is not well tolerated in patients with AH, they often need fat-soluble vitamin supplementation and enteral nutrition. However, there was no significant difference of a 1-mo mortality rate in a previous study comparing enteral nutrition and corticosteroids in patients with severe AH[35]. Nonetheless, further studies are warranted to evaluate the impact of the combination treatment on survival, because early death was more frequent in the enteral nutrition group and late mortality was higher in the steroid-treated group.

In patients with severe AH, renal impairment is a frequently accompanied symptom during hospitalization and also represents an important predictor of infection and survival. The most common cause of acute renal dysfunction is HRS. To prevent HRS, nephrotoxic drugs such as nonsteroidal anti-inflammatory drugs, aminoglycoside, diuretics, and contrast dye should be avoided and volume expanders including albumin and fresh frozen plasma might be administered. Bacterial infection is frequent but difficult to diagnose, since SIRS criteria are often associated with sterile inflammation in ASH. Infection is commonly seen in around 25% of patients with severe ASH at admission and another quarter finally become infected while receiving corticosteroids during admission[36]. Thus, infection and renal failure in ASH have a major impact on survival and should be screened, prevented, or treated at all time-points. Empirical use of antibiotics, although widely instituted, is not routinely warranted. Recent data demonstrated that corticosteroids are not contraindicated for the treatment of ASH after a complete control of infection[36]. Infection developed

during steroid treatment, however, was not the result of immunosuppression by corticosteroids but that of non-response to corticosteroids suggesting severe liver impairment[36]. Such being the case, empirical antibiotic treatment may be more beneficial to steroid responders rather than non-responders by improving survival only in the former.

Specific therapies

Corticosteroids: Apart from general therapeutic measures, specific therapies are indicated for patients with severe AH (MDF ≥ 32) who are at high risk of early death according to clinical prognostic scores[26]. The impact of corticosteroid treatment on survival in those with severe AH has been under debate for the last three decades because of heterogeneity of the study design among different studies and selection bias from ambiguous diagnostic criteria lacking histologic confirmation. Moreover, the mechanisms underlying corticosteroid treatment for AH remain largely unknown. A recent study has carefully examined the effects of prednisolone on liver injury and regeneration in several experimental models regarding alcoholic liver injury[37]. In general, corticosteroids suppress inflammatory and immune-mediated hepatic destruction, but their marked, anti-anabolic effect may suppress regeneration and slow healing by inhibiting expression of genes (*i.e.*, *pSTAT3*) regulating the proliferation and repair of hepatocytes[37]. This study may give some new insights on prednisolone treatment for AH. In a recent meta-analysis, patients allocated to corticosteroid treatment (40 mg/d for 28 d) had a higher 28-d survival rate than those allocated to non-corticosteroid treatment[26]. Corticosteroids have now become a first-line therapy for biopsy-proven, severe ASH. Steroids may be sometimes deleterious in conditions other than ASH, which represent 10%-30% of patients with a clinical diagnosis of AH, dominated by infection-related decompensation. Moreover, the treatment of non-severe forms of AH by corticosteroids is not recommended. Thus, the effect of corticosteroids on survival seems to be restricted to biopsy-proven, severe ASH. Approximately, 60% of patients with advanced forms of ASH might benefit from corticosteroid treatment. Thus, early recognition of non-responders to corticosteroids (40% of the patients) is essential to define stopping rules and minimize the unnecessary exposure to corticosteroids[9]. A Lille score ≥ 0.56 at 7 d upon corticosteroids is defined as non-response to steroids. In non-responders, a 28-d survival rate was no more than 50% despite the continued treatment of corticosteroids[26].

Pentoxifylline: Pentoxifylline shows an antioxidant effect and weakly inhibits tumor necrosis factor-alpha (TNF-α) synthesis. In patients with severe ASH receiving pentoxifylline, a 6-mo survival rate was higher than in those treated with placebo[38]. The survival benefit was attributable to a lower incidence of HRS. However, this beneficial effect was challenged by two recent meta-analyses demonstrating that pentoxifylline decreased the risk

of fatal HRS but did not improve survival significantly, although it remains inconclusive[39,40]. Recently, a Korean multicenter study group has made a head-to-head comparison between pentoxifylline and prednisolone[41]. The results demonstrated that the efficacy of pentoxifylline was not statistically equivalent to that of prednisolone in terms of 6-mo survival, supporting prednisolone as a preferred treatment option for severe AH. However, in patients with severe AH and contraindications to corticosteroids, pentoxifylline still can be considered as an alternative therapeutic option[14,15]. In a recent prospective trial including 270 patients with severe ASH, the combination of pentoxifylline and prednisolone did not bring any significant survival benefit over prednisolone alone[42]. However, a limitation of this study was that they failed to include a treatment arm receiving only pentoxifylline[43]. To overcome this limitation, a large randomized trial with a sufficient sample size is ongoing in the United Kingdom comparing pentoxifylline with corticosteroids or a combination of both in patients with severe AH[44]. Finally, in patients with severe ASH and non-response to corticosteroids based on the Lille model, an early switch to pentoxifylline did not improve the survival outcome[45]. Collectively, pentoxifylline has no additional beneficial effect in combination with corticosteroids in patients with severe ASH and also pentoxifylline alone is ineffectual in non-responders to steroids.

N-acetyl cysteine: Recently, the combination treatment with *N*-acetyl cysteine (NAC), an antioxidant and prednisolone significantly reduced a 1-mo mortality rate compared with prednisolone alone by preventing HRS and infection, although the difference was no longer statistically significant at 3 and 6 mo[46]. However, given the trend toward improved survival in those treated with NAC, additional studies are required to determine the optimal dosing schedule and treatment duration of NAC.

Anti-TNF agents: Since strong evidence supported a central role for TNF-α in several experimental models of ALD, a randomized controlled study in patients with severe ASH tested infliximab in combination with corticosteroids[47]. In fact, the treatment aimed at blocking TNF-α, compared to placebo, was associated with a higher probability of severe infection and mortality[47,48]. Presumably, prolonged or excessive TNF blockade may cause profound immunosuppression and negatively impact liver regeneration[49-51].

Liver transplantation: AH is not considered as a usual indication for liver transplantation (LT). This is related both to the fact that most patients with AH will recover for at least 6 mo after abstinence, and to the "6-mo abstinence rule"[52]. The 6 months' abstinence rule, although socially acceptable and associated with low harmful alcohol relapse, can be replaced with other elements predictive of abstinence such as social and familial support and absence of psychiatric, addictive disorders[52]. The Lille model now allows the early identification of non-re-

Table 2 Summary of potential molecular targets and novel targeted therapies for alcoholic hepatitis

Key element of the pathogenesis	Treatment	Effect	Clinical trial
FXR dysregulation	OCA[67]	FXR agonist	Moderately severe AH (placebo *vs* OCA)
Altered gut integrity	Zinc[68]	Restoration of gut integrity	Severe AH
	LGG[69]	Probiotic effect	Mild to moderate AH (placebo *vs* LGG)
	Rifaximin[70]	Intestinal decontamination	Severe AH (steroid *vs* steroid + rifaximin)
Innate immune activation	Imm 12-E[71]	Anti-LPS antibody	Severe AH (steroid *vs* steroid + low/high dose Imm 12-E)
	Anakinra[57,58,72]	IL-1RA	Severe AH (steroid *vs* anakinra + pentoxifylline + zinc)
	Rilonacept[57,58]	IL-1 inhibitor	Severe AH with response to steroid at day 7 (steroid *vs* steroid + rilonacept)
	Mycophenolate mofetil	IMPDH inhibitor	Severe AH without response to steroid at day 7 (standard of care *vs* steroid + mycophenolate)
Sterile necrosis and apoptosis	Emricasan[54]	Pancaspase inhibitor	Severe AH with steroid contraindications (placebo *vs* emricasan)
Impaired regeneration	G-CSF[63,64]	HPC mobilization	Severe AH without response to steroid at day 7 (placebo *vs* G-CSF)
	IL-22[59,73,74]	Hepatoprotective effect	Only preclinical studies

FXR: Farnesoid X receptor; OCA: Obeticholic acid; AH: Alcoholic hepatitis; LGG: Lactobacillus GG; LPS: Lipopolysaccharide; IL-1RA: Interleukin-1 receptor antagonist; IL-1: Interleukin-1; IMPDH: Inosine-5′-monophosphate dehydrogenase; G-CSF: Granulocyte-colony stimulating factor; HPC: Hepatic progenitor cell; IL-22: Interleukin-22.

sponders to steroids, only 25% of whom being alive at 6 mo. Recently, an early LT concept was suggested to those with a first episode of severe ASH not responding to steroids[53]. Explicit improvement of survival was observed in patients who received early LT compared to historical controls without response to steroids[53]. Obviously, early LT in ASH may be relevant only in highly selected patients with a first episode of severe ASH, a favorable addiction profile, and not responding to medical therapy.

Novel therapies

Despite the current specific therapies against AH, the overall prognosis of severe AH remains dismal. Owing to the scarcity of available therapeutic resources, undoubtedly, there is an urgent need for novel and innovative therapies to combat against severe AH. Over the past several decades, we have made great progress in grasping the clinical course of AH but not been capable of successfully identifying therapeutic targets. The failure of most clinical trials in AH results from a poor knowledge of the key disease drivers. Secondly, systemic large-scale studies are required before we can engage into targeted, therapeutic trials. Finally, all animal models used to test targets represent mild ALD but not severe liver disease that characterizes AH.

Thus, to settle the aforementioned issues, we are increasingly encouraged to conduct multi-center collaborative trials that use common protocols, include biomarkers, and address the spectrum of AH. To that end, recently, National Institute on Alcohol Abuse and Alcoholism has decided to support four AH consortia, which will explore translational studies and clinical trials for AH[54]. Clinical studies will collect and bank genetic or other biologic samples and consents to allow translational studies of basic mechanisms, genetics, epigenetics, and systems biology of AH severity and of treatment response. In parallel with that, several interventional trials are ongoing through multi-institutional consortia to test proof-of-concept for new therapies (Table 2)[54].

Scientific integration for developing new biomarkers and novel therapies for AH mainly focuses on several key elements of the pathogenesis of AH. Firstly, inflammation cascade and innate immune activation are demarcating features of severe AH compared to mild to moderate ALD[55-58]. The syndrome of AH results from severe inflammation and cytokine dysregulation[59,60]. Secondly, gut integrity is significantly altered in AH allowing pathogen-associated molecular patterns to enter the liver and systemic circulation and induce innate immune activation[61,62]. Gut-derived endotoxins and other bacterial products that trigger inflammation are a consequence of increased permeability and altered gut barrier function[62]. Thirdly, cell survival and death pathways contribute to liver dysfunction and the release of damage-associated molecular patterns that further fuel inflammation including hepatocellular apoptosis, sterile necrosis, and injury[61]. Finally, hepatocellular regeneration is profoundly impaired in patients with severe ASH with liver failure. In this regard, it is therapeutically important to characterize the mechanisms of the poor hepatocyte regeneration and promote the differentiation of progenitor cells into functional mature hepatocytes[63-66].

CONCLUSION

There is a pressing need for better definitions to distinguish AH from other clinical syndromes. The definitions need to be related to risk and outcomes, to improve clarity of taxonomy, reduce problems with basic *vs* clinical classification, and aid in treatment decisions. To standardize the nomenclature of AH, we should compare the clinical, analytical, and molecular characteristics of early ASH that is completely asymptomatic with those of classical AH that appears in patients with jaundice and/or decompensation. Alcohol abstinence is the sine qua non of therapy for AH, and, in the milder forms, is prerequisite to clinical recovery. Severe ASH may progress to multi-organ failure and, in particular, renal impairment and infection are the most worrisome complications requiring screening, prevention, and treatment. Clinical

prognostic scores such as MDF and MELD are useful tools to determine whether to initiate steroids and the Lille model at day 7 can be applied to assess responsiveness to steroids as stopping rules. Pentoxifylline can be alternatively used as a first-line therapy in severe ASH patients with contraindications to steroids. However, pentoxifylline provides no additional beneficial effect to patients with severe ASH receiving corticosteroids. Early switch to pentoxifylline either does not significantly improve survival in non-responders to steroids. Convincingly, future studies should include homogenous population and direct to AH patients with intermediate severity and partial or non-responders to steroids. Strategically, we should explore novel therapeutic targets to restore altered gut mucosal integrity, suppress inflammation based on cytokine profiles, promote hepatic regeneration, and limit innate immune responses in severe ASH.

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TOPIC HIGHLIGHT

WJH 6th Anniversary Special Issues (6): Liver transplantation

Difficulties in diagnosing acute kidney injury post liver transplantation using serum creatinine based diagnostic criteria

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Abstract

Renal function in patients with advanced cirrhosis is an important prognostic factor for survival both prior to and following liver transplantation. The importance of renal function is reflected by the introduction of the model for end stage liver disease (MELD) score, which includes serum creatinine. The MELD score has been shown to predict the short term risk of death for transplant wait listed patients and is currently used by many countries to allocate liver transplants on the basis of severity of underlying illness. Changes in serum creatinine are also used to stage acute kidney injury. However prior to liver transplantation the serum creatinine typically over estimates underlying renal function, particularly when a colorimetric Jaffe based assay is used, and paradoxically then under estimates renal function post liver transplantation, particularly when immunophyllins are started early as part of transplant immunosuppression. As acute kidney injury is defined by changes in serum creatinine, this potentially leads to over estimation of the incidence and severity of acute kidney injury in the immediate post-operative period.

Core tip: Acute kidney injury is defined and severity graded based on changes in serum creatinine. Increasing concentrations of bilirubin interefere with laboratory determination of creatinine and reduce creatinine estimations. Post transplantation serum creatinine increases due to a combination of fall bilirubin and the loading doses of calcineurin inhibitor immunosupressants. This combination leads to an over estimation of the lesser grades of acute kidney injury post liver transplantation.

WHY IS PERI-OPERATIVE RENAL DYSFUNCTION IMPORTANT IN LIVER TRANSPLANT RECIPIENTS?

Renal dysfunction is strongly associated with increased risk of mortality in patients with advanced chronic liver disease both awaiting liver transplantation (LT) and also peri-operatively[1-4]. Indeed renal function as determined by estimation of the serum creatinine concentration has been included in the model for end stage liver disease (MELD) score, which predicts the likelihood of death within 3 mo for patients wait listed for liver transplanta-

Table 1 Definitions of acute kidney injury using changes in serum creatinine between the Risk Injury Failure EndStage[7], Akute Kidney Injury Network[8] and Kidney Disease Improving Global Outcomes[9] criteria

Criteria	RIFLE[7]	AKIN[8]	KDIGO[9]
Date of release	2004	2007	2012
Time interval	Diagnosis and Staging: Within 1-7 d and sustained more than 24 h	Diagnosis: Within 48 h Staging: 1 wk	Diagnosis: 50% increase within 7 d or $\geq$ 0.3 mg/dL (26.5 μmol/L) within 48 h
Stage 1 or R	Increased SCr 1.5-1.9 times baseline	Increased SCr 1.5-1.9 times baseline or $\geq$ 0.3 mg/dL ($\geq$ 26.5 μmol/L) increase	Increased SCr 1.5-1.9 times baseline (7 d) or $\geq$ 0.3 mg/dL ($\geq$ 26.5 μmol/L) increase (48 h)
Stage 2 or I	Increased SCr 2.0-2.9 times baseline	Increased SCr 2.0-2.9 times baseline	Increased SCr 2.0-2.9 times baseline
Stage 3 or F	Increased SCr 3.0 times baseline, or Increase in SCr $\geq$ 4.0 mg/dL (350 μmol/L) with an acute rise of $\geq$ 0.5 mg/dL (44 μmol/L)	Increased SCr 3.0 times baseline, or Increase in SCr $\geq$ 4.0 mg/dL (350 μmol/L) with an acute rise of $\geq$ 0.5 mg/dL (44 μmol/L)	Increased SCr 3.0 times baseline, or Increase in SCr $\geq$ 4.0 mg/dL (350 μmol/L)

SCr: Serum creatinine; RIFLE: Risk Injury Failure EndStage; AKIN: Akute Kidney Injury Network; KDIGO: Kidney Disease Improving Global Outcomes.

tion and is used by several countries to preferentially allocate organs to those with more severe disease[2]. Serum creatinine is also part of the United Kingdom End Stage Liver Disease (UKELD) score which similarly predicts 12 mo waiting list mortality[5]. As such accurate assessment of renal function is important, particularly for patients with underlying chronic kdney disease, for example, patients with non-alcoholic steatohepatitis (NASH), due to coexisting diabetic, hypertensive micro or macrovascular renal disease[4,6]. Hence these patients then develop more renal dysfunction after LT, which is associated with increased mortality.

CURRENT DEFINITIONS OF ACUTE KIDNEY INJURY

In order to standardize the definition of acute renal failure, now termed acute kidney injury (AKI), the risk injury failure loss of function and end stage renal failure (RIFLE) guideline criteria were developed[7]. These were subsequently revised by both the acute kidney injury network (AKIN)[8] and more recently by the kidney disease improving global outcomes (KDIGO) group[9] (Table 1). Although all three classifications define stages of severity of AKI by both urine ouput and serum creatinine concentration, in practice most studies have retrospectively used changes in serum creatinine to determine both the incidence and severity of AKI in peri-operative LT transplant recipients. Although these AKI classification systems report increasing mortality with increasing AKI severity, in keeping with other patient groups[10], the question arises as to whether they accurately detect acute kidney injury. In theory the diagnosis of AKI based on these classifications should be relatively straight forward as to whether patients post LT have a 50%, 200%, 300% increase in serum creatinine or an absolute rise above a critical threshold to make the diagnosis of AKI and award an AKI classification (Table 1) Whereas the major hurdle in general medical or surgical practice is determining the "true" baseline serum creatinine measurement upon which to evaluate subsequent changes, all LT patients will have a pre-operative measurement, and initial daily post-operative serum creatinine estimations. So it

would appear a simple matter of cataloguing changes in serum creatinine post-operatively to determine the incidence and severity of AKI post LT.

However serum creatinine estimations typically over estimate "true" renal function pre-operatively[11], and then under estimate renal function post-operatively so potentially increasing the reported incidence of AKI.

WHY DOES SERUM CREATININE OVER ESTIMATE RENAL FUNCTION PRE-OPERATIVELY?

Creatinine is non-enzymatically converted from creatine in muscle. Creatinine is predominantly synthesized in the liver. As such patients with chronic liver disease awaiting LT typically have reduced creatine synthesis due to the combination of reduced dietary protein intake and chronic liver disease. The conversion of creatine through to creatinine depends upon both muscle mass and muscle turnover. Patients wait listed for LT are at increased risk of sarcopenia (muscle wasting) and typically take less exercise than healthy controls, so have a lowered conversion of creatine to creatinine[12]. In addition as creatinine is measured as a concentration, then as many patients with chronic liver disease have oedema, with ascites this results in a larger volume of distribution of creatinine in the body and a lower serum creatinine concentration[13]. Serum creatinine may also be affected by the concomitant prescription of drugs, such as calcitriol which affect the renal tubular secretion of creatinine[14].

Serum creatinine estimations tend to overestimate glomerular filtration rate (GFR) in patients with chronic liver disease, as the most commonly used laboratory method is a colorimetric assay which is subject to interference by chromogens, including bilirubin (both conjugated and unconjugated). As such these chromogens lower the measurement of creatinine, so making serum creatinine an even less precise surrogate of GFR in jaundiced patients. There have been several attempts to improve the accuracy of the Jaffe assay in an attempt to reduce interference from chromogens, such as bilirubin, glucose, uric acid, ketoacids, pyruvate, and some antibiot-

Table 2 Cohort of 329 adult patients transplanted for advanced cirrhosis			
	RIFLE[7]	AKIN[8]	KDIGO[9]
Stage 1 or R	53	93	97
Stage 2 or I	28	28	28
Stage 3 or R	8	8	8
Stage 3 initiation of RRT	17	17	17

Changes in renal as assessed bt RIFLE, AKIN and KDIGO criteria for acute kidney injury for changes in serum creatinine. RIFLE: Risk Injury Failure EndStage; AKIN: Akute Kidney Injury Network; KDIGO: Kidney Disease Improving Global Outcomes; RRT: Renal replacement therapy.

ics[15]. These include acid blanking and absorption techniques with Fuller's earth or Lloyd's reagent, and delayed rate reactions. Initially these were laborious and time consuming so unsuitable for routine use. However the newer generations of chemical pathology laboratory multichannel analyzers now often routinely incorporate modified delayed rate or blank correction creatinine assays. Another modification, the kinetic alkaline picrate method produces a differential rate of colour change between creatinine and non-creatinine chromogens. Enzymatic methods to determine serum creatinine, using creatininases and creatininase hydrolases have been shown to be more reliable and less affected by chromogens[16], but are generally much more expensive and as such has not been widely introduced into routine clinical practice. To put this into clinical perspective[17] the interference that occurs with serum bilirubin concentrations > 62 μmol/L (3.68 mg/dL), result in significant differences in reported serum creatinine values between different methods (modified Jaffe, compensated kinetic Jaffe, enzymatic and standard Jaffe), resulting in significantly different MELD scores. If differences in MELD score are only 1-2 points, then this would have little clinical consequence, but differences of 3 or 4 points which are seen with bilirubin concentrations between 100 μmol/L (5.85 mg/dL) and 200 μmol/L (11.6 mg/dL) are clinically relevant. At even higher serum bilirubin concentrations (> 23.4 mg/dL), *i.e.*, those with the highest priority for LT. then this interference can result in differences in up to 7 MELD points As such the method used to estimate serum creatinine used in MELD scoring should be taken into account, as some patients inadvertently will be discriminated against with respect to others, in terms of priority for LT, when allocation is based on MELD score.

A further problem associated with accuracy and precision of serum creatinine measurements is a lack of universal standard for creatinine. For example in the United Kingdom there were 34 variations of the standard Jaffe reaction used by United Kingdom National Health Service (NHS) clinical chemistry laboratories. To standardize assays, all NHS laboratories were sent isotope dilution mass spectroscopy (IDMS) standards to develop their own correction factors for their creatinine assays. However IDMS standards do not allow for interfering chromogens and as such marked differences remain in serum creatinine estimations between UK NHS laboratories serving liver transplant centres[17,18].

Perhaps not surprisingly because of these multiple limitations of serum creatinine in estimating renal function in patients with advanced chronic liver disease a meta-analysis proposed that GFR estimation by inulin clearance was the only way for accurate assessment of renal function[19], but unfortunately inulin clearance remains impractical for routine clinical use.

WHY DOES SERUM CREATININE UNDER ESTIMATE RENAL FUNCTION POST-OPERATIVELY?

Although creatinine excretion is predominantly by glomerular filtration, there is an additional amount of creatinine secreted by the renal tubule. As such drugs which cause a reversible reduction in glomerular filtration can lead to an increase in serum creatinine, without necessarily causing renal damage. In the post-operative LT patient these would include non-steroidal anti-inflammatories given for post-operative analgesia, and on-going prescription of pre-operative antihypertensive medications, not just angiotensin converting enzyme inhibitors, angiotensin receptor blockers and renin inhibitors. However the drugs most likely to reduce GFR in the immediate post-operative period are the immunophyllins, particularly tacrolimus. In addition to reducing GFR, immunophyllins also reduce renal tubular creatinine secretion by inhibiting cyclooxygenase 2 in the renal medulla so causing renal tubular ischaemia[20].

Hence serum creatinine tends to overestimate renal function prior to LT, and then under estimate renal function post operatively. Thus when using the current definitions of acute kidney injury based on changes in serum creatinine there is a tendency to overestimate both the incidence and severity of acute kidney injury post LT (Figure 1, Table 2). This is most marked for lesser degrees of acute kidney injury, and most noticeable between the RIFLE and other scoring systems (Table 2), due to the differences in definitions with RIFLE requiring a 50% increase compared to AKIN and KDIGO which only require an absolute increase of 0.3 mg/dL. So that switching from a high to a low serum bilirubin post transplantation and starting immunophyllin immunosuppression may be sufficient to cause a minor increase in measured serum creatinine to be classified as acute kidney injury stage 1 by AKIN and KDIGO, but less than the 50% increase required by RIFLE.

Altering peri-operative immunosuppression protocols to delay or avoid the initial use of immunophyllins may help to reduce kidney injury, by using monoclonal antibodies, such as simulect and CAMPath-1, particularly in those with NASH and other patients with pre-existing chronic kidney disease. Lower targets for tacrolimus trough doses have also recently been shown to improve graft survival and reduce both acute and chronic renal impairment[21,22]. Thus, risk modification is needed to optimize renal function in the pre, peri and postoperative

Figure 1 Cohort of 329 adult patients undergoing liver transplantation at the Royal Free Hospital. Serum creatinine measured using an enzymatic method shows a significant increase between the pre-operative and 1st post-operative day and the 3rd and 7th post-operative days respectively. Only 2.4% developed acute kidney injury stage 3 on serum creatinine criteria alone, suggesting that the most probable cause for the significant increase was due to changes in serum creatinine measurement due to a reduction in bilirubin and other chromagens, and the use of immunophyllins as immunosuppressive agents.

management of LT candidates: which may prevent or delay post-LT end stage renal disease[22].

WHAT ARE THE ALTERNATIVES TO MEASURING SERUM CREATININE?

Exogenous markers which are only cleared by the kidney are the most accurate methods for determining renal function. Inulin clearance remains the gold standard for measurement of renal function but cost and technical difficulties limit its use for routine practice[19]. Other direct methods of measuring GFR include exogenous radiolabelled substances (^{51}Cr-ethylene diamine tetra acetic acid (EDTA), ^{99m}Tc-diethylenetriamine pentaacetate (DPTA) and [51]I-iothlamate) or non-radioactive agents (iohexol or iothalamate)[23]. However these methods have typically not been extensively validated in patients with cirrhosis and ascites. As such the British Nuclear Medicine Society Guidelines stated that liver failure, ascites, oedema and low clearance status may produce inaccurate clearance values[24]. Following injection there will be an initial redistribution from plasma into the ascites, and then a later re-equilibration from the ascites back into the plasma. As such ascites has been reported with increased clearances of 16-20 mL/min based on compartmental models[24]. To overcome these difficulties delayed sampling has been used to improve the calculation of the decay slope and time zero[25]. These isotope and radiocontrast techniques correct measurements of glomerular filtration for body surface area, which is calculated using equations based on height and weight. The presence of ascites and changes in body composition[26-28], with loss of muscle and fat mass change the normal relationship between calculated body surface area and muscle mass, and so add errors to the determination of GFR. Ideally these methods can be used for pre-operative assement of renal function, which

can then be used to stratify patients for risk of acute kidney injury post LT and individualise immunosuppression policies. However there use in the immediate post LT period is unclear when renal function is changing.

ESTIMATION OF CREATININE CLEARANCE BY URINE COLLECTIONS

Creatinine clearance, using 24 h urine or shorter timed collections was the traditional method for assessing renal function. However, creatinine clearance underestimates GFR in children and when the serum creatinine levels are high the relative proportion of creatinine secreted by the renal tubules is greater[29] (Table 3). In healthy adults, creatinine clearance typically overestimates "true" GFR based on inulin clearance. Limitations are associated not only with the use of serum creatinine, measurements but also tubular creatinine secretion, which increases with underlying chronic kidney disease, proteinuria, drugs and also extra-renal elimination of creatinine by micro-organisms in the gastro-intestinal tract[14]. Pre-operatively, there may be up to 25% variation in GFR estimation based on creatinine clearance[29], due to incomplete urine collections, timing errors, errors in urine volume measurement, variations in tubular excretion or re-absorption of creatinine, serum creatinine dilution due to increased fluid retention and other unpredictable factors. Due to these multiple errors, there is no evidence that creatinine clearance is superior to serum creatinine in determining renal function in cirrhosis.

MATHEMATICAL ESTIMATIONS OF GLOMERULAR FILTRATION RATE

To overcome some of the limitations of 24 h urine collection, a number of different mathematical formulae have been developed, which incorporate serum creatinine to provide an estimate of GFR (eGFR). However these formulae were developed from a stable chronic kidney disease population, and not for patients with chronic liver disease, or for patients with changing renal function in the post-operative LT period. Although these formulae are increasingly being used in the intensive care setting they have not been validated. Currently used formulae include the Cockcroft-Gault (C-G)[30] and Modification of Diet in Renal Disease (MDRD)[31] formulae. The C-G formula requires serum creatinine, weight, gender and age whereas the MDRD formula incorporates serum creatinine, ethnicity, gender and age (MDRD-4), or creatinine, ethnicity, gender, age, albumin and urea (MDRD-6). Thus, in contrast to C-G formula, a body weight variable (which is difficult to assess as lean body mass in ascitic and malnourished patients) is not needed, and the MDRD equations use ethnicity, gender and age and then adjusts for 1.73 m[2] body surface area (without any assessment of height or weight). In cirrhosis, although there is discrepancy when compared to ^{125}I-iothalamate[32], the MDRD-6 equation is considered a more accurate for-

Table 3 Comparison of the established methods for assessing renal function in clinical practice

	Advantages	Disadvantages
Serum marker		
Creatinine	Widely available	Influenced by several factors unrelated to renal function, including dehydration and volume expansion, dietary protein, muscle mass, physical activity and thyroid hormones renal tubular secretion affected by chronic kidney disease, proteinuria and drugs not an early biomarker of acute kidney injury
Clearance of exogenous marker	"Gold standard"	Absence of standardization of the laboratory methods for jaundiced patients technical difficulties and expense make impractical for routine clinical practice stable renal function
		Less reliable in patients with oedema, ascites, pleural effusions and sarcopenia
Creatinine Clearance f (24 h urine collection)	? more accurate compared to Cr	Inconvenient for outpatientsoverestimates GFR in proteinuria chronic kidney disease influenced by muscle metabolism and diet, inflammatory disease and malnutrition
		Unexplained variation due to incomplete urine collection and errors in urine volume measurement overestimation of GFR in patients with cirrhosis
Mathematical formulae based on Cr	Easier method compared to 24 h urine collection	Not validated for patients with changing renal function (acute kidney injury, muscle wasting disorders)
		Does not overcome the limitations in serum creatinine
C-G formula	Requires only gender, age, body weight	Difficult to determine body weight in patients with ascites and post LT
MDRD formula	Body weight is not needed ethnicity, gender and age are taken into account	Has not been validated in patients with chronic liver disease
		6-variables formula: needs albumin, urea
		Only validated in stable chronic kidney disease patients

GFR: Glomerular filtration rate; Cr: Serum creatinine; C-G: Cockcroft-Gault; MDRD: Modification of Diet in Renal Disease; CKD: Chronic kidney disease; LT: Liver transplantation.

mula, compared to C-G, possibly because it incorporates urea and albumin, which are abnormal in cirrhotics and it excludes body weight, a variable which may be difficult to determine in malnourished patients with ascites[28]. However the MDRD-4 formula is the formula reported by most laboratories, as it was equally accurate as the original six-variable formula in screening for patients with chronic stable kidney disease. In cirrhosis, C-G and both MDRD formulae typically overestimate true GFR, particularly in those patients below 50 years old or those with ascites[33]. Due to inaccuracies of the MDRD-4 equation in determining renal function in patients with an eGFR > 60 mL/min, a new creatinine-based equation known as the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation, using the same variables with MDRD-4 formula, has been proposed, but its superiority in patients with cirrhosis has not been validated[34]. Nevertheless, the use of such formulae does not overcome the limitations in serum creatinine measurement. It has been recommended that creatinine results used for calculating eGFR should be traceable to an IDMS reference method[34], but the IDMS standards do not correct for the effects of chromogens. To overcome the problem that these formulae were derived from cohorts without liver disease new formulae for patients with cirrhosis have been proposed including adding the Child Turcot Pugh (CTP) score and ascites into the formula[35]. These newer formulae have been reported to show better agreement with "true" GFR, compared to the MDRD formulae, but require further external validation before they can be introduced into clinical practice.

ALTERNATIVES TO CREATINNE

Serum cystatin C

Serum cystatin C is an extracellular inhibitor of cysteine proteases[36]. It was originally thought that cystatin C was uniformly produced and secreted by all nucleated cells, but actually has a greater diurnal variation than serum creatinine. Cystatin C is freely filtered by the renal glomeruli and then taken up and catabolized in the proximal tubules. It was initially considered a more sensitive indicator of renal function compared to creatinine[37], in several disease groups including cirrhosis[38,39]. Consequently several Cystatin C based GFR equations, were derived[40,41]. More recently cystatin C has been recognized to be affected by numerous factors including inflammation[42] body composition, proteinuria, cardiovascular risk factors[43,44] infection, thyroid dysfunction, underlying malignancy, smoking and a number of drugs; including corticosteroids, cotrimoxazole, angiotensin converting enzyme inhibitors, and calcineurin inhibitors. Cystatin C has been reported to increase with severity of chronic liver disease[45] as it correlates with bilirubin, INR and CTP stage, and negatively with serum albumin and peripheral platelet count[46]. As cirrhosis evolves the increasing cystatin C values may be related to increased production, secondary to inflammation, or decreased clearance due to reduced renal function. The original cystatin C equations were all derived from non-liver disease populations[47,48]. Recentstudies have evaluated cystatin C GFR formulas in patients with cirrhosis[35]. One reported that although cystatin C formulas were more accurate than the creatinine formulas[49]. GFR estimations were significantly different

to inulin clearance. In the second study serum cystatin C formulas not only significantly overestimated renal clearance compared with ^{51}Cr-EDTA but did not provide any advantage over serum creatinine formulas[35], and serum cystatin C values were significantly affected by the presence of ascites. Although a third study reported cystatin C to more accurately represent renal function than serum creatinine[50]. There has recently been standardisation of serum cystatin C assays and more studies are required to try and develop specific GFR formulae for cystatin C in patients with cirrhosis. However as cystatin C is increased by inflammatory states, changes in cystatin C performs no better than serum creatinine in determing acute kidney injury in the immediate post operative lT period.

NEUTROPHIL GELATINASE ASSOCIATED LIPOCALIN

Acute kidney injury is a potentially life threatening complication in patients with cirrhosis. Neutrophil Gelatinase associated Lipocalin (NGAL) has been recently introduced as an early marker of tubular dysfunction in acute kidney injury. Several studies have reported that NGAL increases in urine and plasma shortly after injury to renal tubular cells and it can be used to aid the differential diagnosis between acute tubular necrosis and volume responsive causes of acute kidney injury in patients with chronic liver disease. Urinary and serum NGAL not only reflect renal tubular injury but are also markers of the host systemic inflammatory response, as NGAL is part of the innate immune response designed to restrict iron availability to invading micro-organisms. After initial promising reports of the superiority of NGAL to other acute kidney injury biomarkers including creatinine[51] more recent reports have failed to substantiate the earlier studies, especially when studies include patients with pre-existing chronic kidney disease.

NGAL has been evaluated in patients with cirrhosis[52]. Patients with kidney dysfunction irrespective of aetiology had greater serum NGAL levels compared to those without kidney dysfunction irrespective of the presence of ascites. Urinary NGAL levels were also increased significantly in patients with cirrhosis and acute tubular necrosis (median values 417, range 239-2242 μg/g creatinine) compared to those with other causes of acute impairment of kidney function, for example hepatorenal syndrome (not associated with active infections), pre-renal azotemia secondary to volume depletion, and chronic kidney disease. However, plasma levels of NGAL were not helpful in the differential diagnosis of kidney dysfunction, in particular reversibility of acute kidney injury. Urinary NGAL levels were found to be significantly increased with urinary tract infections, whereas plasma NGAL was not different in patients with and without bacterial sepsis. As such, NGAL did not aid the differential diagnosis between acute tubular necrosis and hepatorenal syndrome precipitated by infection, as NGAL levels increased in both groups. Thus, larger multicentre trials are awaited to determine whether urinary NGAL, and newer markers of acute kidney injury, such as kidney injury molecule 1 (KIM-1) and urinary IL-18 excretion have a role in diagnosing acute kidney injury in patients with chronic liver disease. Similarly studies in patients following LT have reported that NGAL rises in patients who develop acute kidney injury[53]. Although n serum NGAL may rise earlier than creatinine post LT, this may simply reflect the severity of the ischaemia-reperfusion injury and the initial dilutional effect of intra-operative fluid administration on serum creatinine. Additional studies are warranted to determine whether there is a clinical role for these newer biomarkers in the diagnosis of acute kidney injury following LT.

CONCLUSION

Renal dysfunction increases the risk for mortality in patients with chronic liver disease both prior to and post liver transplantation. Changes in serum creatinine are now used to define acute kidney injury. As such, although small changes in serum creatinine are linked to adverse outcomes, changes in serum creatinine concentration can be influenced by changes in hydration status[54], and in particular for the patient with cirrhosis a falling serum bilirubin post liver transplant can lead to an apparent increase in serum creatinine, simply due to loss of interference with the colorimetric assay, and secondly due to changes in intra-renal perfusion associated with immunphyllins, without necessarily implying acute kidney injury. As serum creatinine is likely to remain the routine clinical marker of kidney function, additional biomarkers are required to help differentiate between assay interference and reversible changes in renal function on one hand and acute kidney injury on the other.

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REVIEW

Laser ablation for small hepatocellular carcinoma: State of the art and future perspectives

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Abstract

During the last two decades, various local thermal ablative techniques for the treatment of unresectable hepatocellular carcinoma (HCC) have been developed. According to internationally endorsed guidelines, percutaneous thermal ablation is the mainstay of treatment in patients with small HCC who are not candidates for surgical resection or transplantation. Laser ablation (LA) represents one of currently available loco-ablative techniques. In this article, the general principles, technique, image guidance, and patient selection are reported. Primary effectiveness, long-term outcome, and complications are also discussed. A review of published data suggests that LA is equivalent to the more popular and widespread radiofrequency ablation in both local tumor control and long-term outcome in the percutaneous treatment of early HCC. In addition, the LA technique using multiple thin laser fibres allows improved ablative effectiveness in HCCs greater than 3 cm. Reference centres should be equipped with all the available techniques so as to be able to use the best and the most suitable procedure for each type of lesion for each patient.

Core tip: The aim of this review is to describe the basic principles, results in terms of safety and efficacy, and recent advancements in laser ablation (LA). This mini-invasive technique is a less known and few employed procedure as compared to radiofrequency ablation (RFA). However, according to published studies LA is as safe and effective as RFA. In the review the technique and potential advantages of LA are described. Our ambition is to provide the hepatologists, and other physicians, with an updated approach to this ablative technique.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a global health problem, ranking as the sixth most common malignancy and the third most frequent cause of cancer-related death worldwide[1-3]. Its incidence is rising, mostly due to the diffusion of hepatitis B or C virus infection, alcohol-related cirrhosis, and nonalcoholic steatohepatitis[2,4]. Its incidence increases with advancing age and is more common in males[5,6]. Thanks to semiannual surveillance of the high-risk population by ultrasound and alpha-feto protein, HCC is increasingly detected at early stage, when curative treatments can be employed[3,4,7,8]. Resection is the mainstay of treatment for patients with HCC solitary tumours, preserved liver function, or mild portal hypertension not suitable for liver transplantation (LT)[9-11],

the latter being the only cure of both HCC and underlying cirrhosis[12]. However, resection may be associated with significant morbidity as well as tumour recurrence, which occurs in about 70% of patients at 5 years[13-17]. When surgery is unfeasible, percutaneous or laparoscopic tumour ablation is the most widely used treatment that can achieve the complete local control of the disease in properly selected candidates[4,18,19]. This procedure is also cost-effective as compared to surgical treatments because it destroys only a minimal amount of liver parenchyma whilst reducing the number of hospitalizations[18,20,21]. Among the available local ablative techniques, laser ablation (LA) is a less investigated and little-used treatment. Our ambition is to provide hepatologists and other physicians with an updated approach to this ablative technique.

GENERAL PRINCIPLES

Laser source

In 1983, Bown[22] described for the first time the use of laser light to ablate liver tumours. Laser devices transform electrical energy into light energy, which interacts with tissue to produce heat and cause cell death[23]. Laser light can be delivered precisely and predictably into any location of the liver. Laser is an acronym for "light amplification by stimulated emission of radiation", a principle based on the spontaneous emission of characteristic photons by excited atoms. Because laser light is coherent and monochromatic, it can be highly collimated and focused and large amounts of energy can be transmitted over long distances without significant losses. The light produced is of a specific wavelength and defines the properties of the laser system and the extent of tissue penetration. Due to the optimal penetration of light in the near-infrared spectrum, neodymium-doped yttrium aluminium garnet (Nd:YAG) lasers with a wavelength of 1064 nm and diode with a wavelength of 800-980 nm are preferred for percutaneous LA[24]. The optical (scattering, reflections, and absorption), thermal (conductivity and thermal storage), and blood flow characteristic of the tissue govern thermal diffusion processes and define the temperature map within the laser-exposed area[25]. The extent and completeness of tumour necrosis depends on a balance between the power applied and tissue charring[26].

Laser transmission

Laser light is transmitted from the source to the patient through flexible optic fibres that have specially designed diffuser tips. An important role is played by the shape, size, and design of the fibre[27-29]. The most common types of fibre currently used are the bare-tip[30,31] and cylindrical diffusing quartz fibres[29]. For the ablation of large masses or multiple tumours located at different sites, beam-splitting devices allowing the simultaneous delivery of light into multiple fibres can be used[24,32,33]. Multi-fibre systems have a synergistic effect by reduced heat dissipation between fibres[24,32]. The use of water-cooled laser application sheaths allows operating at higher powers and makes large lesions faster. Lesion diameter approaches 5-8 cm[33-35] with minimal charring and carbonization[26,29,36-41]. Because there is no destruction of the fibre, multiple applications are generally quite easy and longer lesions can be generated by simply pulling the fibre back in the applicator or advancing it forward.

Role of imaging guidance

The ablation procedure is performed under conscious sedation and local anaesthesia. Real-time ultrasound (US), computed tomography (CT), or magnetic resonance imaging (MRI) are employed to guide either one or multiple thin needle-fibres[42,43] or a coaxial guide needle through tissue and into lesion[44,45]. Most patients are treated as day-cases in outpatient clinics. The number of treatment sessions varies according to the size and number of lesions. Follow-up evaluations are performed within 24 to 48 h or within 4 wk from procedure, and then at 3 to 6 mo intervals as is usual with other thermal techniques[42,44].

US is used for targeting and monitoring during the procedure, while CT is mainly used for post-treatment assessment. Heated tissue becomes hyperechoic because of water loss; this is most pronounced when there is tissue charring[46,47], particularly evident when using uncooled devices as in the laser technique with thin needle-fibres[31,42]. As it is well known, the main disadvantage of US guidance is that it is not suitable to accurately evaluate the temperature or the size of the ablative zone being created[47,48]. Otherwise, the contrast-enhanced US is useful to detect residual disease during procedure[49,50].

Real-time CT is unreliable for the detection of the early signs of laser-induced tissue injury. However, contrast enhanced CT 24 h after the procedure identifies coagulation zone as a not perfused area and correlates precisely with histology. The main role is the detection of residual or recurrent tumour following LA. Within a few days from treatment, the edges of ablation zone become indistinct due to inflammatory changes. During follow-up, local recurrences are easily visualized as contrast-enhancing foci adjacent to the necrotic area[30,51-53].

In contrast, MRI performed during LA allows the monitorization of the actual size and temperature of the ablation zone. MRI is the most accurate method for planning, monitoring, controlling, and assessing laser-induced coagulative necrosis[54-56]. LA power settings and session-treatment durations can be adjusted to obtain appropriate temperature elevations beyond tumour margins, thereby achieving a sufficient safety margin of necrosis. MRI is well suited to detecting residual undamaged tissue or local recurrence in the transition area[57]. This procedure is mainly used in combination with the high power water-cooled laser systems so that the treatment can be performed safely and with a better control of the extent of the ablated area[40,58]. MRI images can be acquired in near real time in any arbitrary plane. This has advantages in optimal planning of the procedure and in more accurately targeting the treatment volume and avoiding damages to critical structures[59]. After treatment delivery, changes

in parameters such as tissue perfusion or diffusion may be used in addition to routine relaxation mechanisms (T1 and T2 weighting) to visualize the extent of ablation. Thus, because modern LA delivery aims to generate lesions rapidly in tissue that has many connective heat sinks and critical structures (such as brain and prostate), the ability to visualize and often quantify tissue temperature changes can be crucial feedback to the safety, efficacy, and overall outcomes of the thermal procedures[24,59,60].

Indications

According to the procedure used and the accessible facilities, selection criteria vary among centers[31,42,44,53,61-65] being established on size, number, and site of HCC in patients who are considered not good candidates for resection or liver transplantation. Although lesions of up to 6 cm have been treated[43], patients eligible for LA are those whose tumours are in accordance with the Milan criteria, irrespective of their location[66-68]. In fact, cancers located near major vessels, bile ducts, bowel, or diaphragm can be ablated with caution with RFA[69] but they can be more safely treated both with MRI-guided technique[44] and, more easily, with very thin devices with a calibre of 0.7 mm (21 gauge)[42,43,53,65-68]. MRI-guided technique allows confident ablation of high-risk located lesions using the real time thermometry and multiplanar MRI targeting[44,58,61,63].

Effectiveness and outcome data

Several retrospective cohort studies have shown that LA is a safe and feasible procedure for the treatment of HCC[31,42-44,53,61-68]. Using multiple bare fibres introduced through 21-gauge needles positioned under US-guidance, the reported complete response rate ranges from 82% to 97%[66-68]. In lesions in high-risk sites, complete response is 95.5%[65]. In patients with monofocal HCC ≤ 4 cm or three nodules ≤ 3 cm each, reported cumulative survival rates at 3 and at 5 years range from 52% to 68% and from 15% to 34%, respectively[53,66-68]. Tumor size, tumor location, and complete ablation were the main factors affecting the outcomes. In a multicenter study, Child's class A patients had a 5-year cumulative survival of 41%; the median survival time was 65 and 68 mo in patients with tumor size ≤ 3 cm and ≤ 2 cm, respectively. The authors stated that the ideal candidates for LA are younger patients with serum albumin within the normal range and a tumor size ≤ 2 cm in whom it is very likely that complete ablation will be achieved. The median time to recurrence was 24 mo and the median disease-free survival time was 26 mo[68]. Like RFA and microwaves ablation (MWA), LA resulted safe and effective also in the treatment of cirrhotic patients awaiting liver transplantation[70].

Promising results have been reported with the use of water-cooled higher power MRI-guided LA. A very low local recurrence rate and a complete response rate reaching up to 98% in nodules ≤ 5 cm has been achieved

with this technique[44,61]. In a study on 39 patients with 61 HCCs a complete ablation rate of 98% and a mean survival rate of 4.4 years were observed[61]. More recently, the same authors confirmed the high percentage of complete response in a cohort of 113 patients with 175 HCCs ≤ 5 cm followed for a period of over 15 years; 75% of the lesions were located at high-risk sites and median survival was 3.5 years[44].

To date there is only one controlled study comparing LA with RFA in treating a small cohort of patients (81 cirrhotic patients with 95 biopsy-proven HCCs) with early stage HCC (nodule ≤ 4.0 cm or three nodules ≤ 3.0 cm each). Thin multiple fibre technique to perform LA and single or cluster 17-gauge cool-tip electrodes for RFA were employed. The authors found LA and RFA to be equally effective; but fewer treatment sessions were needed in RFA group to achieve complete response. Neither significant differences in survival rates between the two methods nor significant complications were observed in both groups[71].

In a randomized prospective trial in a single centre with three years of follow-up being evaluated for final publication, the authors treated 140 patients with 157 biopsy-proven HCCs to compare LA and RFA (70 patients with 77 nodules and 70 patients with 80 nodules, respectively). Median follow up in RFA and LA groups was 21 and 22.5 mo, respectively. Complete response was observed in 97.2% and in 95.8% of RFA and LA group patients, respectively. Median time to tumour recurrence was 25.6 and 37.8 mo in RFA and in LA groups, respectively (P = 0.129). Estimated probability of survival at 1, 2, and 3 years was 94%, 88%, and 66% in RFA group and 94%, 81%, and 59% in LA group, respectively (P = 0.693). No major complications or significant treatment-related morbidity were observed in both groups. The authors concluded that LA was non inferior to RFA either in obtaining the complete ablation of HCC nodules or in long-term outcome[72].

Use in combination with other treatment

Multi-ablation therapy consisting of LA before trans-arterial-chemo-embolization (TACE) has been effective in large HCCs with a mean diameter of 5.2 cm (range, 3.1-9.6 cm)[73], with complete response achieved in 90% of the large tumors. Fifteen additional synchronous small HCC ≤ 3 cm in 11 patients were completely ablated (100%) with LA alone. The survival rate was overall 40% at 3 years and 60% in Child class A patients. The 1-, 2-, and 3-year local recurrence rate for the main tumors was 7% annually while the 1- and 2-year cancer-free survival rates were 74% and 34%, respectively. The rationale of this study was that LA reduces tumor volume within the range of TACE effectiveness and at same time can achieve complete destruction of large lesions with a lower number of TACE sessions (in 70% of patients only a single TACE session was done). Recently, the introduction in clinical practice of a novel needle guide system makes it possible to achieve complete ablation of nodules

Table 1 Studies reporting the outcome of Laser Ablation for small hepatocellular carcinoma

Ref.	Pts/Tumors no	Tumor size (cm) mean	Complete ablation[a], %	Local recurrence rate, %	Overall survival %		3-yr disease-free survival %	Major complication rate[b], %	Mortality rate, %	P value
					3-yr	5-yr				
Giorgio et al[31] (2000)	77/85	≤ 4.0[c]	82[f]	1.1				3.9[d]	1.3[d]	
Pacella et al[73] (2001)	30/30	> 5.0	90[f] (+ TACE)	7	40			0	0	
	30/15	≤ 3.0	100[f]	0				0	0	
	Child-Pugh A				60					
	Child-Pugh B				0					0.001
Pacella et al[66] (2001)	74/92	≤ 4.0[c]	97[f]	6	68	15		0	0	
	Child-Pugh A				73	31				0.052
	Child-Pugh B[7,8,9]				68	0				
Eichler et al[61] (2001)	39/61	≤ 5.0[e]	97.5[g]	0	4.4 yr			0	0	
Pacella et al[42] (2005)	82/99	≤ 4.0[c]	90.9[f]	8.8				1.5	0	
Francica et al[64] (2007)	148/169	≤ 4.0[c]	82[f]	14.7	52	27		0.6	0.6	
		≤ 2.0	95[f]	5						
		≤ 3.0	89[f]	15						
		> 3.0	74[f]	26						0.001
	Well-differentiated	≤ 3.0		0	58					
	Poorly-differentiated			25						0.008
Pacella et al[68] (2009)[1]	432/548	≤ 4.0[c]	79.6[f]	20[2]	61	34		1.6	0.2	
		≤ 2.0	85.1[f]		41					
	Child-Pugh A				63					
	Child-Pugh A	≤ 2.0			68[3]					
Francica et al[53] (2012)	106/116[4]	≤ 4.0[c]	92.2[f]	10.6				0.9	0.5	NS
	58/66	≤ 4.0[c]	95.5[f]							
Francica et al[65] (2012)	116/132	≤ 4.0[c]	100[f]	18.0	57	29				0.029[5]
				2.2[5]						
Eichler et al[44] (2012)	113/175	≤ 5.0[e]	98[g]	1.1	54	30		0	0	
Di Costanzo et al[43] (2013)	104/116	≤ 6.0	87.6[f]	16						
		≤ 5.0	91.7[f]							
Di Costanzo et al[72] (2013)[6]	70/80 (LA)	≤ 5.0[6]	96.3[f]	23.9	66		42	0	0	
	70/77	≤ 5.0[6]	97.4[f]	25.7	59		43	0	0	0.693

[a]Calculated per tumor; [b]Calculated per patient; [c]Single tumor ≤ 4 cm or ≤ 3 nodules each ≤ 3 cm; [d]In patients with Child-Pugh C; [e]Single tumor ≤ 5 cm or multiple ≤ 5; [f]With bare fibers; [g]With water-cooled fibers; [1]Multicentric retrospective study; [2]Local and distant; [3]In pts Child-Pugh class A well-differentiated tumor; [4]At risk site; [5]Only if the ablative margin was ≥ 7.5 mm; [6]RCT with Milan criteria; [7,8,9]Refers to the Child-Pugh class. NA: Not available; NS: Nonsignificant; CR: Complete response.

up to 5 cm in a single session in 91.7% of cases without resorting to combined treatment[43]. All data reported above are summarized in Table 1.

LA may also be combined with other modalities to achieve an increased volume of tumor necrosis. Zou *et al*[74] demonstrated that combined therapy with PEI immediately followed by LA resulted in a significantly larger volume of coagulation zone with reduced residual tumor volume on rabbit VX2 liver tumors. These authors hypothesized that tissue destruction by ethanol may have resulted in increased thermal conduction. In addition, the sclerosis and/or destruction induced on small vessels by PEI causes a reduction of the heat-sink effect and thus an enhancement of laser ablation effect. To date there are no clinical trials with this technique.

Complications

Arienti *et al*[75] performed a multicenter study involving nine centers in Italy with 520 patients who underwent 1064 nm laser sessions for 647 HCCs. Analyzing 90 factors for each record, including tumour characteristics, the authors reported a major complications rate of 1.5% (0.8% death rates) and a minor complications rate of 6.2%. These authors enrolled in their retrospective and prospective study patients with HCC nodules of any size [387 (60%) small, 180 (28%) intermediate, and 74 (11%) large] including 29 (5.9%) patients in Child's class C and 72.1% of patients with portal hypertension. Major complications were associated with excess energy deposition and high-risk nodule locations. Minor complications proved to be associated with excess energy, high bilirubin level, and low prothrombin time. The authors who use MRI-guidance and high-calibre water-cooled devices reported no major complications or cases of mortality in 152 patients treated using large bore water-cooled devices[44,61]. In these series, no case of tumour seeding was observed.

Costs

Using multiple small-bore needles, the price of each laser disposable kit including a needle and a fiber is about €300 (US$ 400). Therefore, the cost of a single LA session varies in relation to the number of devices used: one kit is required for nodules ≤ 1.0 cm; 2 kits for nodules ranging from 1.0 to 2.0 cm, and 4 kits for larger nodules (Figure 1). Treatment can be performed in outpatient surgery by an operator, a nurse, and an anaesthesiologist and requires about 30-45 min (from targeting to final US assessment).

Figure 1 Representative case of complete ablation of large nodule with multi-fibre technique. A: Computed tomography (CT) scan before laser ablation (LA) session shows a nodular lesion 6 cm in maximum diameter (hepatocellular carcinoma moderately differentiated) localized in the S6 with exophytic growth (exophytic component > 40%); B: CT scan performed 4 wk after LA procedure shows complete necrosis of the tumor. Four illuminations were performed using the pullback technique and the treatment lasted 24 min. The procedure was well tolerated and the patient was discharged from the hospital 24 h after the procedure. The only side effects were mild pain and self-limiting fever lasting for 7 d.

TRANSLATING ALL THE AFOREMENTIONED INTO CLINICAL PRACTICE: TOWARDS A PATIENT-TAILORED APPROACH

Before commenting on the role that laser technology plays in percutaneous ablation of HCC, we should briefly summarize the recommended indications commonly accepted by the scientific community so far. On the basis of published data, RFA is now the first-line ablative technique whenever possible[76-81]. PEI has less local control effectiveness but still has a role in achieving complete response when the residual untreated viable tissue is minimal or when location at risk-sites implies serious adverse events or severe complications[82-85]. For solitary HCC ⩽ 2 cm, RFA should be considered the first-line treatment for its lower mortality and morbidity, shorter hospitalization, and lower costs (compared to surgery), and should be preferred to PEI due to its greater effectiveness and predictability of treatment results[86]. Survival outcomes of patients with HCC < 3 cm treated by percutaneous approach are competitive with those of surgery. However, a careful multidisciplinary evaluation of the age and comorbidities of the patients and of the location of these tumours is needed[82,84]. In HCC > 3 cm resection or combined treatment (TACE + RFA or PEI) has been suggested to improve survival[87,88], but available studies do not yet provide useful conclusions as the enrollment criteria of patients was too stringent[88]. Studies are needed to define which population can benefit from the combined treatments.

As RFA effectiveness is size-dependent, to obtain complete necrosis the upper limits must not exceed 2.5-3.0 cm[89,90]. To overcome this limitation and obtain larger volumes of necrosis, a variety of devices[91,92] of different shapes and designs[93,94] used either with different algorithms[95] or activated in different modes (consecutive, simultaneous, or switching) has been developed[96-98]. In the treatment of large HCC (⩾ 5 cm), conventional

RFA is limited mainly by incomplete ablation, with reported complete ablation rate of 74% after single session in lesions between 3 and 5 cm and of 62% in tumours > 5 cm after multiple sessions[99]. Using three internally cooled bipolar electrodes complete, ablation rates was 81% in patients with large HCC[100].

Therefore, multiple heat sources are needed to obtain large volumes of necrosis; the laser technique with multiple thin needle fibres and simultaneous approach[42] satisfies this need. Indeed, LA obtains interesting results with thin, very simple devices that are much less sophisticated and less expensive than those used by RFA. According to the size and shape of the lesions, one to four fibers are used. Two laser fibers for nodules ⩽ 2.0 cm and four fibers with tips arranged in a square configuration for larger nodules are used. For a single illumination, laser light is employed for 4-6 min. For nodules > 3.0 cm, multiple illuminations and the pullback technique are employed. The introduction of the novel needle guide has made it possible to obtain a complete ablation of lesions up to 5 cm[43]. No specific methods are used for treating lesions in high-risk (*i.e.*, near gallbladder, main biliary duct, hepatic hilum, adjacent hollow viscera, or exophytic location) and/or hard-to-reach locations (*e.g.*, in the dome of the liver, in the caudate lobe)[42,43,65]. Additionally, this technique makes it is relatively easy to obtain a safety margin ⩾ 5 mm in a higher percentage of cases (62%)[53] than that reported by other authors with RFA[101-105]. Furthermore, thin devices makes it possible to treat multiple lesions of the liver of different sizes and in different locations in the same LA session without increasing the complications rate[43]. Therefore, it is possible to customize the ablative treatment according to the size and location of the lesion to be treated. Laser techniques can be used effectively in patients with very early and early HCC (BCLC 0 and A) because of their high percentage of complete response. The reported local effectiveness and long-term outcomes obtained with LA are comparable with those of RFA. Specifically, in the subgroup of Child's class A cirrhotic patients with lesions ⩽ 2 cm (BCLC 0-A)

Figure 2 Representative case of complete ablation of hepatocellular carcinoma of 5 cm with combined treatment (laser ablation followed by trans-arterial-chemo-embolization). A: Computed tomography (CT) scan before Laser ablation (LA) shows a lesion 5 cm in diameter beneath the capsule in S8 during arterial phase; B: CT scan after LA shows an area of necrosis larger than basal lesion with small viable foci (white arrowhead) within the zone of coagulation; C: CT scan shows compact retention of iodized oil in the residual viable tissue (black arrowheads) after trans-arterial-chemo-embolization (TACE) session; D: CT scan shows marked volume reduction of treated area and clear shrinkage of viable tissue (black arrowhead) 6 mo after the combined procedure.

without contraindications to surgery treated by LA, 5-year survival was equivalent to that of RFA[68,78] as reported above. Finally, thanks to thin needles and to the more effective tumoricidal action of heat compared to ethanol, we believe that this technique could replace PEI in the treatment of nodules at high-risk sites when RFA is not technically feasible, as has been recommended by some authors[106].

As for the water-cooled laser applicators, it must be emphasized that their main advantage is their MRI compatibility, which allows pre-procedure planning and intra-procedure treatment monitoring using a variety of temperature-sensitive techniques[107,108]. The Frankfurt group has provided compelling long-term survival data in patients treated with this method for the ablation of hepatic metastases[109] and has recently published two papers on primary liver lesions in cirrhotic patients with a high percentage of complete response and low local recurrence[44] (Table 1). However, to achieve these excellent results, the authors used a large cross-sectional probe diameter (3 mm) that requires large bore cannula (9 gauge) for percutaneous treatment. In addition, the diffusion of MRI-guided LA is restricted by machine availability and by complexity of the procedure, requiring between 60 and 120 min to be completed[110,111]. New MRI-compatible applicators permit the execution of the whole procedure within the MR suite, reducing the procedural time and increasing technical effectiveness[112]. However, we think that although interventional MRI guidance is undoubt-edly more accurate than US for monitoring ablation, its use would greatly limit the number of centers capable of performing tumor ablation, with ablation procedures being relegated to only those facilities with such specialized equipment. Thus, given that US is readily available, its use has proven to be successful on a practical level in these last 20 years, compared to the potential benefits of less available technologies. In short, these data show that touted advantages of a particular system do not have equal weight in the clinical scenario. Last but not least, we must add the costs of this option to its overall complexity.

A new ablation laser system consisting of 980-nm diode laser with a power of 15-W and diffuser-tipped optical fiber inserted through a 17-gauge internally cooled catheter was recently introduced in field practice. This system achieves a large, well-circumscribed ellipsoid ablation zone up to 2.0 cm × 2.3 cm in a single application lasting about three minutes, and up to 3.7 cm × 3.2 cm with two parallel applicators placed 1.5 cm apart[60]. Due to its characteristics, this system has been applied thus far to focal malignant lesions of the prostate and the brain[24,59]; research and clinical applications on hepatic focal lesions are underway (oral communication). Therefore, the limitations of the previous system, which used high-calibre devices, can be overcome by this technical solution. Further, the execution time of the entire ma-noeuvre can be shortened significantly by using real-time RM guidance.

Again, *ex vivo* and *in vivo* studies are underway (unpub-

lished data) using diffuser-tipped optical fiber that can be placed in the target area through flexible internally cooled catheter under US guidance. It is possible to produce areas of necrosis of about 3.5 cm × 3.0 cm× 3.0 cm in diameter in about 20 min. If these data are confirmed by clinical studies, we will have made good use of the advantages of US guidance in combination with those deriving from a caliber similar to that of RFA- and MWA-cooled electrode. Therefore, with laser technical improvements such as the new small cylindrical diffuser[60] or the novel needle guide system[43], it possible to employ an array of applicators to increase the ablation zone without increasing invasiveness, procedural complexity, times of ablation, or costs. In clinical practice, a trade-off must be made between these multiple factors and the operator's skill, the available technology, and the biology of the tumor.

While the reported outcome data with combined treatment (LA plus TACE) are interesting, they were obtained with a technique that is the opposite[73] of what is commonly used in referral centers. When surgery is unfeasible, a combined/sequential approach (PEI plus RFA, TACE plus PEI, RFA or MW) should be considered on an individual basis for multinodular nodules and for nodules > 3 cm, after multidisciplinary evaluation[85]. Recently, a meta-analysis of RFA following TACE reported no significant difference in survival rates between RFA plus TACE and RFA for small HCC. On the contrary, this sequential treatment improved overall survival rate in patients with intermediate and large HCC[113]. Therefore, the main indication of combined therapies is for lesions > 3 cm and < 8 cm. Both the PEI and TACE with different mechanism cause a reduction of the blood flow through the tumor, thereby facilitating a larger ablation zone.

LA before TACE, instead, reduces the tumor burden and brings the lesion back within the range of TACE effectiveness. In other words, LA results in a minimal amount of tumor tissue, which can be destroyed with selective TACE using a lesser amount of embolizing material (Figure 2). Because it is possible to destroy lesions up to 5-6 cm with laser technique (Figure 1) we think that this combined method might be effective in treating lesions larger than 6 cm both in cirrhotic patients and in non-cirrhotic patients, thereby avoiding surgery, as currently suggested by some authors[114].

The safety of the procedure was investigated in a multicenter study sufficiently representative both of the type and of the number of possible complications when using either multiple thin needles[75] or large water-cooled devices[44]. The data reported above compare favourably with those of the tested and much more widely used RFA technique and with those of the MWA technique. The mortality rates of RFA range from 0% to 1.5% of cases and major complications from 1.5% to 5.8% of cases[69,115-118]. The mortality and major complications rates of MWA have been reported as 0% to 5.1% and 2.6% to 5.1%, respectively[119-122].

Finally, a few words regarding the MWA technique: its safety profile appears good, but there is still no con-firmation on large series of cirrhotic patients. In the only comparative study with RFA, MWA showed comparable therapeutic efficacy and complications rates than RFA, but required more treatment sessions. Furthermore, adequate clinical data are lacking[123].

CONCLUSION

Given that there is not a single method available that meets all the requirements of an ideal ablation system, based on what has been discussed above and on data from the vast literature available, we can reasonably draw some conclusions: (1) the differences between the techniques in terms of the results are modest; (2) one technique may be more difficult than another and more rapid than another. In other words, there are differences in the ease and duration of the various procedures; and (3) while some energy sources may be better suited to certain applications, none has proven suitable for all applications. The laser technique developed in the United Kingdom has been used in the last two decades mainly in German and in Italy but has not been commercialized and sponsored in the rest of the world[124,125]. We hope that in the future a greater availability of the applicators will facilitate their use in clinical practice. The technique has been sufficiently tested and the recent RCT trial should validate it. The fine needle technique offers maximum flexibility, thereby allowing a tailored approach to the characteristics of each nodule in any location of each patient. More in general, we think that the reference centres that treat more than 50 patients/year should be equipped with all the available techniques so as to be able to use the best and the most suitable for each type of lesion for each patient.

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REVIEW

Targeting the insulin-like growth factor pathway in hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide. Only 30%-40% of the patients with HCC are eligible for curative treatments, which include surgical resection as the first option, liver transplantation and percutaneous ablation. Unfortunately, there is a high frequency of tumor recurrence after surgical resection and most HCC seem resistant to conventional chemotherapy and radiotherapy. Sorafenib, a multi-tyrosine kinase inhibitor, is the only chemotherapeutic option for patients with advanced hepatocellular carcinoma. Patients treated with Sorafenib have a significant increase in overall survival of about three months. Therefore, there is an urgent need to develop alternative treatments. Due to its role in cell growth and development, the insulin-like growth factor system is commonly deregulated in many cancers. Indeed, the insulin-like growth factor (IGF) axis has recently emerged as a potential target for hepatocellular carcinoma treatment. To this aim, several inhibitors of the pathway have been developed such as monoclonal antibodies, small molecules, antisense oligonucleotides or small interfering RNAs. However recent studies suggest that, unlike most tumors, HCC development requires increased signaling through insulin growth factor II rather than insulin growth factor I. This may have great implications in the future treatment of HCC. This review summarizes the role of the IGF axis in liver carcinogenesis and the current status of the strategies designed to target the IGF-I signaling pathway for hepatocellular carcinoma treatment.

Key words: Hepatocellular carcinoma; Insulin; Insulin-like growth factor; Insulin-like growth factor receptor; Therapy; Tyrosine kinase inhibitor; Antibody therapy

Core tip: It is mandatory to develop alternative therapies for the successful treatment of hepatocellular carcinoma (HCC). One of the key drivers of hepatocarcinogenesis is the insulin-like growth factor (IGF) system. Therefore, several inhibitors of this pathway have been developed and their therapeutic potential is being tested in patients with HCC. However, recent studies suggest that IGF-II, a member of the pathway, may be more relevant for hepatocarcinogenesis than its close homologue IGF-I. The purpose of this review is to summarize these facts within a detailed description of the IGF axis and the alterations of the pathway that lead to HCC. The strategies designed to target the IGF-I signaling pathway for HCC treatment are also reviewed.

INSULIN GROWTH FACTOR SYSTEM

The insulin-like growth factor system is formed by three ligands, three receptors and at least six high affinity binding proteins that work cooperatively to regulate cellular metabolism, proliferation, differentiation and apoptosis in most cells[1]. The three ligands are insulin, insulin growth factor (IGF)- I and -Ⅱ. Each has the highest affinity for a specific receptor named after itself: insulin receptor (IR), IGF- I receptor (IGF-IR) and IGF-Ⅱ receptor (IGF-Ⅱ R). Furthermore, the IGF axis is composed of IGF high affinity binding proteins (IGFBPs) and IGFBP proteases[2].

IGF- I and IGF-Ⅱ are single chain polypeptides, approximately 7 kDa in size, that share 62% of the amino acid sequence[3]. They were first named somatomedins, because they mediate the activity of growth hormone (GH), also named somatotropin[4]. Later, they were renamed to highlight their similarity with insulin[5]. Insulin is a small hormone secreted by pancreatic beta-cells that maintains normal glucose levels in blood by regulating carbohydrate, protein and fat metabolism. Several excellent reviews on insulin and the insulin pathway have been published recently and therefore, this ligand will not be dealt with great detail in this article[6-8]. IGF ligands are bound with high affinity to IGFBPs. IGFBPs regulate the half-life and bioavailability of IGF- I and IGF-Ⅱ and modulate their accessibility to the receptor[9]. IGFBP activities are closely regulated by post-translational modifications and IGFBP proteases. Most IGFBPs also have functions unrelated to the IGF system[10].

Most of the intracellular activity of IGF- I and IGF-Ⅱ is mediated by the tyrosine kinase IGF-IR whereas insulin exerts its biologic functions mainly through IR[11]. These receptors are homologous because they derive from a common ancestor gene[12]. Despite the fact that IR and IGF-IR share most of their downstream mediators, it has been commonly accepted that IGF-IR activation promotes proliferation and differentiation and IR activation promotes metabolic signaling[11]. Surprisingly, the IGF-Ⅱ R differs largely from IR and IGF-IR, and sequesters IGF-Ⅱ to internalize it for degradation[13].

IGF-I

IGF- I is the main mediator of GH function in normal embryonic development and postnatal growth[14]. GH is produced and secreted by the pituitary gland to induce body growth[15]. GH binds to the GH receptor in the liver and activates a signaling pathway that leads to transcription of several genes, including IGF- I [16]. Human IGF-I gene can be transcribed from two alternative promoters[17-19]. Furthermore, different mature IGF- I transcripts are produced by alternative splicing and polyadenylation[17,19-22]. These transcripts encode for different pre-proteins that undergo post-translational modifications and mature by proteolytic cleavage at both ends[23], resulting in a single polypeptide of 70 amino acids (7.5 kDa) cross-linked by 3 disulfide bonds[24,25]. Currently, the impact on IGF- I functionality of such a complex mRNA

and protein processing is unclear.

IGF- I is produced by several tissues, including the liver, bone, muscle and brain[26]. The IGF- I produced in these organs acts locally, with the exception of the liver, which produces most of the secreted hormone[27]. Hepatocytes are the main producers of IGF- I in the liver while non-parenchymal cells make a minimal contribution[28]. Liver secretion is possible because IGF- I is not sequestered by liver IGF-IR, which is almost undetectable in healthy hepatocytes[29], and it is only expressed in the liver in non-abundant non-parenchymal cells such as Kupffer cells, hepatic stellate cells (HSCs) and myofibroblasts[28]. Circulating IGF- I levels increase from birth to puberty when they reach their maximum value and then decline with age thereafter[30]. When circulating IGF- I increases, it inhibits the synthesis of GH and IGF- I production is then controlled by negative feedback[31].

IGF- I has similar functions to insulin, since both regulate glucose uptake and their production is affected by nutritional status. IGF- I exerts its function by binding with high affinity to its principal receptor, IGF-IR (Figure 1). However, it can also bind to IR with 100-fold less affinity[32]. IGF- I binding to IGF-IR promotes anabolic processes such as DNA, RNA, protein and glycogen synthesis and results in proliferative and differentiating effects[33].

IGF-II

Unlike IGF- I , IGF-Ⅱ expression is not regulated by GH[34]. In fact, the main regulator of IGF-Ⅱ transcription is still unknown. The *IGF-Ⅱ* gene is generally an imprinted gene expressed only from the paternal allele[35,36]. However, in the liver this control is only maintained at the fetal stage, as IGF-Ⅱ expression becomes biallelic in the adult liver[37]. This is not due to a real loss of imprinting but to the activation of a biallelic adult liver specific promoter responsible for producing 50% of liver IGF-Ⅱ [38]. The standard imprinted IGF-Ⅱ promoters are still active in the adult liver and account for the remaining expression of IGF-Ⅱ from the paternal allele[37,39]. The *IGF-Ⅱ* gene encodes a pre-pro-IGF-Ⅱ protein of 180 amino acids that transforms to a 156 amino acid-long pro-IGF-Ⅱ upon peptide signal loss[40]. Most of the pro-IGF-Ⅱ is cleaved and glycosylated to yield the 67 amino acid-long mature IGF-Ⅱ [41].

IGF-Ⅱ can be produced by several tissues, but most comes from both parenchymal and non-parenchymal liver cells[28]. IGF-Ⅱ expression reaches maximal values during the fetal stage, as IGF-Ⅱ plays a crucial role in fetal development[42]. After birth, IGF-Ⅱ levels decrease to 400-600 ng/mL (about 4-fold higher than IGF- I) and remain constant for the rest of life[43]. Despite the higher amount of IGF-Ⅱ than IGF- I , the function of IGF-Ⅱ is gradually replaced by IGF I after birth[2]. Similar to IGF- I , IGF-Ⅱ is able to bind with high affinity to IGF-IR, to regulate cell proliferation and differentiation (see below)[34]. Furthermore, IGF-Ⅱ can bind to IGF-Ⅱ R, which induces IGF-Ⅱ internalization and degradation[44]. Finally, IGF-Ⅱ can also bind to insulin receptor subtype

Figure 1 Insulin-like growth factor receptors and their ligands. The insulin-like growth factor (IGF) system is composed of three receptors: IGF-IR, IGF-ⅡR and insulin receptor (IR). IGF-IR is the main receptor of the IGF system. It can bind IGF-Ⅰ and IGF-Ⅱ with high affinity. IGF-ⅡR is a negative regulator of the pathway that binds IGF-Ⅱ and promotes IGF-Ⅱ degradation. Finally, IR mediates insulin signaling. Two IR isoforms exist: the adult IR-B isoform that binds insulin and the fetal IR-A isoform, that can bind IGF-Ⅱ in addition to insulin promoting mitogenic signaling. The IR-A isoform is commonly overexpressed in hepatocellular carcinoma (HCC). IR isoforms can form IR-A/IR-B hybrids which behave as IR-A receptors. Moreover, HR-A or HR-B hybrid receptors can be formed between IGF-IR and IR-A or IR-B respectively. These hybrid receptors lack high affinity binding to insulin and act, similar to the IGF-IR receptor, promoting proliferation and survival.

A (IR-A) to display mainly mitogenic effects[45,46]. Interestingly, both IR-A and IGF-Ⅱ are upregulated in several tumors[47].

Insulin

Synthesis and secretion of insulin is mainly regulated by glucose levels, but other stimuli can also influence these processes[6]. Upon binding to the IR, insulin plays a key role in maintaining normal glucose levels in blood by regulating carbohydrate, protein and fat metabolism[48]. While IR activation after insulin binding promotes mainly metabolic events, recent evidence supports the hypothesis that IR can also mediate mitogenic effects[49,50].

IGFBPs and IGFBP proteases

Six high affinity IGFBPs (IGFBP1-6) have been described which share 36% homology[51,52]. There are other IGFBP-related proteins (designated IGFBP-rP1-10) which bind IGF-Ⅰ and IGF-Ⅱ with lower affinity than classical IGFBPs[52]. IGFBP structure is composed of three domains. The N-terminal and the cysteine rich C-terminal domains are involved in IGF ligand binding and are common to all IGFBPs. The intermediate domain is different for each IGFBP and is probably involved in IGF-indepen-

dent functions[10]. IGFBPs transcription is cell specific and is tightly regulated by hormones and by growth factors[2]. IGFBP levels are also controlled post-transcriptionally by proteolysis. There are three types of IGFBP proteases: serine proteinases, matrix-metalloproteinases (MMPs) and aspartyl proteinases[30]. IGFBP proteases are relatively specific for each IGFBP because the site of cleavage is inside the hyper-variable domain of the IGFBPs.

IGFBPs are widely expressed, but each tissue preferentially produces one or two classes[10]. The principal source of IGFBPs is the liver. There, hepatocytes express IGFBP1, 2 and 4 while non-parenchymal cells express IGFBP3[28]. After tissue secretion, IGFBPs circulate in blood and extravascular fluids and all of them bind IGF-Ⅰ and IGF-Ⅱ ligands with high affinity (10^{-10} M)[10]. IGFBP2, 5 and 6 have a special preference for IGF-Ⅱ[53]. Interestingly, IGFBPs do not bind insulin because the specific amino acids that confer IGF binding affinity are not conserved in the insulin sequence[25,54]. Ninety-nine percent of circulating IGF-Ⅰ is bound by IGFBPs[2]. This high efficiency of IGF-Ⅰ binding is due to the excess of IGFBPs (50 times higher than IGF-Ⅰ) and to the high binding affinity[55]. Note that the affinity of IGFBPs for IGF ligands is similar or even higher than the affinity of IGF ligands for

their receptors[52].

IGF binding to IGFBPs increases ligand half-life but decreases IGF availability for signaling through IGF receptors. Both IGF-Ⅰ and IGF-Ⅱ are able to form binary complexes of approximately 50 kDa with IGFBPs or ternary complexes of approximately 150 kDa with IGFBP3 (or IGFBP5 to a lesser extend) and the acid-labile subunit (ALS) protein[9]. Almost 75% of the bound IGF forms ternary complexes[56]. When bound to IGFBP3, IGF-Ⅰ half-life increases from 8 to 30 min and when bound to IGFBP3 and ALS, IGF-Ⅰ half-life increases from 30 min to 15 h[57]. However, the IGF-I-IGFBP3-ALS ternary complex is too large to pass through the vascular endothelium to reach the IGF-IR[58]. Therefore, plasmatic proteases are required to break tertiary into binary complexes, able to cross the vasculature. Subsequent proteolysis of IGFBPs by plasmatic or tissue specific proteases releases IGFs and allows IGF signaling[55].

In general, it can be considered that IGFBP1, 3 and 5 activate IGF signaling while IGFBP2, 4 and 6 are inhibitory. However, the same IGFBP can potentiate or inhibit IGF signaling depending on post-translational modifications or binding to other factors[10,59-61].

IGF-IR

IGF-IR is a transmembrane tyrosine kinase receptor expressed ubiquitously. The mature receptor is composed of 2 homodimers, *i.e.*, two α and two β subunits, cross-linked by disulfide bridges (Figure 1). The α subunit (130-135 kDa) is located extracellularly and contains the IGF binding domain, while the β subunit (90-97 kDa) crosses the membrane and reaches the cytoplasm where the tyrosine kinase domain is located[62].

IGF-1R is mainly activated by IGF-Ⅰ. However, it can also bind IGF-Ⅱ and insulin with 2-5 fold and 100-1000 fold less affinity, respectively[63] (Figure 1). Following ligand binding, IGF-IR suffers a conformational change that activates the tyrosine kinase domain, leading to autophosphorylation of specific tyrosines and recruitment of specific docking proteins, including insulin-receptor substrate proteins (IRS-1 to -4) and Shc[64]. Thus, different signaling cascades are activated (Figure 2): (1) IRS-1 phosphorylation activates the phosphatidylinositol-3 kinase (PI3K)-AKT-mTOR pathway that leads to increased glucose uptake and protein synthesis, cell survival and apoptosis inhibition[65]. Following IRS-1 phosphorylation, PI3K is activated by phosphorylation, leading to activation of AKT/PKB[66]. AKT/PKB inhibits apoptosis by activating by phosphorylation anti-apoptotic proteins such as Bcl2, Bclx and NFκB, and by inhibiting by phosphorylation pro-apoptotic proteins such as the Bcl-2 family member Bad, members of the fork head transcription factor (FOXO) family, Fas ligand (FasL) and caspase 9[62,67]. Furthermore, AKT/PKB induces glucose uptake and glycogen synthesis through inhibition of glycogen synthase kinase-3 (GSK-3β) activity[68] by phosphorylation of the serine 9 residue[69,70]. Finally, AKT/PKB phosphorylates the DNA repair protein p21. Phosphorylated p21 does not bind PCNA leading to cell

cycle progression[71]; and (2) Phosphorylation of the Shc protein activates the RAS-RAF-ERK pathway, related to cell differentiation, proliferation and migration[72]. Phosphorylated Shc complexed with Grb2 and SOS proteins leads to the activation of RAS[73]. RAS induces ERKs, which in turn inhibit apoptosis in a similar way to AKT and induces cell proliferation and migration[74].

Besides these major pathways, IGF-IR may also: (1) activate p38 mitogen-activated protein kinase (p38MAPK), leading to cellular growth and differentiation[75]; (2) bind apoptotic signal-regulated kinase 1 (ASK1), which impedes c-Jun N-terminal kinase (JNK) activation, that in turn, inhibits the apoptosis mediated by death-inducing receptors[76]; (3) lead to the 14-3-3-dependent mitochondrial translocation of Raf, maintaining the mitochondrial integrity, and thus protecting cells from apoptosis[77]; phosphorylate IRS-2 which influences integrin expression, that, together with the IRS-1-dependent decreased cell-cell contact, potentiates cell motility and anchorage independent growth[78]; and (4) affect JAK/STAT-3-mediated inhibition of apoptosis[79]. Thus, in summary, IGF-IR activation leads to differentiation or to increased cell proliferation and migration.

IGF-Ⅱ R

The human *IGF-Ⅱ R* gene is imprinted in rodents, where it shows maternal expression[80,81]. Surprisingly, expression in humans is polymorphic: most humans are biallelic but some show imprinted expression[82,83]. *IGF-Ⅱ R* gene expression results in a transmembrane protein of 2491 amino acids located in the Golgi apparatus (approximately 90%) and in the cell surface (approximately 10%)[84]. The extracellular domain consists of 15 homologous tandem repeats, able to bind with different affinities to mannose 6-phosphate (M6P)-containing proteins or M6P free factors[85]. M6P factors that bind IGF-Ⅱ R include leukemia inhibitory factor, cathepsin D and latent TGF. M6P free proteins bound by the receptor are urokinase-type plasminogen activator receptor (uPAR), retinoic acid and IGF-Ⅱ[86]. It has been shown that IGF-Ⅰ can also bind to IGF-Ⅱ R but with very low affinity, while insulin does not bind at all[87] (Figure 1).

The main function of IGF-Ⅱ R is to transport extracellular and Golgi derived-acid hydrolases and other ligands to lysosomes[86]. Upon IGF-Ⅱ binding, the entire complex is internalized in clathrin-coated vesicles that travel to the endosomal compartment, where the ligand is degraded and the receptor is recycled to the cell membrane[88]. Therefore, in the IGF axis, IGF-Ⅱ R acts as a scavenger receptor lacking intrinsic signaling. Thus, several studies have demonstrated that IGF-Ⅱ R may act as a tumor suppressor gene[80,89,90].

Interestingly, some authors have described that IGF-Ⅱ R may be cleaved from the cell membrane to act as a truncated soluble form of 270-280 kDa[91]. This soluble receptor is detected at very low concentrations (0.1 nmol/L) in the serum and other fluids of several mammalian species[86]. However, it efficiently sequesters circulating IGF-Ⅱ[92].

Figure 2 Insulin-like growth factor-I receptor signaling pathway. Insulin-like growth factor-I (IGF-I), IGF-II and to a lesser extent, insulin, can bind to IGF-IR and promote a conformational change that leads to the autophosphorylation and activation of the IGF-IR. Phosphorylated receptor recruits specific docking proteins including IRS1-4, Shc and 14-3-3 which trigger mainly the PI3K/AKT/mTOR and the RAF/MEK/ERK signaling pathways. Activation of the IGF-IR induces protein and glycogen synthesis, glucose uptake, cell proliferation, migration and survival and cell differentiation depending on the cell type. See text for more details. GSK-3β: Glycogen synthase kinase-3; mTOR: Mammalian target of rapamycin; PTEN: Phosphatase and tensin homolog; PI3K: Phosphatidylinositide 3-kinase; AKT/PKB: Protein kinase B; NFKB: Nuclear factor kappa-light-chain-enhancer of activated B cells; Bcl-2: B-cell lymphoma 2; IRS-2: Insulin receptor substrate 2; FOXO: Forkhead box protein O1; PCNA: Proliferating cell nuclear antigen; RAF: Rapidly Accelerated Fibrosarcoma; MEK: Mitogen-Activated Protein Kinase; ERK: Extracellular-signal-regulated kinase; ELK1: ETS-Like kinase; ELK1: ETS-Like kinase.

IR

IR and IGF-IR share almost the same signaling pathway. Insulin binding to the IR induces a conformational change that results in the autophosphorylation of the IR and the recruitment of IRS proteins or Shc, leading, respectively, to the activation of PI3K-AKT-mTOR and metabolic effects or to the activation of RAS-RAF-ERK and mitogenic effects[6]. In fact there are two isoforms of the IR. The standard receptor is IR-B, expressed in adult liver, muscle and adipose tissue and involved in binding to insulin to regulate glucose homeostasis[62]. Alternative splicing regulation leads to a mature transcript that encodes for IR-A, which lacks 12 amino acids from exon 11 and is expressed by fetal tissues and some tumors[47]. IR-A can bind to insulin, IGF-II and proinsulin with different affinities to promote mainly proliferation, migration and inhibition of apoptosis, but also possess some metabolic activating capacity[62,93] (Figure 1). Insulin binding to IR-A seems to induce more metabolic effects than IGF-II binding[94,95]. IR-A binds insulin with 1.5 fold higher affinity than IR-B and possesses a higher dissociation and internalization rate[95]. Therefore, in cells with increased IR-A:IR-B ratios, most insulin signals through IR-A[7]. This is in line with the higher risk of

HCC when insulin serum levels increase[96]. Proliferation can also result from IR-A binding to IGF-II, which interacts with 3-10 fold lower affinity than insulin, or to proinsulin[97].

IR-A and IR-B can form IR-A/IR-B heterodimers that behave similarly to IR-A[7]. It seems that insulin and IGF-II can bind IR-A/IR-B hybrids with the same affinity as IR-A homodimers (Figure 1). IGF-I also binds IR-A/IR-B hybrids with lower affinity[98]. When IR-A is overexpressed, most IR-B forms IR-A/IR-B hybrids, leading to decreased metabolic signaling and increased proliferation[7].

IGF-IR/IR hybrid receptors

Due to the high homology between IGF-IR and IR, IR can also form heterodimers or hybrid receptors (HR) with IGF-IR[99,100]. The cellular content of HR depends only on the molar concentrations of each receptor because IGF-IR/IR heterodimers and homodimers are formed with similar efficiency[101]. Depending on the IR isoform, HR can be IGF-IR/IR-A (HR-A) or IGF-IR/IR-B (HR-B)[7]. HR-A is activated by IGF-I, IGF-II and, to a lesser extent, by insulin, while HR-B is activated mainly by IGF-I but also by IGF-II with lower affin-

ity[102] (Figure 1). Functionally, HRs behave more like IGF- I R than like IR[48].

It is still unclear how the activation of the different IR isoforms, IGF-IR and HR by insulin, IGF- I and IGF- II leads to different biological effects despite the fact that they share most downstream mediators. Differences in ligand binding, internalization or dissociation rates, protein structure and the presence of cell or tissue specific factors could explain this phenomenon[7,11].

HEPATOCELLULAR CARCINOMA

Given its role in cell proliferation, the IGF system is one of the pathways deregulated in cancer. As IGF- I protein is highly expressed in the liver, hepatocellular carcinoma (HCC) has been traditionally linked with increased IGF activity. HCC is the third cause of cancer-related deaths worldwide[103]. The most relevant risk factors in the development of HCC are those that induce liver cirrhosis, as 90% of HCC develops in a cirrhotic liver[104]. Liver cirrhosis is the result of chronic liver disease, due mainly to prolonged alcohol abuse, genetic predisposition, obesity and viral infections with HBV and HCV[105]. These agents induce chronic inflammation that leads to the death of hepatocytes and the activation of hepatic stellate cells (HSCs)[106]. Activated HSCs secrete collagen leading to liver fibrosis and ultimately, the breakdown of liver architecture and functionality. Hepatocyte death and proliferation results in the formation of regeneration nodules, characteristic of the cirrhosis stage[107]. The inflammation coupled with the high proliferation level of cirrhotic hepatocytes leads to the accumulation of mutations and to the loss of epigenetic control that may result in HCC initiation and progression[108]. The genetic and epigenetic events that lead to HCC include somatic mutations, telomere shortening, changes in gene expression profiles and RNA editing and genomic alterations[109,110]. These alterations result in a deregulation of several signaling pathways including PI3K/AKT/mTOR, RAS/RAF/MAPK, WNT, HGF/c-MET, EGFR, IGF-IR and PDGF, leading to hepatocarcinogenesis[103,111]. In the next section, the involvement of the IGF system in the development of HCC will be dealt with in detail. The contribution of other signaling pathways or the IGFI-R activated factors PI3K/AKT/mTOR and RAS/RAF/MAPK has been extensively reviewed by other authors and will not be described in this review[111-116].

Once an HCC is diagnosed, surgical resection is the primary curative treatment followed by liver transplantation and percutaneous ablation[117]. However, only 30%-40% of patients are eligible for these treatments. Moreover, there is a high frequency of tumor recurrence after surgical resection[118]. Unfortunately, most HCC seem resistant to conventional chemotherapy and radiotherapy[119]. The poor efficacy of antitumor agents is also due, at least in part, to the inefficient drug delivery and metabolism exerted by the cirrhotic liver that host the tumor[109]. In the clinical trials searching for alternative therapies for HCC, patients may suffer from unbearable drug

toxicity and the treatment must be withdrawn leading to the therapeutic failure of compounds that are promising for the treatment of other tumors.

Thus, the development of novel therapies against HCC is urgently required. To this aim, the identification of oncogenic addiction loops or primary "gatekeeper" and "driver" mutations that would allow for HCC initiation and progression, respectively, is mandatory[109,111]. Furthermore, better therapeutic responses could be obtained after a correct patient stratification. Under the name of HCC there are tumors with different etiologies and tumors generated in response to a broad spectrum of deregulated pathways. Therefore, different HCC may respond differently to different therapies. There is a great need for establishing accurate HCC classifications not only for prognostic purposes but also to select the best therapeutic option for each HCC subtype.

To date, Sorafenib is the only drug approved by the FDA available for patients with advanced HCC. Sorafenib is a multikinase inhibitor that blocks PDGFR, VEGFR and RAF phosphorylation[120] resulting in decreased cell proliferation, activation of apoptosis and inhibition of angiogenesis[121]. In patients with advanced HCC, Sorafenib administration produces a statistically significant increase in the overall survival and a decrease in the time to progression of the disease[122]. Many other agents for HCC treatment are under development. Some drugs such as Sunitinib and Brivanib, showed negative results in phase III trials, as first-line or second-line therapies, respectively[109]. Other agents such as Tivantinib, a c-Met inhibitor against the HGF pathway, have shown promising results in patients with HCC[123]. Tivantinib, is particularly efficient in those HCC with high c-Met expression levels[124], highlighting the need for performing personalized medicine with proper HCC molecular analysis to aid in the choice of successful therapies. The combination of different therapies can also increase success. In fact, Sorafenib used in combination with other techniques or other molecules had synergistic effects in preclinical and clinical models of HCC[121,125]. In this review we will focus on therapies related to the IGF system, as other authors have recently reviewed therapies that affect different signaling pathways[114,125-127].

IGF SYSTEM ALTERATIONS IN HEPATOCARCINOGENESIS

The IGF axis is one of the most commonly deregulated signaling pathways that contribute to cancer development. Alterations have been found in almost all members of the pathway. Here, we review the most important alterations that have been associated with hepatocarcinogenesis.

IGF- I
IGF- I is a mayor ligand of the IGF pathway, highly expressed in the liver and highly protumorigenic for several cancers. Surprisingly, several experiments suggest that

IGF- I expression may be antitumorigenic in the case of HCC. Several results support this hypothesis: (1) In situations of chronic liver damage and functional insufficiency, such as liver cirrhosis, the secretion of liver derived molecules including IGF- I is reduced or even totally suppressed in the most severe cases[128]. As the cirrhotic liver is the substrate for HCC development, decreased IGF- I levels could contribute to hepatocarcinogenesis. In fact, in patients with chronic hepatitis, decreased levels of IGF- I are associated with HCC incidence[129]; (2) Patients with HCC also display lower levels of circulating IGF- I when compared with healthy controls[130]. In fact, the development of HCC is preceded by a significant reduction in IGF- I levels, independently of the degree of impairment of liver function. Thus, a precocious diagnosis of HCC could be performed based on a decrease in serum IGF- I levels[129]. Furthermore, transcriptome analysis reveals that IGF- I mRNA levels are decreased in HCC human samples compared to matching adjacent tissue[131]. This can also be observed when liver tumors develop in mouse models after a single exposure to DEN hepatotoxic. In this case, mouse HCC is induced in a non-cirrhotic liver; and (3) decreased levels of IGF- I are associated with higher tumor invasiveness and poor prognosis[132]. The combination of low IGF- I and high VEGF predicts median overall survival of 2.7 mo compared with 19 mo for patients with higher IGF- I and lower VEGF. Serum IGF1 levels also predict tumour progression and overall survival in patients with HCC who undergo transarterial chemoembolization[133]. Also, the lack of liver IGF- I mRNA increases the risk of HCC recurrence after curative resection[134,135].

IGF-II

Excessive IGF-IR signaling is a characteristic feature of liver tumors. Since IGF- I levels are reduced in most HCC, the ligand of the pathway should be insulin or IGF- II. In fact, overexpression of IGF- II has been estimated to occur in 16%-40% of human HCC[136]. Furthermore, in both *in vivo* and *in vitro* models of HCC, IGF- II overexpression correlates with higher cell proliferation[137,138] while IGF- II inhibition promotes apoptosis and decreases cellular proliferation[139,140]. Accordingly, miR-615-5p, a miRNA that targets IGF- II expression directly, induces a decrease in proliferation and migration of HuH7 and HepG2 human hepatoma cell lines[141]. In patients with HCC, increased intratumoral IGF- II mRNA levels are associated with higher metastatic potential whereas increased serum IGF- II levels correlate with the presence of extrahepatic metastasis[142,143].

Overexpression of IGF- II has been shown to be the result of increased transcription[143]. As IGF- II is required for fetal growth, it is expressed mainly during development by the potent paternally imprinted P3 promoter[144]. After birth, transcription of liver IGF- II is gradually shifted from initiation at the imprinted promoter to initiation at a biallelic less active P1 promoter. This maintains low levels of liver IGF- II throughout adulthood[144]. However, alteration in IGF- II imprint-

ing has been described in many tumors[36,145-147]. In HCC, 50%-90% of human biopsies analyzed show a gain of IGF- II imprinting[37,148]. This imprinted phenotype results in increased transcription of IGF- II from the P3 promoter and decreased transcription from the P1 promoter by hypermethylation resulting in IGF- II overexpression[144,149,150]. Furthermore, IGF- II hypomethylation at exon 8-9 is found in 90% of HCV-cirrhotic patients analyzed and correlates with higher risk of developing HCC[151].

Other factors may also lead to IGF- II overexpression, such as Aflatoxin B1 (AFB1), a potent hepatocarcinogen present in food in developing countries[152]. The tumorigenic effect of AFB1 seems mediated by tumor suppressor genes such as p53 and by an overactive IGF signaling due to overexpression of IGF-IR and IGF- II[153,154]. Interestingly, p53mt249, a p53 mutant produced after AFB1 administration, can increase IGF- II transcription[154]. Also, the IGF- II polymorphism +3580AA, has been associated with higher serum levels of IGF- II and has been recently linked to higher risk of HCC in humans[51,155].

Insulin

Little is known about the role of insulin in HCC development. It has been reported that increased insulin serum levels are associated with higher risk of cirrhosis[156] and HCC[96].

IGFBPs and IGFBP proteases

In general, IGFBPs limit bioavailability of IGF ligands, attenuating IGF-IR signaling. Thus, some IGFBPs exert antiproliferative effects in human hepatocarcinoma cell lines. The addition of IGFBP3 to the HepG2 hepatoma cell line is able to counteract the mitogenic effect induced by administration of exogenous IGF- I[157]. Similarly, the administration of IGFBP1-4 results in decreased PLC cell proliferation[158]. Accordingly, the expression of antiproliferative IGFBPs such as IGFBP1, 3 and 4, is downregulated in human HCC[159].

The levels of IGFBP3 are also reduced in cirrhotic patients, but not as much as in HCC samples. Unfortunately, IGFBP3 levels are unable to distinguish between different HCC stages[160]. ALS, which forms a trimeric complex with IGF- I and IGFBP3 incapable of passing through the vasculature and activating IGF-IR[161], has been recently found to be downregulated in human HCC due to genomic loss and hypermethylation[162]. Downregulation of IGFBP3 in human HCC samples has also been linked to promoter hypermethylation[163]. On the other hand, p53, a potent antiproliferative protein, increases the secretion of IGFBP3[164].

IGFBP-rP1, also known as IGFBP7, is a low affinity IGFBP that has been recently identified as a tumor suppressor gene in HCC[13]. Expression of IGFBP-rP1 is dramatically downregulated by astrocyte elevated gene-1 (AEG-1), a novel oncogene that is overexpressed in 90% of the HCC analyzed[165]. In some patients, there is a complete deletion of the *IGFBP-rP1* gene[166]. In others, silencing of IGFBP-rP1 may result from promoter

methylation, which might be used as a biomarker for HCC diagnosis[167]. When IGFBP-rP1 is overexpressed in human HCC cells or tumors, cell growth is inhibited. Interestingly, an inverse correlation between IGFBP-rP1 expression and HCC stage has been found[166].

However, not all IGFBPs display antitumor effects in HCC. Some IGFBPs, such as IGFBP2 and 5, are associated with IGF activation. Accordingly, elevated levels of IGFBP2 have been reported in HCC patients[168]. There are no data on IGFBP5 levels in patients with HCC but inhibition of IGFBP-5 expression exerts antiproliferative effects in the Huh-7 hepatoma cell line[169]. Similarly, as IGFBP proteases release IGF ligands from IGF-IGFBP complexes leading to overactivation of the IGF pathway, they contribute to HCC development. Therefore, increased plasma levels of Cathepsin D, an acidic protease that degrades IGFBP3, have been found in cirrhotic and HCC patients[170]. Moreover, TIMP-1, an inhibitor of MMPs, displays antitumor effects by inhibiting IGFBP3 degradation and IGF-Ⅱ bioavailability[171].

IGF-IR
Signaling through IGF-IR plays an important role in tumorigenesis because of its ability to promote proliferation, protect from apoptosis and potentiate cell migration[29]. IGF-IR overactivation is one of the hallmarks of HCC and can be mediated by increased levels of IGF-IR protein and/or excess of IGF ligands[172]. Healthy mature hepatocytes do not express IGF-IR. In liver cirrhosis the situation is unclear as some authors report that IGF-IR is upregulated while others claim it is downregulated[173]. Most hepatoma cell lines express detectable levels of IGF-IR mRNA and protein[28]. In HCC samples, upregulation of IGF-IR is one of the most common alterations occurring in 30% of the patients[174].

Expression of several downstream components of IGF-IR has been found altered in some HCC samples. IRS-1, the main substrate of IGF-IR activation, is implicated in hepatocarcinogenesis. In fact, 90% of HCC overexpress IRS-1 and IRS-1 overexpression correlates with tumor growth[175]. IRS-2 is also deregulated in HCC. Upregulation of IRS-2 has been found in early and late stages of hepatocarcinogenesis. IRS-2 and IRS-1 have overlapping and specific functions[176]. They are co-overexpressed in 76% of HCC samples and overexpressed independently in 24%[177]. Some studies suggest that a high IRS2/IRS1 ratio may correlate with tumor aggressiveness. In fact, it has been shown that AFB1 increases the levels of IRS-2 but decreases the levels of IRS-1, leading to increased cell migration[178].

IGF-Ⅱ R
Overactivation of IGF-IR signaling by excessive IGF-Ⅱ molecules can be counteracted by IGF-Ⅱ R, which decreases IGF-Ⅱ levels through lysosomal degradation[13]. In fact, increased levels of IGF-Ⅱ may result from decreased expression of IGF-Ⅱ R. Indeed, tumor suppressor characteristics have been attributed to IGF-Ⅱ R in several tumor types. This has been the subject of a recent review[179]. Thus, inhibition of IGF-Ⅱ R expression increases cellular proliferation both *in vitro* and *in vivo*[180,181]. Conversely, overexpression of full length IGF-Ⅱ R into IGF-Ⅱ R deficient cells decreases cell growth and increases apoptosis *in vitro*[182,183] and decreases tumor growth *in vivo*[183,184]. However, overexpression of IGF-Ⅱ R has also been associated with an increase in cell number[185]. This is not surprising. It should be taken into consideration that IGF-Ⅱ is not the only ligand for IGF-Ⅱ R. Overexpression of IGF-Ⅱ R may affect the signaling of other relevant molecules, such as TGFβ, resulting in proliferative effects[179]. Antiproliferative effects of overexpressed IGF-Ⅱ R may only occur in cell lines or tumors whose increased proliferation depends on increased IGF-Ⅱ levels.

Given its role as a tumor suppressor protein, IGF-Ⅱ R is usually found downregulated in cancers, including HCC[186]. Low levels of IGF-Ⅱ R in HCC result from different alterations such as imprinting, loss of heterozygosity (LOH) and/or mutations[179,180,187-189]. There is a small subset of individuals carrying a paternally imprinted IGF-Ⅱ R allele[82]. Thus, they only have maternal expression of IGF-Ⅱ R, as happens with rodents. These individuals, together with rodents, should be more susceptible to developing HCC, as they only require mutations or decrease of gene expression from the active allele to suppress IGF-Ⅱ R functionality or production[190]. LOH caused by allelic deletion at the 6q26 locus, where the IGF-Ⅱ R gene locates, has been found in 54.5% of human HCC samples and in a smaller proportion of dysplastic liver lesions[179]. This LOH has also been observed in cirrhotic nodules suggesting that loss of the IGF-Ⅱ R gene could be an early event in hepatocarcinogenesis[187]. Furthermore, 55% of HCC with IGF-Ⅱ R LOH present mutations in the remaining allele[189]. Interestingly, while some mutations occur at the IGF-Ⅱ binding site of IGF-Ⅱ R, most occur in repeats 9 or 10, which are important for M6P-binding[179]. This finding indicates that the binding of M6P-containing proteins to IGF-Ⅱ R may have antitumoral effects. In fact, the M6P-bearing protein CREG can inhibit cell proliferation by stimulation of lysosomal IGF-Ⅱ degradation[191]. Generally, mutations in IGF-Ⅱ R lead to the formation of truncated proteins. Interestingly, using a truncated form of IGF-Ⅱ R derived from a reported splicing mutation[89], it has been demonstrated that truncated proteins can bind IGF-Ⅱ and M6P-containing proteins and are able to form heterodimers with the full length IGF-Ⅱ R[188]. Surprisingly, these heterodimers are rapidly cleaved and liberated from the cell membrane by MMPs, leading to a great decrease in the amount IGF-Ⅱ R bound to the cell membrane[188]. These data indicate that truncated proteins can act as dominant negative regulators of IGF-Ⅱ R contributing to cancer development.

IR
It has recently been published that the IR-A/IR-B expression ratio markedly increases in intratumoral HCC sections but not in adjacent tissues[7]. The relative abun-

Figure 3 Insulin growth factor system alterations in hepatocellular carcinoma. Healthy hepatocytes secrete high amounts of insulin growth factor (IGF)- I and express IGF-Ⅱ R and IR-B, but do not express IGF-IR. Many HCC are characterized by increased IGF signaling. Such IGF system overactivation is mediated by higher levels of ligands and/or receptors. Levels of functional IGF-Ⅱ increase in many hepatocellular carcinoma (HCC): (1) by reactivation of the fetal promoter of IGF-Ⅱ that leads to IGF-Ⅱ overexpression; (2) by a decrease in circulating IGFBPs that chelate IGF-Ⅱ; or (3) by decreased degradation through IGF-IIR, which is expressed at lower levels due to aberrant imprinting, loss of heterozygosity or gene deletions. Furthermore, IGF-IR and/or IR-A may be overexpressed in HCC. Overexpression of IGF-IR and IR-A leads to an increase in the formation of homodimeric HR-A or heterodimeric IR-A/IR-B receptors that bind IGF-Ⅱ with high affinity resulting in decreased metabolic signaling and increased proliferation.

dance of IR-A, the fetal IR isoform, *vs* total IR mRNAs in normal liver is 5% while in hepatoma cell lines it reaches 50%-75%[192]. The increase of the aberrant splicing that leads to the IR-A isoform is a consequence of the activation of the EGF pathway, one of the most relevant dysregulated pathways in HCC[193]. Interestingly, production of IR-A after EGFR activation only occurs in transformed but not in healthy hepatocytes. High affinity binding of IR-A by IGF-Ⅱ induces mitogenic and anti-apoptotic effects leading to HCC development. Tumors overexpressing both IR-A and IGF-Ⅱ should be resistant to conventional therapies that target IGF-IR.

IGF-IR/IR hybrid receptors and IR-A/IR-B heterodimers

As many HCC overexpress IGF-IR and IR-A and HR formation depends on the concentration of each receptor, these HCC should display an increase in HR-A. Concomitantly, an increase in HR-B and in IR-A/IR-B, which promote proliferation through IGF ligands could also occur together with a decrease in IR-B/IR-B homodi-

mers, responsible for insulin mediated metabolic activity[7]. Interestingly, some cancers have shown an increased fraction of hybrid receptors unrelated with their relative concentrations, suggesting that other factors may be implicated in hybrid receptor formation[194].

In summary, increased proliferation in HCC is generally characterized by an increase in the bioavailability of IGF-Ⅱ, which signals through increased IGF-IR, IR-A homodimers and HR-A leading to increased proliferation and decreased apoptosis (Figure 3). The increase in available IGF-Ⅱ may result from increased *IGF-Ⅱ* gene expression but also from a decrease of IGF-Ⅱ degradation by IGF-Ⅱ R or IGF-Ⅱ sequestration by IGFBPs.

TARGETING THE IGF AXIS IN HCC TREATMENT

Compelling evidence exists to involve the IGF system in hepatocarcinogenesis. Therefore, several strategies that target different IGF components are being studied with

Figure 4 Insulin growth factor targeting strategies in hepatocellular carcinoma treatment. Different strategies can be used to inhibit insulin growth factor (IGF) signaling. They can be ligand based or receptor based therapies. Ligand based therapies affect signaling through all IGF receptors, but these strategies should not be therapeutic in tumors with activating mutations in IGF-IR receptor or downstream proteins. Growth hormone (GH) analogues reduce IGF-I expression by blocking GH-GHR interaction. Specific ASOs and miR-615-5p decrease IGF-II expression. Soluble forms of IGF-II R and IGFBPs can decrease IGF-II bioavailability. Ligand directed neutralizing antibodies impede binding to receptors. Receptor based therapies focus mainly on the inhibition of IGF-IR signaling. Antisense oligonucleotides (ASOs) and siRNAs decrease IGF-IR expression and IGF-IR blocking antibodies impede ligand mediated activation. These strategies do not affect IR-A and IR-A/IR-B, which could be crucial in the progression of some hepatocellular carcinoma. Tyrosine kinase inhibitors inhibit insulin receptor (IR)-A, IGF-IR and hybrid receptor autophosphorylation and subsequent activation but commonly they also inhibit IR-B receptor which is essential for the metabolism of normal cells. IGFBPs: IGF high affinity binding proteins.

the aim of developing new therapeutic drugs for HCC (Table 1)[28]. The most promising strategies include the inhibition of IGF-IR signaling using monoclonal antibodies against IGF-IR, IGF-II and/or IGF-I and small molecule tyrosine kinase inhibitors (TKIs) that inhibit IGF-IR activation and signaling (Figure 4). Other approaches such as the inhibition of IGF-IR or IGF-II expression using siRNAs or antisense oligonucleotides (ASO) and the modulation of the IGFBP activity are under preclinical investigation. Some strategies that target the IGF system may be effective as monotherapies but others may be more effective in combination with chemotherapy and can be used as chemosensitizing agents.

These different strategies should be tested exclusively in HCC cells with an altered IGF-I axis. Furthermore, the expression and functionality of the different members of the pathway should be evaluated prior to treatment. This should ensure that the therapy is targeting an IGF pathway molecule relevant for the growth of the particular HCC to be treated. Such personalized medicine is essential to obtain therapeutic effects. To help in this analysis, an antibody array has been recently developed that detects ten members of the IGF system (IGF-I, IGF-IR, IGF-II, IGF-IIR, IGFBP1, IGFBP2, IGFBP3,

IGFBP4, IGFBP6 and insulin)[195]. Unfortunately, antibodies for IR-A and IR-B isoforms are not included in the array, despite their importance in HCC development.

Targeting IGF-I

Despite the capability of IGF-I to activate IGF-IR signaling, IGF-I is decreased in liver tumors and in the serum of patients with HCC[130,131]. Furthermore, high circulating levels of IGF-I are associated with less aggressive HCC, with a better prognosis[132] and with a better outcome in patients with advanced HCC receiving Sorafenib[196]. The reason for this is unclear.

Alternatively, IGF-I could lead to increased differentiation in hepatocytes and decreased proliferation. In fact, IGF-I has a therapeutic role in human liver cirrhosis, the substrate of HCC development. In a pilot study, the intravenous administration of recombinant IGF-I in patients with advanced liver cirrhosis improved liver functionality[197]. Moreover, we have demonstrated that the administration of IGF-I from viral vectors before the induction of liver cirrhosis prevents the development of the disease in rat models[198]. Furthermore, administration of viral vectors expressing IGF-I into rat cirrhotic livers leads to the complete reversion of liver cirrhosis[198,199].

Table 1 Agents targeting insulin-like growth factor system

Compound	Company	Description	Target	Treatment	Status	Ref./Clinical trial	Unaffected mitogenic IGF proteins/metabolic signaling status
Octreotide	Novartis Farmaceuticals	Somatostatin homologue	IGF-I	Monotherapy	Preclinical	[200,201]	IGF-II and insulin interaction with IGF-IR, IR-A, IR-B/IR-A and HRs/not affected
DX-2647		Neutralizing antibody	IGF-II and proIGF-II	Monotherapy	Preclinical	[202]	Insulin interaction with IR-A, IR-B/IR-A and HR-A/not affected
MEDI-573	AstraZeneca (MedImmune)	Neutralizing antibody	IGF-II and IGF-I	MEDI-573 + Sorafenib	Phase I	NCT01498952; [192,203]	
Cixutumumab (IMC-A12)	ImClone Systems Inc	Blocking antibody	IGF-1R	Monotherapy	Phase II	NCT00639509; [53,215,216]	IGF-I, IGF-II and insulin interaction with IR-A, IR-B/IR-A and HRs/not affected
				Cixutumumab + Sorafenib	Phase I	NCT01008566; NCT00906373	
AVE1642	Sanofi-Aventis	Blocking antibody	IGF-1R	Monotherapy	Phase I	NCT00791544; [125]	
				AVE-1642 + Sorafenib	Phase II	NCT00791544	
				AVE-1642 + Erlotinib	Phase II	NCT00791544	
BIIB022	Biogen-Idec	Blocking antibody	IGF-1R	BIIB022 + Sorafenib	Phase I	NCT00956436; [53,223]	
Figitumumab (CP-751,871)	Pfizer	Blocking antibody	IGF-1R, HRs	Monotherapy	Preclinical	[224,225]	IGF-II and insulin interaction with IR-A, IR-B/IR-A/not affected /impaired.
Linsitinib (OSI-906)	OSI Pharmaceuticals	TKI	IGF-1R, IR	Monotherapy	Phase II	NCT01101906; [226,227]	
				OSI-906 + Sorafenib	Phase II	NCT01334710	
AG1024 (Tyrphostin)		TKI	IGF-1R, IR	Monotherapy	Preclinical	[231]	
NVP-AEW541	Novartis Farmaceuticals	TKI	IGF-1R, IR	Monotherapy	Preclinical	[232,233]	
BMS-536924	Bristol-Myers Squibb	TKI	IGF-1R, IR	Monotherapy	Preclinical	[234]	
GSK1904529A	GlaxoSmithKline	TKI	IGF-1R, IR	Monotherapy	Preclinical	[235]	

IGF: Insulin-like growth factor; IGF-IR: IGF-I receptor; IR: Insulin receptor; TKI: Tyrosine kinase inhibitor.

This therapeutic effect correlates with IGF-I mediated activation of an anti-inflammatory and anti-fibrogenic program. Furthermore, IGF-I expression increases differentiation of cirrhotic hepatocytes and liver functionality. Using our model, overexpression of IGF-I in healthy or cirrhotic livers for more than a year did not lead to detectable liver tumors. Given that IGF-I displays anti-inflammatory and hepatoprotective effects, IGF-I deficiency caused by liver cirrhosis may create an intrahepatic microenvironment that allows for hepatocyte dedifferentiation and facilitates HCC emergence. Alternatively, high IGF-I levels could mark for functional hepatocytes, which are more difficult to transform and easier to cure. Finally, it cannot be ruled out that IGF-I could have an unexpected antitumoral effect on its own. If this is the case, IGF-I and IGF-II signalling through IGF-IR should lead to different responses in HCC. This has never been shown experimentally. Therefore, even if IGF-I could exert some antitumoral effect, it would be risky to overexpress IGF-I once a HCC with altered IGF axis has developed.

Even if downregulation of IGF-I together with upregulation of IGF-IR, IR-A and IGF-II are common events in HCC, suggesting that IGF-I has a limiting role in hepatocarcinogenesis, there are ligand-based therapies that specifically target IGF-I without affecting IGF-II. Theoretically, blocking IGF-I could favor binding of IGF-II to IGF-IR and increase cell proliferation. This has not been observed with Octreotide, a cyclic octapeptide used as GH analogue in the treatment of several types of cancers. Octreotide competes with GH for binding to its receptor and inhibits GH-GHR interaction and signaling leading to a decrease in IGF-I synthesis by the liver, but also affecting the expression of many other molecules[200]. Furthermore, decreased IGF-I should result in increased GH levels. Octreotide treatment showed good results in prostate cancer, but not in HCC. In a recent meta-analysis of all randomized controlled clinical trials using Octreotide for HCC patients, there was an improvement in the overall survival of the treated patients compared with non-treated controls, which was not statistically significant when compared with placebo controls[201].

Targeting IGF-II
IGF-II is overexpressed in human HCC and it activates

proliferation and migration through IGF-IR and IR-A receptors. IGF-Ⅱ based therapies must be designed to decrease or normalize IGF-Ⅱ levels and/or to inhibit its interaction with both receptors without altering insulin signaling. To this aim, blocking antibodies and strategies to decrease IGF-Ⅱ expression are under development.

Human antibody DX-2647 binds to IGF-Ⅱ and pro-IGF-Ⅱ with high affinity impeding their interaction either with IGF-IR and IR-A and suppressing proliferation in several HCC cell lines. Moreover, DX-2647 administration delays tumor growth and inhibits angiogenesis in xenograft models of HCC. DX-2647 also reacts with IGF-Ⅰ but with a 200-fold lower affinity[202]. This antibody remains in a preclinical status. Similarly, human monoclonal antibody MEDI-573 binds IGF-Ⅱ and IGF-Ⅰ (with 150-fold lower affinity than IGF-Ⅱ) without interacting with insulin[203]. MEDI-573 impedes IGF binding to IGF-IR, IR-A, and IGFBP3[192]. MEDI-573 administration reduces proliferation in cells expressing either IGF-IR or IR-A receptors but also in mixed populations of cells expressing both receptors in which an IGF-IR-specific antibody was totally ineffective. These results were obtained in several mouse embryonic fibroblast cell lines overexpressing specific human proteins and were then validated in xenograft tumors[203]. Two phase Ⅰ clinical trials designed to determine the effect of MEDI-573 administration on patients with solid tumors have recently finished but the results have not been yet published. Interestingly, a new clinical trial will test MEDI-573 administration in combination with Sorafenib in unresectable or metastatic HCC.

Strategies to decrease IGF-Ⅱ expression, such as antisense (ASO) or methylated (MONs) oligonucleotides, are under preclinical investigation. ASOs are short single-stranded DNA molecules complementary to a chosen mRNA sequence. ASO binding to the target mRNA results in mRNA expression inhibition as a result of RNase H-mediated mRNA degradation and translation blockage[204]. Downregulation of IGF-Ⅱ expression using ASOs that target IGF-Ⅱ mRNA inhibits cellular growth in hepatoma cell lines, but only in those that overexpress IGF-Ⅱ[205]. MONs that bind to the IGF-Ⅱ P4 promoter results in target DNA methylation and in turn, downregulation of fetal IGF-Ⅱ expression in the Hep3B human hepatoma cell line and in Hep3B derived tumors leading to enhanced survival[140]. Further development will be required to deliver these agents to most tumor cells.

Decreased IGF-Ⅱ availability can also be achieved by increased IGF-ⅡR expression. The administration of the soluble form of IGF-ⅡR (sIGF-ⅡR) to myeloid cell lines leads to a decrease in proliferation and survival[206]. Moreover, IGF-Ⅱ-induced DNA synthesis can be counteracted in hepatocytes and fibroblast using sIGF-ⅡR[92]. However, this soluble receptor can also bind other proteins and may have undesirable side effects. Therefore, therapeutic effects should be evaluated using a soluble form of IGF-ⅡR that only contains the IGF-Ⅱ binding domain.

Targeting IGFBP

IGFBPs modulate IGF signaling by regulation of IGF bioavailability. Most IGFBPs inhibit IGF signaling by limiting ligand access to IGF receptors, with the exception of IGFBP2 and IGFBP5. Therapies based on the administration of inhibitory IGFBPs or the inhibition of activating IGFBPs could be developed. In fact, the effect of increased levels of IGFBP3 and IGFBP-rP1 has already been evaluated.

As IGFBP3 represents 90% of serum IGFBPs[53], IGFBP3 downregulation in cancer significantly increases IGF ligand bioavailability. It has already been shown that the administration of exogenous IGFBP3 inhibits cell proliferation in hepatoma cell lines[157,158]. Interestingly, IGFBP3 expression can be re-induced in liver cancer cells by histone deacetylase inhibitors such as Trichostatin A[207,208]. An ongoing phase Ⅰ clinical trial combines Vorinostat, the first histone deacetylase inhibitor approved by the FDA, with different chemotherapy agents in patients with upper gastrointestinal cancers including liver cancer. Finally, as overexpression of IGFBP-rP1 (IGFBP7) decreases the tumorigenic potential of HCC cell lines[166], the antitumoral properties of an adenovirus expressing IGFBP7 have been recently demonstrated in both in vitro and *in vivo* models of HCC[209].

Targeting IGF-IR and IR/IGF-IR hybrids

Different strategies have been described to block IGF-IR signaling including blocking antibodies, siRNAs, antisense oligonucleotides, small molecule inhibitors and tyrosine kinase inhibitors.

Monoclonal antibodies: The administration of monoclonal antibodies against IGF-IR induces apoptosis and decreases proliferation in HCC[210]. Some of the monoclonal antibodies that have demonstrated promising results in preclinical models are: cixutumumab or IMC-A12 is a human IgG1 monoclonal antibody that selectively binds to IGF-IR, preventing the binding of its natural ligands[211]. The antibody also activates internalization and degradation of IGF-IR, leading to decreased levels of this receptor. Thus, IMC-A12 treatment inhibits downstream signaling in several tumors without altering insulin signaling[212-214]. *In vitro* and *in vivo* studies using different HCC models showed that blockage of IGF-IR by IMC-A12 decreases cell proliferation and increases apoptosis, resulting in prolonged survival and delayed tumor growth[215]. On the basis of these results, a phase Ⅰ clinical trial was performed in patients with advanced solid tumors. However, only partial responses were obtained[53]. In a subsequent phase Ⅱ study, administration of IMC-A12 as monotherapy in patients with advanced HCC displayed no antitumoral activity[216]. Instead, half of the patients developed hyperglycemia and 62% of the patients required initiation or increase in active therapy for diabetes. Besides, several patients showed reduced liver function indicated by elevated transaminases and bilirubin and decreased albumin, suggesting that

by blocking IGF-IR a protective effect of IGF-I on liver function had been lost. The mayor outcome of the study is that increased levels of IGFBP-1 correlated with progression free survival and with overall survival. The lack of therapeutic effect of IMC-A12 could be explained by the lack of IGF-IR in most of the patients, as IGF-IR expression could only be demonstrated in HCC samples obtained from 21% of the patients. However, the patients whose tumors were positive for IGF-IR did not show correlation with survival when compared with the IGF-IR-negative patients[216]. It needs to be determined whether IMC-A12 is more effective as a chemosensitizing molecule. Therefore, two clinical trials using combination of IMC-A12 and Sorafenib are ongoing.

AVE1642 is a humanized monoclonal antibody against IGF-IR that inhibits growth and metastasis in different human xenograft tumor models when used alone and/or in combination with chemotherapy[217-221]. AVE1642 was first tested in humans with advanced multiple myeloma yielding good tolerability but insufficient activity[222]. A posterior phase I/II clinical trial testing AVE1642 alone or in combination with Sorafenib or Erlotinib in patients with advanced or metastatic liver carcinoma supported the safety of AVE1642 in combination with active doses of Sorafenib[125].

BIIB022 is a human non-glycosylated IgG4.P antibody that blocks IGF-I and IGF-II binding to IGF-IR[223]. Preclinical data suggest that BIIB022 administration inhibits the growth of HepG2-derived tumors without induction of hyperglycemia. As BIIB022 lacks an Fc effector function, it displays less toxicity in normal IGF-IR expressing tissues[53]. A phase I study to evaluate the tolerability and safety of combinatorial therapy with Sorafenib has been completed but the results have not yet been published.

CP-751,871, also known as Figitumumab, is a human IgG2 antibody that inhibits IGF-I and IGF-II mediated autophosphorylation of IGF-IR but not IR, resulting in the internalization of the receptor[224]. It has been tested in 8 HCC cell lines, 2 of which, HepG2 and SNU368, were sensitive to the treatment in a dose-dependent manner. Administration of Figitumumab to HepG2 xenograft tumors leads to substantial growth inhibition[225]. Interestingly, in contrast to the other blocking antibodies, Figitumumab is able to inhibit hybrid receptor signaling. In fact, Figitumumab sensitivity has been associated with the levels of N-linked glycosylated IGF-IR/IR hybrids[225]. This compound has reach a phase III trial in multiple myeloma and non-small cell lung cancer, but it has not yet been tested for liver cancers.

Tyrosine kinase inhibitors: OSI-906 is a dual IGF-1R and IR Tyrosine kinase inhibitors (TKI) that displays antitumoral activity in several human cell lines and xenograft tumor models[226]. The mechanisms that mediate sensitivity to OSI-906 have been tested in a panel of 21 human HCC cell lines. In this study, higher responsiveness to OSI-906 was obtained in cell lines expressing high levels of IGF-II and IR[227]. Thus IGF-II and IR could be used as predictive markers for sensitivity to OSI-906 in HCC patients. OSI-906 evaluation in phase I dose escalation studies, alone or in combination with anti-cancer agents, resulted in good disease control rates and limited toxicity, including hyperglycemia, nauseas, vomiting and fatigue[8]. Two phase II clinical trials testing OSI-906 in patients with HCC have been carried out but were terminated due to the safety issues observed in the phase I study or to company policies. The partial results of the trials have not yet been published.

AG1024 (Tyrphostin) is a selective IGF-IR and IR TKI that is currently in preclinical development. Blockage of IGF-IR with AG1024 exerts antiproliferative and pro-apoptotic effects in several cancer cell lines[228-230]. Recently, AG1024 has been tested in two IGF-IR-expressing HCC, resulting in a significant decrease in cell invasiveness and a slight caspase-3 dependent proapoptotic effect[231].

NVP-AEW541 is a novel small molecule inhibitor of the IGF-IR tyrosine kinase activity. NVP-AEW541 has a 26-fold higher affinity for IGF-IR than for IR[232] and induces cell cycle arrest and apoptosis in HCC cell lines without cytotoxicity. When NVP-AEW541 was combined with chemotherapy, an additive antiproliferative effect was observed[233]. The effect of NVP-AEW541 remains to be tested in *in vivo* models of HCC.

BMS-536924 is a novel orally active, ATP-competitive, tyrosine kinase inhibitor of IGF-IR and IR. BMS-536924 antiproliferative activity has recently been described in HCC cell lines[234].

GSK1904529A is a tyrosine kinase inhibitor that blocks IGF-IR and IR phosphorylation. GSK1904529A has been tested in a wide range of cell lines and human xenograft tumor models resulting in low toxicity and strong antiproliferative and antitumoral effects. Although no HCC samples were included, the authors demonstrated that GSK1904529A inhibits the activity of the IR in liver tissues suggesting that it could be also effective in HCC[235].

The design of TKIs that target specifically IGF-IR signaling without altering IR signaling is difficult because of the high homology between these two receptors[62]. On one hand, targeting of both receptors can be advantageous since specific inhibition of IGF-IR was associated with higher IR signaling. On the other hand, targeting IR could lead to altered insulin signaling and unwanted secondary effects.

Antisense oligonucleotides: Phosphorothioate ASOs, which are more resistant to nuclease degradation than unmodified DNA, have been designed to target IGF-IR and have been evaluated in a model of HCC. In this study, inhibition of IGF-IR expression by ASOs results in a significant reduction of HepG2 proliferation. Systemic administration of IGF-IR ASOs in nude mice with orthotopic human HCC xenografts results in reduced tumor growth, recurrence and lung metastasis[236].

CONCLUSION

Deregulation of the IGF system is a common feature in HCC. Recent studies suggest that downregulation of IGF-Ⅰ together with upregulation of IGF-Ⅱ and over-activation of IGF-IR and IR-A are important events in HCC development. Thus, increased IGF-Ⅱ bioavailability, caused by increased IGF-Ⅱ expression or decreased regulation by IGF-ⅡR or IGFBPs, could be responsible for IGF-IR and IR-A overactivation. Furthermore, mutations in factors located downstream IGF receptors, such as Ras, PI3K or PTEN, could induce cell proliferation in tissues with normal IGF ligands or receptors. This has not been the subject of this review. Insulin and IR-B, by coupling to IGF-IR and IR-A, could also play a role in IGF pathway activation that leads to HCC. Little is known about the role of insulin in HCC development. Increased insulin serum levels have been associated with higher risk of HCC. This is probably caused by insulin binding to IR-A homo or heterodimeric receptors.

The role played by IGF-Ⅰ in HCC should be studied in detail. It is unclear why IGF-Ⅰ deregulation seems relevant for hepatocarcinogenesis. Experiments that address the effect of IGF-Ⅰ overexpression or downregulation in HCC development should be performed in animal models. The results may be relevant for the management of HCC in humans. Also, efforts should be devoted to understand why the binding of IGF-Ⅰ, IGF-Ⅱ, or insulin to a specific receptor of the IGF pathway, such as IGF-IR or IR-A/IR-B derived receptors, results in the activation of different signals. It should be interesting to identify liver-specific factors that modify IGF-IR signaling according to the ligand that has been sensed by the receptor, either IGF-Ⅰ or IGF-Ⅱ.

Given the particular features of IGF deregulation in HCC, the most promising therapies to date for HCC are antibodies that block IGF-Ⅱ or IGF-IR and tyrosine kinase inhibitors. The success of the treatment may depend on following personalized medicine protocols that first ensure that the IGF system is deregulated in the HCC to be treated. Furthermore, these protocols should evaluate the serum levels of IGF-Ⅰ, IGF-Ⅱ and insulin and the levels within the tumor of all the IGF ligands, receptors, binding proteins and signaling pathway factors. Such a detailed study of each tumor is essential to decide on a successful therapy. Thus, IGF-IR blocking antibodies are expected to be effective in tumors with increased IGF-IR and poor IR-A activation (Table 1). If this analysis is not performed, functional drugs may show no therapeutic effects. This may be the reason why IGF-IR antibodies display antitumoral effects in preclinical models but only partial responses in clinical trials. In the case of TKIs, as they are able to block IGF-IR and IR-A signaling, they are expected to be effective in all HCC with altered IGF ligands and receptors. However, TKI can also inhibit other tyrosine kinase receptors causing unwanted effects. TKI interaction with IR-B should lead to altered insulin metabolism.

Future therapies that target the IGF system should be developed for the treatment of HCC and other tumors. Novel specific antibodies or small molecules that affect the stability of IGF-Ⅱ or impede IGF-Ⅱ being sensed by IGF-IR and IRs should be developed. Similarly, design of functional TKI inhibitors or other molecules that affect IGF-IR and IR-A, but not IR-B is mandatory. Moreover, expression of key activators of the IGF pathway could be affected by antisense inhibitors or genome editing strategies. This will require the improvement of delivery techniques that allow the efficient delivery of the drugs to most tumor cells. Furthermore, present and future therapies need to take into consideration the altered drug metabolism of cirrhotic livers. As most HCC develop in a cirrhotic liver, it may be useful to stratify the patients according to liver functionality and liver fibrosis status before analyzing the therapeutic effects of a particular drug. Finally, even if the IGF system is altered in many HCC, it is not a unique tumor driver. It will be interesting to analyze the results of successful but also of non-successful trials to address if the blockade of IGF pathway was effective and whether other signaling pathways have been induced for tumor survival upon IGF system blockage. This may lead to rationalized combination therapies that may be essential for the successful treatment of HCC.

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Lipid-lowering agents in the management of nonalcoholic fatty liver disease

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in developed countries and is associated not only with increased risk for liver disease-related complications but also with higher cardiovascular morbidity. Accordingly, lipid-lowering agents are frequently considered in these patients to reduce cardiovascular risk. However, there have been concerns regarding the safety of these agents in patients with chronic liver diseases. In the present review, we discuss the safety of lipid-lowering agents in patients with NAFLD as well as their effects on both cardiovascular and liver disease in this population. Accumulating data suggest that statins are safe in patients with NAFLD and that they reduce the increased cardiovascular morbidity of this population. However, it is still unclear whether statins are also useful as a treatment for NAFLD *per se*, since there are very limited and conflicting data on their effects on liver histology. There is also very scarce evidence regarding the safety and efficacy of other lipid-lowering agents in patients with NAFLD. Randomized controlled studies are needed to evaluate the role of lipid-lowering agents and particularly statins for the prevention of both cardiovascular and liver disease-related complications in this high-risk population.

Core tip: Accumulating data suggest that statins are safe in patients with nonalcoholic fatty liver disease (NAFLD) and that they reduce the increased cardiovascular morbidity of this population. However, it is still unclear whether statins are also useful as a treatment for NAFLD *per se*, since there are very limited and conflicting data on their effects on liver histology. There is also very scarce evidence regarding the safety and efficacy of other lipid-lowering agents in patients with NAFLD.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is characterized by increased amount of fat in the liver in the absence of increased alcohol consumption[1]. NAFLD covers a wide range of histological disorders, ranging from isolated hepatic steatosis to nonalcoholic steatohepatitis (NASH), which is characterized by the coexistence of steatosis with varying degrees of inflammation and fibrosis, whereas some patients progress further to develop cirrhosis[2,3]. NAFLD is the most common chronic liver disease in developed countries[4-6]. Indeed, 34%-46% of the general population has liver steatosis and 12% has NASH[4,5]. Moreover, almost 75% of patients with persistently elevated transaminase levels have NAFLD[6].

Several cross-sectional studies showed that patients with NAFLD have a greater atherosclerotic burden and a higher prevalence of cardiovascular disease (CVD)[7-9]. Moreover, observational studies suggest that patients with NAFLD have increased cardiovascular risk and that CVD is the leading cause of death in this population[10-13]. Since NAFLD and CVD have many common risk factors (*e.g.*, abdominal obesity, type 2 diabetes mellitus (T2DM), insulin resistance, inflammation and oxidative stress), the increased CVD risk in patients with NAFLD might be partly explained by their shared pathogenesis[14-17]. However, there is increased CVD risk in patients with NAFLD even in the absence of T2DM, suggesting that NAFLD is directly causative of CVD[18].

Given the increased cardiovascular risk of patients with NAFLD, aggressive management of CVD risk factors is an essential part of the treatment of these patients. Lipid-lowering treatment is one of the pillars of CVD prevention strategies and primarily consists of administration of statins aiming at reducing low-density lipoprotein cholesterol (LDL-C) levels. The rationale behind this approach is that elevated LDL-C levels are a major independent cardiovascular risk factor[19] and that LDL-C lowering with statins reduces CVD morbidity and mortality[20]. However, an increase in transaminase levels is the most common adverse effect of statins[21]. Moreover, physicians are reluctant to administer statins in patients with elevated transaminase levels[21]. Similar considerations apply for other lipid-lowering treatments, which can be considered in patients who do not achieve LDL-C levels despite treatment with statins or in patients with elevated non-high density lipoprotein cholesterol (non-HDL-C) levels[22]. On the other hand, preliminary data suggest that statins and other lipid-lowering agents might reduce transaminase levels in patients with NAFLD and might also have beneficial effects on CVD morbidity[23,24].

In the present review, we discuss the safety of lipid-lowering agents in patients with NAFLD as well as their effects on both CVD and liver disease in this population.

STATINS IN PATIENTS WITH NAFLD

Safety

Accumulating data suggest that statins are safe in patients with NAFLD. In an observational study in hyperlipidemic patients with elevated transaminases, the incidence of further increase in transaminase levels during treatment with statins was similar compared with patients who had elevated transaminase levels but were not prescribed a statin[25]. Moreover, the incidence of severe elevations in transaminases did not differ during statin treatment between patients who had elevated transaminase levels at baseline and those who had normal transaminases[25].

Randomized controlled studies also support the safety of statins in patients with NAFLD. The West of Scotland Coronary Prevention Study trial compared the effects on CVD events of pravastatin 40 mg/d and placebo in men without established CVD but with LDL-C levels > 155 mg/dL whereas the Cholesterol and Recurrent Events

and Long-term Intervention with Pravastatin in Ischemic Disease trials compared pravastatin 40 mg/d and placebo in patients with established coronary heart disease (CHD). In a post-hoc analysis of these 3 trials, the risk of further increase in transaminase levels among patients who had elevated transaminase levels at baseline was similar in those treated with pravastatin and those administered placebo[26]. In the Greek Atorvastatin and Coronary Heart Disease Evaluation (GREACE) trial, patients with myocardial infarction (MI) were randomly assigned to receive atorvastatin aiming at LDL-C levels < 100 mg/dL or conventional treatment; only 14% of the latter group received a statin. In a post-hoc analysis of this trial, patients with elevated transaminase levels < 3 times the upper limit of normal (ULN) who were given atorvastatin (mean dose 24 mg/d) experienced a normalization of transaminase levels[22]. In contrast, patients with elevated transaminase levels who did not receive statins did not show any change in transaminase levels[23]. Similar results were observed in the Assessing the Treatment Effect in Metabolic Syndrome Without Perceptible Diabetes trial, where treatment of patients with metabolic syndrome with atorvastatin at a mean dose of 24-34 mg/d resulted in normalization of transaminase levels in the subgroup of patients with elevated transaminase levels at baseline[27].

Very recently, a post-hoc analysis of the Incremental Decrease in End Points Through Aggressive Lipid Lowering (IDEAL) trial also showed that treatment of patients with MI with atorvastatin 40-80 mg/d or simvastatin 20-40 mg/d reduces transaminase levels in patients with elevated levels at baseline[24]. It should be emphasized that the diagnosis of NAFLD in all these studies was not based on liver biopsy but on the presence of fatty liver in ultrasound and on the exclusion of other common causes of chronic liver disease (*i.e.*, chronic hepatitis B or C, increased alcohol consumption)[22,23,26]. Moreover, patients with transaminase levels > 3 times the ULN were excluded from all studies[23,24,27].

Based on these reassuring data regarding the safety of statin treatment in patients with elevated transaminase levels, current guidelines state that mild elevations of transaminase levels (< 3 times the ULN) are not a contraindication for the administration of statins, provided that patients are followed-up regularly[1,28]. Importantly, statins do not interact with agents that are used in the treatment of NAFLD (*e.g.*, vitamin E, pioglitazone, metformin, ursodeoxycholic acid, angiotensin receptor blockers) and therefore, can be safely coadministered with the latter agents[1,29].

Effects on cardiovascular events

Emerging data suggest that statins are not only safe in patients with NAFLD but also decrease the elevated CVD risk of this population[30]. In the GREACE trial, treatment with atorvastatin reduced CVD events by 39% compared with no statin treatment in patients with MI and normal transaminase levels at baseline[23]. In contrast, CVD morbidity was reduced by 68% with atorvastatin

treatment in patients with elevated transaminase levels, a reduction significantly greater than in patients with normal transaminase levels[23]. The IDEAL trial recently confirmed these findings. In IDEAL, atorvastatin 40-80 mg/d reduced major CVD events more than simvastatin 20-40 mg/d in patients with MI and elevated transaminase levels[24]. In contrast, the incidence of major CVD events did not differ between atorvastatin- and simvastatin-treated patients with normal transaminase levels[24]. Despite these promising data on the effects of statins on CVD morbidity in patients with NAFLD and the increased CVD risk of this population, it should be emphasized that current guidelines do not differentiate LDL-C targets between patients with NAFLD and the general population[22]. Accordingly, LDL-C targets are < 70 mg/dL in patients with NAFLD who have established CVD, T2DM or chronic kidney disease. In the absence of the latter comorbidities, LDL-C targets are < 70, < 100 and < 115 mg/dL in patients with NAFLD and SCORE risk ≥ 10, 5-9 and 1%-4%, respectively[22].

Effects on liver histology

There are very limited data on the effects of statins on liver steatosis, inflammation and fibrosis in patients with NAFLD. In small uncontrolled studies (*n* = 4-22), treatment with statins reduced steatosis and ballooning but had no effect on fibrosis; the effect on inflammation was inconsistent between studies[31-35]. In the only randomized placebo-controlled study, the administration of simvastatin for 12 mo in 16 patients with NASH had no effect on liver histology compared with placebo[36]. The interpretation of the findings of these studies is obviously hampered by the small number of patients and the lack of a control group in most of them. Moreover, the follow-up time might have been too short to evaluate the effects of statins on liver fibrosis. Considerably longer follow-up will also be required to assess any benefit of statins on the long-term sequelae of NAFLD, *i.e.*, cirrhosis and hepatocellular cancer (HCC). Notably, observational studies reported a decreased risk of HCC in patients treated with statins regardless of the cause (NAFLD, hepatitis B or C)[37-39]. Indeed, in a recent meta-analysis of 10 studies (*n* = 1459417), statins reduced the risk for HCC by 37%[37]. Given the limited data on the effects of statins on liver histology in patients with NAFLD, recent guidelines mention that statins should not be used as a treatment for NAFLD[1].

OTHER LIPID-LOWERING AGENTS IN PATIENTS WITH NAFLD

Ezetimibe

In patients who cannot achieve LDL-C targets despite treatment with the maximal tolerated dose of a potent statin, ezetimibe can be added to statin treatment[22]. Ezetimibe does not appear to be associated with increased risk for transaminase elevations when administered to patients with transaminase levels within the normal range[40].

In an uncontrolled study in 8 patients with NAFLD, treatment with ezetimibe for 1 year reduced transaminase levels but had no effect on liver steatosis assessed with ultrasonography[41]. In another uncontrolled study in 10 patients with NASH, treatment with ezetimibe for 6 mo reduced transaminase levels and ameliorated steatosis in liver biopsy but had no effect on ballooning, inflammation or fibrosis[42]. In another uncontrolled study in 45 patients with NAFLD, ezetimibe reduced transaminase levels and ameliorated steatosis, inflammation and ballooning in liver biopsy but had no effect on fibrosis after 2 years[43]. In a recent randomized controlled study in 32 patients with NAFLD, ezetimibe combined with diet for 6 mo had similar effects on transaminase levels and on liver histology as diet alone[44]. There are no randomized controlled studies that evaluated whether combination of ezetimibe with statins reduces CVD events more than monotherapy with statins.

Bile-acid binding resins

Another option to achieve LDL-C targets in patients who do not reach them despite treatment with the maximal tolerated dose of a potent statin is to add a bile-acid binding resin (BAS)[22]. These agents lack systemic side effects since they are not absorbed by the gastrointestinal tract and are not associated with increases in transaminase levels[45]. Colesevelam is a newer member of this class and is associated with lower rates of gastrointestinal side effects than other BAS[45]. However, in a recent randomized, placebo-controlled study in 50 patients with NASH, treatment with colesevelam for 24 wk increased liver steatosis assessed with magnetic resonance imaging[46]. Nevertheless, in the subgroup of patients who underwent a second liver biopsy at the end of follow-up (*n* = 31), the effects of colesevelam on liver steatosis, inflammation and fibrosis were similar to those of placebo[46]. An early uncotrolled study in 10 patients with NASH reported a decrease in transaminase levels, steatosis and inflammation but no change in fibrosis after treatment with another BAS, probucol, for 1 year[47]. In contrast, in a more recent uncotrolled study in 26 patients with NASH, treatment with probucol for 6 mo decreased transaminase levels but had no effect on steatosis, ballooning, inflammation or fibrosis[48]. The Lipid Research Clinics Coronary Primary Prevention Trial is the only study that evaluated the effects of BAS on CVD events and showed that treatment of hypercholesterolemic men without CHD with cholestyramine for 7.4 years reduced CHD events compared with placebo[49]. However, no separate analyses of the effects of cholestyramine on CVD events were performed in patients with elevated transaminase levels.

Fibrates

In patients at high or very high CVD risk who have triglyceride levels > 200 mg/dL after achieving LDL-C targets with a statin, fibrates can be added to achieve non-HDL-C targets[22]. The combination of fenofibrate with statins does not appear to increase transaminase or

creatine kinase levels more than statin monotherapy[50]. In contrast, the combination of gemfibrozil with a statin is associated with increased risk for rhabdomyolysis and is contraindicated[22]. Regarding the effects of fibrates on NAFLD, in a placebo-controlled study in 27 patients with NAFLD, fenofibrate had no effect on hepatic triglyceride content[51]. In a larger study in 186 patients with MetS and NAFLD, the combination of fenofibrate and atorvastatin was not more effective than atorvastatin monotherapy in reducing transaminase levels and liver echogenicity[52]. In the only study that evaluated the effects of fenofibrate on liver histology, the administration of fenofibrate for 48 wk in 16 patients with NAFLD decreased transaminase levels and improved ballooning but had no effect on steatosis, inflammation or fibrosis[53]. The only study that evaluated the effects of fibrate and statin combination on CVD events is the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial[50]. In ACCORD, patients with T2DM who were being treated with simvastatin 20-40 mg/d were randomized to receive fenofibrate or placebo[50]. After a mean follow-up of 4.7 years, CVD event rates did not differ between the 2 groups[50]. Again, there have not been performed separate analyses of the effects of fenofibrate on CVD events in patients with elevated transaminase levels who were enrolled in the ACCORD trial.

Omega-3 fatty acids

Another option to reach non-HDL-C targets is to add omega-3 fatty acids to statin treatment[22]. This combination is not associated with increased risk for transaminase elevations[54]. Small uncontrolled studies in patients with NAFLD reported a reduction in transaminase levels during treatment with omega-3 fatty acids[55,56]. In 2 controlled studies (n = 40 and 144, respectively), omega-3 fatty acids combined with diet reduced transaminase levels and hepatic fatty infiltration in ultrasound more than diet alone in patients with NAFLD[57,58]. In the only study that assessed the effects of omega-3 fatty acids on liver histology, treatment with the omega-3 fatty acid eicosipentanoic acid (EPA) for 12 mo reduced transaminase levels in 23 patients with NASH[59]. An improvement in liver steatosis, ballooning, inflammation and fibrosis was observed in 6 out of 7 patients who underwent liver biopsy at the end of follow-up[59]. The only study that evaluated the effects of high doses of omega-3 fatty acids on CVD events is the Japan EPA Lipid Intervention Study (JELIS), in which Japanese patients with hypercholesterolemia were randomly assigned to receive statin alone or statin combined with EPA 1800 mg/d[54]. The addition of EPA reduced CVD events by 19% compared with statin monotherapy[54]. However, this study was performed in a population with increased background fish consumption and it is unclear whether these findings are applicable to other populations[54]. Again, the effects of omega-3 fatty acid and statin combination on CVD events were not analyzed separately in patients with elevated transaminase levels in the JELIS trial.

Nicotinic acid

A final option to achieve non-HDL-C targets is to combine statins with nicotinic acid[22]. However, this combination is associated with increased risk for elevations in transaminase levels compared with statin monotherapy[60,61]. Moreover, there are very limited data on the effects of nicotinic acid in NAFLD. In a placebo-controlled study in 27 patients with NAFLD, nicotinic acid had no effect on hepatic triglyceride content[51]. More importantly, 2 recent studies showed that the combination of nicotinic acid with a statin does not decrease CVD events more than statin monotherapy[60,61]. In the Atherothrombosis Intervention in Metabolic Syndrome with Low HDL/High Triglycerides: Impact on Global Health Outcomes (AIM-HIGH) study, patients with CVD who were on simvastatin 20-40 mg/d, were randomized to receive nicotinic acid or placebo[60]. After a mean follow-up of 3 years, the incidence of the primary end-point (death from CHD, nonfatal MI, ischemic stroke, hospitalization for an acute coronary syndrome, or symptom-driven coronary or cerebral revascularization) did not differ between the 2 groups and an increase in the risk of ischemic stroke was observed in patients who received nicotinic acid[60]. In the Heart Protection Study 2 - Treatment of HDL to Reduce the Incidence of Vascular Events (HPS2-THRIVE) study, patients with established CVD who were on simvastatin 40 mg/d were randomized to receive nicotinic acid or placebo[62]. After a median follow-up of 4 years, the incidence of CVD events did not differ between the two groups[62]. Neither of these studies evaluated separately patients with elevated transaminase levels.

CONCLUSION

Accumulating data suggest that statins are safe in patients with NAFLD and that they reduce the increased cardiovascular morbidity of this population. However, it is still unclear whether statins are also useful as a treatment for NAFLD *per se*, since there are very limited and conflicting data on their effects on liver histology. There is also very scarce evidence regarding the safety and efficacy of other lipid-lowering agents in patients with NAFLD. Randomized controlled studies are needed to evaluate the role of lipid-lowering agents and particularly statins for the prevention of both CVD and liver disease-related complications in this high-risk population.

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MINIREVIEWS

Comprehensive review of post-liver resection surgical complications and a new universal classification and grading system

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Abstract

Liver resection is the gold standard treatment for certain liver tumors such as hepatocellular carcinoma and metastatic liver tumors. Some patients with such tumors already have reduced liver function due to chronic hepatitis, liver cirrhosis, or chemotherapy-associated steatohepatitis before surgery. Therefore, complications due to poor liver function are inevitable after liver resection. Although the mortality rate of liver resection has been reduced to a few percent in recent case series, its overall morbidity rate is reported to range from 4.1% to 47.7%. The large degree of variation in the post-liver resection morbidity rates reported in previous studies might be due to the lack of consensus regarding the definitions and classification of post-liver resection complications. The Clavien-Dindo (CD) classification of post-operative complications is widely accepted internationally. However, it is hard to apply to some major post-liver resection complications because the consensus definitions and grading systems for post-hepatectomy liver failure and bile leakage established by the International Study Group of Liver Surgery are incompatible with the CD classification. Therefore, a unified classification of post-liver resection complications has to be established to allow comparisons between academic reports.

Core tip: The large degree of variation in the post-liver resection morbidity rates reported by previous studies might be due to a lack of consensus regarding the definitions and classification of post-liver resection complications. The Clavien-Dindo classification of postoperative complications is widely accepted internationally. However, it is difficult to apply to some major post-liver resection complications. Therefore, a unified classification of post-liver resection complications has to be established to allow comparisons between academic reports.

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INTRODUCTION

Liver resection has become a safe operation, and its mortality rate is now almost zero, which is much lower than the rate seen a decade ago[1-3]. Liver resection is the best curative option for patients with certain types of liver cancer such as hepatocellular carcinoma[4,5] and metastatic liver cancer[6], as it is cost effective and results in a shorter period of disease-related suffering. To reduce the invasiveness of surgery, laparoscopic procedures have been widely adopted for various types of liver resection[2,7-9]. Preliminary clinical studies have demonstrated that compared with open surgery laparoscopic liver resection results in fewer surgical complications, less intraoperative bleeding, and shorter hospital stays whilst achieving similar oncological outcomes[2,10].

Although the mortality rates described by previous studies were similar, the reported post-liver resection morbidity rates varied markedly due to the use of different definitions for each complication. In fact, the overall morbidity rate of open liver surgery has been reported to range from 4.1% to 47.7%[2,11]. Dindo *et al*[12] attempted to unify the definitions of post-liver resection surgical complications by developing their own grading system (Table 1), which has been widely accepted according to surgical academic reports. However, a classification of the complications seen after hepatobiliary surgery produced by the International Study Group of Liver Surgery (ISGLS)[13] was incompatible with the definitions outlined in Clavien's classification. For example, cases that involve surgical or radiological interventions performed under general anesthesia (categorized as Ⅲb under the Clavien-Dindo classification) are rarely seen in the clinical setting. Furthermore, patients who suffer organ failure usually exhibit multiple complications, and thus, it is difficult to identify a single cause of the organ failure.

Therefore, we reviewed the definitions of post-liver resection surgical complications and have developed a simple grading and classification system to allow academic reports to be compared.

POST-HEPATECTOMY LIVER FAILURE

Liver failure is the most serious complication after liver resection and can be life-threatening[14,15]. The etiologies of post-hepatectomy liver failure (PHLF) include a small remnant liver[16], vascular flow disturbance[17], bile duct obstruction[15], drug-induced injury[18], viral reactivation[19], and severe septic conditions[15]. In 2011, the ISGLS defined PHLF as a postoperative reduction in the ability of the liver to maintain its synthetic, excretory, and detoxifying functions, which is characterized by an increased international normalized ratio and concomitant hyperbilirubinemia on or after postoperative day 5[13]. Treatments for PHLF must be selected carefully based on the etiology of the condition. Since it was proposed, most reports have employed the ISGLS definitions of PHLF (Table 2). In addition to the latter definitions, our grading system also includes information about the management strategies that are typically employed to treat each PHLF grade (Table 2).

BILE LEAKAGE

Bile leakage (BL) is a major complication of liver resection. The incidence of BL is reported to be 4.0% to 17%[20], and a previous meta-analysis did not find any difference in the incidence of BL between open and laparoscopic cases[21]. BL is defined as an increased bilirubin concentration in the drain or intra-abdominal fluid; *i.e.*, a bilirubin concentration at least 3 times greater than the simultaneously measured serum bilirubin concentration[22]. Once BL develops, it can sometimes lead to complications and can become difficult to manage without interventional radiology (IVR). One of our representative Grade C cases is shown in Figure 1. BL is usually managed with extensive IVR, and reoperations are rarely required. The ISGLS has also developed a grading system for BL[22]. Although the different grades of PHLF are well defined based on clinical symptoms and the management strategies employed, the definitions of each BL grade are too subjective. Therefore, our grading system includes clinical examples (Table 3).

ACUTE RENAL FAILURE

Acute renal failure (ARF) is associated with various postoperative complications. Renal failure is closely associated with PHLF and can lead to hepatorenal syndrome (HRS). The International Ascites Club (IASC) defined HRS using the following criteria[23-25]: (1) cirrhosis and ascites are present; (2) the patient's serum creatinine level is greater than 1.5 mg/dL (or 133 mmol/L); (3) no sustained improvement in the serum creatinine level (to a level of 1.5 mg/dL or less) is seen at least 48 h after diuretic withdrawal and volume expansion with albumin (recommended dose: 1 g/kg body weight per day up to a maximum of 100 g of albumin/d); (4) shock is absent; (5) the patient is not currently taking nor have they recently been taking nephrotoxic drugs; (6) parenchymal kidney disease, as indicated by proteinuria of greater than 500 mg/d, microhematuria (> 50 red blood cells/high power field), and/or abnormal renal ultrasonography, is absent (Verna EC1, Wagener G, Renal interactions in liver dysfunction and failure).

On the other hand, post-liver resection ARF is still poorly defined. Therefore, we have proposed a grading system for post-liver resection ARF (Table 4). The management of ARF mainly involves dehydration and the use of diuretics[26]. Most cases of Grade A and Grade B ARF are reversible and manageable via the latter approach. We defined cases in which the patient could not pass urine without continuous diuretic use as Grade B. On the other hand, Grade C cases were defined as those in which the patient required hemodialysis.

Figure 1 Representative grade C case in bile leakage. A 67-year-old man had hepatocellular carcinoma (diameter: 2 cm; A: Axial view; B: Coronal view) in segment S5 of his liver (located at the bifurcation of the bile duct in the hilar plate) (C: Axial view; D: Coronal view). The tumor was resected via enucleation; E: Bile leakage was detected and so endoscopic retrograde cholangiodrainage was performed together with percutaneous drainage of the resected pouch; F: Subsequently, stenosis of the left hepatic duct due to bile duct ischemia occurred. Percutaneous transhepatic cholangiodrainage was performed via the B3 duct; G: Three-dimensional reconstruction based on CT images obtained before the patient was discharged from hospital. CT: Computed tomography.

Table 1 Comparison between the modified grading system and the Clavien-Dindo classification

Modified grades	Clavien-Dindo classification	
Grade A	Grade I	Any deviation from the normal postoperative course that did not require special treatment
	Grade II	Cases requiring pharmacological treatment
Grade B	Grade IIIa	Cases requiring surgical or radiological interventions without general anesthesia
Grade C	Grade IIIb	Cases requiring surgical or radiological interventions performed under general anesthesia
	Grade IVa	Life-threatening complications involving single organ dysfunction
Grade D	Grade IVb	Life-threatening complications involving multiple organ dysfunction
	Grade V	Cases that resulted in death

Table 2 Grading system and representative management strategies for post-hepatectomy liver failure

Grades	Definition	Management strategies
Grade A	No change in the patient's clinical management strategy required or manageable with medication	Diuretics, selective digestive decontamination, lactulose, glucagon-insulin therapy, stronger neo-minophagen C
Grade B	Manageable without invasive treatment	FFP transfusion, hyperbaric oxygen therapy
Grade C	Invasive treatment required	Plasma exchange, artificial liver support, surgery (including liver transplantation)

Artificial liver support is including high-flow hemodialysis with FFP transfusion. FFP: Fresh frozen plasma.

ASCITES

Ascites is a common complication in patients who exhibit liver dysfunction or cirrhosis after liver resection[27]. One of the possible pathogenic mechanisms of the ascites seen after liver resection is portal flow resistance at the sinusoidal level due to a reduction in the volume of the portal vascular bed[28]. Hepatic outflow block can also cause increased portal flow resistance[29]. The acute phase after liver resection tends to involve edema in the interstitial organ space, which leads to increased portal flow resistance. The management of ascites after liver resection

Table 3 Grading system and representative management strategies for bile leakage

Grades	Definition	Management strategies
Grade A	No change in the patient's clinical management strategy required or manageable with simple drainage	Drainage within 7 d Antibiotic administration
Grade B	Manageable with interventional procedures	Drainage for 7 or more day, ethanol injection, fibrin paste injection, single ENBD, single EBD, single PTBD, PTPE, TAE
Grade C	Cases involving pneumoperitoneum, inflammation, multiple organ failure, or reoperation	Complicated IVR (combinations with any Grade Bs) Reoperation

ENBD: Endoscopic nasobiliary drainage; EBD: Andoscopic biliary drainage; PTBD: Percutaneous transhepatic biliary drainage; PTPE: Percutaneous transcatheter portal embolization; TAE: Transcatheter arterial embolization; IVR: Interventional radiology.

Table 4 Grading system and representative management strategies for acute renal failure

Grades	Definition	Management strategies
Grade A	Increase in serum creatinine level of ≥ 0.3 mg/dL from the baseline or 1.5 to 2-fold increase from the baseline Urinary output of less than 0.5 mL/kg per hour for more than 6 h	Dehydration Diuretics
Grade B	Two-fold increase in the serum creatinine level from the baseline Urinary output of less than 0.5 mL/kg per hour for more than 12 h	Continuous mannitol + diuretics
Grade C	Dialysis treatment required (serum K > 6.0 mEq, BE < -10, uremia, hypouresis that lasts for more than three days)	Hemodialysis

Table 5 Grading system and representative management strategies for ascites

Grades	Definition in International Ascites Club (2003)	Definition in International Ascites Club (1996)
Grade A	Detected only on United States	Mild
Grade B	Moderate symmetrical distention of the abdomen	Moderate
Grade C	Marked abdominal distention	Massive or tense

Table 6 Grading system and representative management strategies for ascites

Grades	Definition	Management strategies
Grade A	Requiring any changes in the clinical management strategy or manageable with medication Ascites discharge < 1000 mL/d in the drainage case	Diuretics, sodium restriction
Grade B	Grade A ascites that lasts for more than 2 wk or requires peritoneal puncture Ascites discharge < 2000 mL/d in the drainage case	Peritoneal puncture
Grade C	Invasive treatment required	Denver peritoneovenous shunt, TIPS, PSE, splenectomy

TIPS: Transjugular intrahepatic portosystemic shunt; PSE: Partial splenic embolization.

focuses on decreasing the patient's portal pressure[27,28]. The use of diuretics or sodium restriction can decrease systemic flow volume, and ascites can also be controlled by decreasing edema in the inter-organ space or establishing a systemic shunt. Invasive management aims to decrease the patient's portal pressure through mechanical interventions. The IASC previously released statements containing revised definitions of ascites (Table 5); however, they were too abstract to use in academic studies. So, we proposed a modified grading system for post-operative ascites after liver resection (Table 6).

SURGICAL SITE INFECTIONS (SUPERFICIAL, ORGAN AND DEEP) AND WOUND DEHISCENCE

Surgical site infections (SSI) are common after all types

of surgery and are classified into superficial, deep incisional, and organ/space SSI. Although several classifications of SSI have been proposed[30], the definitions developed by the Centers for Disease Control and Prevention (CDC) are widely used internationally[31]. According to the CDC, SSI are infections that occur within 30 d of surgery or within one year if an implant is present[31]. In addition, one of the following criteria must be met: (1) purulent drainage from an incision (incisional infection) or from a drain below the fascia (deep infection); (2) a surgeon or attending physician diagnosing an SSI; (3) an infective organism being isolated from a culture of fluid or tissue obtained from the surgical wound (for incisional infections); (4) spontaneous dehiscence or a surgeon deliberately re-opening the wound in the presence of fever or local pain, unless subsequent cultures were negative, or an abscess being detected during direct examinations (for deep infections). However, the grading of SSI based

Table 7 Grading system for superficial SSI and wound dehiscence

Grades	Definitions	Management strategies
Grade A	Manageable within 2 wk	Small open wound, outpatient service
Grade B	Requiring any management 2 wk and more	Large open wound, inpatient service
Grade C	Any management required under general anesthesia	

Table 8 Grading system for deep and organ/space surgical site infections

Grades	Definitions	Management strategies
Grade A	Manageable without requiring any additional perioperative management within 2 wk	Antibiotics, simple drainage
Grade B	Requiring any management 2 wk and more	Additional drainage, irrigation
Grade C	Any management required under general anesthesia	

Table 9 Grading system and representative management strategies for coagulation disorders

Grades	Definition	Managements
Grade A	Does not require any change in the clinical management strategy	Vitamin K, ATIII, LMWH, SPI, UFH, and DS
	Plat $< 10 \times 10^4$ (preoperative Plat was within normal range)	
	30% reduction in Plat (preoperative Plat was abnormal)	
Grade B	Medication required for more than 5 d	Platelet transfusion
	Plat $< 5 \times 10^4$ (preoperative Plat was within normal range)	
	60% reduction in Plat (preoperative Plat was abnormal)	
Grade C	Intensive care treatment required and involved the failure of other organs	

Plat: Platelet count; ATIII: Anti-thrombin; LMWH: Low molecular weight heparin; SPI: Synthetic protease inhibitor; UFH: Unfractionated heparin; DS: Danaparoid sodium.

Table 10 Grading system and representative management strategies for pneumonia and respiratory disorder

Grades	Definition	Managements
Grade A	Meet SIRS criteria with imaging findings in less than 50% of the lung field or $PaO_2/FiO_2 < 300$	Antibiotics and oxygen Sputum suction
Grade B	Meet SIRS criteria with imaging findings in 50% and more of the lung field or $PaO_2/FiO_2 < 200$	Antibiotics and oxygen, IPPV, NPPV, bronchoscopy for sputum suction
Grade C	Requiring ventilator support	Ventilator

Systemic inflammatory response syndrome criteria is defined as two or more of the following clinical signs: bodily temperature > 38 ℃ or < 36 ℃, heart rate > 90/min, respiratory rate > 20 /min or $PaCO_2$ < 32 mmHg, WBC > 12000/μL or < 4000 /μL or immature cells > 10%. Pneumonia imaging is any of airspace opacity, lobar consolidation, or interstitial opacities. SIRS: Systemic inflammatory response syndrome; IPPV: Intermittent positive-pressure breathing; NPPV: Nasal positive-pressure ventilation.

on symptoms and the management strategy employed is difficult. Therefore, we proposed that SSI should be graded based on how long they take to cure (Table 7 for superficial SSI and wound dehiscence, Table 8 for deep and organ/space SSI). Using this new grading system, it is very easy and simple to grade SSI objectively.

COAGULATION DISORDERS

Coagulation disorders are a common complication after liver resection[32,33]. Most coagulation and anti-coagulant factors are synthesized by the liver, and the ability to synthesize such factors rapidly deteriorates after liver resection in cirrhotic patients and those who experience marked hepatic volume loss[20]. In addition, most patients who are scheduled to undergo liver resection present with thrombocytopenia due to portal hypertension.

Therefore, a prolonged prothrombin time, a prolonged thrombin time, elevated levels of fibrinogen degradation products, and a low platelet count are common after liver resection[34]. As we have mentioned in the ascites section, portal hypertension can occur after liver resection due to an increase in portal flow resistance[17]. Therefore, coagulation disorders should be divided into two different grades based on whether the patient displays normal or abnormal preoperative platelet levels (Table 9).

PNEUMONIA AND RESPIRATORY DISORDER

Postoperative pneumonia and respiratory disorder (PPN/RD) was rarely seen after liver resection recently except in the elderly cases[35,36]. Definition of the PPN/RD

was shown in Table 10. Clinical sign of the PPN/RD is systemic inflammatory response syndrome with any radiological imaging findings[37]. Management will be taken by administrating susceptible anti-biotics with oxygen supply. Acute lung injury (ALI) is defined by PaO_2/FiO_2 ratios < 300 and acute respiratory distress syndrome (ARDS) is defined by PaO_2/FiO_2 ratios < 200[58]. In our grading, ALI is in Grade A and ARDS is in the grade B (Table 10). Our grading is not only defined PPN/RD after liver resection but also after other general surgery.

CONCLUSION

The complications seen after liver resection are different from those encountered after other types of surgery because the liver produces most serum proteins, which play a major role in maintaining systemic homeostasis, and liver resection affects liver function. Therefore, post-liver resection complications tend to be severe. The risk factors for complications after liver resection depend on the pathological background of the liver itself[39]. In patients with normal liver function, the operative time, fresh frozen plasma transfusion requirement, tumor size, and retinol binding protein levels are independent risk factors for complications[40]. On the other hand, the PT and the indocyanine green retention value at 15 min are independent risk factors for complications in cirrhotic patients[40]. Therefore, consensus definitions and grading systems are necessary to allow comparisons between academic reports. Our grading system incorporates established consensus definitions and statements, such as those for PHLF and BL, and attempts to establish objective definitions for grading other complications. We hope that our grading system will be used to describe the complications experienced after liver resection.

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Role of autophagy in differential sensitivity of hepatocarcinoma cells to sorafenib

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Abstract

AIM: To investigate the role of sorafenib (SFN) in autophagy of hepatocellular carcinoma (HCC). We evaluated how SFN affects autophagy signaling pathway in human HCC cell lines.

METHODS: Two different human HCC cell lines, Hep3B and Huh7, were subjected to different concentrations of SFN. Cell viability and onset of apoptosis were determined with colorimetric assay and immunoblotting analysis, respectively. The changes in autophagy-related proteins, including LC3, ULK1, AMPK, and LKB, were determined with immunoblotting analysis in the presence or absence of SFN. To assess autophagic dynamics, autophagic flux was measured with chloroquine, a lysosomal inhibitor. The autophagic responsiveness between different HCC cell lines was compared under the autophagy enhancing conditions.

RESULTS: Hep3B cells were significantly more resistant to SFN than Huh7 cells. Immunoblotting analysis revealed a marked increase in SFN-mediated autophagy flux in Huh7 cells, which was, however, absent in Hep3B cells. While both starvation and rapamycin enhanced autophagy in Huh7 cells, only rapamycin increased autophagy in Hep3B cells. Immunoblotting analysis of autophagy initiation proteins showed that SFN substantially increased phosphorylation of AMPK and consequently autophagy in Huh7, but not in Hep3B cells.

CONCLUSION: The autophagic responsiveness to SFN is distinct between Hep3B and Huh7 cells. Resistance of Hep3B cells to SFN may be associated with altered autophagy signaling pathways.

Key words: Autophagy; Liver cancer; Sorafenib

Core tip: Hepatocellular carcinoma (HCC) is difficult to treat. Sorafenib (SFN) is one treatment option. Autophagy has been proposed to play a pivotal role in HCC. In the present study we investigated the role of autophagy in SFN-treated HCC cells. We found that the autophagic responsiveness to SFN is markedly distinct between Hep3B and Huh7 cells.

INTRODUCTION

Hepatocellular carcinoma (HCC), typically occurring in the setting of cirrhosis and chronic hepatitis, is fifth most common cancer diagnosed worldwide and more than 600000 patients are dying of this disease each year[1]. The

incidence of HCC is also rising in the United States. Despite recent advances in the screening and management of HCC, treatment of this pernicious disease is still far from complete, mostly due to its complex mechanisms underlying proliferation, tissue invasion and metastasis of HCC.

Therapies for HCC include chemoembolization, ablation, surgical resection and transplantation[2], but these interventions are highly invasive and often require prolonged hospitalization of the patients. Recently, sorafenib (SFN), an oral multi-kinase inhibitor, has been shown to inhibit tumor-cell proliferation and tumor angiogenesis through its inhibition of vascular endothelial growth factor receptor 2 and other receptor tyrosine kinases[3,4]. In a placebo-controlled phase III study, SFN displayed about 3-mo extension of survival in advanced and inoperable HCC cases, leading to Food and Drug Administration (FDA) approval[5]. It is, however, noteworthy that some HCC patients show unresponsiveness or acquired resistance to SFN[6]. It is unclear why the efficacy of SFN is limited, although the activation of survival pathways like PI3K/AKT has been proposed to cause the development of SFN resistance[7].

Autophagy is an evolutionary conserved cellular process that degrades both long-lived cytoplasmic proteins and surplus or dysfunctional organelles by lysosome-dependent machinery[8]. Impaired and insufficient autophagy is causatively linked to pathogenesis of ischemia/reperfusion injury and drug-induced toxicity in the liver[9-11]. Growing evidence is accumulating that autophagy also plays a pivotal role in carcinogenesis, tumor proliferation, and resistance to chemotherapy[12]. In addition, recent studies on an anti-cancerous role of autophagy raise a possibility that the modulation of autophagy could be a new therapy against cancer[13,14]. However, a pro-cancerous role of autophagy has also been suggested[15]. Thus, the precise role of autophagy in HCC is largely yet to be elucidated.

In the present study, we investigated the role of autophagy in HCC using two human HCC cell lines, Hep3B and Huh7 cells. Our results demonstrate that autophagic response to SFN and autophagy signaling pathways are markedly distinct between these two HCC cells.

MATERIALS AND METHODS

Reagents and chemicals

SFN and rapamycin were purchased from LC Laboratories (Woburn, MA) and dissolved in DMSO. Chloroquine was purchased from Sigma Chemical Co (St. Louis, MO) and dissolved in phosphate-buffered saline (PBS; 2.7 mmol/L KCl, 137 mmol/L NaCl 10.1 mmol/L Na_2HPO_4, and 1.8 mmol/L KH_2PO_4, pH7.4). Antibodies against ULK1 were purchased from Sigma Chemical Co (St. Louis, MO). All other primary antibodies were purchased from Cell Signaling Technology (Danvers, MA).

Cell culture

The human HCC cell lines, Hep3B and Huh7 cells,

were purchased from American Type Culture Collection (Manassass, VA) and were cultured in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Manassass, VA) supplemented with 10% fetal bovine serum (FBS; Sigma) and 1% penicillin/streptomycin (Mediatech) in 5% CO_2 at 37 ℃. Cells were used for experiments at approximately 80% confluence. For immunoblotting experiments, cells were plated in 60 mm culture dishes at 6×10^5 cells. DMSO was used for a vehicle control. To induce nutrient depletion and starvation, cells were incubated in Krebs-Ringer-hydroxyethylpiperazine-N-2 ethanesulfonic acid (HEPES) (KRH) medium containing 115 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L $CaCl_2$, 1 mmol/L KH_2PO_4, 1.2 mmol/L $MgSO_4$, and 25 mmol/L HEPES at a pH of 7.4.

MTT viability assay

To determine cell viability, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-biphenyl tetrazolium (MTT) assay was used with the different concentrations of SFN and rapamycin. HCC cells were plated on a 96-well microplate at 5×10^3 cells per well for up to 72 h in DMEM medium. DMSO was used as a vehicle control. The MTT salt dissolved in PBS (5 mg/mL) was added to the medium and incubated for 2 h[16]. The medium was subsequently removed and replaced with isopropyl alcohol. The optical density was read at 562 nm in SpectraMax M2e Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA). The results were shown as a ratio of viability of treated to vehicle groups.

Immunoblotting for autophagy proteins

Whole cell lysates were prepared by extracting proteins with radioimmunoprecipitation (RIPA) buffer (150 mmol/L NaCl, 25 mmol/L Tris-HCl (pH 8, 0.1% sodium dodecylsulfate, 1% sodium deoxycholic acid, 1% TritonX-100 and 5 mmol/L EDTA) with 1% protease and 1% phosphatase inhibitors. Protein concentrations were determined by BCA protein assay kit (Pierce, Rockford, IL). Proteins (10 or 15 μg) were separated by the electrophoresis through 4%-12% polyacrylamide gels (InVitrogen, Carlsbad, CA) or by sodium dodecyl sulfate polyacrylamide gel and transferred to polyvinylidene difluoride or nitrocellulose membranes. The expression of LC3, LKB1, phospho-AMPKα (Thr172), AMPKα, PARP and GAPDH were detected using primary polyclonal antibodies. After overnight incubation with primary antibodies at 4 ℃, the membranes were incubated with donkey anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA) and subsequently visualized by enhanced chemiluminescence. Changes in protein expression were determined using the ImageJ software (National Institutes of Health, Bethesda, MD)

SiRNA-mediated knockdown of AMPK

Small interfering RNA (siRNA) for AMPKα1/α2 (sc-45312) and the siRNA Reagent System (sc-45064) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). In a six well tissue culture plate, Huh7 cells were cultured until approximately 70% confluent. For

Figure 1 Cell death with sorafenib. The effect of varying doses (1, 5 and 10 µmol/L) of SFN on hepatocellular carcinoma cell viability was determined by the MTT assay at 24, 48 and 72 h in (A) Hep3B and (B) Huh7 cells. PARP expression was analyzed by immunoblotting analysis (C). SFN: Sorafenib; RAPA: Rapamycin.

each transfection, 80 pmol of siRNA was diluted into 100 µL of siRNA transfection medium, as suggested by the manufacturer. Huh7 cells were incubated with siRNA transfection reagents. Scrambled siRNA (sc-37007) was used for the control experiments.

Statistical analysis

Results were evaluated using unpaired two-tailed Student's *t* test. Data are expressed as mean ± SE and $P < 0.05$ denotes statistical significance. All values are representative of at least three different experiments per group.

RESULTS

Chemoresistance of Hep3B to SFN

To determine the chemosensitivity of HCC cells to SFN, Hep3B and Huh7 cells were treated with different concentrations of SFN for up to 72 h and cell death was evaluated with the MTT assay (Figure 1). There was a marked difference of cell death between two cells. While 5 µmol/L SFN induced virtually no cell death in Hep3B, this concentration caused a significant cell death in Huh7 in a time dependent manner (Figure 1A). Although 10 µmol/L SFN induced cell death in both cell types, the extent of cell killing was substantially greater in Huh7 cells than in Hep3B cells. To confirm the differential sensitivity to SFN between two cell types, the onset of apoptosis was determined with immunoblotting of poly (ADP ribose) polymerase (PARP) cleavage (Figure 1B). Similar to the results from the MTT assay, SFN substantially induced apoptotic cell death in Huh7 cells. Taken together, these results suggest that Hep3B cells are intrinsically more resistant to SFN than Huh7 cells.

Different autophagic responsiveness between Hep3B and Huh7 cells

Autophagy is a pro-survival mechanism in normal cells and has also been associated with chemoresistance in cancer cells[17-19]. Accordingly, we investigated if the autophagic responsiveness to SFN is different between two

cells. Microtubule-associated protein 1 light chain 3-Ⅱ (LC3-Ⅱ), a mammalian orthologue of Atg8, is a specific autophagy marker[20]. Immunoblotting of LC3 showed that SFN at three different concentrations barely changed the expression of LC3-Ⅱ in Hep3B cells (Figure 2A). However, in Huh7 cells, 10 µmol/L SFN significantly increased LC3-Ⅱ expression, suggesting that this concentration of SFN alters autophagy in Huh7 cells, but not in Hep3B. Autophagy is a dynamic process between autophagosomal entrapment and autolysosomal clearance[8]. Thus, the increase in LC3-Ⅱ by SFN in Huh7 cells could be due to either an increase in autophagosome formation or a decrease in autolysosome formation. To distinguish these two possibilities, we measured autophagic flux with chloroquine (CQ), a lysosomotropic agent that inhibits autolysosomal clearance[10]. Immunoblotting analysis of LC3 in the presence and absence of CQ revealed that SFN substantially increased autophagic flux only in Huh7 cells (Figure 2B). Therefore, these data demonstrate that Huh7 cells have higher autophagic responsiveness to SFN than Hep3B cells.

Starvation or nutrient depletion is a powerful stimulus for autophagy[9]. Next, we examined autophagic response of two cells to starvation. To induce starvation, cells were incubated in amino acid- and serum-free KRH for up to 4 h and changes in LC3 were evaluated with immunoblotting (Figure 2C). Autophagy was rapidly increased in Huh7 cells during 1 h of starvation, as judged by increased LC3-Ⅱ expression. However, this starvation-induced increase in LC3-Ⅱ was not evident in Hep3B cells, implying that starvation fails to induce autophagy in Hep3B cells.

Rapamycin enhances autophagy through mTOR inhibition[21]. To investigate if rapamycin can induce autophagy in HCC cells, either Hep3B or Huh7 cells were treated with 5 nmol/L rapamycin and changes in LC3 were determined in the presence and absence of CQ (Figure 2D). In a striking contrast to both SFN and starvation, rapamycin increased the expression of LC3-Ⅱ and autophagic flux in Hep3B cells as well as Huh7 cells.

Figure 2 Autophagic response to sorafenib. A: LC3 expression in hepatocellular carcinoma cells was analyzed in the presence of SFN for up to 6 h. Representative blot (left panel) and quantitative analysis of LC3-II by densitometry (middle and right panel). [a]*P* < 0.05; B: LC3 expression was analyzed in the presence of SFN with and without CQ to determine autophagy flux after 4 and 24 h; C: Autophagic response to nutrient-rich (control) or nutrient-depleted medium was analyzed by immunoblotting of LC3 in Hep3B and Huh7 cells for up to 4 h; D: LC3 expression was analyzed in the presence of 5 nmol/L RAPA after 4 h treatment with and without CQ to examine autophagy flux. NS: Non-significant; SFN: Sorafenib; CQ: Chloroquine; RAPA: Rapamycin.

These findings suggest that mTOR-mediated autophagy is functional and operative in both Hep3B and Huh7 cells. Collectively, our data demonstrate that the autophagic responsiveness of Hep3B cells to SFN and starvation is markedly distinct from that of Huh7 cells.

Different autophagy signaling between Hep3B and Huh7 cells

Autophagy is a multi-step process consisting of initiation, elongation and completion[8]. At the initiation stage of autophagy, two proteins play an integral role in cargo selection and phagophore formation: 5'-adenosine monophosphate-activated protein kinase (AMPK), a molecular energy sensor, and Unc-51 Like Autophagy Activating Kinase 1 (ULK1)[22], a mammalian homolog of yeast Atg1. To investigate whether SFN affects these proteins, we analyzed changes in AMPK and ULK1 expression with immunoblots (Figure 3). Densitometric analysis revealed a significant difference in the status of phospho-AMPK (p-AMPK) between two cells when SFN was added (Figure 3A and B). While the basal levels of p-AMPK were comparable between two cells, administration of SFN to Huh7 cells significantly increased p-AMPK expression. Changes in p-AMPK in Hep3B in the presence of SFN were, however, minimal. Total AMPK levels remained unchanged in both cells. Interestingly, the basal levels of liver kinase B1 (LKB1), a Ser/Thr kinase phosphorylating AMPK[23], was also significantly higher in Huh7 cells than in Hep3B cells (Figure 3A and C). When Huh7 cells were treated with SFN, the expression of LKB1 gradually decreased but remained higher than Hep3B cells especially during the early period of SFN treatment. On the contrary, the basal levels of ULK1 were significantly higher in Hep3B cells

than in Huh7 cells (Figure 3A and D). Administration of SFN did not change ULK1 expression in both cell types throughout 24 h of treatment. Therefore, these results demonstrate that the initiation signaling pathways of autophagy are noticeably different between two HCC cells.

The role of AMPK in autophagy of HCC cells

To further investigate how AMPK affects autophagy in Huh7 cells, AMPK was silenced with siRNA-mediated approaches (Figure 4A). Knocking down of AMPK substantially increased the levels of LC3-II, suggesting an integral role of AMPK in the basal autophagy of Huh7 cells. Co-addition of SFN further enhanced LC3-II expression more than 300%, as compared to the treatment with siRNA alone, implying that SFN may regulate multiple targets of autophagy process other than AMPK. Notably, CQ did not increase LC3-II under this condition.

Next, we explored the effects of AMPK activation on HCC cell death. HCC cells were treated either with 5 mmol/L metformin, an AMPK activator, or with 10 μmol/L SFN for 24 h. Some cells were treated with both. In Hep3B cells, metformin itself significantly increased cell death, which was further enhanced by SFN (Figure 4B). On the contrary, the addition of metformin failed to increase SFN-dependent cell death in Huh7 cells, suggesting that the responsiveness to metformin in the presence of SFN is distinct between two cells.

DISCUSSION

HCC is prevailing worldwide and more than 20000 new cases are reported in the United States each year[1]. The onset of HCC from hepatitis C virus infection is the one of the fastest-rising cause of cancer-mediated death in

Figure 3 Altered autophagy signaling in hepatocellular carcinoma cells. A: Hepatocellular carcinoma cells were treated with 5 and 10 μmol/L SFN for 4 and 24 h and the expression of autophagy initiating proteins was analyzed with immunoblotting; B: Quantitative analysis of p-AMPK. [a]$P < 0.05$; C: Quantitative analysis of LKB1. [a]$P < 0.05$; D: Quantitative analysis of ULK1. [b]$P < 0.001$. SFN: Sorafenib.

Figure 4 Gene silencing for AMPK. A: Huh7 cells were treated with siRNA for AMPK for 24 h. After 4 h treatment with SFN, LC3 was assessed with immunoblotting assay; B, C: The effect of either Met or SFN on hepatocellular carcinoma cell viability was determined by the MTT assay at 24 h in (B) Hep3B and (C) Huh7 cells. Some cells were treated with both agents. [a]$P < 0.05$, [b]$P < 0.001$. Met: Metformin; SFN: Sorafenib; CQ: Chloroquine.

the United States. During the past two decades, the incidence of HCC in the United States has tripled while the survival for 5 years has remained below 12%[24]. Thus, HCC remains a difficult and challenging cancer to treat. SFN is an oral multi-kinase inhibitor and inhibits tumor-cell proliferation and tumor angiogenesis[3,4]. Although this FDA-approved dug can prolong the survival of advanced and inoperable HCC patients, the average extension of survival is limited to about 3-mo. Furthermore, some patients do not respond to SFN[6]. The mechanisms behind such a limited efficacy of SFN remain unknown. Autophagy is an evolutionary conserved cellular process and has been proposed to play a pivotal role in HCC. In

the present study, using human HCC cell lines, we show that Hep3B cells are resistant to SFN-mediated apoptosis, while Huh7 cells are prone to apoptosis in the presence of SFN. Furthermore, we demonstrate that SFN induces a substantial enhancement of autophagy in Huh7 cells, but not in Hep3B cells, and that autophagy signaling pathways are noticeably distinct between two HCC cells.

Autophagy mainly plays a pro-survival role in normal cells by providing cells and tissues with nutrients. However, autophagy can cause cell death through a selective degradation of essential proteins and constituents in the cells[25]. The SFN-sensitive HCC cell line, Huh7 cells, exhibited a substantial difference in both cell death and au-

tophagic responsiveness, compared to the SFN-resistance cell line, Hep3B cells (Figures 1 and 2). Immunoblotting analysis of LC3 and autophagic flux showed that SFN induced a marked increase in autophagy in Huh7 cells, which was, however, absent in Hep3B cells (Figure 2). Lack of the autophagic responsiveness in Hep3B cells was also observed under the condition of starvation, a powerful stimulus of autophagy[9,12], suggesting that these two HCC cells have an intrinsically distinct autophagy. Furthermore, our results suggest that the enhancement of autophagy by SFN in Huh7 cells may be associated with the activation of p-AMPK, a key protein involved in autophagy initiation[22] (Figure 3). The importance of p-AMPK in SFN-dependent activation of autophagy is further supported by our findings that Hep3B cells fail to increase p-AMPK expression upon treatment of SFN. The necessity of AMPK activation for autophagy induction in HCC has been recently reported[26,27].

The contradictory effects of SFN on Hep3B and Huh7 cells have been reported[28] and may be linked to different autophagic responsiveness to this agent. Our results show that starvation or nutrient depletion fails to induce autophagy in Hep3B cells (Figure 2C). The absence of autophagy enhancement by either SFN or starvation could stem from defective or impaired autophagy in Hep3B cells. However, autophagic flux analysis revealed that Hep3B cells, indeed, have a considerable basal and mTOR-dependent autophagic capacity, as judged by the increase in LC3-Ⅱ either with CQ (Figure 2B) or with rapamycin (Figure 2D). These results imply that the signaling pathways of starvation-mediated autophagy may be altered in Hep3B cells. Although the mechanisms underlying SFN-induced autophagy remain to be elucidated, lack of autophagic response to both starvation and SFN in Hep3B cells led us to speculate that SFN-dependent autophagy requires a similar signaling pathway of the starvation-mediated autophagy. Since the only known difference in signaling mechanism between starvation- and rapamycin-induced autophagy exists in the initiation stage of autophagy process[8], we reasoned that events upstream to autophagy signaling pathways might be altered in Hep3B cells. In agreement with this view, we observed that the activation of AMPK, a critical event in autophagy induction under the condition of starvation, was evident only in Huh7 cells, but not in Hep3B cells, upon SFN administration. The precise mechanisms behind SNF-induced autophagy warrant future studies.

When normal, non-tumorigenic cells are subjected to stresses such as ischemia/reperfusion, alcohol and drug, autophagy becomes activated as an adaptive response to these stresses[12]. In contrast, autophagy can prevent and promote tumor development. These seemingly contradictory roles of autophagy in tumor stem from the complexity of tumorgenesis[12]. Prior to tumor establishment, autophagy clears damaged organelles and proteins, leading to preventing neoplastic transformation. However, when tumor develops, the demand for metabolic supplies is progressively increasing. As a consequence, autophagy becomes fully activated to provide the tumor cells with nutrients and amino acids. Thus, autophagy is a "double-edged sword" in cancer where it initially acts as a tumor suppressor, but later acts as a tumor promoter when tumor is established[15].

In conclusion, we have shown that Hep3B cells responds differently to various autophagy stimuli, compared to Huh7 cells. Although the modulation of autophagy could have a new therapeutic potential against cancer, our study demonstrates that caution should be taken before considering autophagy as anticancer regimes in HCC patients.

COMMENTS

Background
The incidence of hepatocellular carcinoma (HCC) is prevailing worldwide but its treatment is still disappointing. Sorafenib (SFN), an oral multi-kinase inhibitor, is one treatment option for HCC patients but its efficacy is limited. Autophagy is a cellular catabolic process that degrades both long-lived cytoplasmic proteins and surplus or dysfunctional organelles by lysosome-dependent machinery. Autophagy also plays a pivotal role in carcinogenesis, tumor proliferation, and resistance to chemotherapy. However, the role of autophagy in HCC remains unclear.

Research frontiers
HCC is fifth most common cancer diagnosed worldwide and the incidence of this pernicious disease is also rising in the United States. However, the treatment of HCC is still far from complete.

Innovations and breakthroughs
In this study authors evaluated the effects of SFN on autophagy in HCC cell lines. Authors found that individual HCC cells respond quite differently to various autophagy stimuli due to distinct autophagy signaling pathways between HCC cells.

Applications
Although the modulation of autophagy could have a new therapeutic potential against cancer, authors demonstrate here that caution should be taken before considering autophagy as anticancer regimes in HCC patients.

Terminology
The most important terms in this article are: HCC, SFN and autophagy.

Peer review
The authors have an idea to find SFN effects in HCC cell lines. To this end, they analyzed the changes of autophagy-related proteins in the cells, and found the different responsiveness to SFN involves autophagy signaling pathway. The paper is interesting.

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META-ANALYSIS

Predictability of IL-28B-polymorphism on protease-inhibitor-based triple-therapy in chronic HCV-genotype-1 patients: A meta-analysis

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Abstract

AIM: To investigate the predictability of interleukin-28B single nucleotide polymorphism rs12979860 with respect to sustained virological response (SVR) in chronically hepatitis C virus (HCV) genotype-1 patients treated with a protease-inhibitor and pegylated interferon-α (Peg-INF-α) based triple-therapy.

METHODS: We searched PubMed, the Cochrane Library and Web of Knowledge for studies regarding the interleukin 28B (IL-28B)-genotype and protease-inhibitor based triple-therapy. Ten studies with 2707 patients were included into this meta-analysis. We used regression methods in order to investigate determinants of SVR.

RESULTS: IL-28B-CC-genotype patients achieved higher SVR rates (odds 5.34, 95%CI: 3.81-7.49) than IL-28B-non-CC-genotype patients (1.88, 95%CI: 1.43-2.48) receiving triple-therapy. The line of therapy (treatment-naïve or -experienced for Peg-INF-α) did not affect the predictive value of IL-28B ($P = 0.1$). IL-28B-CC-genotype patients treated with protease inhibitor-based triple-therapy consisting of Boceprevir, Simeprevir, Telaprevir or Vaniprevir showed odds of 3.38, 14.66, 7.84 and 2.91, respectively. The odds for CC genotype patients treated with Faldaprevir cannot be quantified, as only a single study with a 100% SVR rate was available.

CONCLUSION: IL-28B-SNP predicts the outcome for chronic HCV genotype-1 patients receiving protease inhibitor-based triple-therapy. The predictive value varies between the different protease inhibitors.

Key words: Hepatitis C virus; Direct antiviral agents; Interleukin 28B; Sustained virological response; Meta-analysis

Core tip: Hepatitis C is a world health problem and represents a dynamic field of research for new therapeutic options. Recently direct antiviral agents such as protease inhibitors have been developed which, in addition to pegylated interferon-α and Ribavirin, obtain higher sustained virological response (SVR) rates. Of note, costs are higher and side effects are more common. The data regarding the predictive value of Interleukin 28B (IL-28B) are controversial. This meta-analysis was conducted on 2707 patients treated with different protease inhibitors. Its aim was to clarify the predic-

tive value of IL-28B on SVR in protease inhibitor-based triple-therapy, allowing the possibility of personalized treatment.

INTRODUCTION

Hepatitis C virus (HCV) is a global health Problem. According to the World Health Organization, approximately 150 million people are chronically infected with HCV, and it is estimated that more than 350 thousand are dying each year[1]. HCV is responsible in Europe and North America for 50% of liver cirrhosis and 25% of hepatocellular carcinoma[2-4].

HCV has 7 genotypes (1 to 7) and approximately 100 subtypes[5]. Genotype 1, which is the most common HCV genotype in Western countries, has the worst prognosis and response to antiviral treatment in comparison to other genotypes[6-8].

In the last years the standard therapy (Standard of care, SOC) for HCV consisted of pegylated Interferon-α (Peg-IFN-α) and Ribavirin (RBV)[9]. Recently, several direct antiviral agents (DAA) were developed, such as the protease inhibitors (PI) Boceprevir (BOC), Telaprevir (TVR), Vaniprevir (VNP), Faldaprevir (FLP) and Simeprevir (SMP)[10-13]. In pivotal studies, patients treated with BOC or TVR and Peg-IFN-α/RBV achieved significantly higher sustained virological response (SVR) rates compared to standard therapy[11-14]. These new treatment options bring new hopes for chronically HCV infected patients but they have more side effects and higher costs[10].

Treatment predictors are important tools for the management of therapy in patients with chronic HCV infection. For the current standard treatment with Peg-IFN-α/RBV in patients with chronic HCV infection, HCV genotypes 2 and 3, low baseline viral load, ethnicity, younger age, low γ-GT levels, low γ-GT/ALT level, absence of advanced fibrosis/cirrhosis, and absence of steatosis in the liver have been identified as independent pretreatment predictors of a SVR[15,16].

After initiation of treatment, rapid virological response (RVR, undetectable HCV-RNA at week 4 of therapy) is the best predictor of SVR independent of HCV genotype[16]. Recently, several genome-wide association studies showed that a single nucleotide polymorphism (SNP) within the interleukin 28B (IL-28B) gene is significantly associated with treatment outcome under standard treatment in chronically HCV genotype-1 infected patients[17-19]. IL-28B rs12979860 is the most investigated allele of IL-28B in Europe and North America. The data about the predictive value of IL-28B-genotype in HCV genotype-1 and triple-therapy are inconsistent. In the studies with VNP, IL-28B-genotype had no predictive value for the treatment[13,20]. In the studies by Poordad *et al*[21], Fried *et al*[22], Bronowicki *et al*[23], Sulkowski *et al*[24] and Akuta *et al*[25], IL-28B-CC-genotype had a favorable prognosis. In the study by Flamm *et al*[26] for Boceprevir, genotype IL-28B-TT had a favorable prognosis and by Jacobson *et al*[27] and Pol *et al*[28], IL-28B-genotype had a limited influence on SVR. However, more information about the predictability of IL-28B-genotype would allow physicians to individualize antiviral HCV therapy.

Therefore, we conducted this meta-analysis to investigate the predictive value of IL-28B rs12979860 (CC *vs* CT + TT) allele for SVR in chronically HCV genotype-1 infected patients treated with a triple-therapy regimen consisting of a DAA (BOC, TVR VNP, FLP or SMP) and Peg-IFN-α/RBV.

MATERIALS AND METHODS

We searched in PubMed, Web of Knowledge and the Cochrane Library databases, for relevant articles (full text and meeting abstracts) up to January 2014 regarding the following the next key words: "Boceprevir" or/and "SCH503034", "Telaprevir" or/and "VX-950", "Ciluprevir" or/and "BILN 2061", "Simeprevir" or/and "TMC435", "Danoprevir" or/and "R7227", "Vaniprevir" ("MK-7009"), "MK-5172", "Faldaprevir" ("BI201335"), "Narlaprevir" ("SCH900518"), "Asunaprevir" ("BMS-650032"), "PHX1766", "GS-9256", "GS-9451", "ABT450", "IDX320", "ACH-1625". All these DAAs were used as search words in order to avoid missing studies which have determined IL-28B polymorphism for a triple therapy. Because a large number of patient samples were retrospectively tested for IL-28B genotype and some of these results were only presented in meetings, we have decided to include also the meeting abstracts in our meta-analysis. In order to identify relevant studies, the references of the articles included were manually searched. We did not find any other articles that corresponded to our inclusion criteria. The studies search was performed using manual search for Cochrane Library and EndNote X7 for PubMed and Web of Knowledge databases.

The inclusion criteria were: studies with human subjects, more than 18 years of age, HCV genotype-1 patients, treatment with triple-therapy (IFN therapy-naïve and -experienced) with determined IL-28B genetic polymorphism for rs12979860 allele. Only articles in English were included. The exclusion criteria were: HCV/HIV or HCV/HBV co-infection, liver transplantation recipients, pediatric studies and IL-28B genetic polymorphism other than rs12979860. SVR was defined as undetectable HCV-RNA 24 wk after end of treatment.

The studies were reviewed independently by two authors (NCM and AA). All differences were resolved by consensus among these two authors. Our analysis was based on the original published data. For consistency we refrained from contacting the authors of the individual studies. From the studies, the following data were ex-

Table 1 Characteristics of included trials

Ref.	DAA type	Patient type	IL-28B SNP (*n*)	DAA SVR (*n*)		DAA Non SVR (*n*)		SOC SVR (*n*)		SOC Non SVR (*n*)	
				CC	Non CC	CC	Non CC	CC	Non CC	CC	Non CC
Akuta *et al*[25]	TVR	Mixed	68	31	10	6	21				
Bronowicki *et al*[23]	TVR	Naïve	141	30	30	2	48	7	6	4	14
Flamm *et al*[26]	BOC	Experienced	146	12	52	7	24	5	6	5	35
Fried *et al*[22]	SMP	Naïve	153	34	56	1	16	12	17	0	17
Jacobson *et al*[27]	TVR	Naïve	454	84	127	11	71	35	25	20	81
Lawitz *et al*[20]	VNP	Experienced	131	14	67	9	16	1	3	2	19
Manns *et al*[13]	VNP	Naïve	65	22	14	3	10	4	6	1	5
Pol *et al*[28]	TVR	Experienced	527	60	209	16	137	5	13	12	75
Sulkowski *et al*[24]	FLP	Naïve	110	22	34	0	14	9	12	2	17
Poordad *et al*[21]	BOC	Naïve	653	107	198	25	106	50	43	14	110
		Experienced	259	39	105	11	52	6	10	7	29

DAA: Direct acting agents; TVR: Telaprevir; BOC: Boceprevir; SMP: Simeprevir; VNP: Vaniprevir; FLP: Faldaprevir; CC or non CC: Genotype of IL-28B.

Figure 1 Flow chart of systematic review of protease inhibitor based triple therapy.

tracted: First author, year of publication, type of patients (IFN therapy-naïve or -experienced), total number of patients, the number of patients with determined IL-28B-genotype, type of DAA, IL-28B genetic polymorphism.

The statistical analysis was performed by CR. We used logistic regression to model the chance of a SVR and investigate potential influential factors. In a logistic regression, binary outcome data are modeled based on the *odds* of events (here: SVR). As is usual regression, the *odds* are then formulated as a function of (potential) explanatory variables. Random effects were included in order to accommodate heterogeneity between studies[29]. As the available data allow to fit a multitude of plausible variations of regression models to the data, we approached the *model selection* problem *via* Bayesian Information Criterion (BIC)[30], which allows to compare and select models based on a single adequacy measure. All analyses were performed using the *R* software (www.r-project.org) and the *lme4* package.

RESULTS

Literature search
Four thousand three hundred and thirty-seven studies were initially identified on the bases of DAAs. After re-

moving duplicate citations, the remaining 1522 studies were searched for data regarding IL-28B polymorphism and qualified for abstract review. Among the remaining studies, 1454 studies had no data regarding IL-28B and were excluded. The rest 68 studies were selected for a "full paper review". Among these remaining 68 studies, five of them were reviews. Four of them included only interferon-free therapy. There were three meta-analyses which were excluded. Five studies described only SOC therapy. Another 41 studies and meeting abstracts, including preliminary and subgroup analysis from large trials data, rs8099917 IL-28B allele and non-human studies, had to be excluded (Figure 1).

This meta-analysis is based on the following 10 studies: 7 full text studies and 3 meeting abstracts with a total of 2707 IL-28B patients. The studies of Akuta *et al*[25], Bronowicki *et al*[23], Jacobson *et al*[27] and Pol *et al*[28] investigated the interaction between IL-28B genotype and SVR in patients receiving TVR based triple-therapy. The study from Akuta *et al*[25] had no patients with IL-28B genotype receiving SOC. The studies of Flamm *et al*[26] and Poordad *et al*[21] analyzed the BOC based triple-therapy. VNP was used as DAA in the studies of Lawitz *et al*[20] and Manns *et al*[13]. For SMP and FLP only one study could be included for each of them (Fried *et al*[22] and Sulkowski *et al*[24]; Table 1).

Comparison of dual and triple therapy
Figure 2 illustrates the estimated *odds* and associated *confidence intervals* of a SVR, contrasting dual and triple therapy, and CC and non-CC genotypes. When using conventional dual therapy, the *odds* for SVR are around 0.34 for non-CC genotype (corresponding to Pr = 25% probability), which increases to 1.98 (Pr = 66%) for CC genotype. For triple therapy the *odds* are more favorable, 1.88 (Pr = 65%) for non-CC and 5.34 (Pr = 84%) for CC genotype. The interaction effect between genotype and type of therapy is significant (*P* = 0.00126), *i.e.*, the *odds ratio* between genotypes differs between therapy types (and vice versa). According to the BIC, this model, including a treatment indicator (double *vs* triple), a genotype effect and their interaction fits the data best models that we investigated. In addition including subsets of the

Figure 2 Odds/probabilities of obtaining a sustained virological response with regard to interleukin 28B-genotype and different therapy regimen. The differences between the shown estimates correspond to the odds ratios. A greater difference of odds between the both IL-28B-genotype corresponds to a more beneficial effect. SVR: Sustained virological response; IL-28B: Interleukin 28B.

Figure 3 Odds/probabilities of a sustained virological response with regard to interleukin 28B-genotype in different protease inhibitor based triple therapy. The differences between the shown estimates correspond to the odds ratios. A greater difference of odds between the both IL-28B-genotype corresponds to a more beneficial effect. SVR: Sustained virological response; IL-28B: Interleukin 28B.

above variables or use a treatment indicator are also differentiating between different types of DAA.

Comparison of individual DAA types

In addition to the results that came out as best-fitting according to the BIC, we also analyzed the analogous results where protease inhibitor-based triple-therapy is broken down into individual subtypes (DAAs). Comparing this model and the previous one (including interactions in both cases) in an ANOVA, the difference between DAAs actually is significant ($P = 0.0013$). The resulting estimates are illustrated in Figure 3. Among the different DAA types, the estimated odds for SVR tend to be larger than for double therapy and greater for CC than for non-CC genotype. The only two exceptions were VNP, where SVRs for both genotypes appeared to be of the same order of magnitude and FLP, where the CC-*odds* could not be quantified. For FLP, our data originate from a single study with a 100% SVR rate (22 out of 22 patients) for CC genotype; so all we can say is that the evidence is supports effectiveness of FLP in CC genotype patients. Otherwise, for the CC genotype, the greatest odds for SVR are estimated for SMP (OR = 14.66, corresponding to Pr = 94%), whilst for non-CC genotypes, the greatest *odds* are estimated for VNP (OR = 3.28, Pr = 77%). As in the previous model, the interaction effect between treatment type and genotype was significant ($P < 0.001$).

Effect of patient type (IFN-α-treatment-naïve vs IFN-α-experienced)

Consideration of the patient type (IFN-α-treatment-naïve patients *vs* patients having previously experienced IFN-α treatment) in the regression model did not improve the model fit. Even in the best-fitting model among the ones including a patient-type effect, the patient type regarding previously IFN-α therapy was not significant ($P = 0.1$).

DISCUSSION

The main results of this meta-analysis are: (1) IL28B-

CC-genotype patients receiving protease inhibitor-based triple-therapy have a higher SVR rate than the IL-28B-non-CC-genotype patients with the same treatment type, (2) considering sub-types of DAAs, the effect appears to be present for BOC, FLP, SMP and TLP, but possibly not for VNP; (3) IL-28B-CC-genotype patients have higher SVR rates both, in IFN-naïve and IFN experienced.

Genome-wide association studies in 2009 showed that different polymorphisms in the region of IL-28B are associated with SVR in patients chronically infected with HCV genotype-1, treated with Peg-INF-α and RBV[17-19]. The IL-28B gene is located on the 19 chromosome. The molecular and immunological mechanism of the IL-28B influence on SVR remains unclear[17-19]. Lately a dinucleotide polymorphism ss469415590 (TT/ΔG) was described to be a better genetic predictor, as IL-28B (INF-λ3) for HCV clearance in chronically HCV genotype-1 infected patients treated with SOC[31-33]. Moreover, only the ΔG of this dinucleotide polymorphism creates a novel type Ⅲ interferon protein, IFN-λ4. Absence of IFN-λ4 protein is thus supposed to favor resolution of HCV infection[31,33].

The determination of IL-28B rs12979860 genotype can help to shorten the therapy duration. Genotyping of IL-28B polymorphisms can further be used to improve patient compliance, to remain on treatment in spite of side effects and to defer treatment in patients with low likelihood of response[34]. The American Association for the Study of Liver Diseases suggests IL-28B polymorphism as a robust predictive marker for treatment decision with Peg-INF-α/RBV or in combination with DAA. Testing is useful if it impacts the treatment decision of either patient or physician. Also in studies with interferon-free therapy regimens IL-28B-CC-polymorphism was associated with better early viral kinetics and higher reduction of viral RNA[35]. Other interferon-free treatment regimens replicated these findings for IL-28B genotypes[36].

Recently, a pangenotypic polymerase inhibitor named sofosbuvir was approved in the United States of America and Europe for the treatment of chronically HCV-infected patients. In selected patients sofosbuvir achieves an SVR rate of approximately 90%. However, a 24-wk therapy with sofosbuvir and ribavirin costs about US$ 169000[37]. Cost-effectiveness analysis show that there is no need to treat patients with IL-28B-CC allelic variation with sofosbuvir urgently because they do not necessarily benefit from such a therapy referring to the SVR rate[38]. Nevertheless, regarding the economic aspects the second-generation protease inhibitors will not be cheaper. For this reason, we need more information about predictive factors in order to detect the individuals who benefit most from an antiviral treatment with polymerase inhibitors. Through the use of predictive factors it will be possible to achieve the highest rate for SVR and the least side effects as well as reducing the cost significantly. Certainly, the IL-28B polymorphisms will play a major role in the future.

Our analyzes showed that IL-28B-CC patients could be treated with a protease inhibitors, either with FLP or SMP. Patients with IL-28B-CC who were treated with either FLP or SMP showed a SVR rate of 100% or 94%, respectively. Therefore, patients with IL-28B-CC genotype could be treated preferably with either FLP or SMP and the IL-28B-non-CC genotypes could be treated preferably with either the polymerase inhibitor sofosbuvir or with a combination of polymerase and protease inhibitors in case of an interferon-intolerance[37].

The difference between the IL-28B SNP predictive effect in triple and dual therapy is significant, suggesting that the effect of IL-28B on the *odds* of a SVR is smaller for triple-therapy than for dual-therapy.

Regarding BOC, the individual studies initially had contradictory results. The study conducted by Flamm *et al*[26] showed that for the IL-28B-TT rs12979860 genotype BOC had a favorable prognosis. However this study had a smaller number of participants than the SPRINT2 and RESPOND2 trials. Poordad *et al*[21] analyzed the data from these studies and showed that IL-28B-CC rs12979860 genotype patients were more likely to achieve a SVR. Our analysis showed that in the case of the patients treated with BOC the CC-genotype has a favorable prognosis.

For FLP and SMP, we could only include one study each, with a relatively small number of participants. For FLP, the *odds* for the CC genotype could not be quantified, due to the fact that our data originate from a single study with 100% SVR rate, indicating a strong beneficial effect.

In the case of SMP, the IL-28B-CC-genotype has the second best *odds* among all DAAs, but with a large *CI* because of the limited number of patients that were included in the study. Therefore, future studies with these DAAs are needed to confirm these results. Our meta-analysis showed that SMP based triple therapy is more likely to produce SVR in CC-genotype patients; therefore we recommend IL-28B genotyping before initiation of

this treatment.

The studies by Pol *et al*[28] and Jacobson *et al*[27] showed that IL-28B-genotype has a limited and non-significant predictive value for a SVR regarding the triple-therapy with TVR. Both of them are analyses of the data from larger trials (Pol *et al*[28] from REALIZE and Jacobson *et al*[27] from ADVANCE US) stipulating that TVR based triple-therapy increase the SVR rate through all IL-28B genotypes, especially for the IL-28B-non-CC genotype patients. In our analysis TVR based regimes included a larger number of studies (*n* = 4). The results were significantly favorable for IL-28B-CC-genotype patients. This result can be explained by the fact that Akuta *et al*[25] studied the predictive value of IL-28B-genotype only in Asian patients infected with genotype 1B, with higher SVR rates while the other studies included wider ranges of ethnicities.

IL-28B SNP has a predictive role for both, IFN-naïve and IFN-previously treated patients. For the SOC-double therapy this meta-analysis did not show evidence for a difference in treatment effect between patient types.

The strong points of our meta-analysis is the large number of patients (*n* = 2707), the included studies were randomized, controlled studies and the inclusion of various number of DAA types (*n* = 5). The limitations of our meta-analysis are the relatively small number of studies for some DAAs types (SMP, FLP), even though both, full text and meeting abstracts were included into the search. Another limitation to our study could be the absence of information on the influence of baseline viral loads on SVR and race in correlation with the IL-28B SNP. No long-term data are available yet. Furthermore, this meta-analysis reflects the methodological problems of the included studies.

In conclusion, the IL-28B allelic variation has a predictive value in the protease inhibitor-based triple-therapy of chronically HCV genotype-1 infected patients and it differs among DAA types. However, the effect on the *odds* of a SVR is smaller than the one regarding IL-28B and SOC. We recommend IL-28B genotyping also in the case of SMP-based triple therapy. VNP based regime was the only triple therapy which was not associated with higher SVR rates for IL-28B-CC-genotype patients. Furthermore, prospective studies need to be conducted for the understanding of IL-28B-genotype predictive role in HCV triple-therapy.

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COMMENTS

Background

Hepatitis C is a world health problem and represents a dynamic field of research for new therapeutic options. The interleukin-28B (IL-28B) single nucleotide polymorphism (SNP) is a predictor of sustained virological response for hepatitis C genotype-1 patients treated with pegylated-Interferon-α and ribavirin as the standard of care. Recently, direct antiviral agents have been developed which, in addition to the standard of care, obtain higher sustained virological

response rates, but with higher costs and side effects.

Research frontiers

IL-28B is a solid genetic predictor in the therapy of hepatitis C patients treated with interferon and ribavirin. In the era of new therapeutic options for hepatitis C, the current research hotspot is to evaluate the predictive value of IL-28B in different protease inhibitor-based triple-therapies.

Innovations and breakthroughs

This meta-analysis demonstrates that IL-28B has a predictive value on protease inhibitor-based triple-therapy. This predictability differs among protease inhibitors.

Applications

This study suggests that IL-28B could be used as a genetic predictive factor for antiviral response in hepatitis C genotype 1 patients treated with protease inhibitor-based triple-therapy.

Terminology

Direct antiviral agents such as protease inhibitors are newly developed drugs against hepatitis C. In combination with Interferon and Ribavirin they constitute the triple therapy for hepatitis C. SNP within the interleukin 28B gene as a genetic marker is associated with sustained virological response in the treatment of hepatitis C.

Peer review

Author guidelines has been followed properly in preparing the manuscript. Literature review is adequate. The references are appropriate and relevant. Table and figures reflect the major findings of the study, and they are appropriately presented.

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WJH 6ᵗʰ Anniversary Special Issues (1): Management of hepatocellular carcinoma

Management of "very early" hepatocellular carcinoma on cirrhotic patients

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Abstract
Due to the advances in screening of cirrhotic patients, hepatocellular carcinoma (HCC) is being diagnosed in earlier stages. For this reason the number of patients diagnosed of very early HCC (single tumors $\leqslant$ 2 cm) is continuously increasing. Once a patient has been diagnosed with this condition, treatment strategies include liver resection, local therapies or liver transplantation. The decision on which therapy should the patient undergo depends on the general patients performance status and liver disease. Anyway, even in patients with similar conditions, the best treatment offer is debatable. In this review we analyze the state of the art on the management of very early HCC on cirrhotic patients to address the best treatment strategy for this patient population.

Key words: Hepatocellular carcinoma; Very early; Liver resection; Liver transplantation; Local therapies

Core tip: Very early hepatocellular carcinoma patients are deemed too early for liver transplantation candidacy, known as the best treatment regarding long-term survival and tumor recurrence. Strategies as surgical resection and radiofrequency ablation have gained popularity. Although resection is considered as the first line of treatment, recent studies claim equal results with ablation techniques. Ablation used as a test of time in patients who remain candidates for liver transplantation is attractive. In this review we will analyze in detail the novel strategy repertoire used in the management of these patients.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver, the sixth most common cancer worldwide and the third largest cause of cancer-related deaths[1-3]. The incidence of HCC is increasing in Europe and in the United States[4] and it is currently the leading cause of death among cirrhotic patients[5].

The management of these tumors has significantly improved over the last few years due to a better knowledge of the natural history of the malignancy and the development of staging systems. One of the most reliable and widely adopted methods for staging HCC is the Barcelona Clinic Liver Cancer (BCLC) system[6], that stratifies patients according to the characteristics of the tumor, underlying liver disease and performance status. According to this system, the presence of an asymptomatic single nodule $\leqslant$ 2 cm, in the absence of vascular invasion or extrahepatic disease, has been defined as very early stage HCC[7-9].

In recent years, thanks to surveillance programs on the cirrhotic population, more patients are being diagnosed with very early HCC[9-11].

There are basically three potential curative modalities of treatment for patients diagnosed of very early HCC: Liver resection (LR), liver transplantation (LT) and radiofrequency ablation (RFA). Although these patients show excellent outcome in terms of survival and recurrence[12] compared to those with more advanced tumors, the debate regarding what is the best treatment option in that scenario still remains[3,13].

Our aim is to review the current management of very early HCC on cirrhotic patients.

DIAGNOSIS OF VERY EARLY HCC

Hepatocellular carcinoma is frequently diagnosed by imaging criteria based on the contrast enhancement pattern. Early detection by surveillance is the only way to diagnose HCC when curative treatments are feasible, being the optimal profile for this endpoint very early HCC[9,10]. Intense contrast uptake in the arterial phase followed by contrast washout in the venous phase, both on computed tomography or magnetic resonance, is considered diagnostic for HCC > 1 cm[9,14]. Nevertheless, on cirrhotic patients, small lesions may be misdiagnosed as being HCC and can in fact be intrahepatic cholangiocarcinomas (iCCA) or mixed hepatocellular-cholangiocarcinomas (HCC-CC), being their frequency much lower[15,16].

If the lesion does not show the typical HCC pattern on imaging, biopsy is mandatory[10]. A prospective study including 89 cases with liver nodules between 0.5 and 2 cm reported that non-invasive criteria had a sensitivity of 30%, being necessary a biopsy for their diagnosis[17]. However, pathological diagnosis is particularly complex for nodules < 2 cm, being difficult the distinction between high-grade dysplastic nodules, intrahepatic cholangiocarcinomas (iCCA) and HCC[17]. It is currently considered that a positive tumor biopsy is clinically useful to diagnose an HCC, while a negative biopsy cannot rule out malignancy[18,19].

Anyway, despite the misdiagnosis of small nodules, current data has shown interesting results on the outcome of patients diagnosed of "very early" iCCA and HCC-CC at pathology. These studies demonstrated excellent post-transplant survival for patients with such tumors on pathology. Nevertheless, future studies must be conducted to confirm these results[15,16].

LIVER RESECTION

Liver resection constitutes the first-line treatment option for patients with very early HCC and compensated cirrhosis in most centers[3,11,20]. As indicated by the BCLC, this is especially true when patients are potential candidates for LT[21,22] as we will analyze later in detail.

Partial LR in cirrhotic patients must be addressed under two contradictory principles: to be a curative resection and to preserve as much liver parenchyma volume as possible to avoid postoperative liver failure[1]. Thanks to recent advances in surgical technique and immediate postoperative care, the modern standards for resection of HCC in cirrhotic patients have improved and include a perioperative mortality less than 1%, blood transfusion requirements below 10% and 5-year survival rates of at least 50%[20]. Anyway, major resections are not recommended even in compensated cirrhotic patients because of the risk of post operative liver failure due to an insufficient remnant liver, which can lead to death[9]. Nevertheless and thanks to the advance in several techniques such as portal vein embolization, some groups perform major hepatectomies for HCC after portal vein embolization if there is a sufficient growth of the liver remnant[23,24].

The discussion between anatomic *vs* non anatomic resection still remains. Most studies defend anatomic resection as a method to avoid or ameliorate local recurrence[25,26]. Other studies have not been able to confirm this[27]. If the invasive phenotype is minor, as in the case of very early HCC, the spread beyond the segment may be low and anatomic resection may provide a benefit[9]. Basically the recommendation would be to perform an anatomic resection whenever possible and safe.

One of the main contraindications for LR in cirrhotic patients is the presence of portal hypertension. The BCLC group identified the absence of clinically relevant portal hypertension and normal bilirubin as the key variables to make a safe selection of candidates for LR. An hepatic venous pressure gradient ≥ 10 mmHg was shown to be a predictor of unresolved hepatic decompensation and, consequently, of poor long-term outcome in Child-Pugh A cirrhotic patients after surgery[14,28]. The presence of esophageal varices detectable at endoscopy, splenomegaly and/or a platelet count less than 100000 were considered indirect signs of portal hypertension[29]. The value of portal hypertension assessment in predicting prognosis has been confirmed also by japanese groups[30]. However, some authors have reported good results for patients resected with portal hypertension. Cucchetti *et al*[31], found in 2009 after one-to-one matching, that the only predictors of postoperative liver failure were model of end-stage liver disease (MELD) score and the extent of hepatectomy and so, did not found portal hypertension as a risk factor[31]. Ruzzenente *et al*[32], also concluded that portal hypertension is not an absolute contraindication to liver resection in Child-Pugh class A cirrhotic patients but noted a worse survival in patients who were resected two or more segments if portal hypertension was present probably showing the higher risk of more extended hepatectomies in the cirrhotic population[32]. Anyhow, most centers would only perform LR if portal hypertension is not present, and so, despite the results of some retrospective studies[33], prospective multicenter studies should be conducted to assess the safety of LR in the presence of portal hypertension. Even though the presence of portal hypertension may not be considered an absolute contraindication for LR, it will significantly affect patients early and late outcome after resection.

One of the principal advantages of LR over other

treatments like local therapies is the pathological examination of resected tumors. Indeed, this may represent a very useful tool to predict the risk of recurrence and to select patients with HCC who are likely to obtain the maximum benefit from LT[1,34]. Accordingly, the BCLC recommend LR in cirrhotic patients with very early HCC who are candidates for LT. Histological features on the LR specimen have been proposed as a guide for selection of LT candidates and as a tool for optimization of the donor pool. In selected cases and according to characteristics in specimen aggressiveness, resection may be considered as a bridge to transplantation[35].

Cillo *et al*[36] reported tumor differentiation as a direct marker of biologic tumor aggressiveness, providing interesting information about the risk of recurrence[36].

The BCLC and other groups have proposed a policy of listing patients for LT without evident HCC based on pathological risk of recurrence after resection, characterized by the presence of vascular invasion and/or satellitosis. They have given the name "ab initio" indication, also known as "de principe" LT[34,36-39]. Both parameters, presence of microvascular invasion and additional nodules, could be used to stratify resected patients in two categories: patients with low risk of recurrence and patients with high risk of recurrence[30,40]. The rate of microvascular invasion increases according to the tumor size and it is present in 20%-25% of HCC less than 2 cm[14,41]. Sala *et al*[34] reported in 2004 the efficacy of this strategy in 6 patients who were transplanted after being diagnosed with high risk recurrence (according to gross and microscopic examination after LR) with good results[34]. Scatton *et al*[35] published a retrospective cohort study in 2008, in which de principe LT was proposed to 6 patients because of poor prognosis histological findings on the resected specimen, reporting that all these patients were alive at the time of publication, with a mean follow-up of 55 mo[35]. On the other hand, other authors have proposed that patients who exceed Milan criteria and present poor histological findings at the time of resection, should be precluded from LT because of the high risk of recurrence, while patients exceeding Milan criteria but with good histological prognostic factors may benefit from de principe LT[34,35].

Some recent studies have proposed a molecular signature to define the level of risk due to the oncogenicity of the cirrhotic liver. This concept still has to be validated in clinical practice[9], but looks very promising.

Recurrence after LR
The main problem after LR for HCC is the high rate of tumor recurrence[1,13,42]. There are several reports indicating that the 5-year recurrence rate is up to 80%-100%[43-46].

The most common site of post-resection recurrence is the remaining cirrhotic liver[47], as the persistent underlying liver disease (main risk factor for the development of HCC) is associated with high rates of intrahepatic recurrence[48]. Basically, two types of tumor recurrence after LR have been described: local recurrence, which usually happens in the first 2 years after resection and may be

the result of inadequate R1 resection or secondary to the progression of microscopic vascular invasion and "de novo" recurrence, which happens more than 2 years after resection and constitutes the development of a new tumor due to the presence of underlying cirrhosis[49].

Patients with very early HCC can achieve 5-year survival rates around 90% after resection and extremely low 3-year recurrence rates have been described (around 8%)[3,50]. Other published studies reported similar survival but the disease free survival was around 40% at five years[50,51]. The largest retrospective experience on the outcomes of LR in very early HCC was reported by Ikai *et al*[52] analyzing 2320 patients and finding a 3- and 5-year survival of 84% and 66% respectively. Lee *et al*[53] also reported similar outcomes, with a 3-year survival of 82.5%. None of these studies specified on the recurrence rate after very early HCC.

Treatment of HCC recurrence after LR is currently based on several strategies that include the use of antineoplasic drugs, RFA, chemoembolization, alcoholization, re-resection and liver transplantation; being the most curative therapies the last two[54].

Re-resection: The applicability of re-resection will be determined by the patient general performance status and liver function at the time of recurrence. Some authors have described a low applicability rate (10%-25%) for re-resection and argue that it should ideally be restricted to "*de novo*" cases and not "local recurrences"[55,56]. Several studies have demonstrated good results after re-resection. Poon *et al*[57] reported a 5-year survival rate after re-hepatectomy of 69.3% and Sugimachi *et al*[56] concluded in another study that despite patients with recurrence treated with re-hepatectomy having a better prognosis compared to patients with recurrence who did not have a repeat hepatectomy, re-resection must be performed in selected patients[56]. Anyhow, whenever possible, re-resection should be considered at the time of recurrence and analyzed in a patient to patients basis.

Salvage LT: As previously stated, LR constitutes the first-line therapy for very early HCC on potential candidates for LT with compensated liver cirrhosis. In these regards, surgeons may have in mind that patients can be transplanted at the time of recurrence[58]. This strategy of secondary LT is called salvage transplantation[27]. Poon *et al*[59] published that 80% of patients with recurrence after a LR for HCC remain eligible for LT. Although some authors have published similar results regarding the applicability of salvage transplantation[60], in clinical practice the real applicability of this policy is low, only 10%-20% of cases, as it has been shown in several studies[61,62]. In a previous study from our department, we reported a series of 17 potential candidates for salvage LT, but could only be performed in 6 patients. Age at the time of recurrence was the main reason for contraindicating LT. In spite of that, we found that results of salvage transplantation were similar to those of primary LT[63]. The main problem with this strategy is related to a high drop out of resected patients

from LT, due to a non-transplantable recurrence, tumor progression during the waiting time or life-threatening complication of underlying cirrhosis, and so, the feasibility of salvage transplantation will be closely related to a strict surveillance after resection and the consequent early diagnosis of intrahepatic recurrence[62]. Although there is conflicting results when comparing primary LT and salvage transplantation and there is a concern on the higher risk of complications in patients that receive a transplant after LR, most studies showed no differences when comparing biliary leaks, vascular complications, re-operation or re-transplantation rates[64,65]. Nevertheless, operative mortality and bleeding have been described to be significantly higher after salvage transplantation in some series[62]. As no randomized controlled trials are feasible in this regard, and methodological pitfalls of current data exist, comparable outcomes are still a matter of debate.

A determining factor when including a patient in the waiting list for salvage transplantation is the time from LR to recurrence. Early recurrence (before 1 year) after LR has been found to be a risk factor for poor outcome after transplant probably indicating the tumors aggressiveness[54].

In patients with very early HCC or small single tumors (< 3 cm), salvage transplantation may be more applicable as recurrence of these tumors can be more limited. This may explain the excellent 10-year survival when comparing patients diagnosed with a very early HCC that are transplanted or resected[13,66].

LOCAL THERAPIES

In the last decade RFA has become one of the standard treatments of very early HCC on cirrhotics[67,68]. This treatment can be included basically in 2 strategies: intended as a definitive curative treatment or as a bridge to LT.

According to the European Association for the Study of the Liver (EASL) and the European Organization for Research and Treatment of Cancer, percutaneous ethanol injection (PEI) and RFA are considered as the standard of care for HCC patients with very early HCC not suitable for surgery[16]. According to the American Association for the Study of Liver Diseases (AASLD), PEI was initially suggested as the standard against which any percutaneous therapy should be compared[22]. However, recent studies demonstrate that RFA has better local control for HCC > 2 cm. In tumors < 2 cm RFA and PEI have equal results[69]. Patients with very early HCC do not afford any priority points on the waiting list and generally have low MELD scores, the probability of attracting an organ is very low[70,71]. Accordingly the debate arises on to what is the best option for these patients: immediate ablation or wait until the tumor grows and then patients afford exception points and have real options of attracting an organ[71].

Several studies have shown the efficacy of local therapies on very early HCC[72-76]. Sala *et al*[77] reported a 50% survival at 5 years in Child A patients and treatment response in 70% of nodules < 3 cm and 50% in nodules

> 3 cm or multi-nodularity, achieving results that could almost be equal to surgical resection in selected patients. Livraghi *et al*[78], conducted a multicenter study enrolling cirrhotic patients with HCC < 2 cm undergoing RFA, of whom 46% where initial candidates for surgery. The estimated 3-year and 5-year survival rates were 76% and 55%, respectively and 65% in the subgroup that could potentially have been resected; thus achieving a 5-year survival rate similar to that achieved after surgical resection[78]. The advantages of RFA over surgery were less invasiveness, complications, hospital stay, blood transfusions and treatment costs. N'Kontchou *et al*[79] evaluated long term outcomes of RFA as the first line treatment and prognostic variables in patients with early-stage HCC defined as tumors < 5 cm and less than 3 tumors. They had a complete radiological ablation in 94.7% of their cohort with estimated overall 3 and 5-year survival rates of 60% and 40% respectively. The estimated 3 and 5-year recurrence-free survival rates were 37% and 18% respectively, with a median recurrence-free survival of 23 mo. The size of the tumor was found to be a predictor of local recurrence, but not of overall or tumor-free survival rates. Recurrences where limited and ablated by additional RFA sessions[80]. RFA has been suggested, according to these studies, as an adequate treatment for small HCC, having less side effects and in case of recurrence, multiple RFA sessions could control the disease without comprising survival.

As previously stated, RFA has emerged as the first line treatment for patients with very early HCC non candidates for LT and as a curative-intent treatment for HCC in some centers, as patients will not be afforded with exception points and then wait very long times for a graft[80-82]. The most important limiting factor to this strategy includes post RFA recurrence of 50%-80% at 5 years. Emergence of new tumors rather than local tumor progression seems to be responsible for these results[77-80,83]. A two-step strategy comprises performing RFA to LT candidates with HCC, leaving transplantation as definite therapy only if recurrence or liver failure occurs. A previous "test of time" would identify those patients with aggressive tumor biology who would carry a high risk of recurrence post LT, thus optimizing the scarce resource of organ donors and reducing the burden of HCC patients on the waiting list[4,79]. As stated in a recent editorial by Yao, patients selected for this strategy should be those who have a high probability of long-term cure with a low risk of recurrence[68]. Limitations to this strategy, include the uncertainty for those patients who do not remain as candidates for salvage transplantation according to the size and number of recurrent tumors. Tsuchiya *et al*[84] published a retrospective analysis of 323 patients undergoing RFA as initial treatment of which 60% of patients suffered recurrence beyond transplantation criteria and only 30% of these patients were eligible for salvage LT. Predictors of HCC recurrence were AFP > 100 ng/mL, tumor size > 2 cm, and early recurrence within 1 year[84]. This has raised the question if the "ablate and wait" strategy may result in a percentage of patients with recur-

rence out of transplantable criteria and then loose the opportunity for LT. N'Kontchou *et al*[79] reported promising results with the "two step" strategy, using RFA as first line treatment in patients eligible for LT. The 3- and 5-year overall recurrence rates were 50% and 58%, respectively. For Child A patients, survival rates at 5 years were comparable to that of patients who were offered LT as first line therapy[79].

We believe that the best candidates for RFA as first line treatment would be those Child A patients with solitary lesions ≤ 2 cm who fail to recruit enough exception points on the waiting list as this patients achieve the best long term survival and best complete initial response[68,78,79]. Anyway, these patients should undergo strict follow-up to diagnose recurrence in an early manner.

LIVER RESECTION *VS* RADIOFREQUENCY ABLATION

According to the clinical guidelines by the AASLD and the EASL, surgical resection is the first line treatment for patients with small solitary HCC Child A cirrhosis and no portal hypertension[22]. RFA is an optional treatment for small HCC, obtaining similar results regarding long-term survival compared to surgical resection. Several meta-analysis have tried to assess the advantages and disadvantages of RFA compared to surgical resection. Conflicting data has been obtained from these studies regarding overall survival and disease free survival due to the retrospective nature of the studies involving heterogeneous cohorts. Moreover most of these studies have not analyzed patients with very early HCC in detail[85,86].

Some randomized controlled trials have been performed to assess this issue; none of them strictly analyzes the subgroup of patients with very early HCC. Huang *et al*[87] assessed, in an intention to treat analysis, overall survival, recurrence free survival and overall recurrence comparing 115 RFA patients with 115 surgical resected patients, (both groups had tumors within the Milan criteria). After a 5-year follow-up, overall survival rates of RFA and surgical resection were 54.78% and 75.65%, respectively. Overall survival and recurrence-free survival were significantly higher in the surgical resection group than in the RFA group. Once stratifying by tumor size, surgical resection still offered an advantage over RFA in patients with early HCC (defined as tumors < 3 cm). RFA had an advantage in terms of less hospital stay and less adverse events[87]. Feng *et al*[88] evaluated survival and recurrence undergoing a randomized controlled trial in an intention to treat basis comparing RFA *vs* surgical resection. Early HCC was defined as tumors with a maximum diameter of 4 cm and up to 2 nodules. The 1, 2, and 3-year overall survival rates were 96.0%, 87.6%, 74.8% and 93.1%, 83.1%, 67.2% for the surgical resection and RFA groups, respectively. Recurrence-free survival was 90.6%, 76.7%, 61.1% for the surgical resection group and 86.2%, 66.6%, 49.6% for the RFA group. No significant differences were seen between the two groups regarding overall and

recurrence-free survival. No stratified analysis regarding tumor size and outcomes on both groups was presented. Again, patients that underwent RFA had less hospitalization stay and less blood transfusion rates. Chen *et al*[89] evaluated a cohort of 90 patients who underwent surgical resection compared to 90 patients who underwent RFA. Early HCC was defined as a solitary lesion less than 5 cm. There were no differences in the overall and disease free survival rates. Stratified analyses of both therapeutic interventions for lesions less than 3 cm revealed no differences[89]. The information from the randomized controlled trials on the outcomes of RFA *vs* LR for very early HCC is not clear and the outcomes comparing these therapies still require further investigations.

On the other hand, several observational retrospective studies do make emphasis on very early HCC and outcomes after RFA and surgical resection, however, they lack randomization and may be biased by covariate distribution[90,91]. Hung *et al*[90] analyzed a cohort of patients with very early HCC that included 66 patients in the RFA group and 50 in the surgical resection group. There were no statistically significant differences in terms of overall survival and recurrence but both groups were heterogeneous[90]. Peng *et al*[92] compared retrospectively the effects of RFA and surgical resection in patients with resectable HCC < 2 cm. Seventy-one patients treated with RFA were compared with 74 surgically treated. Overall survival rates at 1, 3, and 5-years were 98.5%, 87.7%, and 71.9% in the RFA group compared to 90.5%, 70.9%, and 62.1% in the surgical resection group. No differences were found regarding disease-free survival between groups. The main problem with this study was its retrospective nature that leads to several selection bias[92]. Wang *et al*[51] compared in a cohort of very early HCC patients, 51 patients undergoing RFA *vs* 91 patients undergoing surgical resection. There was no significant difference in overall survival between groups; however, patients treated by surgical resection had a better disease free survival than those in the RFA group. They suggested that surgical resection should be the preferred modality in very early HCC when liver transplantation is not feasible[51]. Finally, Takayama *et al*[91] published a large Japanese multicenter study analyzing RFA *vs* surgical resection in a cohort of 2550 patients. Basically half of the patients were treated with RFA and half were operated. Disease free survival was significantly better in the surgical resection group compared to RFA. Overall survival in both groups showed no differences. Again, due to the retrospective nature of their study, several selection bias were found. Percutaneous ablation was more prominent in Child B patients who had more hepatic dysfunction compared to those who underwent resection[91].

Surgical resection continues to be the first line treatment for patients with early HCC suitable for surgical therapy; however, many patients cannot be offered resection because of liver dysfunction at the time of diagnosis. RFA seems to be a suitable modality of treatment for these patients, achieving similar results regarding disease free survival and overall survival according to the available

information. The decision on whether to perform RFA or resection of patients with very early HCC will depend on the type of resection required, the general performance status of the patients and their liver function.

LIVER TRANSPLANTATION

Liver transplantation is accepted as the best treatment modality for HCC, as it efficiently removes the tumor within the liver and the remaining oncogenic cirrhotic tissue caused by the underlying pathology[9,93]. Despite the efforts for assuring transplantability for HCC patients according to the Milan Criteria and expansion of these parameters by the University of California, San Francisco criteria, scarcity of organ donors and the increased number of patients on the waiting list renders patients to undergo other treatment modalities[94-98]. According to the principle of utility, ablation and resection in tumors ≤ 2 cm avoids futile transplantation in these patients[75,95,99,100].

In patients with tumors ≤ 2 cm LT is not considered as first line treatment as these patients are deemed "too early" for transplantation and may not be listed with exception points. Three strategies for management may be considered; RFA, surgical resection or waiting for tumor progression with subsequent listing once the tumor exceeds 2 cm.

The wait and not ablate strategy considers waiting for tumors to exceed 2 cm and then seek exception points for LT. With this strategy 9% of patients progress from T1 to directly beyond T2 before listing, drop-out rates once on the waiting list account for 7%-10% of patients, and 3-year survival rates with transplantation achieve 75%[70,71].

Although LT is the best strategy for the treatment of HCC regarding survival and recurrence, in the setting of very early HCC, RFA and surgical resection continue to be first line treatments. The wait and not ablate strategy seems to have good results, however, robust evidence is still lacking as to how and when to apply it considering patients eligible for other ablative techniques[70].

Nowadays, LT remains as a second line treatment for patients with very early HCC and low MELD scores. The main benefit of LT is the treatment of recurrence after LR or RFA. Anyhow, this statement must be taken with caution as some patients may loose their opportunity to be transplanted if recurrence exceeds each centers transplantation criteria.

LIVER RESECTION *VS* LIVER TRANSPLANTATION

Many publications have compared the results of LR and LT for HCC, in general, they have found similar patient survival with better disease-free survival in patients undergoing LT[48,101-105]. However, there are not many publications that focus on the outcomes of very early HCC.

Bismuth *et al*[103] published in a retrospective study that in case of small uninodular or binodular tumors smaller than 3 cm, LT had much better results than resection, showing a disease free survival of 83% *vs* 18% in resected patients[103].

Cha *et al*[101] concluded that partial hepatectomy in patients with early HCC who are otherwise eligible for LT can be performed with minimal morbidity and can achieve comparable 5-year survival to that reported for LT. They stated that resection should be considered the standard therapy for patients with HCC who have an adequate liver reserve[101].

Another publication by Margarit *et al*[63], comparing outcomes of LR and LT in a retrospective study with 259 patients, found no significant differences in overall actuarial survival, with a median survival of 85 mo in both groups. They reported that HCC recurrence was significantly higher after LR (59%) than LT (11%). However, this study included all the patients who presented tumors smaller than 5 cm and the mean tumor size was 3 cm[63].

The publications listed above did not report longer 5-year follow-up, nor did they distinguish between very early and early HCC.

There are two studies (recently published) that tried to assess the long-term outcome (10 years) for patients resected and transplanted. Adam *et al*[62] compared results of HCC < 5 cm after LR or LT under the policy to prioritize Child A patients with peripheral lesions for resection rather than transplantation, finding better results for transplanted patients. For single HCC smaller than 3 cm, they found that LR achieved comparable 10-year overall survival[13]. In another study published by our department, the outcomes were similar to Adam's paper. We compared patients with HCC < 5 cm who underwent LT or LR, finding a higher tumor recurrence rate in resected patients and better survival in patients who were transplanted. However, when we analyzed those tumors < 2 cm, no significant differences were observed in 1-, 5- and 10-year survival between the two groups[66].

The good outcomes of these publications could be justified because the recurrences in very early HCC are easier to treat, whether by re-resection or especially by salvage transplantation, allowing LR to be the treatment of choice for these tumors.

CONCLUSION

The best approach for cirrhotic patients diagnosed of very early HCC is still debatable as there is a lack of sufficient data. With the available information LR is the best treatment option in the case the patients liver function and performance status permits such approach. Moreover, the location of the tumor will also be part of the algorithm when making a decision on resecting the tumor. Ablative therapies such as RFA are an excellent alternative with good outcomes in case of very early HCC. The main counterpart to these treatments is the high rate of tumor recurrence. In this scenario (recurrence after primary treatment) LT can play an important role in the treatment of very early HCC. With the current allocation systems, patients with these tumors don't get exception

points and another interesting approach would be to wait and not treat until the tumor grows to get exception points. Further investigations on the best management of cirrhotic patients diagnosed of very early HCC are needed.

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TOPIC HIGHLIGHT

WJH 6th Anniversary Special Issues (2): Hepatocellular carcinoma

Mammalian target of rapamycin inhibition in hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related death worldwide. It is associated with a poor prognosis and has limited treatment options. Sorafenib, a multi-targeted kinase inhibitor, is the only available systemic agent for treatment of HCC that improves overall survival for patients with advanced stage disease; unfortunately, an effective second-line agent for the treatment of progressive or sorafenib-resistant HCC has yet to be identified. This review focuses on components of the mammalian target of rapamycin (mTOR) pathway, its role in HCC pathogenesis, and dual mTOR inhibition as a therapeutic option with potential efficacy in advanced HCC. There are several important upstream and downstream signals in the mTOR pathway, and alternative tumor-promoting pathways are known to exist beyond mTORC1 inhibition in HCC. This review analyzes the relationships of the upstream and downstream regulators of mTORC1 and mTORC2 signaling; it also provides a comprehensive global picture of the interaction between mTORC1 and mTORC2 which demonstrates the pre-clinical relevance of the mTOR pathway in HCC pathogenesis and progression. Finally, it provides scientific rationale for dual mTORC1 and mTORC2 inhibition in the treatment of HCC. Clinical trials utilizing mTORC1 inhibitors and dual mTOR inhibitors in HCC are discussed as well. The mTOR pathway is comprised of two main components, mTORC1 and mTORC2; each has a unique role in the pathogenesis and progression of HCC. In phase III studies, mTORC1 inhibitors demonstrate anti-tumor activity in advanced HCC, but dual mTOR (mTORC1 and mTORC2) inhibition has greater therapeutic potential in HCC treatment which warrants further clinical investigation.

Core tip: Advanced hepatocellular carcinoma (HCC) has a poor prognosis with limited therapeutic options. The mammalian target of rapamycin (mTOR) pathway (regulated by mTORC1 and mTORC2) is implicated in HCC pathogenesis. This review examines pre-clinical and clinical data demonstrating that mTORC1 inhibition effectively prevents HCC recurrence post-liver transplantation, and also has a modest anti-tumor effect in advanced HCC. The rationale and preclinical data for utilizing dual mTOR (mTORC1 and mTORC2) inhibition in HCC is also reviewed; a current phase I clinical trial to investigate the efficacy of dual mTOR inhibitors is briefly discussed. mTOR pathway inhibition has therapeutic potential in the treatment of advanced HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer diagnosed world-wide, with 250000 to 500000 cases diagnosed per year, and it is the third leading cause of cancer-related death in the world[1]. In the United States, the incidence of HCC has nearly tripled over the past 3 decades, with approximately 20000 new cases diagnosed annually, largely owing to the growing incidence of chronic Hepatitis C-related cirrhosis. HCC is associated with a poor prognosis, with 5-year survival rate persistently less than 10%. It is potentially curable by surgery or liver transplantation if detected early. Unfortunately, over 85% of cases are diagnosed at late stages when surgical intervention is no longer a viable option. The only available systemic treatment is a multi-targeted kinase inhibitor, sorafenib. In randomized, placebo-controlled phase III clinical trials, sorafenib modestly improves overall survival (OS) for patients with intermediate to advanced stage HCC[2,3]. An effective second-line agent for those with sorafenib failure or intolerance has yet to be identified. This has led to an ongoing search for molecular pathways and novel compounds for the treatment of advanced HCC.

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase downstream of the phophoinositide-3-kinase (PI3K)-related kinase family. It is a central regulator of various oncogenic processes including cell growth, proliferation, metabolism, and angiogenesis. There is growing evidence to suggest that mTOR deregulation plays a significant role in hepatocellular carcinogenesis. Pre-clinical data indicates that deregulated expression of mTOR pathway effectors is present in 40%-50% of HCCs, and activation of the mTOR pathway is associated with less differentiated tumors, earlier tumor recurrence, and worse survival outcomes[4]. Our review focuses on components and functions of the mTOR pathway and its potential role in the treatment of advanced HCC.

MTOR PATHWAY

Components of mammalian target of rapamycin complexes

The PI3K/AKT/mTOR signaling pathway- also known as the "mTOR pathway"- contains two important components: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Figure 1). They are multiprotein complexes, comprised of both shared and unique components.

The mTOR kinase- also known as "mTOR"- is one of three components which is present in both mTORC1 and mTOR2; mammalian lethal with SEC13 protein 9 (mLST8) and DEP domain-containing mTOR-interacting protein (DEPTOR) are two other proteins that are common to both mTORC1 and mTORC2. mLST8 interacts directly with mTOR to enhance its kinase activity, particularly within mTORC2 (its effect within mTORC1 is not clearly understood)[5]. DEPTOR prevents substrate binding to mTORC1 and mTORC2, which leads to inhibition of mTORC1 and mTORC2 activity[6,7].

mTORC1, which is sensitive to the effects of rapamycin, has two unique proteins: regulatory-associated protein of mTOR (RAPTOR) and 40 kDa Pro-rich AKT substrate (PRAS40; also known as AKT1S1). RAPTOR serves as a binding platform where substrates are presented to mTOR for subsequent activation of mTORC1[8]. Conversely, PRAS40, like DEPTOR, is a direct inhibitor of mTORC1 substrate binding which hinders mTORC1 activity[9].

Specific to mTORC2 are rapamycin-insensitive companion of mTOR (RICTOR), mammalian stress-activated map kinase-interacting protein 1 (mSIN1; also known as MAPKAP1) and protein observed with RICTOR (PROTOR) (Figure 1)[10]. There is some evidence that RICTOR contributes to the structural foundation of mTORC2; in the absence of RICTOR, mTORC2 becomes inactive[7]. The functions of mSIN1 and PROTOR remain unclear.

Functions and regulations of mTORC1 and mTORC2

mTORC1: mTORC1 expression is driven by stimulants such as energy status, physiologic stress, and growth factors. Specifically, in the presence of growth factors, insulin receptor substrate 1 (IRS1) activates PI3K. PI3K phosphorylates the second messenger called phosphatidylinositol (4,5)-biphosphate (PIP-2), which becomes phosphatidylinositol (3,4,5)-triphosphate (PIP-3) upon phosphorylation. PIP-3 then promotes the phosphorylation of serine/threonine protein kinase (PKB/AKT) at protein residue Thr308 by 3-phosphoinositide-dependent protein kinase-1 (PDK1). Further downstream signaling through the effector tuberous sclerosis 1-tuberous sclerosis 2 complex (TSC1-TSC2) ultimately leads to the activation of mTORC1.

Activated mTORC1 phosphorylates its two downstream targets, 70S ribosomal protein S6 kinase (S6K1) and the eukaryotic initiation factor 4E binding protein 1 (4E-BP1). S6K1 and 4E-BP1 are major regulators of protein translation; they also drive cell proliferation, angiogenesis, and autophagy[11].

Under normal physiologic conditions, 4E-BP1 binds to the eukaryotic initiation factor 4E (eIF4E) to arrest protein translation. However, when 4E-BP1 is phosphorylated by mTORC1, its binding to eIF4E is interrupted, and this allows protein translation to occur. Concomitantly, phosphorylation of S6K1 by mTORC1 also results in protein translation; however, it also creates a negative feedback loop whereby the phosphorylated S6K1 attenuates PI3K signaling by suppressing IRS1 activity, leading to mTORC1 inhibition. Interestingly, mTORC1 inhibition by rapamycin and its analogues disrupts S6K1-mediated feedback inhibition of PI3K signaling, which allows for increased PKB/AKT phosphorylation (Figure 2).

Figure 1 Mammalian target of rapamycin complex 1, Mammalian target of rapamycin complex 2 and their associated proteins. mTOR: Mammalian target of rapamycin; DEPTOR: DEP domain-containing mTOR-interacting protein; mLST8: Mammalian lethal with SEC13 protein 9; RAPTOR: Regulatory-associated protein of mTOR; PRAS40: 40 kDa Pro-rich AKT substrate; RICTOR: Rapamycin-insensitive companion of mTOR; mSIN1: Mammalian stress-activated map kinase-interacting protein 1; PROTOR: Protein observed with RICTOR.

Furthermore, rapamycin-induced inhibition of mTORC1 leads to an accumulation of phosphorylated AKT which can then activate downstream effectors of alternative pathways to inhibit apoptosis and promote cell proliferation[12].

mTORC2: Similar to mTORC1, mTORC2 activity is also promoted by growth factors. The upstream regulatory mechanisms specific to mTORC2 are poorly understood; however, its role in the phosphorylation of PKB/AKT has been well-characterized. The full activation of PKB/AKT requires two steps of phosphorylation: first, at protein residue Thr308 by PDK1, and second, at residue Ser473 by mTORC2[13]. Therefore, mTORC2 indirectly promotes mTORC1 activity through activation of PKB/AKT (Figure 2). Another less understood function of mTORC2 involves the regulation of actin cytoskeleton organization. Unlike the inhibitory effects on mTORC1, the effects of rapamycin and its analogues on mTORC2 are minimal[14].

Constitutive upstream regulators of the mTOR pathway: PTEN and TSC1-TSC2 complex

Phosphatase and tensin homologue on chromosome 10 gene (*PTEN*) is a multiphosphatase tumor suppressor located on human chromosome 10q23.3. It blocks the downstream activity of PI3K-AKT signaling by degrading PIP-3[11]. Inhibition of PIP-3 by PTEN prevents activation of PKB/AKT which leads to the down-regulation of mTORC1 activity (Figure 2). In the absence of PTEN, activation of mTORC1 is unbridled and hepatocellular carcinogenesis occurs. Watanabe *et al*[15] showed high incidence of HCC (66%) in PTEN-deficient mice at the end of an 80-wk period. Wang *et al*[16] demonstrated that decreased PTEN protein expression in HCC tissue samples compared to paired surrounding tissue samples was associated with higher tumor pathologic grade, TNM stage, and more frequent incidence of metastasis.

The TSC1 and TSC2 proteins (also known as hamartin and tuberin, respectively) are regulators of cell proliferation which have been implicated in HCC carcinogenesis. In its active form, the TSC1-TSC2 complex inhibits mTORC1 activation. Specifically, TSC2 acts as a GTPase-activating protein (GAP) which degrades guanosine triphosphate (GTP) and prevents its binding with Rheb, a GTP-binding protein. As a result, Rheb's ability to inhibit FKBP38, a negative regular mTORC1, is disabled and mTORC1 is inhibited. However, Akt-mediated phosphorylation deactivates the TSC1-TSC2 complex by decreasing its GAP-activity towards Rheb, which permits Rheb-GTP binding[17]. In turn, Rheb carries out its usual function of inhibiting FKBP38, and mTORC1 activation occurs (Figure 2)[17,18]. In fact, loss of either TSC1 or TSC2 promotes autonomous activation of mTORC1. Using liver-specific TSC1 knockout mice, Menon *et al*[19] demonstrated that chronic activation of mTORC1 in the absence of TSC1 induced hepatocyte damage, independent of hepatic steatosis, which leads to the spontaneous development of HCC.

It is important to note that PTEN and TSC1-TSC2 complex also function as integrating hubs for the regulation of mTOR *via* alternative signaling pathways. For example, the Src family kinases (SFKs) and the Wnt protein of the Wnt/β-catenin pathway are direct upstream regulators of PTEN and TSC1-TSC2 complex, respectively. Studies of breast cancer cell lines have shown that SFKs phosphorylate PTEN to inhibit its function[20], which then promotes mTORC1 activation. Conversely, the stimulation of Wnt prevents TSC2 phosphorylation through inhibition of GSKβ3, a protein constituent of Wnt/β-catenin pathway, thus inhibiting mTORC1 activation[17].

CLINICAL EXPERIENCE OF MTOR INHIBITION IN HCC

mTORC1 inhibitor in the prevention of HCC recurrence post liver transplantation

Within the past decade, the role of mTOR inhibition in the prevention of HCC recurrence has been examined more thoroughly in the post-liver transplantation patient population. Recurrence is a major cause of morbidity and mortality among these patients, and the recurrence risk is markedly influenced by explant pathology such as poor tumor differentiation and the presence of microvascular invasion[21].

The traditional immunosuppressants used to prevent

Figure 2 The PI3K/AKT/mTOR pathway. IRS1: Insulin receptor subsrate 1; PI3K: Phophoinositide-3-kinase; PIP-2: Phosphatidylinositol (4,5)-biphosphate; PIP-3: Phosphatidylinositol (3,4,5)-triphosphate; PTEN: Phosphatase and tensin homologue on chromosome 10 gene; PDK1: Phosphoinositide-dependent protein kinase-1; PKB/AKT: Serine/threonine protein kinase; TSC1-TSC2: Tuberous sclerosis 1-tuberous sclerosis 2 complex; 4EBP1: Eukaryotic initiation factor 4E binding protein 1; eIF4E: Eukaryotic initiation factor 4E; S6K1: 70S ribosomal protein S6 kinase; SFKs: SRC family kinases.

liver allograft rejection are calcineurin inhibitors (CNIs) such as tacrolimus and cyclosporine. They have been implicated in tumorogenesis both *in vitro* and *in vivo*[22,23]. In contrast, mTOR inhibitors are capable of effective immunosuppression (by blocking interleukin-2-mediated acute graft rejection) and concomitant prevention of hepatocellular tumorogenesis (through potent inhibition of angiogenesis). These two reasons make them attractive immunosuppresants for post-liver transplantation patients with a pre-transplant diagnosis of HCC[24].

In retrospective and non-randomized prospective analyses, post-liver transplantation HCC patients treated with sirolimus (a rapamycin analogue which selectively inhibits mTORC1) showed decrease in HCC recurrences[25]. In a study of 70 post-liver transplantation HCC patients treated with sirolimus-based immunosuppression, Toso *et al*[26] demonstrated an absolute decrease in recurrence rates by 6% (Milan criteria) and 14% (beyond Milan criteria) compared to studies not using sirolimus. A recent meta-analysis in patients with HCC who underwent liver transplantation indicated that sirolimus-treated patients demonstrated longer 5-year relapse-free survival (RFS) and 5-year OS rates (79%-80% and 80%, respectively) compared to CNI-treated patients (54%-60%; 59%-62%, respectively)[27].

Because of this promising data, a prospective, randomized international clinical trial (the "SiLVER trial") has been developed to assess the role of sirolimus in HCC-free patient survival in liver transplantation recipients with a pre-transplant diagnosis of HCC; the primary endpoint is RFS with a planned 5-year follow-up[28].

mTORC1 inhibitor in advanced HCC

Recently, single-arm phase Ⅰ/Ⅱ studies have shown that everolimus (a second-generation mTORC1 inhibitor), has single-agent activity in de novo or recurrent advanced HCC. In a cohort of 36 patients, everolimus hindered disease progression in patients with advanced HCC when used at maximum tolerated dose of 70 mg weekly[29]. In a subsequent phase Ⅰ/Ⅱ study by Zhu *et al*[30], 28 patients with advanced HCC tolerated everolimus at the dose of 10 mg daily. The median progression free survival was 3.8 mo, suggesting a modest antitumor effect of everolimus in advanced HCC[31].

This study led to the global phase Ⅲ randomized EVOLVE-1 trial, where everolimus was compared to placebo in patients with advanced HCC who discontinued sorafenib due to disease progression or drug intolerance. This trial unfortunately showed no OS benefit for

everolimus in the salvage setting of advanced HCC[32]. As discussed in section 2 (b) of this review, mTORC1 and mTORC2 are two complementary components of the mTOR pathway: when mTORC1 is inhibited, mTORC2 is upregulated. This increase in mTORC2 activity generates a surplus of phosphorylated PKB/AKT which, despite mTORC1 inhibition, inhibits apoptosis and promotes cell proliferation *via* alternative pathways (Figure 2)[33]. This phenomenon may partially explain the unsatisfactory efficacy of everolimus demonstrated in the EVOLVE-1 trial, and suggests a potential mechanism for drug resistance against mTORC1 inhibitors in HCC. Given this theory, dual mTORC1 and mTORC2 inhibition has become an attractive pharmacologic target with therapeutic potential in advanced HCC treatment.

The safety of everolimus in combination with sorafenib has also been evaluated for the treatment of advanced HCC, as it posed the opportunity to target two major pathways involved in HCC pathogenesis. However, phase I studies demonstrated intolerable toxicities with this combination, rendering it infeasible as a therapeutic option[34,35].

POTENTIAL OF DUAL MTOR INHIBITION IN HCC

Pre-clinical studies using second generation mTOR inhibitors (*i.e.*, Pp242, OSI027, AZD8055) in HCC cell lines and xenograft models have demonstrated enhanced antitumor efficacy of dual mTORC1/2 targeting[36-38]. Specifically, CC-223 (CC0482223) is a potent selective inhibitor of both mTORC1 and mTORC2 that impedes tumor resistance by inhibiting AKT phosphorylation. In multiple tumor cell lines, substrates of both mTORC1 and mTORC2 (p-S6RP and pAKT Ser473, respectively) were inhibited by CC-223, whereas rapamycin was a successful inhibitor of its downstream target p-S6RP only.

The therapeutic potential of CC-223 is being tested in a phase I trial of patients with refractory malignancies including HCC. Twenty-seven HCC patients have been enrolled as of June 2013; 93% of them previously received sorafenib. With 45 mg daily dosing of CC-223, 11% of patients exhibited a partial response, and 33% of patients maintained stable disease[39]. Due to this encouraging data, a cohort expansion of CC-223 in HCC patients is ongoing.

FUTURE DIRECTIONS IN MTOR INHIBITION FOR HCC

HCC undergoes constant mutational changes throughout its carcinogenesis and progression; therefore, combination therapy may be of interest. The possibility of non-overlapping pathway inhibition can be considered. For instance, sorafenib and dual mTOR inhibition could be a potentially effective strategy. In addition, epigenetic modification through methylation contributes to therapy resistance in many tumor types and HCC is no excep-

tion[40]. Dual mTOR inhibition combined with demethylating agents could also be a valid scientific approach[41].

Dramatic advances in the treatment of HCC have been achieved with improvement in the understanding of the biology of HCC pathogenesis and progression. The mTOR pathway is clearly critical to the progression of HCC. We anticipate that future data on single-agent dual mTOR inhibitors and combination strategies utilizing mTORC dual inhibition with other novel agents will contribute to the advances in HCC treatment.

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TOPIC HIGHLIGHT

WJH 6th Anniversary Special Issues (2): Hepatocellular carcinoma

Problem of hepatocellular carcinoma in West Africa

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Abstract

The incidence of hepatocellular carcinoma (HCC) is known to be high in West Africa with an approximate yearly mortality rate of 200000. Several factors are responsible for this. Early acquisition of risk factors; with vertical or horizontal transmission of hepatitis B (HBV), environmental food contaminants (aflatoxins), poor management of predisposing risk factors and poorly-managed strategies for health delivery. There has been a low uptake of childhood immunisation for hepatitis B in many West African countries. Owing to late presentations, most sufferers of HCC die within weeks of their diagnosis. Highlighted reasons for the specific disease pattern of HCC in West Africa include: (1) high rate of risk factors; (2) failure to identify at risk populations; (3) lack of effective treatment; and (4) scarce resources for timely diagnosis. This is contrasted to the developed world, which generally has sufficient resources to detect cases early for curative treatment. Provision of palliative care for HCC patients is limited by availability and affordability of potent analgesics. Regional efforts, as well as collaborative networking activities hold promise that could change the epidemiology of HCC in West Africa.

Core tip: It is known that outside the region of East Asia, sub-Saharan Africa has the highest incidence of hepatocellular carcinoma (HCC). Within Africa the West African region remains the focus of significant disease activity. We reviewed the main issues responsible for this pattern. Although intervention efforts, such as primary prevention through hepatitis B vaccination, has led to reductions of chronic hepatitis B infection in some countries such as Gambia and Senegal, there remains a huge gap in secondary prevention, which are responsible for continuing high mortality to incidence ratio of HCC in West Africa. Collaborative clinical care and basic science translational research holds promise towards changing the current trend.

INTRODUCTION

Hepatocellular carcinoma (HCC) constitutes almost 85% of all primary liver cancers, and is known to be the 5th most commonly diagnosed cancer globally[1]. In 2012, about 782000 and 746000 new cases and deaths respectively, had HCC as their primary diagnosis[2]. Sub-Saharan Africa is the most affected region of the world, after Eastern Asia owing to the high prevalence of risk factors for this cancer in these continents. Although a description of the burden of HCC in developing countries, highlighting the sub-Saharan African situation has recently been reported by Kew[3], the countries of West Africa have reported more than average incidence of HCC, a situation deserving in depth understanding. In this article, we systematically reviewed the problem of HCC in West Africa: contributing factors, primary and secondary prevention efforts, as well as the provision of palliative care to patients. This review provides an overview of current efforts and suggests opportunities for strategic intervention.

EPIDEMIOLOGY OF HCC IN WEST AFRICA

There is a high incidence of HCC in West Africa. In countries like Gambia, Guinea-Conakry and Sénégal, the incidence of HCC has been reported to range between 30-50/100000 and 12-20/100000 in men and women, respectively[4]. The West African region comprises 16 countries. It has an area approximating 6.1 million km^2, bordered in the north by the Sahara desert and the east by Mount Cameroon and Lake Chad. Aside from shared economic interests, such as the Economic Community of West African States, there are similarities in the dress, cuisine, music and culture of people living in this geographical enclave. These factors may indeed underlie the way that HCC presents.

The mortality rate of HCC is almost the same as its incidence in this region of the world. Individual national cancer registry data are limited. However, the global cancer registry database has provided estimates of incidence and mortality by gender for primary liver cancer; of which HCC constitutes approximately 85%. Taking into account the incompleteness of cancer registration in this region, these data highlight the high case fatality rate of HCC. The most affected country is The Gambia, followed by Guinea, Liberia and Sierra-Leone in that order (Figure 1).

As most affected persons are middle-aged, HCC contributes to decreased economic development of this re-

gion. Whereas the incidence of HCC in most developed countries show that the highest affected is 75 years and older, and similar patterns among high risk Asian populations, the situation is different in West Africa. There is a significant male preponderance of this tumour, being the most commonly encountered malignancy in men in several West African countries (Table 1). The rates of HCC in men in countries like Gambia and Mali tend to peak at 60 to 65 years while females peak between 65 and 75 years[5]. A study has reported peak age of 40 years from this region[6].

Some reasons for the characteristic epidemiological pattern of HCC in West Africa are discussed as follows.

Failure to identify at risk populations

It is not uncommon for some patients in West Africa to be found to have hepatitis B viral infection, for the first time, when they present to hospitals with decompensated liver disease. This late diagnosis is not only as result of lack of health-seeking behaviour, but likely to be due to some additional factors. As the performance of healthcare delivery is often suboptimal in this region, many hepatitis B surface antigen (HBsAg)-positive patients seek herbal and alternative medications as the initial port of call prior to attending orthodox care. Since few individuals receive adequate management for chronic hepatitis B, there is a tendency to progress to cirrhosis. Malignancy, on the background of poor hepatic reserve, with additional consumption of traditional remedies; of unknown toxicities, tip the patients to liver failure on first hospital presentation.

Low hepatitis B virus immunisation adherence

Significant declines in HBsAg prevalence and low rates of childhood HCCs have been realised in countries that introduced Hepatitis B virus (HBV) vaccine[7]. One study in the region has revealed that HBV vaccination is capable of decreasing chronic HBsAg carriage by up to 83%[8]. This observation has been replicated in studies from Senegal and South Africa[9,10]. However, many countries in the region have ensured complete adherence to whole course of HBV vaccination. The Global Alliance for Vaccines and Immunisation funding and the World Health Organisation (WHO), supporting HBV vaccination programmes, have played an important role in the implementation of HBV vaccine programmes in Africa. Despite this effort, HBV vaccine coverage remains low estimated at 70% according to the WHO/UNICEF 2012 data.

Poor treatment of liver diseases

The treatment of liver diseases is generally inadequate in many countries of West Africa. Large number of patients overwhelms the limited number of trained medical personnel, inadequate infrastructure for training curricula, mass emigration of medical professionals and paucity of clinical guidelines adapted to the local setting are important in this regard. It was not until recently that hepatitis C virus (HCV) treatment guidelines for low and middle

Table 1	Summary of some studies indicating male preponderance of hepatocellular carcinoma relative to other cancers in West Africa		
Country	Liver cancer relative to cancer elsewhere	Source of study population	Coverage
Niger[55]	Most common in men; M:F ratio of 1.4:1	National cancer registry	National
The Gambia[56]	Most common in men; 2nd in women	National cancer registry	National
Ghana[57]	Most common in men, 3rd in women; M:F ratio of 1.2:1	Southern Ghana	Mortality data from a tertiary centre
Nigeria[58]	Most common in men; M:F ratio of 2.4:1	South East Nigeria	Cancer mortality data
Nigeria[59]	Most common in 50-59 years old	South West Nigeria	Pathology reports
Côte d'Ivoire[60]	Second in men; less common in women	Cancer registry	National
Mali[61]	Most common cancer in men; cervical cancer leads in women	Cancer registry	National
Guinea-Conakry[62]	Most common in men; second in women	Cancer registry	Regional

Figure 1 Multiple clustered bar charts labelled by incidence and mortality rates per 100000 population of West African countries (data from Globocan 2012 from International Agency for Research on Cancer[54].

income countries were commissioned by the WHO[11]. Inadequate funding prevents the optimal treatment of those affected, as the cost of these medications is prohibitive for most sufferers in these countries[12]. Patients tend to present to hospitals when they have noticed symptoms or when a close relative gets diagnosed with an associated complication of viral hepatitis.

Inadequate public health intervention

The burden of disease imposed by viral hepatitis has been completely ignored by the international health agenda these last decades as the focus has been put on human immunodeficiency syndrome (HIV), tuberculosis, and malaria, three major infectious diseases issues which have been the main recipient of health care resources and funding[13]. Yet, if the mortality and morbidity from cir-

rhosis and liver cancer were grouped, the burden of viral hepatitis would have to be seriously considered by the international health authorities[14]. The lack of public health campaigns is complicated by a plethora of traditional healers.

There is also a scarcity of coordinated health programmes that could inform governments in the region regarding the problem of liver diseases. With significantly high prevalence of HBsAg and anti-HCV in Nigeria, it was only in 2009 that a guideline for the treatment of HBV was produced. Similarly, the WHO and World Hepatitis Alliance estimate that only 17 countries in the whole of Africa that have designed national guidelines on viral hepatitis, of which only 3 sub-Saharan African countries (Cameroon, Rwanda and Mauritania) have implemented guidelines on HBV mother-to-child transmission. With

Figure 2 Prevalence of hepatitis B surface antigen carriage in some West African countries.

the prohibitive cost of antivirals and a fee-for-service system of healthcare, only those who can afford medication get treated. This ultimately results in a large pool of those who could have been treated for viral hepatitis going on to develop HCC.

HIGH RATE OF RISK FACTORS

Hepatitis

HBV, described as a potent carcinogen, is endemic in West Africa demonstrating varied prevalence. The infection rates of HBV vary between 8%-20% in this region[15].

West Africans have longer durations of HBV infections relative to individuals in the developed world who tend to get the infection much later in life. By age of 10 years, 80% of infected people in Africa have acquired HBV[16] and a high carriage rate of up to 20% (Figure 2). Inadequate data in the literature from this region may actually be modulating the true problem of HBV and its sequelae in the West African region.

HCV infection affects more than 8.4 million people in West Africa[11]. Although the transmission route of HCV in this region is not well established, and most cases are thought to be due to use of unsterile sharps, and receipt of unscreened blood products, sexual transmission may have a modest effect[17]. Owing to incorrect assumptions, anti-HCV serology was not part of routine screening for blood products until early 2000s. However, it is now known that the prevalence of HCV in West Africa varies from 2.5%-9.9%[17]. Although less prominent than HBV, HCV contributes to HCC in West Africa, particularly for those above 60 years[5].

The distribution of HBV genotype differs from one region of the world to another, and which could be a determinant factor in the clinical outcome of HBV infection. For example, in central and West Africa genotype E has been documented to predominate[18,19]. Studies from

Asia have demonstrated an increase in the development of HCC among patients with HBV genotype C, compared to genotype B[20-23]. A study in South Africa has shown that HBV genotype A had a greater hepatocarcinogenic potential than other non-A genotypes[24].

Aflatoxin

This mycotoxin, produced by the fungus *Aspergillus spp.*, grows on mainly legumes and cereals in humid conditions in parts of West Africa. These foods are mostly consumed unprocessed as staples in West Africa. Subsistence farming, poor farm produce storage and suboptimal processing systems facilitate widespread exposure to this toxin (Figure 3). Aflatoxin and HBV infection can synergistically increase the risk of HCC. A possible molecular mechanism has been suggested by studies in HBV transgenic mice[25]. That study suggested that chronic inflammatory damage of the liver alters the expression of carcinogen metabolizing proteins and may thus moderate the binding of aflatoxin to DNA. Further research in the region has confirmed a significant increase in the risk of cirrhosis in patients exposed to aflatoxin[26]. Additional research in this area could be far-reaching; and may enhance policy decisions regarding drying, storage, processing and consumption of foods such as cereals that are consumed in large amounts in the countries within this region.

Alcohol

Although not as affluent as developed countries, alcohol consumption goes on, albeit to an undocumented level in West Africa. Locally-brewed fermented drinks in Africa have been reported to significantly contribute to HCC. These studies postulated that the brewing containers (Figure 4) release iron in consumers of these drinks, which leads to an iron overload syndrome. Almost a tenth of some populations in sub-Saharan Africa have been noted to have iron overload[27,28]. Iron levels have

Figure 3 Fungus-infested malt, a product of cereal used in the fermentation of local alcohol beverage "burukutu". Cereals are widely consumed in West Africa and are a source of aflatoxin, which has been shown to potentiate the hepatocarcinogenicity of hepatitis B virus (Picture by Pantong Mark at Jos, Nigeria).

Figure 4 Iron pots used in brewing local beer in a typical West African setting (Picture by Pantong Mark, Jos, Nigeria).

been reported to be significantly higher among Africans with liver cancer than controls[29]. A genetic polymorphism in the ferroportin-1 has been demonstrated in a southern African study population, and thought to be associated with decreased iron excretion[30]. The interaction between alcohol, HBV and iron overload could be far-reaching to predispose West Africans to HCC. Studies have found that the incidence of HCC is 200 fold in haemochromatosis if the patients are above 55 years of age, have HBsAg seropositivity and who additionally drink alcohol[31,32].

HIV and hepatitis co-infection

The impact of HIV infection on chronic viral hepatitis B and C, as well as on the response to hepatitis B immunisation antibody generation are subjects deserving further studies in this region. Data from developed countries have established definite links between HIV/HBV and HIV/HCV co-infections, as well as HCC[33,34]. Prior to the provision of highly active antiretroviral treatment to Africa, most HIV patients died earlier due to opportunistic infections before they developed complications of HBV or HCV. Recent experience emerging from well monitored HIV centres in West Africa[35] confirms that most co-infected patients are expected to survive longer and the impact on the overall burden of HCC will eventually emerge. Furthermore, the impact of HIV infection on the long-term efficacy of the HBV vaccine in West Africa remains to be determined and might pose serious consequences for the gains already made in places that have attained a wide HBV vaccination coverage[36].

CLINICAL PRESENTATIONS OF HCC IN WEST AFRICA

The natural history of untreated HCC and the associated clinical features have been well characterised from developed countries[37]. Early HCC is often asymptomatic and is devoid of pathognomonic features. Certain features that distinguish HCC presenting in developed countries

relative to West African countries are summarised in Table 2. Whereas 5%-10% of HCC patients in the West and almost 30% in Japan are diagnosed with early disease[38], almost all persons diagnosed with HCC in West Africa are diagnosed very late[5,39]. The presence of a painful right upper abdominal mass and swelling, weight loss and early satiety signify advanced disease[40].

Weight loss is the commonest symptom of HCC, often attributed to "witchcraft" in West African populations. Right upper abdominal pain, abdominal swelling and jaundice are not uncommon. Other symptoms include anorexia and confusion. To ease diagnosis, most clinicians in sub-Saharan Africa recognise a prospective HCC patient either as: one with abdominal pain and a hard nodular hepatomegaly, or "a triad of abdominal pain, swelling and jaundice". A few studies from the region[5,41] have corroborated the stated features (Figure 5).

DIAGNOSTIC CHALLENGES OF HCC IN WEST AFRICA

Challenges of diagnosis of HCC in developing countries have been recently highlighted[3]. According to the international guidelines the diagnosis of HCC relies on specific radiological aspects using computed tomography (CT) or magnetic resonance imaging (MRI) scans and/or histopathological analysis. However, in sub-Saharan Africa, these diagnostic tools are rarely used in clinical practice because: (1) CT or MRI scans with contrast are not available in many countries or are expensive and not free of charge; and (2) liver biopsy is an invasive and costly procedure requiring well trained hepatologists, histopathologists and laboratory technicians, who are not always at post. Moreover most percutaneous liver biopsies are not image-guided and hence there is a high chance of mis-diagnosis. Owing to low sensitivity, serum alpha-fetoprotein (AFP) is no longer recommended by most international guidelines (indeed in some guidelines it is used in combination with radiological features) to be used for this purpose[42], although almost all centres in West Africa still rely on it. As one third of HCC do not secrete AFP,

Table 2 Relative differences in risk factors, clinical features, surveillance and management of hepatocellular carcinoma between West Africa and developed countries		
Parameter	**Developed countries**	**West African countries**
Predominant risk factor	Hepatitis C virus[2,63]	Hepatitis B virus[5]
Predominant co-factor	Alcohol	Aflatoxin B1[64]
Peak incidence	8th decade[65]	5th decade[57]
Stage at presentation	High chance of early stage at diagnosis[38]	Often advanced stage at presentation[3]
Surveillance	Routine; although compliance is about 12% in a study in the United States[66]	Not known and not routine
Median survival	Overall survival of > 16 mo in a study from United States[67]	Most die at initial presentation
Diagnosis	Radiological (multi-phasic dynamic CT or MRI) ± liver biopsy[68]	Tumour markers (occasionally, grey-scale ultrasound scan ± liver biopsy)[12,48]
Treatment	Curative therapies and palliative care; according to guidelines	Mainly palliative; often suboptimal

CT: Computed tomography; MRI: Magnetic resonance imaging.

and most of the tertiary centres use only grey scale ultrasound scan systems, a lot of those patients with hepatic lesions are missed and/or confused with other common inflammatory conditions such as amoebiasis, peritoneal and hepatic tuberculosis, lymphoma, cholangiocarcinoma and secondary hepatic tumours. The import of the foregoing is the fact that the rates of HCC being reported are unlikely to reflect the true picture of the burden of the disease in West Africa.

TREATMENT OF HCC IN WEST AFRICA

Owing to very late presentations and poor health infrastructures, the outcome of HCC in West Africa is generally dismal and curative management is impossible, treatment only relying on palliative care for the most part. Yet, very few centres have proper palliative care, as opiates are often unavailable and healthcare workers are not trained to use them. The vast majority (80%-90%) of cancer patients in sub-Saharan Africa only seek medical attention when cancers have reached an advanced stage, where end-of-life strategies are the only option. In 2009, only 12% of cancer patients in sub-Saharan Africa with moderate to severe pain were estimated to have been treated with opioid analgesics, an essential component of palliative care[43].

The management of pain for palliative patients has been also hampered by lack of knowledge and training and apprehension that opioid analgesics would cause severe digestive side effects and addiction. The so-called "opiophobia", among healthcare professionals is frequently observed in Africa[44] and is known to lead to under-prescription of pain relief medication. In The Gambia, it was found that only 12 HCC patients (48%) of HCC patients receive analgesics without any oral morphine prescription (personal communication).

Figure 5 Summary of most common clinical presentations of hepatocellular carcinoma in three West African communities: Gambia, Northern and Southern Nigeria. Note the similarity of clinical presentation in three study sites in two countries in the region. Number of hepatocellular carcinoma patients in parentheses.

REASONS FOR LOW SURVIVAL OF HCC IN WEST AFRICA

Data on HCC survival in Sub-Saharan Africa are almost non-existent. A recent unpublished study conducted in Nigeria reported a median survival of 4 mo in 100 clinically diagnosed HCC patients[45] and preliminary data from the Prevention of Liver Fibrosis and Cancer in Africa programme in The Gambia found a median survival of 61 d (unpublished data). Worldwide data on cancer survival have shown that the 5-year relative survival was lowest in Uganda and Gambia, relative to other countries such as China[46].

Absent surveillance for HCC

In order to detect cases amenable to curative therapy, well-coordinated surveillance for HCC has to be in place. However, as this is not the case in most West African health centres, most HCC cases are detected at advanced stages[1]. Zhang *et al*[47] have recently reported the advantage of surveillance for HCC in at-risk populations in China, in which they noted a reduced mortality rate by 37% relative to a non-surveyed cohort[47]. For sub-Saharan Africa, serum AFP has been recommended for this purpose by the World Gastroenterology Organisation[48]. However, data on the adherence to this recommendation by physicians and compliance by patients are lacking. Data are available to support the fact that surveillance for HCC could improve therapeutic options for HCC[49].

Lack of treatment facilities for HCC

The advantage that surveillance provides would be confounded if treatment for HCC cannot be offered. Less complicated treatment modalities such as percutaneous ethanol injection of tumours could be offered only if the patients present at a relatively early stage. Liver transplant

services are scarce in West African countries. As fee-for-service continues to be the predominant health system in West Africa, specialists would not embark on skills that are rarely utilised.

Alternative treatment for "hepatitis"

There is a flourishing presence of self-acclaimed healers in West Africa (evangelical churches, as well as traditional religious practices) and claims of miraculous healing are an important contributor for the late presentation, as conventional western medical treatment is often a last resort for many of the afflicted.

CONCLUSION

Outlook and recommendations

HCC is a major cause of death in sub-Saharan Africa, estimated to be responsible for annual deaths of 200000 persons[50]. We have highlighted the direct and remote causes that may be contributing to the pattern of disease presentation in West African patients in this article. International and local efforts are underway to help regarding improving the current bleak outlook of this cancer. Deliberate attempts to reduce exposure to aflatoxin post-harvest had yielded encouraging results, which clinical significance could mean reduction of HCC development in at-risk persons[51]. Improvement of healthcare systems that could attract and retain specialists to tackle the risk factors and improvement in health budgetary implementation towards infrastructural facilities could provide a robust platform to changing the current trend.

In view of the multifactorial aetiological factors in the causation of HCC and the fact that little is in place regarding coordinated control of some of the these risk factors, some authors have predicted that the problem of HCC in West Africa is postulated to increase in the next 40-50 years[52]. However, this appears rather pessimistic and suggests that control efforts would not be in place. Already, some groups, such as the Prevention of Liver Fibrosis and Carcinoma in Africa (www.prolifica.eu) consortium have been investigating the impact of treatment of chronic HBV in reducing the incidence of HCC in this region. Already, this collaborative effort, comprising specialists from European and West African institutions, has led to identification of a validated panel of urinary metabolites[53] that could prove to be useful screening tool for HCC in West African populations in the future. Also, the activities of national professional bodies, such as the Society of Gastroenterology and Hepatology in Nigeria in publishing hepatitis treatment guidelines may only be effective if the West African community of states approach hepatitis in a logistical, programmed fashion, as has been done with HIV. More concerted attention is required to tackle HCC in West Africa in a comprehensive manner, involving public health personnel, hepatologists, oncologists, surgeons and palliative care practitioners.

We have thus presented a synopsis of how important HCC is in the West African region of the world; high-lighting the high incidence, mortality and case fatality. Primary prevention methods such as HBV vaccination has led to reduction in chronic HBV infection, but its impact on reducing the incidence of HCC is yet to be documented in this sub region. Additionally, the contribution of aflatoxin deserves further study, as well as avoidance of its exposure aimed at maximising the prevention of liver cancer in this population should be a priority. There is hope in the horizon as there have been coordinated collaborative efforts to: (1) determine the impact of primary prevention in epidemiological terms; (2) provide primary prevention with programmes such as HBV vaccination (Gambia Hepatitis Intervention Study of the MRC); (3) secondary prevention and treatment of chronic HBV with the PROLIFICA programme; as well as (4) enhancing early detection of incident cases (PROLIFICA) in the region. Local efforts such as: provision of guidelines adaptable to the economic resources of the countries in the region as well as hepatitis awareness campaigns hold promise with assisting in the effort to curb this tumour. Parallel control efforts such as proper storage of cereals prior to consumption hold promise to reducing the synergistic contribution of aflatoxin to those already chronically infected by HBV. Results from these endeavours could potentially provide the platform to persuade governments in this region to facilitate larger scale universal policies.

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TOPIC HIGHLIGHT

WJH 6th Anniversary Special Issues (4): Cirrhosis

Role of vaptans in the management of hydroelectrolytic imbalance in liver cirrhosis

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Abstract

Ascites and hyponatremia are the most common complications in patients with liver cirrhosis and develop as a consequence of a severe impairment of liver function and portal hypertension. Increasing evidences support the central role of renal function alterations in the pathogenesis of hydroelectrolytic imbalances in cirrhotic patients, thus implying a dense cross-talk between liver and kidney in the systemic and splanchnic vascular homeostasis in such subjects. Since Arginin Vasopressin (AVP) hyperincretion occurs at late stage of cirrhosis and plays an important role in the development of refractory ascites, dilutional hyponatremia and finally hepato-renal syndrome, selective antagonists of AVP receptors V_2 (vaptans) have been recently introduced in the therapeutic algorithm of advanced cirrhotic patients. Despite the promising results of earlier phase-two studies, randomized controlled trials failed to find significant results in terms of efficacy of such drugs both in refractory ascites and hyponatremia. Moreover, concerns on their safety profile arise, due to the num-

ber of potentially severe side effects of vaptans in the clinical setting, such as hypernatremia, dehydration, renal impairment, and osmotic demyelination syndrome. More robust data from randomized controlled trials are needed in order to confirm the potential role of vaptans in the management of advanced cirrhotic patients.

Core tip: Increasing evidences support the central role of renal function alterations in the pathogenesis of hydroelectrolytic imbalances in cirrhotic patients. Since Arginin Vasopressin (AVP) plays an important role in the development of refractory ascites, dilutional hyponatremia and hepato-renal syndrome, selective antagonists of AVP receptors V_2 (vaptans) have been recently introduced in the therapeutic algorithm of advanced cirrhotic patients. Despite the promising results of earlier phase-two studies, randomized controlled trials failed to find significant results in terms of efficacy. Moreover, concerns on their safety profile arise. More robust data from randomized controlled trials are needed.

INTRODUCTION

Ascites is the most common complication in patients with cirrhosis and develops as a consequence of a severe impairment of liver function and portal hypertension[1].

In fact, there is substantial evidence that severe portal hypertension is the main disorder in the occurrence of ascites in cirrhosis as ascitic patients have significantly higher portal pressure than those without ascites. In particular, an hepatic venous pressure gradient (estimation of the intrahepatic vascular resistance) of more than 12 mmHg has been found to cause the occurrence of ascites in cirrhotics[2,3].

Increasing evidences support the central role of renal function alterations in the pathogenesis of hydroelectrolytic imbalances in cirrhotic patients, thus implying a dense cross-talk between liver and kidney in the systemic and splanchnic vascular homeostasis in such subjects.

Each step of this cross-talk could represent a potential target for the pharmacological management of cirrhotic patients.

PATHOGENESIS OF ASCITES AND DILUTIONAL HYPONATREMIA

It is now evident that ascites is related more to alterations in the arterial vascular compartment and in kidneys than in the portal venous system[4].

Ascites has been traditionally considered as a consequence of backward transmission of the increased intrahepatic hydrostatic pressure into the splanchnic microcirculation and of the decrease in intravascular oncotic pressure because of the impaired hepatic synthesis of albumin.

More recently, the splanchnic arterial vasodilatation secondary to portal hypertension has been found as the central event of ascites formation in cirrhosis[5]. Such mechanism simultaneously induces two different types of events: a "forward" increase in capillary pressure because of a greater inflow of blood at high pressure into the splanchnic microcirculation with consequent passage of fluid into the peritoneal cavity, and the impairment of systemic hemodynamics and renal function, which leads to sodium and water retention[6].

Splanchnic and systemic vasodilatation is due to the excretion by sinusoids of vasoactive mediators, for instance nitric oxid (NO), together with glucagon and other vasodilator molecules[7]. As a consequence of this process, the efforts made by the organism in order to obviate to the imbalance in favor of vasoactive molecules generate a well described chain of processes leading to a "vicious" circle and hence an impairment of the above cited pathological events. Particularly, the followings steps are reported: (1) "underfilling" of effective arterial blood volume; (2) impairment of renal perfusion and deterioration of glomerular filtration rate; (3) release of catecholamines by sympathetic nervous system (SNS) as response to decreased volemia leading to increase in cardiac output and renal vasoconstriction; (4) hyperactivation of Renin-Angiotensin-Aldosterone system (RAAS) and secondary hyperaldosteronism; and (5) release of Adiuretin (ADH), also called arginin vasopressin (AVP), and decrease in levels of Atrial Natriuretic Peptide.

Initially, as long as cirrhosis is compensated and patients don't develop any hydro-electrolytic imbalance, the retained fluid volume suppresses renal reuptake of sodium and water and resets fluid balance, thus leading, together with the augmented cardiac output and cathecolamine incretion, to a general increase in arterial volemia. However, as the disease progresses the effective arterial blood volume isn't maintained any longer by the aforementioned mechanisms and ascites occurs as a consequence of the "vicious circle" due to the continuous retention of water and sodium by the kidneys[7,8].

Due to the above cited mechanisms, the decompensated cirrhotic patient, although the marked hydrosaline reuptake, presents hypovolemia, periferic arterial vasodilatation and tachycardia.

In the earlier phases of ascites onset, the RAAS and the SNS are not activated, hence the cause of sodium retention in this period is still unclear. Afterward, the two systems increase the release of mediators thus leading to further reduction in urine sodium excretion[7].

Finally, hyperincretion of ADH occurs and this explains the late onset of hyponatremia in decompensated cirrhotic patients. In fact, ADH is less sensitive than the SNS and RAAS to changes in the effective volemia[9].

Finally, in this setting, hepato-renal syndrome (HRS) occurs as extreme consequence of such imbalances (see below). The mechanisms leading to ascites occurrence are described in Figure 1.

RENAL ALTERATIONS IN CIRRHOSIS

As above specified, activation of RAAS has a key role in the occurrence of hydroelectrolytic imbalances in cirrhotics. Such activation, as well that of SNS and the hyperincretion of ADH, is a homeostatic response aimed at maintaining blood pressure at normal levels in cirrhotic patients with ascites. In fact, the infusion of selective antagonists of angiotensin II or antidiuretic hormone (V1 antagonists) to experimental animals or ascitic patients leads to a profound hypotensive response secondary to a decrease in peripheral vascular resistance[10]. Among the main stimuli leading to the activation of these systems, arterial vasodilatation and secondary arterial hypotension play a pivotal role.

Other system implied in the pathogenesis of renal sodium retention is the SNS, which increases sodium reabsorption in the proximal tubule, loop of Henle, and distal tubule[11]. As decompensated liver diseases progresses, patients develop a decreased renal ability to excrete free water. This water dilutes the interior milieu and produces hyponatremia and hypo-osmolality. Water retention and dilutional hyponatremia develop months after the onset of sodium retention and ascites and are secondary to non-osmotic hypersecretion of AVP from the neurohypophysis in response to the reduced effective intravascular volume in cirrhosis[12,13]. Higher levels of AVP are responsible for water reabsorption in the distal collecting duct of the kidney. Water retention in patients with dilutional hyponatremia is a part of the positive fluid balance and contributes to the occurrence of ascites[12].

Interestingly, the renal synthesis of prostaglandin E2 is increased in cirrhotics to counteract the water-retaining effect of AVP and hence Non Steroid Anti-Inflammatory Drugs may worsen the renal excretion of solute-free water in these patients[14,15].

As previously stated, the clinical consequence of solute-free water excretion impairment is the development of hyponatremia. This type of hyponatremia is referred to as *dilutional hyponatremia* because it occurs in the setting of increased total body water and dilution of extracellular fluid volume.

Hyponatremia in cirrhosis and ascites has gained attention as a strong prognostic marker, particularly in patients awaiting liver transplantation, given that several reports indicate that when serum sodium concentration if combined with the Model for End-Stage Liver Disease (MELD) score improves the prognostic accuracy of MELD in patients listed for orthotopic liver transplantation[16-18].

In most patients the degree of hyponatremia is mild and the condition is asyntomatic and needs no specific therapy.

Renal vasoconstriction is the renal functional abnormality that develops later in patients with cirrhosis and ascites[19,20].

The occurrence of renal vasoconstriction in patients with cirrhosis and ascites is clinically relevant for several reasons. First, a significant proportion of these patients have refractory ascites, as sodium and water excretion are markedly impaired. Second, it predisposes to the development of HRS[21].

An increased activity of vasoconstrictor factors (mainly plasma renin activity and norepinephrine) and reduced activity of renal vasodilator factors acting on the renal circulation play the most important role in the pathogenesis of HRS because renal vasoconstriction in cirrhosis occurs in the absence of morphologic changes in the kidney[22].

The pathogenesis of renal vasoconstriction in cirrhosis is also related to changes in systemic hemodynamics. The most accepted theory considers renal vasoconstriction as the consequence of the extreme underfilling of the systemic arterial circulation due to marked vasodilatation of the splanchnic circulation, which activates homeostatic vasoconstrictor systems, whose effect on the kidney vasculature cannot be counterbalanced by either renal or systemic vasodilators[23,24].

Progression of functional renal alterations paralleled with cirrhosis course is shown in Figure 2.

VASOPRESSIN RECEPTOR ANTAGONISTS

Due to the aforementioned circulatory dysfunctions and activation of neuro-humoral systems leading to sodium and water retention, there has been an increasing interest in research on drugs that may improve circulatory and renal function in cirrhotics with refractory ascites and/or hyponatremia. Among such drugs, many affords have been sustained in developing and testing selective antagonists of the V2-receptors of vasopressin, known as vaptans.

AVP is a neuropeptide hormone synthesized by two hypothalamic nuclei (supraoptic and paraventricular nuclei) and secreted by the posterior pituitary in response to an increase in plasma tonicity or decrease in plasma volume[25].

The actions of AVP are mediated by three receptor subtypes: V1a, V1b and V2, all of them being G protein-coupled receptors and classified by their location[26]. V1a receptors are present on vascular smooth muscle cells, myocardium, platelets, and hepatocytes, and mediate vasoconstriction, platelet aggregation, and glycogenolysis[25,27,28]. V1b receptors have little selective distribution in the central nervous system. V2 receptors are expressed in principal cells of the renal collecting duct system. As shown in Figure 3, they mobilize intracellular vesicles of aquaporin 2 to the apical plasma membrane of collecting duct cells causing an increase in the reabsorption of free water. AVP acts on V2 receptors on the basolateral surface of principal cells resulting in activation of adenylyl cyclase. This leads to protein kinase activation resulting in preformed cytoplasmic vesicles called aquaporins getting inserted into the luminal membrane. They span the luminal membrane and permit movement of water down an osmotic gradient. The water absorbed is returned to the systemic circulation across the basolateral membrane. When the effect of AVP has worn off, water channels are removed from the luminal membrane by endocytosis, aggregate within clathrin-coated pits, and are returned to

Figure 2 Progression of the alterations of renal functionality in cirrhotics. HRS: Hepato-renal syndrome.

Figure 3 Mechanism of action of vaptans. In physiologic conditions, binding of arginine vasopressin to its receptor, raises introcytoplasmatic levels of cyclic adenosine monophosphate, thus resulting in increased expression of aquaporin on the luminal membrane of principal cells in collecting duct. Therefore, free water move down gradient *via* aquaporin from ductal lumen to blood. Vaptans, by blocking binding of arginine vasopressin to its receptor, inhibit water reabsorption. cAMP: Cyclic adenosine mono-phosphate.

the cytoplasm[29].

Orally and intravenously active non-peptide vasopressin receptor antagonists are called vaptans. They cause aquaresis, that is, excretion of solute-free urine (Figure 3). They differ from the diuretics as they promote excretion of water without the loss of electrolytes and hence are categorized as aquaretics.

USE OF VAPTANS IN THE MANAGEMENT OF REFRACTORY ASCITES

Two phase-2 studies have recently tested satavaptan associated to diuretics finding a therapeutic benefit[30,31]. Such result was confirmed in the setting of ascites recurrence after large-volume paracentesis (LVP) in another phase-2 study[32]. Unfortunately phase-3 randomized, placebo-controlled studies found a non-superiority and a worse

safety profile of satavaptan in combination with diuretics in ascitic patients with even an increased morbidity and mortality for unknown reasons[33].

Therefore, both European and American guidelines suggest as first-line therapy of refractory ascites repeated LVPs + albumin (8 g/L of ascites removed) and Transjugular Intrahepatic Porto-Systemic Shunt as rescue therapy for patients with very frequent requirement of LVPs[34,35]. Main characteristics of vaptans recently tested in clinical trials are reported in Table 1.

USE OF VAPTANS IN THE MANAGEMENT OF DILUTIONAL HYPONATREMIA

Due to the aforementioned prognostic impact of hyponatremia in cirrhotic patients and the well-known association between low serum sodium and comorbidities (neu-

Table 1 Vaptans tested in trials

Name	Receptor selectivity	Administration	Dose (mg)	Half-life (h)
Conivaptan	$V_{1a}R/V_2R$	Oral, IV	40-80	31-78
Tolvaptan	V_2R	Oral	15-60	6-8
Lixivaptan	V_2R	Oral	50-400	7-10
Satavaptan	V_2R	Oral	5-25	14-17
Mozavaptan	V_2R	Oral	30-60	-

IV: Intravenous.

rological complications, above all), in the last years many efforts in order to define an effective and safe therapeutic algorithm for dilutional hyponatremia have been made.

The main therapy of hyponatremia, consisting of increasing solute-free water excretion, has recently been implemented with the introduction of vaptans[56,37]. A number of evidences show that a short-therapy with vaptans (1 wk to 1 mo) ameliorates solute-free water excretion and leads to the increase in serum sodium levels in 45%-82% of patients without particular side effects on renal function, urine sodium, circulatory function, and activity of RAAS[38-41].

Thirst is the main complication related to vaptans. Other possible consequences of vaptan use in cirrhotics are hypernatremia, dehydration, kidney failure, and osmotic demyelination syndrome due to an unregulated increase in serum sodium levels. On the other hand, these concerns found little confirm in the aforementioned studies and their low frequency makes such considerations more theoretical assumptions than real problems[38-41]. Nevertheless, in light of these reported complications, therapy with vaptans should always be started under medical control with an "in-hospital" regime and serum sodium shouldn't increase of more than 8-10 mmol/L per day[34]. Furthermore, vaptan therapy should be avoided in individuals affected by encephalopathy or who cannot guarantee an appropriate uptake of water due to the risk of dehydration and hypernatremia. The metabolism of these drugs is on charge of hepatic CYP3A enzymes; therefore, an unexpected increase in hematic vaptan levels could be due to drugs or compounds known as strong inhibitors of CYP3A such as ketoconazole, grapefruit juice, and clarithromycin. On the other hand, inducers of the CYP3A system, such as rifampicin, barbiturates, and phenytoin, may lead to a severe impairment of vaptan efficacy.

Tolvaptan was approved in the United States and Europe for the treatment of severe hypervolemic hyponatremia (< 125 mmol/L) due to SIADH, while for other conditions such as cirrhosis, ascites, and heart failure, only the American Food and Drug Administration (FDA) had licensed the drug.

Tolvaptan should be started with 15 mg/d and titrated progressively to 30 and 60 mg/d, if needed, following the serum sodium concentration. In the above reported studies, concerns raised only for some reported cases of gastrointestinal bleeding. Except for the aforementioned

event, the safety profile of the drug resulted acceptable but clinicians should be aware that robust long-term data are lacking. However, in a recent placebo-controlled and open-label extension study of chronically administered tolvaptan in patients with autosomal dominant polycystic kidney disease, three cases of serious liver injury attributed to tolvaptan were observed[42]. Therefore, in a recently published safety announcement, FDA has forbidden the use of tolvaptan in patients with underlying liver disease, including cirrhosis, because the ability to recover may be impaired[43].

Conivaptan has also been licensed by FDA for the short term (5 d) intravenous treatment of hypervolemic hyponatremia but in cirrhosis data form randomized trials are lacking.

Given the narrow therapeutic window of these drugs, current practical guidelines state that management of symptomatic hyponatremia relies on infusion of saline and removal of the underlying etiologic mechanism (usually due to diuretic therapy)[34,35]. Vaptan therapy should be reserved to cases of severe hypervolemic hyponatremia (< 125 mmol/L) and should be introduced under careful medical monitoring in hospital regime. Patients may be discharged when sodium levels' stabilization is reached and no further adjustments of vaptan dose are needed. The proper length of therapy with vaptans is still unclear and concerns raise for long-term courses (> 1 mo)[34].

CONCLUSION

Vaptans represent a modern and promising therapeutic tool in the management of hydroelectrolytic imbalances in cirrhotic patients. Their safety profile and efficacy need further validation by randomized controlled trials.

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WJH 6th Anniversary Special Issues (7): Nonalcoholic fatty liver disease

Pathogenesis and therapeutic approaches for non-alcoholic fatty liver disease

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Core tip: In this review, we summarize the pathogenesis underlying the progression of hepatic steatosis to steatohepatitis and cirrhosis. We also discuss established drugs that are already being used to treat non-alcoholic fatty liver disease, in addition to newly discovered agents, with respect to their mechanisms of drug action, focusing mainly on hepatic insulin resistance. As well, we review clinical data that demonstrate the efficacy of these drugs, together with improvements in biochemical or histological parameters. Furthermore, we introduced future treatment option for non-alcoholic fatty liver disease.

Yoon HJ, Cha BS. Pathogenesis and therapeutic approaches for non-alcoholic fatty liver disease. *World J Hepatol* 2014; 6(11): 800-811 Available from: URL: http://www.wjgnet.com/1948-5182/full/v6/i11/800.htm DOI: http://dx.doi.org/10.4254/wjh.v6.i11.800

Abstract

Non-alcoholic fatty liver disease affects approximately one-third of the population worldwide, and its incidence continues to increase with the increasing prevalence of other metabolic disorders such as type 2 diabetes. As non-alcoholic fatty liver disease can progress to liver cirrhosis, its treatment is attracting greater attention. The pathogenesis of non-alcoholic fatty liver disease is closely associated with insulin resistance and dyslipidemia, especially hypertriglyceridemia. Increased serum levels of free fatty acid and glucose can cause oxidative stress in the liver and peripheral tissue, leading to ectopic fat accumulation, especially in the liver. In this review, we summarize the mechanism underlying the progression of hepatic steatosis to steatohepatitis and cirrhosis. We also discuss established drugs that are already being used to treat non-alcoholic fatty liver disease, in addition to newly discovered agents, with respect to their mechanisms of drug action, focusing mainly on hepatic insulin resistance. As well, we review clinical data that demonstrate the efficacy of these drugs, together with improvements in biochemical or histological parameters.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), the accumulation of lipid within hepatocytes, is a common disease[1]. The worldwide prevalence of NAFLD is estimated to be 20%-30%[2], although increasing to 57%-74% among obese patients[3]. NAFLD refers to a wide spectrum of fatty degenerative disorders of the liver in the absence of alcohol intake, ranging from simple steatosis to steatohepatitis and cirrhosis[4]. Nonalcoholic steatohepatitis (NASH) is histologically characterized by inflammatory cell recruitment. NASH is a significant risk factor for hepatic cirrhosis, compared with simple steatosis[5], and 4%-27% of cases of NASH progress to hepatocellular carcinoma after the development of cirrhosis[6]. In one study, NAFLD was pres-

ent in 75% of obese [body mass index (BMI) ≥ 30 kg/m²] patients, 16% of non-obese patients, and 34%-74% of patients with type 2 diabetes[7]. Another study reported diagnoses of fatty liver in 39% of obese (BMI ≥ 30 kg/m²) patients, 41% of patients with known type 2 diabetes, and 32% of patients with dyslipidemia[8]. Patients with NAFLD are not only insulin resistant, but also tend to present with alterations in plasma triglyceride levels[9]. NAFLD is strongly associated with metabolic syndrome, especially insulin resistance, central obesity, and dyslipidemia. Therefore, NAFLD is regarded as a difficult to treat component of metabolic syndrome[10]. In this review, we investigate the mechanisms of hepatic fat accumulation, focusing on the role of insulin resistance therein, and review current therapeutic options and new candidate drugs for the treatment of NAFLD.

PATHOGENESIS

Insulin resistance - free fatty acid flux and hyperinsulinemia

Hepatic steatosis is caused by an imbalance in triglyceride movement through the liver cell. Triglyceride is composed of free fatty acid (FFA) and glycerol. Total FFA is derived from three sources, the diet (15%), *de novo* synthesis (26%), and circulating FFA (56%)[11]. A high-fat diet is known to lead to the development of hepatic steatosis. However, estimates suggest that approximately 60% of liver fat is derived from circulating nonesterified fatty acids (NEFAs) in individuals who eat a normal fat-containing diet[11]. Obesity is associated with insulin resistance and an elevated leptin level. In particular, increased visceral fat correlates with peripheral and hepatic insulin resistance[12,13]. Insulin resistance in skeletal muscle and adipose tissue results in increased levels of NEFAs through increased lipid oxidation in adipose tissue (Figure 1). Accordingly, NEFA flux plays an important role in hepatic fat accumulation[14]. An increase in hepatocellular diacylglycerol is associated with decreased tyrosine phosphorylation of insulin receptor substrate 2 (IRS-2)[15,16]. In turn, the decreased activity of IRS-2 and PI3K leads to increased hepatic glucose production[17]. Hyperinsulinemia also arises in response to insulin resistance in adipose tissue, leading not only to downregulation of IRS-2 in the liver, but also to a continued increase in the level of sterol regulatory element binding protein-1c (SREBP-1c) *via* the insulin signaling pathway involving AKT2, liver X receptor (LXR) and mammalian target of rapamycin[18,19]. Elevated levels of SREBP-1c up-regulate lipogenic gene expression, increase fatty acid synthesis, and accelerate hepatic fat accumulation[20]. Additionally, overexpression of SREBP-1c represses IRS-2 expression[21]. Glucose-stimulated lipogenesis is mediated by carbohydrate-responsive element-binding protein (ChREBP) in the liver. Like SREBP-1c, ChREBP increases lipogenesis by inducing lipogenic gene expression during consumption of a diet high in carbohydrates[22,23].

Endoplasmic reticulum stress

The endoplasmic reticulum (ER) is an intracellular organ-

elle that plays an important role in the synthesis, folding, and trafficking of proteins. Cellular nutrient status and energy condition highly influence the function of the ER, and dysfunction in the ER causes accumulation of unfolded proteins therein, triggering an unfolded protein response (UPR)[24]. Under stress, such as hypoxia, inflammation and energy excess, UPR is characterized by adaptive cellular processes of increased degradation of proteins and translational arrest of protein synthesis to restore normal function of the ER. As well, UPR mediates metabolic and immune responses that aggravate insulin resistance[25-27]. Both PKR-like kinase and the α-subunit of translation initiation factor 2 (eIF2α), well-known ER stress markers, are increased in hepatocytes of ob/ob mice, compared with control mice[26]. Obesity causes ER stress that leads to suppression of insulin signaling through serine phosphorylation of insulin receptor substrate-1 (IRS-1) and activation of the c-Jun N-terminal kinase (JNK) pathway[26]. Among subjects with metabolic syndrome, those with NASH showed higher levels phosphorylated JNK protein, compared to subjects with simple hepatic steatosis. Furthermore, subjects with NASH did not generate spliced manipulation of X-box–binding protein-1 (sXBP-1), which is a key regulator in ER stress in relation to insulin action[24,26]. Additionally, weight reduction in obese subjects has been shown to induce improvement in ER stress *via* suppression of phosphorylated JNK and eIF2α in adipose tissue and the liver[28].

Role of oxidative stress - mitochondrial dysfunction

The two-hit hypothesis is a key concept of NAFLD pathogenesis. In fatty livers, simple hepatic steatosis (first hit) sensitizes the liver to inflammatory cytokines or oxidative stress (second hit), leading to development of steatohepatitis[29]. Oxidative stress is resulted from a serious imbalance between the limited antioxidant defenses and excessive formation of reactive species such as reactive oxygen species (ROS) or reactive nitrogen species (RNS)[30]. ROS is an integrated term that describes a variety of species of free radicals derived from molecular oxygen, such as superoxide, hydrogen peroxide, and hydroxyl[31]. In cells, mitochondria are a major source of ROS generation. The important factor modulating mitochondrial ROS generation is the redox state of the respiratory chain[32,33]. FFAs are metabolized *via* the mitochondrial β-oxidation pathway and the tricarboxylic acid (TCA) cycle, which generates citrate that in turn inhibits glycolysis. As a result, glucose oxidation and glucose uptake *via* glucose transporter type 4 (GLUT4) in skeletal muscle are reduced[34,35]. To compensate for the excessive fat storage in the liver, increased hepatic FFA uptake stimulates hepatic oxidation of fatty acids in obese individuals. Mitochondrial FFA oxidation is maintained until mitochondrial respiration becomes severely impaired[36,37]. However, accelerated β-oxidation not only causes excessive electron flux in the electron transport chain, but also leads to increased production of ROS, and can lead to mitochondrial dysfunction[38]. Excessive ROS production by mitochondria can lead to oxidative damage to

① Diet
② Exercise
③ Pioglitazone
④ Metformin
⑤ Fenofibrate
⑥ Vitamin E
⑦ Pentoxifylline
⑧ Ezetimibe
⑨ Cilostazol
⑩ MUFA

Figure 1 Mechanism of hepatic insulin resistance and the key pathway of drug action. Delivery of FFAs to the liver and skeletal muscle is increased in insulin resistance conditions, and these are metabolized *via* mitochondrial β-oxidation. Consequently, hyperglycemia and increased hepatic FFA uptake reduce glucose uptake and oxidation in skeletal muscle. Diet and exercise are the main treatment strategies for this pathogenesis; insulin sensitizers and MUFA may contribute to reducing peripheral insulin resistance. Pioglitazone and fenofibrate act on β-oxidation of mitochondria and reduce hepatic steatosis. Accelerated β-oxidation also causes increased production of ROS. Vitamin E can reduce oxidative stress. Adipose tissue inflammation of the liver leads to inflammatory activation of hepatic Kupffer cells via classic response (M1) and produce inflammatory cytokines. This is also associated with decreased adiponectin levels and promotes hepatic steatohepatitis. Pentoxifylline inhibits TNF-α and alleviates steatohepatitis. Hyperglycemia caused by insulin resistance up-regulates lipogenic gene expression, such as SREBP-1c and ChREBP, and induces lipogenesis in hepatocytes. Cilostasol may inhibit SREBP-1c. FFA: Free fatty acid; TG: Triglyceride; CPT- I : Carnitine palmitoyltransferase- I ; ACC: Acetyl-CoA carboxylase; ATGL: Adipose triglyceride lipase; ChREBP: Carbohydrate responsive element binding protein; SREBP-1c: Sterol regulatory element binding protein-1c; TCA: Tricarboxylic acid; ROS: Reactive oxygen species; IRS: Insulin receptor substrate; DAG: Diacylglycerol; G-6-P: Glucose 6-phosphate; TNF-α: Tumor necrosis factor- α; MUFA: Monosaturated fatty acids; M1: Kupffer cells activated *via* classic pathway.

the mitochondrial membrane and DNA and can impair mitochondrial metabolic functions[33]. The increase in hepatic lipogenesis in NASH results in increased production of malonyl-CoA. Inhibition of carnitine palmitoyl-transferase-I (CPT-1) by malonyl-CoA leads to decreased entry of long chain fatty acid into the mitochondria, and causes reduced β-oxidation and enhanced triglyceride accumulation in the liver[38-40]. The nuclear receptor peroxisome proliferator-activated receptor α (PPAR-α) plays an important role in the transcriptional control of many enzymes involved in mitochondrial fatty acid β-oxidation. Peroxisome proliferator-activated receptor-gamma co-activator (PGC) -1α cooperates with PPAR-α and regulates genes that encode mitochondrial fatty acid oxidation enzymes, such as CPT-1 and medium chain acyl-CoA dehydrogenase[40]. Previously, a PPAR-α-deficient mouse model showed a lack of hepatic peroxisome proliferation and dyslipidemia with obesity and hepatic steatosis[41].

Inflammation and adipokines

Overall obesity is correlated with NAFLD, and accumulation of intra-abdominal fat in particular is believed to play an important role in the development of insulin resistance[12,13]. Meanwhile, hepatic fat accumulation is associated with insulin resistance independent of intra-abdominal fat accumulation and overall obesity. Even in normal weight subjects, hepatic steatosis has been shown to be related to various parameters of insulin resistance, such as basal glucose level or serum FFA level[42]. In addition to being a major organ of triglyceride deposition, adipose tissue acts an endocrine organ that secretes several hormones[43]. Adipocytes secrete adiponectin and leptin, in addition to the other adipokines, such as retinol-binding protein, tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), and plasminogen activator inhibitor-1[43]. Adiponectin stimulates phosphorylation of AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC) in the liver and muscles, thereby, increasing glucose utilization and fatty-acid oxidation[44]. In a previous study, serum adiponectin levels decreased with an increases in obesity, in particular increases in intra-abdominal fat mass[45,46]. In another study, adiponectin knockout mice fed a high-fat diet exhibited increased incidences of obesity, hyperinsulinemia, and steatohepatitis. These experimental data indicate that adiponectin may play a key protective role against the progression of NASH[47]. Reportedly, adipose tissue in obese individuals stimulates a shift in macrophage activation from the

Table 1 Treatment outcomes of variable regimens

Study	Treatment group	Control group	No.	Study design	Duration (mo)	Histology	Liver enzymes	US
Life style modification								
Huang *et al*[66]	Diet	-	12	Open label	12	Improved	-	-
Ueno *et al*[65]	Diet/Exercise	Control	15	Open label	3	Improved	Improved	-
Pioglitazone (insulin sensitizer)								
Promrat *et al*[71]	Pioglitazone	-	18	Open label	12	Improved	Improved	-
Belfort *et al*[72]	Pioglitazone and Diet	Placebo	55	RCT	6	Improved	Improved	-
Aithal *et al*[73]	Pioglitazone and diet/Exercise	Placebo	74	RCT	12	Improved	Improved	-
Sanyal *et al*[74]	Pioglitazone	Placebo	163	RCT	24	Improved	Improved	-
Metformin (insulin sensitizer)								
Garinis *et al*[100]	Metformin and Diet	Diet	50	RCT	6	-	-	Improved
Haukeland *et al*[103]	Metformin	Placebo	48	RCT	6	-	-	-
Uygun *et al*[101]	Metformin and Diet	Control	50	Open label	6	-	-	Improved
Bugianes *et al*[99]	Metformin	Diet	53	RCT	12	Improved	Improved	-
Bugianes *et al*[99]	Metformin	Vitamin E	57	RCT	12	-	Improved	-
Vitamin E (antioxidant)								
Bugianesi *et al*[99]	Vitemin E	Diet	55	RCT	12	-	-	-
Sanyal *et al*[74]	Vitamin E	Placebo	167	RCT	24	Improved	Improved	
Vajro *et al*[109]	Vitamin E	Diet	25	RCT	6	-	-	-
Other drugs								
Sanjay *et al*[112]	Pentoxifylline	-	18	Open label	6	-	Improved	-
Yoneda *et al*[127]	Ezetimibe	-	10	Open label	6	Improved	Improved	
Vasilios *et al*[118]	Statin	Control	437	Open label	36	-	Improved	-
Lindor *et al*[130]	UDCA	Placebo	166	RCT	48	-	-	-
Capani *et al*[138]	PUFA	Control	42	RCT	12	-	Improved	Improved

No.: Number; US: Ultrasonography; RCT: Randomized controlled trial.

alternative response (M2) to the classic response (M1), and these classically activated macrophages secrete a variety of inflammatory cytokines, such as TNF-α, IL-6, and NO[48]. Additionally, studies showed that inflammatory activation of hepatic Kupffer cells in ob/ob mice promotes hepatotoxicity, resulting in hepatic insulin resistance and steatohepatitis[49,50]. Thus, increases in TNF-α and IL-6 in obese subjects may play an important role in insulin resistance and hepatic steatosis[51,52].

Gut-microbial alternation and TLRs stimulation

As mentioned above, obesity is often associated with NASH and systemic inflammation characterized increases in inflammatory cytokine levels. Obesity also can cause increased intestinal mucosa permeability and endotoxin levels in portal circulation that can contribute to hepatocellular damage[53,54]. Kupffer cells in the liver play a key role in clearing endotoxin and are activated through Toll like receptor 2,3,4 and 9 signaling in the presence of endotoxin. In particular, activation of Toll like receptor4 (TLR4) is reportedly associated with stimulation of lipopolysaccharide (LPS)[55-57]. Previously, animal model studies showed that TLRs 2, 4 and 9 may contribute to the pathogenesis of NAFLD[55,58]. Activated Kupffer cells induce expression of pro-inflammatory cytokines, such TNF-α, IL-6, IL-18 and IL-12 as well as anti-inflammatory cytokines[59]. TLRs including TLRs 2,4 and 9 are activated *via* a MyD88 dependent pathway. This pathway consists of the activation of serine kinase IL-1R-associated kinase and TBF-receptor-associated factor 6 and is involved in the activation of the transcription factor NF-κB, which is related to inflammatory cytokine production[60].

TREATMENT

Life style modification - diet and exercise

Weight loss due to diet and exercise has been demonstrated to alleviate hepatic steatosis[61]. Body weight reduction and exercise are important independent factors for improvement of hepatic steatosis[62]. In obese women, hepatic fat content measured by magnetic resonance imaging was shown to decrease in response to weight loss interventions[63]. Several studies have shown a significant reduction in alanine transaminase (ALT) levels and improvement in biochemical markers following intervention with a calorie-restricted diet combined with exercise[63,64]. A few studies have also shown histologic improvement with increased exercise and weight reduction[65,66] (Table 1). Exercise improves insulin sensitivity in skeletal muscle *via* GLUT4 expression and increases glucose utilization. Thus, exercise decreases levels of serum glucose and insulin[67]. An improvement in hyperinsulinemia can result in decreased liver fat mass, because hyperinsulinemia stimulates hepatic steatosis *via* the SREBP-1c pathway[19]. In particular, NAFLD patients with metabolic syndrome show a great improvement in hepatic steatosis after weight loss[68].

Insulin sensitizer-thiazolidinedione, metformin

Thiazolidinedione: Thiazolidinediones (TZDs) are insulin-sensitizing agents that have been shown to improve not only hepatic steatosis, but also whole body insulin resistance[69]. Improvements in insulin resistance and histologic and biochemical parameters were reported with TZD treatment[70-74]. Rosiglitazone is one TZD and

is associated with an increased risk of myocardial infarction and cardiovascular death[75]. Meanwhile, pioglitazone is regarded as safe in regards to cardiovascular outcomes and is not associated with increased cardiovascular risk[76,77]. In patients with type 2 diabetes, pioglitazone has been recommended for the treatment of steatohepatitis proven by liver biopsy; however, its role in non-diabetic patients has not been established. The American Association for the Study of Liver Disease (AASLD) introduced pioglitazone as a first-line treatment of NAFLD in patients with type 2 diabetes[78]. TZDs increase glucose utilization of peripheral tissue and improve whole body insulin sensitivity as measured by the hyperinsulinemic euglycemic clamp technique, in patients with type 2 diabetes. Moreover, serum adiponectin levels increase and serum insulin levels decreases after treatment with pioglitazone[79,80]. An increase in serum adiponectin contributes to alleviation of hepatic steatosis and improves hepatic and peripheral insulin resistance[79]. As mentioned above, adiponectin increases lipid oxidation of FFA by ACC phosphorylation in the liver[44], and promotes the activation of anti-inflammatory M2 macrophages rather than M1 macrophages[81]. Obesity is closely related to an increase in NAFLD risk[82]. Increased levels of inflammatory adipose tissue macrophages (ATMs) and their secreted cytokines in a mouse model were shown to be related to systemic insulin resistance, which is associated with NAFLD development[15,83]. According to previous studies, ATMs are increased in obese subjects[84], and pioglitazone treatment results in not only a decrease in ATM content, but also in the inflammatory markers, TNF-α, IL-6, and inducible nitric oxide synthase[85,86]. TZDs also promote the alternative activation of monocytes into macrophages with anti-inflammatory properties, as opposed to the pro-inflammatory phenotype[87]. Although the pathogenesis of NAFLD development is closely related to obesity, the distribution of fat is more important than overall obesity. Excessive visceral fat accumulation plays an important role in the development of insulin resistance and NAFLD by acting as a source of FFA[12]. Pioglitazone is strongly associated with fat redistribution, increases in subcutaneous fat area decreases in visceral fat area (visceral to subcutaneous fat ratio)[88]. Another study showed that the ratio of visceral fat thickness to subcutaneous fat thickness decreases after pioglitazone treatment and is correlated with a change in high sensitivity C-reactive protein levels[89]. TZD treatment results revealed a decrease in serum FFA levels, which in turn reduced FFA supply to the liver and led to a decrease in hepatic triglyceride content[90]. Recent studies have focused on the role of sirtuin-6 (SIRT-6) in the glucose and lipid metabolism associated with TZDs. TZD treatment reduced hepatic fat accumulation and increased expression of SIRT-6 and PGC1-α in rat livers[91]. Also, liver-specific SIRT-6 knockout mice exhibited fatty liver formation[92], leading to NASH[93].

Metformin: Metformin improves insulin resistance and hyperinsulinemia by increasing peripheral glucose uptake and decreasing hepatic gluconeogenesis[94]. Metformin activates AMP kinase *via* a LKB-1 dependent mechanism in skeletal muscle. Also it can activate AMPK by stimulating AMP accumulation in hepatocytes. The increase in AMP interferes with glucagon action and decreases cAMP levels, leading to decreased production of hepatic glucose[95,96]. Activation of AMPK results in decreased hepatic triglyceride synthesis and increased fatty acid oxidation[97], as well as attenuated hepatic steatosis due to decreased SREBP-1c activity[98]. A randomized controlled trial showed that subjects treated with metformin exhibit significant improvement in ALT levels, compared with those who were on a restricted diet or were treated with vitamin E, as well as improvements in histology after a 12 mo of treatment[99,100]. Many studies have shown that metformin treatment normalizes transaminase levels and decreases hepatic steatosis as determined by follow-up ultrasound; nevertheless, histologic data remain limited[100-103]. As NASH is closely associated with development of HCC and liver fibrosis, metformin may be limited in the reduction of these severe outcomes, including mortality[104].

Antioxidant - vitamin E (α-tocopherol), pentoxifylline

As mentioned above, oxidative stress contributes to the progression of NASH from simple hepatic steatosis. A recent study reported that subjects who were treated with vitamin E (α-tocopherol) showed improvement in hepatic steatosis and serum aminotransferase levels compared to a placebo group[74]. Vitamin E (α-tocopherol) has been used to treat non-diabetic NASH patients diagnosed by liver biopsy[78]. Meta-analyses of vitamin E have revealed an increase in all-cause mortality with high dose ($\geqslant$ 400 IU/d) vitamin E supplement use, especially in subjects with chronic disease or at high risk for cardiovascular disease events, such as type 2 diabetes. However, these results are uncertain in healthy subjects[105,106]. Two pilot studies reported improved ALT levels with vitamin E treatment[107,108]. However, two randomized controlled trials failed to show the efficacy of vitamin E treatment in NAFLD[109,110]. Pentoxifylline, a TNF-α inhibitor, has also been considered for the treatment of hepatic steatosis, since it plays an important role in the progression of simple hepatic steatosis to steatohepatitis. In previous studies, administration of pentoxifylline generated improvements in biochemical markers, such as aminotransferase and Homa-IR, in patients with NASH[111,112]. Nevertheless, further study is needed to prove the efficacy of pentoxifylline with respect to histologic improvement of NAFLD.

Lipid-lowering agents - fibrates, ezetimibe and statins

Hypertriglyceridemia is a major component of metabolic syndrome and is strongly associated with NAFLD. Increased FFA delivery to the liver causes accumulation of hepatic fat[9]. Many different lipid-lowering agents have been investigated for the treatment of NAFLD. Patients treated with gemfibrozil, one type of fibrate, showed decreased ALT levels, compared to the control group[113]. However, clofibrate did not show a beneficial effect on

NAFLD[114]. PPAR-α modulates not only FFA transport and β-oxidation to decrease triglyceride in hepatocytes, but also glucose and amino acid metabolism in liver and skeletal muscle. PPAR-α activation is involved in lipoprotein metabolism by increasing lipolysis, thus reducing the production of triglyceride-rich particles[115]. Fenofibrate increased levels of PPAR-α and decreased hepatic steatosis in an APOE2KI mouse model that represented diet-induced NASH[116]. A prospective study using atorvastatin reported significant reductions in serum transaminase level[117,118]. Atorvastatin induces hepatic low-density lipoprotein receptor-related protein 1 (LRP-1) that plays an important role in clearance of circulating triglyceride in the liver[119]. In disposal of chylomicron in hepatocytes, interaction of LRP-1 receptors and apolipoprotein E (ApoE) play important roles[120]. Thus, ApoE-deficient mice showed development of hepatic steatosis even when they were fed a normal chow-diet. Accordingly, ApoE may play a key role in intracellular metabolism and control of VLDL production by hepatocytes[121]. Statins are very important drugs to treat dyslipidemia in subjects with both insulin resistance and NAFLD. However, there is continued concern about the use of statins in subjects with established liver disease. According to several randomized controlled studies and retrospective studies, statin rarely induces serious liver injury[122-125]. Ezetimibe, a potent inhibitor of cholesterol absorption, has been reported to improve hepatic steatosis in obese Zucker fatty rats[126]. In a randomized controlled study, six months of treatment with ezetimibe led to improvements in serum ALT levels and histologic observations[127,128].

Ursodeoxycholic acid

Ursodeoxycholic acid (UDCA) is widely used in subjects with abnormal liver function. Several studies have investigated the efficacy of UDCA as a treatment drug of NAFLD, reporting that UDCA treatment attenuated hepatic steatosis, including histologic improvement[114,129,130]. However, in a placebo controlled, randomized control trial, UDCA exhibited limited efficacy in histologic improvement in subjects with NASH and improvements in liver enzyme did not differ in the UDCA group, compared to the placebo group[130]. Accordingly, AASLD does not recommend UDCA for the treatment of NAFLD[78].

Other treatment options - future candidates

Cilostazol: SREBP-1c is a key regulator of lipogenic gene expression in hepatocytes. Recent data have shown that cilostazol, a selective type Ⅲ phosphodiesterase inhibitor, inhibits SREBP-1c expression *via* the suppression of LXR and Sp1 activity[131]. Cilostazol also decreases serum triglyceride levels by increasing lipoprotein lipase (LPL) activity in STZ-induced diabetic rats[132]. Also, experimental data show that cilostazol stimulates LRP1 promoter activity in hepatocytes, leading to increased hepatic LRP1 expression[133]. In a study that used two experimental NAFLD models, both high-fat/high-calorie (HF/HC) diet mice and the choline-deficient/L-amino acid-defined (CDAA) diet mice, cilostazol generated improvement in

hepatic steatosis in both mice models[134]. Cilostazol exhibits the potential for improvement of hepatic steatosis, and further data on its role in NAFLD are needed.

Polyunsaturated fatty acids and monounsaturated fatty acids: Polyunsaturated fatty acids (PUFAs) are found primarily in safflower, corn, soybean, cottonseed, sesame, and sunflower oils. Omega-3 fatty acids are representative of PUFA. A marked increase in long-chain PUFA n-6/n-3 ratio is observed in NAFLD patients and is associated with increased production of pro-inflammatory eicosanoids and dysregulation of liver and adipose tissue function[135]. PPAR-α activity is impaired in conditions in which levels of circulating n-3 PUFA are decreased and the n-6/n-3 fatty acid ratio is increased[136,137]. Treatment with n-3 PUFA was shown to improve biochemical parameters and alleviated hepatic steatosis by ultrasound follow-up[138,139]. Monounsaturated fatty acids (MUFAs) are comprised in olive oil. In a rat model, supplementation with MUFA resulted in improved insulin sensitivity, compared to rats fed a saturated fatty acid (SFA) diet. Additionally, GLUT4 translocation in skeletal muscle was decreased in rats fed a SFA diet, but not in those fed a MUFA diet. Increased GLUT4 translocation is related to an improvement in insulin sensitivity[140]. In obese rats, MUFA diet attenuated hepatic steatosis and altered hepatic fatty acid levels[141]. The beneficial effects of dietary MUFA in NAFLD patients should be investigated.

GLP-1 analogue: Exenatide is the synthetic form of exendin4 and it stimulates endogenous insulin secretion, leading to decreases in blood glucose. In one animal study, treatment of exendin4 resulted in a decrease of hepatic fat content, as well as reduction of fatty acid synthesis, in the liver of ob/ob mice[142]. In patients with type 2 diabetes, an exenatide treatment group showed greater improvements in liver enzymes, attenuating hepatic steatosis, than the metformin treatment group. However, this study had limitations of a lack of histologic confirmation of the liver[143]. To prove the efficacy of glucagon like peptide-1 (GLP-1) analogue in treatment of NAFLD, randomized controlled trials over a longer period are required.

MK615: MK615 is extracted from Japanese apricots, and can suppress the production of inflammatory cytokines such as TNF-α and IL-6 by inactivating NF-κB[144,145]. MK615 is regarded as a hepatoprotective agent, as it has been shown that a MK615 treatment group exhibited greater decreases in liver enzyme levels, compared with control groups. In rat models, MK615 treatment mice showed more improved liver histology than control mice[146]. Thus, further studies are required to clarify the effects of MK615 in subjects with NAFLD.

CONCLUSION

NAFLD is a common disease that can progress to liver cirrhosis. Moreover, NAFLD is strongly associated with type 2 diabetes and insulin resistance. NAFLD is the

result of complex interactions among diet, metabolic components, adipose tissue inflammation, and mitochondrial dysfunction. The pathogenesis of hepatic steatosis has not yet been fully determined. In this review, we outlined previously known mechanisms of NAFLD, as well as introduced new mechanisms that have been recently discovered. Above all, we reviewed the mechanisms of drugs matched to the pathogenesis of NAFLD. Furthermore, we introduced future treatment option for NAFLD. TZDs play a key role in restoring insulin sensitivity and decreasing adipose tissue inflammation, generating histologic improvements in hepatic steatohepatitis. Pioglitazone can be used to treat NASH in patients with type 2 diabetes with biopsy-proven NAFLD; meanwhile, non-diabetic patients can be treated with vitamin E. Metformin is a well-known insulin sensitizer; however, further study is needed to prove histologic improvements in patients with NAFLD. Additionally, the cholesterol-lowering agent ezetimibe has also shown histologic improvements. Cilostazol acts on SREBP-1c and can improve dyslipidemia; however, further research is needed to clarify the relationship between NAFLD and cilostazol. Finally, there is an outstanding need for effective preventive and therapeutic regimens to overcome NAFLD.

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MINIREVIEWS

Perceptions of post-transplant recidivism in liver transplantation for alcoholic liver disease

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Abstract

Although alcoholic liver disease (ALD) is regarded as a common indication for liver transplantation (LT), debatable issues exist on the requirement for preceding alcoholic abstinence, appropriate indication criteria, predictive factors for alcoholic recidivism, and outcomes following living-donor LT. In most institutions, an abstinence period of six months before LT has been adopted as a mandatory selection criterion. Data indicating that pre-transplant abstinence is an associated predictive factor for alcoholic recidivism supports the reasoning behind this. However, conclusive evidence about the benefit of adopting an abstinence period is yet to be established. On the other hand, a limited number of reports available on living-donor LT experiences for ALD patients suggest that organ donations from relatives have no suppressive effect on alcoholic recidivism. Prevention of alcoholic recidivism has proved to be the most important treatment after LT based on the resultant inferior long-term outcome of patients. Further evaluations are still needed to establish strategies before and after LT for ALD.

Key words: Abstinence; Alcoholic liver disease; Liver transplantation; Six-month rule

Core tip: Prevention of alcoholic recidivism has proved to be the most important treatment after liver transplantation based on inferior long-term outcome of patients. Further evaluations, however, are still needed to establish strategies before and after liver transplantation with alcoholic liver diseases.

Kawaguchi Y, Sugawara Y, Akamatsu N, Kaneko J, Tanaka T, Tamura S, Aoki T, Sakamoto Y, Hasegawa K, Kokudo N. Perceptions of post-transplant recidivism in liver transplantation for alcoholic liver disease. *World J Hepatol* 2014; 6(11): 812-817 Available from: URL: http://www.wjgnet.com/1948-5182/full/v6/i11/812.htm DOI: http://dx.doi.org/10.4254/wjh.v6.i11.812

INTRODUCTION

Alcoholic liver disease (ALD) is regarded as a common indication for liver transplantation (LT), and accounts for approximately 40% of all primary transplants in Europe[1] and 25% in the United States[2]. One of the reasons making LT for ALD a complicated topic of issue is that alcohol abuse is the primary cause for end-stage liver disease development. Patients themselves are viewed as being responsible for their illness as compared to other diseases including cholestatic liver diseases and viral cir-

rhosis. Thus, controversy may exist over organ allocation to ALD patients in deceased-donor liver transplantation (DDLT). Organ allocation to patients with self-inflicted disease is less acceptable to society[3-5], and post-transplant alcoholic recidivism may raise questions on sharing organs as a public resource. By contrast, living-donor liver transplantation (LDLT), which remains the mainstream approach in Asia including Japan, does not conflict with the above-mentioned issues on organ allocation. However, requiring an abstinence period of at least six months (the so-called six-month rule[6]) to soften the controversy may also be debatable because the benefit of such pre-transplant abstinence remains unclear. Nevertheless, prevention of alcoholic recidivism is inevitably the most important factor to enhance medical benefits of LT and to gain more public acceptance as well. In the present article, we review the current status of LT for ALD mainly derived from DDLT cases, and focus on controversies involved in LDLT with the aim to explore the future direction of LT for ALD.

LT FOR ALD

Selection criteria

Selection criteria of LT for ALD, such as pre-transplant abstinence period, participation in rehabilitation program, and consultation with a psychiatrist, have been used in most institutions in addition to common criteria for other original diseases[7-13]. This is presumably because the criteria allow observations needed to determine the recovery odds from potential liver failure[7,14,15] and prevent post-transplant alcoholic recidivism[16-20]. In addition, there is a preponderance of evidence supporting that a pre-transplant abstinence period of six months has become a mandatory selection criterion[8,11-13,19-21], as its benefit was reported by Bird *et al*[6] in 1990. However, there are also reports indicating that an abstinence period of more than six months is not a significant predictive factor for alcoholic recidivism[22-24], along with those demonstrating that LT candidates with ALD barely survive for six months even with no alcohol intake[15,23]. A solid validation for requiring pre-transplant abstinence, as well as optimal duration of abstinence, if necessary, has yet to be established.

Alcoholic recidivism

Alcoholic recidivism has been considered to negatively impact postoperative compliance and long-term outcomes of recipients[21,24-30]. This perception may have encouraged LT professionals to evaluate predictive factors for alcoholic recidivism and therefore, to require specific criteria for ALD patients to prevent alcoholic recidivism in addition to commonly applied criteria. Rates and predictive factors of alcoholic recidivism are summarized according to the previous reports in Table 1[11,19-22,24,31,32]. The rates of alcoholic recidivism ranged widely from 10% to 42% as a result of inconsistent definitions on alcoholic recidivism and follow-up time. In fact, DiMartini *et al*[33] classified post-transplant alcohol consumption patterns into five categories based on time until relapse,

three of which are harmful to the patients: no alcohol use, infrequent/low level of consumption, early onset/moderate and decreased consumption, later onset/harmful level of consumption, and early onset/heavy/increasing consumption. According to this classification, 46% of patients developed alcohol recidivism, with harmful use of alcohol accounting for 19%. In addition to inconsistent definitions on alcoholic recidivism, the fact that its detection is mainly based on statements from patients and/or reports from relatives makes evaluation difficult[11,19-22,24,31,32,34,35]. Random conducting of blood alcohol tests is useful for surveillance of ALD patients[19] as indicated through the resulting reduced rate of pre-transplant recidivism. With respect to predictive factors for alcohol recidivism, the following factors have been indicated in previous reports: abstinence period, presence of psychiatric comorbidity, poor compliance, family history of alcoholism, high-risk alcoholism relapse score (4-6)[36], poor social support, presence of young children, female sex, age < 50 years. An abstinence period before LT has been demonstrated as the predictive factor in most[11,19-21,31], but not all[22,24,32], publications.

Patient outcomes

The long-term survival rates of patients who underwent LT for ALD are reportedly 82%-92% at one year, and 72%-83% at 5 years[1,11,21,37,38]. These results are comparable to those of patients including all etiologies from different parts of the world (79%-83% at one year and 67%-77% at five years)[28,37,39]. Alcohol recidivism has been reported to impair long-term outcome[24,26,27,29-31], presumably due to its negative influence on the recipients, including alcohol toxicity, poor compliance, development of post-transplantation malignancies and occurrence of cardiovascular diseases. Rates of graft loss due to alcoholic recidivism range between 0% and 50%[21,27,30,40,41], and significant association of ALD patients with increased development of post-transplantation malignancy and occurrence of cardiovascular diseases were suggested[1,42].

Concerns on LT for acute alcoholic hepatitis without an abstinence period

Alcoholic hepatitis is a distinct clinical syndrome associated with recent or ongoing alcohol consumption, and its severity leads to high mortality exceeding 50%[35,43-46]. Medical treatment including the use of corticosteroids and/or pentoxifylline reduces the mortality rate to approximately 20%-30%[43,47]. Non-responsive patients suffer high mortality, and thus LT for alcohol hepatitis has been proposed in select patients[35,47,48]. However, alcoholic hepatitis is a controversial indication, or even a contraindication, for LT in most institutions[49,50] due to the high potential for alcohol recidivism, and conceivably due to the lack of pre-transplant abstinence period. A recent prospective multicenter study showed clear improvement on the odds of survival among patients unresponsive to medical therapy and followed with LT for severe alcoholic hepatitis[35]. The six-month and two-year survival rates among LT patients were significantly higher among

Ref.	Year	Alcoholic recidivism	Predictive factors
Gish *et al*[32]	2001	20%	Poor compliance and personality disorder
Jauhar *et al*[22]	2004	15%	Family history of alcoholism
DiMartini *et al*[19]	2006	42%	Alcohol dependence and an abstinence period
De Gottardi *et al*[11]	2007	12%	HRAR high score (4-6), presence of psychiatric comorbidity, and an abstinence period (≤ 6 mo)
Pfitzmann *et al*[21]	2007	19%	An abstinence period (< 6 mo), poor social support, presence of young children, and a poor psychosomatic prognosis
Tandon *et al*[31]	2009	24%	Pre-transplant abstinence
Karim *et al*[20]	2010	10%	An abstinence period (< 6 mo), female sex, presence of psychiatric comorbidity, age < 50 yr
Egawa *et al*[24]	2014	23%	Presence of psychiatric comorbidity

Table 1 Predictive factors for alcoholic recidivism

HRAR: High-risk alcoholism relapse.

non-LT patients (six months: 77% ± 8% *vs* 23% ± 8%, *P* < 0.001; two years: 71% ± 9% *vs* 23% ± 8%, *P* < 0.001). The survival rate of patients who underwent LT was comparable to that of patients who responded to medical therapy (77% ± 8% *vs* 85% ± 4%). The overall recidivism rate with relapse was 12%, with no case of alcoholic relapse within the initial six-month follow-up period after LT. Similar survival rates were reported in a retrospective study comparing LT outcomes for patients with alcoholic hepatitis to those with alcoholic cirrhosis (one year: 93% *vs* 88%; two years: 91% *vs* 84%; five years: 80% *vs* 78%)[48]. However, both studies mentioned an observable difference in society's readiness towards transplants for ALD and other self-inflicted liver diseases, despite their comparable mortality. In fact, criticism from the public is not present in response to LT for patients with fulminant hepatic failure stemming from voluntary acetaminophen poisoning, nor intravenous-drug users with acute hepatitis B virus infection[35,48]. In order to gain public acceptance, some sensitive issues surrounding LT for alcoholic hepatitis need to be addressed even though the medical benefits of LT have been proposed for strictly selected patients.

CONSIDERATIONS ON LDLT FOR ALD

Although there are many reports on DDLT for patients with ALD, there are few concerning LDLT. This is most likely because ALD is not a major primary disease for LT in the regions where LDLT is common, and DDLT is not practical due to the shortage of deceased donors. For instance, ALD accounts for only 2% of all primary transplantations in Japan, where 98% of LT has been performed through LDLT according to the registry by the Japanese Liver Transplantation Society[37]. Nevertheless, ALD is an important indication for LT following an annual increase of ALD recipients in Japan[37]. There are only two published reports on LDLT for ALD patients; one is a single-center study from our own institution[13], and the other is a multi-center questionnaire-based study in Japan[24].

Single-center study
Although the number of patients with ALD was limited in our single-center study, the results indicated a relatively

low recidivism rate (8%) after LDLT for ALD patients selected based on a strict criteria that required the six-month rule, participation in Alcoholics Anonymous or equivalent rehabilitation program, consultation with a psychiatrist, and signed agreement declaring an intention of lifetime abstinence[13]. In addition, the study implied that pre-transplant abstinence was useful to observe possible recovery from liver failure as well as to identify patients who would not abstain from alcohol before and/or after LT. From this, we assumed that the role of abstinence before LDLT is to ensure positive effects on preventing post-transplant alcoholic recidivism even if results are not established and to recompense the potential risks the donor carried.

Multi-center study
In contrast, the rate of post-transplantation relapse in the multi-center study involving 38 institutions in Japan, with selection criteria for ALD patients determined at each institution, ranged from 7% to 95%[24]. The study noted the possibility that relatives who donated their organs, notwithstanding operation risks, may have allowed recipients' alcohol consumption after LT. In fact, recidivism rates of patients whose parents or siblings were donors ranged from 28% to 50%, slightly higher than those whose donors were spouses (13%) or relatives (23%). Considering the relatively high alcoholic relapse rate after LDLT, the study suggested that DDLT may be more suitable for patients with ALD.

Patient outcomes
The long-term survival rate of patients who underwent LDLT for ALD was comparable with that of DDLT[1,11,21,37,38]; 100% at one year and 91% at five years in the single-center study[13], and 81% at one year and 76% at five years from data in the registry of the Japanese Liver Transplantation Society[37]. Similar to DDLT[21,26,27,29,30], the long-term survival rate for relapsing patients was significantly lower than that for abstinent patients (one year: 100% *vs* 100%; three years: 95% *vs* 99%; five years: 90% *vs* 96%, *P* = 0.01)[24].

Public and ethical perspectives on LDLT for ALD
LDLT for ALD may seem to be generally accepted by

society from a public point of view because, unlike with DDLT, it does not conflict with organ allocation issues. Nevertheless, ethical issues remain. First, liver transplantation professionals are confronted with difficult situations caused by the dilemma between strong willingness displayed by the family to donate and compliance with pre-transplant abstinence rule. For instance, professionals working in most institutions feel obliged to inform patients who may have prospective living donors and their family members that the requirement for a six-month abstinence period is still applicable, even when some of the patients are not expected to survive more than six months. Second, recidivism is not readily accepted by society even if the organ is donated by a family member because LT is supported by national- and/or social- welfare systems in general. LDLT for ALD, inseparable from the public opinion, becomes a complicated topic that requires a viewpoint slightly different from DDLT for ALD when addressing their issues.

The extremely limited number of reports on LDLT for ALD led to difficulty in achieving consensus on optimal selection criteria for ALD patients as well as on strategies for preventing alcoholic recidivism after LT. To improve current status of LDLT for ALD and support liver transplantation professionals involved in the treatment for ALD, a significant increase in the number of reports on this topic are essential, not to mention a well-designed prospective study.

CONCLUSION

Alcoholic liver disease remains a commonly recognized indication for LT in Europe and the United States, with an increasing presence in Asia as well. ALD is a self-inflicted disease in which patients may possibly relapse to alcohol consumption after transplantation. These facts still raise questions on sharing organs as a public resource for DDLT. LDLT, unlike DDLT, may not necessarily link to organ allocation issues, but it is nonetheless inseparable from the public eye as an ethical standpoint. Considerable efforts to improve post-transplant outcome are required to recompense the potential risks to living donors.

Prevention of alcoholic recidivism is regarded as the most important post-transplant treatment because alcohol impairs long-term outcome of ALD patients. Although not conclusive, an abstinence period and presence of psychiatric comorbidity are potential predictive factors for post-transplantation recidivism. Organ donations from relatives do not suppress alcoholic recidivism as the recipient's alcohol consumption tends to be tolerated by the donors themselves. Incidentally, recent studies promote the medical benefits of LT for patients with alcoholic hepatitis whose medical therapy was ineffective, but recidivism is anticipated in these patients who likely continue to consume large volumes of alcohol. LT for alcoholic hepatitis is still a highly controversial issue from the public point of view, and needs to be resolved.

Well-designed prospective studies on DDLT/LDLT are essential to resolve the debatable issues on LT for ALD. Establishment of accurate predictive factors for alcoholic recidivism, benefits and optimal duration of pre-transplant abstinence, and appropriate indication criteria of LT for ALD are among high priority issues. Further evaluations on these issues will help to more effectively control alcoholic recidivism and improve, not only the outcome of LT for ALD patients, but also acceptance from society.

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CASE CONTROL STUDY

Circulating microRNA, miR-122 and miR-221 signature in Egyptian patients with chronic hepatitis C related hepatocellular carcinoma

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Abstract

AIM: To explore the potential usefulness of serum miR-122 and miR-221 as non-invasive diagnostic markers of hepatitis C virus (HCV)-related hepatocellular carcinoma (HCC).

METHODS: This prospective study was conducted on 90 adult patients of both sex with HCV-related chronic liver disease and chronic hepatitis C related HCC. In addition to the 10 healthy control individuals, patients were stratified into; interferon-naïve chronic hepatitis C (CH) (n = 30), post-hepatitis C compensated cirrhosis (LC) (n = 30) and treatment-naïve HCC (n = 30). All patients and controls underwent full clinical assessment and laboratory investigations in addition to the evaluation of the level of serum miRNA expression by RT-PCR.

RESULTS: There was a significant fold change in serum miRNA expression in the different patient groups when compared to normal controls; miR-122 showed significant fold increasing in both CH and HCC and significant fold decrease in LC. On the other hand, miR-221 showed significant fold elevation in both CH and LC groups and significant fold decrease in HCC group (P = 0.01). Comparing fold changes in miRNAs in HCC group *vs* non HCC group (CH and Cirrhosis), there was non-significant fold elevation in miR-122 (P = 0.21) and significant fold decreasing in miR-221 in HCC *vs* non-HCC (P = 0.03). ROC curve analysis for miR-221 yielded 87% sensitivity and 40% specificity for the differentiation of HCC patients from non-HCC at a cutoff 1.82.

CONCLUSION: Serum miR-221 has a strong potential to serve as one of the novel non-invasive biomarkers of HCC.

Key words: MiRNA; Hepatocellular carcinoma; Serum

Core tip: In the current study a signature of circulating miRNAs (miR-122 and miR-221) was evaluated. miR-221 was differentially expressed between patients with hepatocellular carcinoma and those without (chronic hepatitis and liver cirrhosis) with lower serum level of miR-221 in former group of patients in comparison to later one. miR-221 yielded 87% sensitivity and 40% specificity in differentiating between both groups at a cutoff 1.82 folds. The present study emphasizes that circulating miR-221 deserves further attention as a potential non-invasive biomarkers for hepatocellular

carcinoma.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver cancer, and the fifth most common cancer worldwide[1]. The last decade has witnessed a significant rise in the incidence of HCC[2-4] with a specially high incidence reported in Egypt[5]. A direct role of hepatitis C virus (HCV) in hepatocarcinogenesis has been suggested[6]. However, it seems that cirrhosis is the common route through which several risk factors act and induce carcinogenesis[7].

Currently available blood tumor markers are far from optimal. Alfa-fetoprotein (AFP), Lens culinaris agglutinin-reactive AFP (AFP-L3) and des-carboxyprothrombin (DCP) perform poorly in the surveillance mode and early detection of HCC[8]. The Practice Guidelines of the American Association for the Study of Liver Diseases (AASLD) (July 2010) rejected AFP whether for the surveillance or the diagnosis of HCC. This highlights the need for other methods that would be minimally-invasive, simple and reliable for the early detection of HCC.

MicroRNA (miRNA) is a non-coding *RNA* gene product that negatively controls gene expression by altering the stability or translational efficiency of its target mRNAs[2]. MiRNAs regulate several biological processes, such as cell differentiation, apoptosis and proliferation. miRNAs have been reported to be aberrantly present in cancers whether through up- or down-regulation in neoplastic cells compared with their normal counterparts[3,4]. What makes miRNAs even more interesting is that several recent studies have demonstrated that miRNAs are detectable and stable in plasma and serum[4-6].

The goal of the present study was to evaluate circulating serum miRNAs (miR-122 and miR-221) expression levels in Egyptian patients with HCC as well as in patients with HCV-related chronic liver disease to explore their potential as novel non-invasive markers for diagnosis of HCV-related HCC.

MATERIALS AND METHODS

During the period between March and June 2012 serum samples were collected from consecutive HCV-infected patients presenting to our outpatient department: 30 with chronic HCV alone (CH), 30 with HCV-related cirrhosis (LC) and 30 with HCV-related HCC. Serum samples were also collected from 10 age and gender-matched healthy volunteers (defined as those with normal transaminases, normal hepatic ultrasound and negative for HBsAg, HBc-Ab and HCV RNA-PCR). All patients were recruited after a written informed consent and the study protocol was approved by the ethics review committee of Cairo University hospital. Exclusion criteria included: patients with chronic HBV infection or any other identifiable cause for chronic hepatitis other than HCV, previous treatment for HCC or antiviral therapy for HCV and any associated malignancies other than HCC.

RNA extraction

For the real-time PCR RNAs were extracted from serum using TRIzol according to the manufacturer's instruction. The RNA purity was assessed by the RNA concentration and quantified by NanoDrop ND-1000 (Nanodrop, United States). Single-stranded cDNAs were generated using the RT kit (Qiagen, Valencia, CA, United States) according to the manufacturer's directions (miSCript miRNA PCR system, miRneasy mini kit for miRna extraction, miScript RT II for miRna reverse transcription, miSCript Primer Assay and miSCript SYBR Green PCR Kit for PCR amplification.

RNA quantification

PCR quantification experiments were performed with PCR (Applied Biosystems; Foster City, CA) using the SYBR Green PCR Master Mix according to the manufacturer's protocol. The primers for microRNA-122, -221 and housekeeping gene were supplied by Qiagene, Germany (catalog numbers 3416, 3857 and 33712). The housekeeping miRNA SNORD68 was used as the endogenous control. Fluorescence measurements were made in every cycle and the cycling conditions used were: 95 ℃ for 30 s, and 40 cycles of 95 ℃ for 5 s and 60 ℃ for 34 s.

Expression of miRNAs was reported as ΔCt value. The ΔCt was calculated by subtracting the Ct values of miRNA SNORD68 from the Ct values of the target miRNAs. As there is an inverse correlation between ΔCt and miRNA expression level, lower ΔCt values were associated with increased miRNA. The resulting normalized ΔCt values were used in calculating relative expression values by using 2- Δ(Ct), these values are directly related to the miRNA expression levels. The 2-ΔΔ (Ct) method was used to determine relative-quantitative levels of individual miRNAs.

Statistical analysis

Patients were categorized into 4 groups; normal, CH, Cirrhosis and HCC. Further comparisons were performed between HCC group and Non-HCC (CH and cirrhosis). Quantitative variables were expressed by mean ± SD or expressed by median and inter quartile range (IQR) for non-parametric data. They were compared by *t*-student or ANOVA test when appropriate. Qualitative variables were compared by χ^2 or Fischer's exact test when appropriate. AFP levels were transformed into their log values

Table 1 Demographic parameters of patients

	HCC (*n* = 30)	Cirrhosis (*n* = 30)	CH (*n* = 30)	Normal (*n* = 10)	*P* value
Age (yr) Mean ± SD	60.27 ± 8.20^C	55.07 ± 7.35^B	38.20 ± 8.21^A	40.89 ± 16.85^A	$\leqslant 0.001$
Gender (male)	25 (83.3)	21 (70)	22 (73.3)	6 (66.7)	0.6
Hb (g/dL)	11.44 ± 2.85^B	10.53 ± 2.00^B	14.23 ± 1.58^A	14.03 ± 2.48^A	< 0.001
WBC × 10^3/mm^3	5.73 ± 2.56^A	7.08 ± 3.77^A	6.15 ± 2.13^A	7.38 ± 2.72^A	0.217
Platelets 10/mm^{3D}	126.00 ± 74.05^B	116.10 ± 68.94^B	228.80 ± 59.74^A	271.3 ± 116.7^A	< 0.001
Total bilirubin (0.1-1.2 mg/dL)	1.88 ± 2.01^A	4.29 ± 6.75^B	0.74 ± 0.26^A	0.79 ± 0.36^A	< 0.001
ALT (0-42 IU/L)	66.59 ± 44.59^B	32.15 ± 23.59^A	66.78 ± 36.56^B	29.16 ± 19.96^A	< 0.001
AST (0-42 IU/L)	119.99 ± 56.12^B	63.81 ± 39.49^A	59.67 ± 45.04^A	45.64 ± 54.32^A	< 0.001
ALP (0-290 IU/L)^D	394.8 ± 282.28^A	304.89 ± 191.85^A	203.58 ± 82.37^{AB}	198.8 ± 139.1^B	0.003
Albumin (3.5-5.5 g/dL)^D	3.16 ± 0.40^C	2.49 ± 0.54^B	4.22 ± 0.36^A	4.09 ± 0.92^A	< 0.001
PC %	69.63 ± 16.25^C	51.58 ± 17.12^B	88.24 ± 10.89^A	97.63 ± 11.77^A	< 0.001
AFP log10 ng/dL	2.50 ± 1.19^B	0.79 ± 0.54^A	0.59 ± 0.38^A	NA	< 0.001

[A,B,C,D]Groups with different letters show significant difference, those with similar letters show no significant difference. HCC: Hepatocellular carcinoma; NA: Not applicable. CH: Chronic hepatitis C.

Table 2 Tumor-related characteristics (*n* = 30)

	Parameter	Number (%)
AFP level (0-10)	Normal	4 (13.4%)
	Elevated	26 (86.6%)
PS	PS 0	24 (80)
	PS 1-2	4 (13.4)
	PS > 2	2 (6)
BCLC	Stage 0	0 (0)
	Stage A	1 (3.8)
	Stage B	19 (73.1)
	Stage C	4 (15.4)
	Stage D	2 (7.7)
Number of focal lesions	Single	17 (56.7)
	Multiple	13 (43.4)
Site of focal lesions	Right lobe	18 (60)
	Left lobe	5 (16.7)
	Both	7 (23.3)
Tumor size by CT	< 3 cm	1 (3.3)
	3-5 cm	12 (40)
	> 5 cm	17 (56.7)
Portal vein invasion	Yes	7 (23.3)
	No	23 (76.7)

PS: Performance status; AFP: Alfa-fetoprotein; CT: Computed tomography.

to undergo parametric statistical tests. Receiver operator characteristic (ROC) curves were constructed to assess the value of miRNA in diagnosing HCC and to assess area under the curve (AUROC). AUROC less than 0.60 with *P* value > 0.05 is considered unreliable for ROC curve. Spearmen and Pearson correlations were done for correlating quantitative variables. In all tests, *P* value was considered significant if less than 0.05.

RESULTS

This study was conducted on 100 participants stratified into: Group1: thirty patients with HCV-related HCC who were diagnosed according to EASL guidelines 2012; Group2: thirty patients with hepatitis C related liver cirrhosis; Group 3: thirty non-cirrhotic patients with chronic hepatitis C viral infection (CH), while Group 4 included ten age and gender-matched healthy volunteers

(defined as those with normal hepatic ultrasound and transaminases and negative for hepatitis B and C by PCR) considered as internal reference.

The demographic and pathologic features of the studied participants are shown in Tables 1 and 2. There was a significant difference between the diseased groups regarding age (*P* < 0.001). Regarding gender difference; males were predominant in HCV related liver disease patients in the three groups and they represented 83.3%, 70%, 73.3% in HCC, cirrhosis and CH groups respectively with no statistically significant difference between the studied groups (*P* = 0.60).

miR-122 serum levels

Analysis of median fold change in expression level of miR-122 in patients' sera in comparison to the normal control group showed that miR-122 displayed significant fold decrease in expression in cirrhosis group (0.8) and significant fold increasing in expression level in both CH (2.1) and HCC (2.15) groups (*P* ≤ 0.01), Table 3. Comparing serum miR-122 expression level between different studied groups displayed an increasing tendency towards statistical significant fold elevation in expression of miR-122 in serum of HCC patients (2.15) in comparison to liver cirrhosis (0.8) with *P* value 0.083 (AUC = 0.646), Figure 1. No significant fold change in miR-122 expression was found between either (HCC *vs* CH groups) or (CH *vs* cirrhosis groups). MiRNA122 showed nonsignificant up-regulation in HCC patients in comparison to non-HCC patients (CH and Cirrhosis); (*P* = 0.21).

miR-221 serum levels

Analysis of fold change in expression level of miR-221 in patients' sera in comparison to the normal control group showed significant fold decrease in HCC group and significant fold increase in expression level in CH and cirrhosis groups in comparison to normal control group (< 0.01), Table 4. There was a statistically significant fold decreasing in serum miR-221 levels of HCC patients (0.92) in comparison to cirrhosis (3.4) and in comparison to CH (1.7) (*P* = 0.05 and 0.06 respectively). On the other

Table 3 MiR-122 serum levels in the different groups

	Fold of change comparing to normal			
Group	HCC $n = 30$	Cirrhosis $n = 30$	CH $n = 30$	*P* value
miR-122 (IQR)	2.15 (7.3)	0.8 (3.7)	2.1 (9)	< 0.01
				0.083[1]
				0.572[2]
				0.417[3]
Group	HCC $n = 30$	Non HCC (CH + cirrhosis) $n = 60$		*P* value
miR-122 (IQR)	2.15 (7.3)	1.75 (6.8)		0.21 (NS)

[1]HCC *vs* cirrhosis; [2]HCC *vs* CH; [3]Cirrhosis *vs* CH. HCC: Hepatocellular carcinoma; CH: Chronic hepatitis C; IQR: Inter quartile range; NS: Non significant; PVP: Positive predictive value; NVP: Negative predictive value.

Table 4 MiR-221 serum levels

	Fold of change in comparison to normal			
	HCC $n = 30$	Cirrhosis $n = 30$	CH $n = 30$	*P* value
miR-221 (IQR)	0.92 (0.88)	3.4 (19.2)	1.7 (2.6)	> 0.01
				0.05[1]
				0.06[2]
				0.214[3]
	HCC	Non HCC		
	0.92 (0.88)	1.81 (7.75)		0.03 (S)

[1]HCC *vs* LC; [2]HCC *vs* CH; [3]LC *vs* CH. HCC: Hepatocellular carcinoma; CH: Chronic hepatitis C; IQR: Inter quartile range; LC: Liver cirrhosis.

hand, there was no statistical significant fold change in serum miR-221 expression level between (CH *vs* cirrhosis groups) ($P = 0.214$).

miRNA-221 displayed significant fold decrease in HCC group (0.92) compared to non-HCC patients (CH and cirrhosis) (1.81) (*P* value 0.03). At a cut-off of 1.82 folds, miR-221 yielded 87% sensitivity and 40% specificity in differentiating between both groups. Figure 2, Table 5.

Correlation of miRNA's with features of HCC

There was no statistically significant correlation between serum expression level of studied miRNAs and serum AFP level in the different studied groups of patients. No significant correlation was found between the two miR-NAs and tumor size, Child-pugh grade in HCC group of patients.

Correlation of miRNA's with severity of hepatic dysfunction

Serum miRNA-122 expression level showed statistically significant correlation with serum necro-inflammatory markers of the liver [aspartate transaminase (AST) and alanine transaminase (ALT) levels) in CH group (*P* value 0.034 and 0.030 respectively), Table 1.

DISCUSSION

Over the last 2 decades it has become common practice

Figure 1 Receiver operator characteristic curve for miR-122 as a discriminant of hepatocellular carcinoma *vs* cirrhosis patients.

Figure 2 Receiver operator characteristic curve for miR-221 as a discriminant between hepatocellular carcinoma *vs* non-hepatocellular carcinoma.

to use tumour markers, mainly AFP, for the screening of HCC. However, performance of tumor markers has not been optimal with the sensitivity and specificity of AFP and PIVKA-II in the range of 39%-64% and 76%-91%, and 41%-77% and 72%-98%, respectively[9,10], the quest for an optimal tumor marker hence continues. miRNAs have been implicated in roles affecting cellular proliferation and oncogenesis[11]. Cellular miRNAs have been linked with HCC[12]. Their availability in the circulation makes them a tempting target for early tumor detection[12]. The aim of the present study was to explore the potential usefulness of serum miR-122 and miR-221 as novel noninvasive markers for diagnosis of HCV related hepatocellular carcinoma in Egyptian patients.

Most of our HCC patients were within Child-Pugh A and B classifications (56.7%, 36.7% respectively), 73.1% were stage B on BCLC scoring system[13]. This could be explained by the fact that most of them were recruited

Table 5 Diagnostic performance of miR-221 for discriminating patients with hepatocellular carcinoma from those without

AUC	*P* value	Best cutoff	Sensitivity	Specificity	PPV	NPV
0.655	0.03	< 1.82	0.87	0.40	0.47	0.83

HCC: Hepatocellular carcinoma; PVP: Positive predictive value; NVP: Negative predictive value.

while being assessed for the possibility of interventional treatment. Another possible explanation for rather good liver condition seen in HCC series could be attributed to that with implementing surveillance programs, allowing detecting tumors at an early stage in well compensated patients. Alfa fetoprotein level was normal (< 10 ng/dL) in 13.4% of recruited HCC patients. Similar finding was observed by Tateishi *et al*[14] who suggested that not all tumors secrete AFP, and serum levels are normal in up to 40% of small HCCs. It was also showed that α-Fetoprotein alone is not recommended for the diagnosis of HCC and studies showed that its cut off value should be set at 200 ng/mL.

Analysis of fold changes in expression level of miR-122 displayed significant fold increase in expression level in chronic hepatitis C group (2.1) and significant fold decrease in expression in cirrhotics (0.8) in comparison to normal controls. miR-122 is present abundantly in hepatocytes with much lower levels in the circulation in healthy subjects. With hepatocyte injury miR-122 is released in the circulation more readily and serum levels rise. With the eventual loss of hepatocytes and development of fibrosis with proliferation of myelofibroblasts and accumulation of extracellular matrix the circulating miR-122 levels drop again[15].

In the current study there was significant fold rise in serum expression level of miR-122 in HCC group in comparison to normal control group (*P* value < 0.01). Matching our results, Trebicka *et al*[15] who studied hepatic miR-122 expression in 43 HCV related HCC in comparison to 3 healthy liver samples using qRT-PCR; miR-122 was strongly up-regulated in malignant liver nodules in comparison to healthy liver. They suggested that miR-122 might down regulate target mRNA of unknown tumor suppressor genes and thus lead to further tumor growth[15].

In a study on hepatitis B patients Xu *et al*[16] suggested that cancer-induced hepatocyte damage would release the abundant intracellular miR-122 into the circulation, the stability of miRNA would be reflected by easily detectable high blood levels[17]. In contrast to our results, significant down regulation of miR-122 in HCC compared to normal liver tissue was reported by Meng *et al*[18], Wang *et al*[19] and Huang *et al*[20] who compared miR-122 expression profile of 3 different pairs of tumor and normal human liver-derived RNA and 20 HCC liver tissues (mixed etiologies) to normal tissues respectively using microarray[18,19,20]. Similarly a significant down regulation in miR-122 in 19 HBV related HCC liver tissue in comparison to paired healthy liver by next-generation sequencing

was reported by Connolly *et al*[21].

Ladeiro *et al*[22] have established significant down expression of miR-122 in 28 HCC liver tissues (mixed etiologies) in comparison to 4 healthy liver tissues by qRT-PCR.

In our series, no statistically significant correlation could be verified between serum miR122 expression level and patient characters (age), liver synthetic functions tests (Albumin, bilirubin and PC), or serum AFP level in HCC *vs* non HCC group (CH and cirrhosis). However, in the chronic hepatitis groups serum miR-122 was correlated with higher AST and ALT levels, further solidifying the theory regarding the initial rise in miR-122 levels due to hepatocyte inflammation and destruction followed by a drop in the levels with the developing fibrosis. Köberle *et al*[23] also reported significant correlation between serum miR-122 expression level and necro-inflammatory markers (AST, ALT), and Albumin but no significant correlation was found with bilirubin in HCC patients[23].

Perhaps the most significant finding in our study was related to miR-221. Analysis of fold change in expression level of miR-221 in patients' sera of HCV associated liver disease (CH and cirrhosis) in comparison to normal control group showed significant fold increase in expression level in CH and cirrhosis groups in comparison to normal control group (< 0.01). Also a significant fold decrease in serum miR-221 in HCC group (0.92) in comparison to normal control was noticed. We assumed that with the progression of liver disease from chronic hepatitis to cirrhosis the increased activity of hepatic stellate cells was associated with increase miR-221 expression level, such high level stimulated tumorigenesis and increase level of miR-221 in tissue, but as miR-221 is anti apoptotic so serum miR-221 didn't show similar increase. In contrast to our results many studies established up regulation of miR-221 in HCC in relation to normal control, *e.g.*,[18,19,20,24]. However, most these studies assessed tissue miR-221 rather than serum levels. The different results could also be explained by technical variations including sampling methods and freezing and RNA isolation procedures. The etiology of liver disease is also variable in different studies including viral and alcoholic. The stage of the disease is also a source of variation especially that it is still not evident how miRNA expression changes with fibrosis progression. Different studies have also used different control samples for normalization, *e.g.*, non-HCC tissue from the same patient, healthy liver tissue from another subject or patients with the same pathology but not HCC, this is especialy relevant to studies assessing tissue miRNA levels[25].

Similar to what was previously reported by Rong *et al*[26], we found no statistically significant correlation could be verified between serum miR-221 expression level and patient characters (age), laboratory values (AST, ALT), liver synthetic functions tests (Albumin, bilirubin and PC), or serum a-fetoprotein level in HCC *vs* non HCC group (CH and cirrhosis) and no statistically significant correlation could be found between the clinic-pathological parameters of hepatic focal lesion, *e.g.*, (number of focal lesions, Child

score, biggest diameter of focal lesion BCLC, and portal vein invasion) and miR-221 expression level ($P \geqslant 0.05$)[26].

Circulating miR-221 level is significantly up-regulated in the serum of HCV infected patients. It has some value in the differentiation between HCV patients with hepatocellular carcinoma and those without with 87% sensitivity and 40% specificity. It may be able to serve as a promising non-invasive diagnostic marker for HCC. Better results could be obtained if combined with other markers and testing a panel of miRNA's collectively could ultimately serve as a reliable diagnostic test for HCC.

COMMENTS

Background

Hepatocellular carcinoma (HCC), the most common type of liver cancer, is amongst the top three leading causes of cancer-related deaths worldwide with a median survival of only six to eight months. This poor outcome is related to the late detection, with more than two thirds of patients diagnosed at advanced stages of disease. Thus, surveillance of populations at-risk may detect tumors at an early stage when curative interventions can be implemented. The performance of available circulating biomarkers in the screening and diagnostic settings of HCC is sub-optimal.

Research frontiers

MiRNAs constitute a large class of genes that encode short RNAs (19-24 nucleotides long), which play key roles in development and differentiation, by the post-transcriptional regulation of protein coding genes. At present, miRNAs have a widely recognized role in human carcinogenesis, including hepatocarcinogenesis, and many experimental evidences indicate that they may act as oncogenes or tumor suppressor genes regulating the expression of crucial protein coding genes. MiRNAs have been proposed as possible novel biomarkers for cancer diagnosis.

Innovations and breakthroughs

In the current study a signature of circulating miRNAs (miR-122 and miR-221) was evaluated. MiR-221 was differentially expressed between patients with HCC and those without (chronic hepatitis and cirrhosis) with lower serum level of miR-221 in former group of patients in comparison to later one. MiR-221 yielded 87% sensitivity and 40% specificity in differentiating between both groups at a cutoff 1.82 folds.

Applications

The present study emphasis that circulating miR-221 deserves much attention as potential non invasive biomarkers for HCC in the diagnostic setting.

Terminology

HCC: Hepatocellular carcinoma; Non HCC: Chronic hepatis C group of patients and patients with liver cirrhosis.

Peer review

The manuscript entitled "Circulating microRNA, miR-122 and miR221 Signature in Egyptian Patients with Chronic Hepatitis C Related Hepatocellular Carcinoma". The manuscript is interesting.

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CASE REPORT

Hemodynamic effects of ambrisentan-tadalafil combination therapy on progressive portopulmonary hypertension

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Abstract

Intravenous epoprostenol is recommended for World Health Organization functional class (WHO-FC) Ⅳ patients with pulmonary arterial hypertension (PAH) in the latest guidelines. However, in portopulmonary hypertension (PoPH) patients, advanced liver dysfunction and/or thrombocytopenia often makes the use of intravenous epoprostenol challenging. Here we report the cases of two WHO-FC Ⅳ PoPH patients who were successfully treated with a combination of two oral vasodilators used to treat PAH: ambrisentan and tadalafil. Oral vasodilator therapy using a combination of ambrisentan and tadalafil may be a safe and effective therapeutic option for WHO-FC Ⅳ PoPH patients and should be considered for selected patients with severe and rapidly progressing PoPH.

Key words: Portopulmonary hypertension; Ambrisentan; Tadalafil; Thrombocytopenia

Core tip: Advanced liver dysfunction and/or thrombocytopenia often hamper the use of intravenous epoprostenol in patients with portopulmonary hypertension (PoPH). However, recent progress in the oral treatment for pulmonary hypertension (PH) has enabled better clinical outcome in severe PH patients. Here we report two World Health Organization functional class Ⅳ patients with PoPH and thrombocytopenia who were successfully treated with ambrisentan and tadalafil. Oral vasodilator therapy using a combination of ambrisentan and tadalafil may be a safe and effective therapeutic option for patients with PoPH and advanced thrombocytopenia.

INTRODUCTION

Reportedly, 2%-6% of patients with portal hypertension also develop pulmonary hypertension (PH); this combined disorder is called portopulmonary hypertension (PoPH)[1-3]. According to the latest guidelines[4], PoPH is classified in the pulmonary arterial hypertension (PAH) spectrum. It is recommended that PoPH patients be managed similarly to those with other forms of PAH, while considering the presence of liver disease and its consequences[5]. Although intravenous epoprostenol treatment is recommended for World Health Organization functional class (WHO-FC) Ⅳ PAH patients, advanced liver dysfunction and/or thrombocytopenia

Figure 1 Transthoracic echocardiography performed before and after treatment. A: Transthoracic echocardiography (TTE) performed before treatment. The parasternal short axis view at the basal level in diastole shows pronounced interventricular septal deviation toward the left ventricle accompanied by pericardial effusion; B: TTE performed after treatment. The parasternal short axis view at the basal level in diastole shows a decrease in the interventricular septal deviation toward the left ventricle. Pericardial effusion has disappeared. RV: Right ventricle; LV: Left ventricle.

Figure 2 Clinical course and treatment of case 1. Plasma brain natriuretic peptide concentration remarkably decreased from 2212 pg/mL to 46 pg/mL by intravenous dobutamin infusion and subsequent oral ambrisentan-tadalafil combination treatment.

often make such invasive management difficult in PoPH patients. Here we describe two WHO-FC Ⅳ PoPH patients who favorably responded to the combined use of ambrisentan and tadalafil, oral vasodilators that have been proven to be effective against PAH.

CASE REPORT

Case 1

In September 2010, a 56-year-old man with hepatitis B virus-related cirrhosis, esophageal varix, and hepatocellular carcinoma was referred to our department with abnormal findings on electrocardiogram. Doppler echocardiography indicated increased systolic right ventricular pressure, estimated by a tricuspid regurgitation pressure gradient (TRPG) of 79 mmHg. Right heart catheterization (RHC) also revealed an increased mean pulmonary artery pressure (PAP) of 40 mmHg and an increased pulmonary vascular resistance (PVR) of 6.38 Wood units. PoPH was diagnosed; however, his clinical course (WHO-FC I) was modest. Accordingly, we decided to employ a careful wait-and-watch approach.

However, 3 mo later, the patient experienced rapid progression of exertional dyspnea, facial edema, and syncope, and he was consequently admitted to our department. He presented with jugular venous distention, pretibial pitting edema, and a pronounced pulmonary component of the second heart sound. Laboratory data revealed advanced thrombocytopenia (5.0×10^4/mL), an increased D-dimer level (9.38 μg/dL), an increased indirect bilirubin level (4.9 mg/dL), a mildly increased transaminase level (aspartate aminotransferase, 69 U/L; alanine aminotransferase, 40 U/L), and an increased plasma brain natriuretic peptide (BNP) level (2212 pg/mL). Transthoracic echocardiography revealed right ventricular dilatation and severe interventricular septal deviation toward the left ventricle, accompanied by pericardial effusion (Figure 1A). The pulmonary artery systolic pressure, estimated on the basis of TRPG, was 120 mmHg. Abdominal computed tomography (CT) revealed dilatation of the splenic, esophageal, and umbilical veins, suggestive of portal hypertension, whereas ventilation/perfusion lung scintigraphy revealed no significant mismatch. These results indicated rapid progression of PoPH; therefore, dobutamine (2 μg/kg per minute) was initiated. RHC revealed that the mean PAP was increased to 55 mmHg, cardiac index (CI) was decreased to 2.49 L/min per square, and PVR was increased to 10.9 Wood units.

Because of comorbid advanced thrombocytopenia and hepatocellular carcinoma, we initiated ambrisentan at a dose of 2.5 mg once daily, added tadalafil at a dose of 20 mg once daily, and subsequently increased the dose of the two agents in turn to reach the maximum dose (ambrisentan, 10 mg/d; tadalafil, 40 mg/d) in 9 d. After a month, the patient's heart failure symptoms and signs had improved and his plasma BNP level had decreased (Figure 2).

At a follow-up assessment 5 mo later, the patient's WHO-FC had improved from Ⅳ to Ⅱ and his plasma BNP level had decreased to 44.4 pg/mL. Echocardiography revealed a decrease in TRPG from 95 to 56.2 mmHg, a less pronounced interventricular septal shift toward the left ventricle, and no evidence of pericardial effusion (Figure 1B). Repeat RHC revealed a decrease in
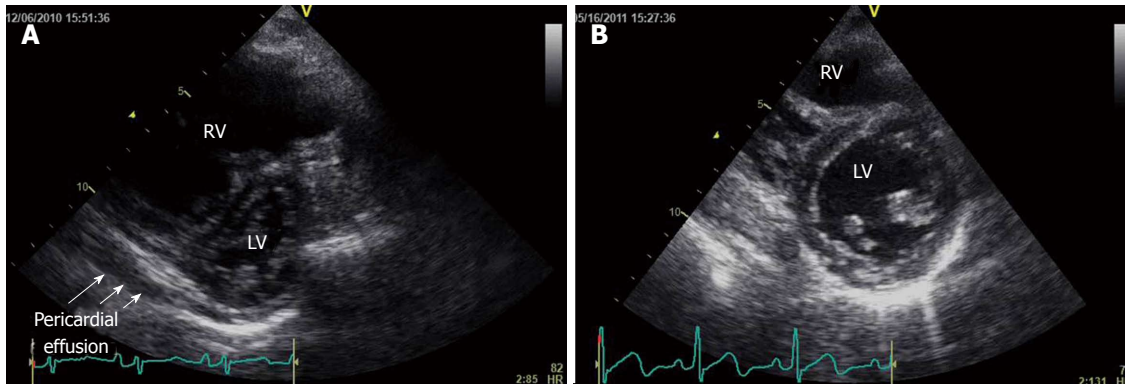
Figure 3 Transthoracic echocardiography performed before and after treatment. A: Pre-treatment. Transthoracic echocardiography (TTE) performed before treatment. The parasternal short axis view at the basal level in diastole shows pronounced interventricular septal deviation toward the left ventricle accompanied by pericardial effusion; B: Five-month after treament. TTE performed after treatment. The parasternal short axis view at the basal level in diastole shows a decrease in the interventricular septal deviation toward the left ventricle. The pericardial effusion has disappeared. RV: Right ventricle; LV: Left ventricle.
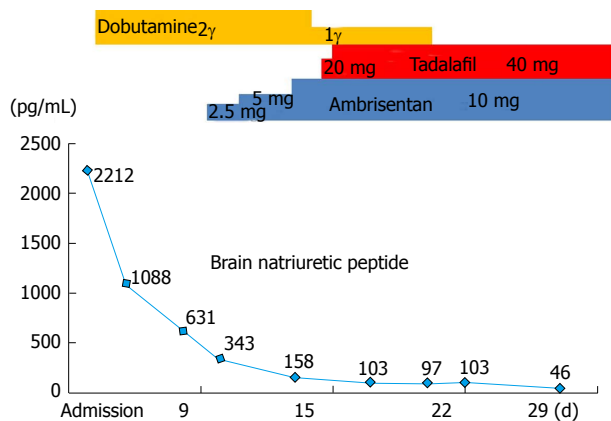

Figure 4 Clinical course and treatment of case 2. Plasma brain natriuretic peptide concentration remarkably decreased from 1897 pg/mL to 139 pg/mL by intravenous dobutamin infusion and oral ambrisentan-tadalafil combination treatment.

mean PAP (34 mmHg) and PVR (6.4 Wood units) and improvement in CI (4.27 L/min per square).

Case 2

In April 2013, a 70-year-old man was referred to our department with rapid worsening of exertional dyspnea (WHO-FC IV) and suspected PH on echocardiography. He had been diagnosed with liver cirrhosis from excessive alcohol consumption, hepatocellular carcinoma, and esophageal varix approximately 9 years previously. He presented with jugular venous distention, hepatomegaly, and splenomegaly as well as a pronounced pulmonary component of the second heart sound and a third heart sound on chest auscultation. Laboratory data revealed advanced thrombocytopenia (3.5×10^4/mL), an increased D-dimer level (4.39 μg/dL), mild hepatic dysfunction (Child-Pugh B), and an increased plasma BNP level (988 pg/mL). Echocardiography revealed right ventricular dilatation and severe interventricular septal deviation toward the left ventricle accompanied by pericardial effusion (Figure 3A). Systolic PAP was 125 mmHg, as estimated by TRPG. CT suggested dilation of the esophageal vein.

Ventilation/perfusion lung scintigraphy revealed a mismatch in the left upper lobe, although it was limited and not compatible with chronic thromboembolic PH. Cardiac magnetic resonance imaging indicated a dilated right ventricle and decreased right ventricular ejection fraction (RVEF; 25.8%). A hemodynamic study with oxygen at 5 L/min revealed an increased mean PAP (62 mmHg), a decreased CI (1.43 L/min per square), and an increased PVR (18.5 Wood units).

Based on findings indicative of portal hypertension and RHC findings, PoPH was diagnosed. We initiated dobutamine at 2 μg/kg per minute to support cardiac function. We then initiated ambrisentan at a dose of 2.5 mg once daily. and added tadalafil at a dose of 20 mg once daily. The doses of these two agents were then increased in turn to reach the maximum dose (ambrisentan, 10 mg/d; tadalafil, 40 mg/d) in 15 d.

Within a month and a half, the patient's plasma BNP level had decreased, right heart failure signs had disappeared, and WHO-FC had decreased to III (Figure 4). At the follow-up assessment conducted 4 mo later, his WHO-FC was III and the plasma BNP level had decreased to 35 pg/mL. Echocardiography revealed that TRPG had decreased to 56.2 mmHg, the degree of interventricular septal deviation toward the left ventricle had decreased, and pericardial effusion was absent (Figure 3B). Cardiac magnetic resonance imaging-derived RVEF improved to 50.8%, and RHC revealed improvement in mean PAP (34 mmHg), CI (2.9 L/min per square), and PVR (4.7 Wood units).

Both patients gave their written informed consent prior to their inclusion in the present study. The present study complied with the Declaration of Helsinki.

DISCUSSION

In the latest guidelines for PH, the recommended treatment strategy for PoPH is similar to that for PAH, while liver transplantation is considered in a selected subset of patients[6]. Based on this strategy, intravenous epoprostenol would be considered for WHO-FC IV patients,

including the present two cases. However, the successful use of oral agents effective against PAH has been reported for PoPH patients[7-9], suggesting a potentially important role of these drugs in PoPH treatment. In the two cases presented here, the use of intravenous epoprostenol was initially considered. However, it could not be used because of comorbid thrombocytopenia, which was thought to increase the risk during Hickman catheter implantation[10], and the probable further deterioration of thrombocytopenia caused by intravenous epoprostenol treatment itself[11]. In addition, both patients had been diagnosed with hepatocellular carcinoma, which further supported the use of a conservative treatment strategy. Considering the severe and rapid progressive state of the disease, we initiated combination treatment using ambrisentan, a dual endothelin receptor antagonist with limited liver toxicity, and tadalafil, a long-acting phosphodiesterase 5 inhibitor.

After the oral combination therapy, the patients' signs and symptoms dramatically improved. In addition, pulmonary hemodynamics improved. In particular, PVR decreased by 41% in case 1 and by 75% in case 2. This degree of PVR reduction was comparable with or greater than that achieved by drugs effective against PAH[12]. Furthermore, WHO-FC, the plasma BNP level, and CI notably improved after treatment in both cases in the present study, suggesting a favorable clinical outcome[13].

Notably, some clinical features were unique to the two cases of PoPH presented here. One was the rapid progression of disease. Pathologically, PoPH cases reportedly show vascular remodeling, as observed in idiopathic PAH[10,11], which is likely to develop gradually. However, in these two cases, PH-related symptoms and signs rapidly progressed during the month prior to admission. In addition, CI is usually increased in PoPH, reflecting the portal systemic shunt. However, in the present two cases, CI decreased. One possible interpretation of these clinical features is a unique pathogenesis of PoPH in these two cases, such as vascular spasm, rather than the gradual progression of pulmonary vasculopathy typically observed in PoPH[14,15]. Such a rapid pathogenesis may explain why oral treatment dramatically improved the clinical features in the short term. In addition, decreased cardiac function, as represented by CI, may have been caused by an unusually rapid elevation of PAP/PVR, which quickly resolved after treatment.

In the Reveal registry, the survival rate of PoPH is reportedly worse than that of idiopathic PAH and familial PAH[16]. Two possible explanations have been proposed for the worse clinical outcome of PoPH. First, comorbid advanced liver diseases such as liver cirrhosis and cancer can negatively impact survival. Second, comorbid liver disease and its complications also impede the optimal use of drugs effective against PAH, such as endothelin receptor antagonists and intravenous epoprostenol. In the present two cases, long-term outcome must be evaluated, despite the short-term outcome (up to 5 mo) being favorable.

In conclusion, we presented two cases of severe PoPH with a favorable clinical response to a combination of ambrisentan and tadalafil. Although this approach cannot be generalized, this combination therapy should be considered in selected patients with severe and rapidly progressive PoPH. Further studies would be required to better understand the pathogenesis and establish optimal treatment strategies for PoPH patients.

COMMENTS

Case characteristics
Two male patients with liver cirrhosis and portal hypertension.

Clinical diagnosis
Rapidly progressive exertional dyspnea.

Differential diagnosis
Progression of liver dysfunction, pulmonary and cardiovascular disease.

Laboratory diagnosis
Case 1: thrombocytopenia (5.0 x 10^4/mL), and increased D-dimer (9.38 μg/dL), transaminases, and plasma BNP levels (2212 pg/mL); Case 2: thrombocytopenia (3.5 × 10^4/mL), and increased D-dimer (4.39 μg/dL) and plasma BNP levels (988 pg/mL).

Imaging diagnosis
Right ventricular dilatation and increase in the estimated systolic pulmonary artery pressure by transthoracic echocardiography in both cases. Abdominal CT scan revealed findings of portal hypertension, whereas ventilation/perfusion lung scintigraphy showed no significant mismatch in both cases.

Treatment
Both patients were treated with ambrisentan–tadalafil combination therapy for rapidly progressive portopulmonary hypertension (PoPH).

Related reports
Successful monotherapy using an oral agent effective against pulmonary artery hypertension has been recently reported for patients with PoPH.

Term explanation
PoPH is a subtype of pulmonary hypertension (defined as a mean pulmonary artery pressure equal to or greater than 25 mmHg) that develops in patients with portal hypertension.

Experiences and lessons
A combination of ambrisentan and tadalafil may be a safe and effective therapeutic option for a certain subset of patients with PoPH and advanced thrombocytopenia.

Peer review
It is a good paper for publication.

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TOPIC HIGHLIGHT

WJH 6th Anniversary Special Issues (1): Management of hepatocellular carcinoma

Role of anti-angiogenesis therapy in the management of hepatocellular carcinoma: The jury is still out

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Abstract

As the leading cause of disease-related deaths, cancer is a major public health threat worldwide. Surgical resection is still the first-line therapy for patients with early-stage cancers. However, postoperative relapse and metastasis remain the cause of 90% of deaths of patients with solid organ malignancies, including hepatocellular carcinoma (HCC). With the rapid development of molecular biology techniques in recent years, molecularly targeted therapies using monoclonal antibodies, small molecules, and vaccines have become a milestone in cancer therapeutic by significantly improving the survival of cancer patients, and have opened a window of hope for patients with advanced cancer. Hypervascularization is a major characteristic of HCC. It has been reported that anti-angiogenic treatments, which inhibit blood vessel formation, are highly effective for treating HCC. However, the efficacy and safety of anti-angiogenesis therapies remain controversial. Sorafenib is an oral multikinase inhibitor with anti-proliferative and anti-angiogenic effects and is the first molecular target drug approved for the treatment of advanced HCC. While sorafenib has shown promising therapeutic effects, substantial evidence of primary and acquired resistance to sorafenib has been reported. Numerous clinical trials have been conducted to evaluate a large number of molecularly targeted drugs for treating HCC, but most drugs exhibited less efficacy and/or higher toxicity compared to sorafenib. Therefore, understanding the mechanism(s) underlying sorafenib resistance of cancer cells is highlighted for efficiently treating HCC. This concise review aims to provide an overview of anti-angiogenesis therapy in the management of HCC and to discuss the common mechanisms of resistance to anti-angiogenesis therapies.

Core tip: Hepatocellular carcinoma (HCC) is a devastating disease with a high mortality rate. For a long period of time, no effective treatment options are available for patients with advanced HCC. During the last decade, molecularly targeted therapies have been introduced into the treatment of advanced HCC. However, the efficacy and safety of molecularly targeted therapies remain controversial. In addition, primary or acquired drug resistance limits the activity of molecularly targeted agents, but the underlying mechanisms have not been fully understood. This concise review aims to

provide an overview of anti-angiogenesis therapy in the treatment of HCC.

INTRODUCTION

Primary liver cancer (PLC) is one of the most common malignancies and the second leading cause of cancer-related deaths around the world. Hepatocellular carcinoma (HCC), the most common type of PLC, accounts for approximate 90% of PLC cases in most countries. In addition, HCC is the 5th and 7th most common cancer in males and females, respectively. The worldwide incidence of HCC is increasing partially due to the rising number of infections caused by hepatitis B virus or hepatitis C virus[1-3]. Recently, while the diagnosis of HCC has been remarkably improved with the use of noninvasive imaging tests, a large number of patients were still diagnosed at the advanced stage due to the lack of symptoms during early stages and the rapid progression of cancer cells[4,5].

The management of HCC depends mainly on tumor stage and liver function reserve. Currently, curative treatments such as surgical resection, liver transplantation, and local ablation can significantly improve the survival of HCC patients at the early stage[2,6]. However for a long period of time, no effective treatment options are available for patients with advanced HCC or who progressed into an advanced stage after other treatments failed. In recent years, molecularly targeted therapies using monoclonal antibodies, small molecules, and vaccines have been widely studied in cancer managements. Given that HCC is a highly vascularized tumor, anti-angiogenic treatments might be highly efficient for the treatment of HCC by inhibiting the formation of blood vessels in cancer tissues through small molecules[7-9].

RESISTANCE TO ANTI-ANGIOGENIC DRUGS OF HCC CELLS

As an oral multikinase inhibitor, sorafenib has both anti-proliferative and anti-angiogenic effects on tumors through blocking Raf and vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) receptor tyrosine kinase signaling. Sorafenib is the first molecular target drug approved for the treatment of advanced HCC. A phase 3, randomized, double-blind, placebo-controlled, multicenter study was performed in 2008 in 21 Western countries to evaluate the effects of sorafenib on the treatment of HCC. This study showed that sorafenib prolonged the median survival and the time to radiologic progression by approximately 3 mo

in advanced HCC patients[10,11]. Cheng *et al*[12] also reported that sorafenib was effective for advanced HCC and was well tolerated in HCC patients from the Asia-Pacific region. In addition, high safety and well-tolerance of sorafenib have been reported in a large phase 4 study including over 1500 patients with unresectable HCC[13,14]. Therefore, sorafenib has been established as the standard first-line monotherapy for patients with advanced HCC[9,15-17]. However, the efficacies of current anti-angiogenesis therapies are still far from satisfactory (Table 1). Currently, the median survival time of HCC patients who received sorafenib treatment is not longer than 1 year even after many years of research[18].

Resistance to molecularly targeted agents including sorafenib is a major reason causing the failure of anti-cancer therapies (Table 2)[17,19,20]. Primary resistance is observed in some HCC patients who are initially not susceptible to sorafenib therapy due to intrinsic indifference. After long-term exposure, tumor cells may gradually become resistant and/or less susceptible to sorafenib, leading to acquired resistance[17]. Both primary and acquired resistance to sorafenib has been commonly reported in HCC patients[21]. Ezzoukhry *et al*[22] found that HCC cells exhibited different susceptibilities to sorafenib. For example, some HCC cell lines such as Hep3B and SNU-449 were inherently resistant to sorafenib. The authors also showed that activation of the epidermal growth factor receptor (EGFR) was a possible determinant of inherent resistance of HCC cells to sorafenib. In an *in vitro* study, Zhang *et al*[23] showed that phosphorylated extracellular signal-regulated kinase was a potential predictor of sorafenib sensitivity in HCC. Similarly, Blivet-Van Eggelpoël *et al*[21] demonstrated that EGFR and human epidermal growth factor receptor-3 reduced the susceptibility of HCC cells to sorafenib.

The exact molecular mechanisms underlying the acquired resistance to sorafenib are largely unknown[17]. In 2011, Chen *et al*[24] reported that activation of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway mediates acquired resistance to sorafenib in HCC cells. Xia *et al*[25] also showed that activation of the transforming growth factor beta-and PI3K/Akt-signaling pathways led to acquired resistance to sorafenib in HCC cells. Recently, a number of studies provided evidence showing that many mechanisms such as cancer stem cells[26-29], epithelial-mesenchymal transition[25,26,28-30], autophagy[31-34], and microenvironment (hypoxic, inflammation, and cytokines)[35-38] were involved in the acquired resistance to anti-angiogenesis therapies of HCC[17,39]. In addition, Zhai *et al*[17] suggested in a review article that sorafenib could simultaneously or sequentially activate the addiction switches and compensatory pathways when its targets were silenced, leading to acquired resistance. Taken together, the exact mechanisms of sorafenib resistance have not been fully elucidated. Therefore, further studies should be conducted to clarify the biological mechanisms, which may further improve the therapeutic effects of sorafenib.

Table 1 Clinical studies on anti-angiogenesis therapy of hepatocellular carcinoma included in this review

Ref.	Year	Phase	Investigational drug	Outcome
Llovet *et al*[10]	2008	Phase 3	Sorafenib	Increased survival
Cheng *et al*[12]	2009	Phase 3	Sorafenib	Increased survival
Lencioni *et al*[13]	2012	Phase 4	Sorafenib	High safety
Lencioni *et al*[14]	2014	Phase 4	Sorafenib	High safety
Johnson *et al*[40]	2013	Phase 3	Brivanib	Less well-tolerated
Cheng *et al*[41]	2013	Phase 3	Sunitinib	Significantly inferior than sorafenib
Zhu *et al*[42]	2012	Phase 3	Sorafenib plus erlotinib	No survival benefit
Llovet *et al*[43]	2013	Phase 3	Brivanib after sorafenib failed	No survival benefit
Zhu *et al*[44]	2014	Phase 3	Everolimus after sorafenib failed	No survival benefit

Table 2 Studies on the mechanisms of anti-angiogenesis therapy resistance in hepatocellular carcinoma

Ref.	Year	Investigational drug	Pathways/genes involved	Effects
Blivet-Van Eggelpoël *et al*[21]	2012	Sorafenib	EGFR and HER-3	Restrict cell response
Ezzoukhry *et al*[22]	2012	Sorafenib	EGFR	Potential determinant of primary resistance
Zhang *et al*[23]	2009	Sorafenib	pERK	Potential biomarker for sensitivity prediction
Chen *et al*[24]	2011	Sorafenib	PI3K/Akt	Mediates acquired resistance
Xia *et al*[25]	2013	Sorafenib	TGF-β-and PI3K/Akt	Mediates acquired resistance
Chen *et al*[26]	2011	Sorafenib	EMT and hedgehog signaling	Drug resistance
Xin *et al*[27]	2013	Sorafenib	CSCs	Drug resistance
Chow *et al*[28]	2013	Sorafenib	EMT	Acquired resistance
Fernando *et al*[29]	2014	Sorafenib	TGF-β pathway	Prediction of low susceptibility
Huang *et al*[30]	2013	Sorafenib	EMT	Drug resistance
Shi *et al*[31]	2011	Sorafenib	Autophagy	Drug resistance
Shimizu *et al*[32]	2012	Sorafenib	Autophagy	Impair antitumor effects
Zhai *et al*[33]	2014	Sorafenib	Autophagy	Acquired resistance
Liu *et al*[34]	2013	Sorafenib	Autophagy	Facilitates resistance
Liang *et al*[36]	2013	Sorafenib	Hypoxia	Drug resistance
Mao *et al*[38]	2014	Sorafenib	microRNA-193b	Enhances cell response
Ebos *et al*[46]	2009	Sunitinib	VEGFR/PDGFR	Accelerate metastasis and decrease overall survival
Pàez-Ribes *et al*[47]	2009	Sunitinib	VEGFR/PDGFR	Increase local invasion and distant metastasis
Xiong *et al*[50]	2009	Sorafenib	TECs	Drug resistance
Li *et al*[53]	2011	Bevacizumab	Dll4-notch signaling	Drug resistance

EGFR: Epidermal growth factor receptor; HER-3: Human epidermal growth factor receptor-3; pERK: Phosphorylated extracellular signal-regulated kinase; PI3K: Phosphatidylinositol 3-kinase; TGF-β: Transforming growth factor beta; EMT: Epithelial-mesenchymal transition; CSCs: Cancer stem cells; TECs: Tumor-derived endothelial cells; VEGFR: Vascular endothelial growth factor receptors; PDGFR: Platelet-derived growth factor receptors; Dll4: Delta-like ligand 4.

The discovery and development of sorafenib have paved the way to the development of new anti-angiogenesis drugs for advanced HCC or for whom sorafenib failed. More recently, many clinical trials are conducted all over the world, but the problem still exists. Due to good results from preclinical and early-phase studies, some other molecularly targeted drugs have been applied as the second-line treatment for advanced HCC when sorafenib treatment fails. In a number of large-scale randomized phase 3 trials, unfortunately, none of them have shown survival benefits in the first-line (brivanib, sunitinib, erlotinib, and linifanib[40-42]) or second-line (brivanib[43], everolimus[44]) setting after sorafenib progression[18,45].

Furthermore, it was proposed that anti-angiogenic therapies may cause tumor progression and metastasis. Ebos *et al*[46] reported that sunitinib (a VEGF receptors/PDGF receptors kinase inhibitor) promoted tumor growth and metastasis after a short-term application. Similarly, Pàez-Ribes *et al*[47] demonstrated that application of angiogenic inhibitors targeting the VEGF signaling pathway elicit malignant progression of tumors to increased local invasion, lymphatic and distant metastasis. Recently, Chow *et al*[28] reported that advanced HCC patients with acquired resistance to sorafenib might have enhanced tumor growth properties or metastatic potentials. Therefore, understanding the molecular mechanisms underlying anti-angiogenesis therapy resistance may allow us to identify key molecular targets for efficient anti-angiogenesis therapy.

NEW MECHANISMS OF RESISTANCE TO ANTI-ANGIOGENIC DRUGS

During the last five years, increasing evidence suggested that tumor-derived endothelial cells (TECs), which exhibit distinct histologic appearance compared to normal endothelial cells (NECs), may contribute to the resistance of anti-angiogenic therapies[48,49]. In 2009, Xiong *et al*[50] reported that TECs in human HCC tissues had higher angiogenic capacity and sorafenib resistance than NECs.

Some researchers have concluded that TECs can acquire molecular cytogenetic abnormalities in tumor microenvironment; however, the molecular mechanisms underlying the resistance of TECs to anti-angiogenic therapies remain largely unknown. Attempts to resolve this dilemma have resulted in the discovery of transdifferentiation of tumor cells to vascular endothelial cells. In 2010, Wang *et al*[51] and Ricci-Vitiani *et al*[52] provided strong evidence showing that a number of TECs that contribute to blood vessels in glioblastoma were transdifferentiated from tumor stem-like cells. Wang *et al*[51] also showed that blocking the VEGF/VEGFR2 signaling pathway inhibited the maturation of tumor endothelial progenitors into endothelia but not the differentiation of tumor stem-like cells into endothelial progenitors, while the initial differentiation of tumor stem-like cells to endothelial progenitor cells was regulated by Notch1. Consistently, Li *et al*[53] reported that Delta-like ligand 4 (Dll4; a novel Notch ligand)-Notch signaling mediated the resistance to VEGF inhibitor bevacizumab and Dll4-expressing tumors were resistant to a VEGFR targeting multikinase inhibitor *in vivo*. Furthermore, it has also been shown that Dll4-mediated Notch signaling played a central role in active vascularization[54] and blockade of Dll4 resulted in tumor growth inhibition even for tumors resistant to anti-VEGF treatments[55].

CONCLUSION

In summary, sorafenib is still the only approved drug for the therapy of advanced HCC. However, the long-term survival benefit from sorafenib treatment is relatively limited. Some other anti-angiogenesis drugs have been evaluated preclinically and clinically for the treatment of HCC, but their effects were not satisfactory. Therefore, identification of novel anti-angiogenic drugs and improvement of the currently available anti-angiogenesis therapies are highlighted for the treatment of HCC.

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TOPIC HIGHLIGHT

WJH 6th Anniversary Special Issues (2): Hepatocellular carcinoma

Role of hepatectomy for recurrent or initially unresectable hepatocellular carcinoma

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Abstract

As a result of donor shortage and high postoperative morbidity and mortality after liver transplantation, hepatectomy is the most widely applicable and reliable option for curative treatment of hepatocellular carcinoma (HCC). Because intrahepatic tumor recurrence is frequent after loco-regional therapy, repeated treatments are advocated provided background liver function is maintained. Among treatments including local ablation and transarterial chemoembolization, hepatectomy provides the best long-term outcomes, but studies comparing hepatectomy with other nonsurgical treatments require careful review for selection bias. In patients with initially unresectable HCC, transarterial chemo-or radio-embolization, and/or systemic chemotherapy can down-stage the tumor and conversion to resectable HCC is achieved in approximately 20% of patients. However, complete response is rare, and salvage hepatectomy is essential to help prolong patients' survival. To counter the short recurrence-free survival, excellent overall survival is obtained by combining and repeating different treatments. It is important to recognize hepatectomy as a complement, rather than a contraindication, to other nonsurgical treatments in a mul-

tidisciplinary approach for patients with HCC, including recurrent or unresectable tumors.

Key words: Hepatocellular carcinoma; Hepatectomy; Repeat hepatectomy; Conversion therapy; Multidisciplinary treatment

Core tip: Previous studies comparing hepatectomy with other nonsurgical treatments for hepatocellular carcinoma (HCC) evaluated which provided superior survival benefit. However, considering the high recurrence rate after curative loco-regional treatment, and limited indications for hepatectomy because of background liver damage, it is important to recognize hepatectomy as a complement to other nonsurgical treatment, rather than a contraindication. A multidisciplinary approach combining and repeating different treatments prolongs patients' survival with HCC, including those with recurrent or initially unresectable tumors.

INTRODUCTION

Liver transplantation is the most promising strategy for radical treatment for hepatocellular carcinoma (HCC) because it eradicates both the tumors and the background damaged liver; hepatectomy is second. However, high perioperative morbidity and mortality, and a shortage of donors limit application of liver transplantation. Poon *et al*[1,2] reported that although the risk of postoperative

Table 1 Repeat resection rate, 5-year recurrence-free survival rate, and overall survival rate after repeat hepatectomy in previous studies

Ref.	Year	Number of primary hepatectomy	Number of second hepatectomy/HCC recurrence after primary hepatectomy	5-yr recurrence free survival after repeat hepatectomy	5-yr overall survival after repeat hepatectomy
Poon et al[4]	1999	244	11/105 (10%)	NA	69%
Nakajima et al[7]	2001	94	12/57 (21%)	Not reached	52%
Sugimachi et al[8]	2001	474	78/300 (26%)	NA	47.50%
Minagawa et al[9]	2003	334	56/183 (31%)	17%	56%
Chen et al[5]	2004	627	34/286 (12%)	NA	56.80%
Taura et al[10]	2006	610	55/465 (12%)	NA	NA
Itamoto et al[11]	2007	483	70/279 (25%)	10%	50%
Shimada et al[12]	2007	319	13/211 (6%)	NA	25%
Tralhão et al[6]	2007	190	16/97 (19%)	NA	31%
Liang et al[13]	2008	NA	73/853 (9%)	10.50%	27.60%
Choi et al[14]	2008	353	9/97 (9%)	NA	78%
Wu et al[15]	2009	1177	149/641(23%)	31.80%	56.40%
Kishi et al[16]	2011	221	8/134 (6%)	NA	37.50%
Huang et al[17]	2012	NA	82/NA	8.20%	22.40%
Tsujita et al[18]	2012	NA	112/NA	NA	67.30%
Yamashita et al[19]	2013	791	163/308 (53%)	29%	60%

HCC: Hepatocellular carcinoma; NA: Not assessed.

tumor recurrence was low after transplantation, the long-term prognosis after transplantation was comparable to patients who underwent hepatectomy among patients with Child-Pugh class A background liver disease. Therefore, hepatectomy remains a reliable and widely applicable surgical treatment; however, the main limitation is that it is not indicated in patients with impaired liver function resulting from cirrhosis irrespective of the etiology of the liver disease. Multimodal therapy combining nonsurgical treatments including local ablation and transarterial chemoembolization (TACE) with hepatectomy and/or liver transplantation have been advocated for recurrent HCC, multinodular HCC, or initially unresectable HCC. This review was aimed to evaluate the role of hepatectomy among the various treatments for recurrent or advanced HCC.

Hepatectomy for recurrent HCC following local treatment

Because HCC usually develops in the injured liver, tumors frequently recur even after curative local treatment. The incidence of intrahepatic recurrence within 2 years after primary hepatic resection is 70%[3]. However, because recurrences occur most commonly in the remnant liver, comprising 85%-90% of initial recurrence sites[3], repeat hepatectomy or other local treatment is indicated. In general, treatments are selected based on the same criteria as the primary HCC. Several studies compared the results of repeat hepatectomy with nonsurgical treatment and showed that repeat hepatectomy was associated with a better prognosis[4-6]. However, these studies were retrospective analyses and may have included the selection bias that the repeat hepatectomy group usually included patients with better background liver function and less multinodular tumors. Repeat hepatic resection is indicated for only a limited proportion of patients (6%-53%) and the 5-year overall survival after second

hepatectomy is reported as 22%-78%[4-19]. The repeat resection rate, 5-year recurrence-free survival rate, and overall survival rate after second hepatectomy in these studies are summarized in Table 1. The difference in the survival rate would probably have been influenced by the difference in the background liver damage, types of recurrence, and tumoral factors such as size, number, and vascular invasions, but precise assessment was difficult due to the insufficient data. A small number of studies reported the outcomes after a third or fourth hepatectomy[15,19]. In the two series evaluating the outcomes of 1117 and 791 patients who underwent primary hepatectomy for HCC, a second, third, and fourth hepatectomy was performed in 23% (149/641) and 53% (163/308), 37% (35/96) and 65% (36/55), and 27% (8/30) and 69% (9/13) of the patients with recurrence, respectively. Five-year overall survival after a second and third hepatectomy was 56% and 59% in Wu et al's[15] series and 60% and 43% in Yamashita et al's[19] series, respectively. Factors related to both primary and recurrent tumors such as tumor size, number, and vascular invasion and also the degree of background liver damage as assessed by Child-Pugh class, indocyanine green retention rate, or platelet counts were reported as prognostic predictors. Recurrence-free interval and/or type of recurrence, multicentric occurrence or intrahepatic metastases[17,20], were also commonly reported to be prognostic predictors in several studies. Intrahepatic metastases usually occur via the portal vein, and are therefore associated with portal vein invasion. Distinction of them is important because intrahepatic recurrence is associated with malignant behavior compared to multicentric occurrence. Differentiation is possible by histopathological examination as defined by the Liver Cancer Study Group of Japan (Table 2)[21] but there is no established method to differentiate intrahepatic metastases vs multicentric occurrence preoperatively, an issue requiring further research.

Figure 1 Recurrent hepatocellular carcinoma adjacent to a radiofrequency ablation scar. A: Computed tomography showing the tumor with unclear borders with arterial enhancement (arrow) adjacent to the scar (arrowhead); B: Cut surface of the resected specimen showing the recurrent tumor (arrow) and radiofrequency ablation scar (arrowhead).

Table 2 Three types of definition of intrahepatic metastases by the Liver Cancer Study Group of Japan[21]

	Definition
1	Tumors clearly growing from portal vein tumor thrombi
2	Tumors surrounding a large main tumor with multiple satellite nodules
3	A small solitary tumor that is near the main tumor and histologically similar to or less differentiated than the main tumor

It is important that hepatectomy and other local treatments be considered complementary and not exclusive. The dissociation between low recurrence-free survival and rather high overall survival shown in Table 1 reflects the slow progression of the disease and the importance of repeating treatment, usually TACE. Repeating locoregional treatment such as ethanol injection (PEI), radiofrequency ablation (RFA), or TACE, for intrahepatic recurrence prolongs patient survival[10,22-25], and provides a comparable prognosis after RFA compared with repeat hepatectomy[7,12,13,24]. Taura *et al*[10] compared the long-term outcomes of 610 patients with HCC who underwent hepatectomy before 1990 and after 1991. There was no change in the disease-free survival (early *vs* late period, 28% *vs* 26%, respectively, at 5 years), but survival after tumor recurrence increased significantly in the later period (12% *vs* 22% at 5 years) and overall survival also improved (39% *vs* 58% at 5 years). The authors concluded that increased application of RFA to solitary intrahepatic recurrence, which was the most common type of recurrence, contributed to the improved prognosis[10]. Kishi *et al*[16] reported that the number rather than the type of treatment for tumor recurrence was associated with prolonged survival.

As was referred in the beginning of the introduction, liver transplantation is the most promising, and salvage liver transplantation for recurrent HCC, which have been reported with 5-year survival rate of 54%-61% could be a choice of treatment because these figures were comparable with that after primary liver transplantation for HCC that was 59%-72%[26-29]. However, shortage of do-

nor organ, expensive medical costs, and contraindication for elderly patients preclude popularization of this strategy. Indication for salvage transplantation have not been established, but various factors including recurrence free survival, microvascular involvement, satellite nodules, as well as tumor number and size at the time of primary hepatectomy and/or transplantation should be considered. Further, intention-to-treat analyses comparing patients who underwent hepatectomy with liver cirrhosis of potentially eligible for transplantation and patients listed for primary liver transplantation showed comparable overall (5-year survival; hepatectomy *vs* listed for transplantation; 66% *vs* 58%; *P* = NS) and disease-free (41% *vs* 54%: *P* = NS) survival mainly due to the influence of waiting period[30]. Another intention-to treat analysis also showed the limited value of salvage transplantation with only 28% of transplantability rate and comparable prognosis with the patients with liver resection[31].

Salvage hepatectomy for refractory HCC after other local treatment

Here, the term "refractory HCC" is defined as HCC recognized as remnant, unresponsive, or locally recurred tumor at the site treated with locoregional treatment such as ablation or TACE. The indications for hepatectomy are dictated by the degree of background liver damage, while the indications for RFA are limited less by the degree of liver damage and more by tumor size and location, especially with respect to major vascular structures. We occasionally experience difficult complete resection after local recurrence or remnant HCC after RFA because of unclear tumor borders (Figure 1). Several studies have shown that locally recurrent HCCs after RFA were more invasive because of lower tumor differentiation grade, capsule invasion, and vascular invasion, resulting in the need for extensive liver resection with increased operation time and blood loss[32-36]. In such cases, repeat RFA is rarely indicated and salvage hepatectomy should be the first-choice treatment. The mechanism of aggressive tumor behavior is not clear. Increased intratumoral pressure by RFA may favor intravascular tumor spread[37,38]. Diffi-

Figure 2 Multinodular hepatocellular carcinomas. Transarterial chemoembolization achieved complete response in one tumor in segment Ⅳ with accumulation of lipiodol showing no arterial enhancement in contrast to the other tumor in segments Ⅵ and Ⅶ that was enhanced in the arterial phase (A) and washed out in the portal phase (B).

culty in early diagnosis of recurrence because of blended necrotic and active areas without a clear delineation may also be a factor[34]. Although recurrence after salvage hepatectomy for these recurrent tumors is frequent, with 5-year recurrence-free survival of 0%-33%, 5-year overall survival is reported as 43%-67%[34-36]. Whether surgical resection or RFA should be selected for HCC that are amenable to both treatments is a controversial issue[39,40], and which is better is still in debate. In a randomized controlled trial by Huang *et al*[41] comparing surgical resection and RFA in patients with HCC meeting the Milan criteria[42], 115 patients were enrolled in each group and both recurrence-free and overall survival was better in the resection group (resection *vs* RFA: 5-year recurrence-free survival, 51.3% *vs* 28.7%, $P = 0.017$; 5-year overall survival, 75.7% *vs* 54.8%, $P = 0.001$)[41]. Hasegawa *et al*[43] reported the results of a Japanese nationwide survey comparing the results of surgical resection, RFA, and PEI in patients with no more than three HCC tumors and with none over 3 cm. A total of 12968 patients with 5361, 5548, and 2059 patients undergoing surgical resection, RFA, and PEI, respectively, were analyzed, and the 5-year recurrence was 63.8%, 71.7%, and 76.9%, respectively (surgical resection *vs* RFA, $P = 0.0001$; RFA *vs* PEI, $P = 0.0001$) and the 5-year overall survival was 71.1%, 61.1%, and 56.3%, respectively (surgical resection *vs* RFA, $P = 0.0001$; RFA *vs* PEI, $P = 0.005$). Although these were the outcomes for the treatment of primary HCC and there have been no established evidence suggesting which of the hepatectomy or ablation is better first choice for recurrent HCC, these results suggest that surgical resection should be selected as a first-line treatment for HCC that is amenable to either surgical resection or ablation, and curative resection should be attempted for local recurrence after ablation for as long as possible.

In the treatment of multinodular HCC, surgical resection can be complementary with other nonsurgical therapies to obtain good long-term prognosis even though TACE is usually indicated for multinodular HCC, rather than surgical resection. The guidelines for HCC treatment from the American Association for the Study of Liver Diseases and the European Association for the Study of the Liver[44,45], based on the Barcelona Clinic Liver Cancer criteria[46] recommend hepatic resection only for patients with solitary tumor without portal hypertension. In the Japanese guidelines, surgical resection is indicated for patients with up to three tumors. For four or more tumors, TACE or transarterial infusion is indicated as the first-choice treatment[47]. We occasionally experience multinodular HCCs treated with repeated TACE showing complete necrosis of a large proportion of the tumors with a small number of remnant viable tumors (Figure 2). It is still unclear whether salvage hepatic resection of the remaining viable tumors is beneficial. A small number of studies have shown benefits with a multimodal approach by combining hepatic resection with simultaneous ablation[48] or reduction surgery followed by ablation and adjuvant TACE or arterial infusion therapy[49]. However, these were retrospective studies with a small number of patients and the details of the exact number of tumors were not provided. Furthermore, differentiation between intrahepatic metastasis and multicentric occurrence is important, as discussed earlier, and criteria as to the number of nodules indicated for hepatectomy remains unclear.

Hepatectomy for down-staged HCC for initially unresectable tumors

In contrast to colorectal liver metastases, in which systemic chemotherapy and/or hepatic artery infusion chemotherapy can convert the unresectable tumor to resectable in > 40% of patients[50-52], HCC conversion therapy has not been established.

Yao *et al*[53] proposed the University of California, San Francisco down-staging protocol inclusion criteria for liver transplantation as: (1) one lesion > 5 cm and up to 8 cm; (2) two to three lesions with at least one lesion > 3 cm and not exceeding 5 cm, with a total tumor diameter up to 8 cm; or (3) four to five lesions with none > 3 cm, with a total tumor diameter up to 8 cm. The authors reported that down-staging was successful in 43/61 patients (71%) and 35 patients underwent liver transplantation with a 4-year survival after transplantation

of 92%[53]. Lei *et al*[54] applied the criteria to hepatectomy and reported the outcomes of 66 of 102 patients (59%) with successful down-staging by TACE and/or RFA. Of the 66 patients, 31 and 35 patients underwent liver transplantation and hepatectomy, respectively, and both recurrence-free (68% and 60% at 5 years, respectively) and overall survival (77% and 69% at 5 years, respectively) were comparable[54]. TACE and/or hepatic artery infusion therapy is usually used as the down-staging treatment. The conversion rate from unresectable to resectable HCC by these modalities was reported as 13%-18%, with a 5-year survival of 49%-56%[55,56].

In contrast to colorectal liver metastases, in which pathologic response is correlated with the prognosis after curative hepatectomy[57], such correlation was not necessarily confirmed in patients with HCC. Of note, Ravaioli *et al*[58] reported that incomplete necrosis by TACE was an independent predictor of poor recurrence-free survival after liver transplantation. Furthermore, several studies showed that preoperative TACE was associated with an increased risk of extrahepatic metastases[59-61]. This might be explained by Adachi *et al*'s[62] hypothesis that viable HCC cells are less firmly attached and likely to spill into the bloodstream during intraoperative manipulation after incomplete response to TACE. Because complete necrosis is rarely obtained, especially for large tumors, the routine application of preoperative TACE for resectable HCC is not recommended. However, based on results showing that a proportion of patients can undergo curative resection following down-staging by TACE and obtain long-term survival, aggressive loco-regional treatment to attempt curative resection should be adopted in patients with initially unresectable HCCs.

The development of other treatment strategies for unresectable HCC such as radioembolization by yttrium-90[63] or systemic treatment combining cisplatin/interferon α-2b/doxorubicin/fluorouracil (PIAF)[64] may increase the rate of conversion. Lau *et al*[65] reported that 49 of 285 patients (17%) underwent salvage surgery following down-staging by intra-arterial yttrium-90 microspheres or PIAF for initially unresectable HCC and obtained a 5-year survival rate of 57%. Notably, 8 of the 49 patients had extrahepatic metastases initially and these patients also obtained long-term survival with a 5-year survival rate > 40% and neither the extension of the disease nor the degree of tumor pathologic response was associated with the prognosis. Although relatively high response rates are obtained with PIAF, frequent adverse events such as neutropenia and thrombocytopenia preclude wide application, especially in patients with cirrhosis[64,66]. In a recent study by Kaseb *et al*[67], an independent predictor of an objective response to PIAF was the use of five or more cycles. The authors suggested that patient selection is important because only responding patients will have an improved prognosis with curative hepatectomy.

To discuss the issue of conversion, it should be noted that the definition of "unresectable" cannot be unanimous and differ according to extension of the tumor, background liver function, and surgeons' judgments. It is also important to recognize that "technically" and "oncologically optimally" resectable are not necessarily the same. It is, however, certain that conversion rate for HCC is still unsatisfactory and the all reports referred above are retrospective studies with small number of patients. Further development of effective treatment for downstaging is expected.

CONCLUSION

Although hepatectomy is indicated for only a small proportion of patients with recurrent or down-staged HCC after primary treatment, an excellent prognosis is obtained if curative resection is achieved, especially for tumors with a multicentric occurrence pattern, rather than intrahepatic metastases. Preoperative differentiation of the two patterns is a future research issue. Even in initially unresectable HCCs, hepatectomy plays a key role in a multidisciplinary approach.

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TOPIC HIGHLIGHT

WJH 6th Anniversary Special Issues (2): Hepatocellular carcinoma

Transarterial chemoembolization for hepatocellular carcinoma: A review of techniques

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common malignant diseases worldwide. While curative therapies, including resection, liver transplantation, and percutaneous ablation (percutaneous ethanol injection and radiofrequency ablation), are applicable for only a portion of the HCC population, transcatheter arterial chemoembolization (TACE) has been recognized as an effective palliative treatment option for patients with advanced HCC. TACE is also used even for single HCCs in which it is difficult to perform surgical resection or locoregional treatment due to systemic co-morbidities or anatomical problems. TACE has become widely adopted in the treatment of HCC. By using computed tomography-angiography, TACE is capable of performing diagnosis and treatment at the same time. Furthermore, TACE plays an important role in the multidisciplinary treatment for HCC when combined with other treatment. In this review, we first discuss the history of TACE, and then review the previous findings about techniques of achieving a locoregional treatment effect (liver infarction treatment, *e.g.*, ultra-selective TACE, balloon-occluded TACE), and the use of TACE as a drug delivery system for anti-cancer agents (palliative, *e.g.*, platinum complex agents, drug-eluting beads) for multiple lesions.

Core tip: Transcatheter arterial chemoembolization (TACE) has become widely adopted in the treatment of hepatocellular carcinoma (HCC). By using computed tomography-angiography, TACE is capable of performing diagnosis and treatment at the same time. Furthermore, TACE plays an important role in the multidisciplinary treatment for HCC when combined with other treatment. In this review, we first discuss the history of TACE, and then review the previous findings about techniques of achieving a locoregional treatment effect (liver infarction treatment, *e.g.*, ultra-selective TACE, balloon-occluded TACE), and the use of TACE as a drug delivery system for anti-cancer agents (palliative, *e.g.*, platinum complex agents, drug-eluting beads) for multiple lesions.

INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for one-third

of cancer-related deaths worldwide, and has become the fourth leading cause of cancer death in Japan and the seventh leading cause of cancer death in the United States. In recent years, liver cancer deaths have decreased due to remarkable progress in the treatment of viral hepatitis in Japan, while HCC deaths remain high in the United States[1].

Underlying liver disease is present in most HCC cases. Development of HCC in a healthy liver is rare; the majority of patients who develop HCC have a background of chronic hepatitis/cirrhosis viral hepatitis, alcohol abuse, and/or non-alcoholic steatohepatitis. HCC frequently recurs after primary treatment due to the underlying liver disease[2,3].

With advances in diagnostic imaging and treatment in recent years, adaptation of radical treatment strategies such as surgical resection and radiofrequency ablation therapy is increasing. However, even in cases in whom curative treatment is selected as initial treatment, a high recurrence rate due to multi-centric carcinogenesis and intrahepatic metastasis makes it difficult for cure to truly be achieved. Transarterial chemoembolization (TACE) has been widely performed as a treatment for multifocal HCC in patients in whom curative treatment is difficult to perform[4-11].

In the Barcelona Clinic Liver Cancer staging system, TACE is indicated for patients with intermediate-stage HCC (four or more tumors), and in the 2010 Japan Society of Hepatology consensus-based treatment algorithm for HCC, TACE is recommended for patients with a Child-Pugh score A or B, tumor diameter of more than 3 cm, or four or more tumors. However, in real clinical conditions, TACE is selected even for single HCCs in which it is difficult to perform surgical resection or locoregional treatment due to systemic co-morbidities or anatomical problems[12,13].

TACE has been widely adopted in the treatment of HCC. Through the use of computed tomography (CT)-angiography, diagnosis and TACE can be performed at the same time. TACE also plays an important role in the multidisciplinary treatment of HCC as it is often combined with other treatments (*e.g.*, with radiofrequency ablation, with percutaneous ethanol injection, with radiation therapy).

In this review, we discuss the history of TACE and review previous findings about techniques for achieving a locoregional treatment effect (liver infarction treatment) and the use of TACE as a drug delivery system for anti-cancer agents (palliative) for multiple lesions.

CHANGES IN HEPATIC ARTERY CHEMOEMBOLIZATION FOR HCC

TACE induces tumor necrosis through "starvation tactics". It takes advantage of the fact that advanced HCCs are fed only by the hepatic artery and is intended to embolize the distal portion of the hepatic artery. The liver receives blood from the portal vein and hepatic artery

at a ratio of 3:1 in the normal liver. Although this ratio varies in cirrhosis, the cirrhotic liver still receives blood flow from both of these vessels. In contrast, classical HCC (moderately-differentiated type) tumors receive nutritional blood flow through the hepatic artery only, and do not depend on portal vein blood flow. By utilizing this property of HCC, TACE was developed by Yamada *et al*[4]. TACE has become widely used for the treatment of HCC since the 1980s. Embolization using adriamycin or mitomycin C and gelatin sponges has been carried out since the first half of the 1980s. Intraarterial injection of lipiodol with anti-cancer drugs before embolic agent results in enhanced embolic effects[14,15]. It also became apparent that using a water-in-oil type emulsion is highly effective for embolization and tumor uptake, and this method is also widely used[16].

Microcatheter insertion into the first three to four branches of the hepatic artery became easily available starting around 1990. Through the use of microcatheter injection of lipiodol into the peripheral branches of the hepatic artery, segmental TACE/subsegmental TACE became a standard treatment. Segmental TACE/subsegmental TACE allows for strong locoregional embolization while stopping the portal blood flow, thereby improving the local treatment effects of TACE[17,18]. In the 2000s, platinum complex agents became available, and treatment effects could be obtained even in HCCs that developed TACE resistance through repeat TACE[19]. Cone beam CT and flat-panel detectors have advanced imaging as they enable more accurate TACE treatment[20,21].

CONVENTIONAL TACE

Intra-tumor concentrations of drugs (particularly polymer drugs) are much higher than those of normal tissue and blood due to the characteristics of blood vessels in solid tumors[14]. In hypervascular HCC, blood returns to the sinusoidal or portal vein; in addition, HCC tissue does not have associated lymph vessels. These features allow stasis of viscous liquid such as lipiodol in the sinusoidal or portal vein in or around HCCs. Nakamura *et al*[22] reported that liver necrosis occurs following injection of lipiodol into the hepatic artery until it is visualized in the portal vein branch. Based on this discovery, Uchida *et al*[15] and Matsui *et al*[18] developed segmental and subsegmental TACE[15,18,22].

The use of water-soluble anti-cancer drugs along with lipiodol as water-in-oil type therapy has been reported to be good for distribution of anti-cancer drugs in HCC[16,23]. Therapy involving selective infusion into tumor vessels of an anti-cancer drug/lipiodol mixture and an embolic agent (gelatin sponge) is generally called conventional TACE (cTACE), and it is widely used as standard treatment worldwide (Table 1).

In 1983 Yamada *et al*[4] reported a 1-year survival rate of 44% for TACE. The 3-year survival rate of segmental TACE reported by Uchida *et al*[15] in 1990 was 67%, Matsui *et al*[18] reported a 4-year survival rate of 67% in

Table 1 Summary of key prospective trials and retrospective studies for the treatment of hepatocellular carcinoma

Ref.	Year	Analysis	No. of patients	Objective response (%)	Overall survival (%)		
					1 yr	2 yr	3 yr
cTACE							
Llovet et al[7]	2002	Prospective	40	35 (at 6 mo)	82	63	29
Lo et al[8]	2002	Prospective	40	39 (at 3 mo)	57	31	26
Takayasu et al[10]	2006	Prospective	8510	NA	82	63	47
DEB-TACE							
Lammer et al[52]	2010	Prospective	102	51.6 (at 6 mo)	NA	NA	NA
Sacco et al[54]	2011	Prospective	33	100 (at 1 mo)	NA	86.8	NA
Song et al[57]	2012	Retrospective	60	81.6 (at 3 mo)	88	NA	NA
Wiggermann et al[58]	2011	Retrospective	22	22.7 (at 8 mo)	70	NA	NA

TACE: Transcatheter arterial chemoembolization; cTACE: Conventional TACE; DEB: Drug-eluting bead; NA: Not available.

1993, and Takayasu *et al*[24] reported a 3-year survival rate of 77% using interventional radiology (IVR)-CT in subsegmental TACE in 2001. Thus, therapeutic outcomes of TACE have improved rapidly along with advances in TACE techniques, drugs, microcatheters, and the adaption of IVR-CT[25].

ULTRA-SELECTIVE TACE

It has recently become possible to insert microcatheters into the distal hepatic artery more safely due to progress in microcatheter and guidewire technology. Ultra-selective TACE aims to achieve a local therapeutic effect through liver infarction. This technique involves insertion of a microcatheter selectively into a peripheral rather than subsegmental branch (subsubsegment artery), thereby wedging the tumor-feeding vessels, and then injecting lipiodol under high pressure into the tumor and surrounding sinusoids.

The local recurrence rate of ultra-selective TACE has been reported to be 7.9% at 12 mo and 17.7% at 24 mo[26]. Ultra-selective TACE allows injection of lipiodol even into hypovascular lesions in well-differentiated HCC, and is reported to have a local control rate of 53.2% in such cases[27].

With respect to pathological background, in a study of patients who underwent liver resection after undergoing ultra-selective TACE through peripheral branches, necrosis of the tumor as well as the surrounding liver parenchyma was observed[28]. With the spread of fine microcatheters as a treatment aimed at liver infarction, injection of lipiodol in the peripheral rather than the subsegmental branches is becoming a standard treatment[29].

BALLOON-OCCLUDED TACE

Irie *et al*[30] reported in 2008 that better lipiodol deposition was obtained by performing selective TACE while preventing the backflow of embolic material proximally using a micro-balloon catheter, called balloon-occluded TACE (B-TACE)[30]. In conventional TACE, lipiodol suspended with an anti-cancer drug is present in the bloodstream. The blood flow may slow before sufficient lipiodol and drug have reached the tumor, and may even

stop flowing. This may occur because when the arterial blood flow is reduced, the backflow of blood to the tumor occurs from the sinusoidal and portal veins. In addition, lipiodol inflow restriction to the normal liver parenchyma is caused by a reduction in peripheral arterial pressure.

In B-TACE, hemodynamic changes caused by balloon occlusion reduce the arterial blood flow by closing the hepatic artery, thereby pushing lipiodol into the tumor under high pressure and enabling the drug to be intensively administered to the tumor, allowing for an enhanced therapeutic effect. In recent years, since micro-balloon catheters with small diameters have become more available, B-TACE has been widely used, primarily in Japan (Figure 1). In performing B-TACE, evaluation of the collateral circulation of the tumor is essential, as a good response rate is obtained if the catheter tip pressure is equal to 64 mmHg or less; the collateral circulation pressure increases above that in many cases[31].

PLATINUM COMPLEX AGENTS

In advanced HCC treatment, it is critical that TACE functions as an efficient drug delivery system. Platinum complex agents are anti-cancer agents that cause DNA damage. Unlike anthracyclines, which are excreted from the bile, platinum complex agents are not metabolized by P450, and are excreted primarily in the urine. Thus, platinum complex agents are considered to be advantageous for patients with liver cirrhosis. Cisplatin, a first-generation platinum complex, and miriplatin, a third-generation platinum complex, are both used in the treatment of HCC[32-39].

Kawamura *et al*[19] reported that a 19.6% response rate was obtained by switching the anti-cancer agent used in TACE to a platinum complex agent in cases in which the tumor number or size increased despite administration of more than one TACE treatment. Use of a platinum complex agent also resulted in a survival benefit in responders[19]. Furthermore, Maeda *et al*[40] also reported the efficacy of TACE using cisplatin in patients with HCC that had not responded to TACE using epirubicin, with a response rate of 27.5%[40].

However, cisplatin is associated with serious side ef-

Figure 1 A patient with unresectable hepatocellular carcinoma who received balloon-occluded transcatheter arterial chemoembolization with miriplatin. A: Micro balloon catheter was inserted into A8. Feeding artery was occluded using micro balloon (arrow). Miriplatin/lipiodol suspension and 1-mm gelatin sponge particles were administrated slowly under balloon occlusion; B: Treated lesion showed a dense accumulation of lipiodol (arrowhead).

fects, including renal failure and anaphylaxis. Kawaoka *et al*[33,41] reported that anaphylaxis occurs more frequently during performance of three or more than three TACE procedure with cisplatin. In recent years, miriplatin has been administered as a third-generation platinum complex that has been developed for hepatic arterial infusion therapy particularly for HCC. Miriplatin {cis-[(1R,2R)-1,2-cyclohexanediamine-N,N')bis(myristato)]-platinum(Ⅱ)monohydrate; Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan} is a novel lipophilic cisplatin derivative that can be suspended in lipiodol. Miriplatin/lipiodol suspension is a stable colloidal emulsion that is deposited within HCC tumors, where active derivatives of miriplatin are gradually released. Also, in a cisplatin-resistant rat hepatoma cell line model, miriplatin did not show cross-resistance with cisplatin[37].

Despite clinical expectations, it is difficult to obtain adequate deposition of miriplatin in HCC, and local recurrences, particularly intra-tumoral recurrences, frequently develop using selective TACE[42]. This may be due to the higher viscosity of miriplatin, since it is suspended in lipiodol; miriplatin may be retained within the artery, so a sufficient amount of the drug does not reach the tumor through narrow blood vessels.

Seko *et al*[43] reported that reduction of the miriplatin/lipiodol suspension viscosity resistance can be obtained by warming it to 40 ℃. Compared to ordinary (room temperature) miriplatin treatment, which has a response rate of 44.3%, efficiency is improved to 70.1% for warmed miriplatin treatment[43]. Kora *et al*[44] also reported similar treatment outcomes for warmed miriplatin.

Miriplatin is known to have less serious side effects and a lower incidence of renal failure compared to other platinum complex agents. Thus, miriplatin is considered to be suitable for repeat treatments, patients with complications, and elderly patients[45,46].

DRUG-ELUTING BEAD

In recent years, beginning in western countries, permanent spherical embolic material (*i.e.*, beads) have been used in TACE with the aim of more efficient drug delivery[47-49]. Unlike conventional gelatin sponges, the particle size of this material is uniform. Prediction of the level of embolism is straightforward, and a sustained embolic effect can be obtained. It is also possible to impregnate anti-cancer drugs into the beads, and the anti-tumor effect is improved due to the slow release of anti-cancer agents into the tumor.

Two formulations of drug-eluting beads (DEB) are available in Japan: Hepasphere[50] and DC Bead[51] are widely used and each have unique features. DC Bead is a raw material derived from polyvinyl alcohol that is capable of impregnation of positively-charged drugs (*e.g.*, epirubicin, doxorubicin, or irinotecan). Its size is slightly decreased, and its hardness is increased by impregnation of anti-cancer drugs. Meanwhile, Hepasphere is a raw material derived from a polymer with high water absorption and can thus be impregnated with water-soluble anti-cancer agents. The size of Hepasphere increases following impregnation, it expands to about four times its size in the blood, and the resulting embolus is highly flexible and molds to the shape of the target vessel.

In a randomized controlled trial (PRECISION V) that compared TACE using lipiodol (cTACE) to TACE using DC Bead, the complete response rate, objective response rate, and disease control rate were superior in the DC Bead group compared to the cTACE group, although these differences were not statistically significant. In addition, response rates were significantly higher in certain sub-groups, such as in patients with a Child-Pugh score B and in those with HCC in bilateral lobes[52]. Vogl *et al*[53] also reported that the incidence of decreased left heart ejection fraction, post-embolization liver enzyme elevation, and hepatobiliary system adverse events were lower in the DC Bead group compared to the cTACE group[53]. Sacco *et al*[54] reported similar results from a randomized controlled trial of DEB-TACE *vs* cTACE for unresectable HCC: post-treatment elevation of alanine aminotransferase was frequently observed in the cTACE group. However, time to progression and survival did not significantly differ between the two groups: the cumula-

tive 2-year survival rates were 86.8% in the DEB-TACE group and 83.6% in the cTACE group[54].

For TACE with epirubicin-eluting Hepasphere, Seki *et al*[55] reported a 1-mo response rate of 56.3% and a 6-mo response rate of 52.6%, using response rates as defined by the EASL criteria[55]. For the treatment of HCCs that became refractory to TACE with epirubicin-eluting Hepasphere, changing the impregnated anti-cancer drug to cisplatin resulted in a response at 6 mo in 40% of patients[56].

Several retrospective studies showed the safety and efficacy in DEB-TACE group were significantly higher than in cTACE group (Table 1)[57-59].

However, clear evidence of DEB-TACE superiority compared to cTACE has not been established to date.

LIMITATIONS

There are limitations in this review. First, this is not a systemic review. Therefore, this article may have the potential biases of the authors. Second, we mostly described Japanese history of TACE for HCC in this review.

CONCLUSION

Improvement of the therapeutic effects of TACE treatment for HCC has been obtained by progression in techniques, drugs, and therapeutic equipment. In a variety of TACE treatments, selecting the anti-cancer agents, treatment methods and equipment for the best therapeutic effect is becoming more important. In the future, it is necessary to clarify the optimal treatment choices for each HCC patient.

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TOPIC HIGHLIGHT

WJH 6th Anniversary Special Issues (3): Hepatitis B virus

How did hepatitis B virus effect the host genome in the last decade?

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INTRODUCTION

There are more than 350 million people who are infected by hepatitis B virus (HBV) throughout the world[1]. HBV is the main cause of some liver illnesses, such as chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC)[2]. The World Health Organization classifies HBV in "group 1" as the consequential oncogenic factor after tobacco smoking. It has been estimated that due to late diagnosis and limited treatment options, after lung and stomach cancer, HCC is third prominent cause of cancer related death and nearly 53% of HCC cases has a connection with HBV. The incidence of HCC changes according to geographical conditions. Chronic HBV infection is the most important risk factor for HCC in the world. Other risk factors such as chronic hepatitis C virus (HCV), hepatitis D virus or human immunodeficiency virus infection, aflotoxin B_1 exposure, metabolic factors as obesity and diabetes and alcohol abuse increase the comparative endanger for tumor progression when coexist with HBV infection. Moreover, demographic factors such as Asian or African ancestry, male sex or advanced age are the synergistic effects that have been reported to raise the possibility of HCC in chronic HBV-infected individuals[3-7].

Even though many pathways and factors contributing to HCC development have been identified, many features of hepatocellular carcinogenesis and direct role of viral factors are difficult to define[3]. However, HBV infection is the main risk factor for HCC development. Not only in HCC, but also in chronic HBV patients and chronic HBV carriers some mutagenic effects of HBV on somatic cells

Abstract

The principal reason of chronic liver disease, cirrhosis and hepatocellular carcinoma is chronic viral hepatitis all over the world. Hepatitis B virus (HBV) has some mutagenic effects on the host genome. HBV may be exhibiting these mutagenic effects through integrating into the host genome, through its viral proteins or through some epigenetic mechanisms related with HBV proteins. This review aims to summarize the molecular mechanisms used by HBV for effecting host genome determined in the last decade. The focus will be on the effects of integration, HBV proteins, especially HBV X protein and epigenetic mechanisms on the host genome. These interactions between HBV and the host genome also forms the underlying mechanisms of the evolution of hepatocellular carcinoma.

Key words: Hepatitis B virus; Host genome; Integration; Hepatitis B virus proteins; Epigenetic

Core tip: Hepatitis B virus (HBV) has some mutagenic effects on the host genome. This review aims to summarize the molecular mechanisms used by HBV for effecting host genome determined in the last decade.

are detected. For example in our study, we proofed the genotoxic effects of HBV on peripheral blood lymphocytes of chronic HBV patients and chronic HBV carriers[8]. Ucur *et al*[9] showed the increased sister chromatid exchange frequency and low mitotoic index; Bolukbas *et al*[10] and Grossi *et al*[11] demonstrated DNA damage using the alkaline comet assay, in peripheral blood lymphocytes. HBV may harm to host DNA in many ways. But simply it can be categorized in at least 3 different mechanisms: First, the viral DNA integration in the host genome can induce chromosome instability, although HBV usually persists as an episome and the integrate genomes are dead and can no longer drive HBV replication. Second, insertional mutations of *HBV* are known to activate many genes and promote genetic alterations in the host genome. The third mechanism is based on viral proteins and gene products of sporadically truncated *HBV* genes from integrated HBV DNA[3,5]. While a variety of manuscripts have been published about these mechanisms separately, here a short review describing effects of HBV on host genome in the last decade is given. The focus of this review will be on integration, proteins and epigenetic mechanisms of HBV.

DIRECT EFFECTS OF INTEGRATION ACTIVATED BY THE HBV DNA ON THE HOST GENOME

Integration is not necessary for the viral replication but it enables viral genomic persistence. Long term chronic inflammation related to continuous cycles of cell death and proliferation increases the amounts of DNA ends in host genomic DNA, thus supporting the viral integration. Cellular topoisomerase I is a crucial factor in the linearization and integration of viral replicative mediators[3,7]. Several kinds of changes in the sequence of HBV genome have been identified, but inverted duplications and the deletions are the most common alterations[12]. Disclosure to oxidative stress or mutagenic agents, loss of DNA repair capacity, high hepatocyte turnover due to inflammation and/or coinfection with other viruses may be the reason of HBV DNA integration[3,7]. In these conditions the genome is more unstable and tend to the devolopment of deletions, single- or double-stranded breaks or rearrangements[12]. HBV integration can induce rearrangements and/or partial deletions at the integration site of the host chromosome[6]. Integration happens by chance in the contex of human genomes and may ocur at the various places of different chromosomes[7]. Translocations, production of fusion transcripts, chromosomal deletions and generalized genomic instability may be caused by these integrations, and alterations probably cause the choosing of hepatocyte clones that have a growth profit[3,12]. Liver carcinogenesis may occur as a result of HBV integration that cause a significant increase in an anti-apoptotic or oncogenic signal[12]. Using a polymerase chain reaction (PCR) based approach (*i.e.*, inverse PCR, Alu PCR, restriction site PCR)[12], it was confirmed that insertion of HBV into cellular genome is an event that happens during HBV infection even after acute self-limiting hepatitis[6]. In 85%-90% of HBV-related HCC, integrated viral DNA has been detected[3,7].

Most of the integration events reported occur near or within common fragile sites (large genomic regions that are liable to deletions, breaks, chromosomal rearrangements and gene amplifications)[12], in genes managing proliferation control, cellular signal transduction cascades, cell viability[6] or Alu sequences and microsatellites (other repetitive human genomic regions) that are liable to instability in carcinogenesis development and growth. Integrated viral DNA into coding regions or cellular regulatory regions of the genome may alter gene expression (cis-activation) or change the structure and function of the produced cellular proteins which possibly cause malignant transformation[3,7]. Insertion of viral DNA may also cause hepatocellular malign transformation *via* the production of mutated viral proteins such as preS/S proteins or truncated X proteins which may trigger signalling cascades in carcinogenesis (trans-activation)[7]. Moreover, HBV genome has enhancer elements that can activate heterologous promoters in a orientation- and position-independent manner[13]. The study of many groups suggest that the HBV enhancers are able to transactivate cellular genes up to 100 kb distant from the integration site[12].

In the late 1970s and early 1980s, the primer investigations defining cellular genomic regions of HBV integrations were carried out[12]. In humans, the recurrent integration of the viral genome into or in the proximity of a host genome has been suggested for HBV in 1987 and in the development of HCC in 2000s. In fact, woodchuck hepatitis virus causes liver cancer by targeting myc oncogenes (*N-myc*, *c-myc*, and *N-myc2*) and some cases of HBV integration into important cellular genes have been informed in human liver tumors (*e.g.*, retinoic acid receptor beta, cyclin A). However, HBV integration regions targeting host genes have not been defined in the past[13]. But in the last decade, there have been many investigations focusing on the integration sites of HBV and insertional mutagenesis seen in HCC.

Paterlini-Bréchot *et al*[13] have isolated nine DNA integration regions from nine hepatocellular carcinomas, demonstrating that the viral genome make mutations in the important regulatory host genes by using HBV-Alu PCR. These genes have a role in cell proliferation and/or differantiation and/or survival: interleukin (IL)-1R-associated kinase 2 gene, neurotropic tyrosin receptor kinase 2 gene, inositol 1,4,5-triphosphate receptor type 2 (*IP3R2*) gene, p42 mitogen-activated protein kinase 1 (*p42MAPK1*) gene, alpha 2,3 sialytransferase gene, *IP3R1* gene, EMX2-like gene, human telomerase reverse transcriptase (*hTERT*) gene and thyroid hormone uncoupling protein gene (Table 1). Different genes, which were located at the HBV DNA integration region, have a specific key cellular function or share common cell signalling cascades. Also, they found that HBV targets both the telomerase gene

Table 1 Genes targeted by hepatitis B virus DNA integration

Gene	Description	Ref.
NTRK2	Neurotropic tyrosin receptor kinase 2	Paterlini-Bréchot et al[13]
IRAK2	Interleukin-1R-associated kinase 2	Paterlini-Bréchot et al[13]
MAPK1	Mitogen-activated protein kinase 1	Paterlini-Bréchot et al[13]
IP3R2	İnositol 1,4,5-triphosphate receptor type 2	Paterlini-Bréchot et al[13]
IP3R1	İnositol 1,4,5-triphosphate receptor type 1	Paterlini-Bréchot et al[13]
ST3GAL VI	Alpha 2,3 sialytransferase	Paterlini-Bréchot et al[13]
TRUP	Thyroid hormone uncoupling protein	Paterlini-Bréchot et al[13]
EMX2-like	Empty spiracles 2-like	Paterlini-Bréchot et al[13]
hTERT	Human telomerase reverse transcriptase	Paterlini-Bréchot et al[13]
WBSCR1	Williams-Beuren syndrome critical region 1	Kimbi et al[14]
AXIN1	Axis inhibitor 1	Minami et al[16]
BBX	Bobby sox	Minami et al[16]
CTNND2	Catenin delta-2	Minami et al[16]
EYA3	Eyes absent 3	Minami et al[16]
ODZ2	Odd Oz 2	Minami et al[16]
TERT	Telomerase reverse transcriptase	Sung et al[18]
MLL4	Mixed-lineage leukemia 4	Sung et al[18]
CCNE1	Cyclin E 1	Sung et al[18]
FN1	Fibronectin 1	Ding et al[21]
SMAD5	SMAD family member 5	Ding et al[21]
PHACTR4	Phosphatase and actin regulator 4	Ding et al[21]

and *IP3R* gene in two different tumors. This data shows viral integration may target some preferential regions, especially *hTERT* gene.

Kimbi *et al*[14] amplified HBV and chromosomal DNA from the sera of five patients with uncomplicated acute hepatitis B and one with fulminant disease. In one patient with uncomplicated disease, HBV DNA was integrated into host chromosome 7q11.23 in the Williams-Beuren syndrome critical region 1 gene (Table 1). This gene contains a high abundance of Alu repeats, repetitive elements that have shown to be the preferred sites for recombination and for HBV DNA insertion. Moreover, the integrant is within the region commonly deleted in patients with Williams-Beuren syndrome, giving rise to loss of heterozygosity. The investigators also mentioned that clonal expansion of an integrant is required for tumor formation and early integration of HBV DNA might have a role in HBV-induced hepatocarcinogenesis. It is because the integrated viral DNA in early infection is in accordance with the observation, that importation of linear DNA into the nucleus is necessary for insertion of viral DNA into chromosomal DNA, and that such importation is known to occur during the initiation of infection. This data was also examined by Murakami *et al*[15] in the study of detecting the possible persistence of integrated genomes in peripheral blood mononuclear cells (PBMCs) and the exact position of the viral genome, after the clearence of serum HBV surface antigen (HBsAg). Their results showed that HBV genome integrates early during acute viral infections and persists in an integrated form in PBMCs. Another study providing the proof of HBV integration at an early stage of chronic infection in hepatocytes was carried out by Minami *et al*[16]. They examined virus-cellular gene junctions in chronic hepatitis tissues without HCC and by analysing six patients 42 independent viral-host junctions have been obtained

and chromosomal locations for 20 of the 42 junctions have been shown. Each integration evidently influenced a single clone in six clones. Among these six genes, axis inhibitor 1 is believed to function as a tumor suppressor; eyes absent 3, homolog of odd Oz 2 and homolog of bobby sox are human homolog of drosophila genes that are critical for organ development, but their roles are still mysterious; catenin delta-2 is an oncogene (Table 1). Moreover, in contrast to the previous claim that HBV integration happens randomly, this study suggests that the integration of HBV into chromosome 3 is preferential.

Mixed-lineage leukemia (*MLL*) 2 and 4 genes are also suggested as preferential targets for HBV DNA integration[2,17]. In addition to TERT and cyclin E 1 genes, Sung *et al*[18] showed integration at *MLL4* gene as previous studies (Table 1). MLL4 locates on chromosome 19q13.1 where an amplification or a frequent rearrangement has been shown in solid tumors. After integration, site specific expression such as HBV X (HBx)/MLL4 proteins and chimeric HBx/MLL4 transcripts proposing an insertional mutagenesis that could functionally have a connection with liver carcinogenesis[2].

New accesses have been improved to identify unique integration sites in high-throughput manner using the next generation sequencing (NGS) technologies, which prevented biased identification and preferential amplification of unique integration sites. In the researches of Sung *et al*[18], Fujimoto *et al*[19] and Jiang *et al*[20] they used complete genomic sequencing to identify genome wide HBV integration by the advantages in NGS technologies. Considering that, whole genome sequencing is overpriced for sequencing wide amounts of specimen, Ding *et al*[21] have improved an optional method for deep sequencing and amplification that joins ligation mediated PCR to Illumina's paired-end adapters. This effective and cheaper method have been called massive anchored parallel se-

quencing (MAPS) method. By this method, in addition to two familiar recurrent target genes, fibronectin 1 and TERT1, they identified novel target genes for HBV integration such as actin regulator 4, SMAD family member 5 and phosphatase (Table 1). They also found that HBV integration prefered chromosome 17 and mostly integrated into human transcriptional sites.

Occult HBV infections (OHBI) have the presence of HBV DNA but are short of available serum HBsAg. Occult infection mechanism is still mysterious, however, many acceptable pathways, such as maintenance in PBMCs and integration into human genomic regions exist. Bhargava et al[22] planned to research the molecular pathways lying beneath the DNA damage response activated as a result of OHBI in host cells in their investigation. They found that OHBI causes DNA damage in peripheral blood lymphocytes. It was also found that there was a strong relationship between OHBI and oxidative stress. On the other hand, Pollicino et al[23] reported a case of 43 years old man seronegative for HBV and HCV infections and positive for HFE-haemochromatosis, who developed HCC in the lack of severe liver damage. In this study they tried to evaluate the occult HBV infection. HBV-Alu PCR showed HBV integration. This integrant was placed upstream of the partitioning-defective-6-homolog-gamma gene (PARD6G) and this gene had overexpression in tumor tissues when we compare it to non-tumor liver tissues. Being a target of transforming growth factor-beta in the tumor invasion and metastasis, PARD6G is included in the polarized migration of cells, establishment of cell polarization. These two studies show that OHBI lead to deregulation of gene expression and may alter the oncogenic pathways.

HBV integration effectively surveys the human genome, exerting insertional mutation pressure, and thus may expand the oncogenic opportunities for patients infected by HBV. The most dominant HBV integration sites occur MLL and hTERT genes. Moreover, there are many candidate genes such as 60S ribosomal protein genes, platelet-derived growth factor receptor, calcium signalling related genes[20,24]. Bok et al[25] proposed 3 different models for gene activation in HBV DNA integration on chromosome 11q13 in the SNU cell line: (1) viral integration induces genetic changes and activation of gene expression at the integration site without gene amplification; (2) viral DNA induces gene amplification, causing overexpression during integration and rearrangement; and (3) gene activation is related to gene amplification, regardless of viral integration. These models might also be available for all other gene activation mechanisms in HBV DNA integration. The target sites and integration mechanisms will give information for key genes and pathways included in development of not only HBV and but also non-HBV-induced cancers.

EFFECTS OF HBV PROTEINS ON THE HOST GENOME

Several studies have reported about the procarcinogenic effects of HBV proteins or their randomly truncated transcripts after integration. This part will focus on the effects of HBV proteins, especially X protein, on the host genome in the last decade.

HBx protein

HBx, is a X open reading frame encoded small polypeptide of 154 amino acids, usually produced at very limited amounts during chronic and acute HBV infection. HBx can be found in the cytoplasm of infected hepatocytes and at low level in the nucleus. A variety of HBx functions are still enigmatic[3,6].

The clinical importance of HBx starts with the integration of HBV DNA into the chronic HBV carriers' hepotocytes genome. X gene is generally preserved in the integrants, and HBx is frequently seen in malignant hepatocytes of chronic HBV carriers[26]. The integrated HBx often have rearranged forms and may show many deletions, truncation with fusion to cellular DNA or point mutations[7]. One significant information derived from the researches of HBV integration was that 3'-end X gene was frequently deleted in HCC cells, and this causes the COOH-terminal truncated HBx protein. This protein, rather than the full length HBx, is needed and adequate to cause HCC[27]. So as to understand the relation between HBV integration and HCC development, Wang et al[28] isolated and characterized integrated HBV in 14 primer cases of HCC. The findings showed that C-terminal X protein caused by 3'-deleted X gene was observed in 10 samples as a result of HBV integration. These deletions lead to the losses of transcription factor Sp1 binding site, p53-dependent transcriptional repression binding site, and growth-suppressive effect domain, causing cell transformation and proliferation. This result proposes that 3'-deleted X gene may have a significant role in the HCC development.

HBx has some controversal effects like pro-proliferative effects and induction of cell cycle arrest or prevention and initiation of apoptosis[3,6]. HBx effects the expression of several genes that are included in signal transduction pathways, metastasis, transcriptional regulation, immune response, metabolism, control of the cell cycle, proliferation and the apoptosis[6,26].

HBx changes expression of cellular gene by triggering cytoplasmic signal transduction pathways [e.g., ras, nuclear factor kappa B (NF-κB), src, activator protein-1 (AP-1), Jak/STAT, PI3K/Akt, Wnt] and by binding to nuclear transcription factors [e.g., activating transcription factor 2 (ATF-2), cAMP responsive element-binding protein (CREB), Oct-1, basal transcription factors], and they both help cell growth and survival[1]. HBx localizations (cytoplasm and nucleus) are associated with different functions. HBx, placed in the nucleus, is proposed to interfere directly with transcription factors or to use a function like a transcription factor. A direct relationship between ATF-2 and CREB concluding in their raised DNA binding affinity[6]. HBx placed in the cytoplasm, where it interferes with and stimulates protein kinases, including IKK, protein kinase C, Jak/STAT, PI3K, pro-

tein kinaseB/Akt, and stres activated protein kinase/Jun N-terminal kinase[26].

HBx is an activator of transcription factor NFϰB. HBx stimulated NF-κB promotes liver cells to survive against Fas-mediated apoptosis[26]. However, Zhang et al[29] showed another function of NF-κB in their study. Calpain small subunit 1 (Capn4) is included in the HCC metastasis and upregulated in the tissues of HCC. They supposed that HBx might assist migration of hepatoma cell by Capn4. Their results revealed that HBx could up-regulate the Capn4 expression at the mRNA and protein levels, and increase Capn4 promoter activity. Interestingly, they found that the inhibition of NF-κB could attenuate the upregulatin of Capn4. Thus, they concluded that HBx upregulate Capn4 through NF-κB/65 to promote migra-tion of hepatoma cells. In another study, Zhou et al[30] showed the migration of leukocytes in a NF-κB related pathway. Interferon-γ inducible protein 10 (IP-10) in-volves in cellular immune damage and inflammatory cell recruitment during virus infection. In their study, Zhou et al[30] demonstrated that HBx increases IP-10 expression and the effect of HBx on IP-10 induction is blocked by the addition of the NF-κB inhibitor. Consequently, they reported that HBx affects NF-κB pathway which leads to IP-10 promoter transactivation and then increases leuko-cyte migration, thus causes immune pathological injury of liver.

HBx interacts with transcription machinery, in ad-dition, there is evidence that HBx involves in the stages of apoptosis. HBx affects the regulation of apoptosis through its role on survivin, caspases, and mitochondria. It has been shown that HBx blocks caspase 3 activity[26]. The elevation of cytosolic calcium signals seems to play a possible role in stimulation of cell proliferation and transcription pathways. Direct interaction of HBx with endoplasmic reticulum (ER) and mitochondria as well as integration events of the X open reading frame were reported to alter intracellular calcium homeostasis[3]. Having a role in caspase-3-dependent pathway, HBx pertubs homeostasis of intracellular Ca^{2+}. This is an im-portant effect in the control of HBx-related apoptosis. HBx possibly have a contact with Bcl-2 during hepatic apoptosis. Proapoptotic activity of HBx bypasses or gets over the Bcl-2 inhibitory effect[26]. Survivin is a apoptosis preventer protein and is overexpressed in a majority of human tumors. HBx can upregulate the expression of survivin in hepatic tumor cells[31]. Moreover, several fac-tors, for example transforming growth factor (TGF)-β, induce PI3K and its downstream target, protein kinase B/Akt, to inhibit apoptosis. HBx downregulates TGF-β-induced apoptosis in hepatocytes by stimulating activity of PI3K[26].

UV-damaged DNA binding protein 1 (DDB1) works as an E3 ubiquitin ligase complex subunit[32] and has been shown to help cell cycle regulation and DNA repair[26,32]. HBx binds to DDB1 and by this way replication of HBV genome is stimulated in the nuclear compartment of cells. HBx needs this nuclear interaction with DDB1 also for interfering with cell viability. It has been demon-strated that HBx triggers lagging chromosomes during mitosis, which then causes arrangement of abnormal mitotic spindles and cells with multinucleus. These for-mations demand the binding of HBx to DDB1. Thus, this binding may induce genetic instability in regenerating hepatocytes; therefore causes to HCC development[32].

The human p53 genes' transcriptional repression is caused by HBx and it has capacity to bind to the p53. HBx C-terminal region is needed for sustaining the p53 in the cytoplasm and blocking the p53-mediated apopto-sis. However, a tremendous excess of p53 is found when it is compared to HBx in the hepatocytes[6,26]. It seems that the anti and proapoptotic effects of HBx depends on the status of hepatocyte differantiation[3].

Telomerase which adds repetitive DNA sequences to the telomeres, the ends of the chromosomes, is a ribonu-cleoprotein. By this way it prevents telomere shortening and cell death[33]. Telomerase activation has been implied in immortalization and malignant transformation of cells in vitro and is a vital step in tumor and cellular senes-ence[3,26]. Although telomerase is a complex comprising a catalytic subunit (hTERT) and an RNA component (hTER), hTERT is the crucial factor of telomerase activ-ity in human cells[34]. High levels of hTERT mRNA in HCC of several grades were found by researchers and they originate in cells which have gone through the mo-lecular changes of the first steps of hepatocarcinogen-esis[33]. It has shown that HBx gene can up-regulate the transcriptional expression of hTERT mRNA[35]. In the study of Su et al[36], it was found that by transcriptionally repressing its promoter, HBx down-regulated the human telomerase expression. They evaluated human telomerase promoter and identified myc-associated zing finger pro-tein (MAZ) as a transcriptional repressor of the promot-er in order to find out the molecular mechanism. It was found that the physical association of HBx with MAZ, supresses human telomerase by enhancing MAZ binding to its consensus sequence in the promoter. In this situa-tion, HBx acts as a transcriptional corepressor.

HBx can also lead to both stabilization of hypoxia-inducible factor-1 and overexpression of vascular endo-thelial growth factor gene. It seems HBx causes carcino-genesis via the alteration of angiogenic pathways[7].

In addition to being involved in angiogenic pathways, HBx can contribute to tumor cell invasion by the way that includes the up-regulation of heat shock protein 90 alpha (HSP90alpha). HSP90alpha isoform is an ATP-dependent molecular chaperone which sustains the effec-tive structure of client oncoproteins in tumor cells. Li et al[37] showed that HBx triggers expression of Hsp90alpha at the transcriptional level. HBx is directly included in the HSP90alpha transcriptional activation mediated by c-myc. Moreover, by activation of Ras/Raf/ERK1/2 cascades HBx triggers c-Myc expression, which causes c-Myc-mediated HSP90alpha promoter activation first and then HSP90alpha expression up-regulation. HSP70 and HSP60 have also been shown as a HBx cellular targets[26].

In conclusion, the HBx protein is a multifunctional and very important viral protein in the initiation of he-

patocellular transformation and cell survival during the HBV infection. It interacts with alot of molecules and involves in many cellular pathways. Variations in the role of HBx in hepatocarcinogenesis may be due to hepatocyte differantiation, different functions of truncated X protein and amount of expressed HBx. More information about HBx might highlight many mechanisms involved in HCC and give insights and tools for therapeutic means.

Surface proteins

Large hepatitis B (LHBs) and Middle hepatitis B (MHBs) virus surface proteins are encoded by the preS1/preS2 sequences of HBV. Experimental data revealed that HBV *preS/S* genes truncated at the 3' end and integrated into the cellular genes have a transcriptional activator function and encode proteins that accumulate in the ER. These *preS/S^t* genes encoded for C-terminally truncated surface proteins (MHBS^t) exhibit regulatory functions, such as the transactivation of host genes involving c-Ha-ras, c-myc, and c-fos oncogenes and the precise activation of the c-Raf-1/MEK/Erk2 signaling essential for AP-1 and NF-κB activation. Described processes result in increased hepatocyte proliferation[3,7].

HBV surface proteins accumulate in ER. Accumulation of proteins in ER is known to trigger apoptosis in the presence of prolonged and severe stress due to an induction of an oxidative stres[3]. PreS-mutant LHBs might also accumulate in ER and with induction of genomic instability and oxidative DNA damage, they become the reason of stress-signalling pathways. This also causes defective DNA damage response and repair in liver cells expressing HBV surface antigen[38]. Moreover, centrosome multiplication and the overexpression of both cyclin A and cyclooxygenase 2 might be caused by pre-S2 mutant proteins, therefore inducing cell cycle progression, chromosome instability and proliferation of hepatocytes in HBV related HCC[3,7]. Churin *et al*[39] also demonstrated that the expression of HBV surface proteins in the liver of transgenic mice induces phosphorylation of eukaryotic initiation factor 2α (eIF2α) and protein kinase like ER kinase activation. eIF2α phosphorylation resulted in activation of ER stress markers and a proapoptotic protein. Morover, by searching on two groups of mice with different genetic backround, they showed that hepatic HBV surface protein expression induced tumor development and fibrosis depends on host genetic backround.

HBV surface proteins may also alter the expression of some host genes with known functions as proved in the research of Rao *et al*[40]. It has shown that HBV-encoded small surface protein (SHBs) has an influence on hepatic cell expression of host genes related to fatty acid synthesis and decomposition.

Core protein

The HBV core protein (HBc) is a 21-22 kDa protein that affects the human immune response. HBV genome encode for the core protein to form the viral capsid[3]. HBc may interact with the human genome in HBV in-

fected liver cells. HBc was demonstrated to suppress the the human carcinogenesis related genes expression, p53 and interferon beta; inhibits tumor necrosis factor-related apoptosis-inducing ligand induced apoptosis in hepatocytes *via* blocking gene expression of the pathway-associated death receptor[41].

However, it is not known how HBc interacts with human genome. Guo *et al*[41] examined the distribution of HBc binding to promoters in the human genome and assessed its effects on the associated genes' expression. It was shown that HBc antibody immunoprecipitated nearly 3100 human gene promoters. The high CpG density promoters were found most commonly among this set of gene promoters. HBc is able to bind to 41 gene promoters of the WNT/β-catenin signalling pathways and 64 gene promoters of the MAPK pathways. These are two most important pathways involved in HBV-related HCC. Moreover, HBc is able to increase host NF-κB DNA-binding ability, thus in order to enhance or inhibit other host nuclear proteins' transcriptional activator functions, HBc may bind to and interact with them. As a result, HBc can bind to a great amount of human gene promoters throughout the whole human genome. This key finding may represent one of the pathogenic mechanism of HBV infection.

EPIGENETIC EFFECTS OF HBV ON THE HOST DNA

According to the recent researches virus and host interactions also happen epigenetically. Interactions between epigenetic machinery and the viral proteins may cause changes in the epigenetic landscape of the cell thus causing cancer. Histone modifications and DNA methylation are epigenetic mechanisms which have an important role during HBV replication[42].

Bisulfite sequencing is the gold standart technique to analyze the methylation state of individual cytosines. In order to acquire complete DNA methylomes of double-stranded DNA viruses like HBV, Fernandez *et al*[43] joined the use of model organisms and bisulfite genomic sequencing of multiple clones. Most importantly, they found that DNA methylated *HBVgp2* and *HBVgp4* genes, which respectively code for the S and C viral proteins, have lost their expression. It was also shown in their sequencing data that the vast majority of the HBV genomes kept the *HBVgp3* gene coding for the X protein in an unmethylated condition. Interestingly, HBX protein might regulate DNA methyltransferase (DNMT) activity.

The expression of DNMTs, which catalyze the addition of a methyl group to the cytosine ring of the 5'-CpG dinucleotide, is often raised in livers infected with HBV and also in HCC[7,44]. The roles of the major methyltransferases have the following roles: DNMT1 adds methyl groups to the hemimethylated CpG dinucleotides and has an important function in the supply of methylation during cell division; DNMT2 lacks DNA methyltransferase capabilities and seems to take part in adding methyl

groups to the structural RNA; DNMT3a and DNMT3b is able to methylate not only unmethylated, but also hemi-methylated CpG dinucleotides[45]. Several studies have reported that both HCC and HBV-infected cells exhibit increased levels of DNMT1, DNMT3a and DNMT3b and aberrant DNA methylation[44].

It was reported that the overexpression of HBx protein induces transcription of *DNMT1*, *DNMT3a* and *DNMT3b* genes and directly interacts and activates the *de novo* methyltransferase DNMT3a. It suggests that this viral protein may be responsible for methylator phenotype in HBV related HCC[7,44].

In their study, Vivekanandan *et al*[45] have revealed that HBV infection up-regulates DNMTs, which then causes the methylation of HBV DNA by DNMT3a. This methylation causes the production of pregenomic RNA, viral mRNA, and a decreased production in viral protein. However, in the same cells, the up-regulation of DNMTs also causes methylation of host CpG islands overlapping gene promoters related to carcinoma[45]. It was demonstrated that specific gene promoters are methylated in HBV infected cells such as metallothionein-1F, IGFBP3, SUFU, and TIRAP[44,45]. HBx may also inhibits transcription of E-cadherin[46], p16[INK4A] and glutathione S-transferase P1 *via* CpG methylation of the regulatory elements[7]. Some immunoregulatory genes active against HBV can also be methylated. For instance, HBV replication is able to cause *de novo* methylation and decrease the IL-4 expression, that favors the virus because HBV replication is repressed by the IL-4 expression[45].

In addition to methylation processes, it has also been shown that HBx associates with components of histone modification machinery, such as HDAC and CBP/p300 HAT. So it has an effect on gene expression[42].

In summary, HBV infection up-regulates DNMTs in hepatocytes. This up-regulation causes methylation of viral DNA, however specific and critic genes in the host genome can also be methylated. It is certain that, these modifications are significant factors in the development of HCC.

CONCLUSION

At present, in terms of the experimental approaches used, such as PCRs, gene clonings, microarrays, immunohistochemical methods, it is possible to explore the effects of HBV on the host genome. In the last decade, the investigations about the pathogenic effects of HBV on the host genome have mostly focused on HCC development. During this development period, a dominant oncogene isn't encoded by HBV genome, however it uses multifactorial pathogenic mechanisms. In addition to direct mechanisms, which are mainly represented by integration of HBV DNA into the cellular genes and by the production of proteins with transforming capacities, indirect mechanisms involving HBV proteins disrupting vital molecular pathways may be seen in HCC development. Although it is thought that HBV DNA

integrations are random and there is no specific region of integration, it is not fully incidental but instead seems to be partly optional. Special integration sites change the expression of various components of the cell cycle, signalling, transcription and apoptotic pathways. It is also shown that the formation of truncated HBV proteins, the activation of host genes by integrated HBV enhancer sequences and modifications in the epigenetic machinery of the host cell are important carcinogenic mechanisms in HCCs.

Although the main risk factors for HBV-induced hepatocarcinogenesis are well known, we still lack a deeper understanding of molecular pathways disrupted by HBV and the relationship between key molecules in these pathways. Therefore, molecular understanding of the mechanisms in HBV-related HCC is necessary for defining risks and identifying novel therapeutic approaches.

Cloning and fully characterizing HBV integrations, phage library construction and sequencing are still the gold standards. Recently, several new methods have been developed using NGS technologies avoiding optional amplification and biased identification of unique integration sites. For example, complete genomic sequencing and an efficient, cost effective method, MAPS, have been developed in high-throughput manner. In the future, novel easy and not time consuming experimental approaches and new HCC animal models might be developed to invent promising therapeutic approaches. The specific antiviral new drugs reducing the chance of integration, decreasing or preventing the harmful epigenetic effects and blocking HBV proteins related with HCC development might provide basis for future and might give hope to the patients.

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WJH 6th Anniversary Special Issues (3): Hepatitis B virus

Occult hepatitis B virus infection

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Abstract

Occult hepatitis B virus (HBV) infection (OBI) refers to the presence of HBV DNA in the absence of detectable hepatitis B surface antigen. Since OBI was first described in the late 1970s, there has been increasing interest in this topic. The prevalence of OBI varies according to the different endemicity of HBV infection, cohort characteristics, and sensitivity and specificity of the methods used for detection. Although the exact mechanism of OBI has not been proved, intrahepatic persistence of viral covalently closed circular DNA under the host's strong immune suppression of HBV replication and gene expression seems to be a cause. OBI has important clinical significance in several conditions. First, OBI can be transmitted through transfusion, organ transplantation including orthotopic liver transplantation, or hemodialysis. Donor screening before blood transfusion, prophylaxis for high-risk organ transplantation recipients, and dialysis-specific infection-control programs should be considered to reduce the risk of transmission. Second, OBI may reactivate and cause acute hepatitis in immunocompromised patients or those receiving chemotherapy. Close HBV DNA monitoring and timely antiviral treatment can prevent HBV reactivation and consequent clinical deterioration. Third, OBI may contribute to the progression of hepatic fibrosis in patients with chronic liver disease including hepatitis C. Finally, OBI seems to be a risk factor for hepatocellular carcinoma by its direct proto-oncogenic effect and by indirectly causing persistent hepatic inflammation and fibrosis. However, this needs further investigation. We review published reports in the literature to gain an overview of the status of OBI and emphasize the clinical importance of OBI.

Key words: Occult hepatitis B virus infection; Transmission; Reactivation; Chronic liver disease; Hepatocellular carcinoma

Core tip: Occult hepatitis B virus infection (OBI) is defined by the presence of hepatitis B virus (HBV) DNA without detectable hepatitis B surface antigen. The prevalence of OBI varies according to the different endemicity of HBV infection, cohort characteristics, and detection methods. Increasing research on OBI has been conducted with respect to the following: (1) transmission through transfusion, organ transplantation, or hemodialysis; (2) reactivation in an immunosuppression state; (3) contribution to the progression of chronic liver disease; and (4) increased risk for hepatocellular carcinoma. Further studies are needed to establish its clinical significance and management.

INTRODUCTION

Hepatitis B virus (HBV) infection is an important global

public issue. Approximately 2 billion people have serologic markers of HBV worldwide, 360 million of whom have chronic HBV infection. The natural course of HBV infection is determined by the interactions between the host and the virus. Chronic HBV infection is characterized by persistent HBV surface antigen (HBsAg) positivity and viremia. In the past, clearance of HBsAg expression in patients with chronic hepatitis B was considered as disease remission and disappearance of viral DNA[1]. However, the "occult" or "silent" form of HBV infection was first reported in the late 1970s in blood donors with anti-hepatitis B core (anti-HBc) antibody without HBsAg who transmitted hepatitis B[1,2]. The meaning of this clinical entity was reviewed in 1998 by a panel of European and US scientists as a part of the serological pattern "anti-HBc" alone, although the term occult was not used at that time[3]. Increasing data showed persistent low levels of HBV DNA in serum and liver tissues after HBsAg clearance was observed during acute self-limited or chronic HBV infection. Demonstration of this clinical entity has brought about the concept of "occult" or "silent" HBV infection, indicating the presence of HBV DNA in the absence of detectable HBsAg[3,4]. Owing to the development of highly sensitive molecular biology techniques, the clinical and virologic features of occult hepatitis B virus infection (OBI) have been revealed, and its clinical significance has been highlighted recently[5-7]. In this paper, we reviewed the status of OBI with respect to its definition, epidemiology, diagnosis, and mechanism. We also focused on the clinical importance of OBI by focusing on 4 processes: transmission, reactivation, contribution to the progression of hepatic fibrosis, and hepatocellular carcinoma (HCC) occurrence, based on results of available reports.

DEFINITION AND CLASSIFICATION

Several definitions of OBI have been suggested. In the 2008 international workshop held in Italy, OBI was defined as the presence of HBV DNA in the liver (with or without HBV DNA in serum) without HBsAg as determined by using the currently available assays[8]. Serum HBV DNA can be either detectable or undetectable, and when detectable, the level of HBV DNA is usually very low (< 200 IU/mL). When serum HBV DNA levels are comparable to those usually detected in cases of overt HBV infection, "false OBI" should be considered. False OBI is usually due to rare infection with *S* gene escape mutants, which produce a modified HBsAg that is not recognized by routinely used detection assays[9,10]. Defining OBI according to the presence of liver HBV DNA expression is the most accurate because HBV DNA in the liver can be detected even when HBV cannot be detected in serum[11]. However, obtaining hepatic HBV DNA is difficult in clinical practice, and assays for the detection of HBV DNA in liver tissue have not been well standardized[7].

Detection assays for HBV DNA in serum have been used with sufficient sensitivity; therefore, OBI is often defined as the presence of serum HBV DNA without detectable HBsAg in clinical practice and in many studies[7,8,12]. Bréchot *et al*[9] proposed to define occult HBV infection as the detection of HBV DNA by using PCR or other amplification assays in HBsAg-negative individuals. Allain[13] also defined OBI as the presence of HBV DNA without detectable HBsAg, with or without anti-HBc or anti-HBs antibody outside the pre-seroconversion window period. This definition of OBI according to the presence of serum HBV DNA is most commonly used in clinical practice.

OBI has also been defined as a serological condition characterized by the presence of isolated hepatitis B core antigen (anti-HBc) in the absence of HBsAg and anti-HBs antibody[3,14]. Detection of anti-HBc antibody, a surrogate marker of OBI, is useful when an HBV DNA test is not available or when intermittent viremia is suspected[8,15]. However, when OBI is defined according to the presence of anti-HBc antibody alone, false anti-HBc positivity and negativity in the detection of OBI should be considered. Not all anti-HBc-positive subjects are HBV DNA-positive. In addition, the absence of anti-HBc antibody does not exclude seronegative OBI. As mentioned earlier, the definition of OBI slightly differs between studies; thus, cautious interpretation should be exercised when comparing study results about OBI.

OBI can be classified into 2 groups, seropositive OBI [anti-HBc and/or anti-hepatitis B surface (anti-HBs) positive] and seronegative OBI (anti-HBc and anti-HBs negative), on the basis of the HBV antibody profile. Seropositive-OBI develops when serum test results for HBsAg become negative after acute hepatitis or when HBsAg is cleared during the course of chronic hepatitis B. In fact, annual HBsAg seroclearance rates are reported to be 0.50%-2.26% per year in chronic hepatitis B patients, and persistent HBV DNA in the liver was detected in some of these patients[16,17]. Seronegative-OBI is caused by primary occult of anti-HBs or anti-HBc from the beginning of the infection because of the mutation or due to progressive loss of anti-HBs[8,18]. Most OBIs are seropositive OBIs, but > 20% of patients with OBI are seronegative for OBI, representing a population negative for all serum markers of HBV infection[19].

EPIDEMIOLOGY

The prevalence of OBI is reported to range from 1% to 95% worldwide. These prevalence rates are influenced by several factors as follows: (1) geographic differences (endemicity); (2) different patient characteristics, including the presence of comorbid diseases such as chronic hepatitis C; and (3) and the different diagnostic techniques used, which have different sensitivity[8,20,21].

The prevalence of OBI differs according to the endemicity of HBV infection. OBI was reported at a higher rate in an HBV endemic area such as East Asia where 41%-90% of the population had prior exposure to

HBV, and less frequently in the low endemic areas such as North America, where only 5%-20% of the population had previous exposure[22]. Several studies on blood donors with similar cohorts have reported a 0.1%-1.05% prevalence of OBI in HBsAg-negative, anti-HBc-positive donors from North America, 0%-1.59% in donors from Europe, and up to 6% in donors from an endemic area[5,23,24].

The prevalence of OBI differs according to the cohort characteristics. OBI is more commonly noted in patients at high risk for parenterally transmitted infections such as hepatitis C virus (HCV) infection or immunosuppression condition such as human immunodeficiency virus (HIV) infection[25]. In particular, OBI prevalence is high in patients with HCV infection, with HBV DNA detected in approximately 33% of patients with HBsAg-negative HCV infection in Italy and > 50% patients in East Asian countries[19,26,27]. OBI prevalence was also high in patients with other chronic liver diseases at 20%-30%[25,28], in hemophilia patients in Japan at 51%[29], and in intravenous drug users at 45%[30]. Among HIV-infected patients in Turkey, 19.1% of subjects had isolated anti-HBc (considered a marker of OBI) compared to only 2.4% in blood-donor controls[31]. The effect of highly active antiretroviral therapy, clusters of differentiation (CD)4 cell count, and HIV RNA load on OBI prevalence is still controversial[24,32,33].

The prevalence of OBI also differs according to the sensitivity of HBV DNA or HBsAg testing. There are various amplification methods for detecting HBV DNA, and the HBV genome target sites are also different. Some commercial assays are more sensitive than others at detecting HBsAg mutants. The type of sample used (liver or serum) or number of samplings can also have some effect on the diagnosis of OBI. Indeed, as serum HBV DNA levels seem to fluctuate in OBI, serial sample is more useful to identify OBI[21].

Since the study population differs significantly based on the above-mentioned factors, prevalence was hard to compare directly among studies. Therefore, caution should be exercised when interpreting the prevalence of OBI in different studies.

MECHANISM

Some researchers insist the lower sensitivity of the HBsAg immunoassay compared to that of polymerase chain reaction (PCR) for the detection of HBV DNA is responsible for development of OBI. However, this difference in the assay sensitivity cannot explain the characteristic lower replication rate of HBV observed in OBI. The precise underlying mechanisms of OBI are not well understood, which could be multifactorial. Both host and viral factors seem to have roles in suppressing viral replication and infection control[5,19].

Host factors

OBI is characterized a low rate of HBV replication *in*

vivo; however, occult HBV strains are replication-competent *in vitro*. This suggests that host, rather than viral, factors are more responsible for OBI[34]. Similarly, many clinical observations indicate that OBI reactivation sometimes occurs under immunosuppressive conditions, such as during cancer chemotherapy treatment, HIV infection, or hematopoietic stem cell transplantation[25,35]. This can be explained by a break in the balance between the host's immune system and the virus that occurs during occult infection caused by change in immune system function, resulting in reactivation of OBI. These findings strongly indicate the critical role of the host's immune system in development of OBI.

Other *in vitro* studies showed vigorous antiviral T-cell responses several years after clinical recovery from acute hepatitis B. This suggests persistent synthesis of minute undetectable amounts of virus by HBV covalently closed circular DNA (cccDNA) or other viral transcripts in OBI, maintaining the HBV-specific memory T-cell response[36]. Therefore, these findings also indirectly emphasize the role of the host immune system in the development and maintenance of OBI[25,37,38].

In addition to memory T-cell immune reaction, innate immune system or cytokines such as tumor necrosis factor-α and interferon-γ also have been reported to be associated with OBI[39,40]. Furthermore, epigenetic regulation, including DNA methylation of the HBV genome and posttranslational modification of histones, has been reported to be related to the OBI[41].

Viral factors

Although there is no sufficient evidence, viral factors also seem to have some effect on development of OBI. Several possible mechanisms explaining the low viral replication rate in OBI have been demonstrated. Mutations of the X region of HBV reduce the ability of the X protein to transactivate host cellular proteins that are essential for viral replication, which led to the suppression of replication and expression of HBV DNA, and resulted in negative seropositivity for HBsAg[42]. Escape mutation of the S region was another possible viral factor associated with OBI, which also decreases reactivity in HBsAg detection assays[43]. In addition, a large number of mutations were reported which can reduce HBsAg expression, decrease immune recognition of the virus, and impair HBV packaging. However, cautious interpretation is necessary, as most of these studies lacked a control group or mutations appeared not only in patients with OBI but also those with overt HBV infections. Further studies should be conducted[44].

DIAGNOSIS

Several methods using liver tissue, DNA extracts from liver or blood, or other serologic markers such as anti-HBc IgG have been used to diagnose OBI. The gold standard for OBI diagnosis is the detection of HBV DNA in the DNA extraction from the liver, as cccDNA

persists in the hepatocytes and HBV DNA is sometimes detected in the liver in the absence of HBV DNA in the serum. However, obtaining liver tissue is an invasive procedure; therefore, obtaining hepatic HBV DNA is difficult in clinical practice. In addition, real-time PCR-based assays for serum (or plasma) HBV DNA detection have been used with sufficient sensitivity to detect OBI in many cases; hence, serum HBV DNA assays are widely used to diagnose OBI[8].

DNA should be extracted from samples using the most efficient procedure. A higher rate of HBV DNA detection has been obtained with snap-frozen liver tissue than with paraffin-embedded liver tissue. When a blood sample is used, at least 1 mL of serum should be collected to improve the sensitivity of the test. DNA extracts should be amplified by highly sensitive nested PCR or by a real-time PCR technique that can detect fewer than 10 copies of HBV DNA using the oligonucleotide primers specific for different HBV genomic regions and complementary to highly conserved nucleotide sequences. Appropriate negative and positive controls should be included in each PCR experiment. In addition, periodic testing for HBV DNA will improve diagnosis of OBI especially in high-risk patients, as intermittent viremia can occur in occult HBV infection[8,18,21,22,45].

When highly sensitive HBV DNA testing cannot be performed, anti-HBc could be used as a possible surrogate marker for identifying potential seropositive OBI in cases of blood and organ donation or those receiving immunosuppressive therapy. In this case, seronegative OBI or false-negative anti-HBc in an immunocompromised host should also be considered[45].

CLINICAL SIGNIFICANCE

Transmission of OBI

Transfusion: Although the risk of HBV transmission through blood transfusion has decreased owing to the development of sensitive and specific diagnostic assays, transfusional transmission of HBV still occurs. Transmission of HBV by transfusion occurs in 3 situations: (1) blood from a donor with OBI; (2) blood from patients in the infectious window period of HBV infection; or (3) blood from a donor infected with S-escape mutant HBV infection not detected by the routinely used diagnostic HBsAg assay. The prevalence of OBI in blood donors is variable depending on the geographic area, and is higher in HBV endemic areas[46]. In an Australian study analyzing 2673521 blood donors, the incidence of OBI was approximately 5.55 per 100000 donors compared to the 1.06 per 100000 donors with an acute serologic window period infections[47]. In China, the pooled prevalence of OBI among donors was 0.094%[48]. In Europe, OBIs are detected in 1:2000 to 1:20000 samples donated[45,49-52].

The infectivity of OBI by transfusion is determined not only by the viral load or the volume of plasma but also by the HBV serological status (anti-HBc and/or anti-HBs). The risk of HBV transmission may depend on the presence of anti-HBsAb. Among occult HBV-infected donors, those with high anti-HBs levels (recovered) are unlikely to transmit the infection, whereas those without anti-HBs (anti-HBc only) may transmit the infection[53,54]. However, the infectivity of anti-HBs-containing blood components in immunodeficient or immunosuppressed recipients has not been systematically explored. Considering that immunocompromised hosts represent a substantial proportion of transfusion recipients, caution should be exercised when anti-HBc-positive blood is transfused to immunocompromised recipients, even when anti-HBs positive[45,54].

Nucleic acid testing (NAT) for donor screening detects HBV infection in the window period (before the appearance of HBsAg) as well as OBI, indicating the presence of HBV DNA in the absence of HBsAg. Therefore, the introduction of NAT has further decreased the risk of HBV transmission through blood transfusion. However, cost effectiveness and availability of NAT should be considered before clinical application. Where HBV DNA testing is not available, such as in developing countries, testing for anti-HBc is strongly recommended[8,45,55].

Organ transplantation: OBI in a transplantation donor is important because there is a risk of HBV transmission from an OBI-seropositive donor, and severe HBV reactivation can occur in some of these cases during immunosuppression. As the hepatocytes are the reservoir of HBV cccDNA, the rate of transmission is higher in orthotopic liver transplantation compared to other organ transplantations such as kidney, bone marrow, and heart[25,56]. The transmission of HBV infection from HBsAg negative/anti-HBc positive (considered OBI) donors to recipients were reported at a rate of 17%-94%[57-59]. Because of this high risk of transmission, prophylaxis is recommended to prevent HBV reactivation. Although not directly compared in randomized controlled trials, the combination of antiviral and hepatitis B immunoglobulin seems to be superior to treatment with antiviral or hepatitis B immunoglobulin monotherapies as prophylaxis. Lamivudine is the most widely used antiviral, and studies using newer antivirals such as entecavir, adefovir, and tenofovir are few[57]. It is uncertain whether OBI is transmitted from HBV-seronegative donors.

OBI in liver transplant recipients is also important. The etiology of *de novo* HBV infection after liver transplantation was traced to OBI in both donors and recipients[60].

Hemodialysis: Hemodialysis patients are at increased risk of parenterally transmitted infections because they are in an immunosuppressed state and exposed to invasive procedures, share the same dialysis machine, and receive more transfusions than the general population. The relatively low acceptance and response rates to the HBV vaccine among dialysis patients also likely contributes to OBI transmission in hemodialysis patients[55,61]. The prevalence of OBI in hemodialysis patients varies from 0%

to 54% according to the diagnostic techniques or HBV endemicity[62,63], and several studies suggest that OBI could be a source of viral spread both to other patients and staff within the hemodialysis units[61,62]. Therefore, patients and staff need HBV vaccine boosts to maintain levels of protective antibody to HBsAg (anti-HBs). Strict dialysis-specific infection-control programs, including avoidance of dialyzer reuse and use of dedicated dialysis rooms and machines, should be implemented. Staff for infected patients should be educated on preventive method to limit HBV transmission within dialysis units. Furthermore, regular screening for HBV DNA with sensitive PCR-based assays in all dialysis patients should be considered, and more attention should be given to patients who receive immunosuppressant drugs after renal transplantation[62,64].

Pregnancy and OBI transmission: Kwon *et al*[65] studied the possibility of transmission of OBI to the fetus in 202 healthy pregnant women. Among these, six (3%) women were OBI positive. When cord blood of 4 of these 6 women was evaluated for HBV DNA, all were HBV-DNA negative. This result suggests that vertical transmission through the cord blood is negligible, but this needs to be investigated further[65].

Reactivation

HBV reactivation after systemic chemotherapy was first reported in the mid 1970's, and thereafter, HBV reactivation has been reported not only in HBsAg-positive patients but also in OBI patients[35,66]. Although, reactivation in OBI occurs more rarely than in HBsAg positive patients, HBV reactivation is quite a frequent event in immunocompromised OBI patients when including not only symptomatic hepatitis but also HBsAg re-seroconversion in the reactivation of OBI[66-68]. This finding is clinically important because it can be associated with liver dysfunction, sometimes causing life-threatening fulminant hepatic failure, and often requires interruption of chemotherapy[20,69]. The underlying mechanism of reactivation is thought that chemotherapy induced immunosuppressive state triggers rapid viral replication because of the loss of the immunological control. After immune system reconstitution, cytotoxic T-cell-mediated hepatocyte injury may occur, leading to the development of hepatic inflammation and concomitant hepatic necrosis.

Hematological malignancies, hematopoietic stem cell transplantation, liver transplantation from anti-HBc positive donors, and treatment with anti-CD20 (rituximab) seem to be the factors associated with the highest risk of OBI reactivation[25,70-73]. Other immunosuppressive conditions, including HIV infection, kidney or bone marrow transplantation, systemic chemotherapy, and rheumatologic diseases or inflammatory bowel disease treated with biological agents or high-dose steroids for prolonged treatment, also have been reported as possible causes of viral reactivation in OBI patients[37].

While prophylactic antiviral treatment to prevent re-

activation is well established in HBsAg-positive patients undergoing immunosuppressive therapies, its use in OBI patients is debatable[25,70]. In highly endemic areas, 20% of cancer patients have been reported to be HBsAg-negative and anti-HBc positive[74]; thus, prophylactic antiviral use for all OBI patients is unlikely to be cost-effective[68]. On the contrary, delayed treatment with an antiviral may be fatal, and frequent monitoring for reactivation in OBI patients is sometimes difficult. Therefore, use of antivirals is recommended for OBI patients with highest risk of reactivation (previously suggested) regardless of HBV DNA presence and when HBV DNA monitoring is unavailable in routine practice[70,73,75,76]. HBsAg-negative and anti-HBc-positive patients with undetectable or low levels of HBV DNA without highest risk of reactivation should be carefully monitored using alanine aminotransferase (ALT) and HBV DNA levels, with adequate intervals before and during immunosuppressive treatments, and also for several months after stopping treatment. In this case, antiviral treatment should be started as soon as HBV reactivation is detected, before ALT level elevation, since the objective of this strict surveillance is to identify HBV reactivation early before liver injury to prevent acute hepatitis[68,75]. Among antivirals, lamivudine seems to be effective in patients with no or very low serum HBV DNA levels[77]. More potent nucleoside analogues should be chosen when reactivation is confirmed or lamivudine resistance is suggested[73]. Currently, there is no consensus about the optimal duration of preventive antiviral therapy[78]. However, several reports suggest the start of prophylaxis 1-2 wk before the start of immunosuppressive therapy and prolonged antiviral therapy at least 6-12 mo after completion of chemo- or immunotherapy to prevent delayed reactivation of HBV[79,80]. However, further studies should be performed to determine the optimal duration of treatment.

Progression of chronic liver disease

It has been shown that HBV genomes may persist for a long time in the liver, inducing mild necro-inflammation in patients after complete clinical recovery from acute self-limited hepatitis B[81]. An *in vivo* study of a woodchuck model showed similar results; animals that recovered from acute woodchuck hepatitis virus (the rodent HBV-like hepadnavirus) showed lifelong existence of viruses replicating at low levels, inducing mild persistent liver necroinflammation[82]. These results suggest the role of OBI in the progression of chronic liver disease, and there has been much interest in the clinical impact of OBI, both as a mono-infection and as co-infection with HCV, on the course of chronic liver disease.

As HBV and HCV share the same transmission route, OBI is highly prevalent in patients with HCV-related chronic hepatitis. Thus, many cross-sectional studies investigated the influence of OBI on the outcome of chronic hepatitis C. Previous studies showed OBI as a risk factor for more severe liver disease[26,83]; however, the cross-sectional nature of most of these studies could

have biased patient selection. A recent longitudinal Italian cohort study by Squadrito *et al*[84] showed that among chronic hepatitis C patients, patients with OBI had higher risk of progression to cirrhosis, development of HCC, and increased risk of liver-related death compared to OBI-negative patients[84]. Other studies additionally showed the association of ALT level flares with detection of HBV DNA in HCV-OBI co-infected patients, indicating that transient HBV reactivation might be involved in liver injury in these patients[85,86]. A recent meta-analysis of both OBI mono-infection and co-infection with HCV showed that OBI is associated with chronic liver disease, with an overall 8.9-fold increased risk compared to individuals without OBI. Subgroup analysis comparing HCV-positive and -negative subjects showed that HCV-positive as well as HCV-negative patients (cryptogenic liver disease) had increased risk for chronic liver disease[87].

Conclusively, when OBI is present with HCV or with other chronic liver diseases, hepatic inflammation induced by a mild immune response to OBI may accelerate liver injury. In most healthy subjects under immune control, it is not determined yet whether OBI can cause clinically relevant hepatic damage[70,88].

Hepatocellular carcinoma occurrence

HBV infection is known to be one of the most important risk factors in the development of HCC. Although the mechanism by which HBV infection causes HCC is not completely known, HBV causes HCC both indirectly and directly. HBV infection causes hepatic inflammation, regeneration, and fibrosis associated with cirrhosis, which indirectly contribute to HCC development. In a direct pathway, HBV integrates into the host genomes, produces proteins with pro-oncogenic activities, such as X protein and mutant preS-S proteins, and causes genetic and epigenetic alterations that may directly induce hepatocyte transformation[89,90].

Considering that OBI is characterized by intrahepatic persistence of viral cccDNA, OBI can be a risk factor for HCC development in a similar way. In epidemiologic studies, a significantly higher prevalence of OBI was observed in HCV-positive HCC patients than in HCV-positive populations without HCC. Similar results were reported in the HCV-negative patients with cryptogenic liver disease or alcoholic liver disease[37,91-94]. An *in vivo* experimental study also demonstrated that woodchucks, after serological recovery from acute woodchuck hepatitis virus infections, are at high risk of developing HCC even after apparent clearance of the virus[95]. In prospective studies, the cumulative probability of developing HCC was significantly higher among patients with OBI than among HBV DNA-negative patients, both in the presence[96-99] or absence of HCV infection[100]. In addition, a recent meta-analysis demonstrated that OBI increases the risk of HCC in both HCV and non-HCV infected patients[101].

Although these results support the idea that OBI is a risk factor for HCC, caution should be exercised during interpretation[102]. First, as most of the study subjects had chronic liver disease of various etiologies, these results indicate OBI as a co-carcinogen of HCC in addition to other suggested carcinogens such as previous HBV infection, HCV, or alcohol. The role of OBI *per se* in the occurrence of HCC should be further investigated. Second, further studies should be performed to confirm the role of OBI in cryptogenic liver disease. Previous studies have considered heterogeneous definitions of cryptogenic liver disease and non-B non-C liver diseases, (*e.g.*, not differentiating nonalcoholic fatty liver disease and sometimes including alcohol or autoimmune liver disease in cryptogenic liver disease); therefore, it is difficult to interpret the results. Third, several other studies did not find an association between OBI and HCC, and ethnic and epidemiologic differences should be considered in the interpretation of the results[103].

CONCLUSION

OBI, defined as the presence of HBV DNA without detectable HBsAg, has recently gained increasing attention. Although the exact mechanism of OBI has not been determined, OBI can be transmitted, cause reactivation of HBV, and contribute to the development of progressive liver disease and HCC. Thus, physicians should focus on the appropriate management of these patients, and further studies to clarify the clinical significance of OBI are needed.

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TOPIC HIGHLIGHT

Functional foods effective for hepatitis C: Identification of oligomeric proanthocyanidin and its action mechanism

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Abstract

Hepatitis C virus (HCV) is a major cause of viral hepatitis and currently infects approximately 170 million people worldwide. An infection by HCV causes high rates of chronic hepatitis (> 75%) and progresses to liver cirrhosis and hepatocellular carcinoma ultimately. HCV can be eliminated by a combination of pegylated α-interferon and the broad-spectrum antiviral drug ribavirin; however, this treatment is still associated with poor efficacy and tolerability and is often accompanied by serious side-effects. While some novel direct-acting antivirals against HCV have been developed recently, high medical costs limit the access to the therapy in cost-sensitive countries. To search for new natural anti-HCV agents, we screened local agricultural products for their suppressive activities against HCV replication using the HCV replicon cell system *in vitro*. We found a potent inhibitor of HCV RNA expression in the extracts of blueberry leaves and then identified oligomeric proanthocyanidin as the active ingredient. Further investigations into the action mechanism of oligomeric proanthocyanidin suggested that it is an inhibitor of heterogeneous nuclear ribonucleoproteins (hnRNPs) such as hnRNP A2/B1. In this review, we presented an overview of functional foods and ingredients efficient for HCV infection, the chemical structural characteristics of oligomeric proanthocyanidin, and its action mechanism.

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Key words: Hepatitis C virus; Blueberry leaves; Functional foods; Oligomeric proanthocyanidin; Heterogeneous nuclear ribonucleoproteins

Core tip: An infection by hepatitis C virus (HCV) causes chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. While the combination of pegylated α-interferon and ribavirin is used for the elimination of HCV, a new anti-HCV drug is required due to the poor efficacy and serious side-effects associated with this combination therapy. We searched for new anti-HCV agents from natural products and then identified oligomeric proanthocyanidin from blueberry leaves. Further investigations suggested that several heterogeneous nuclear ribonucleoproteins may be the candidate proteins involved in the proanthocyanidin-mediated inhibition of HCV subgenomic expression. Oligomeric proanthocyanidin isolated from blueberry leaves may have potential usefulness as an anti-HCV compound.

INTRODUCTION

Hepatitis C virus (HCV) is a major cause of viral hepatitis and currently infects approximately 170 million people worldwide[1,2]. An infection by HCV causes high rates of chronic hepatitis (> 75%) and progresses to liver cirrhosis and hepatocellular carcinoma ultimately[3]. A total of 27% and 25% of individuals that develop liver cirrhosis and hepatocellular carcinoma worldwide, respectively, arise in HCV-infected people[4]. The World Health Organization reported that between 350000 and 500000 people die from HCV-related diseases each year. However, there is no effective vaccine against HCV infection at present.

Currently, the combination of pegylated α-interferon and a broad spectrum antiviral drug, ribavirin, is used as the standard therapy for chronic HCV infection[2,5,6]. However, its option is unfortunately limited by efficacy, tolerability, and significant side-effects. Therefore, it had been required to establish a new therapeutic modality without serious adverse effects. Recently, direct-acting antivirals (DAAs) that inhibit HCV-specific proteins have be clinically investigated[7,8]. For example, boceprevir and telaprevir are new DAAs that were first approved by the United States Food and Drug Administration (FDA) in 2011[9]. DAAs are expected to provide new promising treatment options in hepatitis C patients; however, at present, they face difficulties to disseminate worldwide due to high costs. Therefore, new anti-HCV agents that are safe, economical, and complementary with present therapies, are still required.

Since the development of HCV-related liver cirrhosis and hepatocellular carcinoma requires a prolonged period (20-30 years), the progression of this disease may be influenced by a diet including dairy products. Interest in functional foods and their ingredients as natural resources for cancer prevention and treatment is increasing[10,11]. Eating habits, foods, nutrients contained in them, and other food constituents play important roles on the development of several types of cancer and 35% of cancer deaths are estimated to be possibly related to dietary factors[12]. Polyphenols derived from various fruits and vegetables have recently been suggested to be effective in the prevention of cancer. The South Kyushu region of Japan, including the prefecture of Miyazaki, has been recognized as a high prevalence area of HCV and it emerges as a social issue. Therefore, attempts were made to identify functional food ingredients having suppressive activities against HCV replication as an industry-academia-government collaboration study[13]. By screening of 1700 samples from 283 agricultural products in Miyazaki prefecture, we found that oligomeric proanthocyanidin, a polyphenolic ingredient abundantly contained in the leaves of the blueberry plant, suppressed the expression of HCV subgenomic RNA in an HCV replicon cell system[13].

In this review, we presented an overview of functional foods and ingredients efficient for HCV infection, the chemical structural characteristics of oligomeric proanthocyanidin, and its action mechanism.

HCV LIFE CYCLE AND ANALYTICAL TOOL

HCV belongs to *Hepacivirus* genus of the *Flaviviridae* family and has a positive-sense single stranded RNA of 9.6 kb wrapped with enveloped membrane[14]. After their adsorption on the surface of host cells, HCV particles are internalized into endocytic compartments and viral genomic RNA is then released into the cytoplasm by fusion of the viral envelope and cellular membrane. Genomic RNA serves as mRNA for viral proteins and is translated into a single polyprotein (3011 amino acids), resulting in 4 structural proteins (Core, E1, E2, and p7) and 6 non-structural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) by post-translational processing (Figure 1A). It also serves as a template for viral genome replication. Non-translated regions (NTRs), 5'NTR and 3'NTR, are connected with the HCV polyprotein-coding region, and modulate viral protein synthesis and genome replication. The assembly of these viral components occurs on the endoplasmic reticulum (ER) membrane. Viral proteins and genomic RNA assemble on the cytoplasmic side of the membrane and then progeny virions bud into the ER lumen, followed by their release to the extracellular space. In the life cycle of HCV, each viral protein functions as described below[14]. Core is a highly basic protein that encapsidates HCV genomic RNA. E1 and E2 are glycoproteins integrated into the viral envelope. p7 functions as an ion channel and an antiviral drug, amantadine, is the p7 ion channel blocker[15]. Importantly, several steps of HCV infectious process are coordinated by NS proteins. NS2 and NS3 are a cysteine protease and serine protease, respectively, that play roles in the post-translational processing of viral proteins. NS3 serine protease activity requires NS4A as a cofactor. NS4B and NS5A have been suggested to serve in viral assembly on the ER membrane and NS5B is an RNA-dependent RNA polymerase. Many studies to date have reported that these viral proteins are associated not only with viral replication, but also pathogenicity *via* interactions with various host proteins. The identification of host proteins associated with the HCV life cycle is very important for anti-HCV drugs, and the HCV replicon cell system has contributed significantly to the development of these drugs[16,17]. This system consists of the human hepatocellular carcinoma line Huh-7 in which the transfected luciferase gene connected with HCV subgenomic RNA including the downstream coding regions of NS3 and the expression of HCV subgenomic RNA can be quantified by luciferase activity (Figure 1B). It provides a useful tool for HCV drug development

Figure 1 Structure of the hepatitis C virus genome and cell system for anti-hepatitis C virus drug discovery. A: HCV genomic RNA and viral proteins. HCV genomic RNA encodes a single polyprotein of 3011 amino acids. After being translated, the polyprotein is processed into 4 structural proteins (Core, E1, E2, and p7) and 6 non-structural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B). The polyprotein-coding region is flanked by 5' and 3'NTRs. Viral RNA also serves as a template for viral genome replication and both NTRs modulate viral protein synthesis and genome replication; B: The HCV replicon cell system. Huh-7 cells were transfected with the luciferase gene connected with HCV subgenomic RNA including the downstream coding regions of NS3. The expression of HCV subgenomic RNA could be quantified by luciferase activity. HCV: Hepatitis C virus; NTRs: Non-translated regions.

and the elucidation of mechanisms underlying HCV genome replication[17]. We have used this HCV replicon system to screen functional foods with anti-HCV activity.

THERAPEUTIC OPTIONS FOR CHRONIC HCV INFECTION

Currently, the combination of pegylated α-interferon and a broad spectrum antiviral drug, ribavirin, is used as the standard therapy for chronic HCV infection[2,5,6]. However, the HCV genotype is an important determinant of its efficacy and tolerability. Whereas the virological response to this combination therapy is more than 70% for genotypes 2 and 3, it is less than 50% for genotype 1[18-20]. Furthermore, this therapy causes significant side-effects such as thrombocytopenia, flu-like symptoms, fever, rash, anorexia, and thyroid dysfunction. Depression and irritability that are expressed as neuropsychological disorders during therapy impair quality of life universally. Therefore, it had been required to establish a new therapeutic modality without serious adverse effects.

Recently, DAAs that inhibit HCV-specific proteins have been clinically investigated[7,8]. Two DAAs, boceprevir and telaprevir first came to the HCV drug market and were approved by FDA in May 2011. Boceprevir or telaprevir was used as triple therapy with pegylated α-interferon and ribavirin for hepatitis C patients with genotype 1[9]. These DAAs are inhibitors against HCV NS3/4A serine protease and bind covalently with active

site of the enzyme[21-23]. The triple therapy using boceprevir or telaprevir significantly increased the rate of sustained virological response (SVR) for naive or previous treated hepatitis C patients with HCV genotype 1[24-29]. After that, next generation DAAs, ABT-450/r, simeprevir, and faldaprevir, which are also NS3/4A protease inhibitors, have been reported to have advantages of their convenience and improved side effects profile[30-32]. Further, daclatasvir and sofosbuvir, which are an NS5A replication complex inhibitor and a nucleotide analogue NS5B polymerase inhibitor, respectively, also increased SVR rate[33-35]. Notably, the combination of these DAAs only was the highly effective treatment for patients with HCV genotype 1[36,37] and it is feasible to treat HCV without interferon and ribavirin.

While patients with hepatitis C can be treated by above mentioned DAAs without significant side-effects, it requires high medical costs and limits access to the therapy in cost-sensitive countries[38]. Of the 20 countries with the high prevalence of HCV, 12 are categorized as low or lower-middle income countries[39]. Therefore, new anti-HCV agents that are safe, economical, and complementary with present therapies, are still required and we focus attention on functional foods and their ingredients.

FUNCTIONAL FOOD INGREDIENTS EFFECTIVE FOR HCV

The development of HCV-related liver cirrhosis and hepatocellular carcinoma requires a prolonged period (20-30 years). Therefore, the progression of the disease and HCV infectivity may be influenced by a diet including dairy products. Functional foods and their ingredients are known to be capable of modulating various biological processes such as apoptosis and have been attracting interest as natural resources for the prevention and treatment of cancer[10,11,40]. Dietary polyphenols derived from various fruits and vegetables have been suggested to be effective in cancer prevention. Although the importance of functional food ingredients as DAAs against HCV is not fully recognized, these findings suggest that they contribute to the elimination of the virus.

Several functional food ingredients have been reported to interfere with different steps of the HCV life cycle. Epigallocatechin-3-gallate (EGCG) (Figure 2A) and curcumin (Figure 2B), which are ingredients of green tea (Camellia sinensis) and the Indian spice turmeric (Curcuma longa), respectively, inhibit the entry of HCV into host cells[41,42]. Quercetin (Figure 2C), a flavonoid that is abundantly contained in onions, apples, berries, and red wine, has been shown to inhibit NS3 protease activity[43]. Punicalagin (Figure 2D) and its related substance punicalin from the pomegranate (Punica granatum L.) reduced the replication of HCV[44]. Naringenin (Figure 2E) from the grapefruit (Citrus X paradisi Macfady.) has been identified as an ingredient that interferes with viral assembly[45,46]. Diosgenin (Figure 2F) and epicatechin (Figure 2G), which are contained in yams (Dioscorea spp.) and

Figure 2 Chemical structure of functional food ingredients with anti-hepatitis C virus activities. A: Epigallocatechin-3-gallate; B: Curcumin; C: Quercetin; D: Punicalagin; E: Naringenin; F: Diosgenin; G: (-)-epicatechin.

green tea, respectively, also affect the signal transduction pathways of host cells and inhibit HCV replication *via* the signal transducer and activator of transcription 3 and cycloxygenase-2 pathways, respectively[47,48]. The finding that curcumin and quercetin also inhibited HCV replication by associating with sterol regulatory element binding protein-1 and heat shock proteins, respectively, indicated the existence of multifunctional ingredients[49,50]. Silymarin, which is an extract from milk thistle (Silybum marianum) and consists of at least 7 flavonoid compounds, was also found to interfere with several steps of HCV infectious process, such as NS5B polymerase activity and virus entry and transmission[51]. As shown in Figure 2, most ingredients are polyphenol compounds and, EGCG (A), quercetin (C), naringenin (E), and epicatechin (G) have similar chemical structures. There may be a characteristic structure modulating viral proteins and their associations with host proteins.

A

B

Figure 3 Chemical structures of a flavan-3-ol and proanthocyanidin. A: (+)-catechin; B: An example of a procyanidin B-type polymer with an (-)-epicatechin based structure.

Clinically, the supplementation of vitamin group has been reported to increase SVR rates in chronic hepatitis C patients who underwent the standard therapy with pegylated α-interferon and ribavirin[52-54]. Regarding significant side-effects of the standard therapy, a tomato-based functional food abundant in natural antioxidants alleviated the severity of anemia caused by ribavirin and improved the tolerance to the drug[55].

OLIGOMERIC PROANTHOCYANIDIN FROM BLUEBERRY LEAVES HAS SUPPRESSIVE ACTIVITY AGAINST HCV SUBGENOME REPLICATION *IN VITRO*

To identify functional food ingredients effective for hepatitis C, we comprehensively screened the extracts of commonly ingested agricultural products (1700 samples from 283 species) grown in Miyazaki prefecture, Japan using an HCV replicon cell system[13]. Samples having high antioxidative activities were first selected irrespective of edible part or non-edible part, and then the inhibitory

activities against HCV subgenomic RNA replication were examined using the system. We found that extracts of blueberry leaves significantly suppressed the replication. Furthermore, by comparing the inhibitory activities using leaves from various kinds of blueberry species, it was found that the leaves of rabbit-eye blueberry (Vaccinium virgatum Aiton) had the highest activity[13]. Rabbit-eye blueberry is cultivated in a region with a warm climate, such as the southern areas of Japan, including Miyazaki prefecture. Its leaves have been also reported to be good sources of polyphenols and natural antioxidants[56].

We identified oligomeric proanthocyanidin as the blueberry leaf-derived inhibitor of HCV subgenomic RNA replication[13]. Proanthocyanidin is a polyphenol and has polymerized structures in which more than two flavan-3-ol units such as catechin (Figure 3A) and epicatechin (Figure 2G) are covalently linked. Figure 3B shows an example of the chemical structure of proanthocyanidin. Proanthocyanidin possesses two interflavan bonds, in which the A-type and B-type have two bond linkages (C4→C8 and O7→C2) and one linkage (C4→C8 or C4→C6), respectively[57], and both types co-exist in proanthocyanidin from the rabbit-eye blueberry plant[13]. While catechin, epicatechin, EGCG, and dimers such as procyanidin B2 did not exhibit inhibitory activity against HCV subgenomic expression in our experimental system, proanthocyanidin oligomer having polymerization degree of 8 to 9 markedly inhibited this expression[13]. This finding suggested that the HCV inhibitory activity of oligomeric proanthocyanidin in the replicon assay may require an oligomerized structure.

Proanthocyanidins are abundantly contained in various plants and foods[58] and contribute to organoleptic properties such as bitterness and astringency[59]. Proanthocyanidin-containing foods and nutritional supplements are known to have benefits in health promotion. United States Department of Agriculture Database reported proanthocyanidin contents of various foods, showing that apple peel, red kidney beans, pinto beans, cacao beans, cocoa, grape seeds, several nuts (almonds, hazelnuts, pecans, and pistachios), sorghum, and cinnamon are proanthocyanidin-rich[60]. Blueberry fruits are also relatively proanthocyanidin-rich; however, the fruits did not show significant HCV inhibitory activity compared to the leaves (unpublished data). In the fruits, proanthocyanidin contents of monomer, dimer, trimer, 4-6mer, 7-10mer, and polymer with degrees of polymerization greater than 10mer are 3.46, 5.71, 4.15, 19.57, 14.55, and 129.05 mg per 100 g edible portion, respectively[60]. As the inhibitory activity required the oligomeric structure of proanthocyanidin having a polymerization degree of 8 to 9 but not polymer and fresh blueberry leaf contained 3000-4000 mg proanthocyanidins per 100 g total extracts[13], leaves but not fruits from blueberry are likely suitable for the prevention of HCV-related diseases. With regard to the oral uptake, oligomeric proanthocyanidin seems to elute off by boiling for cooking as shown with pint beans[60]. Therefore, oligomeric proanthocyanidin from blueberry

Figure 4 Identification strategy of candidate proteins involved in the proanthocyanidin-mediated inhibition of hepatitis C virus subgenomic expression[13]. Total proteins were extracted from hepatitis C virus (HCV) replicon cells and then proanthocyanidin-binding and catechin-binding proteins were purified by affinity chromatography using sepharose beads coupled with pro-anthocyanidin and catechin, respectively. Purified proteins were separated by two-dimensional electrophoresis followed by detecting spots of proteins having higher affinity to proanthocyanidin than catechin (arrows). Mass spectrometric analysis and further screening by a siRNA-based replicon assay showed that hnRNP A2/B1, A/B, K, and L are candidate proteins involved in the oligomeric proanthocyanidin-mediated inhibition of HCV subgenomic expression. hnRNP: Heterogeneous nuclear ribonucleoprotein.

leaves might be ingested as a hot water extract such as herbal tea. However, absorption efficiency of oligomeric proanthocyanidin in the intestine may be very low.

Proanthocyanidin has also been reported to possess anti-viral activity against other viruses, herpes simplex virus and human immunodeficiency virus type 1[61-65]. To the best of our knowledge, we first reported that the pro-anthocyanidin oligomer inhibited the expression of HCV subgenomic RNA[13]. However, the effects of oligomeric proanthocyanidin on HCV replication in hepatocytes *in vivo* currently remain unknown.

ACTION MECHANISM OF OLIGOMERIC PROANTHOCYANIDIN IN HCV REPLICON CELLS

The suppression of HCV subgenomic RNA replication by oligomeric proanthocyanidin has been attracting increasing attention. Polyphenolic compounds gener-

ally have high antioxidant activities[10,11,58]. Therefore, the nonspecific antioxidant activity of polyphenols may contribute to the suppression of HCV subgenomic RNA replication by oligomeric proanthocyanidin. However, we examined other polyphenolic compounds in our HCV replicon assay, and found that constitutional units such as catechin and epicatechin did not display suppressive activity, which requires the oligomerized structure of proanthocyanidin[13]. While it currently remains unknown whether proanthocyanidin oligomer can be translocated within the cells in spite of the structure, the ingredient has been reported to be absorbed from the digestive tract[66,67], implying the internalization into cells. Oligomeric proanthocyanidin appears to suppress HCV subgenomic RNA replication *via* a specific association with certain intracellular molecules.

Proteomic approach using two-dimensional differential gel electrophoresis combined with mass spectrometry provides a powerful tool to determine the cellular response to functional foods[40]. To clarify the action mechanism of oligomeric proanthocyanidin in HCV replicon cells, we performed proteomic analysis of proanthocyanidin-binding proteins purified by affinity chromatography[13]. Then, cellular proteins from replicon cells having higher affinity to proanthocyanidin than catechin were identified by a mass spectrometric analysis, and whether the proteins identified were associated with HCV RNA expression was further examined using a siRNA-based replicon assay (Figure 4). Four heterogeneous nuclear ribonucleoproteins (hnRNPs), hnRNP A/B, A2/B1, K, and L, were suggested to be possible cellular binding proteins of oligomeric proanthocyanidin. While siRNA targeting hnRNP A/B, K, and L showed weak inhibitory activities, the knockdown of hnRNP A2/B1 significantly suppressed HCV subgenomic replication[13].

HnRNPs comprise a family of RNA-binding proteins that are involved in diverse RNA-related biological processes[68]. They are multifunctional proteins composed of major and minor hnRNP proteins, and hnRNP A/B, A2/B1, K, and L that we identified belonged to the major hnRNPs[69]. Previous studies demonstrated that these hnRNPs regulated the metabolism of RNA such as pre-mRNA splicing and transcription[70-76]. For example, hnRNP A2/B1 was shown to affect the alternative splicing of several tumor suppressors and oncogenes in glioblastoma cells[72]. Furthermore, several studies reported interactions and cooperation between these hnRNPs[77-79]. hnRNP A2 and hnRNP L have also been shown to exist as a complex and regulate the expression of glucose transporter-1 by binding to mRNA 3'NTR[80,81].

In the HCV life cycle, hnRNPs are associated with HCV genome RNA and regulate its replication. hnRNP A1, which exhibits high homology with hnRNP A2/B1, was shown to facilitate HCV replication *via* binding to the HCV 5' and 3'NTRs (Figure 1), and the replication was significantly suppressed by the double knockdown of hnRNP A1 and hnRNP A2[82]. hnRNP K and hnRNP L are also NTR-binding proteins[83-85]. Furthermore, all

the hnRNPs we identified as the target protein candidates of oligomeric proanthocyanidin were included in HCV 3'NTR-binding proteins[86]. Collectively, these findings suggested that a complex composed of hnRNP A2/B1, A/B, K, and L may serve in HCV genome replication by binding to NTRs and oligomeric proanthocyanidin is an inhibitor of the replication complex. This possibility should be addressed in a further study.

CONCLUSION

Currently, a combination of pegylated recombinant interferons and ribavirin is used as the standard therapy for hepatitis C patients. Recently emerged DAAs are expected to provide new promising treatment options in hepatitis C patients. However, their high medical costs may make difficult to disseminate worldwide. We demonstrated that extracts of blueberry leaves suppressed HCV subgenome replication *in vitro*, and their active ingredient was oligomeric proanthocyanidin[13]. Investigations into the underlying action mechanism suggested that proanthocyanidin may be an inhibitor of several hnRNPs such as hnRNP A2/B1[13]. On the other hand, it currently remains unknown whether the oligomeric form of proanthocyanidin, which is required for the inhibition of HCV replication, can be efficiently absorbed from the digestive tract to maintain effective plasma concentrations *in vivo*. However, further basic research on the action mechanism of oligomeric proanthocyanidin against HCV replication may open ways to develop novel anti-HCV drugs and supplements for hepatitis C patients worldwide.

ACKNOWLEDGMENTS

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TOPIC HIGHLIGHT

WJH 6th Anniversary Special Issues (7): Non-alcoholic fatty liver disease

Involvement of the TAGE-RAGE system in non-alcoholic steatohepatitis: Novel treatment strategies

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is a major cause of liver disease around the world. It includes a spectrum of conditions from simple steatosis to non-alcoholic steatohepatitis (NASH) and can lead to fibrosis, cirrhosis, liver failure, and/or hepatocellular carcinoma. NAFLD is also associated with other medical conditions such as obesity, diabetes mellitus (DM), metabolic syndrome, hypertension, insulin resistance, hyperlipidemia, and cardiovascular disease (CVD). In diabetes, chronic hyperglycemia contributes to the development of both macro- and microvascular conditions through a variety of metabolic pathways. Thus, it can cause a variety of metabolic and hemodynamic conditions, including upregulated advanced glycation end-products (AGEs) synthesis. In our previous study, the most abundant type of toxic AGEs (TAGE); *i.e.*, glyceraldehyde-derived AGEs, were found to make a significant contribution to the pathogenesis of DM-induced angiopathy. Furthermore, accumulating evidence suggests that the binding of TAGE with their receptor (RAGE) induces oxidative damage, promotes inflammation, and causes changes in intracellular signaling and the expression levels of certain genes in various cell populations including hepatocytes and hepatic stellate cells. All of these effects could facilitate the pathogenesis of hypertension, cancer, diabetic vascular complications, CVD, dementia, and NASH. Thus, inhibiting TAGE synthesis, preventing TAGE from binding to RAGE, and downregulating RAGE expression and/or the expression of associated effector molecules all have potential as therapeutic strategies against NASH. Here, we examine the contributions of RAGE and TAGE to various conditions and novel treatments that target them in order to prevent the development and/or progression of NASH.

Key words: Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Advanced glycation end-products; Toxic advanced glycation end-products; Receptor for advanced glycation end-products; Toxic advanced glycation end-products-receptor for advanced glycation end-products system; Diabetes mellitus; Cardiovascular disease; Dietary fructose; Dietary advanced glycation end-products

Core tip: Toxic advanced glycation end-products (TAGE) synthesis is increased by non-alcoholic steatohepatitis (NASH), and patients with NASH exhibit significantly increased serum and hepatic TAGE concentrations. Interactions between TAGE and the receptor for advanced glycation end-products (RAGE) have been suggested to cause oxidative stress and increase the fibrogenic potential of cultured human hepatic stellate cells. Therefore, TAGE signaling *via* RAGE and the resultant synthesis of reactive oxygen species might play a role in the worsening of hepatic pathology seen in NASH. These observations led us to suggest that extracellular and intracellular TAGE are involved in the pathogenesis of NASH.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a major cause of chronic liver disease in developed countries, and hence, is becoming a global public health issue[1]. NAFLD includes a range of conditions, from simple steatosis to non-alcoholic steatohepatitis (NASH)[2-4]. NASH has the potential to progress, which can result in cirrhosis, liver failure, and/or hepatocellular carcinoma[2-4]. NAFLD is regarded as a hepatic symptom of metabolic syndrome (MetS) and is associated with visceral obesity, abnormalities in glucose and lipid metabolism, insulin resistance (IR), and hypertension[5-7]. In NAFLD patients, underlying metabolic conditions such as those described above result in worsening liver dysfunction and a higher incidence of liver fibrosis and are also involved in the development of cardiovascular disease (CVD)[8,9].

Advanced glycation end-products (AGEs) might be involved in the mechanism that links NASH and diabetes mellitus (DM). Accumulating evidence indicates that in diabetic patients chronic hyperglycemia upregulates the production of AGEs (senescent macroprotein derivatives) *via* non-enzymatic glycation (the Maillard reaction). It has been demonstrated that the binding of AGEs to their receptor (RAGE) induces oxidative stress followed by inflammatory and/or thrombogenic responses in a variety of cell types. Furthermore, in diabetes such binding is considered to be involved in the pathogenesis and worsening of angiopathic conditions[10-16]. In our previous study, the most abundant type of toxic AGEs (TAGE); *i.e.*, glyceraldehyde-derived AGEs (Glycer-AGEs), were found to make a significant contribution to the development of angiopathic conditions in DM[17-20]. In addition, there is a growing consensus that TAGE-RAGE interac-

tions affect gene expression, intracellular signaling, and the secretion of pro-inflammatory factors and induce reactive oxygen species (ROS) production in various cell types including hepatic stellate cells (HSC) and hepatocytes[21,22]. Thus, TAGE-RAGE interactions might play a role in the pathological changes associated with lifestyle-related diseases, particularly NASH. TAGE synthesis is increased in NASH, and NASH patients were found to exhibit significantly higher hepatic and serum TAGE concentrations than individuals with simple steatosis or healthy controls[23]. TAGE-RAGE interactions have also been found to be associated with the induction of oxidative stress and increases in the fibrogenic potential of cultured human HSC[22]. Therefore, it is suggested that TAGE signaling through RAGE and the subsequent ROS production play a role in the worsening of hepatic pathology observed in NASH.

Accordingly, inhibiting the binding of TAGE to RAGE and TAGE synthesis and downregulating RAGE expression and/or the expression of its effectors have potential as treatment strategies for NASH. Here, we examine the contributions of RAGE and TAGE to various conditions and novel treatments that target these molecules in order to prevent the development and/or progression of NASH.

AGEs

The Maillard reaction, in which the N-terminal α-amino or ε-amino regions of protein lysine residues react non-enzymatically with the ketone or aldehyde moieties of reducing sugars, *e.g.*, fructose, glucose, *etc.*, is responsible for synthesizing AGEs. AGEs are known to be involved in protein aging and the pathological complications associated with DM[10-13,17-20,24-27]. In hyperglycemic DM patients, the first step in this process involves the conversion of reversible Schiff base adducts to more stable covalently bound Amadori rearrangement products, which subsequently undergo further rearrangement to produce irreversibly bound moieties (AGEs), and this process can range in duration from days to weeks.

Initially, AGEs were identified based on their fluorescent yellow-brown appearance and their ability to produce cross-links with and between amino groups. However, the term AGEs now refers to numerous products associated with the advanced stages of the glycation process, including *N*-(carboxyethyl)lysine, *N*-(carboxymethyl)lysine (CML), and pyrraline, which are colorless and can not form cross-links with proteins[24-29]. *In vivo* AGE production is affected by the sugar concentration, the rate of turnover of the chemically modified target, and the time available. Increases in the glucose concentration were previously considered to have a major influence on the Maillard reaction; however, glucose is one of the least reactive sugars found in biological organisms[24,30]. As well as extracellular AGE synthesis, the rapid intracellular production of AGEs from intracellular precursors such as trioses, dicarbonyl compounds, and

Figure 1 Alternative *in vivo* advanced glycation end-product synthesis routes. Reducing sugars, such as glucose, fructose and glyceraldehyde, are known to react non-enzymatically with the amino groups of proteins to form reversible Schiff bases and Amadori product/Heyns products. These early glycation products undergo further complex reactions such as rearrangement, dehydration, and condensation to become irreversibly cross-linked, heterogeneous fluorescent derivatives termed advanced glycation end-products (AGEs). Glu-AGEs: Glucose-derived AGEs; Fru-AGEs: Fructose-derived AGEs; Glycer-AGEs: Glyceraldehyde-derived AGEs; Glycol-AGEs: Glycolaldehyde-derived AGEs; MGO-AGEs: Methylglyoxal-derived AGEs; GO-AGEs: Glyoxal-derived AGEs; 3-DG-AGEs: 3-deoxyglucosone-derived AGEs; CML: N-(carboxymethyl)lysine; P-NH2: A free amino residue; HbA1c: Hemoglobin A1c; TAGE: Toxic AGEs; HFCS: High-fructose corn syrup; AR: Aldose reductase; SDH: Sorbitol dehydrogenase; FK: Fructokinase.

fructose has been gaining attention[31,32]. Due to the great degree of variation in the structures of the AGEs found *in vivo* and the complex nature of the reactions required for their synthesis, only some AGEs have had their structures identified[33]. Furthermore, even the structures of cytotoxic AGEs are yet to be elucidated.

In a previous study, we found that α-hydroxyaldehydes (glycolaldehyde and glyceraldehyde), fructose, glucose, and dicarbonyl compounds (glyoxal and methylglyoxal, 3-deoxyglucosone) all contribute to protein glycation[27,34-37]. A total of 7 immunochemically distinct AGEs classes [methylglyoxal-derived AGEs; Glycer-AGEs; fructose-derived AGEs; glucose-derived AGEs (Glu-AGEs); 3-deoxyglucosone-derived AGEs; glyoxal-derived AGEs; and glycolaldehyde-derived AGEs] were found in serum samples collected from hemodialysis patients with type 2 DM (T2DM)[27,34-37]. Accordingly, we suggested that the *in vivo* formation of AGEs occurs *via* a process involving the Maillard reaction, sugar autoxidation, and sugar metabolism pathways (Figure 1).

PATHWAY FOR THE *IN VIVO* SYNTHESIS OF GLYCER-AGEs

In vivo, two different pathways are responsible for glyceraldehyde (GLA) production, (1) the fructose metabolic pathway (fructolysis) and (2) the glycolytic pathway (glycolysis)[18-20,38]. In pathway (1) under hyperglycemic conditions a rise in the intracellular glucose concentration stimulates the production of fructose *via* the polyol pathway in insulin-independent tissues, such as nerve tissue, the kidneys, the lens of the eye, red blood cells,

and the brain[39-42]. In addition, fructose is a constituent of sucrose and high-fructose corn syrup (HFCS), and hence, is included in many people's diets[43,44]. Fructokinase phosphorylates fructose to fructose 1-phosphate, which is then broken down into dihydroxyacetone phosphate and GLA by aldolase B[45,46]. Next, the resultant GLA is transported (or leaks passively) across the cell membrane. GLA induces TAGE synthesis in the both intracellular and extracellular compartments; as for pathway (2) the enzyme glyceraldehyde 3-phosphate (G3P) dehydrogenase (GAPDH) usually breaks down the glycolytic intermediate G3P. However, reductions in GAPDH activity lead to the intracellular accumulation of G3P. As a result, G3P metabolism starts to occur *via* an alternative pathway, leading to a rise in the concentration of GLA, which promotes the synthesis of Glycer-AGEs, a major form of TAGE. This indicates that a positive feedback mechanism is in operation; namely, that the inhibition of GAPDH activity by TAGE promotes TAGE synthesis (Figure 2).

DIETARY FRUCTOSE

It is suspected that fructose is at least partially responsible for the obesity epidemic affecting developed countries. The greater prevalence of fructose in people's diets results in greater glucose flux and elevated fructose metabolism in hepatocytes. Fructose used to be considered to be a beneficial dietary substance due to the fact it does not stimulate insulin secretion; however, as insulin signaling plays a key role in the development of NAFLD, this property of fructose might be undesir-

Figure 2 Routes for *in vivo* glyceraldehyde-derived advanced glycation end-products synthesis. The glycolytic intermediate glyceraldehyde 3-phosphate (G3P) is usually catabolized (glycolysis) by the enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH). However, reductions in GAPDH activity lead to the intracellular accumulation of G3P. As a result, G3P metabolism starts to occur *via* an alternative pathway, leading to a rise in the concentration of glyceraldehyde, which promotes the synthesis of TAGE. Fructokinase phosphorylates fructose to fructose 1-phosphate, which is then broken down into dihydroxyacetone phosphate and glyceraldehyde by aldolase B (fructolysis). The resultant glyceraldehyde is transported (or leaks passively) across the cell membrane. Glyceraldehyde promotes the formation of TAGE both intracellularly and extracellularly. AGEs: Advanced glycation end-products; TAGE: Toxic AGEs (glyceraldehyde-derived AGEs); HFCS: High-fructose corn syrup; AR: Aldose reductase; SDH: Sorbitol dehydrogenase; FK: Fructokinase; G6P: Glucose 6-phosphate; F6P: Fructose 6-phosphate; F1,6DP: Fructose 1,6-diphosphate; F1P: Fructose 1-phosphate; P-NH2: Free amino residue.

able[47-49]. In adolescents, increased fructose consumption is linked with various CVD risk factors. However, visceral obesity might be responsible for these associations. In the United States, fructose consumption is considered to be associated with the recent rise in the prevalence rates of obesity, fatty liver, and T2DM. The liver is extremely sensitive to variations in dietary content and plays the primary role in the metabolism of simple sugars, such as fructose and glucose[47,48].

The number of calories an individual consumes each day can have a significant influence on their risk of developing NAFLD because excessive energy intake results in obesity, leading to a greater risk of NAFLD. However, the development and progression of NAFLD are also affected by dietary composition. Of all carbohydrates, fructose plays an especially important role in NAFLD progression[50-53]. For example, it has been suggested that fructose consumption is associated with hepatic fat accumulation, fibrosis, and inflammation[54]. The accumulation of visceral adipose tissue and higher plasma triglyceride concentrations have also been linked with fructose con-

sumption[55,56]. Thus, fructose has an important influence on the development of fatty liver disease[57].

Particular dietary sugars (especially fructose) are considered to play a role in the development and progression of NAFLD. The sugar additives (usually HFCS or sucrose) found in beverages and processed foods are widely viewed as the main source of the increased amounts of fructose consumed in developed countries. Dyslipidemia, obesity, and IR have all demonstrated strong associations with greater fructose consumption, and evidence indicating that fructose is involved in the development and progression of NAFLD is accumulating. Human studies have linked fructose consumption to hepatic fat accumulation, fibrosis, and inflammation. At present, it is unclear whether fructose can cause NAFLD on its own or whether it only promotes the condition when consumed in excessive amounts by individuals with a sedentary lifestyle, IR, and/or a positive energy balance. However, there is enough evidence to support a recommendation that the consumption of foods and drinks that are high in added fructose-containing sugars should

be limited[54,58].

Although we need to increase our knowledge regarding the influence of fructose on NAFLD, the links between excessive fructose consumption and hypertriglyceridemia, IR, and the accumulation of visceral adipose tissue are sufficiently clear to support a clinical recommendation that NAFLD patients decrease the amount of fructose in their diets.

AGE RECEPTORS

A variety of signaling pathways are activated by AGE synthesis *via* a series of cell surface receptors. Among AGE receptors, the multi-ligand receptor RAGE has been studied most extensively[59-63]. In addition, various other AGE receptors such as AGE-receptor complexes (AGE-R1/OST-48, AGE-R2/80K-H, and AGE-R3/galectin-3)[64,65] and certain members of the scavenger receptor family (SR-A[66], SR-B:CD36[67,68], SR-BI[69], SR-E:LOX-1[70], FEEL-1, and FEEL-2[71]) have been reported. It was reported that the expression of these AGE receptors varies between different types of cells or tissues and is influenced by metabolic changes, *e.g.*, changes associated with hyperlipidemia, DM, or aging[72]. *In vivo* and *in vitro* experiments examining the mechanisms responsible for the effects of AGEs and the factors that regulate their actions, *e.g.*, soluble RAGE (sRAGE), it was suggested that these molecules have significant pathobiological effects[63,73]. A variety of different cell types, such as neurons, hepatocytes, endothelial cells (EC), HSC, microglia, and pericytes, express RAGE[59-61].

In recent *in vitro* and *in vivo* studies, we found that protein amino moieties readily react with GLA to produce TAGE[18-20]. Furthermore, TAGE induce vascular inflammation and ROS production, and hence, promote the development of atherosclerosis in DM[74,75]. As TAGE display the greatest affinity for RAGE[74,75] and the binding of TAGE to RAGE adversely affects the vasculature of diabetic patients[18-20], TAGE might contribute to the greater CVD incidence rates seen in DM patients and impaired glucose tolerance (IGT) patients that display postprandial hyperglycemia. Furthermore, we have recently reported that in DM patients TAGE make significant contributions to the pathogenesis of angiopathy[19,20]. Accumulating evidence indicates that TAGE-RAGE interactions induce oxidative stress in various cell types, such as HSC and hepatocytes.

THE TAGE-RAGE SYSTEM IS INVOLVED IN LIVER DISEASE

As for the effects of TAGE on hepatocytes, we demonstrated that in Hep3B cells, a human hepatocellular carcinoma cell line, TAGE-RAGE interactions upregulated the hepatic production of C-reactive protein (CRP) by activating Rac-1[76]. The latter study indicated that at least two CRP expression-inducing signaling pathways are in operation in TAGE-treated Hep3B cells: the nuclear fac-

tor kappa B (NF-κB)-Rac-1-induced signal transducer and activator of transcription 3-dependent pathway, which is not directly affected by ROS, and an NADPH oxidase-mediated ROS-dependent pathway involving Rac-1[76]. During the induction of CRP expression by TAGE, the early stages of the process might be ROS-independent, whereas the latter stages might involve a ROS-mediated pathway. In Hep3B cells, the phosphorylation of insulin receptor substrate-1 (IRS-1) at its serine-307 residue and of c-Jun N-terminal kinase (JNK), c-JUN, and IκB kinase were promoted by TAGE. The increased phosphorylation of IκB kinase was associated with reductions in the concentration of IκB[77]. These effects of TAGE on Hep3B cells were abrogated by the overexpression of the dominant negative form of Rac-1. Treatment with curcumin, an inhibitor of NF-κB, or a JNK inhibitor decreased the phosphorylation of IRS-1 at its serine-307 residue in Hep3B cells. In addition, TAGE downregulated the tyrosine phosphorylation of IRS-1, weakened the affinity of the p85 subunit of phosphatidylinositol 3-kinase for IRS-1, and decreased glycogen synthesis in insulin-treated Hep3B cells. All of these effects were abrogated by treatment with NF-κB or JNK inhibitors[77]. Taken together, these results suggest that TAGE activate Rac-1, leading to the induction of the JNK- and IκB kinase-dependent serine phosphorylation of IRS-1, which in turn contributes to hepatic IR.

As the main producers of extracellular matrix molecules in the liver, HSC are important contributors to liver fibrogenesis[78]. In a previous study, we found that TAGE promoted the expression of genes and proteins associated with fibrogenesis or inflammation, *e.g.*, collagen type Iα2, monocyte chemoattractant protein-1 (MCP-1), and transforming growth factor-β1, in cultured HSC *via* NADPH oxidase-dependent ROS generation[22]. These results increase our knowledge of the role played by TAGE in the pathogenesis of NASH.

INTRACELLULAR TAGE ARE INVOLVED IN LIVER DAMAGE

GLA is a precursor of TAGE. Two GLA-forming pathways are considered to be in operation in the liver: (1) the glycolytic pathway and (2) the fructose metabolic pathway[18-20,38]. As a result, the liver tends to accumulate GLA to a greater extent than other organs.

Abnormalities in fructose and glucose metabolism can result in elevated intracellular GLA levels, which in turn can lead to upregulated intracellular TAGE synthesis, and such processes might play a role in the development of NASH. We found that in Hep3B cells GLA caused the intracellular TAGE concentration to rise and induced apoptosis in a concentration- and time-dependent manner[79]. Conversely, intracellular TAGE production was downregulated and GLA-induced apoptotic cell death was prevented by the addition of aminoguanidine, which inhibits AGE synthesis. Hepatocyte apoptosis was reported to be a characteristic of NASH in previous studies[80,81].

Figure 3 Proposed model for toxic advanced glycation end-products-mediated responses in the liver. HFCS/sucrose and dietary AGEs, which are normally found in sweetened beverages and commercial food products, are taken into the body, where they enhance the production/accumulation of TAGE, upregulate RAGE mRNA expression, and increase serum TAGE concentrations, leading to TAGE-RAGE interactions. The interaction between TAGE and RAGE alters intracellular signaling, gene expression, and the release of pro-inflammatory molecules and also induces oxidative stress in hepatocytes and hepatic stellate cells, which might contribute to the pathological changes observed in NAFLD/NASH. The formation of intracellular TAGE is associated with protein dysfunction followed by inflammation and cell death. Extracellular TAGE induce inflammation and fibrosis/cancer malignancy *via* RAGE signaling. AGEs: Advanced glycation end-products; TAGE: Toxic AGEs; RAGE: Receptor for AGEs; Hsc70: Heat shock cognate 70; ROS: Reactive oxygen species; VEGF: Vascular endothelial growth factor; HFCS: High-fructose corn syrup; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis.

We identified a TAGE-modified protein (approximately 70 kDa) that was initially observed and tended to accumulate in GLA-treated hepatocytes as heat shock cognate 70 (Hsc70)[79]. Hsc70 might be important for GLA-induced cytotoxicity, as TAGE modifications have been demonstrated to have deleterious effects on protein function[82,83]. Furthermore, we found that the mRNA expression level of the acute phase reactant CRP was up-regulated by intracellular TAGE[79]. Recently, it was demonstrated that NASH patients have higher plasma high-sensitivity CRP (hs-CRP) concentrations than healthy subjects or patients with simple steatosis[84,85]. Interestingly, in NASH patients a strong correlation was detected between the plasma hs-CRP concentration and the severity of liver damage[84,85]. In addition, intracellular TAGE were reported to induce inflammation, which is a characteristic of NASH. Taken together, these results indicate that intracellular TAGE make a significant contribution to the pathogenesis of NASH and might have potential as targets of treatments for NASH (Figure 3).

SERUM TAGE CONCENTRATIONS AND LIVER DISEASE

We measured the serum concentrations of three AGEs (Glu-AGEs, CML, and TAGE) in 66 patients with histologically defined-NASH who were free from liver cir-

rhosis, 10 patients with simple steatosis, and 30 control subjects to examine whether evaluating circulating AGE concentrations is useful for differentiating between NASH and simple steatosis[23]. The results of the latter study suggested that serum TAGE concentrations are involved in the pathogenesis of NASH and might be useful as biomarkers for differentiating between NASH and simple steatosis as: (1) The NASH patients exhibited significantly increased serum TAGE concentrations compared with the patients with simple steatosis and the healthy controls. According to receiver operating characteristic curves of the subjects' circulating TAGE concentrations, the optimal cut-off value for predicting NASH was 8.53 units/mL, which resulted in sensitivity and specificity values of 66.7% and 88.9%, respectively; (2) The subjects' homeostatic model assessment of insulin resistance (HOMA-IR) values and serum adiponectin concentrations (adiponectin is synthesized by adipose tissue and is an anti-inflammatory adipokine that can increase insulin sensitivity) exhibited positive and inverse correlations with their serum TAGE concentrations, respectively; (3) The subjects' serum TAGE concentrations were not correlated with the severity of their hepatic steatosis or fibrosis, nor were they influenced by the subjects' glucose tolerance status. The serum TAGE concentrations of the normal and IGT patients did not differ; (4) The NASH patients' hepatocytes contained TAGE,

whereas those belonging to the patients with simple steatosis exhibited negligible TAGE concentrations; and (5) The subjects' Glu-AGE and CML concentrations did not differ among the groups[23]. The above results indicate that serum TAGE concentrations are useful biomarkers for assessing residual liver function.

PUTATIVE MOLECULAR MECHANISMS RESPONSIBLE FOR THE ASSOCIATION BETWEEN NAFLD AND CARDIOVASCULAR DISEASE

Endothelial progenitor cells (EPC) help to maintain the structure and function of the endothelium, and hence, facilitate angiogenesis and vascular repair. In addition, the number of circulating EPC and their activity levels were found to be inversely correlated with atherosclerotic risk factors. Thus, the number and activity levels of EPC might be useful biomarkers for predicting cardiovascular events. In a recent study, Chiang *et al*[86] demonstrated that compared with the controls NAFLD patients had significantly fewer circulating EPC and the function of their EPC was impaired. Thus, in NAFLD patients reductions in the number of EPC or their activity might increase the likelihood of cardiovascular events.

In recent studies, we found that: (1) the serum concentration of TAGE, but not CML, was independently associated with HOMA-IR in non-diabetic subjects[87]; (2) in T2DM patients, the serum TAGE concentration, but not those of Glu-AGEs or hemoglobin A1c (HbA1c), can be used as a biomarker of cumulative postprandial hyperglycemia[88]; (3) the serum concentration of TAGE, but not those of HbA1c or CML, was demonstrated to be an independent predictor of vascular inflammation (as evaluated by [18F] fluorodeoxyglucose-positron emission tomography in outpatients who visited Kurume University Hospital)[89]; (4) in healthy subjects, the serum TAGE concentration was found to be independently associated with a reduction in the number of circulating EPC and the impairment of the migratory activity of EPC[90]; and (5) a Japanese trial assessing the utility of pitavastatin and atorvastatin as treatments for acute coronary syndrome reported that high baseline TAGE concentrations were associated with plaque progression[91]. These results suggested that the serum TAGE concentration, but not those of HbA1c, CML, or Glu-AGEs, might be a useful biomarker for predicting atherosclerosis progression and future cardiovascular events. Thus, TAGE-RAGE system activation is considered to lead to a greater risk of cardiovascular events and contribute to the progression of liver damage, which would provide a mechanism link between CVD and NAFLD/NASH.

NOVEL TREATMENT STRATEGIES

The majority of studies about NASH have attempted to assess the relationships between NAFLD/NASH and T2DM, CVD, or chronic kidney disease (CKD)[92]. The results outlined above strongly suggest that the TAGE-RAGE system is involved in the development and progression of NASH. As a result, several therapeutic strategies that target this system, *e.g.*, inhibiting TAGE synthesis, downregulating the expression of RAGE or molecules involved in its downstream pathways, and blocking TAGE-RAGE interactions, have been developed as potential treatments for NASH.

The inhibition of TAGE synthesis: Acarbose

Whilst there are many drugs that are able to improve glycemic control, including patients' postprandial plasma glucose concentrations, some drugs specifically target postprandial hyperglycemia.

The absorption of carbohydrates from the small intestine can be delayed by treatment with the α-glucosidase inhibitor acarbose, and T2DM patients that were administered acarbose displayed less severe postprandial hyperglycemia[93]. A recent study found that in patients with T2DM or IGT acarbose treatment reduced the rate at which the intimal media of the carotid arteries thickened and led to a lower incidence of CVD[93], indicating that acarbose ameliorates postprandial hyperglycemia, and hence, inhibits the development and progression of CVD. In an *in vivo* study, we found that protein amino moieties readily react with GLA to produce TAGE, leading to oxidative stress and vascular inflammation. These observations suggested that in DM GLA plays a role in promoting the development of atherosclerosis[18-20]. In a study involving T2DM rats, we demonstrated that the serum concentration of TAGE, but not HbA1c, is a marker of cumulative postprandial hyperglycemia[94]. Based on the abovementioned results, we suggest that acarbose reduces serum TAGE concentrations, which could at least partially explain its cardioprotective effects *in vivo*. In a previous study, 50 mg acarbose (dosing schedule: thrice a day for a 12-wk period) were administered to 13 Japanese T2DM patients who were free from inflammatory conditions, atherosclerotic heart disease, and microangiopathy and had never taken oral hypoglycemic agents. The patients' serum TAGE concentrations as well as their serum levels of other biological molecules were assessed before and after the administration of acarbose[88]. The DM patients' serum free fatty acid and TAGE concentrations had fallen significantly after 12 wk' acarbose treatment. Acarbose also reduced their postprandial plasma glucose concentrations. These results indicate that HbA1c concentrations might not accurately reflect the ameliorative effects of acarbose on postprandial hyperglycemia. Furthermore, they suggest that serum TAGE concentrations might be useful biomarkers for assessing cumulative postprandial hyperglycemia in T2DM patients. As TAGE have adverse effects on CVD[20], acarbose might be useful for preventing CVD in NASH patients with T2DM or postprandial hyperglycemia.

Inhibiting the binding of TAGE to RAGE using sRAGE

RAGE was found to contribute to acute liver damage in

numerous studies, and the blockade of RAGE was demonstrated to reduce cholestatic, toxic, and ischemic liver damage[95-98].

Patients with chronic liver injuries were found to exhibit significantly higher hepatic RAGE expression levels[99], and in NAFLD patients a correlation was detected between the severity of fibrosis and the patients' serum TAGE concentrations, indicating that RAGE and TAGE make significant contributions to the development of liver disease[23]. In addition, DM, which upregulates AGE synthesis and RAGE expression, has been found to accelerate the progression of fibrosis in a number of human liver conditions, including chronic hepatitis C and NAFLD[100]. Recently, we found that TAGE-RAGE interactions promote inflammation, affect the expression levels of various genes and the activity of intracellular signaling pathways, and induce oxidative stress in various kinds of cells. These effects might be involved in the pathological changes seen in various chronic diseases[17-20].

Endogenous sRAGE has recently been detected in humans[74]. It has been suggested that it is synthesized *via* the cleavage of a splice variant of RAGE (a type of secretory RAGE exhibiting C-terminal truncation) or full-length cell surface RAGE[74]. Patients with T1DM or T2DM display increased total endogenous sRAGE concentrations[101-104]. In addition, we and others have detected positive correlations between the total serum sRAGE concentration and serum TAGE concentrations in both non-DM and DM subjects[104,105]. Furthermore, body mass index-, sex-, and age-adjusted TAGE concentrations were found to increase significantly in proportion to the rise in the serum sRAGE concentration in non-DM subjects[104,105]. These results indicate that *in vivo* circulating sRAGE, which functions as a decoy receptor, is unable to bind to and remove the TAGE present in the blood in an efficient manner. As TAGE promote RAGE expression, the blood sRAGE concentration might be a marker of RAGE production within tissues. Furthermore, it might change in response to variations in the serum concentration of TAGE in order to ameliorate TAGE-induced tissue damage including NASH[106-109].

An angiotensin II type 1 receptor blocker: Telmisartan

It has been suggested that the TAGE-RAGE axis interacts with the renin-angiotensin system. In a previous study, we suggested that the angiotensin II type 1 receptor blocker telmisartan reduces RAGE expression *via* its ability to modulate the peroxisome proliferator-activated receptor-γ (PPAR-γ)[21,110]. We came to this conclusion due to the following observations, which were obtained in experiments involving Hep3B cells: (1) whilst telmisartan downregulated ROS synthesis, TAGE-induced RAGE expression, and CRP expression, candesartan did not induce any of these processes; (2) the PPAR-γ inhibitor GW9662 abrogated the telmisartan-induced inhibition of the expression of RAGE and its associated effector molecules; (3) the effects of ciglitazone and troglitazone, which are full agonists of PPAR-γ, were similar to those

of telmisartan; and (4) the administration of curcumin, an inhibitor of NF-κB, or antioxidants abrogated the upregulation of CRP mRNA expression induced by TAGE. Due to its unique ability to modulate PPAR-γ, telmisartan is increasingly considered to be a useful cardiometabolic sartan[21,110,111]. In addition, it has been demonstrated that thiazolidinediones downregulate endothelial RAGE expression *via* NF-κB suppression[112]. These results suggest that telmisartan has anti-inflammatory effects on TAGE signaling; *i.e.*, it reduces hepatic RAGE expression by activating PPAR-γ, and might also help to protect against NASH.

A hydroxymethyl-glutaryl-CoA reductase inhibitor: Atorvastatin

In a recent study, we found that in Hep3B cells the hydroxymethyl-glutaryl-CoA reductase inhibitor atorvastatin reduced TAGE-induced ROS synthesis in a dose-dependent manner[113]. In addition, atorvastatin and the antioxidant N-acetylcysteine downregulated CRP expression at both the mRNA and protein levels in TAGE-treated Hep3B cells[113]. These results showed that the antioxidative effects of atorvastatin abrogate CRP expression-associated TAGE signaling. Furthermore, they indicate that statins protect blood vessels from damage and abrogate the adverse effects of TAGE by downregulating the activity of their effector molecules.

The consumption of fructose-containing beverages is associated with a greater risk of MetS-related conditions, including NAFLD. Despite the fact that caloric restriction and weight loss is the only effective treatment for NAFLD, it has been demonstrated that atorvastatin is safe for use in NAFLD patients and results in improvements in their hepatic histology. In a previous study, we found that atorvastatin reduced the serum TAGE concentrations of 43 patients with a combination of biopsy-proven NASH and dyslipidemia[114]. After 12 mo atorvastatin treatment (10 mg daily), all of the patients demonstrated significant reductions in their hepatic transaminase (aspartate aminotransferase and alanine aminotransferase (ALT) and γ-glutamyl transpeptidase concentrations. In addition, by end of the treatment their plasma tumor necrosis factor-α (TNF-α) and plasma adiponectin concentrations were reduced by 31% and elevated by 16%, respectively. The patients' HOMA-IR values were slightly reduced. The patients' liver/spleen ratios rose significantly from 0.54 ± 0.26 at the baseline to 0.94 ± 0.24 at the end of the treatment; however, their visceral fat area values were unchanged. During the treatment, the patients' serum TAGE concentrations fell significantly (they were 10.4 ± 3.8, 5.9 ± 3.3, and 2.5 ± 1.1 units/mL before the treatment and after 6 mo and 12 mo treatment, respectively). Correlations were detected between the patients' serum TAGE concentrations and their serum concentrations of thiobarbituric acid reactive substances (TBARS), TNF-α, procollagen type III propeptide, ALT, and type IV collagen 7S[114].

The administration of atorvastatin to Sprague-Dawley

male rats that had consumed a liquid fructose solution (10% w/v) abrogated the inflammatory and metabolic changes induced in the liver by fructose. These beneficial effects were considered to be due to the anti-inflammatory activity of atorvastatin and its downregulation of the hepatic expression of fructokinase, which inhibits fructose metabolism in the liver[115]. Reduced synthesis of GLA (a TAGE precursor and a fructose metabolite) leads to a drop in TAGE synthesis. Atorvastatin is able to reduce the serum TAGE concentration without altering glucose metabolism and does so in a cholesterol-lowering-independent manner. In the abovementioned study, the serum TAGE concentrations of the NASH patients with dyslipidemia fell significantly after the atorvastatin treatment, but their glucose metabolism was unaffected[114]. In conclusion, atorvastatin was demonstrated to be an effective treatment for NASH patients with dyslipidemia who did not respond adequately to diet and exercise therapy. In addition to improving their serum TAGE concentrations, atorvastatin also improved their histological and biochemical data. As atorvastatin decreased the serum TAGE concentrations of NASH patients with dyslipidemia, TAGE might be useful biomarkers for the treatment of NASH[114]. Controlled trials should be performed to further examine the clinical utility of TAGE as biomarkers in NASH.

Dietary AGEs: Kremezin

A study involving mice produced found that AGEs facilitate the progression from simple steatosis to NASH and liver fibrosis[116]. In the methionine choline-deficient rat model of NAFLD, high dietary consumption of AGEs results in elevated hepatic AGE concentrations and increased fibrosis, liver damage, and inflammation. The latter effects are considered to be mediated *via* the RAGE- and oxidative stress-dependent profibrotic effects of AGEs on activated HSC[117]. The above observations indicate that pharmacological and dietary strategies that target the AGE-RAGE system are able to slow the progression of NAFLD.

In a recent study, we detected increased hepatic expression levels of vascular endothelial growth factor (VEGF) and RAGE in rats that had been administered Glu-AGE-rich beverages. This suggested that dietary AGE consumption is associated with the hepatic expression of liver fibrosis-related genes[118]. Moreover, the abovementioned rats' livers were found to contain TAGE- and Glu-AGE-positive cells[118]. These results indicate that the consumption of Glu-AGE-rich beverages leads to upregulated hepatic expression of RAGE and VEGF and encourages the build-up of TAGE and Glu-AGEs, resulting in the binding of TAGE to RAGE. Thus, it is important to consider the amounts of Glu-AGEs present in foods to prevent liver disease, especially in people that are at risk of CKD, CVD, NAFLD/NASH, or DM.

It has been demonstrated that Kremezin, an oral adsorbent that consists of porous spherical carbonic particles, is able to attenuate the progression of chronic renal failure (CRF) by removing uremic toxins, *e.g.*, indoxyl sulfate precursors, from the intestine[119]. In CRF patients without DM, 3 mo Kremezin treatment (6 g/d) resulted in markedly reduced serum TAGE and Glu-AGEs concentrations, while the concentrations of these molecules were unaffected in renal function- and age-matched CRF patients that did not receive the drug[120]. The EC in the post-treatment serum samples collected from the Kremezin-treated patients exhibited markedly lower concentrations of MCP-1, vascular cell adhesion molecule-1, and RAGE mRNA than those found in the serum samples collected before treatment[120]. These findings indicate that the pathogenesis of vascular damage is influenced by dietary Glu-AGEs in TAGE-RAGE-related conditions and that reducing the amount of dietary Glu-AGEs taken into the body might represent a useful strategy against NAFLD/NASH.

Further clinical studies might provide insights into whether restricting the consumption of Glu-AGEs would be beneficial for preventing or slowing the progression of NAFLD/NASH and whether Glu-AGEs represent a novel therapeutic target for treatments that aim to reduce the risk of liver disease.

CONCLUSION

TAGE formation and accumulation are known to increase in various tissues during normal aging and to occur at a markedly accelerated rate in DM patients[18-20]. An increasing body of evidence suggests that TAGE are involved in the pathogeneses of various disorders including hypertension, Alzheimer's disease, diabetic vascular complications, CVD, NAFLD/NASH, and cancer growth and metastasis[7,8,18-23,79,87-91,114,121-126]. We found evidence that TAGE are involved in the pathogenesis of NASH in humans[7,23,114]. TAGE stimulated the proliferation and activation of HSC *in vitro via* RAGE, which resulted in hepatic inflammation and fibrosis[22]. In addition, NASH patients exhibited significantly higher serum TAGE concentrations than patients with simple steatosis or healthy controls[7,23]. Atorvastatin reduced the serum TAGE concentrations of NASH patients with dyslipidemia, and correlations were detected between the patients' serum TAGE concentrations and their serum TNF-α, ALT, type IV collagen 7S, procollagen type III propeptide, and TBARS concentrations[114]. In a recent study, we found that non-B or non-C hepatocellular carcinoma (NBNC-HCC) patients had significantly increased circulating TAGE concentrations compared with NASH subjects without HCC and the control subjects[127]. The findings outlined in the present review indicate that TAGE contribute to the pathogenesis of NBNC-HCC and that they might be useful biomarkers for discriminating between NBNC-HCC and NASH.

In conclusion, an increasing amount of evidence indicates that TAGE and RAGE both make important contributions to liver disease. TAGE might play a role in the development and progression of NASH and could be
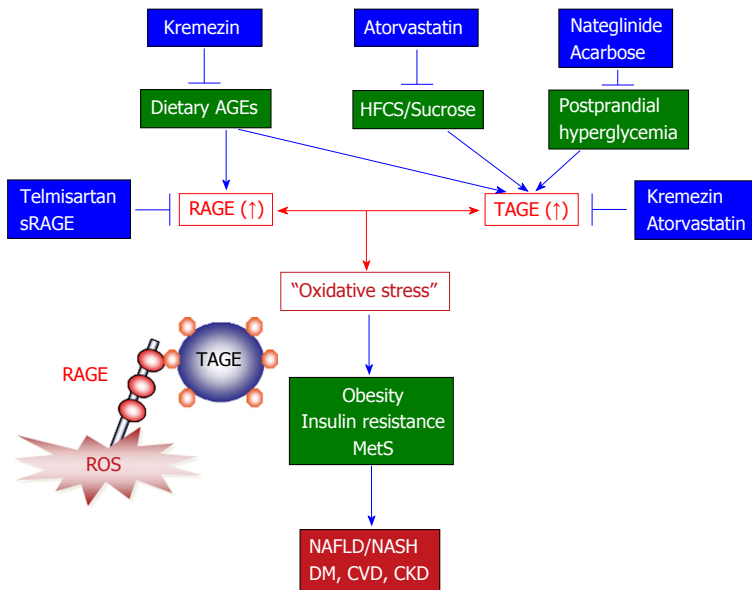
Figure 4 The toxic advanced glycation end-products-receptor for advanced glycation end-products system and novel treatments that target this system to prevent the development and/or progression of non-alcoholic steatohepatitis. Accumulating evidence suggests that TAGE-RAGE interactions affect intracellular signaling, gene expression, and the release of pro-inflammatory molecules and also induce oxidative stress in numerous types of cells, all of which have the potential to contribute to the pathological changes associated with lifestyle-related diseases including NAFLD/NASH. Since TAGE display the strongest binding affinities for RAGE and have adverse effects on diabetic vessels through their interactions with RAGE, TAGE might be partly responsible for the increased risk of cardiovascular disease (CVD) seen in diabetes mellitus (DM) patients and the impaired glucose tolerance observed in patients with postprandial hyperglycemia. NAFLD is considered to be a hepatic symptom of metabolic syndrome (MetS) and is strongly associated with insulin resistance, obesity, and abnormalities in glucose and lipid metabolism. It is important to consider the amounts HFCS/sucrose and AGEs present in foods to prevent liver disease, particularly in individuals that are at high risk of developing NAFLD/NASH, DM, CVD, or chronic kidney disease (CKD). Taken together, the present study suggests that TAGE could be used as novel therapeutic targets for the prevention of lifestyle-related diseases. Therefore, inhibiting the formation of TAGE, blocking TAGE-RAGE interactions, and suppressing the expression of RAGE or its downstream effectors all have potential as therapeutic strategies against lifestyle-related disease including NAFLD/NASH. AGEs: Advanced glycation end-products; TAGE: Toxic AGEs; RAGE: Receptor for AGEs; sRAGE: Soluble form of RAGE; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; HFCS: High-fructose corn syrup.

useful biomarkers for differentiating between NASH and NAFLD or between NBNC-HCC and NASH. Further clinical and experimental studies are required to elucidate the mechanisms by which the TAGE-RAGE system affects the development and progression of lifestyle-related conditions including NAFLD/NASH (Figure 4).

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TOPIC HIGHLIGHT

WJH 6th Anniversary Special Issues (7): Non-alcoholic fatty liver disease

Transitions of histopathologic criteria for diagnosis of nonalcoholic fatty liver disease during the last three decades

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is the hepatic manifestation of metabolic syndrome, and is the most common type of chronic liver diseases in the majority of developed countries. NAFLD shows a wide spectrum of disorders including simple steatosis, nonalcoholic steatohepatitis (NASH), and cirrhosis. While simple steatosis is recognized to be benign and stable, NASH is considered to be an aggressive form of the disease progressing to cirrhosis. Currently, differentiation between NASH and simple steatosis can be done only by liver biopsy. Despite many proposals and revisions, the histological criteria for the differentiation have not been perfected yet. In this review article, the changes in the histopathologic criteria of NAFLD during the last three decades are summarized, and perspectives of the future changes are demonstrated. The discussion focuses on how pathologists have been dealing with "hepatocellular ballooning". Loose criteria, in which hepatocellular ballooning was not required for the diagnosis of NASH, were applied in many clinical studies published in around 2000's, whereas a strict criterion based on the presence/absence of hepatocellular ballooning was approved recently. Hence, simple and reliable methods

of identifying ballooned hepatocytes are being sought. Clinical and pathological predictors of NAFLD-related hepatocarcinogenesis will also be sought in the future.

Core tip: The differentiation between nonalcoholic steatohepatitis and simple steatosis can be done only by liver biopsy. Through many proposals and revisions, the histological criteria for the differentiation have been changed. The changes in the criteria during the last three decades are exhibited in this review article, with a special interest in "hepatocellular ballooning".

Ikura Y. Transitions of histopathologic criteria for diagnosis of nonalcoholic fatty liver disease during the last three decades. *World J Hepatol* 2014; 6(12): 894-900 Available from: URL: http://www.wjgnet.com/1948-5182/full/v6/i12/894.htm DOI: http://dx.doi.org/10.4254/wjh.v6.i12.894

INTRODUCTION

In newly proposed disease entities, or even in already established ones, the definitions and diagnostic criteria may be revised repeatedly. The revisions are led by alterations in recognition of the disease, changes in morbidity and social healthcare strategy in each era, elucidation of the pathologic mechanisms, *etc*. Hence, these changes probably occur more frequently in a disease of unknown etiology. Nonalcoholic fatty liver disease (NAFLD) and its aggressive form, nonalcoholic steatohepatitis (NASH),

Table 1 Histological criteria for diagnosis of nonalcoholic steatohepatitis used in the previous studies (1980-preset)							
Ref.	Year	Steatosis	Inflammatory cell infiltration	Hepatocellular necrosis	Hepatocellular ballooning	Mallory-Denk body	Pericellular fibrosis
Ludwig et al[1]	1980	≥ Moderate	+	+			
Falchuk et al[23]	1980	≥ Moderate				+	+
Diehl et al[26]	1988	≥ Mild		+		+1	+1
Nagore et al[25]	1988	≥ Mild	+		+	+	+
Lee et al[35]	1989	≥ Mild	+				+
Powell et al[28]	1990	≥ Moderate	+				
Wanless et al[29]	1990	≥ 5%			+		
Bacon et al[36]	1994	≥ Minimal	+				
Laurin et al[37]	1996	≥ Minimal	+				
George et al[38]	1998	≥ Minimal	+				
Younossi et al[32]	1998	> 1/3	+	+			
Matteoni et al[33]	1999	> 1/3	+	+	+2		
Brunt et al[41]	1999	> 0%	+				
Dixon et al[44]	2001	≥ Mild	+	+	+1		+1
Neuschwander-Tetri et al[2]	2003	≥ 5%	+		+		
Bedossa et al[51]	2012	> 5%	+		+3		

1Either one; 2Modified in 2009[34]; 3Normal-sized hepatocyte with clear reticular cytoplasm. +: Required; Blank: Not required.

are representative examples.

Although NASH/NAFLD have generally been accepted as independent diseases since Ludwig's monumental publication in 1980[1], minor revisions regarding definition, criteria (mainly histopathologic features and a cutoff level of alcohol consumption) and diagnostic algorithm have continued to be made. A goal of the revisions is establishment of accurate selection criteria to extract NAFLD cases that are most likely to progress to cirrhosis or to hepatocellular carcinoma (HCC). The selected patients become subjects of follow-up and therapeutic interventions[2,3]. NAFLD is considered to be the most common chronic liver disease in the majority of developed countries, and clarification of the high-risk group of NAFLD patients is the most critical issue in current hepatology.

Noninvasive clinical methods, which can evaluate the degree of steatosis and can diagnose NAFLD in some cases, have been developed[4,5]. However, since they cannot evaluate inflammatory activity, the diagnosis of NASH still requires histological examination[4]. It is not possible to perform liver biopsy in every NAFLD patient, and thus, the detailed pathobiological and clinicopathologic characteristics of NASH/NAFLD have not yet been elucidated. Consequently, the histopathologic criteria for the diagnosis of NASH/NAFLD have changed repeatedly. The ambiguous and wandering criteria have confused general pathologists.

What are the reliable histopathologic markers of true NASH? No one can provide an appropriate answer to this substantial query. I review the 30-year history of the revision process that contained many trials and errors (Table 1). This review may not only introduce a clue to the answer, but also provide a direction for future studies on NASH/NAFLD.

BEFORE "LUDWIG" (-1979)

The proposal of NASH as a new disease entity by Ludwig et al[1] in 1980 was truly the first milestone in NASH/NAFLD research. Historically, many pathologists prior to Ludwig focused on fatty livers and cirrhosis associated with morbid obesity or diabetes[6-13]. Histologic pictures shown in the earlier reports were of NASH/NAFLD according to the current diagnostic criteria. They had noticed even some morphological features of this type of fatty livers rather different from alcoholic fatty livers, such as low percentages of Mallory-Denk body and siderosis and frequent nuclear glycogen[11-13]. However, due to the facts that most fatty livers did not progress to fibrosis and cirrhosis[13,14], and that livers could physiologically store a certain amount of lipid, there had been for a long time controversy regarding the pathologic significance of lipid accumulation in livers. In other words, fatty change was considered as an innocent bystander, not harmful, and an accompanying phenomenon caused by hepatotoxic pathogens[15].

There was no obvious definition of the physiological level of hepatic fat. Galambos et al[16] studied hepatic histopathologic findings corresponding to abnormal laboratory test results in obese patients. In that study, the authors defined > 33% fatty change as an abnormal/pathologic condition. There was no explanation about how the authors determined 33% as the normal limit. This fact indicates that the value of 33% was acceptable without any explanations as the normal limit at that time.

Undiscovered hepatitis C virus (HCV)[17] might have disturbed to recognize NASH/NAFLD as independent hepatic disorders. Especially HCV genotype 3 is now known to be able to cause prominent hepatic steatosis as well as necroinflammation[18]. Pathologists might have misunderstood NASH as viral hepatitis, and simultaneously, HCV-related hepatitis as primary steatotic liver disease. The potential overlap of NASH/NAFLD and HCV-related hepatitis is still a focus of debate[19,20].

In that era, earlier than Ludwig's, many reports concerning NASH/NAFLD were published from Japanese institutes[7,21,22]. Although the medical interest had not
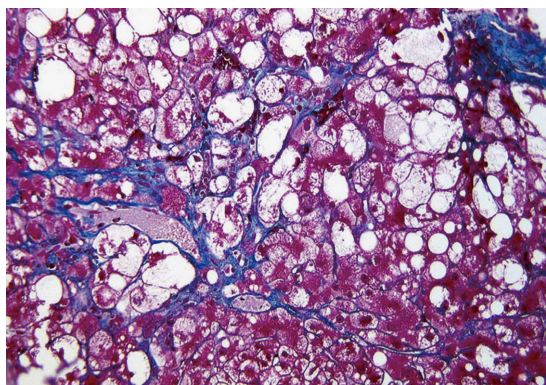
Figure 1 Typical case of nonalcoholic steatohepatitis showing hepatocellular ballooning and perivenular/pericellular fibrosis (Azan-Mallory stain; Original magnification, × 200).

been directed to metabolic syndrome, Japanese pioneer researchers investigated fatty liver disorders with keen observations and deep insights. Surprisingly, they suggested that fatty change was a first step of NAFLD progression to cirrhosis and dysfunctions of hepatocellular organelles including endoplasmic reticulum were pivotal[7,22]. These are completely identical with the present recognitions of pathological mechanisms of NASH/NAFLD.

AFTER "LUDWIG" (1980-1999)

The current disease concept and terminology of NASH were established only by a single pathologic report entitled "Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease" written by Ludwig et al[1] in 1980. At present, it is well recognized that the contribution of this breakthrough article to hepatology is too large to be estimated. The report consisted of clinicopathologic reviews of twenty cases of NASH. Their inclusion criteria, namely diagnostic criteria of NASH, were extremely simple and clear: non-habitual drinkers with liver damage that was indistinguishable from alcoholic injury histologically. The authors proposed to categorize all types of liver damages fulfilling the criteria into one disease entity named NASH. A little confusion might have arisen because NASH included fatty liver disorders associated with nutritional disturbances and even drug-induced damage as well as those associated with morbid obesity and diabetes. In addition, the definition of "nonalcoholic" became a big issue; they excluded only obvious alcohol abusers.

In the same year (1980), Falchuk et al[23] published their article entitled "Pericentral Hepatic Fibrosis and Intracellular Hyalin in Diabetes Mellitus", and suggested that an inflammatory hepatic disorder associated with diabetes mellitus was an intermediate illness between fatty liver and cirrhosis. The contents of the papers by Ludwig et al[1] and Falchuk et al[23] complemented each other, and emphasized the independence and importance of NASH among chronic liver diseases. However, the etiopathology of NASH was not elucidated, and the concept of NASH

did not gain complete acceptance for about 20 years. As proof, some articles very similar to the earlier studies than 1980 were published, and different terms such as diabetic hepatitis and fatty liver hepatitis were used instead of NASH[24,25]. A study seeming to have repeated the Ludwig's original work also appeared. It was not surprising that the results of those studies validated the presence of the new disease, NASH, and its progressive nature[25,26].

A growing interest produced one substantial question: what kind of fatty liver disorders is truly progressive? Two scientific streams, which still influence today's research trends, sprang from the query. The diagnostic criteria in the streams have been gradually modified.

One of the scientific streams was to highlight specific findings of NASH/NAFLD. Clain et al[27] systematically reviewed previous papers on NAFLD, and concluded that the presence of perivenular/pericellular fibrosis (Figure 1) potentially indicated a progressive disease[9]. Powell et al[28] confirmed that NASH was actually a slowly progressing disease, and tried to classify NASH on the basis of steatosis, inflammation, Mallory-Denk bodies and fibrosis. However, they could not find a relationship with prognosis. Wanless et al[29] accentuated the importance of hepatocellular ballooning (Figure 1), and made a diagnosis of NASH according to the presence of steatosis and ballooning. In that article, histologically abnormal lipid accumulation was defined as fatty change that affected more than 5% of hepatocytes. There was no obvious evidence for the definition of "more than 5%". A previous biochemical analysis revealed that normal livers (livers of healthy persons) could store lipids comprising less than 5% of liver weight[30]. Accordingly, "more than 5%" has been used as a standard value for defining pathologic hepatic lipid accumulation until now. Teli et al[31] defined NASH as hepatic steatosis with lobular inflammation, hepatocellular ballooning, Mallory-Denk bodies, and hepatocellular necrosis. They suggested that non-NASH NAFLD (namely, simple steatosis, and steatosis with portal inflammation) did not progress to NASH and cirrhosis. This stream led to the NAFLD classification of Younossi et al[32] and to Matteoni's classification[33]. Hepatocellular ballooning was a key finding for their classification. Later they insisted on the necessity of hepatocellular ballooning in diagnosis of NASH (Table 1)[34]. In their original classification, however, they used a term "steatohepatitis" for type 2 NAFLD, which was steatosis with lobular inflammation but without ballooning.

The other scientific stream was to establish a score system based on semi-quantitative analyses of the histological severity of liver damage. The trial was initiated by Diehl et al[26], followed by Lee et al[35]. They evaluated the degree of steatosis, inflammatory cell infiltration, hepatocellular damage, Mallory-Denk bodies, and fibrosis using four- or five-step scales. Unfortunately, they failed to find a relationship between the scores and prognoses. Bacon et al[36] also performed a semi-quantitative analysis using a system similar to Diehl's system, but did not described its relationship with prognosis. Whilst the cutoff alcohol
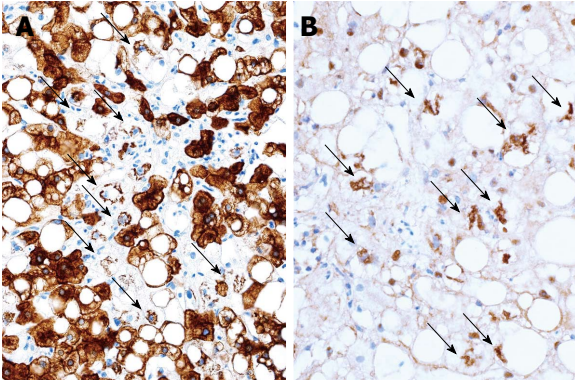
Figure 2 Typical immnohistochemical findings of nonalcoholic steatohepatitis. A: Cytokeratin (CK)18; B: Ubiquitin. Stained (brown) small aggregates are seen in ballooned hepatocytes with CK18-negative cytoplasms (arrows) [Immunoperoxidase; original magnifications, (A) × 150 and (B) × 200].

consumption ensuring "nonalcoholic" was strict (less than 20 g ethanol/d; it is almost same as the current standard, < 30 g for men and < 20 g for women[3]), they used a very loose histological criterion for diagnosing NASH. The minimal diagnostic requirements were only steatosis and inflammatory cell infiltration into lobular areas. Various clinical studies of NASH using loose diagnostic criteria similar to theirs were published thereafter[37,38]. Finally, even simple steatosis was recognized to be one aspect in the spectrum NASH and to be an ongoing change potentially progressing to more severe NASH or cirrhosis[39]. The well-known "two-hit theory" presented in the same year was generated through analogous hypothetical thinking[40]. Brunt's system[41] was established and published in such research background. They evaluated the severity of NASH using a histological score composed of inflammation grade and fibrosis stage similar to the METAVIR score[42]. The NASH severity score was used in many clinical studies, and contributed to the subsequent flowering of research on NASH. Unexpectedly, however, fairly mild fatty liver disorders were implicated in NASH cohorts by being diagnosed as grade 1 NASH. The original purpose of Brunt's system was to show a standard for evaluation of the severity of NASH. They did not seem to intend primarily to present a diagnostic criterion of NASH[43]. The expanded understanding (or misunderstanding) of NASH had rapidly spread, apart from the inventors' idea.

AFTER BRUNT AND MATTEONI (2000-PRESENT)

About ten years after the flowering of NASH/NAFLD research in Western countries, Japanese hepatologists also began to study NASH/NAFLD aggressively, due to the wide prevalence of metabolic syndrome in the beginning of the 21st century. Many NASH/NAFLD researchers in Japan understood that the minimum requirements for diagnosis of NASH were Brunt's grade 1 (without hepatocellular ballooning) and Matteoni's type 2 NAFLD. Their recognitions had been conserved even after the decision of the AASLD Single Topic Conference in 2002[2],

in which hepatocellular ballooning was officially recommended as a factor in the diagnosis of NASH. They did not notice the controversy about NASH diagnostic criteria[44] and the kaleidoscope changes of the criteria in that period.

In 2005, the NAFLD activity score (NAS), which was considered as a type of modified Brunt's system, was developed and published by Kleiner et al[45] This also led to a simplistic recognition that a NAS of over 5 points is NASH[46], and led to further confusion in the laboratory and clinical settings.

On the other hand, Younossi et al[47] confirmed that hepatocellular ballooning was a predictor of liver-related death in their follow-up study, and insisted that Matteoni's classification was superior to Brunt's system or NAS. Brunt et al[48,49] also cautioned about the presence of cases of non-NASH NAFLD showing NAS ≥ 5 and cases of NASH showing NAS ≤ 4, to avoid misusage of NAS. Through their discussions and controversies[49], the diagnostic criteria of NASH/NAFLD were revised and standardized hastily. Hepatocellular ballooning finally became the most important finding in the diagnosis of NASH.

However, the difficulty in correctly identifying hepatocellular ballooning subsequently became a critical issue[50]. To overcome this problem, Bedossa et al[51] proposed a semi-quantitative method in which all hepatocytes with clear reticular cytoplasm were defined as ballooning and graded by the cellular size. The same research group has recently published a new diagnostic algorithm in which hepatocellular ballooning is a root node of the binary tree[52].

Examination of specific markers for ballooning has been recommended as a method to determine it objectively. Hepatocellular ballooning is a result of degeneration and fragmentation of cytoskeleton intermediate filaments, cytokeratin (CK) 8/18, and an aggregates of the degenerated CK 8/18 is a Mallory-Denk body[53]. Immunohistochemical staining for CK 18, ubiquitin and p62 can be applied to detect hepatocellular ballooning. A negative result for the presence of CK 18 in hepatocytes can be interpreted as degenerative disappearance of CK 18, and the presence of ubiquitin-, p62-, and CK 18-positive intracellular inclusions indicates aggregation of degenerated CK 18 (Figure 2). The usefulness of ubiquitin immunohistochemistry in the diagnosis of NASH was first suggested in 2000[44,54]. Recently, the significance of these special stainings has been reconfirmed[55,56].

Alternatively, controversy about such excessive weighting to hepatocellular ballooning in the diagnosis may arise. Perivenular/pericellular fibrosis (Figure 1) should be highlighted more because of its close association to hepatocellular ballooning and its potential linkage to cirrhosis[9]. The diagnostic criteria of NASH/NAFLD still remain to be improved.

FUTURE DIRECTIONS

Although the histological criteria of NASH/NAFLD have been revised during the last three decades, there has

Figure 3 Case of hepatocellular carcinoma (A) associated with simple steatosis (B)[59] [upper row, histology with immunoperoxidase for a peroxidation marker (inset), original magnifications, (A) × 100 and (B) × 100; lower row, a macroscopic photo of the sample].

been an absolute premise: cirrhosis is a final advanced form of NAFLD. However, recent reports of HCC associated with NAFLD may deny the central dogma of the NASH/NAFLD concept. Surprisingly, considerable numbers of such HCCs arose from non-cirrhotic steatotic livers or even from livers with simple steatosis (Figure 3)[57-59]. Accumulation of cellular damage without major morphological changes and acceleration of cellular senescence may lead to hepatocarcinogenesis[59,60]. The facts will possibly lead to a paradigm shift in medical strategies for NAFLD.

How do we select which NAFLD patients to follow up? What is a true prognostic factor of NAFLD? Is it a histological finding? These are the ultimate themes for NAFLD researchers.

CONCLUSION

While reviewing the 30-year history of changes in the histological criteria for the diagnosis of NASH/NAFLD, the importance of hepatocellular ballooning in the diagnosis of NASH, imperfectness of the present criteria, and necessity of exploring new predictors of hepatocarcinogenesis were elucidated. Further collection of evidence is necessary to solve these problems, and pathologists will play central roles in this process.

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REVIEW

Vitamin D deficiency in chronic liver disease

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Abstract

Vitamin D is an important secosteroid hormone with known effect on calcium homeostasis, but recently there is increasing recognition that vitamin D also is involved in cell proliferation and differentiation, has immunomodulatory and anti-inflammatory properties. Vitamin D deficiency has been frequently reported in many causes of chronic liver disease and has been associated with the development and evolution of non-alcoholic fatty liver disease (NAFLD) and chronic hepatitis C (CHC) virus infection. The role of vitamin D in the pathogenesis of NAFLD and CHC is not completely known, but it seems that the involvement of vitamin D in the activation and regulation of both innate and adaptive immune systems and its antiproliferative effect may explain its importance in these liver diseases. Published studies provide evidence for routine screening for hypovitaminosis D in patients with liver disease. Further prospectives studies demonstrating the impact of vitamin D replacement in NAFLD and CHC are required.

Core tip: (Vitamin D and liver disease) vitamin D deficiency has been frequently reported in many causes of chronic liver disease and has been associated with the development and evolution of non-alcoholic fatty liver disease (NAFLD) and chronic hepatitis C (CHC) virus infection. The role of vitamin D in the pathogenesis of NAFLD and CHC is not completely known, but it seems that the involvement of vitamin D in the activation and regulation of both innate and adaptive immune systems and its antiproliferative effect may explain its importance in these liver diseases.

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INTRODUCTION

Vitamin D insufficiency and deficiency are prevalent in almost half the healthy population of developed countries[1]. Most experts define vitamin D insufficiency as a 25(OH)D level below 75 nmol/L (30 ng/mL) and deficiency as levels below 50 nmol/L (20 ng/mL). It is estimated that one billion people suffer from deficiency or insufficiency of vitamin D[2]. In the United States, between 25% and 50% of the adult population has vitamin D deficiency[3]. In patients with chronic liver diseases, the prevalence of vitamin D deficits is much higher and practically universal[4]. Up to 93% of patients with chronic liver disease have insufficient vitamin D levels, and almost one-third of these show severe deficiency[5].

The outcome of vitamin D deficiency in terms of osteoporosis, osteomalacia and increased fracture risk is well known[6,7]. Furthermore, the association between vitamin D deficiency and the development of infections, cardiovascular, autoimmune and degenerative diseases and several types of cancer (colon, prostate and breast cancer) has also been reported[8]. Vitamin D is important

Figure 1 Vitamin D synthesis. VDR: Vitamin D receptor; DBP: Vitamin D-binding protein; UVB: Ultraviolet radiation; RXR: Retinoid X receptor.

in calcium homeostasis and has also been implicated in the mechanisms of cellular proliferation, differentiation and immunomodulation[9]. These effects are noted in the pathogenesis and treatment of many chronic liver diseases. In this review, we will focus on vitamin D functions involved in the development of chronic liver disease and on the relationship between vitamin D deficiency and the two main causes of chronic liver disease: chronic hepatitis C (CHC) virus infection and non-alcoholic fatty liver disease (NAFLD).

An evidence-based approach was used for this review. MEDLINE search was performed to September 2014 using the following MeSH terms: liver diseases, vitamin D, cholecalciferol, hepatitis C, Chronic, nonalcoholic fatty liver disease. Searches were limited to English language articles. References of suitable articles were searched for other appropriate articles.

VITAMIN D SYNTHESIS

Under normal conditions, biogenesis from epidermal cells is the main source of vitamin D. In the skin, ultraviolet radiation from sun exposure transforms 7-dehydrocholesterol, a metabolite of cholesterol, into pre-vitamin D_3, which is transformed into vitamin D_3 (cholecalciferol). A small portion of vitamin D comes from dietary sources, such as milk and eggs, in the form of vitamin D_2 (ergocalciferol) and D_3 that is absorbed in the intestine by biliary acids[1,10]. Vitamin D synthesized from skin and from dietary sources may be stored in the adipocytes, or it may undergo hepatic 25-hydroxylation. This latter process is mediated by isoforms of the P450 cytochrome (CYP2R1, CYP27A1), the 25-hydroxylases, which produce 25-hydroxyvitamin D [25(OH)D] or calcidiol. The metabolite 25(OH)D, most abundant in blood, is an inac-

tive form of vitamin D. It has a half-life of 2-3 wk and is a useful measure of vitamin D levels because it reflects the total amount of vitamin D from dietary sources, sun exposure and conversion from fatty deposits of the liver, and its concentration in plasma is the most reliable indicator of vitamin D status[11]. This vitamin D metabolite, like others, is a low-solubility lipophilic molecule that moves through the bloodstream attached to plasmatic proteins, the most prevalent of which is vitamin D-binding protein (DBP), also known as Gc. Up to 88% of serum 25(OH)D is attached to a DBP, a protein synthesized mainly in the liver that has anti-inflammatory and immunomodulating functions independent of its role as a vitamin D transporter[12,13]. 25(OH)D is hydroxylated in the proximal tubules of the kidney by 1α-hydroxylase (CYP27B1) that form $1\alpha,25(OH)_2D$ or calcitriol, the most biologically active and powerful metabolite of vitamin D[1]. CYP27B1 activity has been observed in the kidney and other tissues, including the liver, fat tissue and the cells of the innate immune system[14]. Finally, 24-hydroxylase, which is most abundant in the intestine and the kidney, catabolizes the calcitriol into an inactive metabolite that is excreted in bile[15] (Figure 1).

$1\alpha,25(OH)_2D$ has a half-life of 4 h. It is transported *via* attachment to plasmatic proteins such as DBP and, as mentioned previously, conducts most of the biological effects of vitamin D by directly and indirectly controlling the expression of over 200 genes linked to angiogenesis, apoptosis, proliferation, differentiation and immunomodulation[1,16,17]. The biological effects of vitamin D are mediated by binding to the vitamin D receptor (VDR), belongs to the superfamily of nuclear steroid hormone receptors, which is expressed in more than 30 tissues, including the liver, the pancreatic islet cells, the epithelial cells of the gastrointestinal tract and the immune system

cells[18]. Hence, vitamin D deficiency may be involved in several processes, such as cancer, diabetes mellitus (DM) and cardiovascular and autoimmune diseases[19-26]. Furthermore, the immune system cells, including macrophages, dendritic cells, and T and B lymphocytes, express CYP27A1 or CYP27B1 enzymes and thus can metabolize 25(OH)D to calcitriol. Calcitriol will then have an autocrine or paracrine function[19,20]. Vitamin D favors the innate response of the immune system and has a "self-regulatory" effect by limiting the adaptive response. On one hand, it stimulates the synthesis of antimicrobial peptides (cathelicidin and beta-defensin) and the chemotaxis and phagocytosis of the macrophages. On the other hand, it decreases the expression of class Ⅱ complex molecules, co-stimulating molecules and the synthesis of Th1, Th2 and Th17 cytokines[19,20]. Finally, in addition to acting as a transcription factor, VDR seems to induce fast non-genomic responses by activating cellular signaling pathways. In this sense, has been shown presence of VDR in plasma membranes of intestinal, lung, kidney, muscle cells and osteoblasts, where it efficiently binds $1\alpha,25(OH)_2D$[16,27,28].

REGULATORY MECHANISMS OF VITAMIN D SYNTHESIS

The synthesis process of vitamin D includes regulatory mechanisms in each step, as follows: (1) in the skin, excess of vitamin D_3 is destroyed by sunlight, thus preventing vitamin D_3 intoxication from excessive sun exposure[29]; (2) the 25-hydroxylation of vitamin D is under-regulated. The levels of 25(OH)D increase according to the intake of vitamin D; thus, plasmatic levels of 25(OH)D are used to regulate vitamin D status; (3) in contrast, 1α-hydroxylase is highly regulated. Different factors are involved in its activity and expression, including serum calcium and phosphate, parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23). An elevated calcium serum concentration suppresses 1α-hydroxylase directly and indirectly by decreasing the PTH levels[30]; elevated plasmatic phosphate also decreases the expression and activity of 1α-hydroxylase through a mechanism that is not yet understood. This increase in serum phosphate seems to trigger an increase of FGF23 that inhibits $1\alpha,25(OH)_2D$ synthesis[31]. Furthermore, the synthesis and degradation of $1\alpha,25(OH)_2D$ is also controlled by local factors such as cytokines and growth factors, although this local production has no effect on the blood levels[32,33]. In the case of the macrophages, the expression of CYP27B1 and synthesis of $1\alpha,25(OH)_2D$ are induced by inflammatory cytokines, such as interferon (IFN)γ, and by toll-like receptor (TLRs) ligands, such as the lipopolysaccharide (LPS); (4) the 25-hydroxyvitamin D-24-hydroxylase (CYP24A1) catabolizes $1\alpha,25(OH)_2D$ to calcitroic acid, a biologically inactive bile-excreted metabolite[15]. The activity and expression of this enzyme, which is most abundant in intestine and kidney, is controlled by the levels of $1\alpha,25(OH)_2D$, phosphate and

PTH[34,35]; (5) the DBP protein may buffer the levels of free vitamin D which is correlated with the levels of active vitamin D, this prevents intoxication[36]. Additionally, DBP prevents catabolism and excretion of the hormone. The DBP levels decrease in liver disease, nephrotic syndrome and malnutrition; despite this modification, the concentration of $1\alpha,25(OH)_2D$ remains constant; and (6) $1\alpha,25(OH)_2D$ controls its own synthesis not only through the increase of CYP24A1 expression, as mentioned above, but also by directly or indirectly inhibiting CYP27B1 expression and providing a negative feedback pathway.

Therefore, we can conclude that multiple factors regulate vitamin D metabolism. The intake of vitamin D through diet or sun exposure is only one of many variables that determine its activity, another of these variables are DBP levels, the local synthesis of $1\alpha,25(OH)_2D$ (the autocrine or paracrine effect) and VDR expression.

VITAMIN D AND CHRONIC LIVER DISEASE

As discussed previously, vitamin D plays an important role in reducing the risk of chronic diseases, including DM type 2, several types of cancer, and cardiovascular, autoimmune and infectious diseases. This role most likely results from the local production of $1\alpha,25(OH)_2D$ and its autocrine and paracrine actions in cellular proliferation and differentiation, apoptosis, insulin and renin secretion and interleukin (IL) and bactericidal protein production[1,16,17,19-23]. These effects may also be relevant in the pathogenesis of chronic liver diseases.

Vitamin D deficiency is extremely common in chronic liver disease patients. Up to 93% of these patients have some degree of vitamin insufficiency[4,5]. Even patients with mild liver disease are affected, although liver cirrhosis patients more commonly suffer from severe deficiency.

Several studies in general populations have shown that low levels of 25(OH)D significantly increase the risk of mortality from all causes, including cardiovascular diseases[37,38]. Regarding patients with chronic liver disease of varying etiologies, vitamin D deficiency has been associated with increased mortality[39,40], bacterial infections[41], portal hypertension complications[42] and fibrosis severity[43,44]. However, because the liver plays an important role in the metabolism and pleiotropic functions of vitamin D, the question is whether vitamin D deficiency is a consequence of liver disease or a contributor to the liver dysfunction.

Severe liver disease decreases vitamin D hydroxylation and albumin and DBP production, all of which are linked to low levels of 25(OH)D. Nevertheless, the vitamin D deficiency in chronic liver disease is only partly the result of a synthesis dysfunction of the liver, as evidenced by the fact that vitamin D deficiency is highly prevalent in non-cirrhotic patients[4]. The levels of 25(OH)D in cirrhotic patients normalize after vitamin D treatment, which indicates that the 25-hydroxylation is pre-

served[45,46]; and although DBP is moderately decreased in cirrhosis[47], vitamin D metabolites require only 5% of the DBP binding sites[48], indicating that liver dysfunction must be severe to decrease the DBP levels and contribute to vitamin D deficiency. Therefore, vitamin D deficiency in chronic liver disease requires several causes in addition to those mentioned above, including inadequate sun exposure, insufficient food intake, steroid use, jaundice-related deterioration of vitamin synthesis on the skin and decreased vitamin D absorption caused by intestinal edema secondary to portal hypertension or to cholestasis-induced bile salt disruption.

The observed association between vitamin D and liver disease is insufficient to establish a causal effect between vitamin D deficiency and the severity of chronic liver disease. Recent systematic and umbrella reviews has cast doubt on any causal link between vitamin D deficiency and non-skeletal health outcomes, suggesting that vitamin D deficiency is a marker of ill-health, rather than an important factor implicated in the pathogenesis of disease[49]. However, there is growing evidence that vitamin D is involved in the decrease of inflammation and fibrosis[43,50,51]. Proinflammatory signals in monocytes and macrophages may regulate the local metabolism of vitamin D, auto-inducing the expression of CYP27B1 and the local production of $1\alpha,25(OH)_2D$, and thus controlling the excessive inflammatory response[33,52]. Almost 90% of the tissue macrophages are in the liver[53], which suggests that the liver production of active vitamin D is affected during inflammatory diseases of the liver. Furthermore, VDR is expressed in both macrophages and other non-parenchymal cells and biliary epithelial cells[54]. After activation, these cells increases the expression of cathelicidin, an antimicrobial peptide with anti-endotoxin activity[55], and inhibits the synthesis of biliary acids, thus protecting the hepatocytes from these acids[56,57]. Therefore, the relationship between vitamin D and hepatic physiopathology may result from signaling disruptions in non-parenchymal liver cells or extrahepatic cells[58].

It is important to mention that, together with diet intake and sun exposure, genetic factors substantially contribute to variations in 25(OH)D levels[59,60]. Several simple nucleotide polymorphisms of genes involved in the metabolism of VDR and vitamin D, such as DHCR7 (encode the 7-dehydrocholesterol reductase enzyme), CYP2R1, CYP24A1 and GC (encode DBP), have been strongly linked with the serum levels of 25(OH)D and its efficacy[59-62]. A recent study community-dwelling black Americans, as compared with whites, had low levels of total 25(OH)D and DBP, resulting in similar concentrations of estimated bioavailable 25(OH)D. Racial differences in the prevalence of common genetic polymorphisms provide a likely explanation for this observation[63]. Therefore, such genetic variations may be associated with the severity of chronic liver disease, and several polymorphisms of the *VDR* gene associated with primary biliary cirrhosis, autoimmune hepatitis, CHC and hepatocellular carcinoma have been identified[64-69].

The available data suggest that vitamin D supplements could be beneficial in terms of morbimortality[70,71]. Most experts consider of at least 75 nmol/L (30 ng/mL) as the most advantageous 25(OH)D level for reducing the risk of fractures, prevention of cancer and the risk of hypertension, and between 90-120 nmol/L (36-48 ng/mL) as the most optimal level[71]. In fact, a recent meta-analysis that included 73 cohort studies (849412 participants) and 22 controlled and randomized studies with over 30716 participants showed that vitamin D₃ supplements significantly reduced mortality from any cause among older adults[72]. Few published prospective studies have examined the effects of supplements in chronic liver disease, and the results to date are contradictory, most likely because of issues with study designs, the quantity of vitamin D administered, the pre- or post-treatment measurements used and the presence of genetic polymorphisms that influence the biological activity of vitamin D. Nonetheless, vitamin D supplements are currently recommended to decrease the skeletal effects of vitamin D deficiency. In fact, the latest recommendation suggest that a 25(OH)D level over 20 ng/mL is sufficient to meet the vitamin D requirement[73]. However, the Endocrine Society Clinical Practice Guideline (ESCPG) suggested that vitamin D requirements may be greater for sick patients than for healthy individuals and blood level above 30 ng/mL may have additional health benefits in reducing the risk of various disease conditions[74]. In addition, the ESCPG suggest that 25(OH)D should be measured in chronic liver disease patients to identify those with levels under 20 ng/mL who would benefit from vitamin D supplements to reduce the risk of bone fracture[74]. Similarly, the guidelines of the European Association for the Study of the Liver recommend calcium (1000-1200 mg/d) and vitamin D (400-800 UI/d) supplements for cholestatic liver disease patients, although supplement use is supported by limited clinical data[75]. In fact, despite the frequency of vitamin D deficiency in liver disease patients, their calcium and PTH serum concentration levels are normal, which contradicts the possibility that regulatory mechanism of calcium metabolism is affected[76,77]. Our group has confirmed these results in cirrhotic patients of different etiologies; these patients showed vitamin D deficiencies[78] but had free vitamin D levels similar to those of healthy subjects (unpublished data). Consequently, the unaffected free vitamin D may be involved in the lack of correlation between the levels of 25(OH)D and calcium and PTH and may maintain calcium homeostasis without causing secondary hyperparathyroidism[79]. For this reason, several authors indicate that the levels of total and free 25(OH)D should be measured to identify the vitamin D status in chronic liver disease patients[76]. Nonetheless, these patients have a high prevalence of bone mass loss that can be explained by the previous data of vitamin D deficiency and by other interfering factors, such as the increase in pro-inflammatory cytokines[80-82], hypogonadism[83], elevated bilirubin levels[84] and steroid treatment[85].

VITAMIN D FUNCTIONS AND THEIR IMPLICATIONS IN LIVER DISEASES

Vitamin D maintains the normal skeletal architecture and plays roles in the cardiovascular[86,87] and nervous systems[88,89] and cellular proliferation and differentiation[90,91]. Furthermore, vitamin D may be relevant in the physiopathology of chronic liver diseases because of its effect on the immune system and its anti-fibrotic effect[51,92,93].

Several research lines suggest that vitamin D has beneficial effects in liver diseases by activating and regulating innate and adaptive immunity.Vitamin D increases innate immunity[23], stimulating the mechanisms associated with the elimination of pathogen agents through the secretion of antibacterial proteins, such as cathelicidin and beta-defensin, and favoring chemotaxis and macrophage phagocytosis[19,20,94,95]. An excessive immune response can cause tissue damage; in this sense, vitamin D promotes an adequate innate immune response by regulating the expression of several TLRs and by decreasing the production of proinflammatory cytokines[52]. An inverse relationship between vitamin D levels and the expression of TLR2, TLR4 and TLR9 in monocytes has been observed, as has a decrease in the expression of these innate immunity receptors after the administration of $1\alpha,25(OH)_2D$[52,96,97]. These three TLRs are primarily related to the inflammation and fibrosis of the liver. A high-fat diet, alcohol consumption and structural changes in the intestinal mucosa resulting from chronic liver diseases (*e.g.*, the loss of epithelial attachment, vascular congestion, defects of the mucosal immune system) alter the permeability of the mucosa, promoting an increase in intestinal bacteria translocation[98-100] and bacterial products, such as LPS, through the bloodstream; there, these bacteria bond to the TLRs, mainly TLR4, that are present such immune cells as hepatocytes, biliary epithelial cells, dendritic cells and hepatic stellate cells, triggering the synthesis of proinflammatory cytokines and fibrogenesis that ultimately result in liver damage[98,101]. However, vitamin D is involved not only in the regulation of TLR expression but also in intestinal permeability; it plays a role in intestine epithelial cell differentiation and in improving cell bonding[102,103], thus decreasing the bacterial products in the liver.

Regarding adaptive immunity, vitamin D seems to control an excessive immune response by decreasing the expression of class Ⅱ HLA complex molecules and co-stimulator molecules and by modulating the T cell response[19,20,104]. The activation of naïve T cells has been shown to be vitamin D-dependent[105]; furthermore, it inhibits the development of Th1 (IL-2 and interferon-gamma proinflammatory cytokine producers) and Th9 and increases the number of Th2 cells (IL-4, 5 and 10 anti-inflammatory cytokine producers), thus affecting the polarization of T helper cells[106-108]. Additionally, $1\alpha,25(OH)_2D$ prevents the development of Th17 cells by inhibiting IL-6 and IL-23 production from the dendritic cells, and it induces the differentiation and expansion of regulatory T cells that secrete the anti-inflammatory cytokines IL-10 and transforming growth factor beta (TGF-β)[107,109,110]. This ability to modulate the adaptive immune system may explain the association between vitamin D deficiency and the risk of autoimmune diseases and liver damage.

Moreover, *in vitro* and *in vivo* studies of mouse models with liver fibrosis have reported that vitamin D has an anti-fibrotic effect due to ability to affect the pathological process of liver fibrosis at several stages, such as: inhibition of injury trigger, suppression of hepatic stellate cells activation and proliferation, reduction in accumulation of extracellular matrix and even degradation of collagen metalloproteinases activation and tissue inhibitor matrix metalloproteinases (TIMPs) inhibition[92,93]. Moreover, Ding *et al*[111] revealed an intersecting VDR/SMAD genomic circuit that regulates hepatic fibrogenesis and define a role for VDR as an endocrine checkpoint to modulate the wound-healing response in liver and VDR ligands as potential therapy for liver fibrosis[111]. In this regard, a recent study in mice showed that the active metabolite of vitamin D-$1\alpha,25(OH)_2D$ may prevent liver fibrosis in the *in-vivo* model. However, it cannot ameloriate establish cirrhosis in an animal model[112].

VITAMIN D AND CHRONIC HEPATITIS C VIRUS INFECTION

Epidemiological studies show that vitamin D deficiency may increase the risk of acquiring viral infections such as influenza, human immunodeficiency virus and respiratory infections[113]. Chronic hepatitis C virus (HCV) infection is one of the main causes of chronic liver disease; it is estimated to affect 130 to 150 million people worldwide, a significant number of whom also develop cirrhosis and hepatic cancer[114]. A high percentage of these patients (46% to 92%) have low vitamin D levels[50,115-117], and more than 25% suffer from severe deficiency[50,115,117]. It has been hypothesized that the high incidence of vitamin D deficiency in these patients may be caused by HCV's effect on direct or indirect 25-hydroxylation through cytokine induction or oxidative stress[118,119] and that the virus may suppress 25(OH)D levels due to a disruption in lipid metabolism; as shown a recent study where HCV decreases the production of 7-dehydrocholesterol, the endogenous precursor of vitamin D[120].

As discussed previously, vitamin D inhibits fibrosis and modulates the innate and adaptive immune response, increases the production of antimicrobial peptides and inhibits proinflammatory cytokines. The anti-inflammatory action of vitamin D[19,20,50,94,95,104,106-110] can explain the improved therapeutic results of IFN and ribavirin (RBV) after the administration of vitamin D supplements[121-123], as some data indicate that proinflammatory cytokines and chemokines promote the persistence of HCV[124]. In this respect, a low Th1/Th2 ratio is an independent sustained viral response (SVR) factor in the treatment of the HCV genotype 1[125], and $1\alpha,25(OH)_2D$ favors Th2 in this balance, as mentioned previously[108]. Furthermore, several

Table 1 Studies regarding vitamin D and hepatitis C virus

Ref.	Year	Design	*n*	HCV genotype	Vitamin D deficiency	Outcome	*P*
Petta *et al*[50]	2010	Cohorts	197	1	73%	Vitamin D levels (ng/mL): SVR: 26.6 No SVR: 23.7	0.05
Bitetto *et al*[121]	2011	Cohorts	42	1 and no 1	Not stated	SVR according to the vitamin D levels (ng/mL): ≤ 10 ng/mL: 10% > 10 and ≤ 20 ng/mL: 30% > 20 ng/mL: 50%	< 0.05
Bitetto *et al*[136]	2011	Cohorts	211	1-5	46.4%	SVR according to the vitamin D levels (ng/mL): ≤ 10 ng/mL: 50% > 10 and ≤ 20 ng/mL: 60.9% > 20 ng/mL: 69%	0.038
Lange *et al*[115]	2011	Cohorts	468	1-3	66%	SVR (genotype 2/3): Vitamin D deficit (< 10 ng/mL): 50% Without deficiency: 81%	< 0.0001
						SVR (genotype 1) Vitamin D deficit: 60% Without deficiency: 54%	0.45
Nseir *et al*[133]	2011	Cohorts	80	1	Not stated	Vitamin D levels (ng/mL): SVR: 42.1 No SVR: 27.3	< 0.001
Jazwinski *et al*[134]	2011	Cohorts	82	1	Not stated	Vitamin D levels (ng/mL): SVR: 23.3 No SVR: 19.3	0.82
Abu-Mouch *et al*[123]	2011	Randomized prospective	72	1	59% (with vitamin D supplementation) 60% (control group)	SVR: With vitamin D: 86% Control group: 42%	< 0.001
Nimer *et al*[122]	2012	Randomized prospective	50	2-3	60% (with vitamin D) 50% (control group)	SVR: With vitamin D: 95% Control group: 77%	< 0.001
Lange *et al*[116]	2012	Cohorts	269	1-4	74%	No significant association between SVR and 25(OH)D serum levels	0.13
Kitson *et al*[137]	2013	Cohorts	274	1	48%	Vitamin D levels (ng/mL): SVR: 76.6 No SVR: 84.7	0.03
Esmat *et al*[140]	2014	Randomized prospective	101	4	95%	SVR: With vitamin D: 44% Control group: 68.6%	0.22
Yokoyama *et al*[142]	2014	Randomized prospective	84	1b	Not stated	SVR: With vitamin D: 64.3% Control group: 50%	0.19
Grammatikos *et al*[138]	2014	Cohorts	398	1	Not stated	Vitamin D levels (ng/mL) SVR: 15.8 No SVR: 17.6	0.09

HCV: Hepatitis C virus; SVR: Sustained viral response.

in-vitro studies have considered vitamin D a direct HCV antiviral agent[126-128]. Gal-Tanamy *et al*[127] showed that vitamin D increases VDR expression and inhibits HCV replication in human hepatocytes by inducing the expression of IFN beta and the IFN-stimulated gene (*MxA*) with different antiviral properties, thus producing a synergic effect with antiviral treatment[127]. In the same study, vitamin D or calcitriol added to the antiviral treatment had a synergic effect in the inhibition of HCV. In addition, in recent clinical studies have described an association between VDR polymorphisms on the response to IFN/RBV therapy in CHC[129,130].

The relevance of vitamin D in CHC has been reported in numerous studies that associated vitamin D deficiency with a greater degree of necrosis and fibrosis[40,50,68,131,132] and with a lower likelihood of a SVR to IFN-based therapies[50,115,121,123,133-135]. In fact, all of the patients who showed severe vitamin D deficiency had hardly any SVR, while 50% of those with normal levels or almost normal levels had SVR[50,121,123,136]. However other studies failed to find ant relationship between baseline vitamin D level and SVR and fibrosis[116,137-140] (Table 1). In addition, conflicting conclusions have been reached in two recent meta-analysis[130,141]. This may be due to limitations of the studies included: (1) the small number of patient; (2) majority had a cross-sectional studies that are subject to bias due to the possibility of reverse causation; (3) lack of vitamin D level assessment during therapy;

and (4) characteristic of vitamin D assessment (seasonality, cut off values, methodology of vitamin D determination, ethnicity). In contrast, vitamin D has been shown to increase the probability of SVR when it is added to the antiviral treatment[121-123,142] (Table 1). Thus, futher clinical investigation on the effect of vitamin D supplementation in treating CHC are needed to confirm this item.

Furthermore, Bitteto *et al*[136] provided additional information in their study of the rs12979860 C/T polymorphism of IL28B. In their study, vitamin D levels were complementary to the rs12979860 C/T polymorphism of the IL28B for predicting SVR in CHC patients infected with difficult-to-treat genotypes (1, 4, 5). Another polymorphism, the CYP27B1-1260 polymorphism is also known to decrease the intracellular concentration of calcitriol in mononuclear cells and T lymphocytes[134] and is a known cofactor in immune response disruption in these cells. In fact, the study by Lange and colleagues confirms the lack of SVR in patients infected with the HCV 1, 2 and 3 genotypes who have this polymorphism[115]. This study also hypothesized that genotype 3 patients had low 25(OH)D levels, in contrast with previously published data[50,136]. We should, however, note that the definition of vitamin D deficiency differed among the three studies, a factor that should be considered when interpreting these results.

Vitamin D also favors the HCV response by improving the sensitivity to insulin[143-145]. Insulin resistance (IR) is considered one of the most important factors in predicting HCV patients' response to IFN and RBV[146], and vitamin D is known to prevent DM type 2[144]. As β-pancreatic cells contain VDR, vitamin D deficiency may alter the balance between the intra- and extracellular calcium and interfere with insulin release[147].

Therefore, in theory, vitamin D deficiency may be linked to a lack of response to anti-viral treatment, while vitamin D supplementation may potentiate SVR.

VITAMIN D AND NAFLD

NAFLD is a pathological clinical entity that includes a broad spectrum of liver conditions from steatosis to nonalcoholic steatohepatitis (NASH) and cirrhosis[148] and NAFLD is one of the main causes of chronic liver disease in developed countries, affecting 20% to 30% of the population[149,150]. Some NAFLD patients develop NASH and cirrhosis, while most others do not experience disease progression; however, the reason for these differences in progression are not known. NAFLD is generally related to at least one metabolic syndrome characteristic; in fact, liver conditions are considered part of the syndrome, and although their pathogenesis is not yet known, IR is a key factor in its development[151,152]. Several studies show a negative correlation between vitamin D levels and obesity, glucose intolerance, IR, metabolic syndrome and body mass index (BMI)[24-26,153-155]. Furthermore, vitamin D deficiency stimulates PTH, which has been linked to IR and an increase in the acute-phase reactant[156]. In sup-

port of this hypothesis, some studies show that vitamin D administration improves insulin secretion[145,157-160] and that its use decreases IR in patients with end-stage renal disease[161]. Moreover, VDR polymorphisms have been associated with IR and have an effect on insulin secretion and on the fasting glucose concentration[162]. Additionally, previous studies have shown that VDR knock-out mice developed hepatic steatosis[163]. Finally, studies have shown that vitamin D administration in mice activates the fibroblastic intestinal growth factor 15 (FGF15) (human ortholog FGF19). This intestinal hormone prevents IR and high-fat diet-induced obesity by inhibiting CYP7A1, an essential enzyme in the physiopathology of liver dyslipidemia[164]. This evidence suggests that vitamin D is linked to the development of NAFLD *via* its role in glucose metabolism by accelerating the conversion of proinsulin to insulin, while vitamin D deficiency has been associated with pancreatic β cell dysfunction and a greater prevalence of type 2 DM[153,164-167].

As in the case of CHC, vitamin D levels are lower in patients with NAFLD compared with healthy controls[43,167-174]. In addition, vitamin D deficiency in obese patients has been attributed to the accumulation of the vitamin D in adipose tissue[175-177]. Furthemore, vitamin D levels are inversely correlated with the severity of steatosis, necroinflammation and fibrosis independent of age gender, BMI, Homeostatic Model Assesment of IR score and presence of metabolic syndrome[43,168,178]. In a recent clinical study of adults with NAFLD, Targher *et al*[43] showed that the vitamin D levels had an effect on the development of hepatic steatosis and in the severity of the histological lesion. In fact, their hypothesis stated that patients with greater inflammation and fibrosis had lower vitamin D levels independent of other components of the metabolic syndrome. This observation was later confirmed in pediatric patients[179,180] (Table 2). Still, an association between vitamin D and NAFLD has been demonstrated that is independent of BMI or IR and metabolic syndrome[43,157,162]. Although causal conclusions are difficult to obtain from these studies, their results suggest that vitamin D deficiency plays a role in the development and progression of fatty liver, especially in terms of its anti-inflammatory potential. In fact, vitamin D reduces the risk for NAFLD in healthy men[181] and attenuates high fat diet-induced hepatic steatosis in rats by modulating lipid metabolism[182].

Vitamin D deficiency has been linked to a systemic increase in inflammation markers[183,184], and systemic inflammation may play a central role in the pathogenesis and progression of NAFLD[185,186]. Increases in visceral adiposity promote the release of fatty acids and proinflammatory cytokines and activate inflammation pathways in the liver, prompting proinflammatory cytokine secretion that leads to liver damage[187]. Moreover, the obesity promotes the onset of NAFLD due to increased hepatic lipid synthesis secondary to excess free fatty acids; subsequent association with oxidative stress on mitochondrial and with the increase of proinflammatory

Ref.	Year	Design	n	NAFLD diagnosis	Vitamin D levels (ng/mL)	P
Table 2 Studies regarding vitamin D and non-alcoholic fatty liver disease						
Targher *et al*[43]	2007	Cohorts prospective	120	Liver biopsy	Controls (60): 29.8 ± 6	0.001
					Steatosis (10): 23.72 ± 8	
					NASH (50): 14.8 ± 9.2	
Manco *et al*[179]	2010	Cohorts prospective	64	Liver US	Without necroinflamation: 26.1 ± 10	0.16
					With necroinflamation: 19.9 ± 9.8	< 0.001
					Without fibrosis: 27.7 ± 10.3	
					With fibrosis: 17.1 ± 7.4	
Barchetra *et al*[168]	2011	Cohorts prospective	262	Liver US	Without NAFLD (100): 20.5 ± 9.7	< 0.001
					NAFLD (162): 14.8 ± 9.2	
Jablonski *et al*[169]	2013	Cohorts retrospective	1214	Liver US	Controls (607): 34 ± 8	< 0.001
					NAFLD (607): 30 ± 7	
Kasapoglu *et al*[171]	2013	Cohorts prospective	613	Liver US	Controls (275): 26,4 ± 9.8	< 0.05
					NAFLD stage 1 (133): 20 ± 9.2	
					NAFLD stage 2 (106): 13.3 ± 6.7	
					NAFLD stage 3 (99): 8.8 ± 7.4	
Black *et al*[170]	2014	Cohorts prospective	994	Liver US	Without NAFLD (838): 30.8 ± 9.6	< 0.001
					NAFLD (156): 26.8 ± 8.8	
Yildiz *et al*[174]	2014	Cohorts prospective	101	Liver US	Without NAFLD (43): 16.4 (IQR 12.4-24.8)	0.005
					NAFLD grade 1 (41): 14.2 (IQR 9.5-21.2)	
					NAFLD grade 2 (17): 11.5 (IQR 7.5-16.7)	
Dasarathy *et al*[178]	2014	Cohorts prospective	148	Liver biopsy	Controls (39): 35.7 ± 6	< 0.01
					Steatosis (67): 25 ± 11.3	
					NASH (81): 18.1 ± 8.4	
Nobili *et al*[180]	2014	Cohorts prospective	73	Liver biopsy	NASH (49) was associated with lower VD levels,	< 0.001
					i.e., -9.0 pg/mL when compared with that in	
					children without NASH (24)	
Küçükazman *et al*[173]	2014	Cohorts prospective	211	Liver US	Without NAFLD (57): 20 ± 13.6	< 0.001
					NAFLD (154): 12.3 ± 8.9	

US: Ultrasonography; IQR: Interquartile range; NAFLD: Non-alcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis.

cytokines can definitely trigger a progression of steatosis to NASH and cirrhosis[188]. Studies *in vivo* and *in vitro* have clearly documented that steatosis reduces oxidative activity controlled by cytochrome P450[189]. These inflammatory processes may be blocked by increasing the levels of 25(OH)D, and the development and progression of NAFLD may stop. In fact, vitamin D supplements have been shown to decrease inflammation markers[190-193] and increase anti-inflammatory cytokines[190]. It is known that vitamin D's effects in the liver are not only exerted on the hepatocytes, given that these cells express very little VDR mRNA. In contrast, sinusoidal cells, Kupffer cells, hepatic stellate cells and immune system cells express VDR mRNA that is functionally active. Therefore, vitamin D deficiency may affect the activity/expression of macrophages, dendritic cells and T and B lymphocytes by favoring oxidative stress and the production of proinflammatory cytokines that lead to subclinical inflammation[18,19]. Furthermore, fibrosis is induced by TGF-β secretion that results from the increased secretion of the matrix metalloproteinase 9 inhibitor (TIMP-1)[194]. In fact, cell cultures show that vitamin D has an anti-inflammatory and an antifibrinolytic effect on hepatic stellate cells. Finally, animal models show that more severe histological lesions of NAFLD are associated with higher levels of mRNA of TLR2, 4 and 9, proinflammatory cytokines and oxidative stress markers in rats with a high-fat diet and deficient in vitamin D[195]. A recent study of experimentally NAFLD-induced rats showed that ultraviolet light exposure decreased hepatic stellate cell activity and TGF-β synthesis and stimulated the production of apolipoprotein E and adiponectin. Together, these findings translate into a beneficial effect on NAFLD, and a decrease in IR, steatosis, apoptosis, inflammation and intrahepatic fibrosis was hypothesized[196]. Thus, given the above-mentioned findings, we can conclude that extrahepatic signaling affects fibrosis and inflammation[187] and that the vitamin D-VDR axis may play a role in the initiation and progression of NAFLD.

Therefore, although the mechanisms of vitamin D's control over hepatic lipid homeostasis and its link with inflammation are not fully known, recent research lines provide a more comprehensive understanding of its immune modulation capacity and of new therapeutic interventions for NAFLD.

CONCLUSION

The pleiotropic effects of vitamin D indicate a relationship between its deficiency and numerous chronic diseases, such as DM, cardiovascular, autoimmune and infectious diseases, several types of cancer and chronic liver diseases. In the case of chronic liver diseases, vitamin D seems to modulate the innate and adaptive immune system, which explains the association. Specifically, vitamin D deficiency has been associated with a greater risk of portal hypertension complications, mortality and increased histological severity in NAFLD and CHC, and

a lower probability of viral response to HCV treatment with IFN based therapies. In fact, clinical studies suggest that these parameters may improve with vitamin D supplementation; however, prospective, randomized and placebo-controlled studies are required to establish firm conclusions.

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Recombinase polymerase amplification as a promising tool in hepatitis C virus diagnosis

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Abstract

Hepatitis C virus (HCV) infection represents a significant health problem and represents a heavy load on some countries like Egypt in which about 20% of the total population are infected. Initial infection is usually asymptomatic and result in chronic hepatitis that give rise to complications including cirrhosis and hepatocellular carcinoma. The management of HCV infection should not only be focus on therapy, but also to screen carrier individuals in order to prevent transmission. In the present, molecular detection and quantification of HCV genome by real time polymerase chain reaction (PCR) represent the gold standard in HCV diagnosis and plays a crucial role in the management of therapeutic regimens. However, real time PCR is a complicated approach and of limited distribution. On the other hand, isothermal DNA amplification techniques have been developed and offer molecular diagnosis of infectious dieses at point-of-care. In this review we discuss recombinase polymerase amplification technique and illustrate its diagnostic value over both PCR and other isothermal amplification techniques.

Core tip: Recombinase polymerase amplification (RPA) shows many advantages over both real time polymerase chain reaction and other isothermal Amplification methods. In this review we show the importance of molecular detection methods and how isothermal amplification techniques offer molecular point-of-care diagnosis. RPA shows unique characteristics among isothermal approaches that makes it a promising tool in the molecular diagnosis. Because hepatitis C virus is an endemic viral infection, we suggest that RPA may play an important role and save much time in screening infected individuals and managing the therapeutic course.

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HEPATITIS C VIRUS

Hepatitis C virus (HCV) is a positive-sense single-stranded RNA virus that was first cloned in 1989 and classified as a member of the family Flaviviridae[1]. This viral infection is characterized by high replication rate. It is estimated that about 10^{12} virions per day are produced in a given individual[2]. In addition, its genome exhibits a high degree of sequence variation caused by its error prone RNA polymerase. However, there are 6 characterized genotypes of HCV, 52 subtypes within these genotypes[3]. Humans are the only reservoir for HCV infection; which often leads to an asymptomatic chronic state in 80% of cases with subsequent development to acute liver disease.

An estimated 2%-3% of the world's population is living with HCV infection and each year more than 350000

die of HCV-related complications, including cirrhosis, liver failure or hepatocellular carcinoma[4].

Although hepatitis C is considered to be endemic disease worldwide, there is a high degree of geographical variation in its distribution[5-9]. The prevalence of HCV infection is low, in most European countries where it represents 0.5%-2% of the general population[10,11], Americas, Australia, and South Africa (0.2% to 0.5%)[10]. Intermediate prevalence is reported in Middle East, India and Brazil[7,10]. Egypt recorded the highest prevalence of HCV in the world with about 20% of the population[7,9].

HCV is a blood prone infection, modes of transmission that have been reported include; transfusion of contaminated blood products, organ transplantation from infected donors, intravenous drug use, sexual transmission, public shaving, acupuncture, and invasive hospital procedures with contaminated equipment[12-16]. In Egypt where the highest prevalence in the world has been recorded, the major route of HCV infection was *via* an antischistosomal treatment program, with more than 35 million injections given over a 20-year in the period (1960-1980)[17].

The current standard treatment for chronic hepatitis C is a combination of pegylated interferon alfa and ribavirin. Sustained Virological Response (SVR) represents the endpoint of the treatment regimen, which indicates undetectable HCV RNA 24 wk post treatment[18].

Due to the lack of a vaccine or some form of post-exposure prophylaxis, the number of infected individuals will continue to increase, and in turn HCV-related morbidity and mortality, in the absence of effective care and treatment programs. The management of hepatitis C infection should not only focus on the treatment, but also prevention of infection to reduce the reservoir of infected individuals who can transmit the virus[19,20].

HCV DIAGNOSIS TECHNIQUES

The current laboratory techniques used for HCV diagnosis include: (1) Serological assays (*e.g.*, the enzyme-linked immunosorbent assay, recombinant immunoblot assay, *etc.*); and (2) Molecular assays: Depends on nucleic acid testing (NAT): qualitative [*e.g.*, reverse transcriptase polymerase chain reaction (RT-PCR), TMA, *etc.*] and quantitative (*e.g.*, real time PCR, *etc.*).

Advantages and limitation of serologic assays

The ease of automation and cost-effectiveness made serologic assays the most practical tool in HCV diagnosis[21]. However, antibody detection exhibits many disadvantages including that; detection is limited during the early stages of infection, poor sensitivity (false negative) in hemodialysis patients, immunocompromised patients[22-25], an abundance of false-positives[26] (because recovered patients may stay anti-HCV positive for years) and variability in accuracy between deferent commercial kits.

NAT

NAT detect and quantify HCV RNA and are now con-

sidered the gold standard in the diagnosis of HCV infection. In this approach, HCV RNA is extracted from the sample and reverse transcribed into the complementary DNA, which is then amplified into a large number of detectable copies by the polymerase chain reaction (PCR). Unlike antibody detection that could be positive for years after resolving infection, the presence of HCV RNA indicates active infection and it can be detected in 1-2 wk post-infection[27,28]. NAT offers accurate and sensitive diagnosis of HCV without any additional confirmatory test and can be used to diagnose individuals with acute HCV infection. In addition, NAT play a crucial role in the management of antiviral therapies by monitoring HCV RNA level. It determines the basal viral load and monitors the treatment response[29]. Till now, fully automated real-time PCR is the most promising approach in NAT as it is faster, more sensitive and is not prone to contamination.

ADVANTAGES AND LIMITATION OF NAT

The importance of NAT arises from its ability to detect and quantify HCV RNA and in turn detecting the active infection (in contrast to anti-HCV). In addition, it can determine the level of the virus replication. Furthermore, it plays an important role in the antiviral treatment regimens and determines whether a virological response has been occurred or not[30].

However, molecular techniques for HCV diagnosis have many limitations including that; it is of complex procedures, time consuming and technically demanding as it cannot be carried out except in a highly equipped molecular biology laboratory (high cost analytical instruments).

ADVANCES IN HCV DIAGNOSIS

Every day the world takes a step towards NAT which becomes more practical than it was before. The competition between the commercial products enforces the companies to produce more simple, easy to use and cheap assays. In addition to the growing dependence on NAT, the significant advances in HCV diagnosis include using point-of-care (POC) alternatives instead of the routine venous puncture. POC can use specimen matrices such as oral fluid or finger-stick blood. Most existing POC are immunoassays and are now widely used for different applications. POC represent an ideal approach for the management of hepatitis C infection as it can reaches remote areas where the high equipped molecular biology laboratories are limited and in turn shorten the time of results which extend HCV screening. For instance, the development of a molecular point-of-care assay would represent a significant improvement in the field of HCV diagnosis.

ISOTHERMAL DNA AMPLIFICATION

Molecular analytical techniques gain a growing interest. According to the mentioned limitation combining conventional molecular methods, especially real time PCR,

Table 1 Characters of some isothermal amplification techniques[51]

	NASBA	LAMP	SDA	RCA	HDA	RPA
Template	DNA, RNA	DNA[1]	DNA[1]	DNA[1]	DNA[1]	DNA[1]
No. of primers	2	4-6	4	1	2	2
No. of enzymes	3	1	2	2	2	2
Temperature (℃)	41	60-65	37	37	65	30-42
Reaction duration (min)	90-120	60-90	120	60	75-90	20
Denaturation step	Y	N	Y	N	N	N
Inhibition tolerance	N	Y	N	N	Y	Y
Product detection	GE, RT	GE, RT, TE	GE, RT	GE	GE, RT	RT
Multiplex	Y	N	Y	N	Y	Y
Point-of-care	Y	Y	Y	N	Y	Y

[1]RNA can be amplified after the introduction of a reverse transcription step. NASBA: Nucleic acid sequence-based amplification; LAMP: Loop-mediated isothermal amplification; SDA: Strand-displacement amplification; RCA: Rolling circle amplification; HDA: Helicase-dependent amplification; RPA: Recombinase polymerase amplification; GE: Gel Electrophoreses; RT: Real Time; TE: Turbidity; Y: Yes; N: No.

Table 2 Advantages/disadvantages of some isothermal amplification methods

Technique	Advantages	Disadvantages
NASBA	Specifically designed to detect RNA and in turn RNA viruses Power saving (41 ℃)	Denaturation step Less efficient in Amplifying RNA targets out of the range 120-250 bp
LAMP	Highly specific (utilizes 4-6 primers spanning 6-8 distinct sequences) Tolerance to biological substances Could be detected by a cheap turbidity-meter	Primer design is complex Unable to perform multiplex amplification
SDA	Power saving (37 ℃)	Sample prep. needed Nuclease selection is complex Inefficient in long target sequences
RCA	Power saving (37 ℃) Specific enough to allow SNP analysis	Primer is complex RNA amplification is complex Works only with a circular nucleic acid template
HDA	Simple primer design Robust to biological substances No initial heating step	Expensive enzymes
RPA	Power saving (37 ℃) Simple primer design Extremely quick (20 min) No initial heating step Robust to biological substances	

NASBA: Nucleic acid sequence-based amplification; LAMP: Loop-mediated isothermal amplification; SDA: Strand-displacement amplification; RCA: Rolling circle amplification; HDA: Helicase-dependent amplification; RPA: Recombinase polymerase amplification.

there was a demand to develop a simple, sensitive and cost effective technology.

Isothermal DNA amplification is an alternative to PCR-based technique and developed for point-of-care diagnosis[31,32]. In isothermal techniques, amplification reactions are performed at a constant temperature and hence there is no need for expensive thermal cycling instrument. Major practiced isothermal amplification techniques include; nucleic acid sequence-based amplification, loop-mediated isothermal amplification (LAMP)[33], strand-displacement amplification (SDA)[34], rolling circle amplification (RCA)[35], helicase-dependent amplification (HDA)[36] and recombinase polymerase amplification (RPA)[37]. Isothermal DNA amplification techniques are simple, rapid and cost effective with equivalent specificity and sensitivity to PCR, enabling point-of-care diagnostics without the need to high costing equipment[31,32]. However, isothermal amplification approaches differed from each other in terms of operating temperature, reaction

duration, mechanism, strengths and weaknesses. Table 1 summarizes the characters of the major practiced isothermal amplification methods.

As a competition between isothermal amplification techniques to perform molecular diagnosis at point-of-care, RCA will be kicked out of the race because it is incompatible with point-of-care diagnosis. Complex primer designing and the inability to perform multiplex amplification eliminates LAMP. The need to a denaturation step and the inability to tolerate inhibitory biological components exit both NABSA and SDA. Finally, RPA beats HAD in being faster and cheaper. Table 2 shows advantages and disadvantages of the major practiced isothermal amplification techniques.

RPA

RPA is an isothermal DNA amplification and detection method[37]. The amplification depends on a specific
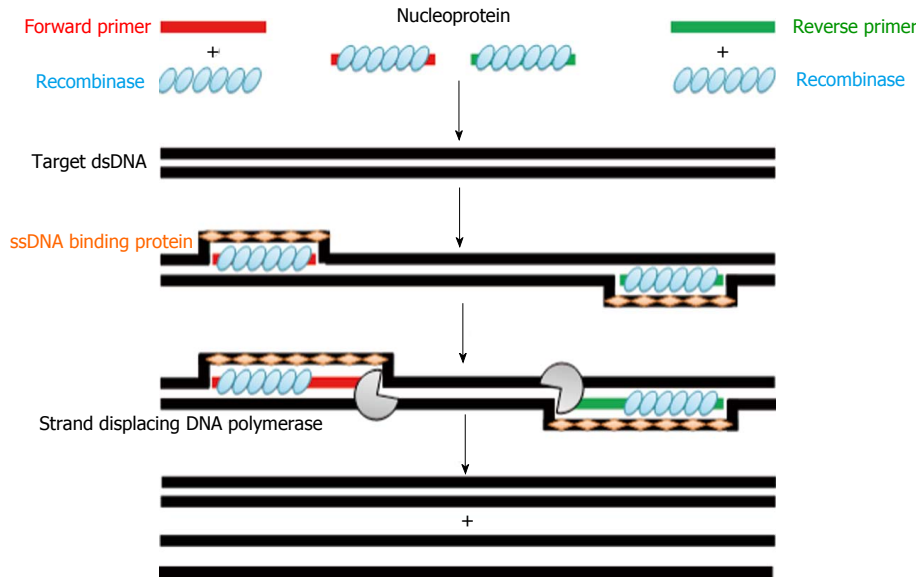
Figure 1 Recombinase polymerase amplification technology amplification cycle (for details, see the text above). dsDNA: Double-stranded DNA.

combination of enzymes and proteins (recombinase, single strand binding protein, and strand displacing DNA polymerase) used at a constant temperature and yielding a result in maximum 10 min. At first, RecA coat a single-stranded DNA (primers) to form nucleoprotein filaments. These filaments can then scan targeted double-stranded DNA for sequences complementary to those of coated primers. Then, the nucleoprotein filaments initiate a 5'-strand invasion at the site of homology (Figure 1) forming what is known as D-loop. The strand invasion is stabilized by single strand binding protein. After that, strand extension takes place at the free 3'-end of the nucleoprotein filaments by a strand displacing DNA polymerase to synthesize a new complementary strand. During strand extension, the new synthesized strand displaces the originally paired strand.

Real-time detection of RPA amplicons is possible *via* specific probes (Figure 2). Development of fluorescence depends on the separation of fluorophore and quencher *via* Exonuclease Ⅲ cleaving at an internal abasic site mimic [tetrahydrofuran (THF)] of the hybridized exo-probe (Figure 2)[38,39]. Fluorescence signal can be measured in real-time *via* a simple point-of-care scanner.

RPA technique is not restricted for amplification of the double stranded DNA targets, but also it could be used for amplification of RNA targets, as in the case with RT-PCR. Ahmed Abd El Wahed *et al*[40], 2013 had developed reverse transcriptase RPA (RT-RPA) assay for the detection of corona virus. The assay showed rapid kinetics with equal sensitivity and specificity of the real-time RT-PCR. The author suggested the diagnostic importance of the RT-RPA assay during the Hajj for the point-of-care detection of MERS-CoV infected cases to prevent the spread of the virus. Euler *et al*[39], 2012 have developed a qualitative real-time RPA assay for detection of *Francisella tularensis* and the assay showed results comparable to real-time PCR. In another wider study by Euler *et al*[41], 2013 RPA based assays were developed for

the detection of Gram-negative (*Francisella tularensis* and *Yersinia pestis*) and Gram-positive bacteria (*Bacillus anthracis*), DNA viruses (variola virus), whereas RT-RPA assays were developed for RNA viruses including Rift Valley fever virus, Ebola virus, Sudan virus and Marburg virus. The authors found analytical sensitivity and specificity equal to PCR with no cross-detection among respective targets. Also, Ahmed *et al*[42], 2014 have developed RPA based assay for the detection of *Leptospira* and the method showed fast and less sensitivity to amplification inhibitors. Another competitive character compared to PCR based protocols had been reported by Kersting *et al*[43], 2014 study in which RPA have been used for multiplex detection (detection multiple targets in the same reaction) of *Neisseria gonorrhoeae*, *Salmonella enterica* and *Staphylococcus aureus*. The author concluded that the kinetic performance of RPA was faster than PCR with no loss in sensitivity and specificity[43]. In the light of the above mentioned results it is clear that, RPA show competitive results as compared with PCR as regard to sensitivity and specificity, whereas RPA exceeds PCR as regard to the reaction kinetics.

In another study, RPA showed an impressive results in which the amplification reaction was conducted under a broad range of conditions from 30 ℃-45 ℃ with high inhibitory concentration of known PCR inhibitors in just 15 min[44].

ADVANTAGES AND DISADVANTAGES OF RPA

RPA overcomes the technical difficulties posed by current molecular techniques.

At first; it demonstrates a rapid kinetics, the process begins operating the immediately when the sample is contacted to the reagents and there is no need for melting the double-stranded DNA target.
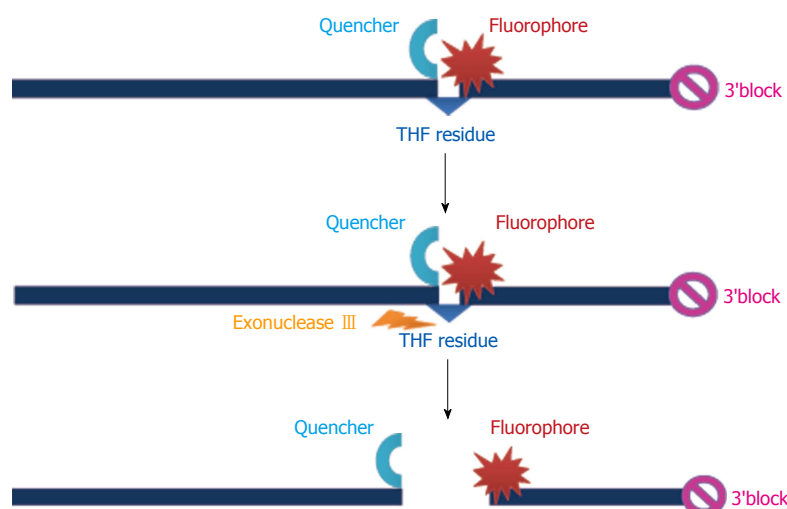
Figure 2 Example for the specific probe of the recombinase polymerase amplification assay. THF: Tetrahydrofuran.

Second, it operates at a constant temperature (30-42 ℃, optimum nearly 37 ℃), which give the advantage of being an energy saving technique and cost saving (there is no need for thermal cycler).

In addition, the target can be either DNA, or RNA, making RPA suitable for the detection and diagnosis of RNA viruses, like HCV.

Furthermore, the combination of probe-based detection, RPA represents a significant advance in the development of portable and accessible nucleic acid-based tests.

Unlike PCR in which the amplification reaction is controlled by temperature, digital RPA suffers from undesired reactions because the amplification could proceeds at room temperature if the nucleic acid sample is premixed with initiation reagents prior to compartmentalization and thus increasing the target count. However, any low-temperature non-specific pre-amplification reaction can be eliminated by compartmentalization of the nucleic acid template prior to adding initiation reagents[45,46].

Another drawback of low temperature amplification results from the interaction between primers even when well-designed. These interactions can create noise that defeats the analysis. However, this drawback could be avoided by using Self Avoiding Molecular Recognition System (SAMRS)[47]. SAMRS are nucleotide analogues that can bind to natural DNA but not to other SAMRS species. Therefore, primers built from SAMRS not interfere with each other. The concept of SAMRS was introduced over a decade ago[48,49] and was exploited to fix interactions between primers in PCR and multiplex PCR[50].

CONCLUSION

Early diagnosis and treatment of HCV infection can reduce the risk of long-term complications and prevent further transmission as well. NAT represent the gold standard for the diagnosis of HCV infection. Detection of HCV RNA level is an important factor in antiviral regimens especially for determination of SVR. RPA combines the advantages of serologic and Molecular tech-

niques and overcome limitations of both. It represents a simple, accurate and cost effective diagnostic tool and can be carried out at remote areas. In turn, it could improve the management of HCV infection by screening carrier individuals and stop transmission. The demand for the development of nucleic acid based point-of-care assay is increasing alongside with increasing the number of HCV infected patients. RPA based HCV diagnosis would represent a significant advance in the management of HCV.

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MINIREVIEWS

Palliative external-beam radiotherapy for bone metastases from hepatocellular carcinoma

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Abstract

The incidence of bone metastases (BMs) from hepatocellular carcinoma (HCC) is relatively low compared to those of other cancers, but it has increased recently, especially in Asian countries. Typically, BMs from HCC appear radiologically as osteolytic, destructive, and expansive components with large, bulky soft-tissue masses. These soft-tissue masses are unique to bone metastases from HCC and often replace the normal bone matrix and exhibit expansive growth. They often compress the peripheral nerves, spinal cord, or cranial nerves, causing not only bone pain but also neuropathic pain and neurological symptoms. In patients with spinal BMs, the consequent metastatic spinal cord compression (MSCC) causes paralysis. Skull base metastases (SBMs) with cranial nerve involvement can cause neurological symptoms. Therefore, patients with bony lesions often suffer from pain or neurological symptoms that have a severe, adverse effect on the quality of life. External-beam radiotherapy (EBRT) can effectively relieve bone pain and neurological symptoms caused by BMs. However, EBRT is not yet widely used for the palliative management of BMs from HCC because of the limited number of relevant studies. Furthermore, the optimal dosing schedule remains unclear, despite clinical evidence to support single-fraction radiation schedules for primary cancers. In this review, we outline data describing palliative EBRT for BMs from HCC in the context of (1) bone pain; (2) MSCC; and (3) SBMs.

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Core tip: Due to a lack of clinical data, external-beam radiotherapy (EBRT) for bone metastases (BMs) from hepatocellular carcinoma (HCC) is still not widely used as a palliative therapy component, and the optimal dosing schedule remains unclear. BMs from HCC typically occur as expansive, bulky soft-tissue masses; they exhibit expansive growth that compresses the peripheral nerves, spinal cord, or cranial nerves, causing both bone and neuropathic pain, and neurological symptoms. In this review, we outline the data describing palliative EBRT for BMs from HCC to treat bone pain, spinal compression, and skull base metastases.

Hayashi S, Tanaka H, Hoshi H. Palliative external-beam radiotherapy for bone metastases from hepatocellular carcinoma. *World J Hepatol* 2014; 6(12): 923-929 Available from: URL: http://www.wjgnet.com/1948-5182/full/v6/i12/923.htm DOI: http://dx.doi.org/10.4254/wjh.v6.i12.923

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in men worldwide[1]. The rate of bone metastases (BMs) in extrahepatic metastasis is reported to be approximately 20%[2]. The incidence of BMs in HCC patients has historically been low compared with those of other cancers, but has recently increased[3]. Previously,

Table 1 Incidence of bone metastases in clinical studies *n* (%)

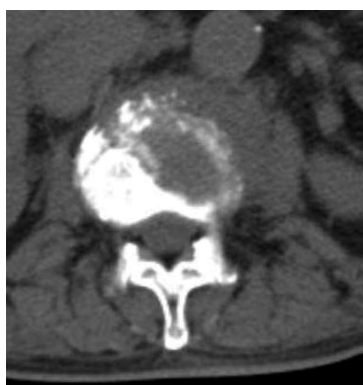| Ref.	Study period	Patients	Extrahepatic Ms	Incidence of BMs	Rate of BMs in extrahepatic Ms
Kuhlman *et al*[13]	1979-1985	300		22 (7.3)	
Liaw *et al*[14]	1983-1887	395		20 (5)	
Katyal *et al*[15]	1992-1997	403	148 (36.7)	41(10.2)	28
Fukutomi *et al*[3]	1978-1987	269		12 (4.5)	
	1988-1997	404		52 (12.9)	
Natsuizaka *et al*[5]	1995-2001	482	65 (13.5)	25 (5.2)	38.50
Uchino *et al*[2]	1990-2006	2386	342 (14.3)	87 (3.6)	25.40
Senthilnathan *et al*[16]	2000-2008	209	51 (18)	5 (2)	10

BMs: Bone metastases; Ms: Metastases.

Figure 1 Typical computerized tomography image of a lumbar spinal bone metastasis from hepatocellular carcinoma. The bone metastasis shows osteolytic, destructive, and expansive components with soft-tissue masses.

clinicians did not focus on BMs in advanced HCC because of their low incidence, and the prognosis of these patients was generally poor[4]. Recently, the prognosis and management of HCC have improved as a result of novel imaging techniques and multidisciplinary treatment approaches. BMs are now diagnosed more frequently in HCC patients with extrahepatic metastases[5]. BMs themselves rarely affect patient survival; however, they are the most common source of moderate and severe cancer pain[6,7] and can cause neurological symptoms[7]. In patients with spinal BMs, the consequent metastatic spinal cord compression (MSCC) causes paralysis. Skull base metastases (SBMs) with cranial nerve involvement can cause neurological symptoms. External-beam radiotherapy (EBRT) can effectively relieve bone pain[7,8] and the neurological symptoms caused by BMs[7,9]. However, EBRT has not been widely used in the palliative management of BMs from HCC because only a few reports have been published that focus on its use to relieve pain and neurological symptoms. In this review, we will outline the data pertaining to the use of EBRT for BMs from HCC.

INCIDENCE OF BMs

HCC is accompanied by BMs in 6%-20% of patients in autopsy studies[10-12]. The incidence of BMs in HCC patients has been reported to be relatively low; 2%-12.9% in clinical studies, and 7.3%-38.5% in patients with ex-

trahepatic metastases (Table 1)[2,3,5,13-16]. However, BM incidences of 10.2% and 12.9% were reported by Katyal *et al*[15] and Fukutomi *et al*[3], respectively, which are higher than previously reported rates. According to Katyal *et al*[15], this difference may have been the result of current treatment regimens that utilize combined chemotherapy and chemoembolization to prolong survival. Fukutomi *et al*[3] compared the incidence of BMs in two chronological periods (1987-1997 and 1998-1997). BMs were found in 4.5% of patients in the first decade and 12.9% of patients in the second decade. The increased incidence of bone metastasis was attributed to the prolonged survival of HCC patients due to improved diagnosis and treatment. Bone scintigraphy and computed tomography (CT) have been used widely as imaging modalities for BMs, and magnetic resonance imaging (MRI) has often been performed in recent studies. MRI is useful in cases in which the bone scan is negative and the BMs have properties of soft tissue masses[17]. In three recent studies, the incidence of BMs was reported to be 2%-5.4%[2,5,16]. However, the rate of BMs in patients with extrahepatic metastases is relatively high (25.4%-38.5%)[2,5], except in one report from the United States[16]. In a recent study, Natsuizaka *et al*[5] found that extrahepatic metastases were relatively common, and more than 65% of the study patients had early-stage tumors that would not be expected to metastasize.

RADIOLOGICAL FEATURES

Most BMs from HCC are located in the vertebrae, followed by the pelvis, ribs, sternum, limb bones, and cranium[18], which is a similar distribution to the BMs of other tumor types[19]. The distribution of BMs in HCC is similar to that observed in previous studies[3,4,8]. The lower-thoracic and lumbar vertebrae are common sites for vertebral BMs[18]. The reported incidence of skull metastases varies widely; 3.5%-30%[4,7,8,14].

Radiographically, typical BMs from HCC appear as expansible, destructive findings with large soft-tissue masses[13]. Most BMs are osteolytic and thus detectable using CT[14,19] (Figure 1). However, HCC patients rarely exhibit either purely osteolytic or osteoblastic lesions[13,19]. Soft-tissue masses are unique to BMs of HCC and have been observed in 39%-85.4% of patients[8,18-20]. These

Table 2 Studies of external radiotherapy to treat painful bone metastases from hepatocellular carcinoma

Ref.	Patients (*n*)	Sites (*n*)	Fraction dose	Pain relief (CR)	Dose-response relationship	Comments
Roca *et al*[27]	26	37	MFs 30-50 Gy	79% (44%)	NR	11 lesions (with CTx)
Kaizu *et al*[28]	57	99	MFs 20-65 Gy	83.8% (33%)	Better pain relief TDF ≥ 77	16 lesions (with TAE) Tumor volume (NS)
Matsuura *et al*[29]	38	44	MFs 26-60 Gy	91% (32%)	NR	Tumor regression (> 40 Gy)
Seong *et al*[7]	51	77	MFs 12.5-50 Gy	73%	Better pain relief BED > 43 Gy	With neurological symptoms (25%)
He *et al*[8]	205	205	MFs 32-66 Gy	99.5% (29.80%)	NR	Higher retreatment rate (with soft-tissue masses)
Hayashi *et al*[30]	28	48	MFs 20-52 Gy SF 8 Gy	83% (17%) MFs: 87% (17%) SF: 75% (17%)	NR	Longer pain relief (> 36 Gy) No spinal compression No neuropathic pain

MFs: Multiple fractions; SF: Single fraction; CR: Complete relief; NR: No dose response; TDF: Time, dose, and fractionation factor; BED: Biologically effective dose; NS: No significant difference; CTx: Chemotherapy; TAE: Transcatheter arterial embolization.

soft-tissue masses can replace the normal bone matrix and exhibit expansive growth, frequently within the vertebral body. Paravertebral masses have been shown to grow inward to encapsulate and destroy the bone matrix[18]. These masses often compress peripheral nerves, the spinal cord[21], or cranial nerves[22], causing not only bone pain but also neuropathic pain[6] and neurological symptoms.

RADIOTHERAPY FOR BONE PAIN

EBRT is prescribed most frequently to relive pain from BMs and the efficacy of EBRT for treating BMs has been well established[23]. Generally, pain relief is obtained in 60%-90% of treated patients, but the sites of primary tumors from these reports were mainly in the lung, breast, and prostate[23-26]. There have only been a few studies of EBRT for HCC BMs, but they show that 73%-99.5% of patients obtained overall pain improvement and 17%-44% of patients achieved complete pain relief (Table 2)[7,8,27-30]. Except for a report from China[8], these studies were retrospective analyses with a small sample size; however, the reported results for overall pain relief are similar to those obtained for BMs of other primary tumors. HCC is often complicated by liver failure, and narcotic drugs may induce hepatic coma. Therefore, EBRT may play an important role in relieving the pain from BMs, thus minimizing the use of narcotic drugs for pain relief.

Soft-tissue masses with BMs often cause neuropathic pain, spinal compression, and pathological fractures, and these issues were evaluated simultaneously with bone pain relief in some previous reports. The pain assessments differed among the studies; two recent studies[8,30] evaluated pain relief using the International Bone Metastases Consensus Working Party Guidelines[31,32] with some modifications. Even after considering the differences among studies, it has been shown that EBRT is equally effective for the relief of pain caused by BMs from HCC, as well as metastases from other primary tumors.

Various EBRT dosage and fractionation schedules have been used to treat pain, ranging from an 8 Gy single fraction (SF) to multiple fractions (MFs). An SF of 8 Gy delivered in one day is more convenient for the patient and more cost effective compared with schedules employing MFs. However, MFs that deliver a higher total dose than an SF may have increased biological effects on the tumor. In a randomized controlled trial conducted by the Radiation Therapy Oncology Group (RTOG 9701)[33] in which an SF (8 Gy) was compared with MFs (30 Gy in 10 fractions over 2 wk), it was demonstrated that both schedules provided equivalent pain relief. Furthermore, the RTOG trial found a significantly lower rate of acute toxicity with an SF compared to MFs, although there was no significant difference in late toxicity (*e.g.*, pathologic fractures). Similar findings concerning the pain relief after treatment based on an SF or MFs have also been reported[26,34]. Similarly, according to other recent studies including a meta-analysis, both SF and MF-based treatments have provided equivalent pain relief, although SF treatment often requires re-treatment[23-25,35]. In terms of pain relief, most previous studies failed to show a dose-response relationship for BMs from other primary cancers. For BMs from HCC, Roca *et al*[27], Matsuura *et al*[29], and He *et al*[8] also found no apparent dose-response relationship for pain relief. In contrast, Kaizu *et al*[28] and Seong *et al*[7] did find a dose-response relationship, although in the former study, 15 of the 57 patients analyzed[28] underwent transcatheter arterial embolization of BMs in addition to EBRT, and 25% of the patients in the study by Seong *et al*[7] had neurological symptoms with bone pain. He *et al*[8] also found no dose-response relationship for pain relief, but higher complete pain relief rates were obtained using higher radiation doses. Furthermore, they observed that the re-treatment rate was higher among patients with expansible soft-tissue masses and noted an increased presence of residual cancer cells in these patients relative to those lacking soft-tissue extension. Matsuura *et al*[29] reported a lack of observed tumor regression at doses < 40 Gy and that 3 patients treated

with doses $\geq$ 40 Gy (40 Gy, 46 Gy, and 60 Gy) survived for > 6 years without recurrence. Pain caused by BMs can originate directly from the bone, or as a result of nerve root compression, or muscle spasms in the lesion area (*i.e.*, neuropathic pain)[7]. In a randomized trial of radiotherapy for neuropathic pain caused by BM, Roos *et al*[36] (Trans-Tasman Radiation Oncology Group) compared the efficacy of SF (8 Gy) to MFs (20 Gy/5 fraction) treatment and concluded that an SF was not as effective as MFs; the outcomes with SF treatment were generally poor, although the difference was not statistically significant. In that study, the most frequent primary tumor sites were the lung and prostate. For patients with BMs from HCC that cause neuropathic pain through nerve root compression, a higher radiation dose may be needed to shrink the soft-tissue mass and provide pain relief.

Nearly all previous studies[7,8,27-29] involving BMs of HCC used MF schedules and evaluated both bone pain and neuropathic pain. We conducted a retrospective evaluation of the palliative efficacy of EBRT, excluding cases with spinal cord compression or neuropathic pain[30] and assessed different dosing schedules for BMs from HCC with soft-tissue masses. Our analysis included a relatively small number of patients (28 patients, 42 sites), and the overall response rates were 75% and 87% for SF and MF treatment, respectively; this difference was not significant. Patients undergoing high-dose MFs ($\geq$ 36 Gy in total) achieved on average a significantly longer duration of pain relief than those undergoing SF or moderate-dose MF therapy ($\leq$ 30 Gy in total). The median durations of overall pain relief for MFs were 3.8 and 1.8 mo after SF treatment. These results were similar to those reported for other series involving different primary cancers[24,25,34]. In our study, we found that EBRT effectively palliated painful BMs from HCC, that an 8 Gy SF and MFs resulted in equivalent pain relief, and that high-dose MF schedules may result in longer lasting pain relief.

Soft-tissue masses are unique to BMs from HCC and often cause both bone and neuropathic pain. In HCC patients with neuropathic pain, higher RT doses using an MF schedule are usually necessary because of the presence of soft-tissue masses. It is critical to discriminate between these different pain types, and large-scaled cohort studies are necessary to determine an optimal radiotherapy plan in terms of doses and fractions for each.

RADIOTHERAPY FOR SPINAL BMs

Spinal BMs often cause not only pain but also MSCC, which primarily develops in one of three ways[37]: (1) the continued growth and expansion of vertebral BMs into the epidural space; (2) neural foraminal extension *via* a paraspinal mass; and (3) the destruction of vertebral cortical bone, leading to vertebral body collapse and the displacement of bony fragments into the epidural space. SCC secondary to BMs from HCC can develop in any of these ways because typical these metastases have an expansible and destructive nature and give rise to soft-tissue

masses[13].

MSCC is estimated to occur in approximately 5%-10% of all cancer patients[37]. The most common primary sites are the breast and lung[37,38]; however, the rate of MSCC resulting from BMs from HCC is unclear. MSCC that diminishes motor function and causes paraplegia is considered an oncological emergency requiring urgent treatment[38]. Conventionally, MSCC has been managed with corticosteroids and high-dose EBRT. EBRT is an effective treatment for MSCC and has been included in standard care. Rades *et al*[38] retrospectively analyzed the use of 5 radiotherapy schedules (8 Gy, 20 Gy/5 fractions, 30 Gy/10 fractions, 37.5 Gy/15 fractions, and 40 Gy/20 fractions) for MSCC treatment and found that motor function improved by 26%-31%, post-treatment ambulation was achieved in 63%-74% of cases, and that all 5 schedules provided similar functional outcomes. Rades *et al*[38] therefore recommended a schedule of 8 Gy for patients with a poor survival prognosis and 30 Gy/10 fractions for other patients. Maranzano *et al*[39] reported that 76% of patients achieved full recovery, or at least were still able to walk, after EBRT with doses > 30 Gy over a 2 wk period in combination with steroids, and that the most important response predictors were an early diagnosis and favorable histology. For MSCC specifically caused by HCC, Maranzano *et al*[39] reported a median duration of improvement of only 1 mo, which was shorter than the duration observed for other cancers, including breast cancer, for which it was 12 mo. Nakamura *et al*[9] reported a retrospective series of 24 ambulatory patients with MSCC derived from HCC. Five patients (21%) underwent salvage therapy and 4 (21%) had become non-ambulatory by the last follow-up. The ambulatory rates at 3 and 6 mo were 85% and 63%, respectively. Nakamura *et al*[9] concluded that EBRT with a biologically effective dose range of 39-50.7 Gy (total radiation dose range, 30-39 Gy) was not sufficient to prevent MSCC-related paralysis and that dose escalation *via* a precise radiation technique should be evaluated. In MSCC caused by BMs from HCC, it will probably be important to shrink and control the soft-tissue masses. Therefore, higher radiation doses are needed to prevent MSCC-related paralysis. For patients with a good survival prognosis, high-precision radiation therapy [intensity-modulated radiotherapy (IMRT) or stereotactic irradiation (STI)] should be considered for the delivery of higher radiation doses, whilst sparing the spinal cord and reducing the risk of radiation myelitis. In addition to EBRT, surgery is being re-evaluated for the palliative management for MSCC. The results of a randomized trial reported by Patchell *et al*[40], showed that direct decompressive surgery with postoperative EBRT was more effective at restoring ambulation than EBRT alone.

Vargas *et al*[41], Somerset *et al*[42] and Doval *et al*[21] showed in case reports that patients with MSCC derived from HCC who were treated with laminectomy or resection of the epidural lesion had a good clinical course. Vargas *et al*[41] concluded from their case report that surgi-

cal therapies such as direct decompression of the tumor with postoperative EBRT or vertebral body resection with stabilization should be considered in patients for whom surgery could be expected to succeed.

RADIOTHERAPY FOR SBMs

SBMs occur in 4% of cancer patients[43] and often cause pain or cranial nerve palsies. Because of their rarity, SBMs have received limited attention in the published medical literature. Their clinical manifestation depends on the metastatic cranial nerve invasion site. In a review by Laigle-Donadey *et al*[43], the most common primary cancers from which SBMs originated were prostate (38%) and breast cancer (20%). The incidence of SBM from HCC has been reported to be 0.4%-1.6%[44-47] and until 2009, only 25 such cases had been reported[38]. However, the incidence of SBM from HCC increased significantly during the period between 1990 and 2001[39]. SBM without other osseous metastases is an unusual finding and cranial nerve deficits are found in 96% of cases in which the SBM was derived from HCC[38]. Radiotherapy is usually the standard treatment for SBM and has been used to treat 70% of patients[43]. EBRT provides excellent pain relief and often leads to the regression of cranial nerve dysfunction, which lasts until death in most cases. There is consensus that the rate of neurological improvement is closely related to the length of time to EBRT following the onset of symptoms[43]. Vikram *et al*[48] reported that 87% of patients for whom EBRT was initiated < 1 mo after the onset of symptoms in contrast to 25% for whom EBRT was initiated ≥ 3 mo after the onset of symptoms. However, the appropriate doses and fractions to use in EBRT for SBMs have still not been agreed upon. Discrepancies with respect to the dose-response relationship can be explained by the different radiosensitivity of each primary tumor. In cases involving SBMs from HCC, higher radiation doses are needed to improve the neurological symptoms by resolving the compression and invasion of cranial nerves caused by soft-tissue masses. Nozaki *et al*[47] reported a case in which multiple SBMs from HCC were successfully treated with EBRT. They found that slightly higher radiation doses (50 Gy/20 or 25 fractions) were delivered and improvements in neurological symptoms and tumor regression were achieved. However, EBRT places certain organs at risk, including the brain stem, optic nerve, and optic chiasm. Stereotactic radiosurgery (SRS) and stereotactic radiotherapy (SRT) are more recent therapeutic options for SBMs. They are high-precision radiation therapies in which delivery is accurate to within one to two millimeters and are performed in a non-surgical procedure that delivers precisely targeted radiation at much higher doses than traditional radiotherapy in a single dose (SRS) or fractionated regimen (SRT). This treatment is only possible because of the development of highly advanced radiation technologies such as the gamma knife, and high-precision linear accelerators that permit maximum dose delivery within the target while minimizing the dose to organs at risk. In

a report of HCC cases treated by gamma knife radiosurgery, the clinical symptoms improved in 61% of the patients after treatment and tumor control was achieved in 67% of cases[49]. Gamma knife radiosurgery is particularly useful for small tumors (diameter < 30 mm)[36]. Stereotactic radiotherapy *via* Novalis, a high-precision linear accelerator, can administer high doses to tumors while sparing normal structures and organs at risk, thus being a useful EBRT technique for SBM treatment[50]. In a study utilizing Novalis, all 11 cases (including 1 HCC case) achieved and maintained local control until the end of the follow-up period or death. SBM remains a challenge with respect to EBRT planning and delivery.

SURVIVAL ASSOCIATED WITH BMs

The 1-year survival rate after EBRT initiation or the diagnosis of BMs has been reported to be 13.8%-32.4%, with a 5-7.4 mo median survival time[7,8,13,29,30]. Unfavorable significant prognostic factors of patients with BMs have been reported as lower performance status, multifocal BMs, tumor stage IVA, metastasis to other organs, higher tumor marker levels, uncontrolled intrahepatic tumors, and ascites at the initial presentation[4,7,8,29].

The prognosis of patients with MSCC is worse than for patients with only BMs. According to Nakamura *et al*[9], the median observed survival duration for all patients was 5.1 mo and the overall 6-mo survival rate was 38%.

SBMs are generally late events and occur at a stage when many patients have already developed disseminated disease, particularly other BMs[43]; 71% of these patients were reported to have died within a short period of between 11 d and 9 mo after the onset of neurological symptoms[40].

CONCLUSION

Soft-tissue masses are unique to BMs from HCC and often cause both bone and neuropathic pain, and neurological symptoms. EBRT is effective for the relief of painful symptoms resulting from BMs, MSCC, and SBMs from HCC. However, the optimal dose and fraction schedules for bone pain palliation remain unclear. Large-scaled cohort studies are necessary to determine the optimal radiotherapy doses and fractions to treat both bone pain and neuropathic pain. MSCC and SBMs remain a challenge for EBRT. High-precision radiation therapy (IMRT or STI) should be considered for the delivery of higher radiation doses with sparing of normal tissues.

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RETROSPECTIVE STUDY

Evaluation of hepatocellular carcinoma development in patients with chronic hepatitis C by EOB-MRI

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Abstract

AIM: To evaluate the efficacy of ethoxibenzyl-magnetic resonance imaging (EOB-MRI) as a predictor of hepatocellular carcinoma (HCC) development.

METHODS: Between August 2008 and 2009, we studied 142 hepatitis C virus-infected patients (male 70, female 72), excluding those with HCC or a past history, who underwent EOB-MRI in our hospital. The EOB-MRI index [liver-intervertebral disc ratio (LI)] was calculated as: (post-liver intensity/post-intervertebral disc intensity)/(pre-liver intensity/pre-intervertebral disc intensity).

RESULTS: The median follow-up period was 3.1 years and the patients were observed until the end of the study period (31 December, 2012). In the follow-up period, HCC occurred in 21 patients. The cumulative occurrence rates were 2.1%, 9.1%, and 14.1% at 1, 2, and 3 years, respectively. Using the optimal cut-off value of LI 1.46, on univariate analysis, age, aspartate amino transferase (AST), α-fetoprotein (AFP) ≥ 10, albumin, total cholesterol, prothrombin time, platelets, and LI < 1.46 were identified as independent factors, but on multivariate analysis, LI < 1.46: risk ratio 6.05 (1.34-27.3, P = 0.019) and AFP ≥ 10: risk ratio 3.1 (1.03-9.35, P = 0.045) were identified as independent risk factors. LI and Fib-4 index have higher area under the receiver operating characteristic curves than other representative fibrosis evaluation methods, such as Forn's index and AST-to-platelet ratio index.

CONCLUSION: LI is associated with the risk of HCC occurrence in hepatitis C patients. LI may be a substitute for liver biopsy when evaluating this risk and its combined use with Fib-4 is a better predictive method of HCC progression.

Core tip: This manuscript addresses a method of hepatocellular carcinoma (HCC) prediction by using a new technique that evaluates hepatic fibrosis using a non-invasive method (reported recently). This is the first reported study to consider a possible substitute for liver biopsy by using an magnetic resonance imaging (MRI) method (a widespread method in public medical services) for evaluating the risk of occurrence. We propose that this method will become one of the most popular and precise noninvasive methods to predict the occurrence of HCC, and the combination of this MRI method and Fib-4 index may provide a better predictive method of HCC progression.

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INTRODUCTION

The major cause of cirrhosis globally is chronic hepatitis C. The risk of hepatocellular carcinoma (HCC) development is related to this, as reported in several papers[1-3], and advanced fibrosis increases the risk of carcinogenesis[1]. The prognosis of HCC is not good, even when detected and treated at an early stage[4-6]. Thus, it is important to determine outpatients' fibrotic stage in order to identify the risk of HCC occurrence in the management of patients with chronic liver disease. Even now, to determine the grade of fibrosis, the gold standard is liver biopsy, but it is associated with certain problems such as sample error and severe complications[7-10]. Previously, noninvasive methods to evaluate fibrosis were reported, such as Forn's index[11], the Fibro index[12], and aspartate amino transferase-to-platelet ratio index (APRI)[13]. Using laboratory data, it has been reported that the Fibrotest is a useful prognostic factor for hepatitis C patients[14]. On the other hand, specific methods, such as transient elastography[15], magnetic resonance (MR) elastography[16], and acoustic radiation force impulse[17], have been reported to evaluate fibrosis as surrogates of liver biopsy. Transient elastography is reported to indicate a wide-ranging risk of HCC incidence. We recently reported the accuracy of staging fibrosis in chronic hepatitis in hepatitis C virus (HCV) infection using ethoxibenzyl-MR imaging (EOB-MRI)[18], but there are no reports about a predictor of HCC incidence using this new method. Here, we report a study to evaluate the efficacy of EOB-MRI as a predictor of HCC development.

MATERIALS AND METHODS

Patients

Between August 2008 and December 2009, we studied 142 HCV-infected patients, excluding those with HCC or a past history, who underwent EOB-MRI in our hospital. Clinical data were obtained within one month of EOB-MRI information being obtained. The definition of HCV infection was determined by a positive anti-HCV antibody and detection of quantitative or qualitative HCV RNA. Exclusion criteria were as follows: (1) infection with hepatitis B or human immunodeficiency viruses; (2) alcohol abuse; (3) the presence of numerous liver tumors; and (4) having previously undergone partial splenic arterial embolization or splenectomy. During the follow-up period, the history of interferon (IFN) therapies and associated responses was examined. We defined a sustained virological response (SVR) as undetectable HCV-RNA for at least 24 wk after IFN therapy. The study protocol conformed to the ethics guidelines of the 1975 Helsinki Declaration and was approved a priori by the institution's human research committee. All blood tests were performed within 1 wk before or after MRI.

Follow-up of patients and HCC diagnosis

The screening of HCC occurrence was carried out by enhanced MRI or enhanced computed tomography (CT). Outpatients were followed up with blood tests, tumor markers for HCC, and image analysis, such as ultrasonography, enhanced CT, or enhanced MRI, every 3 to 6 mo. The diagnosis of HCC was determined by enhanced CT or enhanced MRI, considering enhancement in the arterial phase and washout in the earlier delayed venous phase as a classical sign of HCC[19,20]. When the diagnosis of HCC was not clear in CT or MRI, a histological diagnosis was performed by tumor biopsy[21]. Cases that were diagnosed as HCC within 6 mo from the first MRI trial were excluded because there should have been only small HCC when the first MRI was performed. This study was continued until December 31, 2012.

MRI techniques

A 1.5-Tesla MR system (Philips Co., Amsterdam, The Netherlands) was used: 0.025 mmol/kg body weight gadoxetate disodium was intravenously injected and quantitative measurements were performed using unenhanced and gadoxetate disodium-enhanced imaging at 20, 35, 70, and 180 s, and the imaging at 15, 20, and 25 min was obtained as hepatobiliary phases. Imaging parameters were as follows: repetition time/echo time = 4.17/2.05 ms. Then, 1-2 cm^2 regions of interest of the mean signal intensity value of the liver were measured. At each MRI, the means of three different regions of right anterior, right posterior, and left lateral segments of the liver devoid of large vessels or severe artifacts were calculated. Using the liver to intervertebral disk signal intensity (LISI) and liver signal intensity/intervertebral disk signal intensity, we calculated the post-enhanced LISI/pre-enhanced LISI [described as liver-intervertebral disc ratio (LI)], as detailed in our previous report[18]. We used hepatobiliary phase data at 20 min because this is most commonly used globally and the data showed no significant difference from the value at 25 min. As we reported previously, because cut-off values of 1.31 and 1.80 are representative values of liver cirrhosis and significant fibrosis of the liver, we divided all patients into < 1.31, 1.311 to 1.38, 1.381 to 1.50, 1.501 to 1.60, and > 1.601. Age, sex, aspartate aminotransferase (AST), alanine aminotransferase (ALT), serum albumin level, total bilirubin (T.Bil), gamma-glutamyl transpeptidase (γGTP), total cholesterol, and platelet count (Plt) were examined. The prothrombin time (PT) was measured as a percentage of the daily internal control.

Statistical analysis

Baseline data are presented as the mean ± SD with the range in parentheses for quantitative variables. The best models derived from the categorical variables were compared by the χ^2 or Fisher's exact test, whereas Wilcoxon rank sum test (nonparametric) for continuous variables and the unpaired Student's t test (parametric) were used to evaluate differences in age, sex, albumin, T.Bil, PT, Plt, AST, ALT, γGTP, total cholesterol, and α-fetoprotein (AFP) at the time of entry. The results are reported as

Variables	Mean ± SD
Age (yr)	66.1 ± 12.4 (28-87)
Male (M/F)	70/72
AST (U/L)	48.9 ± 23.4 (11-155)
ALT (U/L)	51.7 ± 34.1 (10-228)
Serum albumin (g/dL)	4.1 ± 0.5 (2.4-5)
Gamma-GT(IU/L)	67 ± 92 (14-811)
ALP (U/L)	331 ± 171 (141-1206)
T.Chol (mg/dL)	175 ± 36 (90-280)
T.Bil (mg/dL)	0.86 ± 0.42 (0.3-2.9)
PT (%)	93 ± 15.2 (55.2-134)
Platelet ($\times 10^3/\mu$L)	136 ± 60 (42-338)
AFP (ng/mL)	14.5 ± 27.5 (1.6-235)
LI	1.51 ± 0.19 (1.11-2.15)
Patients who received IFN, *n* (%)	39 (27.5)
Patients who achieved SVR, *n* (%)	27 (19.0)

AST: Aspartate amino transferase; ALT: Alanine aminotransferase; Gamma-GT: Gamma-glutamyl transpeptidase; T.Chol: Total cholesterol; T.Bil: Total bilirubin; PT: Prothrombin time; AFP: α-fetoprotein; LI: Liver-intervertebral disc ratio; IFN: Interferon; SVR: Sustained virologic response; ALP: Alkaline phosphatase; F: Female; M: Male.

hazard ratios with 95%CI. *P* < 0.05 in a two-tailed test was considered significant for all analyses. Patients were censored when they died without HCC development, when they stopped visiting, or when the study period ended. Cumulative occurrence curves were analyzed using the Kaplan-Meier method and tested by Wilcoxon's method. The Cox proportional hazard regression model was used to estimate the risk factors for hepatocarcinogenesis using the following variables in univariate and multivariate analyses: sex, albumin, T.Bil, PT, Plt, AST, ALT, γGTP, alkaline phosphatase (ALP), total cholesterol, AFP ($\geqslant$ 10 ng/mL), LI (< 1.46) at the time of entry, and the history of IFN therapy (with or without, and SVR or non-SVR).

All statistical analyses were performed using IBM SPSS Statistics 21 software (IBM, Chicago, IL, United States).

RESULTS

Patient characteristics
A total of 145 patients who had undergone EOB-MRI were examined. Three patients were excluded because they developed HCC within 6 mo.

Patient characteristics at the time of EOB-MRI are shown in Table 1. There were 70 men and 72 women, with a mean age of 66.1 ± 12.4 years. The mean AFP level was 14.5 ng/mL and the median was 5 ng/mL. Thirty-seven patients (26%) had an AFP level of $\geqslant$ 10. Thirty-nine patients received IFN and 27 patients achieved SVR in the follow-up period.

Occurrence of HCC and patient follow-up
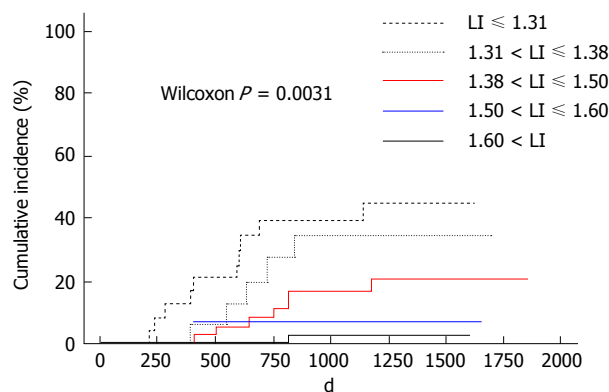The median follow-up period was 3.1 years, during which 14 (9.8%) patients were lost to follow-up and were cen-

Figure 1 Cumulative incidence of hepatocellular carcinoma occurrence stratified by liver-intervertebral disc ratio. Cumulative occurrence rates increased gradually in an LI-independent manner. LI: Liver-intervertebral disc ratio.

sored at the time of the last visit. Nine patients died of liver failure, one died of gastroenterological varices rupture, and nine died of liver-unrelated causes, and they were censored when they died. The remaining patients were observed until the end of the study period (31 December, 2012). During the follow-up period, HCC occurred in 21 patients. The cumulative HCC occurrence rates were 2.1%, 9.1%, and 14.1% at 1, 2, and 3 years, respectively, by the Kaplan-Meier method. Baseline characteristics were compared in patients with and without HCC occurrence (Table 2). There were no significant differences between the no-HCC occurrence group and the HCC occurrence group in terms of age, sex, ALT level, gamma-GT, T.Bil, the performance of IFN therapy, and the achievement of SVR, while AST, ALP, and AFP were higher and albumin, total cholesterol (T.Chol), PT, platelets, and LI were lower in the HCC occurrence group than in the no-HCC occurrence group.

Occurrence rate of HCC stratified by LI
The cumulative occurrence rates at 1, 2, and 3 years in each LI group were 0%, 0%, and 2% in patients with LI $\geqslant$ 1.601; 0%, 5.8%, and 5.8% in patients with LI 1.501-1.600; 0%, 7.1%, and 14.3% in patients with LI 1.381-1.500; 0%, 11.8%, and 23.5% in patients with LI 1.311-1.380; and 12.5%, 29.2%, and 33.3% in patients with LI $\leqslant$ 1.310, respectively (Figure 1). The occurrence rates differed significantly among the 5 LI groups (*P* = 0.0031), increasing with decreasing LI.

The receiver operating characteristic curve (ROC) curve was used to evaluate the cumulative incidence of LI and a cut-off value of 1.46 was determined [area under the ROC (AUROC): 0.765 ± 0.05, 0.669-0.861] by calculating the highest accuracy value (0.63) and likelihood ratio (2.19). The use of this cut-off value resulted in sensitivity: 90.5%, specificity: 58.7%, positive predictive value: 27.5%, and negative predictive value: 97.3%. We compared these results with several representative fibrosis evaluation methods reported previously (Figure 2). The AUROC for each was: Forn's index, 0.733 ± 0.05,
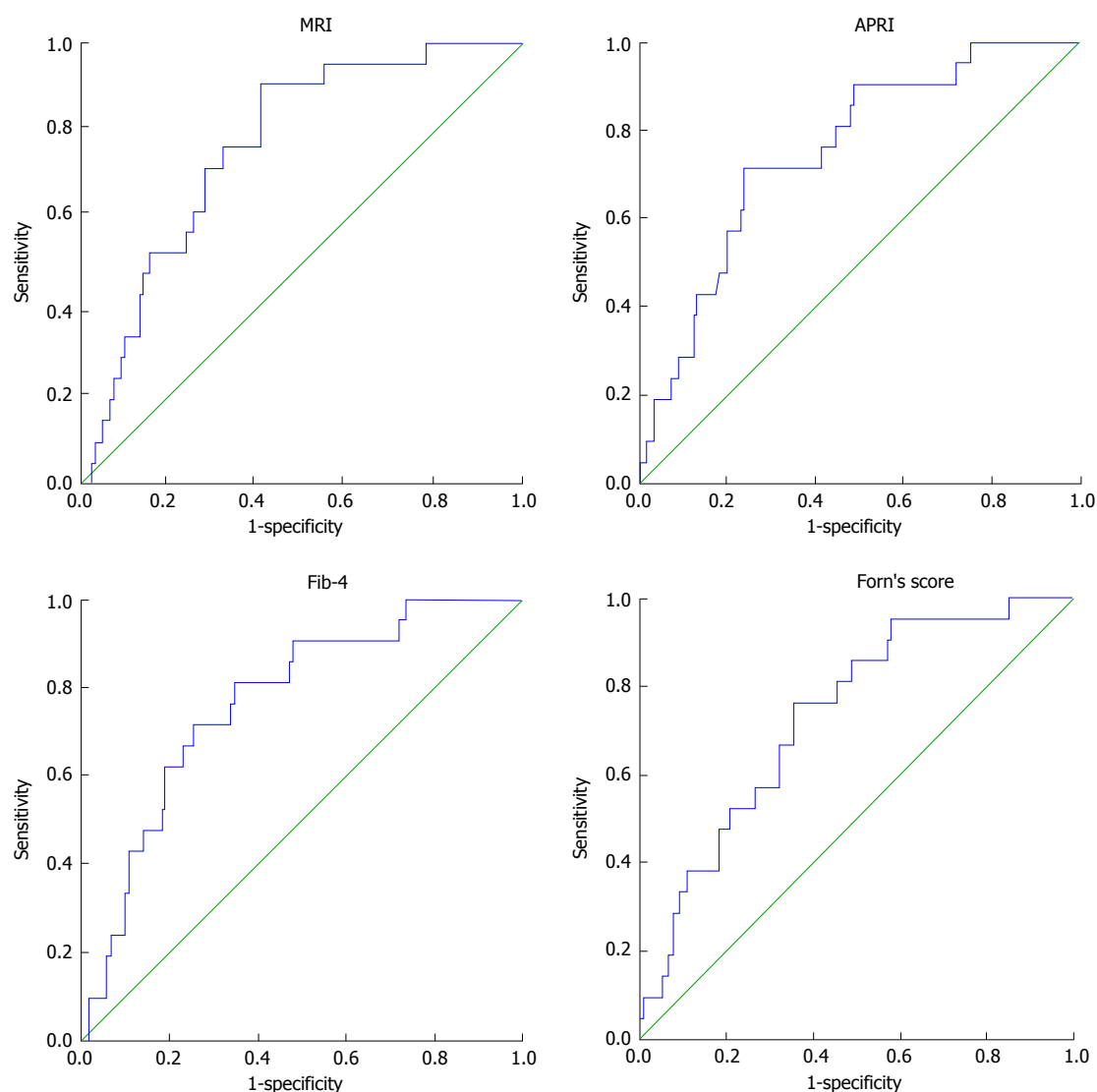
Figure 2 Receiver operating characteristic curve evaluating the cumulative incidence of liver-intervertebral disc ratio, aspartate amino transferase-to-platelet ratio index, Fib-4, and Forn's index. APRI: Aspartate amino transferase-to-platelet ratio index; MRI: Magnetic resonance imaging.

Table 2 Comparison of baseline characteristics between patients who have no hepatocellular carcinoma occurrence and hepatocellular carcinoma occurrence

Variables	No HCC occurrence n = 121	HCC occurrence n = 21	P value
Age (yr)	65.4 ± 12.9 (28-87)	70.3 ± 7.9 (51-79)	0.094
Sex (M/F)	59/62	11/10	0.759
AST (U/L)	46.0 ± 20.4 (11-110)	65.5 ± 31.6 (34-155)	0.000
ALT (U/L)	49.5 ± 32.5 (10-228)	64.4 ± 40.5 (31-205)	0.063
Serum albumin (g/dL)	4.1 ± 0.5 (2.4-5)	3.8 ± 0.5 (2.6-4.7)	0.030
Gamma-GT (IU/L)	67 ± 97 (14-811)	62 ± 43 (16-226)	0.829
ALP (U/L)	315 ± 158 (141-1206)	426 ± 212 (150-1006)	0.006
T.Chol (mg/dL)	178 ± 36 (90-280)	159 ± 35 (93-260)	0.029
T.Bil (mg/dL)	0.86 ± 0.42 (0.3-2.9)	0.86 ± 0.42 (0.3-2.9)	0.287
PT (%)	94 ± 15.5 (55.2-134)	86.8 ± 12.0 (67-110)	0.045
Platelet (× 10^3/μL)	142 ± 61 (42-338)	104 ± 40 (46-166)	0.006
AFP (ng/mL)	11.7 ± 26.3 (1.6-235)	30.3 ± 31.6 (4.2-116)	0.004
LI	1.53 ± 0.20 (1.11-2.15)	1.37 ± 0.10 (1.23-167)	0.000
Patients who received IFN, n (%)	37 (30.6)	2 (9.5)	0.062
Patients who achieved SVR, n (%)	25 (20.7)	2 (9.5)	0.366

HCC: Hepatocellular carcinoma; AST: Aspartate amino transferase; ALT: Alanine aminotransferase; Gamma-GT: Gamma-glutamyl transpeptidase; ALP: Alkaline phosphatase; T.Chol: Total cholesterol; T.Bil: Total bilirubin; PT: Prothrombin time; AFP: α-fetoprotein; LI: Liver-intervertebral disc ratio; IFN: Interferon; SVR: Sustained virologic response; F: Female; M: Male.

Table 3 Risk factors contributing to hepatocellular carcinoma incidence

Variable	Univariate analysis			Multivariate analysis		
	Risk ratio	95%CI	*P*-Value	Risk ratio	95%CI	*P*-value
Age (per 1 year old)	1.04	1.01-1.09	0.045	1.05	0.99-1.11	0.139
Sex (F)	0.74	0.31-1.73	0.483			
AST (U/L)	1.02	1.01-1.03	< 0.001	1.01	0.99-1.03	0.200
ALT (U/L)	1.01	0.99-1.02	0.12			
Serum albumin (g/dL)	0.27	0.12-0.60	0.001	0.62	0.17-2.26	0.469
Gamma-GT (IU/L)	1.00	0.99-1.01	0.942			
ALP (U/L)	1.002	1.001-1.004	0.006	1.00	0.99-1.01	0.504
T.Chol (mg/dL)	0.98	0.97-0.99	0.01	0.99	0.98-1.01	0.483
T.Bil (mg/dL)	2.01	0.77-5.25	0.153			
PT (%)	0.97	0.94-0.99	0.018	1.01	0.97-1.05	0.621
Platelet ($\times 10^3$/μL)	0.98	0.97-0.99	0.003	0.99	0.98-1.01	0.281
AFP ($\geqslant$ 10 ng/mL)	7.39	2.97-18.37	< 0.001	3.10	1.03-9.35	0.045
LI (< 1.46)	11.63	2.71-49.9	0.001	6.05	1.34-27.3	0.019
$\geqslant$ 1.601	1.00					
1.501 to 1.60	2.68	0.17-42.9	0.48			
1.381 to 1.50	7.24	0.89-58.9	0.06			
1.311 to 1.38	11.5	1.2-103	0.02			
$\leqslant$ 1.31	17.34	2.16-138.7	0.007			
Patients who received IFN	0.20	0.04-0.87	0.032	1.09	0.21-5.62	0.917
Patients who achieved SVR	0.35	0.81-1.51	0.158			

AST: Aspartate amino transferase; ALT: Alanine aminotransferase; Gamma-GT: Gamma-glutamyl transpeptidase; ALP: Alkaline phosphatase; T.Chol: Total cholesterol; T.Bil: Total bilirubin; PT: Prothrombin time; AFP: α-fetoprotein; LI: Liver-intervertebral disc ratio; IFN: Interferon; SVR: Sustained virologic response; F: Female.

Table 4 Analyses of liver-intervertebral disc ratio contributions to hepatocellular carcinoma occurrence risk divided by other risk factors

	Subgroup	*n*	Risk ratio	95%CI	*P*-value
Age	$\geqslant$ 69	75	12.51	1.63-95.82	0.015
	< 69	67	9.2	1.11-76.58	0.041
Sex	Male	70	8.4	1.08-65.18	0.042
	Female	72	7.024	1.49-33.14	0.014
Platelet ($\times 10^3$/μL)	< 120	67	4.48	1.01-19.89	0.048
	$\geqslant$ 120	75	14.96	1.89-118.2	0.013
Albumin (g/dL)	< 4.2	72	9.7	1.27-74.24	0.029
	$\geqslant$ 4.2	70	10.79	1.29-89.7	0.028
ALT (U/L)	$\geqslant$ 50	88	10.98	1.39-86.7	0.023
	< 50	54	12.7	1.62-99.63	0.016
IFN	-	103	13.35	1.78-100.1	0.011
	+	39	3.498	0.22-55.96	0.376
SVR	-	115	15.98	2.13-119.7	0.007
	+	27	3.795	0.28-60.74	0.346

ALT: Alanine aminotransferase; IFN: Interferon; SVR: Sustained virologic response.

0.627-0.840; APRI, 0.752 ± 0.05, 0.648-0.856; and Fib-4, 0.765 ± 0.05, 0.665-0.861. Comparing these results, MRI is as effective as the Fib-4 method and more effective than Forn's index and APRI.

Prognostic Factors of HCC occurrence risk by univariate and multivariate analyses

On univariate analysis, LI < 1.46, AFP $\geqslant$ 10, age (per year of age), AST (per 1 U/L), serum albumin (per 1 g/dL), ALP (per 1 U/L), T.Chol (per 1 mg/dL), PT (per 1%), platelets (per 1 $\times$ 10^3/μL), and receiving IFN were identified as risk factors for the occurrence of HCC. The risk of HCC occurrence increased in accordance with LI decrease. On multivariate analysis, LI < 1.46 (*P* =

0.019) and AFP $\geqslant$ 10 ng/mL (*P* = 0.045) were identified as independent factors; LI: risk ratio: 6.05 (1.34-27.3, *P* = 0.019) and AFP: 3.1 (1.03-9.35, *P* = 0.045) (Table 3). The LI contributions to HCC occurrence risk were also evaluated in subgroup analyses. We investigated whether higher LI was a significant risk factor with several other factors (Table 4). High LI was a significant risk factor even with low or high values of age, Plt, albumin, ALT, and male or not, IFN-treated or not, and SVR achieved or not. The LI contribution was greater at age $\geqslant$ 69 (older group) and with platelets $\geqslant$ 120 $\times$ 10^3/μL (less fibrosis). In the IFN-untreated group and the SVR-unachieved group, there was a significant risk in low LI, but in the IFN-treated group and SVR not-achieved group, there
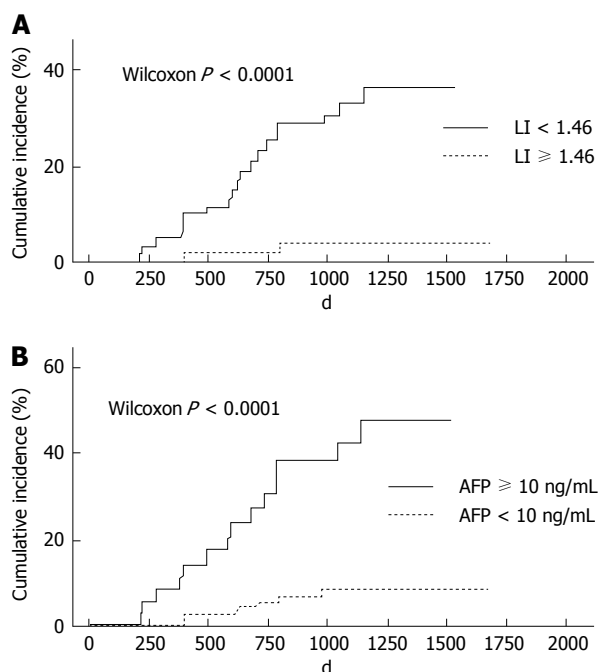
Figure 3 Relationship between cumulative occurrence rates and liver-intervertebral disc ratio (A), cumulative occurrence rates and serum α-fetoprotein level (B). A: Occurrence rates with LI < 1.46 were significantly higher than those with LI ≥ 1.46; B: Occurrence rates with serum AFP ≥ 10 ng/mL were significantly higher than in those with serum AFP < 10 ng/mL. LI: Liver-intervertebral disc ratio; AFP: α-fetoprotein.

were no significant differences because the sample numbers were very small.

Relationship between occurrence rate and LI or AFP

The occurrence rate in patients with LI < 1.46 was significantly higher than in those with LI ≥ 1.46 (Wilcoxon $P < 0.0001$) (Figure 3A); in addition, in those with serum AFP ≥ 10 ng/mL, it was significant higher than in those with serum AFP < 10 ng/mL (Wilcoxon $P < 0.0001$) (Figure 3B).

DISCUSSION

It is known that liver fibrosis is the strongest prognostic factor of chronic liver disease and liver biopsy is now recognized as the best method for evaluating this condition[22], although it has problems such as complications. Several risk factors for HCC occurrence or recurrence have been reported, such as age, sex[1], serum albumin level[23,24], AFP level[25], and high transaminase[25]. Our study showed almost the same results as these previous reports. In particular, the progression of fibrosis may increase the risk of HCC incidence, so it is very important to determine the stage of liver damage[26,27]. Various methods have been reported for the evaluation of liver fibrosis and have been divided into two groups: ultrasonographic methods[15,17,28,29] and others[16,18]. Although gadolinium ethoxybenzyl diethylene triamine pentaacetic acid (Gd-EOB-DTPA)-enhanced MRI is one of the most

sensitive methods to detect HCC development, it is also a very important method to evaluate liver fibrosis as a noninvasive investigation[19,30]. We used Gd-EOB-DTPA-enhanced MRI and the LI:EOB-MRI index = (post-liver intensity/post-disc intensity)/(pre-liver intensity/pre-disc intensity) because it has the highest accuracy of all of the calculation methods using EOB-MRI[18]. In this study, we used the 20-min hepatobiliary phase because many institutions accept the hepatobiliary phase as being 20 min after injection and it has also been accepted by consensus of the International Forum for Liver MRI[31]. In our previous study, the data between 20 and 25 min showed no significant difference (data not shown).

LI constantly decreased as the fibrosis stage progressed to a higher stage, but many values overlapped between close fibrous stages, so we decided that the best cut-off point was 1.46 on the ROC curve by calculating the accuracy value and likelihood ratio. Using this cut-off value, LI < 1.46 always showed a high risk, with both low and high risks for several other factors, showing that lower LI is a strong independent risk factor and can complement other risk factors. LI may reflect not only the fibrosis stage but also functional aspects of the liver because it is decided by various factors, such as decreased hepatocytes, deficient hepatocyte function, and indocyanine green clearance[32-34]. The uptake and excretion of gadoxetate disodium are carried out by the anion-transporting polypeptides Oatp1 and Mrp2[35]. The balance of these effects may regulate the signal intensity of liver parenchyma in the hepatobiliary phase followed by a decrease of its signal upon hepatic damage or deteriorating cirrhosis[36-38]. Viewed from this perspective, LI could be an outstanding predictor that reflects the occurrence of HCC and prognosis, in comparison to other methods that can assess only fibrosis.

In the present study, two patients developed HCC in the higher-LI group. According to their clinical data, both had significant splenomegaly and varices, and their actual pathology obtained from surgery was F4. OATP1B1/1B3 are hepatocyte-specific transporters determining the uptake of Gd-EOB-DTPA during MR, and genetic polymorphisms of their polypeptides might influence hepatic enhancement[39], but their actual influence is relatively small and the intensity in the second case was extremely high, so it was thought to be difficult to explain this discrepancy completely. In particular, one of the two patients achieved SVR during observation but developed HCC. AFP of the two patients did not change even when HCC developed. The occurrence of HCC after IFN therapy is a rare but important problem, as some studies have reported recently[40,41]. Chang et al[40] advocated calculating the HCC prediction score after IFN therapy and, using this method, the score of our case was 5 in the so-called medium-risk group. Because the AUROC value of Fib-4 is as high as that of LI, the cut-off value (4.0) of Fib-4 was determined because the highest accuracy (0.669) was obtained, with sensitivity: 80.9%, specificity:

64.5%, positive predictive value: 28.3%, and negative predictive value: 95.1%. Using this cut-off value, 4 patients developed HCC at < 4. Interestingly, although LI and Fib-4 have similar ROC, there are relatively weak correlations between these two methods (Pearson, $r = -0.303$, $P = 0.0002$), so it is thought that they complement each other. Our two cases in which HCC development initially could not be predicted were finally predicted using Fib-4. Therefore, a combination of these two methods is a better predictive method than using a single predictive method as they complement each other and, in addition to information on clinical advanced liver fibrosis, such as low Plt, splenomegaly, and the existence of obvious varices, they will enable more accurate prediction of HCC progression.

Methods such as LI using EOB-MRI and transient elastography may be strong predictors of the HCC occurrence risk. Fibroscan is more cost-effective than MRI, but the equipment is very expensive and is restricted for use in specific hospitals because it can be used only to evaluate tissue elasticity and is ineffective in patients who are obese or have ascites. On the other hand, MRI can evaluate patients who have ascites and/or are obese and is used in many general hospitals, so it is a widely available method.

Our study revealed that the EOB-MRI index is associated with the risk of HCC occurrence in hepatitis C patients and may become a substitute for liver biopsy when evaluating the risk in these patients, even when their condition is not appropriate for other noninvasive methods, and the combination of EOB-MRI index and Fib-4 may become a better predictive method of HCC progression.

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COMMENTS

Background
The major cause of cirrhosis is chronic hepatitis C and it is well known that the risk of occurrence of hepatocellular carcinoma increases as fibrosis progresses. Therefore, it is important to reveal the fibrosis stages of outpatients.

Research frontiers
The gold standard test to investigate the fibrous stage of the liver is needle biopsy, but it is potentially harmful, so other noninvasive methods are needed and several have been reported.

Innovations and breakthroughs
This method can predict the hepatocellular carcinoma (HCC) incidence noninvasively and has the advantage of being suitable for some individuals for whom other methods are unavailable due to several factors, such as the presence of ascites.

Applications
This method uses pictures that are typically obtained to detect HCC in outpatients, so it does not require the preparation of special equipment.

Peer review
The authors evaluated the development of HCC in hepatitis C virus patients using ethoxibenzyl-magnetic resonance imaging (EOM-MRI), and observed that EOM-MRI is a highly sensitive and predictive method. This study was well performed, and the manuscript is overall well written and easy to understand.

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