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ABOUT COVER

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REVIEW

Clinical implications, diagnosis, and management of diabetes in patients with chronic liver diseases

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Abstract

Diabetes mellitus (DM) negatively affects the development and progression of chronic liver diseases (CLD) of various etiologies. Concurrent DM and CLD are also associated with worse clinical outcomes with respect to mortality, the occurrence of hepatic decompensation, and the development of hepatocellular carcinoma (HCC). Unfortunately, early diagnosis and optimal treatment of DM can be challenging, due to the lack of established clinical guidelines as well as the medical complexity of this patient population. We conducted an exploratory review of relevant literature to provide an up-to-date review for internists and hepatologists caring for this patient population. We reviewed the epidemiological and pathophysiological associations between DM and CLD, the impact of insulin resistance on the progression and manifestations of CLD, the pathogenesis of hepatogenic diabetes, as well as the practical challenges in diagnosis and monitoring of DM in this patient population. We also reviewed the latest clinical evidence on various pharmacological antihyperglycemic therapies with an emphasis on liver disease-related clinical outcomes. Finally, we proposed an algorithm for managing DM in patients with CLD and discussed the clinical and research questions that remain to be addressed.

Key Words: End stage liver disease; Diabetes mellitus; Liver cirrhosis; Insulin resistance; Non-alcoholic fatty liver disease; Liver diseases

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Core Tip: Diabetes is an independent risk factor for the development and progression of chronic liver disease (CLD) of various etiologies. Concurrent diabetes and CLD predict worse clinical outcomes, including hepatic decompensation, hepatocellular carcinoma (HCC), and complications following liver transplantation. Traditional glycemic markers, including fasting glucose, oral glucose tolerance test, and hemoglobin A1c, are not accurate in patients with severe CLD. Metformin and α-glucosidase inhibitors are associated with significant benefits beyond glycemic control, including reductions in HCC risk and incidence of hepatic encephalopathy. Glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors may exert a hepatic protective effect irrespective of the degree of glycemic control.

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INTRODUCTION

The liver plays a major role in maintaining blood glucose homeostasis, including glycogenesis and lipogenesis under feeding conditions as well as glycogenolysis and gluconeogenesis under fasting conditions. The liver is also the primary site of insulin clearance. Not surprisingly, diabetes mellitus (DM), a metabolic disease characterized by impaired blood glucose regulation and altered insulin sensitivity, is strongly associated with the development, progression, and consequence of chronic liver diseases (CLD), as illustrated in Figure 1.

DIABETES AND CHRONIC LIVER DISEASES

Insulin resistance, occurring in the context of metabolic syndrome, is a well-established independent pathophysiological driver for the development of non-alcoholic fatty liver disease (NAFLD)[1]. An early study on non-obese patients revealed that fasting insulin level and index of insulin resistance were nearly double in those with NAFLD compared to healthy controls[2]. Subsequent cross-sectional studies have shown that as much as 69%-87% of patients with type 2 DM (T2DM) have evidence of NAFLD on imaging or histology[3,4], while 21%-45% of patients with NAFLD were also found to have DM[5]. It is generally accepted that the presence of T2DM increases the risk of developing NAFLD by 2-5 folds[6]. Indeed the most recent practice guidelines from the American Diabetes Association (ADA) now make specific recommendations regarding screening patients with prediabetes or T2DM for NAFLD[7], representing a step up from prior guidelines published by the American Association for the Study of Liver Diseases (AASLD) and the European Association for the Study of the Liver (EASL), which only included a qualified recommendation about screening high-risk patients due to significant gaps in knowledge concerning the treatment of NAFLD at the time[8,9].

The impact of insulin resistance and DM holds true for other etiologies of CLD as well. Insulin resistance, for example, was found to be an independent predictor of fibrosis in non-diabetic patients with chronic hepatitis C virus (HCV) infection[10]. Hyperinsulinemia and hyperglycemia have also been associated with the development of more severe fibrosis in patients with chronic HCV infection[11,12]. A recent Taiwanese nationwide cohort study has further identified new-onset DM as an independent predictor for cirrhosis and decompensation in patients with chronic hepatitis B virus (HBV) or chronic HCV[13,14]. In alcoholic liver disease (ALD), BMI and fasting blood glucose levels have been demonstrated to correlate with fibrosis score after adjusting for daily alcohol intake and the total duration of alcohol use[15].

The relationship between DM and HCV infection is particularly noteworthy because of its possible two-way association. There is epidemiological evidence to suggest that diabetic patients are nearly 3.5 folds more likely to acquire HCV infection[16]. Conversely, HCV infection is associated with a 1.5-1.7 fold excess risk of new-onset T2DM development[17,18]. Although the exact mechanisms of action remain elusive, it is

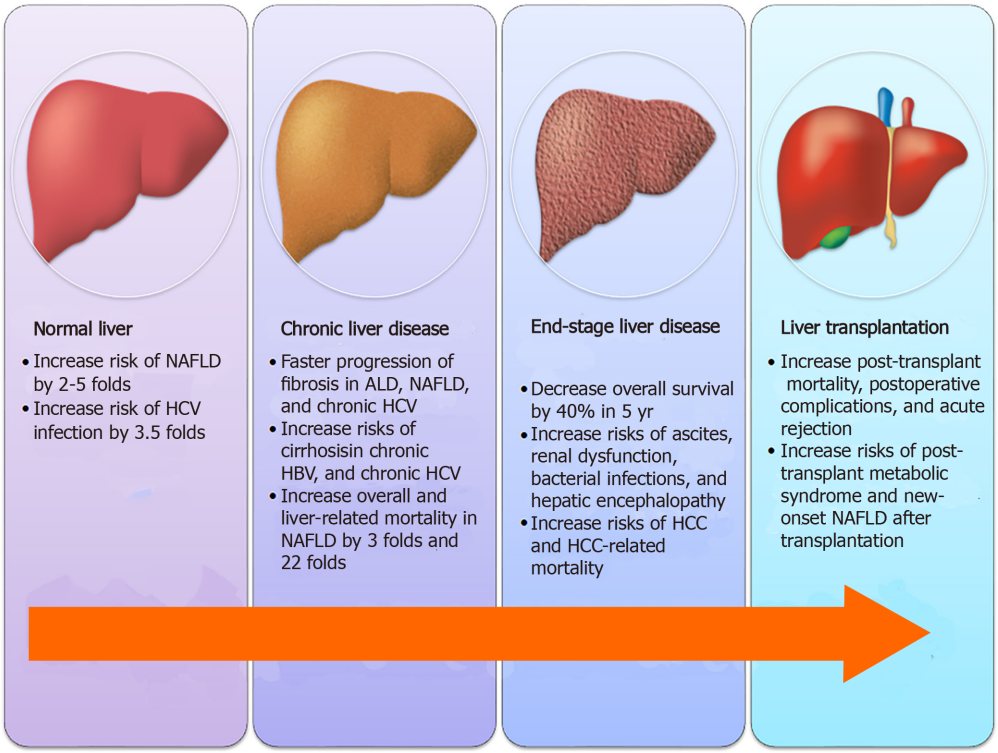

Figure 1 Impact of diabetes on various stages of chronic liver diseases. NAFLD: Non-alcoholic fatty liver disease; HCV: Hepatitis C virus; ALD: Alcoholic liver disease; HCC: Hepatocellular carcinoma.

postulated that the metabolic disturbances associated with DM may favor HCV survival as evidenced by reports that insulin resistance positively correlates with HCV viral load[10]. Meanwhile, insulin resistance and DM are also increasingly being recognized as metabolic extrahepatic manifestations of chronic HCV infection. HCV may exert a direct cytopathic effect on pancreatic islets and induce β-cell death *via* a caspase 3-dependent pathway[19,20]. In addition, HCV core protein has been shown to promote ubiquitination of insulin receptor substrate (IRS)-1 and -2 in transfected hepatoma cells and a transgenic mouse model[21,22], thereby interrupting hepatic insulin signaling resulting in hepatic insulin resistance. Finally, dipeptidyl peptidase-4 (DPP-4) expression was found to be upregulated by HCV nonstructural protein while interferon therapy led to decrease serum DPP-4 activity in HCV-infected patients[23,24], providing support for another mechanism of HCV-induced insulin resistance *via* the glucagon-like peptide-1 (GLP-1) pathway[25].

DIABETES AND END-STAGE LIVER DISEASES

Once CLD progresses to end-stage liver disease (ESLD), the presence of DM continues to predict more severe diseases and worse clinical outcomes. On the one hand, the prevalence of DM positively correlates with the severity of liver disease based on the Child-Pugh (CP) class or Model for End-Stage Liver Disease (MELD) score[26,27]. On the other hand, multiple prospective studies on patients with compensated/decompensated cirrhosis of various etiologies have consistently demonstrated lower survival, as much as a 40% reduction in 5 years, in the diabetic group compared to the non-diabetic group[28-30]. There is evidence that even the presence of subclinical impaired glucose tolerance (IGT) predicts worse short-term and long-term survival in cirrhotic patients[29,31,32]. It is noteworthy that the higher mortality in these diabetic patients is due not to the classical DM-related micro-/macrovascular diseases, but complications of liver failure[33]. Clinically, baseline DM status was shown to be independently associated with the development of ascites, renal dysfunction, and bacterial infections during long-term follow-up in a cohort of cirrhotic patients with chronic HCV infection[34]. DM is also associated with more severe hepatic encephalopathy in cirrhotic patients independent of the severity of liver disease[35].

Most importantly, the incidence of hepatocellular carcinoma (HCC) and HCC-

specific mortality are nearly double in patients with preexisting DM based on a meta-analysis of 28 prospective epidemiological studies[36]. The risk for HCC is further increased by up to 10 folds when combined with alcohol consumption and/or viral hepatitis[37,38]. The impact of DM on the risk for HCC is particularly dramatic in patients with certain single nucleotide polymorphisms in the patatin-like phospholipase domain containing 3 (PNPLA3) gene, which was known to increase the risk for HCC in alcoholic cirrhosis[39]. A case-control study of nearly 500 HCC patients revealed an adjusted odds ratio of 19.11 for diabetic patients with the GG genotype compared to that of 2.65 for non-diabetic controls with the same genotype[40]. Remarkably, cirrhosis is only present in 50% of patients with NAFLD-related HCC[41], suggesting that the mechanism of hepatocarcinogenesis in the context of metabolic syndrome may be different from the classical mechanism involved in cirrhosis. It is postulated that hyperinsulinemia is central to the pathophysiological effects of DM on HCC development by upregulating growth signaling pathways[42], promoting an inflammatory milieu[43], activating hepatic progenitor cells[44], and stimulating angiogenesis[45].

Regardless of the etiology of underlying CLD, the diagnosis of HCC carries a poor prognosis with median survival measured in the order of months[46]. A recent meta-analysis of over 9000 HCC cases concluded that DM status is associated with lower overall/disease-free survival in patients receiving hepatic resection as well as lower overall survival in patients receiving non-surgical treatments[47]. This reduction in overall survival may partly be attributable to an increased risk of postoperative complications, including hepatic decompensation, intractable ascites, and intraperitoneal infection, as demonstrated by several regression studies on HCC patients undergoing hepatic resection[48,49]. Similarly, there is evidence that diabetic patients undergoing transarterial chemoembolization (TACE) are at an increased risk for hepatic decompensation, liver abscess formation, and prolonged acute renal failure[50,51].

HEPATOGENIC DIABETES

It is worth noting that not only is DM a risk factor for ESLD but it can also be a complication of ESLD. Hepatogenic diabetes is characterized by the development of hyperinsulinemia, insulin resistance, and β-cell dysfunction after the onset of cirrhosis, often in the absence of traditional risk factors, such as a family history of DM or obesity. As illustrated in Figure 2, it is postulated that hyperinsulinemia occurs in cirrhosis as a result of decreased insulin clearance by the damaged liver and increased portosystemic shunting. This notion is supported by evidence that hyperinsulinemia worsens after transjugular intrahepatic portosystemic shunt (TIPS) placement in diabetic patients[52], yet improves with portosystemic shunt occlusion by balloon-occluded retrograde transvenous obliteration (BRTO)[53]. There is also evidence that cirrhotic patients may develop exaggerated β-cell responsiveness to glucose as well as pancreatic islet hypertrophy, resulting in increased insulin secretion[54].

Sustained hyperinsulinemia can, by means of an array of negative feedback mechanisms[55-57], give rise to insulin resistance in peripheral adipose tissues and skeletal muscles, resulting in reduced insulin-stimulated non-oxidative glucose disposal[58]. The hyperglycemic effect of reduced glucose disposal can be further amplified by cirrhosis-induced sarcopenia and impaired glucose effectiveness[59]. Moreover, certain etiologies of CLD may exert additional disease-specific mechanisms of insulin resistance, in a similar fashion to HCV-induced DM[21]. Chronic alcohol exposure, for instance, may inhibit insulin signaling pathways by altering intracellular calcium level within hepatocytes[60], resulting in impaired insulin-mediated suppression of hepatic glucose production[61]. Finally, there is evidence that hepatic expression of DPP-4 is increased and GLP-1 is decreased in both chronic HCV and NAFLD[25,62,63], resulting in hepatic insulin resistance and IGT.

As in the pathogenesis of T2DM, the development of pancreatic β-cell dysfunction signals the body's diminishing capacity to compensate for worsening insulin resistance and marks the progression from IGT to frank DM[27]. Similar to the development of insulin resistance, a number of disease-specific mechanisms of β-cell dysfunction may be operating even before the onset of cirrhosis. Chronic alcohol consumption and hereditary hemochromatosis, for instance, cause down-regulation of glucokinase and increased oxidative stress, respectively, leading to increased β-cell apoptosis and decreased glucose-induced insulin secretion[64,65]. In NAFLD, the combination of chronic hyperglycemia and elevated free fatty acid levels lead to

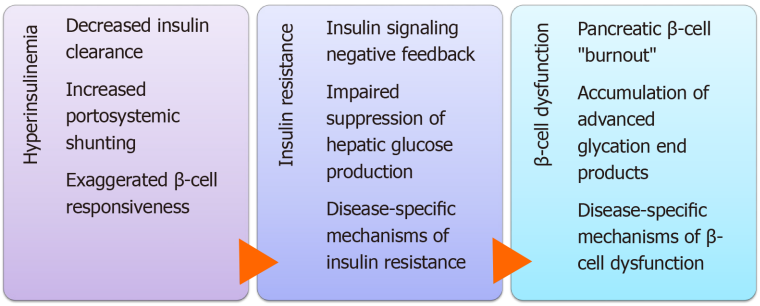

Figure 2 Mechanisms of action of hepatogenic diabetes.

dysfunction and death of pancreatic β-cells by glucolipotoxicity[66]. Meanwhile, chronic HCV infection is thought to induce pancreatic islet destruction *via* a combination of autoimmune-mediated and direct cytopathic mechanisms as abovementioned[19,20,67]. Eventually, the development of cirrhosis and hepatic decompensation further exacerbates β-cell dysfunction due to the accumulation of advanced glycation end products[68], which can inhibit glucose-stimulated insulin secretion and induce apoptosis of β-cells[69,70].

DIABETES AND LIVER TRANSPLANTATION

In many cases, orthotopic liver transplantation (OLT) remains the only curative option for patients with ESLD. Given the pathogenic effects of insulin resistance on oxidative and inflammatory stresses, it is not surprising that multiple large-scale retrospective studies have repeatedly demonstrated pre-transplant DM to be associated with decreased post-transplant survival, independent of BMI or MELD score[71-73]. Pre-transplant DM is also an independent predictor for postoperative complications, including cardiovascular events, infections, renal dysfunction, and acute graft rejection[71]. There is even evidence that the donor's history of DM correlates with increased recipient's mortality as well as increased risk of graft failure[73], suggesting that DM can exert biologically-significant damages to the liver without causing clinically-detectable hepatic dysfunction.

Similarly, the presence of post-transplant DM, which occurs in about 30% of patients undergoing OLT[74], has been linked to increased mortality as well as a higher incidence of postoperative complications and acute rejection[73,75,76]. Although preexisting overt DM may disappear in more than half of the patients after OLT[77], pre-transplant DM remains one of the most important predictors for post-transplant DM[78]. This observation is consistent with the findings that peripheral insulin resistance is improved, but not completely normalized, after OLT[79]. Other recognized risk factors for new-onset DM after transplantation, which affects 7%-18% of OLT patients[77,80], include male gender[78,80], use of tacrolimus[80,81], and HCV infection[78,81]. Besides DM, metabolic syndrome and its other individual components, namely hypertension, dyslipidemia, and obesity, are also found to be highly prevalent post-transplant[82]. Not only does it increases the incidence of cardiovascular events[83], but the presence of post-transplant metabolic syndrome may also contribute to the development of new-onset NAFLD after transplantation for non-NAFLD cirrhosis[84]. While there have been case reports of combined liver and islet transplantation in cirrhotic patients with concurrent type 1 DM[85], the safety and utility of this technique in patients with hepatogenic diabetes or T2DM is yet to be determined[86].

TRADITIONAL GLYCEMIC MARKERS FOR PATIENTS WITH LIVER DISEASES

Given the abovementioned detrimental effects of DM on the development and progression of CLD as well as the manifestation and management of ESLD, it is reasonable to hypothesize that early diagnosis and optimal treatment of DM in patients with CLD would be beneficial. Nonetheless, IGT and DM were identified in as much as 38.5% and 19.2%, respectively, of patients with normal fasting plasma glucose

(FPG) and compensated cirrhosis of various etiologies in a small prospective study[87], which alluded to the practical challenges of managing DM in this patient population. The first challenge is to establish an accurate diagnosis and to assess disease severity. As summarized in Figure 3, the utility and accuracy of most glycemic markers are restricted in patients with CLD.

Fasting plasma glucose

FPG is a simple and inexpensive test for diagnosing DM but its widespread adoption is limited by the need for prolonged fasting and stringent processing requirements[88]. Although the test offers a reasonable intra-individual reproducibility[89], FPG can be affected by stress, acute illness, and alcohol consumption. Moreover, the optimal FPG threshold for diagnosing DM in patients with CLD/ESLD remains controversial. Certain studies have reported that using thresholds of 100-125 mg/dL and ≥ 126 mg/dL, the sensitivities of FPG in detecting prediabetes and DM in high-risk populations were as low as 28.9% and 55.7%, respectively[90,91]. In a study on patients with HCV cirrhosis, 21% of patients with FPG < 110 mg/dL met the diagnostic criteria for DM based on oral glucose tolerance test (OGTT) and regression analysis showed that a threshold of 107 mg/dL, instead of 126 mg/dL should be used for diagnosing DM in these patients[92]. It is unclear if the same threshold can be applied to patients with other etiologies or severities of liver diseases.

Oral glucose tolerance test

OGTT is often considered the gold standard for diagnosing DM. It is the preferred method for detecting gestational DM, cystic fibrosis-related DM, and post-transplant DM[93]. Furthermore, it is the only way to formally diagnose IGT, which may be particularly relevant to cirrhotic patients because of the implications on short-term and long-term prognosis as abovementioned[29,31,32]. The major drawbacks of OGTT are the need for prolonged fasting as well as the length and complexity of the test, which may contribute to the poor reproducibility of OGTT in clinical practice[94]. Most importantly, OGTT cannot be used to assess disease severity or treatment effectiveness because of practical limitations and the lack of established OGTT-based glycemic targets.

Hemoglobin A1c

Since it was first proposed as a measure of glucose intolerance in 1976[95], glycated hemoglobin (A1c) has evolved into one of the most broadly deployed tests for diagnosing and monitoring DM. Its major advantages are the absence of a fasting requirement and the relative ease of sample handling. A1c is particularly useful as a marker for treatment effectiveness because it reflects average blood glucose over a period of months instead of a single point in time. While it remains a useful glycemic marker in most patients with mild liver diseases, the accuracy and validity of A1c in patients with advanced liver diseases remained controversial. A small study involving 15 patients with compensated cirrhosis found 40% of subjects to have A1c results below the non-diabetic reference range[96]. Another retrospective study on a cohort decompensated cirrhotic patients undergoing liver transplantation evaluation revealed a similar discordance between measured A1c and average blood glucose[97]. The poor diagnostic performance of A1c is attributable to the well-described curvilinear correlation between A1c and erythrocyte turnover[98], which can occur in patients with CLD/ESLD as a result of hemorrhage related to portal hypertension and coagulopathy, hemolysis caused by splenomegaly as well as impaired erythropoiesis due to marrow suppression and nutritional deficiency[99]. A1c value can also be affected unpredictably by blood transfusion[100,101]. More importantly, the inherent biochemical characteristics of the glycation process limit the ability of A1c to capture the excessive blood glucose lability exhibited by patients with CLD, as demonstrated in a recent study that compared A1c against continuous glucose monitoring in diabetic patients with cirrhosis[102]. This shortcoming may have crucial clinical implications since glycemic variability was shown to be an independent predictor for fibrosis in NAFLD as well as cardiovascular disease and all-cause mortality in the general population[103,104]. Finally, there is evidence that alcohol consumption is independently associated with lower A1c values, even adjusting for confounding factors such as FPG, obesity, and anemia[105-107], likely as a result of an increase in the size of the hemoglobin A1 fraction[108].

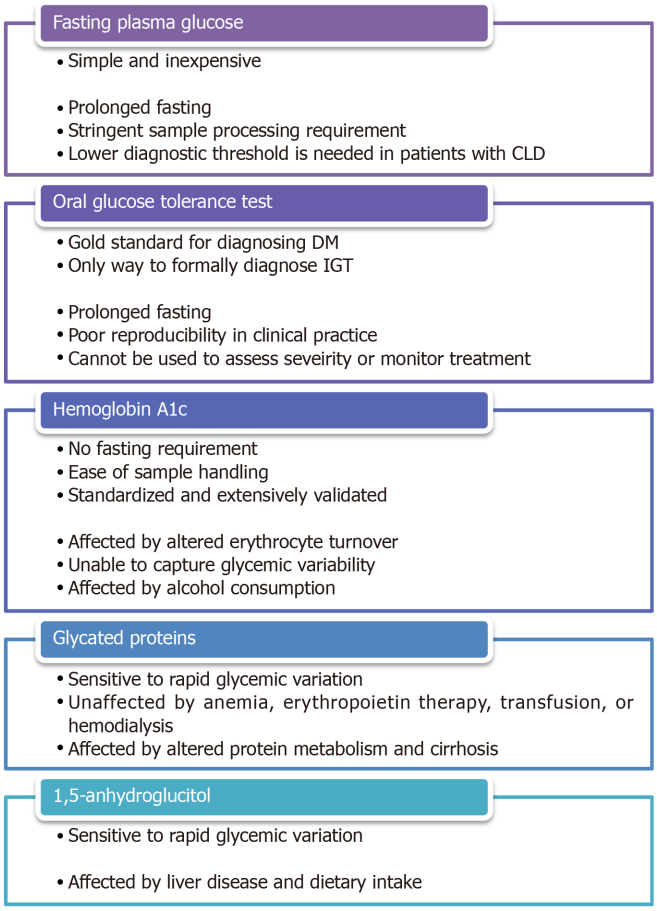

Figure 3 Pros and cons of various glycemic markers. CLD: Chronic liver diseases; DM: Diabetes mellitus; IGT: Impaired glucose tolerance.

NON-TRADITIONAL GLYCEMIC MARKERS FOR PATIENTS WITH LIVER DISEASES

Glycated proteins

Glycated albumin (GA) and fructosamine are ketoamines that are formed by non-enzymatic glycation of glucose to serum proteins in a similar fashion to the glycation of hemoglobin. Due to the shorter half-life of albumin and other serum proteins, the faster rate of albumin glycation compared to hemoglobin glycation, and the direct exposure of serum proteins to serum glucose, GA and fructosamine are typically taken to reflect glycemic control over 2-3 wk[109,110]. GA has proven to be a particularly useful glycemic marker in patients with chronic kidney disease and/or on dialysis because, unlike A1c, GA level is not affected by anemia, erythropoietin therapy, blood transfusion, or hemodialysis[111,112]. Not surprisingly, the accuracy of GA and fructosamine is negatively impacted by disease states that affect protein metabolisms. Indeed, an older study on a small cohort of cirrhotic patients demonstrated an inverse correlation between fructosamine levels and serum albumin levels [113]. A more recent study comparing fructosamine against continual glucose monitoring confirmed the poor association in diabetic patients with cirrhosis[102]. Several entities, including albumin-corrected fructosamine, total protein-corrected fructosamine, and CLD-A1c, have been proposed in an attempt to improve the diagnostic performance of GA and fructosamine by correcting for variations in serum protein concentrations[114,115]. Unfortunately, the validity of these entities outside the study populations has not been externally or prospectively verified.

Serum 1,5-anhydroglucitol

Serum 1,5-anhydroglucitol (1,5-AG) is a dietary monosaccharide that is normally reabsorbed by the proximal renal tubules, but its reabsorption is competitively inhibited by glucosuria in the setting of hyperglycemia. Serum 1,5-AG was found to be a sensitive day-to-day glycemic marker in diabetic patients as well as a sensitive diagnostic test in those at high risk of DM[116,117]. Nonetheless, there is evidence that 1,5-

AG metabolism is affected in patients with liver diseases[118,119]. There is also a concern that dietary intake may affect serum 1,5-AG level[120], which is particularly relevant to the cirrhotic population due to the high prevalence of malnutrition.

MANAGEMENT OF DIABETES IN PATIENTS WITH LIVER DISEASES

The second challenge in the management of DM in patients with liver diseases is to identify a safe and effective treatment strategy for this medically complicated population, especially those with decompensated cirrhosis. It is well accepted that basic lifestyle interventions, such as healthful diet, physical activity, alcohol/smoking cessation as well as psychosocial support are fundamental to the medical management of both DM and liver diseases. Nonetheless, antihyperglycemic medications are often needed when patients fail to achieve targeted glycemic control through lifestyle interventions alone. Attention must be paid to consider the unique mechanisms of action, the side effect profiles, and the implications on liver diseases associated with the use of these medications, as summarized in Figure 4. An up-to-date summary, with a focus on liver-disease related outcomes, of the major clinical trials involving these medications is provided in Supplementary Table 1.

Metformin

Metformin, which is often considered first-line oral therapy in T2DM due to its favorable safety profile and cardioprotective effects[121], has been studied in both diabetic and non-diabetic patients with biopsy-proven NAFLD[122-128]. Despite the limitations of these small, open-label, or non-randomized published trials, the overall evidence indicates that metformin use is associated with improvement in insulin resistance, aminotransferase levels, and liver morphology[129]. Furthermore, several large retrospective studies on diabetic patients with CLD of various etiologies have demonstrated a 50%-70% reduction in HCC risk among those treated with metformin[130-132], with one recent case-control study showing a dose-dependent association[133]. Similar results, including a reduction in 5-year HCC incidence, liver-related mortality, and transplantation, were obtained in a prospective study involving specifically diabetic patients with HCV cirrhosis[134]. Additionally, metformin has been shown to reduce the incidence of overt hepatic encephalopathy by 8 folds through inhibition of glutaminase activity[135]. Most strikingly, a retrospective study on 250 diabetic patients with cirrhosis of various etiologies revealed that the continuation of metformin after cirrhosis diagnosis nearly doubles the overall median survival across all Child-Pugh classes[136].

Despite its remarkable morbidity and mortality benefits, metformin is often withheld from patients with liver diseases due to an exaggerated concern for metformin-associated lactic acidosis (MALA). It is worth noting, however, that metformin is not intrinsically hepatotoxic and can be safely administered even in the context of mildly abnormal transaminases[137]. In fact, animal studies have revealed a protective potential of metformin against acetaminophen-induced hepatotoxicity[138,139]. Additionally, MALA is an exceedingly rare condition with an estimated incidence of < 10 per 100000 patient-years of exposure in patients without significant renal impairment[140]. A recent retrospective study of over 132000 patients with newly diagnosed T2DM found no significant difference in the risk of lactic acidosis in patients prescribed metformin compared to those prescribed other antihyperglycemic medications or no medication[141]. This is in contrast to its predecessor, phenformin, which carries a significantly higher risk of lactic acidosis even at therapeutic drug levels[142]. Finally, it is important to bear in mind that although metformin can increase lactate production by promoting intestinal glucose utilization *via* the anaerobic pathway as well as inhibiting hepatic lactate uptake and gluconeogenesis[143,144], lactate is not toxic in itself and hyperlactatemia does not directly cause the clinical condition of lactic acidosis[145].

Pioglitazone

The thiazolidinedione pioglitazone, an insulin-sensitizing peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist, is another extensively studied antihyperglycemic agent that shows promise in the treatment of nonalcoholic steatohepatitis (NASH). A small pilot study on non-diabetic patients with biopsy-proven NASH resulted in normalization of aminotransferase levels in 72% of patients as well as improvement in histological features of hepatic steatosis, hepatocellular injury, parenchymal inflammation, and fibrosis in 67% of patients after 48 wk of

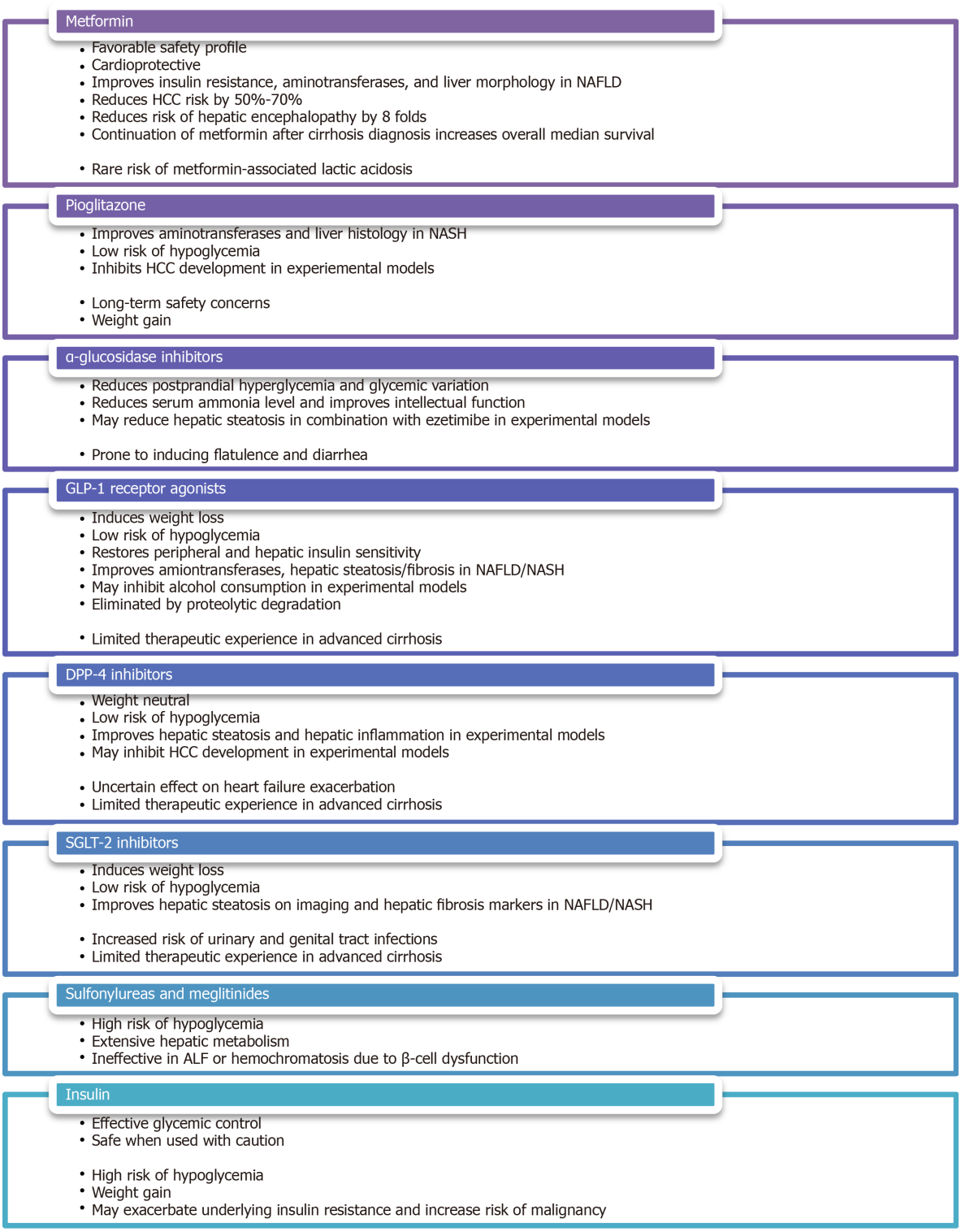

Figure 4 Pros and cons of various antihyperglycemic medications. NAFLD: Non-alcoholic fatty liver disease; HCC: Hepatocellular carcinoma; NASH: Nonalcoholic steatohepatitis.

treatment[146]. Similar results except for a decrease in fibrosis score, which failed to achieve statistical significance in two studies[147,148], were replicated in subsequent randomized, placebo-controlled trials of various study designs involving both diabetic and non-diabetic patients[147-150]. It is interesting to note that although its beneficial effects are greater in patients with T2DM, pioglitazone use is associated with a reduction in NAFLD activity score and resolution of NASH in 46% and 26%, respectively, of patients without frank DM[151]. Given its low risk of inducing hypoglycemia, pioglitazone may be uniquely suited in the treatment of selected NASH patients with normoglycemia at baseline. Finally, there is limited *in vitro* and *in vivo*

data suggesting that pioglitazone may inhibit HCC development[152,153], but these findings have not been confirmed in human studies[154].

Lingering concerns regarding the long-term safety of thiazolidinedione therapy stemmed from potential troglitazone-related hepatotoxicity, rosiglitazone-related cardiovascular risks, and pioglitazone-related bladder cancer[155-157], remain a barrier to the widespread use of pioglitazone in clinical practice. This is particularly relevant in the face of findings that long-term rosiglitazone or pioglitazone therapy does not impart additional improvement in metabolic or hepatic parameters compared to short-term therapy[150,158]. A recently published expert panel statement, however, does recommend the use of pioglitazone, either alone or in combination with a statin or ezetimibe, for the primary or secondary prevention of cardiovascular disease as well as the avoidance of cirrhosis, liver transplantation, or HCC in patients with NAFLD/NASH[159]. Another barrier to the use of pioglitazone is its propensity to cause significant weight gain, which may be dose-dependent[160], as demonstrated by multiple systematic reviews and meta-analyses[161,162]. Finally, other than a small pilot study that showed a reduction in hepatic steatosis on imaging in patients with human immunodeficiency virus (HIV)/HCV coinfection[163], the use of pioglitazone in other etiologies of CLD has not been thoroughly investigated.

α-glucosidase inhibitors

Although α-glucosidase inhibitors, such as acarbose, voglibose, and miglitol, enjoy limited adoption amongst the general diabetic population due to its gastrointestinal side effects of flatulence and diarrhea, they deserved to be recognized as a pluripotent antihyperglycemic agent in patients with CLD. A randomized, placebo-controlled trial of acarbose in diabetic patients with compensated cirrhosis, who are prone to postprandial hyperglycemia partly due to decreased hepatic glucose uptake and impaired glycogenesis[164], demonstrated a significant reduction in fasting glycemia, postprandial glycemia, mean glycemic variation, and A1c in the treatment group[165]. By favoring saccharolytic, instead of proteolytic, intestinal bacterial flora, acarbose treatment has also been shown to reduce serum ammonia level as well as to improve intellectual function in a randomized, placebo-controlled trial, crossover study[166], making it an effective regimen for the consolidated treatment of hyperglycemia and mild hepatic encephalopathy. In a small pilot study of diabetic patients with biopsy-confirmed NASH, miglitol was found to reduce aminotransferase levels, hepatic steatosis, and histological inflammation after 12 months of therapy[167]. Moreover, voglibose was found to be effective in minimizing pioglitazone-induced weight gain in the general diabetic population[168]. It is important to note that although there are rare case reports of acarbose-related acute hepatitis[169], the medication is generally felt to be safe in patients with CLD/ESLD since it is exclusively metabolized in the gastrointestinal tract and has a very low systemic bioavailability[170]. This notion is supported by a Taiwanese nationwide retrospective study, which discovered no increased risk of liver injury amongst diabetic patients with severe renal insufficiency regardless of the presence or absence of CLD[171].

GLP-1 receptor agonists

GLP-1 receptor agonists, such as exenatide and liraglutide, constitute an increasingly popular class of incretin-based therapy for the treatment of T2DM thanks to their ability to induce weight loss and their lower risk of hypoglycemia. The observations that glucose-induced GLP-1 secretion and hepatic GLP-1 receptor expression are deficient in NAFLD patients suggest GLP-1 agonism may exert a direct effect on hepatocytes and may play a role in the treatment of NAFLD[62,172,173]. Several small open-label studies have demonstrated reductions in hepatic steatosis, aminotransferase levels, and liver fibrosis score after treatment with liraglutide in diabetic patients with imaging findings of hepatic steatosis[174-176]. These findings were further supported by a Japanese single-arm, open-label study, and a British double-blinded, randomized, placebo-controlled trial of liraglutide on patients with biopsy-proven NASH. The former showed significant improvement in histological inflammation and fibrosis in 60%-70% of subjects after 96 wk of therapy[177], while the latter showed histological resolution of NASH in 39% of subjects after 48 wk of therapy[178]. A reduction in hepatic triglyceride content was also observed in a small, open-label, randomized study of exenatide in diabetic patients with imaging findings suggestive of NAFLD[179]. With regard to other etiologies of CLD, GLP-1 receptor agonists were found to exhibit an inhibitory effect on alcohol consumption in animal models[180,181]. It has also been reported that fasting serum GLP-1 levels were decreased in patients with chronic HCV, but not those with chronic HBV[25]. It is worth noting, however, that patients with HBV-, HCV-, or alcohol-related liver diseases were

excluded from the original Liraglutide Effect and Action in Diabetes trials[182]. The effects of GLP-1 agonism on other liver disease-related clinical outcomes, such as encephalopathy and HCC development, have yet to be thoroughly evaluated. Since GLP-1 receptor agonists are eliminated by proteolytic degradation and glomerular filtration, no dosage adjustment is necessary for patients with any degree of hepatic impairment[183]. Caution is advised in patients with advanced cirrhosis given limited therapeutic experiences in this vulnerable population.

DPP-4 inhibitors

DPP-4 is a ubiquitously expressed membrane-bound and circulating glycoprotein that acts enzymatically on a variety of substrates, including GLP-1, gastric inhibitory polypeptide (GIP), insulin-like growth factor-1 (IGF-1), neuropeptide Y and substance P, as well as interacts non-enzymatically with many ligands, including adenosine deaminase, caveolin-1, and CD45[184]. As such, DPP-4 inhibitors, such as linagliptin, saxagliptin, sitagliptin, and vildagliptin are thought to act upstream of GLP-1 by slowing its degradation, but they may also exert diverse metabolic, immunologic, and neurologic effects *via* other GLP-1-independent pathways. Similar to GLP-1 receptor agonists, DPP-4 inhibitors are considered weight neutral and carry a low risk of hypoglycemia[185], but they have the added advantage of being oral agents. In the context of liver diseases, hepatic DPP-4 expression was found to be upregulated in patients with NAFLD while serum DPP-4 activity was reported to correlate with the histopathologic grade of NASH as well as serological markers of liver damage[186-188]. In addition, DPP-4 inhibition by specific inhibitors was demonstrated in several animal models of NAFLD to attenuate hepatic steatosis[189,190]. There were two open-label trials of sitagliptin, one reported a reduction in intrahepatic lipid content in diabetic patients with clinical NAFLD, another reported improvements in hepatic steatosis and ballooning in patients with biopsy-proven NASH irrespective of DM status[176,191]. Unfortunately, improvements in hepatic steatosis, liver fibrosis, or NAFLD activity score were not observed in other randomized, placebo-controlled trials of sitagliptin[192,193]. It is worth noting that the hepatic protective effects of DPP-4 inhibitors, which may be mediated through direct actions on hepatocytes *via* GLP-1 receptors[172], appear to occur irrespective of the degree of glycemic control[187]. Moreover, the observations in obese patients of increased expression of membrane-bound DPP-4 on visceral adipose tissue-derived dendritic cells as well as increased release of soluble DPP-4 as adipokine from visceral adipocytes support the notion that DPP-4 inhibition may play a role in modulating adipose tissue inflammation and regulating adipose tissue metabolism[194,195], likely through a GLP-1-independent pathway[196]. Finally, alteration in DPP-4 expression was observed in rat hepatoma cell lines and human HCC specimens[197,198], raising the possibility that DPP-4 activity may influence HCC development. It is prudent, however, to mention that although DPP-4 inhibitors were shown to be protective against cardiovascular diseases by improving endothelial function and reducing inflammation[199,200], a recent meta-analysis of five clinical trials revealed a slightly increased risk of hospital admission for heart failure attributable to DPP-4 inhibitor use amongst diabetic patients with existing cardiovascular risk factors[201]. Most DPP-4 inhibitors, except vildagliptin, are considered safe in patients with mild or moderate hepatic impairment, although caution is advised in patients with severe hepatic impairment due to limited therapeutic experiences[183].

Sodium-glucose cotransporter-2 inhibitors

Instead of directly altering insulin availability or insulin sensitivity, sodium-glucose cotransporter-2 (SGLT-2) inhibitors, including canagliflozin, dapagliflozin, empagliflozin, and ertugliflozin, exert their antihyperglycemic effect by blocking proximal renal tubular glucose reabsorption, thus leading to increased glucose excretion in the form of glucosuria. SGLT-2 inhibitors carry a low risk of hypoglycemia and are capable of inducing modest weight loss as well as lowering blood pressure[202]. They have been demonstrated in several animal models of NAFLD/NASH to improve hepatic steatosis and liver fibrosis[203,204]. Improvement in hepatic steatosis as well as reductions in various serological or imaging parameters of liver injury were also observed in clinical trials of several SGLT-2 inhibitors in diabetic NAFLD patients[205-211]. Most impressively, preliminary evidence indicates SGLT-2 inhibitors may slow the proliferation of hepatoma cell lines and hinder xenograft tumor growth in nude mice independent of their antihyperglycemic effect, suggesting that SGLT-2 inhibitors may attenuate HCC development *via* some unidentified mechanisms of action[212]. The use of SGLT-2 inhibitors in other etiologies of CLD has not been thoroughly investigated. Long-term safety data on the use of SGLT-2 inhibitors in

cirrhotic patients is relatively scarce given limited therapeutic experiences, but canagliflozin and ertugliflozin are generally considered safe in patients with mild or moderate hepatic impairment[213,214]. Limited data is showing that empagliflozin can be used without dosage adjustment across all degrees of hepatic impairment[215], while dose reduction may be necessary for dapagliflozin in patients with severe hepatic impairment[216].

Sulfonylureas and meglitinides

The second- and third-generation sulfonylureas, including glipizide, glyburide, gliclazide, and glimepiride, and the meglitinides, repaglinide, and nateglinide, are insulin secretagogues that stimulate insulin secretion from pancreatic β-cells. While these medications are highly effective antihyperglycemic agents capable of lowering A1c by 1%-2%[217], they also carry a high risk of hypoglycemia[218]. Patients with hepatic impairment are particularly susceptible due to reduced drug inactivation and elevated free drug concentration because both sulfonylureas and meglitinides are extensively metabolized by the liver and are tightly bound to serum proteins. The risk of hypoglycemia is further exacerbated in patients with cirrhosis as a result of impaired hepatic gluconeogenesis, reduced hepatic insulin clearance, and peripheral hyperinsulinism[219]. Alcohol consumption can predispose patients to hypoglycemia in a similar fashion by impairing gluconeogenesis and glycogenolysis[220]. Conversely, these medications may be rendered ineffective in chronic ALD or hemochromatosis due to β-cell dysfunction and apoptosis[64,65]. Most notably, several case-control studies have meta-analyses revealed an increased odds of HCC development by up to 3 folds amongst patients with T2DM treated with sulfonylureas[131,221-223], possibly as a result of hyperinsulinemia. Expert opinions advise that insulin secretagogues be avoided or used with extreme caution in patients with CLD/ESLD[224].

Insulin

Despite the ever-expanding list of antihyperglycemic medications, insulin and insulin analogs remain the safest and most effective glycemic therapy in patients with DM. They can be used, with caution, even in patients with decompensated cirrhosis or severe hepatic impairment[225,226]. Not surprisingly, hypoglycemia is a major limiting side effect. On the one hand, cirrhosis can induce or exacerbate a state of hyperinsulinemia and insulin resistance as abovementioned[219], which may increase a patient's insulin requirement. On the other hand, cirrhotic patients may exhibit an exaggerated response to insulin due to impaired hepatic gluconeogenesis and sarcopenia[227]. These opposing mechanisms of actions, together with symptoms of nausea, bloating, and poor appetite experienced by many patients with CLD, make it difficult to predict a patient's day-to-day exogenous insulin requirement. Furthermore, some cirrhotic patients, who are on β-blockers for variceal bleeding prophylaxis, may experience an impaired hormonal counterregulatory response to insulin, resulting in delayed recovery from hypoglycemia[228]. As such, expert opinions recommend the use of short-acting insulin analogs as well as frequent dose adjustment and close glucose monitoring to minimize the risk of hypoglycemia[229].

Weight gain of 3 to 9 kg associated with the initiation of insulin therapy, attributable to the intrinsic anabolic properties of insulin, the central effects of insulin on appetite and reward regulation, the non-physiological pharmacokinetics of exogenous insulin, and the behavioral changes related to fear of hypoglycemia[230], is a well-documented phenomenon amongst the general diabetic population[231]. This weight gain can be particularly detrimental to patients with metabolic syndrome as it is predominantly represented by fat mass and is likely to further exacerbate the underlying insulin resistance, oxidative stress, and systemic inflammation[232], leading to worsening metabolic diseases as well as increased risk of malignancy. Similar to the case of sulfonylureas, data from observational studies suggest an association between insulin therapy and HCC development amongst patients with T2DM[130,131]. Further studies are needed to further delineate the oncogenic risk of insulin therapy versus the cardiometabolic risk of uncontrolled hyperglycemia. Nonetheless, it may be prudent to reserve insulin therapy in patients with CLD to those who are unable to receive or inadequately managed by other antihyperglycemic medications.

GLYCEMIC TARGETS IN PATIENTS WITH LIVER DISEASES

After selecting an appropriate glycemic marker and formulating an effective antihyperglycemic therapy plan, the third challenge in the management of DM in

patients with CLD is to determine a reasonable glycemic target. For the general non-pregnant adult population, the ADA recommends an A1c goal of < 7%[233], which is largely based on several landmark studies demonstrating decreased rates of microvascular complications, such as retinopathy, neuropathy, and nephropathy, in patients undergoing intensive glycemic control[234,235]. It is important to note, however, that the higher mortality in patients with concurrent DM and CLD is predominantly attributable to complications of liver failure, not DM-related micro-/macrovascular diseases[33]. Unfortunately, except for a small cohort study on NAFLD patients showing an association between improvement in A1c and improvement in liver fibrosis on biopsy[236], the impact of glycemic control on the natural history of various CLD etiologies has not been thoroughly investigated. It is also unclear if the degree of glycemic control directly correlates with the incidence or severity of liver disease complications. Although the ADA does recommend that less stringent A1c goals be considered in selected patients with limited life expectancy, extensive comorbid conditions, advanced micro-/macrovascular complications, or difficult to manage diabetes[233], there is little data to guide the clinical decision-making process in practice. Further studies are desperately needed to help determine the optimal glycemic targets, in relation to the etiology of liver disease and the degree of decompensation, in patients with CLD.

CONCLUSION

Given the abovementioned evidence, it is clear that DM plays a significant role in the development and progression of CLD. DM can also occur as a consequence of or be exacerbated by CLD. Most importantly, concurrent DM and CLD are associated with worse clinical outcomes, including reduced survival, more severe liver failure-related complications, and a higher incidence of HCC and HCC-specific mortality. It is, therefore, imperative that practitioners astutely identify and closely monitor the development of DM in any patient with CLD, bearing in mind that A1c may not be accurate in patients with advanced liver diseases. Alternative glycemic markers, such as FPG, OGTT, or CLD-A1c, may be needed to establish the diagnosis in patients with moderate-to-severe anemia, frequent transfusion requirement, chronic alcohol consumption, and/or fluctuating A1c levels. Once the diagnosis of DM is confirmed, efforts should be made to incorporate the management of DM into the patient's overall treatment plan, with aims to slow the progression of CLD and to reduce the risk of liver failure-related complications. A proposed treatment algorithm is presented in Figure 5. Similar to the general diabetic population, lifestyle interventions involving a calorie-restricted diet and moderate-intensity exercise should be considered first-line treatment. If antihyperglycemic pharmacotherapy is deemed necessary, metformin should be included as the backbone, unless otherwise contraindicated, given its favorable safety profile, chemopreventive effect, and mortality benefit. GLP-1 receptor agonists, DPP-4 inhibitors, and SGLT-2 inhibitors may be considered, even in patients with mild-to-moderate hepatic impairment, given its low risk of hypoglycemia, weight-neutral metabolic profile, and protective effect on hepatic steatosis and hepatic fibrosis. α-glucosidase inhibitors may be useful in patients with concurrent postprandial hyperglycemia and mild hepatic encephalopathy as a way to minimize polypharmacy. Insulin therapy should be reserved for patients who failed other antihyperglycemic medications and should entail frequent dose adjustment and close glucose monitoring to minimize the risk of hypoglycemia. Sulfonylureas and meglitinides should be avoided in most instances.

The aforementioned evidence also highlighted the importance of recognizing the impact of insulin resistance and DM on other etiologies of CLD besides NAFLD. A recent consensus statement endorsed by a panel of international experts has revitalized the decades-long effort to revise the definition and nomenclature of NAFLD[237], acknowledging the heterogeneity of the disease in relation to its natural history, risk factors, clinical manifestations, and responses to treatment[238]. The newly proposed definition of metabolic dysfunction-associated fatty liver disease (MAFLD) no longer requires the exclusion of alcohol consumption or other concomitant liver diseases. It also serves to promote research on patients with coexisting metabolic and alcoholic liver disease, who were previously excluded from most NAFLD studies but are in fact at high risk of disease progression[239].

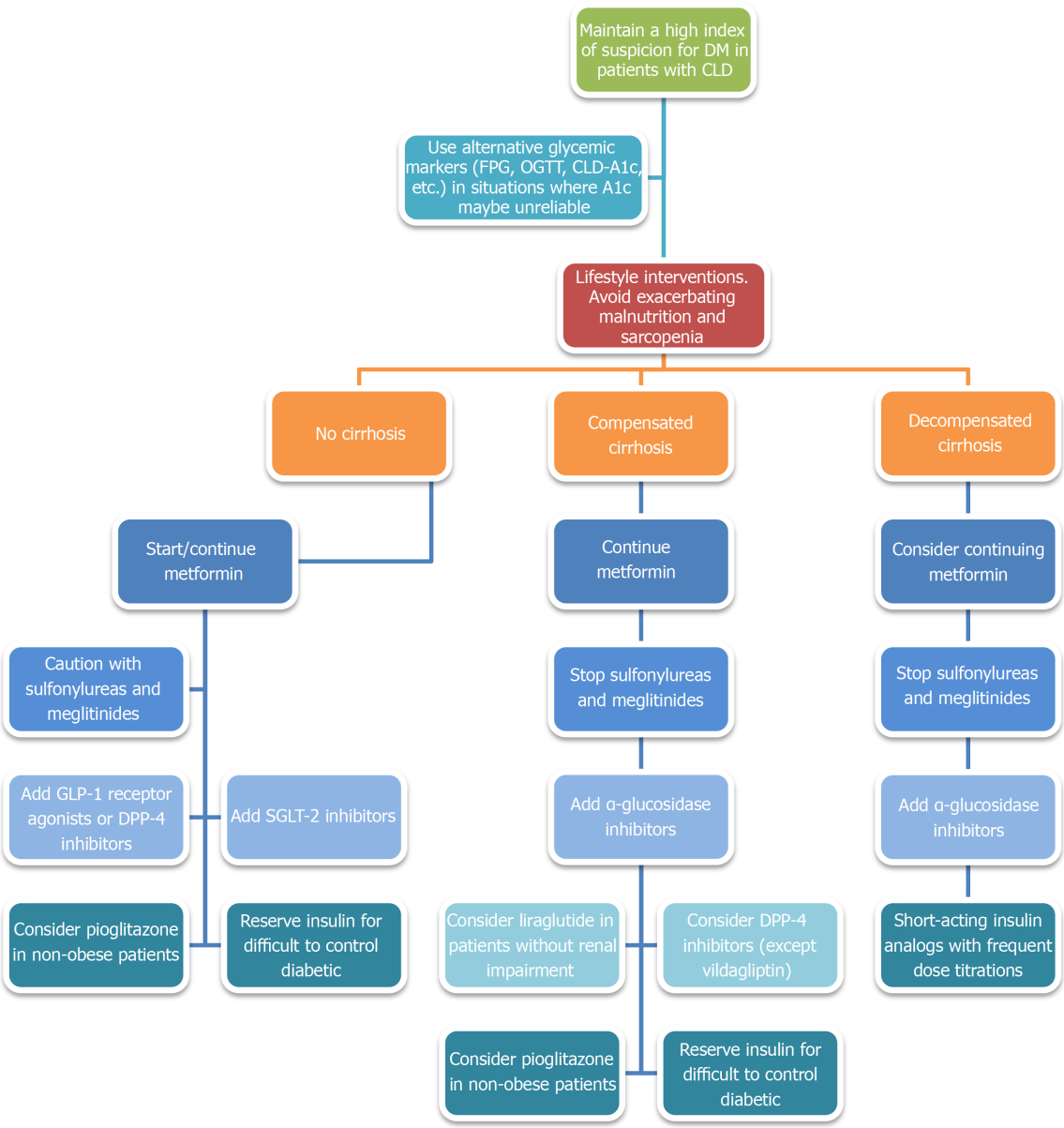

Figure 5 Proposed diabetes treatment algorithm in patients with chronic liver diseases. CLD: Chronic liver diseases; DM: Diabetes mellitus; FPG: Fasting plasma glucose; OGTT: Oral glucose tolerance test; SGLT-2: Sodium-glucose cotransporter-2; GLP-1: Glucagon-like peptide-1; DPP-4: Dipeptidyl peptidase-4.

FUTURE DIRECTIONS

Despite our improved understanding of the interplay between DM and CLD, thanks largely to research in the pathophysiology and management of NAFLD, many pressing clinical questions remain to be addressed. First, an alternative glycemic marker, whose diagnostic accuracy is not affected by altered erythrocyte turnover or excessive glycemic variability, is desperately needed for diagnosing and monitoring DM in patients with advanced liver diseases. Ideally, the test could be performed without prolonged fasting and the result could be easily converted back to an A1c-equivalent value. Second, the optimal glycemic target for slowing CLD progression and preventing liver disease complications while minimizing the risk of hypoglycemia needs to be established. It is reasonable to suspect that patients with various degrees of decompensation would benefit from different glycemic targets. Third, a serological marker for DM-related liver diseases akin to the use of urine albumin excretion to screen for diabetic nephropathy should be investigated. A recent study has shown, for example, that the circulating level of osteopontin (OPN), a soluble pluripotent glycophosphoprotein and a proinflammatory cytokine, may be useful in diagnosing NAFLD in patients with DM[240]. Fourth, given the impact of DM on the progressive of CLD and liver disease complications, it would be interesting to see if the inclusion of a

glycemic marker in the calculation of the MELD score would improve its predictive value for the short-term survival and liver disease severity. Fifth, the long-term safety and efficacy of the novel antihyperglycemic medications, including GLP-1 receptor agonists, DPP-4 inhibitors, and SGLT-2 inhibitors, need to be thoroughly investigated, especially in the non-NAFLD patient populations and with regard to liver disease-related clinical outcomes, such as encephalopathy and HCC development. Sixth, the oncogenic risk of insulin therapy in the context of insulin resistance and chronic hyperinsulinemia must be further evaluated. Seventh, considering the medical complexity of patients with CLD, the risk for drug-induced liver injury from polypharmacy, and the impact of glycemic control on transplant survival and complications, it is worth debating if DM in patients with CLD would be best managed by internists, endocrinologists, or hepatologists.

It is also important to acknowledge and address a number of systemic barriers in order to facilitate advances in this area of research. Clinical studies on the management of DM have traditionally focused on the micro-/macrovascular complications of prolonged hyperglycemia instead of liver disease-related clinical outcomes. In addition, patients with CLD are often excluded from clinical trials of novel medications due to overwhelming concerns regarding hepatic dysfunction and drug-induced liver injury. Conversely, there is an emphasis on the manifestations of portal hypertension as well as the relatively short-term changes in morbidity and mortality, instead of the systemic and long-term effects of insulin resistance, in the management of CLD. While the rapidly worsening epidemic of metabolic syndrome has certainly called to attention the impact of DM on the natural history of liver diseases, most research efforts, especially in terms of novel treatment options, remain restricted to the traditional definitions of NAFLD/NASH. We hope that the evolution from NAFLD to MAFLD would help broaden future studies in this area to include other etiologies of CLD as well over the next 5 to 10 years.

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REVIEW

Biomarkers for hepatocellular cancer

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Abstract

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide. If diagnosed early, curative treatment options such as surgical resection, loco-regional therapies, and liver transplantation are available to patients, increasing their chances of survival and improving their quality of life. Unfortunately, most patients are diagnosed with late stage HCC where only palliative treatment is available. Therefore, biomarkers which could detect HCC early with a high degree of sensitivity and specificity, may play a crucial role in the diagnosis and management of the disease. This review will aim to provide an overview of the different biomarkers of HCC comprising those used in the diagnosis of HCC in at risk populations, as well as others with potential for prognosis, risk predisposition and prediction of response to therapeutic intervention.

Key Words: Biomarkers; Hepatocellular carcinoma; Liver cancer; Cancer; Review; Serum; Plasma; Scoring models; Algorithm; Genetic; Micro-RNA; miRNA; Diagnosis; Prognosis; Liquid biopsy.

Core Tip: The use of ultrasound with/without alpha-fetoprotein in the context of screening patients with chronic liver disease for the development of early stage hepatocellular carcinoma that is treatable, remains problematic. Consequently there has been considerable work done to examine biomarkers either individually, or in combination to

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address this deficiency. Whilst there are several promising targets (discussed in this manuscript) for this indication it appears that Gender, Age: *Lens culinaris* agglutinin-reactive of alpha-fetoprotein, Alpha-fetoprotein, and Des-γ-carboxy prothrombin, which has been established in Europe and Japan, and remains to be so in North America, may be clinically the best performer available.

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INTRODUCTION

Liver cancer is the sixth most common type of cancer globally and is ranked third for the most cancer-related deaths[1]. Liver cancer is more prominent in men being the second leading cause for cancer-related deaths and sixth in women[1]. Hepatocellular carcinoma (HCC) accounts for 85%-90% of primary liver cancer cases[2]. The prevalence of HCC is disproportionately high in areas with a high incidence of hepatitis B virus (HBV). These areas mainly include sub-Saharan Africa and Eastern Asia[3]. It is estimated that almost 80% of all HCCs are viral in etiology induced by both HBV and hepatitis C virus (HCV)[4]. Other risk factors include chronic alcohol consumption, non-alcoholic steatohepatitis (NASH) and cirrhosis arising from a variety of other causes.

The development of HCC is recognized to be a multistep progress with dysplastic macronodules transforming into early and then more aggressive tumors. A number of driver mutations are associated with this process (Figure 1) including TERT (most frequent, 60%), CTNNB1, TP53, CDKN2, ARID1A, AXIN1 as well as DNA gene amplifications involving VEGFA (6p21) and FGF19/CNND1 (11q13), with continuing in-depth exome sequencing turning up novel mutational signatures and risk associations[5,6]. Unfortunately, none of these have any demonstrated value as biomarkers for early disease.

The most widely used classification system, used for treatment decision making is the Barcelona Clinic Liver Cancer (BCLC) algorithm[7]. Ideally, screening would enable pick-up of lesions at early stages (O, A). Disappointingly, HCC is often diagnosed during the advanced stages of the disease as the tumour is often asymptomatic until it progresses and becomes large and infiltrating (stages B, C and D). Curative treatment options such as liver resection, transplantation, and radio-frequency ablation are then precluded due to the poor prognosis of the disease. Advanced-stage HCC non-operative patients are often prescribed a vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor β (PDGFR-β) tyrosine kinase inhibitor, of which sorafenib is the prototypical drug. Sorafenib inhibits tumour angiogenesis and increases median survival on average three months longer than placebo, as reported in a phase 3, double-blind study[8].

A meta-analysis study revealed a median survival of less than one year for patients with HCC diagnosed at advanced stage. However, if diagnosed early, the five-year survival rate of HCC is estimated to be over 70%[9]. There is also a need for screening patients who undergo successful treatment/resection, as HCC disease recurrence and death occur in upto 70% and 50% respectively, after 5 years. Currently, transabdominal ultrasound is the recommended modality for surveillance of patients with cirrhosis[10], however this is of limited sensitivity for smaller lesions, where repeated US scanning (USS) and/or further imaging in the form of either CT or MRI may be required. On-going surveillance of high-risk individuals such as those who have cirrhosis or chronic viral hepatitis can help increase the survival rates of those diagnosed with HCC[7,11].

A biomarker, as defined by the World Health Organisation is: "Any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease[12]". Biomarkers can be broadly categorized into four types, each of which have received attention in HCC: Diagnostic, prognostic, predisposing/risk and predictive[13]. Diagnostic biomarkers aim to detect, at an earlier stage, the presence of a disease or condition. Prognostic biomarkers are used to identify the likely outcome of disease progression or recurrence. Predisposition

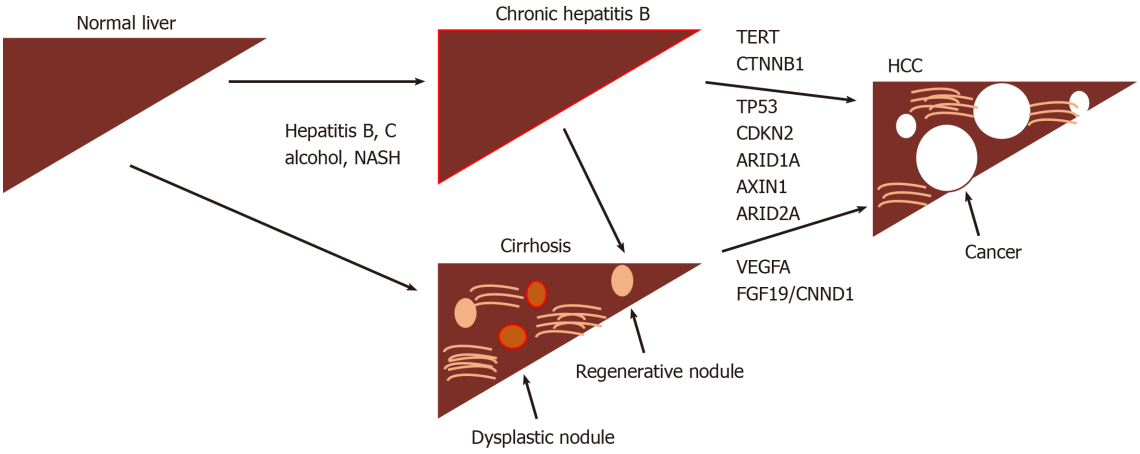

Figure 1 The development of hepatocellular carcinoma is recognized to occur through intermediate steps involving inflammation or fibrosis (cirrhosis) *via* a variety of mechanisms. NASH: Non-alcoholic steatohepatitis; TERT: Telomerase reverse transcriptase; CTNNB1: Catenin beta 1; TP53: Tumor protein 53; CDKN2: Cyclin-dependent kinase inhibitor 2A; ARID1A: AT-Rich Interaction Domain 1A; ARID2A: AT-Rich Interaction Domain 2A; VEGFA: Vascular endothelial growth factor A; FGF19: Fibroblast growth factor 19; CNND1: Cyclin D1; HCC: Hepatocellular carcinoma.

biomarkers are used to indicate the likelihood of developing a certain condition or disease, most commonly through identification of genetic mutations or subtypes. Lastly, predictive biomarkers aim to evaluate the likelihood of a specific medical intervention to have a favourable or unfavourable effect.

Markers for diagnosis of HCC

Alpha-fetoprotein: Alpha-fetoprotein (AFP), a glycoprotein and an oncofetal antigen has been the most widely used biomarker to aid in the diagnosis of HCC[14]. Aberrant production of AFP is observed in almost 50% of all HCCs[15]. However, this biomarker is not specific for HCC, and can also be upregulated during chronic liver disease, pregnancy, other malignancies of the gastrointestinal tract, as well as of the gonadal region[16]. A meta-analysis report found that AFP assays for HCC diagnosis had a pooled sensitivity of 51.9% and a specificity of 94%, yielding an area under the curve (AUC) value of 0.81[17], (Table 1). Unfortunately, it lacks specificity at low levels (20 ng/dL), and may fail to detect small HCCs. AFP levels of 400 ng/dL or greater in plasma are thought to be diagnostic of HCC and may indicate medical complications such as greater tumor size, portal vein thrombosis, and an overall lower median survival rate[18]. Due to these limitations, as well as being impacted by liver inflammation thereby hindering the test's ability to predict a true positive or a true negative, active research to find a better alternative has yielded several potential alternatives.

AFP-L3: Aberrant glycosylation of proteins is a known hallmark of cancer[19]. Lens culinaris agglutinin-reactive AFP (AFP-L3) is the glycosylated isoform of AFP, which has been found to be more sensitive than the widely-used AFP biomarker, when AFP is elevated. The AFP-L3 value is reported as a percentage of the total AFP with a cut-off value of more than 10% commonly used[20]. Twelve articles included in a meta-analysis reported a sensitivity and a specificity of 48.3% and 92.9%, respectively and a summary receiver operating characteristic (SROC) of 0.7564[21].

AFP-L3 has also been found to be a prognostic biomarker after hepatic resection to determine the likelihood of recurrent HCC. Individuals with a low AFP-L3 level 15% after the second hepatic resection were found to have a 5-year survival rate of 91.7%. In contrast, individuals with an AFP-L3 level 15% had a 5-year survival rate of 23.8%[22].

Des-gamma carboxy-prothrombin: Des-gamma carboxy-prothrombin (DCP), also known as "protein induced by vitamin K absence or antagonist-II" (PIVKA-II), is an abnormal prothrombin protein which lack γ-carboxy residues[23]. A cross-sectional study was used to compare the commonly used clinical marker, AFP to DCP. Sensitivity and specificity values of 89% and 95%, respectively, have been reported with a DCP cut-off value of 125 mAU/mL[24]. A meta-analysis based on 20 publications reported sensitivity and specificity of 71% and 84%, respectively, with a SROC of 0.8930[25]. Elevated DCP levels have been associated with a high prevalence of portal vein invasion[26].

Table 1 Diagnostic markers for hepatocellular carcinoma

Marker	Comparison groups	Sensitivity	Specificity	AUC	Ref.
AFP	Meta-analysis	51.9	94	0.81	Xu *et al*[17]
AFP-L3	Meta-analysis	48.3	92.9	0.76	Yi *et al*[21]
DCP	Meta-analysis	71	84	0.89	Zhu *et al*[25]
Glypican-3	Meta-analysis	55	58	0.78	Xu *et al*[34]
AKR1B10	HCC *vs* controls	72.7	95.7	0.9	Ye *et al*[36]
HMGB3	HCC *vs* controls	75.6	85.6	0.79	Zheng *et al*[41]
GALAD	HCC *vs* controls	91.6	89.7	0.97	Berhane *et al*[49]

AUC: Area under the curve; AFP: Alpha-fetoprotein; AFP-L3: Isoform of AFP binding lectin L3; DCP: Des-gamma carboxyprothrombin; AKR1B10: Aldo-keto reductase family 1 member 10; HMGB3: High mobility group box 3; GALAD: Gender, Age: *Lens culinaris* agglutinin-reactive of alpha-fetoprotein, Alpha-fetoprotein, and Des-γ-carboxy prothrombin.

DCP has been found to be a predictive marker of response to treatment with the pharmaceutical tyrosine kinase inhibitor, gefitinib, which targets epidermal growth factor receptor (EGFR) and other proteins such as c-Met and hepatocyte growth factor (HGF)[22]. Gefitinib was found to induce apoptosis in HCC cells, however when treated in the presence of elevated DCP levels, an antagonizing effect was observed reducing the gefitinib-induced apoptosis of the tumorous hepatocytes[27], which occurred through its ability to upregulate EGFR, c-Met and HGF. High DCP has also been shown to be associated with tumor recurrence, metastases, and overall large tumour burden[28,29].

Golgi protein 73: Golgi protein 73 (GP73) is a type II Golgi transmembrane protein has received attention as a diagnostic marker for HCC. A study with 68 patients diagnosed with HCC were evaluated for GP73 levels 2 d prior to transcatheter arterial chemoembolization (TACE) and 7 and 30 d following the procedure. Using ELISA, the protein expression was observed to be markedly higher on average in patients with HCC compared to the controls, 152.5 μg/L *vs* 49.3 μg/L, respectively. Two days following TACE the levels decreased to 99.2 μg/L. After 30 days, the levels were 115.2 μg/L in those with a good response (CT evidence of good lipiodol retention and no active lesions) *vs* 183.2 μg/L where there was a poor response. In regards to the Barcelona clinic liver cancer stages, increasing GP73 concentrations were observed for progressively more advanced stages of the disease[30], BCLC stage A, 92.1 μg/L, stage B, 122.9 μg/L, and stage C, 162.6 μg/L.

Glypican-3: Glypican-3 (GPC3), a cell surface protein, with a 70 kDa core protein mass has been shown to display diagnostic and therapeutic utility for HCC[29,31]. GPC3 has been found to be highly expressed in HCC's – a study which analyzed the GPC3 mRNA transcript, found 74.8% of HCC samples *vs* 3.2% non-tumor liver control samples expressed this[32]. A meta-analysis analyzing the prognostic abilities of GPC3 found overexpression to be an indicator of poor overall survival with a hazard-ratio (HR) of 2.18 (95%CI: 1.47-3.24), poor disease-free survival (HR = 2.05, 95%CI: 1.43-2.93), tumor vascular invasion with an odds-ratio (OR) of 2.74 (95%CI: 1.15-6.52), and hepatic cirrhosis (OR = 2.10, 95%CI: 1.31-3.36)[33].

Although GPC3 has proven to be a good prognostic biomarker, its diagnostic abilities on the other hand are below par. A meta-analysis study comparing the performance of AFP and GPC3 found the latter marker to be inferior than the commonly used marker, AFP. The pooled sensitivity and specificity of GPC3 was found to be 0.55 (95%CI: 0.52-0.58) and 0.58 (95%CI: 0.54-0.61), whereas AFP's sensitivity and specificity was 0.54 (95%CI: 0.51-0.57) and 0.83 (95%CI: 0.80-0.85), respectively. The combination of GCP3 and AFP increased the tests' sensitivity and specificity to 0.85 (95%CI: 0.81-0.89) and 0.79 (95%CI: 0.73-0.84), respectively[34].

Aldo-Keto Reductase family 1 member 10: The aldo-keto reductase family 1 member 10 (AKR1B10) has been linked as a potential biomarker indicating diagnostic and prognostic value[35]. AKR1B10, is a part of a family of NAD(P)H linked oxidoreductases; found on chromosome 7 (7p33), AKR1B10 is involved in the reduction of aldehyde to alcohol, converting retinal to retinol. A large multicenter study from three independent hospitals in China recruited a total of 1224 participants

to validate the role of AKR1B10 in the diagnosis of HCC[36]. Serum levels of AKR1B10 were assessed in the cohort and found an AUC of 0.896 (95%CI: 0.867-0.921) a sensitivity of 72.7%, and a specificity of 95.7% with a diagnostic cutoff value of AKR1B10 at 267.9 pg/mL. Interestingly, although knockdown of AKRB1B10 has been found to decrease cell proliferation, invasiveness, and tumour growth, a high expression of AKR1B10 unexpectedly indicated better overall and disease-free survival[37]. Furthermore, it is worth noting that AKR1B10 expression is increased in the early-stage HCC[38].

High mobility group box 3: The high mobility group box 3 (HMGB3) is a part of the high mobility group (HMG) family of chromosomal proteins involved in chromatin replication, recombination, transcription, DNA repair and stability[39]. Downregulation of microRNA-200b, which is a direct target of HMGB3, occurs in HCC and increases the proliferation and migration of cells in HCC[40]. In a study of 225 patients, the serum HMGB3 levels were assessed at a cutoff value of > 2.0 ng/mL. The AUC for HMGB3 was found to be 0.791 (95%CI: 0.730–0.853) with a sensitivity of 75.6%, and a specificity of 81.6%. This was found to be slightly better than the clinical marker commonly used with an AUC of 0.743 (95%CI: 0.679-0.808), a sensitivity of 56.7%, and a specificity of 76.5% at a cut-off value of 20 ng/mL[41]. High HMGB3 expression was also correlated with poor overall-survival and disease-free survival.

Dickkopf 1: Serum levels of Dickkopf 1 (DKK1), which is a secretory antagonist of the Wnt pathway, have been investigated in HCC, cirrhosis, chronic hepatitis B and healthy controls. Good sensitivities and specificities were reported in both the test and validation cohorts, with DKK1 being reported as positive in early stage disease (< 2 cm tumors) as well as in AFP negative patients. Unfortunately, despite a correlation between DKK1 and tumor size there was none seen with BCLC stage[42].

SALL4: A promising biomarker SALL4, which like AFP is an oncofetal protein, has been correlated with outcomes in HCC, in separate cohorts of patients from Hong Kong and Singapore[43]. This marker appears to be associated with a progenitor, more aggressive form of HCC, and the findings have been confirmed independently[44]. Of possible therapeutic importance is SALL4's property of recruiting the nucleosomal remodeling complex ((NuRD) thereby repressing tumor suppressors such as PTEN. This interaction has been exploited and an inhibitory peptide found with a target affinity of 23nM which has been demonstrated to have significant antitumor effects in xenograft mouse models (85% growth reduction)[45].

Phe-Trp and GCA: Using a modification of LC-MS, Luo *et al*[46] have reported that a biomarker panel comprising phenylalanyl-tryptophan (Phe-Trp) and glycocholate (GCA) performed well in distinguishing HCC from cirrhosis and healthy controls. In particular, the panel could detect AFP negative HCC as well as small HCC (S-HCC), defined as a solitary HCC nodule, or at most 2 nodules less than 3cm in diameter.

Composite markers for HCC

BALAD: The BALAD model was first introduced in 2006 by Toyoda *et al*[47] to aid in the staging of HCC using five serum markers: Bilirubin, Albumin, *Lens culinaris* agglutinin-reactive of alpha-fetoprotein, Alpha-fetoprotein, and Des-γ-carboxy prothrombin (BALAD). A multicenter study recruited 2600 HCC patients while excluding those on warfarin or Vitamin K, as these may alter the serum DCP levels[47]. Cutoff values of 400 ng/dL for AFP, 15% for AFP-L3, and 100 milli-arbitrary unit/mL for DCP were found to optimally predict patient survival. Although the system seems promising, further studies need to be conducted to validate this model.

The BALAD-2 model is refined from the previous BALAD model, which combines raw data with the previous Japanese cohort along with a newly added United Kingdom cohort[48]. The major difference between the BALAD and BALAD-2 model is the statistical analysis; the BALAD-2 model assumes the variables to be continuous rather than assuming a linear relationship. In addition, the original BALAD model divided cohorts into six classes (0-5), whereas the BALAD-2 model divided the cohort into four classes (1-4). A study by Berhane *et al*[49] aimed to validate the BALAD-2 model by collecting patient outcomes of 2,430 individuals diagnosed with HCC, 4404 individuals diagnosed with chronic liver disease, 229 individuals diagnosed with hepatobiliary tract cancer, and 92 healthy individuals. The Formula 1 is used to group individuals into four prognostic groups.

AFP and DCP were modelled as per 1000 units, measured in ng/mL where AFP was capped at 50000 units. Bilirubin and albumin were measured in mmol/L and

Formula 1

$$\text{Linear predictor} = 0.02 \times (\text{AFP} - 2.57) + 0.012 \times (\text{AFPL3} - 14.19) + 0.19 \times (\ln[\text{DCP}] - 1.93) + 0.17 \times ([\text{bilirubin}]^{1/2} - 4.50) - 0.09 \times (\text{albumin} - 35.11)$$

g/LS, respectively. The 4 prognostic groups were based on score > 0.24 (risk 4, high), between 0.24 and > -0.91 (risk 3), from -0.91 to -1.74 (risk 2) and < -1.74 (risk 1, low).

GALAD: The GALAD model was established to aid in the diagnosis of HCC, first developed by Johnson *et al*[50] through a United Kingdom cohort. Similar to the BALAD model, the GALAD model uses three tumour serological markers: *Lens culinaris* agglutinin-reactive of alpha-fetoprotein, Alpha-fetoprotein, and Des-γ-carboxy prothrombin. However, the GALAD model replaces the two liver function tests with Gender and Age[50]. The GALAD score is calculated using the Formula 2.

Where gender is assigned an arbitrary score of 0 for females and 1 for males.

The aforementioned study by Berhane *et al*[49] found a GALAD score cut-off of -0.63 to yield an AUC of 0.97 (95%CI: 0.96–0.98) with a sensitivity and specificity of 91.6% and 89.7%, respectively in the United Kingdom cohort. The Japanese cohort was found to demonstrate optimal performance at a GALAD score cut-off of -1.95 with an AUC of 0.93 (95%CI: 0.92–0.94) and sensitivity and specificity of 81.4% and 89.1%, respectively[49].

This score has been used to investigate early HCC developing in patients with NASH in centres in Germany and Japan. In a case control study involving 125 patients with HCC and 231 patients with NASH from 8 centres in Germany, as well as 389 patients under surveillance in Japan, of whom 26 patients developed HCC, it was found that GALAD identified HCC patients with a significantly greater AUC than any of AFP, AFP-L3 or DCP[51].

Markers indicating predisposition towards HCC

Death receptor 4: Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis by binding to the TRAIL receptor 1 (TRAILR1) also known as the death receptor 4 (DR4)[52]. Genetic alteration of DR4 suggest a higher susceptibility to a number of cancers such as bladder, ovarian, and HCC[52-54]. A study conducted by Körner *et al*[52] examined SNPs at C626G (Thr209Arg, rs20575) and A638C (Glu228Ala, rs20576) in individuals affected with HBV and HCC (n = 56); HCV, cirrhosis, and HCC (n = 159); HCV, cirrhosis, without HCC (n = 75); HCV without cirrhosis and HCC (n = 159); HCV (n = 234); and healthy controls (n = 359). Overall the study found an increased risk of HCC in individuals that carried the 626C allele and the homozygous 638AA genotype mutant who were infected with HCV genotype 1 with an odds ratio of 1.975 (95%CI: 1.205-3.236) (P = 0.007). Another study, conducted in an Egyptian population examined the A1322G SNP of the *DR4* gene and found an odds ratio of 2.34 (95%CI: 1.56-3.51) and 3.51 (95%CI: 2.33-5.28) for the AG genotype and GG genotype, respectively with an increased risk of individuals affect with HCV-related HCCs[55] (Table 2).

Kinesin family member 1B: Kinesin Family member 1B (KIF1B), part of the kinesin superfamily, is involved in axon myelination, growth, and transport of organelles, proteins, and RNAs to specific locations in the cell[56]. With two alternative splice isoforms, KIF1Bα and KIF1Bβ, KIF1B is found on chromosome 1 (1p36.22)[57]. KIF1Bβ has been found to function as a haplo-insufficient tumour-suppressor gene inducing apoptosis, independent from p53[58]. The downregulation of KIF1B mRNA has been shown to correlate with poor prognosis of HCC in different clinicopathologic situations such as vascular invasion, recurrence, and overall-survival[59]. A genome-wide association (GWAS) study by Zhang *et al*[57] identified an intronic SNP – rs17401966 – in the KIF1B gene. Samples were collected from 1962 individuals with 1430 HBV-related HCC cases and 159 family trios of Chinese ancestry. The study identified this polymorphism has a protective effect on HCC, decreasing the likelihood of developing HCC with an odds-ratio of 0.61 (95%CI: 0.55–0.67). However, conflicting studies examining the KLF1B polymorphism in individuals derived from Saudi Arabian, Japanese, and Thai populations found no significant associations[60-62]. A meta-analysis study of the KLF1B polymorphism determined that the polymorphism decreases the risk of HCC for Chinese populations[57].

Although HCV may be eradicated after sustained virologic response (SVR) is achieved, a likelihood of developing HCC exists in cases with more advanced fibrosis. A study by Nagata *et al*[63] examined the risk of developing HCC after SVR using

Table 2 Selected determinants of risk for hepatocellular carcinoma development

Marker	Comparison groups	SNP	Genotype	OR	*P* value	Ref.
DR4	HCC/HBV, HCC/HCV, HCV, controls	C626G/A638C	626C + 638AA	1.98 (1.20-3.24)	0.007	Körner *et al*[52]
DR4	HCC/HCV, HCV, Controls	A1322G	AG	2.34 (1.56-3.51)		Zayed *et al*[55]
			GG	3.51 (2.33-5.28)	0.001	
TLL1	HCV-treated	rs17047200		2.37 (1.74-3.23)	2.66×10^{-8}	Matsuura *et al*[64]
MHC class 1	HCC/HCV, Controls	Rs2596542G>A		1.39 (1.27-1.52)	4.21×10^{-13}	Kumar *et al*[66]
(MICA)	(Japanese cohort)					
MHC class II	HCC-HCV, HCV		DQB1*03:01 (HCV-Geno 1)	0.65 (0.48-0.89)	0.007	Lee *et al*[70]
			DQB1*06:02	3.03 (1.18-7.74)	0.02	
			(HCV-non-Geno1)			

HCC: Hepatocellular carcinoma; HBV: Hepatitis C virus; HCV: Hepatitis C virus; TLL1: Tolloid-like 1; MHC: Major histocompatibility complex; MICA: MHC class I polypeptide-related sequence A gene.

Formula 2

$$Z = -10.08 + 0.09 \times age + 1.67 \times sex + 2.34 \times \log_{10}(AFP) + 0.04 \times AFPL3 + 1.33 \times \log_{10}(DCP)$$

interferon-therapy and interferon-free therapy and found the probability of novel HCC development to be 2.5% and 1.1%, respectively.

Tolloid-like 1: A GWAS identified a SNP variant in the gene tolloid-like 1 (TLL1) found on chromosome 4 within the intronic region (rs17047200)[64]. TLL1 was originally found to play a role in the formation of the interventricular septum of the heart and is now a marker of interest in HCC[65]. The study recruited 457 patients in Japan who underwent SVR through interferon-based treatments and found an odds-ratio of 2.37 (95%CI: 1.74-3.23; $P = 2.66 \times 10^{-8}$)[64].

Major histocompatibility complex class 1: A study by Kumar *et al*[66] in 2011 identified a susceptibility locus for individuals who developed HCV-related HCC through a GWAS conducted in a Japanese cohort. DNA was genotyped in 721 individuals with HCV-related HCC and 2890 HCV-negative controls which identified eight SNPs ($P < 1 \times 10^{-5}$). In the replication stage, 673 cases of HCC and 2596 HCV-negative controls were genotyped at the eight SNPs and identified a polymorphism found on the 5′ flanking region of the major histocompatibility complex (MHC) class I polypeptide-related sequence A gene (MICA) on chromosome 6 (6p21.33). The polymorphism, rs2596542G>A, with an OR of 1.39 (95%CI: 1.27–1.52) ($P = 4.21 \times 10^{-13}$), was found to be associated with a susceptibility to develop HCC from chronic hepatitis-C. It is worth noting that although the polymorphism is associated with developing HCC from chronic hepatitis C, it is not associated with a susceptibility to develop chronic hepatitis C. The polymorphism was also associated with a decrease in expression of the MICA protein in the HCV-related HCC cohort. Conversely, a study by Lange *et al*[67] conducted in Switzerland found the rs2596542G>A to have a protective effect on HCC in patients with HCV throughout the Caucasian population (OR = 0.52, 95%CI: 0.26-1.08). A meta-analysis study analyzing a total of 11 articles with 4528 HCC cases and 16625 controls, found the A/A allele to increase the risk among Asian and African population and an increased risk of HCC in the G/G allele among the Caucasian populations[68]. Increased risk of HCC with individuals who carry the polymorphism was not found in HBV-related HCC's.

MHC class II: The human leukocyte antigen (HLA) is the human MHC, which is found on chromosome 6 (6p21) and is divided into three classes, class I, class, II, and class III. Class II contains the DQ gene family made up of the α and β chains, DQA1 and DQB1, respectively[69]. A GWAS genotyped 502 HCC patients and 749 controls identifying a SNP present in the *HLA* gene, specifically the *HLA-DQB1* gene. An additional 994 HCV seropositive participants were genotyped, specifically in the *HLA-*

DQB1 gene and found that *DQB1*03:01* had protective effects for individuals with HCV genotype 1 with an odds-ratio of 0.43 (95%CI: 0.23-0.81) (P=0.0095). However, a *DQB1*06:02* indicated a risk of developing HCC for non-genotype 1 HCV patients with an odds-ratio of 3.03 (95%CI: 1.18-7.74) (P = 0.0208)[70].

For postoperative HCC, Nault *et al*[71] have described a 5-gene signature consisting of HN1, RAN, RAMP3, KRT19, and TAF9 (of which 4 were upregulated in tumor) that had prognostic ability for postoperative tumor recurrence and survival. The effect was apparent whether a Western cohort of hepatitis C- or an Eastern cohort of hepatitis B-related HCC were analyzed.

Emerging markers for HCC, non-coding RNA and CircRNA

The human genome encodes for many more RNA molecules than proteins, which are known as non-coding RNAs (ncRNA). These comprise both short molecules between 20 to 30 nucleotides long, known as mi-RNA, si-RNA and pi-RNA, together with long non-coding RNA (lncRNA) which are greater than 200 nucleotides. Both types have been investigated in HCC and are discussed below. This section will also briefly describe circRNAs that are formed through the back-splicing of the 3′ and 5′ ends to form a loop that can sequester miRNAs and proteins to affect gene expression.

Mi-RNAs in diagnosis: Micro-RNAs are usually 21-23 nucleotides in length and function *via* the RNA-induced silencing complex (RISC) to regulate gene expression through mRNA degradation, or alternatively by translational repression. They are recognized to be highly useful tools in the diagnosis and prognosis, as well as serving as therapeutic targets for diseases[72]. Aberrant production or alterations of mi-RNAs have been associated with a number of pathologies including cancer, diabetes and cardiovascular diseases[73].

Initial studies utilized different methodologies to investigate miRNA expression in hepatitis B, and HCC, with findings in one study[74] where miR-25, miR-375 and let7f could significantly separate HCC from controls (AUC = 0.997), were not reproducible in another[75]. The seven miRNAs discovered in the latter study, miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a and miR-801 were shown to distinguish between HCC and all of healthy controls, hepatitis B and cirrhosis. Moreover, the changes persisted across the range of BCLC stages O, A, B and C. Of interest, 4 of the identified miRNAs (26a, 223, 21 and 122) had also been previously reported as capable of achieving this. Notably, 2 of these, miR-21 and miR-122, have been underscored as useful biomarkers in a subsequent meta-analysis involving 50 studies that included 3423 cases of HCC, 2403 cases of chronic hepatitis and 1887 healthy controls[76]. The pooled analyses indicated that they were slightly better at distinguishing HCC from controls than those with chronic hepatitis. Of these two molecules miR-122 appeared to be particularly compelling since mice with a genetic deletion of this in the liver were found to be prone to the development of NASH, fibrosis and HCC with expression of oncofetal molecules such as AFP and IGF2[77,78].

Moshiri *et al*[79] have used RNA sequencing of plasma to approach the problem differently. After identification of 38 differentially expressed miRNAs with at least a 3-fold change between HCC and cirrhosis and/or controls, 9 were chosen for further validation steps by droplet digital PCR (ddPCR) technology. Of these, miR-101-3p, miR-1246, miR-106b-3p, miR411-5p were evaluated in independent cohorts. Whether they analyzed plasma or serum, diagnostic accuracies of well over AUC > 0.90 were achieved for miR-101-3p, miR-1246 and miR-106b-3p, individually or in combination.

Role in treatment of HCC: Previous work with sorafenib did not identify any biomarkers that were predictive of treatment response in patients with HCC, however, baseline levels of angiopoietin 2 and VEGF were independent predictors of survival[80]. A more recent study has shown an inverse correlation between levels of miR-221 and sorafenib resistance in animal models and a small cohort of patients[81]. Bruix *et al*[82] and Teufel *et al*[83] investigated tissue and baseline plasma samples in patients involved in a trial to investigate the response to regorafenib, which is another multikinase inhibitor. Levels of miR-30a, miR-122, mir-125b, miR-200a, miR-374b, miR-15b, miR-107, miR-320 and miR-645 were all associated with survival time with regorafenib treatment. The study also found survival time associations with reduced baseline levels of Ang1, cystatin B, LAP-TGFb1, Lox-1, MIP1a, after treatment.

The objective of using screening in a cirrhotic population is to detect HCC early, however, in an analogous situation, what happens to patients that undergo HCC resection and is there a way of then predicting recurrence? Mi-RNAs may be useful in this scenario as shown by Fu *et al*[84]. By analyzing data on 318 patients from The Cancer Genome Atlas (TGCA) they uncovered a 7-miRNA signature correlated with

5year survival which comprised miR-187, miR-9-3, miR-490, miR-1258, miR-3144, miR-551-a and miR-665. These findings will require replication in larger prospective cohorts to validate them further.

LncRNAs in HCC: In a search for novel genes in HCC, Panzitt *et al*[85] discovered the lncRNA HULC (highly upregulated in liver cancer). This has been investigated in HCC and found to be associated with clinical stage and intrahepatic metastasis[86]. Other lncRNAs found to be overexpressed in HCC and associated with a poor prognosis include ZEB1-AS1 and DANCR which function to repress cadherin expression and CTNNB1 degradation respectively[87,88]. Conversely, other lncRNAs exhibit reduced expression in HCC and affect tumor progression by EMT[89].

CircRNAs in HCC: With respect to CircRNAs, several have been shown to affect key aspects of tumor biology. As an example, CircMAT2B has been implicated in altering tumor metabolism under hypoxia; it does so by sequestering miR-338-3p which leads to increased PKM which is involved in glycolysis[90]. Another is CircASAP1 which affects miR-326 and miR-532-5p thereby enhancing MAPK (mitogen-activated protein kinase) signaling and TAM infiltration. This has been shown to be associated with a poor prognosis[91].

New concepts for biomarker development

A 'liquid biopsy', performed through a blood collection, may be the simplest means whereby physicians can collect information from patients in a minimally invasive manner[92]. It may become an alternative to the time-consuming surgical biopsies which place patients at risk of developing complications. The risk of needle-track tumour seeding is another disadvantage of surgical biopsies, which precludes their routine use to evaluate suspected liver cancer. Moreover, serial collections would be feasible, allowing evaluation of tumour progression in real time[93]. A liquid biopsy analyzes a range of molecular data such as circulating tumor cells (CTCs), cell-free circulating tumor DNA (ctDNA), and exosomes released from necrotic tumor cells, thus providing insight into tumour behaviour.

Circulating tumor cells in HCC: Circulating tumor cells are extremely rare, estimated to be as low as 10 cells in 10 mL of blood, making them difficult to detect[94]. However, CTCs can provide a wealth of information on multiple DNA abnormalities, gene fusion transcripts, and RNA expression of the cancerous cells when isolated. Flow cytometry is commonly used to search for CTCs through fluorescently labelled cellular tags, many which target stem cell markers such as: Epithelial cell adhesion molecule (EpCAMs), CD133, CD90, CD44, CD13, and cytokeratin 19[95,96]. A study by Sun *et al*[97] demonstrated the clinical significance of CTCs in 123 HCC patients by analyzing EpCAM, which showed a high probability of tumour recurrence in individuals with ≥ 2 CTCs in 7.5 mL of blood. Recurrence was found in 26 of 51 patients with ≥ 2 CTCs, whereas only 15 of 72 patients with < 2 CTCs showed recurrence after curative resections. The mean follow-up time was reported to be 15.1 ± 2.3 mo[97]. Perioperative analysis of CTCs for individuals with HCC may provide insight on prognosis and can tailor clinical treatment decisions.

Circulating tumor DNA: Cell-free circulating tumor DNA (ctDNA) is typically 180-200 basepairs (bp) in length (approximately the size of mononucleosomal unit), released when tumour cells are phagocytosed or undergo apoptosis. The difficulty of analyzing ctDNA is due to its low concentration in the blood. Moreover, cell-free DNA (cfDNA) is released by normal cells further decreasing ctDNA concentrations. Healthy subjects are found to have a peripheral cfDNA concentration of 10 ng/mL to around 100 ng/mL, with a half-life between 16 min and 2.5 h[95]. Quantitative analysis from several studies have revealed the mean concentration of cfDNA to be 3-4 times elevated in HCC patients as compared with chronic hepatitis patients, and almost 20 times higher compared to healthy controls[98]. Clinico-pathological parameters such as tumor grade, size of tumor, shorter overall survival, and metastatic ability have been found to correlate with elevated cfDNA levels[98]. Aberrant epigenetic alterations, through DNA methylation, have been found to be one of the universal hallmarks of cancer which is being investigated in ctDNAs[99]. A study by Chan *et al*[100] analyzed the hypermethylation of the RASSF1A [Ras association (RalGDS/AF-6) domain family member 1A] gene, observed in 93% of HCC patents *vs* 58% of HBV patients, and 8% of the healthy controls. Interestingly, with a cut off value of 1×10^6 copies/L of the hypermethylated RASSF1A, 50% of AFP-negative HCCs are identified. This may indicate a role for its use in promising combinatorial techniques to help in the diagnosis of HCC. Other features of elevated RASSF1A concentrations may predict

poor disease-free survival.

DISCUSSION

The basic aims of HCC biomarker research are to find novel molecules, as well as optimizing use of existing ones, to be able to diagnose the disease earlier in at risk populations, and furthermore, to be able to predict disease outcome in response to treatment, as well as provide prognostic information (Figure 2). At the same time the process needs to safeguard patients against unnecessary testing and follow-up where there are abnormal biomarker findings but no defined algorithms for further management, given that only a fraction of the at-risk population will eventually develop the disease[101]. A systematic review has indicated that USS-based screening is indeed capable of improving mortality associated with HCC[102]. Conversely, recent work has questioned the validity of any screening for patients with cirrhosis, in a matched case control study of the VA health care system, using either USS, AFP or both, where no difference was found for HCC-related mortality[103]. The finding of significant heterogeneity in approaches to HCC management in 18031 patients from 14 countries, together with distinct demographics and outcomes, indicates a need for earlier diagnosis[104].

Serum biomarkers such as AFP allow for a minimally-invasive and rapid evaluation of at risk patients. However, there are no recommendations for its regular use outside Japan, where in conjunction with USS, and AFP-L3 and DCP, it is used every 6 months. A recent meta-analysis utilizing 32 studies and 13367 patients showed that US, with AFP *vs* without, exhibited greater sensitivity at detecting early stage HCC (63%, 95%CI: 48-75% *vs* 45%, 95%CI: 30%-62%, P = 0.002). However, US alone was more specific (RR = 1.08; 95%CI: 1.05-1.09) and detected any stage HCC with a sensitivity of 84%[105].

Some markers of more aggressive HCC may evolve into developing management algorithms and SALL4 may be one such example. This may also offer some insight into disease management, as demonstrated with the aid of molecular reconstruction and subsequent investigation of interfering with its interaction with NuRD[45]. Similarly, in evaluating markers indicative of advanced HCC it was uncovered that Ang2 and Vegf independently marked cases with more rapidly progressive disease but had no bearing on sorafenib response[80]. In comparable work conducted with regorafenib, survival changes correlated with levels of several miRNAs including miR-30a, miR-122 and miR-200a.

As discussed in this article, there are a number of other serological markers such as GP73, GPC3, AKR1B10, which appear promising but all require further validation. It may be that there is no advantage for any of these over AFP alone, as demonstrated in a meta-analysis for another interesting biomarker, osteopontin[106]. Alternatively, they may require to be combined with other markers such as albumin, to improve their performance.

Micro-RNAs can be used as diagnostic or prognostic tools and may also serve as therapeutic targets for HCC. Unfortunately, despite the wealth of data generated in this area, this approach has failed to show consistency for the molecules assessed. Micro-RNAs appear to exhibit variability according to whether measured in plasma or serum hence in part explaining the discrepancies observed in numerous earlier studies. It appears that mi-RNAs are found in higher concentrations in plasma *vs* serum, with platelet mediated degradation during the clotting process speculated to be a possible explanation for this.

Of those that were initially found to be promising, specifically miR-21 and miR-122, these were not reported to be differentially expressed in an analysis utilizing RNA sequencing. The work by Moshiri *et al*[79] has shown that some additional mi-RNAs that may have potential greater accuracy. However, given the lack of overall reproducibility of findings so far in this field, these observations remain preliminary and will require follow-up. The same conclusion may apply to the analysis of CTCs in HCC. In one study 95% of 195 HCC patients demonstrated a correlation between hybrid and mesenchymal CTCs (EpCAM/Twist/Snail) with BCLC stage, AFP, recurrence and metastasis[107]. However, a retrospective study that analyzed 113 HCC patients before curative treatment and 143 HCC patients after curative treatment, found no correlation of total CTCs or the EMT phenotype with AFP, BCLC stage, tumor size or vascular invasion[108].

From a practical perspective, perhaps the composite scoring systems GALAD and BALAD-2 currently exhibit the most favorable diagnostic accuracy over conventional

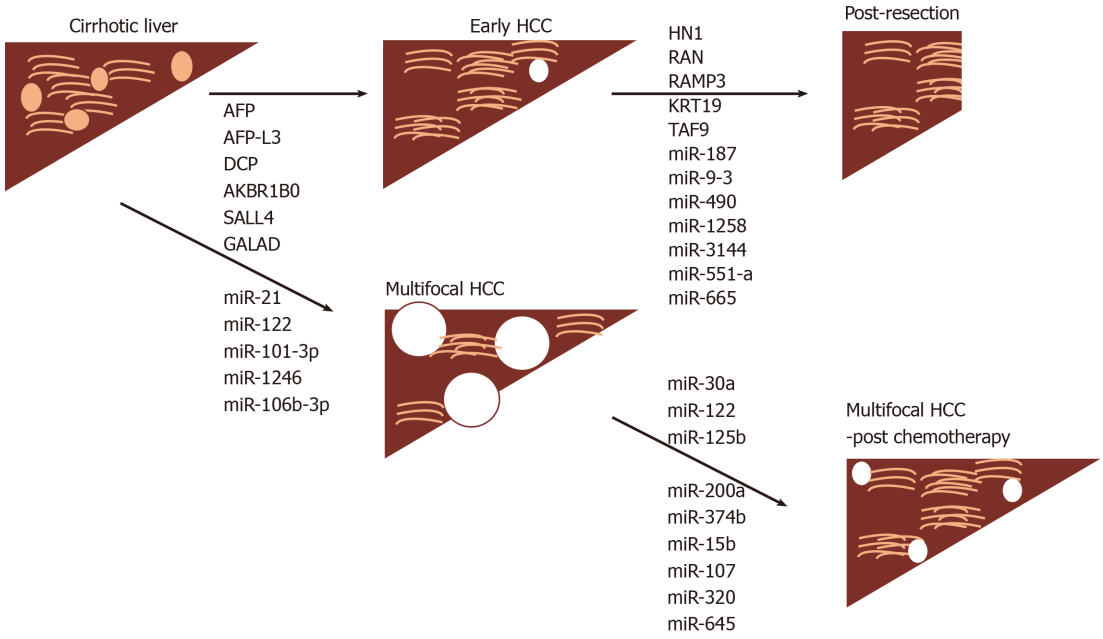

Figure 2 Markers associated with cancer development and those associated with response to surgical intervention or chemotherapy.
HCC: Hepatocellular carcinoma; AFP-L3: Isoform of AFP binding lectin L3; DCP: Des-gamma carboxyprothrombin; AKBR1B0: Aldo-keto reductase family 1 member 10; SALL4: Sal-like protein 4; GALAD: Gender, Age: *Lens culinaris* agglutinin-reactive of alpha-fetoprotein, Alpha-fetoprotein, and Des-γ-carboxy prothrombin.

methods. The findings have been replicated in cohorts of patients in both Europe and Japan, where the etiology of HCC differs. Further prospective analysis in North America may help to establish this approach as shown by the recent work for GALAD in NASH related HCC[51]. A retrospective analysis has also indicated its superiority over USS in detecting HCC including patients with negative AFP, and its performance remained excellent for early stage HCC[109].

CONCLUSION

In conclusion, despite the plethora of studies so far, and the promise of different classes of biomarkers, there appears to be no specific one that currently fulfils the need to pick up early HCC (BCLC stage O/A) with any advance over USS with or without AFP. Similarly, patients undergoing curative resection or chemo-/immuno-therapy may benefit from comparable analyses. Future prospective multicentre trials are required in defined at risk populations for HCC to assess the various classes of agents discussed.

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MINIREVIEWS

Comprehensive review of hepatotoxicity associated with traditional Indian Ayurvedic herbs

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Abstract

With growing antipathy toward conventional prescription drugs due to the fear of adverse events, the general and patient populations have been increasingly using complementary and alternative medications (CAMs) for managing acute and chronic diseases. The general misconception is that natural herbal-based preparations are devoid of toxicity, and hence short- and long-term use remain justified among people as well as the CAM practitioners who prescribe these medicines. In this regard, Ayurvedic herbal medications have become one of the most utilized in the East, specifically the Indian sub-continent, with increasing use in the West. Recent well-performed observational studies have confirmed the hepatotoxic potential of Ayurvedic drugs. Toxicity stems from direct effects or from indirect effects through herbal metabolites, unknown herb-herb and herb-drug interactions, adulteration of Ayurvedic drugs with other prescription medicines, and contamination due to poor manufacturing practices. In this exhaustive review, we present details on their hepatotoxic potential, discuss the mechanisms, clinical presentation, liver histology and patient outcomes of certain commonly used Ayurvedic herbs which will serve as a knowledge bank for physicians caring for liver disease patients, to support early identification and treatment of those who present with CAM-induced liver injury.

Key Words: Ayurveda; Complementary and alternative medicines; Drug induced liver

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Core Tip: Ayurvedic herbal medications (AHM) can cause liver injury ranging from an asymptomatic elevation of liver enzymes to cirrhosis and portal hypertension. Patients who develop AHM-related liver injury have a history of consumption of complex polyherbal formulations. In most cases, identification of the offending hepatotoxic agent is difficult due to the number and complexity of herbs involved. However, multiple observational studies, quality case series, and well-performed case studies have demonstrated the hepatotoxic potential associated with certain herbs used in Ayurvedic practice. The commonly utilized and over-the-counter available Indian herbs or their extracts, such as Ashwagandha, Aloe vera, Guggul, Puncture vine, Turmeric, Gotu-kola, Bakuchi, Senna, Noni, Malabar tamarind, and Gurmar have been associated with various types of liver injury ranging from acute self-limiting hepatitis, chronic hepatitis, prolonged cholestasis, hepatic sinusoidal obstruction syndrome, cirrhosis, and portal hypertension and can present clinically as acute severe liver injury, acute liver failure, acute decompensation of cirrhosis or acute on chronic liver failure. Physician knowledge regarding regional and local complementary and alternative practices among the general and patient population is essential in identifying those who develop complications of liver disease secondary to herbal hepatotoxicity, to make optimal treatment decisions, and for early prognostication.

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INTRODUCTION

Ayurveda, an ancient traditional system of medicine, originated in the Indian sub-continent. According to Ayurvedic principles, the Universe is composed of five elements, namely, *Vayu* (Air), *Jala* (Water), *Aakash* (Space or ether), *Prithvi* (Earth) and *Teja* (Fire) that form the three elemental humors of the human body in varying combinations - the *Vata dosha*, *Pitta dosha*, and *Kapha dosha*, collectively called the "*Tridoshas*" that, along with five sub-categories for each of the primary *doshas* are thought to control all of the essential physiological functions of the human body. Even though practitioners of Ayurveda believe it to be a complete system of medicine, Ayurvedic science lacks the rigorous and ideal scientific approach required for disease diagnosis and treatment. This is underscored by the fact that prospective, well-designed, good quality controlled trials are deficient in the current literature concerning Ayurvedic practice[1]. In contrast, integrative approaches toward practices in Traditional Chinese Medicine (TCM) has been exemplary, improving our understanding of beneficial active components in Chinese herbs which have been ultimately utilized in the management of lethal and resource burdening diseases such as malaria. Ayurvedic herbal medicines (AHM) are broadly divided into non-proprietary or classical and proprietary drugs. In the former, manufacturing methodology follows principles and guidelines as per approved classical texts of Ayurveda (such as *Charak Samhita* or *Susrut Samhita*); while in the latter, private drug manufacturers decide on the content, composition and preparatory methods involved in the preparation of the AHM (examples include Himalaya® Liv 52™ or Charak® Livomyn™ syrup). Das *et al*[2] in a study from North-East India, found that unknown herbal medications were a significant cause of mortality among patients with acute liver failure (ALF). Similarly, Udayakumar *et al*[3] demonstrated that traditional indigenous herbal medications prescribed by South-Indian Tamil healers led to ALF with high mortality in affected patients[3]. In a large single-center series from South-India, Devarbhavi *et al*[4] found that Indian Ayurvedic medicines caused drug-induced liver injury (DILI) in 1.3% of patients in whom almost half of those affected died due to progressive liver failure. In a pioneering study, Philips *et al*[5] addressed clinical

outcomes and analyzed component toxicology of AHMs causing severe DILI. In this study, patients were prescribed AHMs mainly for non-specific gastrointestinal symptoms. The overall mortality was approximately 19% and most of the formulations were unlabelled polyherbals, with high levels of arsenic and mercury which were significantly associated with death on follow-up[5]. The same authors showed that among cirrhosis patients consuming AHMs, 35.7% presented with severe DILI leading to acute on chronic liver failure (ACLF) with an overall mortality of 53%. In this series, the most common culprit leading to AHM-DILI were unlabelled polyherbal preparations followed by proprietary Ayurvedic drugs[6]. A recent multicentre study spearheaded by the Asian-Pacific Association for the Study of Liver (APASL) demonstrated that ACLF in Asia-Pacific countries was predominantly due to CAMs (in approximately 72% of patients) inclusive of Ayurvedic herbals and herbal and dietary supplements[4]. Components implicated in DILI related to Ayurvedic medicines are difficult to ascertain due to mislabelling or un-labelling of the product, presence of potentially toxic adulterants or contaminants, and importantly, the complex polyherbal nature of preparations. In this context, a precise knowledge regarding certain potentially hepatotoxic herbs is of utmost importance for the clinician while dealing with patients with AHM-related DILI. In this review, we discuss exhaustively from current literature, the hepatotoxicity of common Indian Ayurvedic herbs that are utilized in pure form or as mixtures; explore pertinent clinical presentations and outcomes with real-life patient examples and summarize to provide future directions on Ayurvedic herbs-related DILI.

AYURVEDIC HERBS WITH POTENTIAL HEPATOTOXICITY

Withania somnifera (Ashwagandha)

Ashwagandha is a herbal extract derived from the roots of *Withania somnifera*, an evergreen shrub endemic to India and Southeast Asia, commonly known as the Indian ginseng or winter cherry. It is claimed to have neuroprotective and anti-inflammatory properties and has been used to treat a variety of symptoms and diseases ranging from fatigue, stress, epilepsy and arthritis to cancer chemoprevention. Some Ayurvedic practitioners and drug manufacturers proclaim that Ashwagandha has "anti-aging" properties. Multiple studies performed on small animal-models and published in anecdotal journals have alleged a "liver safe" profile[7,8]. Apart from a few randomized clinical trials with small patient numbers but with penurious methodology, and short follow-up, there have been no rigorously performed, scientifically sound, prospective studies confirming the efficacy and safety of Ashwagandha in the current literature[9]. The bioactive compounds identified in Ashwagandha include steroidal lactone triterpenoids (called withanolides), alkaloids such as cuscohygrine, and anahygrine, flavonoids, phytosteroids and coagulins. The terpenoid Withaferin A is purported to have hepatoprotective properties. The first report of possible Ashwagandha-related DILI was from Japan. Inagaki *et al*[10] described a 20-year-old man with anxiety disorder who used twice the recommended dose of Ashwagandha bought online in combination with multiple antianxiety drugs. The pattern of DILI was of "highly possible" cholestatic type as per the Digestive Diseases Week – Japan 2004 (DDW-J) diagnostic criteria. The liver biopsy showed severe intrahepatic cholestasis with extensive canalicular bile plugs. The patient recovered uneventfully within 2 mo after treatment with ursodeoxycholic acid, phenobarbitone, and withdrawal of the offending drug. A drug-induced lymphocyte stimulation test revealed reactivity and drug interactions between Ashwagandha, propranolol, and alprazolam[10]. Björnsson *et al*[11] reported on a series of patients from Iceland and the United States Drug-Induced Liver Injury Network (DILIN) with liver injury due to Ashwagandha. The authors described five patients, mostly males with a mean age of 43 years, who developed cholestatic jaundice after consuming a herbal supplement ranging from 2 to 12 wk. Liver injury was cholestatic or mixed type and liver biopsy showed severe cholestatic hepatitis. The clinical course was prolonged, ranging from 5 to 20 wk and normalization took up to 5 mo. None of the patients developed liver failure. Chemical analysis using liquid chromatography coupled to quadrupole time-of-flight mass spectrometry on the retrieved products confirmed the presence of Ashwagandha, the absence of other toxic compounds or the presence of potentially hepatotoxic conventional drugs. In one patient, the additional consumption of *Rhodiola rosea* (golden root or roseroot) was suspected to have caused a herb-herb interaction resulting in the liver injury[11]. Ashwagandha-containing herbal medications can result in severe cholestatic liver injury, which may be prolonged, but self-liming without the

development of chronic DILI or ALF (Figure 1).

Bacopa monnieri and Centella asiatica (Brahmi/Gotu Kola)

The herb Brahmi, used synonymously with Gotu Kola is extensively utilized in Ayurveda due to its seemingly beneficial effect on neurocognitive functions. Both have distinct biochemical component properties, pharmacokinetics, and pharmacodynamics, and are considered separate herbs in the ancient Ayurvedic texts. Nonetheless, both are commonly used as memory "boosters" or cognition enhancers. Brahmi is essentially *Bacopa monnieri*, a perennial non-aromatic creeping herb, also called the water hyssop. The primary bioactive constituents of Brahmi include steroidal saponins called bacosides, alkaloids such as Brahmine, herpestine, and nicotine. Gotu Kola or *Centella asiatica* commonly called the Indian pennywort, is a perennial flowering plant that is indigenous to the Indian subcontinent and Southeast Asia. Similar to Brahmi, the bioactive components in Gotu Kola are saponin-triterpenoids, which include asiaticosides, brahmoside, and Brahminoside as well as the glycosides isothankuniside and thankuniside[12,13]. In their report on severe hepatotoxicity due to Ayurvedic herbal products, Teschke and Bahre described an older woman who developed a severe hepatocellular type of DILI after consuming multiple herbal products for 9 mo for vitiligo. All other competing causes were systematically excluded by the authors. On structured causality assessment using the Council for International Organizations of Medical Sciences (CIOMS) scale on individual products, the authors found that primary hepatotoxicity was possibly due to "Bakuchi" tablets (score 8+), containing extracts of *Psoralea corylifolia* (also called babchi or purple fleabane; discussed later). However, the CIOMS score was 6+ (possible) for Brahmi tablets. Properly conducted structured studies or pharmacovigilance on organ-specific toxicity of Brahmi is deficient in current literature[14]. Gotu Kola is also implicated in contact dermatitis (due to the presence of madecassoside) and infertility due to bioactive components, isothankuniside, and thankuniside[15,16]. An Argentinian group of researchers were the first to describe a series of women in whom ingestion of Gotu Kola for weight loss for approximately one to two mo resulted in severe cholestatic hepatitis. Liver biopsy revealed acute granulomatous hepatitis, with marked necro-inflammatory activity and eosinophilic degeneration mainly in zone 3, along with a lymphoplasmacytic infiltrate in all patients, and additional features of chronic hepatitis with prolonged cholestasis and progression to cirrhosis in one patient. In the patient with chronic DILI-related cirrhosis, acute decompensation developed with repeat intake. In other patients, drug withdrawal, along with a short course of corticosteroids and ursodeoxycholic acid therapy resulted in a complete reversal of liver injury at one year follow-up. In the first patient, repeat consumption of Gotu Kola seven mo later led to recurrence of DILI with similar biopsy features to the initial biopsy. In both instances, autoantibodies were positive favouring the possibility of immune-mediated DILI[17]. In another report, Gotu Kola intake for 6 wk for acne treatment led to ALF in a 15-year old girl with complete resolution of illness after drug withdrawal[18]. Phytochemicals such as alkaloids and cyclic compounds undergo biotransformation in the liver leading to the generation of metabolites that cause direct damage to hepatocytes and cholangiocytes or generate antigens, which trigger immune-mediated liver injury. In Gotu Kola, the bioactive compounds such as asiaticoside are triterpenoids which belong to the same family of hepatotoxic saponosides found in other herbs such as germander and Chinese skullcap. These bioactive compounds induce apoptosis through alteration of cellular transport at the cell membrane level, which characteristically could result in eosinophilic degeneration and cellular necrosis seen on liver histopathology[14-17]. Brahmi or Gotu Kola related DILI presents with cholestatic granulomatous hepatitis and severe autoantibody mediated necroinflammation (Figure 2). A strong suspicion for the intake of these herbs should be considered in patients presenting with acute onset cholestatic hepatitis with autoantibodies and biopsy features of granulomatous hepatitis after excluding other causes.

Curcuma longa (Turmeric)

Turmeric and its bioactive compound curcumin are derived from the root of *Curcuma longa*, a perennial plant that is native to India, belonging to the ginger family. In Ayurvedic practice, it is heavily used as an anti-inflammatory and antioxidant as well as for digestive system-related symptoms and diseases such as pharyngitis, inflammatory bowel disease and fatty liver disease. Turmeric contains 3%-6% polyphenolic compounds (curcuminoids) such as curcumin, desmethoxycurcumin, and bisdemethoxycurcumin, which are responsible for its bioactivity. Turmeric is also rich in phytosteroids and bioactive compounds that have immunomodulatory

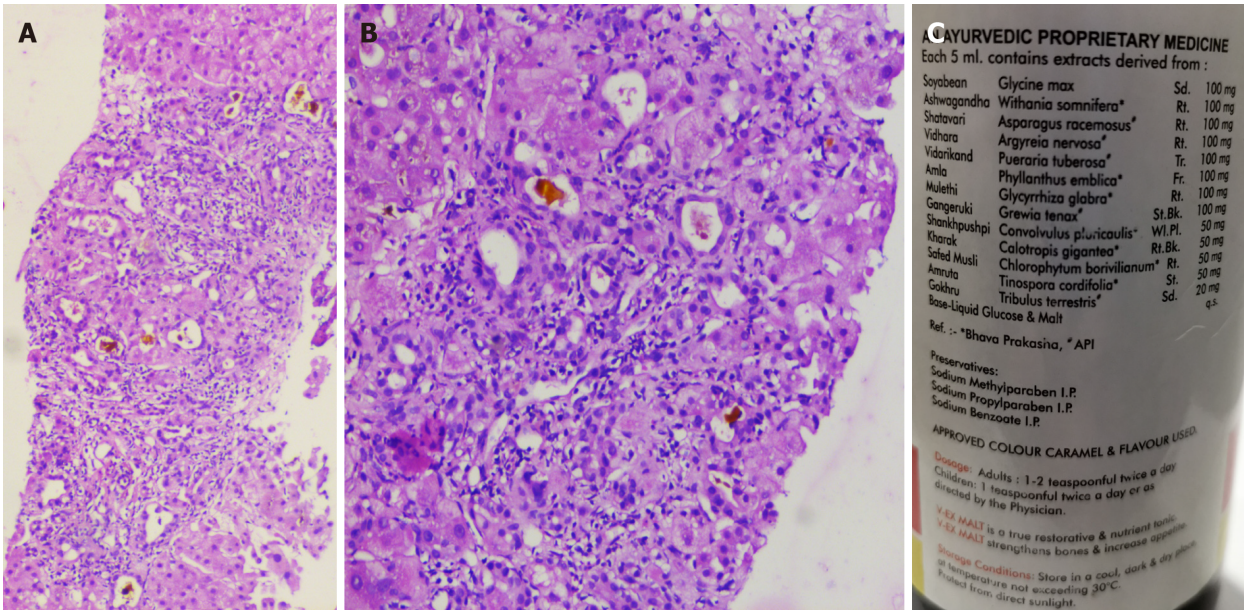

Figure 1 Liver histopathology of acute liver injury in a patient ingesting Ashwagandha. A and B: Percutaneous liver biopsy revealed severe canalicular cholestasis along with moderate portal inflammation [A, hematoxylin and eosin stain (H&E), 40 × and B, H&E stain, 400 ×)]; C: The polyherbal formulation containing Ashwagandha, retrieved from the patient is shown in C. The patient had an uneventful course with resolution of symptoms and normalization of liver tests at 2 mo.

properties[19,20]. Even though Ayurvedic practitioners vouch for the multisystemic beneficial effects of turmeric, a strong scientific basis for its efficacy and safety has not been proven through meticulous clinical studies. The majority of the beneficial effects of turmeric or its bioactive agents are still confined to small animal model studies without adequate translation to human diseases[21]. Lukefahr *et al*[22] described the case of highly probable drug-induced autoimmune hepatitis (AIH) ascribed to ingestion of turmeric dietary supplement for 10 mo in a 76-year old woman. The immunoglobulin G levels were elevated along with positive autoantibody titres for atypical perinuclear anti-neutrophil cytoplasmic antibodies and antibody to smooth muscle actin. Liver histopathology was compatible with AIH, and other causes of acute hepatitis were ruled out. After withdrawing the turmeric supplement, liver injury improved within 1 mo and returned to normal within a year and remained so at the three year follow-up[22]. Similar reports of AIH-like severe hepatitis associated with turmeric use were reported by Suhail *et al*[23] and Lee *et al*[24]. Imam *et al*[25] reported the case of a 78-year old female who ingested an over-the-counter curcumin supplement for dyslipidemia for 1 mo. She presented with progressive jaundice without cholestasis. The DILI was of hepatocellular type and other competing causes of acute hepatitis were ruled out. After stopping the supplement, the liver tests showed approximately 50% improvement at the end of one week and complete resolution by day 42. The authors did not review or analyse the curcumin supplement for adulterants and contaminants, and a liver biopsy was not performed given clinical improvement[25]. Luber and co-workers described two cases of curcumin-related severe hepatocellular DILI in their paper. The first patient was a woman in her early fifties with cholestatic hepatitis on Ancient Wisdom™ High Potency Turmeric (375 mg curcuminoids + 4 mg black pepper) for 1 mo before the onset of symptoms. Her liver biopsy revealed severe lobular mixed inflammation and interface hepatitis. After resolution of liver injury within 2 mo, she restarted the turmeric supplement (1125 mg curcuminoids per day) for 3 wk and presented with severe cholestatic hepatitis again. Further withdrawal resulted in complete resolution of symptoms. Analysis of the sample did not reveal any adulterants, toxins, heavy metals, or synthetic drugs. The second case was of DILI with autoantibodies in a 55-year-old male with metabolic syndrome after ingestion of turmeric-based supplements for cardiovascular health[26]. Similar biopsy findings and clinical outcome related to turmeric-induced severe liver injury was reported by Chand *et al*[27]. The patient developed additional features of severe myalgia, skin rash, and arthritis which resolved with normalization of liver tests after turmeric supplement withdrawal. We noted that in a middle-aged female patient with compensated hepatitis B virus-related cirrhosis, the use of turmeric capsules (1g per day for 3 wk) resulted in severe spur-cell anemia requiring blood transfusion, which

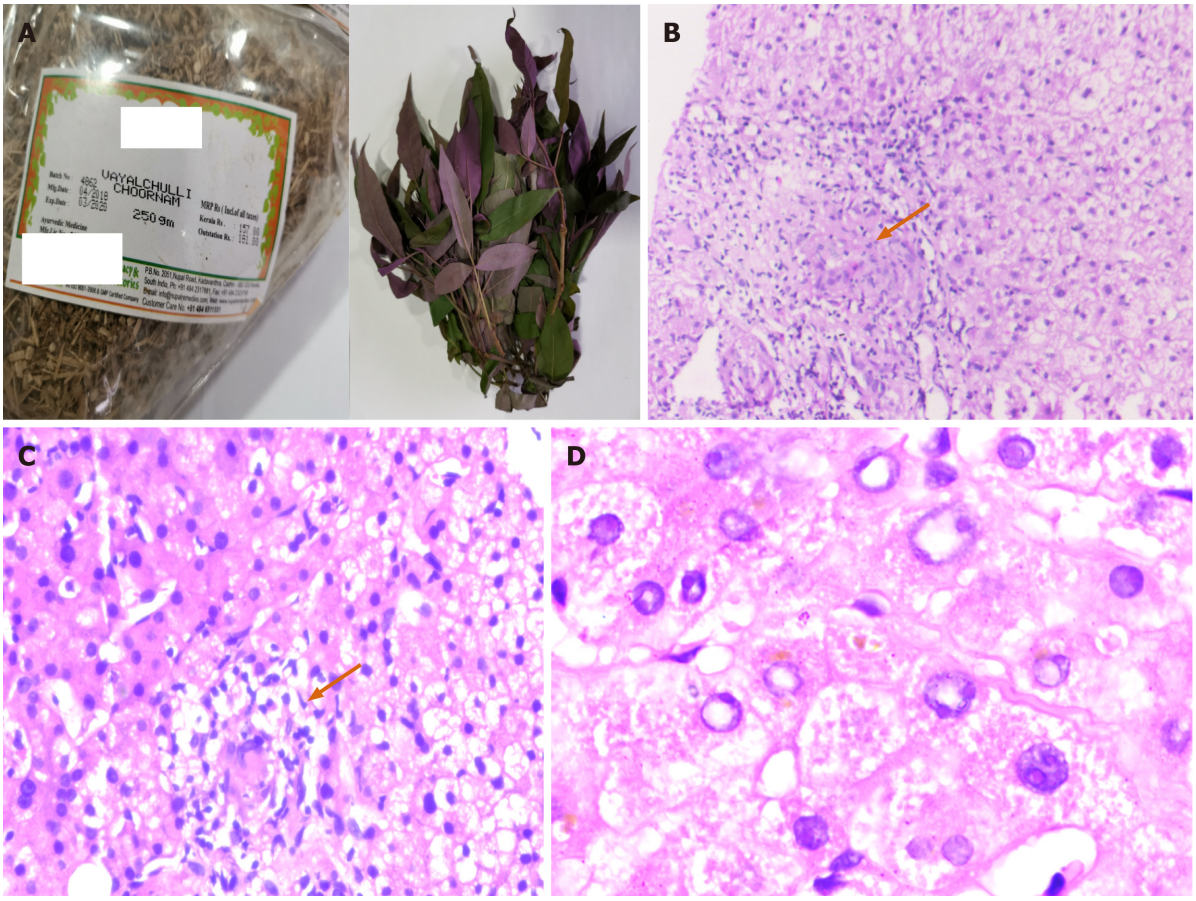

Figure 2 Granulomatous hepatitis in a patient consuming multiple Ayurvedic herbs. The retrieved herbs implicated in acute liver injury with granulomatous hepatitis are shown in A (Hygrophila auriculate, also called the Marsh Barbel and an unknown coloured herb). Transjugular liver biopsy was performed in view of ascites in this patient. The liver histology revealed large vague as well as well-formed lobular and portal based granulomas and associated eosinophilic and lymphocytic inflammation within the sinusoids [B and C; hematoxylin and eosin (H&E), 100 × and 400 × respectively]; severe feathery degeneration of hepatocytes associated with hepatocellular cholestasis was also notable (D, H&E, 400 ×). This patient died after 6 mo follow-up due to portal hypertensive events complicated by septic shock.

resolved spontaneously on drug withdrawal, but reappeared after rechallenge (same dose for 2 wk) to resolve again after halting the turmeric supplement (Figure 3). To summarize, turmeric and its bioactive compounds have been implicated in rare but severe autoimmune hepatitis-like liver injury and rarely, severe self-limiting cholestasis with the potential to recur with drug rechallenge.

Commiphora wightii or mukul and Boswellia serrata (Guggul, Guggulu or Gugulipid)

Guggul is the gum resin procured from the white sap of various plants used in Ayurvedic practice. Two plants, *Commiphora wightii* (Indian bdellium or myrrh tree) and *Boswellia serrata* (Salai or Sallaki guggul or Shallaki from Indian olibanum or frankincense tree) are commonly utilized to prepare guggul. Guggul is considered to have beneficial effects on multiple organ systems and is used to treat symptoms ranging from leg swelling and non-specific ulcers to diseases such as inflammatory bowel disease and aggressive malignancies. The main ingredients of guggul are guggulsterone, guggulsterol, boswellic acid and an ethyl-acetate soluble fraction called guggulipid consisting of highly bioactive phytochemicals. A large number of *in vitro* and *in vivo* studies have shown that guggul and its bioactive components act on multiple molecular targets leading to anti-inflammatory, antioxidant, and anti-apoptotic activity. This has led to the use of guggul for conditions such as arthritis, in fat-burners, for dyslipidemia and cardiovascular health. However, studies on the safety and clinical efficacy of guggul or its specific bioactive components are non-existent in published literature[28]. Grieco *et al*[29] described the case of a 63-year-old woman who consumed an over-the-counter lipid-lowering Ayurvedic product called "Equisterol®" (containing guggul sterol, sitosterol, chlorogenic acid, policosanol, multivitamins and red yeast rice derived monacolin) for 6 mo which was followed by the development of acute severe hepatitis. Liver biopsy revealed extensive

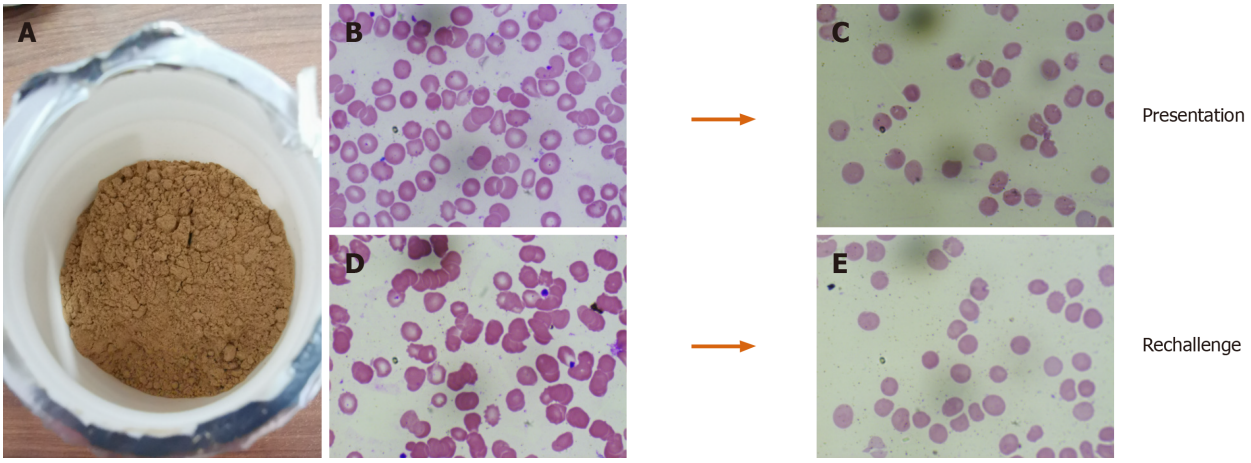

Figure 3 Turmeric-induced spur cell anemia in a patient with compensated cirrhosis. The retrieved turmeric powder supplement is shown in A; B and C (Wright Giemsa stain, 40 ×) shows the presence of spur cells and resolution of spur cells on turmeric withdrawal. On rechallenge (D and E; Wright-Giemsa stain, 40 ×) with turmeric supplement, spur cells reappeared without the need for blood transfusion, only to resolve after withdrawing the offending agent again.

necroinflammation with eosinophilic infiltration of hepatic lobules. Drug withdrawal and supportive care led to complete resolution of symptoms and normalization of liver tests within 10 d. The liver injury could have been due to the monacolin (with statins like activity) in red yeast, even though herb-yeast interaction was not ruled out[29]. Yellapu *et al*[30] described a female bodybuilder who consumed a multi-ingredient fat burner supplement leading to ALF. The supplements (Somalyz and Lipolyz, Species Nutrition, United States) contained usnic acid, L-carnitine, choline and ethanolamine, gamma-aminobutyric acid, vitamin E, green tea extract, guggulsterone Z, and guggulsterone E. She underwent cadaveric liver transplantation and was discharged uneventfully. Explant histopathology revealed massive hepatic necrosis and parenchymal collapse with a few areas of ductular regeneration. Even though various known plant-derived hepatotoxins (such as usnic acid, green tea extracts) were components of the supplement, the presence of guggul and its interactions were not ruled out. Guggul use has been implicated in the development of skin rash, diarrhea, headaches, nausea, and liver toxicity with high doses[30]. As reported by Polavarapu and co-workers, a 44-year old male developed fatigue, malaise, and jaundice after consuming a fat-burner product (Lipo-6™ containing guggulsterones and green tea extract) for 1 mo. Withdrawal of the herbal supplement resulted in complete clinical resolution after 1 mo[31]. Dalal *et al*[32] described a middle-aged woman who developed severe hepatocellular jaundice due to the intake of three different Ayurvedic herbal and Homeopathic medications (punarnaya mandur, extract from the *Boerhavia diffusa* and kanchnar guggulu, extract from *Bauhinia variegate*). Liver biopsy demonstrated mild portal chronic inflammation and interface activity with grade 3 bridging fibrosis, presence of ceroid-laden Kupffer cells, and conspicuous eosinophils suggestive of herbal-induced liver injury. Analysis of the retrieved herbal products and other medications did not reveal known hepatotoxic components, and the patient improved after a short follow-up[32]. Guggul and its bioactive compounds have been implicated in possible and probable DILI with hepatocellular pattern of liver damage, which is usually self-limiting. However, there have been reports of ALF requiring liver transplantation when guggul compounds have been part of multiherbal fat-burner products, a herb-herb interaction that remains unexplored (Figure 4).

Psoralea corylifolia (Bakuchi)

Psoralea corylifolia, commonly known as purple fleabane, is a popular herb used in Ayurvedic medicine for the treatment of various skin disorders. It is a perennial plant with growth throughout the plains of the Indian continent. Seeds of the plant (known as *Fructus Psoraleae, FP*) are mainly used for treating leprosy (hence the Sanskrit name, "kushtanashini" or destroyer of leprosy), psoriasis, leukoderma, and vitiligo. The monoterpenoid phenol called bakuchiol is the most important phytochemical component. Other constituents include coumarin compounds such as active psoralens, benzofuran derivatives and flavonoids[33]. A case of severe acute cholestatic hepatitis associated with *FP* was reported by researchers in South Korea in a post-menopausal woman consuming more than the recommended dose of the extract for osteoporosis. The liver biopsy findings were suggestive of zone 3 necrosis, cholestasis, and mixed

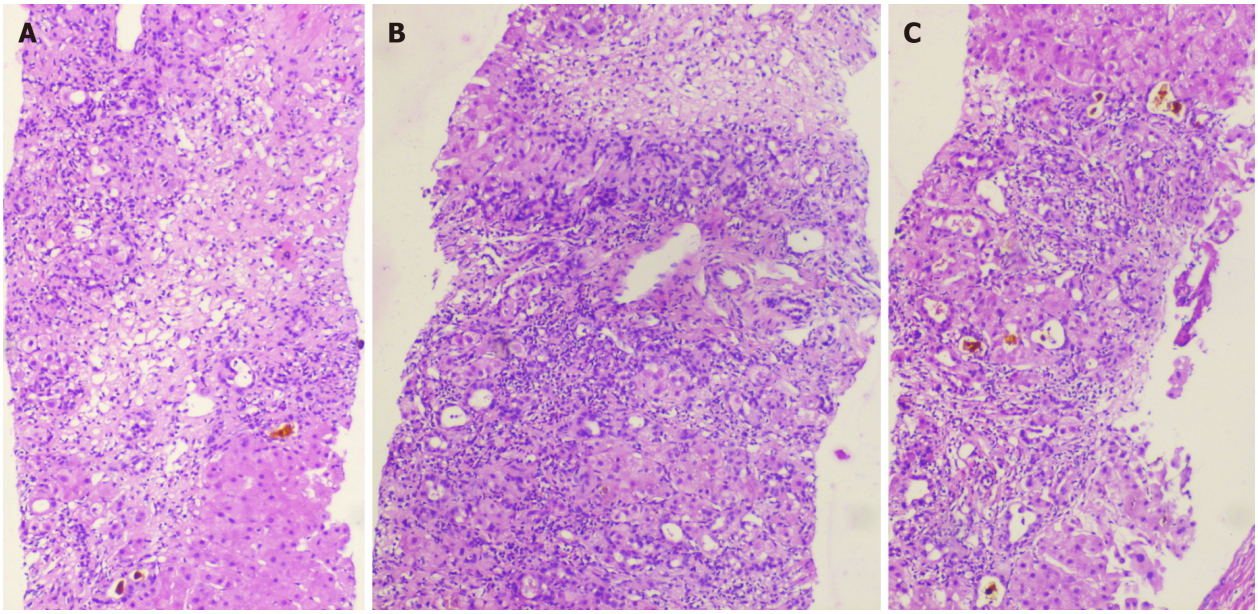

Figure 4 Guggul-related liver toxicity. The liver histopathology showed zone 3 necrosis extending to the portal region [A, hematoxylin and eosin (H&E) stain, 40 ×] with mixed neutrophilic and eosinophilic inflammation of the portal region associated with severe ductular reaction and early fibrosis (B, H&E stain, 100 ×). Associated moderate to severe canalicular cholestasis is also noted (C, H&E stain, 40 ×).

inflammation in the lobules[34]. Three further cases of severe hepatotoxicity due to *FP* was reported by Cheung *et al*[35]. The analysis of the retrieved proprietary medicines revealed psoralens and bakuchiol[35]. In another report, Bakuchi tablets were considered the cause of severe hepatocellular type liver injury in an older woman consuming Ayurvedic medicines for vitiligo[32]. A similar case was reported by Smith *et al*[36], of a 52-year old female who presented with a one-week history of jaundice and severe pruritus with abdominal pain after ingesting Bakuchi seeds for vitiligo. The liver biopsy revealed centrilobular necrosis with collapse, extensive cholestasis with mixed lobular inflammation. Clinical improvement and normalization of liver chemistries were notable at 3 mo follow-up[36]. Recently, Li and colleagues described a case of severe cholestatic hepatitis leading to fatal ALF due to the consumption of Bakuchi seeds over 7 mo in a 53-year-old with vitiligo. Studies have shown multiple mechanisms for Bakuchi-related liver toxicity. The psoralen-induced inhibition of the mTOR signalling pathway, mitochondrial injury, and impairment in liver regeneration with deleterious effects on liver lipid metabolism (such as reduction of mRNA expression of CYP7A1, HMG-CoA reductase, PPARα and increased expression of BSEP) along with dose and frequency related accumulation of psoralen has been shown to promote hepatotoxicity in small animal and *in vitro* studies[37-39]. To summarize, prolonged use of Bakuchi is associated with severe cholestatic hepatitis, mostly related to hepatotoxic psoralen compounds and bakuchiol, that can lead to ALF and death in predisposed patients.

Cassia angustifolio (Indian Senna)

Cassia angustifolia or the Indian or Tinnevelly senna (containing tinnevellin glucosides) belong to the legume family of Fabaceae, which comprise mostly ornamental plants. Leaves and sometimes flowers and fruits of the senna plant are used in herbal teas and as laxatives in Ayurvedic and Egyptian or Alexandrian (species known as *Cassia acutifolio*; containing 6-hydroxymusicin glucoside) traditional medicine. Some consider senna to be a safe herbal alternative for weight loss. The major bioactive components are anthracoids (sennoside A and B), which are primarily anthraquinone glycosides[40]. Beuers *et al*[41] described a 26-year old nurse who presented with severe cholestatic hepatitis after consuming high doses of senna-based medicines for 1 mo (senna fruit extracts corresponding to 100 mg sennoside daily along with 10 g of folia sennae laxative herbal tea twice weekly). Her liver biopsy showed extensive necrosis around the central veins, moderate portal and lobular infiltration of lymphohistiocytes and occasional plasma cells in the absence of autoantibodies. After drug withdrawal, liver tests, and clinical symptoms normalized, only to recur after resuming senna medications after 2 mo, and improving again, after drug withdrawal. Sennosides are converted into rhein anthron in the intestine most commonly by *Escherichia coli*, which

is then absorbed through the intestinal mucosa, glucuronidated, or sulfated and excreted through feces and urine. Rhein anthron is structurally similar to danthron, which is a well-known hepatotoxic laxative. The anthraquinones in rhubarb of which rhein anthron is a major component has been shown to be associated with liver injury[41]. A similar case was reported by Sonmez *et al*[42] in a 77-year old male in whom liver biopsy revealed bridging hepatocellular necrosis and canalicular cholestasis with slow but steady normalization of DILI on long-term follow-up. Seybold *et al*[43] reported the case of a young woman with CYP2D6*4 homozygous variant in whom, the repeated use of comparatively small quantities of senna herbal tea led to severe acute hepatitis which resolved after drug withdrawal. The authors concluded that "poor metabolizers" of phase I hepatic detoxification were predisposed to severe liver injury due to senna[43]. Vanderperren *et al*[44] demonstrated that chronic ingestion of large amounts of senna fruits in the form of herbal tea led to ALF associated with acute kidney injury and severe coagulation failure in a 52-year old woman. Investigations revealed large amounts of cadmium on urine toxicology consistent with possible contamination of the herbal product with heavy metals[44]. Cadmium is a well-known cumulative nephrotoxic and hepatotoxic agent, demonstrated in multiple Ayurvedic herbal products as a contaminant or adulterant and associated with severe liver injury[4,5,45,46]. Severe hepatotoxicity leading to fatal ALF was noted in an older woman consuming *Senna occidentalis* (*S. occidentalis*) herb for constipation. *S. occidentalis* is a common weed that is considered highly toxic to cattle and small herbivores. Recurrent annual outbreaks of a hepato-myo-encephalopathy syndrome in children in western Uttar Pradesh in India due to *S. occidentalis* poisoning was also reported by Vashishtha *et al*[47,48]. In the absence of proper regulatory standards, oversight, and poor manufacturing practices associated with Ayurvedic medicines, contamination of classical senna preparations with similar but more toxic herbs remain a possibility for causes concerning sporadic liver injuries[49]. Portal vein thrombosis due to consumption of boiled, dried Indian senna leaves in a 42-year old woman without underlying comorbid disease or prothrombotic conditions was also reported in the literature[50]. To summarize, senna is associated with severe hepatocellular and cholestatic liver injury and possible renal injury due to the presence of anthraquinone alkaloids in predisposed patients. The clinical course is usually self-limiting with liver toxicity proven on re-challenge; however, in rare instances, fatal ALF has been reported.

Aloe barbadensis mille (aloe vera)

Aloe vera is a perineal, xerophytic, succulent, cactus-like shrub belonging to the Lily family used for centuries in traditional medicine for the management of skin diseases, wound healing; and orally as an anti-oxidant. Aloe is derived from the Arabic word "Alloeh, " meaning "shining bitter substance" while "vera" is Latin for "true" and called by Egyptians as the "plant of immortality. " The aloe leaf contains glucomannans such as acemannan, the anti-inflammatory glycoprotein alprogen and multiple anthraquinones such as aloin and emodin and the plant hormones auxins and gibberellins[51]. The first report of aloe-induced acute hepatitis was published by Rabe *et al*[52] from Germany in 2005. In their paper, a middle-aged woman developed acute severe cholestatic jaundice after ingesting aloe supplements (500 mg tablets) for 4 wk to delay "aging". The liver biopsy revealed portal and lobular lymphoplasmacytic infiltrates and eosinophilic granulomas with bridging necrosis and bilirubinostasis. The liver injury and symptoms abated after stopping the aloe tablets. The authors contemplated that the liver injury was possibly due to the presence of bioactive aloe alkaloids acting on the cytochrome P450 system resulting in detoxification process interference and direct action of biotransformation end metabolites causing hepatocytotoxicity[52]. Since its original description, multiple reports, including patient series from Korea and Sweden and an extensive literature review on aloe-induced hepatitis, have been published in the literature. Liver biopsy findings in subsequent studies also featured severe hepatocyte ballooning, apoptosis, extensive cholestasis, and the presence of eosinophils predominant lobular inflammation[53,54]. Apart from hepatotoxicity, additional systemic toxicity in the form of severe intraoperative bleeding due to probable interaction between aloe plant-derived prostaglandins and sevoflurane during leg surgery in a young woman; renal failure due to over-dosing on aloe products; aloe-induced Henoch-Schonlein purpura and cathartic melanotic colon with adenomas are reported with aloe vera use[55]. Patient series of herbal and dietary supplements induced liver injury, mostly due to Herbalife® products, containing aloe-vera extracts leading to severe liver injury are also well documented in certain pharmacovigilance registries such as the Spanish DILI registry[56]. Even though *in vitro* and *in vivo* studies have demonstrated the detoxifying and hepatoprotective potential

of aloe vera, well-conducted translational clinical trials in humans confirming this aspect are still lacking in published literature. Aloe-related acute decompensation of cirrhosis in a chronic hepatitis C virus patient, presenting with acute severe cholestatic jaundice, was reported by Vázquez-Fernández *et al*[57]. To summarize, oral aloe vera use ranging from 2 wk to 24 wk, reportedly leads to severe cholestatic hepatitis among predisposed patients as well as acute severe decompensation in patients with underlying chronic liver disease. The injury is mostly of the hypersensitive type and self-limiting but may have a prolonged morbid course (Figure 5).

Morinda citrifolia (Indian mulberry or Noni juice)

Morinda citrifolia, commonly known as noni, belongs to the coffee family cultivated throughout the tropical countries. Due to its strong and pungent odor, it is also known as cheese fruit or vomit fruit. Noni-related products in the form of juice or extracts are considered by traditional medicine practitioners to benefit diseases from mouth ulcers to diabetes and HIV-AIDS even though high quality and reproducible studies demonstrating alleged clinical efficacy are non-existent in the literature. Published data on the efficacy and safety of noni juice and extracts are limited to *in vivo* and *in vitro* studies that are widely (and inaccurately) quoted by manufacturers and sellers of noni juice as strong evidence for its practical and safe use. Compositional analysis of noni demonstrated alkaloids (xeronine), polysaccharides, anthraquinones (damnacanthal, morindone), and glycosides such as citrifolinoside[58]. Austrian researchers described a 45-year-old man who consumed noni juice for 3 wk as a "prophylactic antioxidant," and developed acute hepatitis. The liver biopsy revealed severe mixed inflammation of the portal tracts along with marked eosinophils, hepatocellular cholestasis in zone 3, and histiocytic infiltration of the sinusoids. Complete resolution occurred within ten days of stopping noni juice[59]. Stadlbauer *et al*[60] described the occurrence of ALF requiring liver transplantation in a 29-year-old man and severe acute hepatitis due to consumption of noni juice for 3-mo in an elderly woman with a self-limiting disease course in the latter. The biopsies revealed severe mixed inflammation of portal tracts and lobules along with extensive centrilobular necrosis and severe ballooning associated with mixed inflammation[60]. Nonetheless, the development of ALF in the former patient cannot be solely attributed to noni juice since multiple other potentially toxic herbal supplements such as Skullcap (a known hepatotoxic Chinese herb), and green tea extracts were also consumed during the same period. Further reports of liver injury due to noni juice have been published from Croatia, Germany, and recently the United States. In these cases, where liver biopsy was performed, the findings were similar to previously described cases – a predominant hepatocellular form of liver injury with a mixed pattern of inflammation with marked eosinophilic infiltration of portal tracts, perivenular hepatocellular zonal necrosis with hepatocellular or canalicular cholestasis. All had a self-limiting disease course with complete clinical resolution within weeks to months[61]. Manufacturers and proponents advocating the benefits of noni juice with vested business interests have been keen to publish their retaliation against published literature on noni juice-related hepatotoxicity. However, almost all of their justifications have been biased and based on anecdotal and narrative evidence especially the efficacy and safety aspects of noni juice since idiosyncratic hepatotoxicity as a possibility has never been ruled out[62]. Further to this, multiple instances of the United States Food and Drug administration serving warning letters to manufacturers for making false claims on the curative and health benefits of noni has been in the limelight recently[63]. Current literature on the hepatotoxicity of noni juice demonstrates a possible self-limiting idiosyncratic type of herb-induced liver injury, probably due to anthraquinones, presenting with a severe hepatocellular type of liver damage with a predominance of eosinophilic portal inflammation, central necrosis, and cholestasis.

Ayurvedic herbs containing pyrrolizidine alkaloids

Alkaloids belong to the group of amino acid-derived, nitrogen-bearing molecules displaying a wide range of biochemical activities. Pyrrolizidine alkaloids (PAs) are those derived from ornithine and are commonly found as esters formed by a necine base (amino alcohols) and one or more necic acids (mono- or aliphatic dicarboxylic acids). Accordingly, based on the structure of the necine base, PAs may be sorted into four groups: Retronecine-, heliotridine-, otonecine- and platynecine-types. The following plant species - *Heliotropium sp., Trichodesma sp., Symphytum sp.* (known as Comfrey), *Senecio sp.* (used in Bush teas), *Eupatorium sp., Crotalaria sp.* (commonly used in Indian Ayurveda), *Chelidonium majus sp.*, and *Castilleja sp.* are known to be rich in PAs. In 1968, Mattocks was the first to describe the hepatotoxicity mechanism of PA, which leads to hepatic veno-occlusive disease, currently known as hepatic sinusoidal

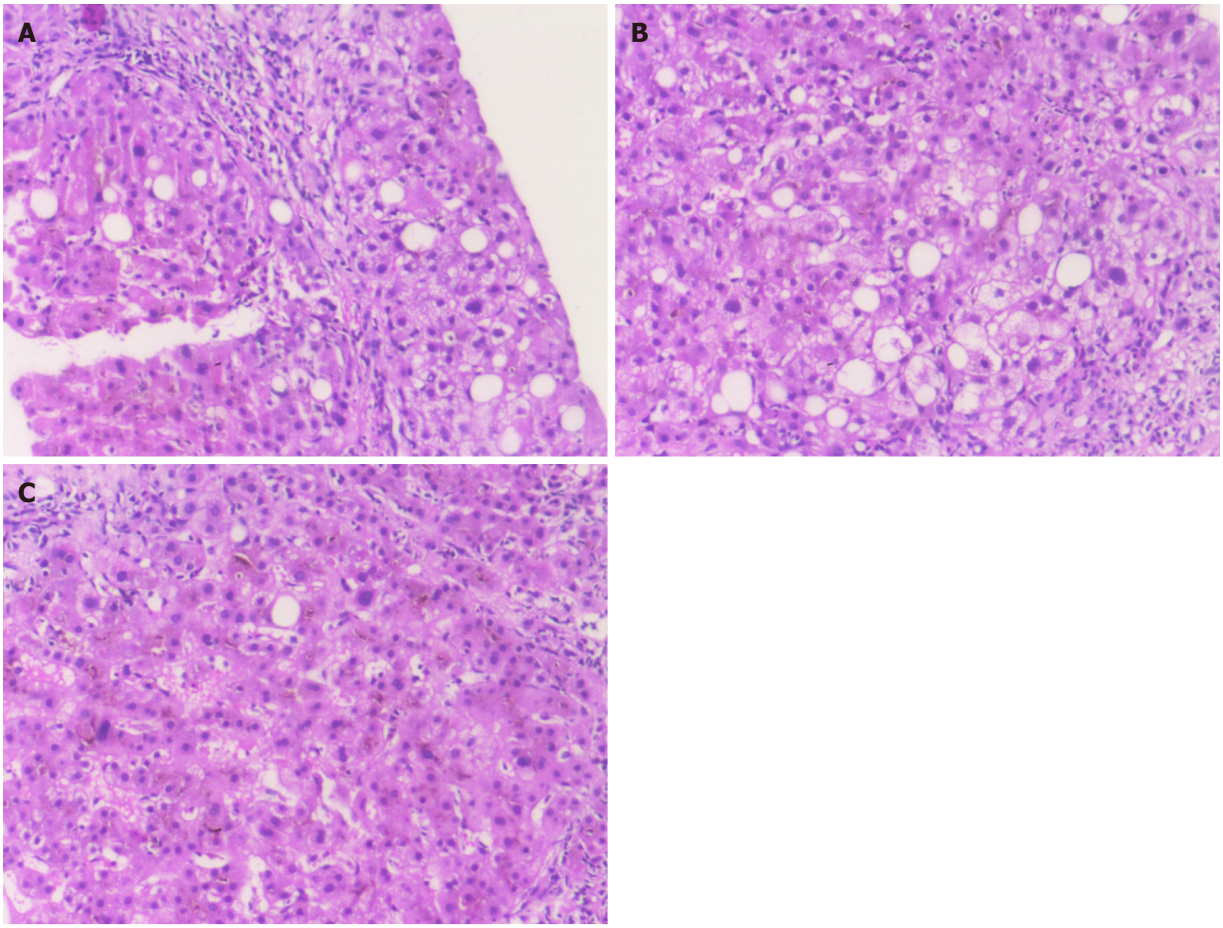

Figure 5 Aloe vera-induced liver injury in a middle-aged diabetic woman. The percutaneous liver histopathology showed porto-portal bridging necrosis [A, hematoxylin and eosin (H&E) stain, 200 ×] associated with moderate to severe ballooning of hepatocytes, hepatocyte fall out and neutrophilic satellitosis (B, H&E stain, 400 ×). Severe intrahepatic and canalicular cholestasis is also noted (C, H&E stain, 400 ×).

obstruction syndrome (HSOS), a condition that can lead to liver failure potentiating organ transplantation or to cirrhosis and portal hypertension[64]. PAs are transformed and activated into intermediate metabolites of which dehydropyrrolizidine alkaloids (DHPAs) are of significance. DHPA bind with groups containing sulfur, nitrogen, and oxygen present in proteins to form adducts that penetrate the nucleus and react with DNA, ultimately causing DNA cross-links and DNA-protein cross-links resulting in genotoxicity and abnormal cellular function primarily in the liver, leading to hepatocyte damage. These adducts pass to the adjacent space of Dissé as well as the sinusoidal lumen, where they injure the sinusoidal cells leading to HSOS. Apart from the liver, DHPA can also reach the pulmonary arterioles leading to secondary pulmonary hypertension and, in the long-term, congestive heart failure. Tricodesmine, a PA metabolism byproduct is neurotoxic and can cause encephalitis, vertigo, delirium and coma. The case of HSOS in a newborn of a woman who consumed herbal tea prepared from *Tussilago farfara* L showcasing the teratogenicity of PA is also reported[65]. The cognizance that PA-containing herbs can promote HSOS development comes from the identification of a large number of sporadic cases from Africa and the Indian subcontinent among those consuming herbal teas and ingesting traditional healer concoctions. Cross-contamination of herbal teas with PA-rich herbs leading to liver toxicity is also described. In India, consumption of honey processed (during pollen drying) using PA containing *Crotalaria juncea* L has been demonstrated to promote PA toxicity[66]. Figure 6 demonstrates the serial liver biopsy of a young male who developed HSOS leading to cirrhosis and portal hypertension at one year follow-up, after consuming over-the-counter capsule extracts of *Holarrhena antidysenterica (pubescens)* for "indigestion" for 1 mo. *H. antidysenterica* contains PAs, and rats fed with extracts demonstrated liver toxicity in the form of injury to centrilobular veins, centrilobular sinusoidal hemorrhage, congestion and centrilobular and focal hepatocellular necrosis compatible with PA-induced damage[67]. Thorough knowledge of herbs promoting chronic and sub-acute liver injury, and careful assessment of

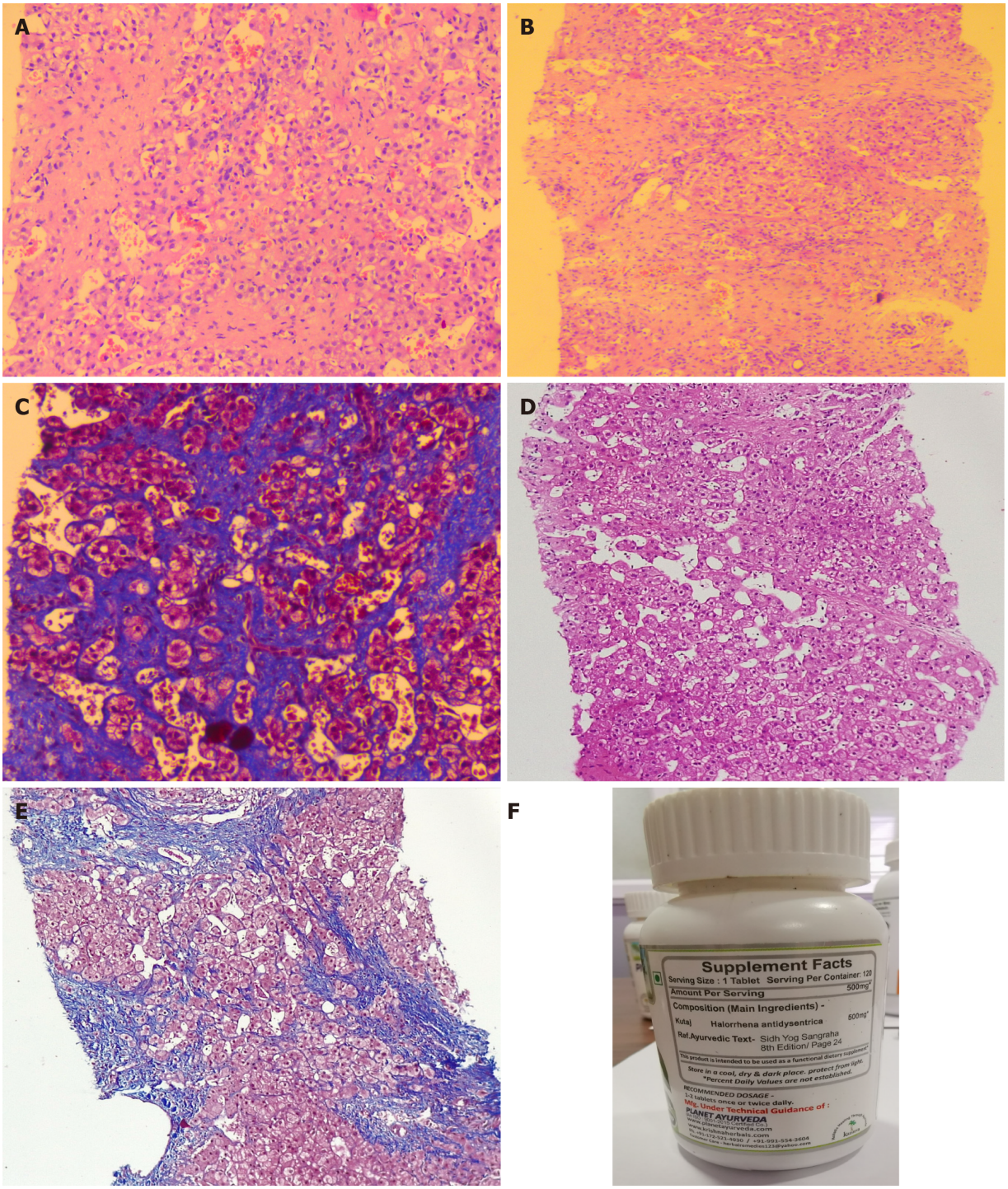

Figure 6 Hepatic sinusoidal obstruction syndrome in a young male who consumed *Holarrhena antidysenterica*, known to contain pyrrolizidine alkaloids. Transjugular liver biopsy was performed in view of ascites. The liver histology showed fairly preserved portal areas with extensive sinusoidal dilatation in all zones associated with early sinusoidal and peri-sinusoidal fibrosis [A and B, hematoxylin and eosin (H&E) stain, 400 ×]; the sinusoidal fibrosis is quite evident on Masson-trichrome stain (C, 400 ×); after one year follow-up, in view of recurrence of ascites and acute variceal bleeding, a repeat liver biopsy was performed which revealed prominent sinusoidal dilatation with sinusoidal and portal fibrosis (D, H&E stain, 200 ×) and cirrhosis changes with bridging fibrosis and partial nodule formation (E, Masson-trichrome stain, 200 ×). The retrieved over-the-counter herbal supplement is shown (F).

herbal components in polyherbal medications is mandated by physicians treating probable cases of DILI due to Ayurvedic drugs to diagnose rare but possible causes of herbal hepatotoxicity that may progress to chronic liver disease and portal hypertension.

Miscellaneous Ayurvedic Herbs

***Garcinia cambogia* (Malabar Tamarind):** Currently known as *Garcinia gummi-gutta* (L) Roxb, the fruit rind is extensively used as a flavouring agent in many parts of the

Indian subcontinent. In Ayurvedic medicine, it has been purported to be a safe remedy for constipation, rheumatic diseases, helminthic infestation, and as a weight-loss agent due to its appetite suppressing activity. Hydroxy-citric acid (HCA), an α-,β-dihydroxy tricarboxylic acid, is the major component present in the fruit rind, which may be responsible for the anorexic property. Garcinia fruit contains approximately 10% to 30% HCA, which reduces weight gain by inhibiting adenosine triphosphate (ATP)-citrate lyase, the enzyme responsible for catalytic reactions during fatty acid synthesis. Further appetite suppression occurs through increased release of serotonin neurotransmitter that dissipates eating behaviour. Multiple small animal model studies have demonstrated the anti-diabetic, anti-obesity, hypolipidemic, anti-inflammatory, antimicrobial, anti-parasitic activity as well as hepatoprotective effects of *Garcinia*. However, none of these have translated into clinical practice or confirmed in randomized studies. Those endorsing the safety and efficacy of *Garcinia* uphold their beliefs on the basis of low quality studies[68]. A randomized double-blind controlled trial on *Garcinia* failed to show significant change in fat mass and body weight compared to placebo, while a recent meta-analysis demonstrated an uncertain overall effect on long-term weight[69,70]. A total of 24 case reports and 8 case series reporting adverse events among 66 patients after *Garcinia* extract consumption was published in the literature. Of these, 17 studies out of 32 describe cases of acute liver injury, liver failure, and hepatotoxicity, observed among 50 patients consuming pure *Garcinia* or supplement mixtures. The latency period lasted from a few days to one year with continued ingestion in some patients. Two patients died, eight required liver transplantation, while one developed chronic DILI leading to cirrhosis[71,72]. Hepatotoxic adulterants and contaminants such as high levels of chromium, cadmium, and thallium in marketed *Garcinia* supplements leading to acute liver injury have been reported in the literature[73]. *Garcinia cambogia* has been implicated in acute self-limiting hepatitis, as well as ALF requiring liver transplantation and progression to cirrhosis. Furthermore, poor manufacturing practices resulting in adulteration and contamination of proprietary *Garcinia* supplements add to the hepatotoxic potential of *Garcinia* independently or synergistically.

***Gymnema sylvestre* (Cow plant or "Gurmar"):** Known as the "sugar destroyer," this perennial woody climbing herb belonging to the milk-weed family is commonly used for treating diabetes mellitus. The most important phytochemical constituents include triterpene saponins, which are collectively called gymnemic acids, the polypeptide gurmarin, and the alkaloid conduritol. Gymnemic acid type A is the most potent hypoglycemic component, highly concentrated in the shoot tips and least in the seeds. Some of the alkaloids and saponins also act as appetite suppressants. Shiyovich *et al*[74] described the case of a 60-year-old female who developed severe acute hepatitis after consuming "gurmar tea" for diabetes for one week. The work-up for other competing causes of acute hepatitis was non-contributory. The liver histopathology revealed lobular disarray with severe necroinflammatory changes in the hepatic lobules, marked ductular proliferation, and neutrophilic infiltration of portal areas. A short course of corticosteroids and "gurmar" withdrawal resulted in complete resolution of symptoms[74]. *Gymnema* is also one of the components of the well-described hepatotoxic herbal and dietary weight loss supplement, Hydroxycut®[75]. Physicians caring for patients with DILI and patients with diabetes mellitus opting for additional alternative therapies must be made aware of the potential liver-damaging effects of herbs such as *Gymnema* (Figure 7).

***Tribulus terrestis* ("Gokshura" / Goat's head / Devil's weed or Puncture vine):** *Tribulus terrestris* (TT), a tap-rooted herb belonging to the caltrop family, is native to regions with a tropical climate. It is known to be a noxious weed with a woody prickly fruit. Its use in Ayurveda for impotence and sexual disorders, and its use as a dietary supplement among bodybuilders and athletes, stems from the belief that it increases testosterone level, has anabolic steroid properties, and improves muscle strength – efficacy that lacks scientific proof through quality studies. The main biologically active phytochemicals in TT are steroidal saponins (mainly protodioscin, prototribestan, and dioscin), flavonoids, Harman alkaloids and lignan amides. TT ingestion is known to produce tribulosis (renal and liver injury) associated with the accumulation of phylloerythrin and beta-carboline alkaloids in the blood of cattle. It also causes staggers (neuromuscular ataxia) and geeldikkop (cholestatic liver injury and bile cast nephropathy) in sheep[76]. Talasaz *et al*[77] described a 28-year-old man who, after ingesting "Tribulus Water," developed severe liver (transaminases > 40 times the upper limit of normal) and kidney dysfunction, and seizures leading to hypertensive crisis and advanced azotemia due to acute renal tubular dysfunction requiring

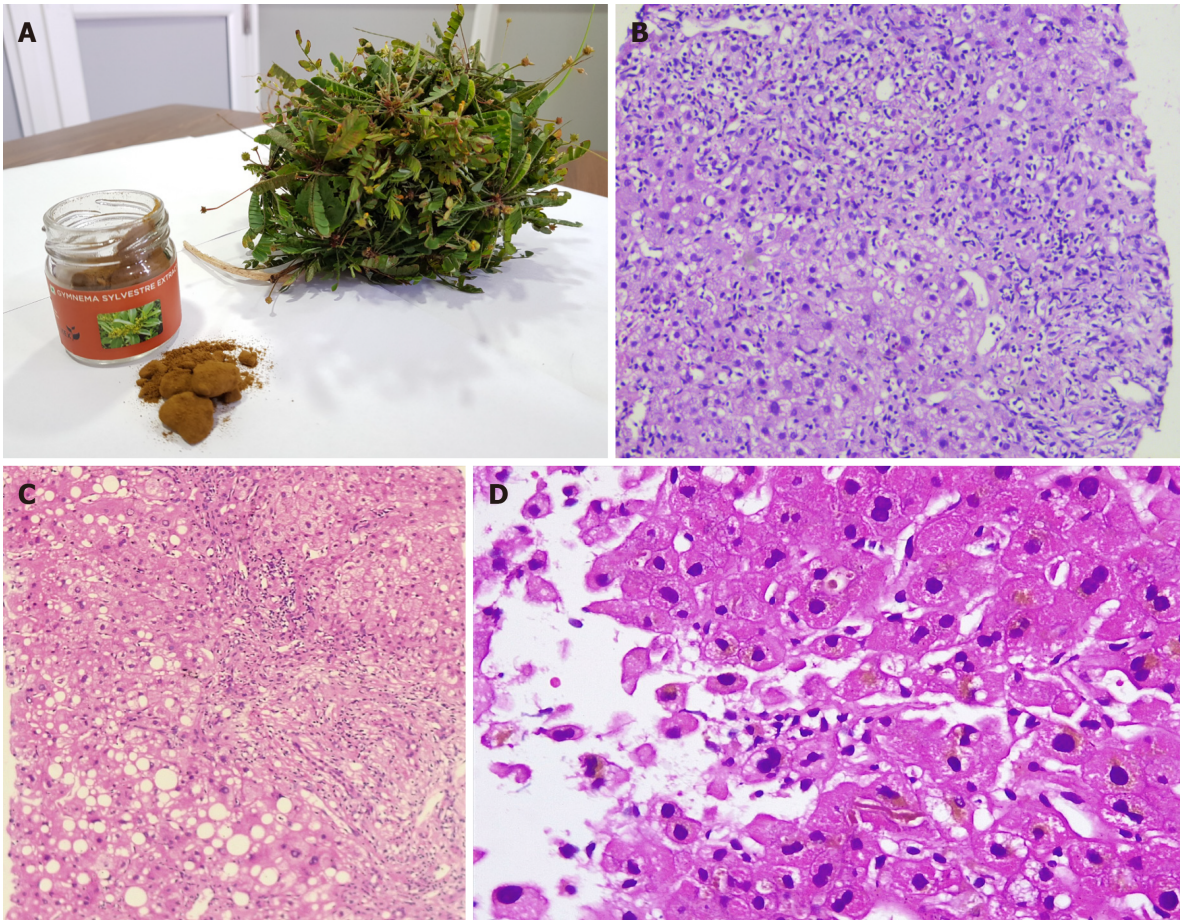

Figure 7 Acute cholestatic hepatitis due to *Gymnema sylvestre* (gurmar or sugar destroyer) herb. The retrieved herbal supplement and an unknown herb is shown in A. Liver biopsy revealed moderate to severe neutrophil predominant interface hepatitis [B, hematoxylin and eosin (H&E) stain, 200 ×]; micro and macrovesicular steatosis, lobular inflammation and severe ductular reaction (C, H&E stain, 200 ×) and intrahepatic cholestasis with eosinophilic degeneration of hepatocytes (D, H&E stain, 400 ×).

haemodialysis. After a stormy 2 wk hospital course, both liver and renal parameters improved, and the patient was discharged[75]. Similarly, Ryan *et al*[78] described a young, healthy male who ingested TT herbal tablets for a few months as part of body-building, who presented with severe jaundice and pruritus, followed by acute renal failure with bile-casts in the renal tubules on histopathology evaluation. A percutaneous liver biopsy showed only severe cholestasis, well-preserved hepatic lobules without inflammation supporting evidence for cholestasis-induced renal damage in the patient, possibly due to TT use[78]. These reports shed light on the role of herb-induced non-serious liver injury, such as bland cholestasis indirectly leading to severe extrahepatic organ failure.

***Valeriana officinalis* (Valerian):** Valerian is an herbal root extract form of *Valeriana officinalis*, a perennial flowering plant growing in Europe and Asia. The name is derived from the Latin verb "valere" meaning "strong, healthy." Valerian is known to contain multiple bioactive phytochemicals such as actinidine alkaloid, valerianine, valerene and, gamma-aminobutyric acid. It is used as an anxiolytic and sedative in Ayurvedic medicine. Potential mechanisms for sedation are not yet fully identified as phytochemicals such as valeric acid, and other root-specific components did not demonstrate sedative properties in experimental models. It is believed that valerenic acid by acting on the 5-HT_{5A} serotonin receptor modulates the sleep-wake cycle. The doubtful use of valerian as a sedative is underscored by a report on overdose in which the patient presented with mild symptoms that resolved within 24 h after consuming 20 times the recommended dose[79,80]. Valproic acid is a derivative of valeric acid. A small number of case reports have showcased hepatotoxicity associated with valerian. MacGregor *et al*[81] were the first to report on the potential hepatotoxic effects of valerian in 1989 in a series of four patients, all of whom consumed over-the-counter sleeping pills that also included other herbs. The presence of Chinese skullcap was

thought to be the major inciting agent in these patients, but caution was advised against using valerian-based herbal combinations[81]. Cohen *et al*[82] described a young woman who developed self-limiting severe acute cholestatic jaundice after consuming capsules of valerian root extract 300 mg twice daily for 3 mo in the absence of other competing causes. Vassiliadis *et al*[83] described a 50-year-old woman with valerian extracts and tea consumption for 3 wk, after which she developed acute hepatitis. The liver biopsy showed mild portal fibrosis, lymphocytic, and eosinophilic inflammation of portal tracts, inflammatory changes of the small bile duct with ductular reaction and necrosis in the perivenular zone. Liver injury resolved after 10 mo on conservative management[83]. In a study on herb-induced liver injury in the Berlin Case-Control Surveillance program, possible causality for valerian-induced DILI was notable in five (four females) patients. The liver injury was hepatocellular and cholestatic in type, and two patients developed jaundice. In two patients, biopsy revealed extensive necrosis while in one, it was perivenular (zone III) in nature, with all showing severe lobular and portal-based mixed type of inflammation. The disease course was self-limiting with symptom resolution and improvement of liver dysfunction on follow-up[84]. In Figure 8 we illustrate the baseline and follow-up liver histopathology of a young woman who developed severe acute cholestatic hepatitis that progressed to chronic DILI and chronic liver disease at 6 mo follow-up, after consuming Indian valerian (*Valeriana wallichi*) root extract (Tagara®, The Himalaya Drug Company) for three to seven days. A summary of all discussed Ayurvedic herbs and their hepatotoxic details is shown in Table 1.

CONCLUSION

Ayurvedic herb-related hepatotoxicity and liver injury can present as asymptomatic minor transaminase elevations, acute and chronic hepatitis, granulomatous hepatitis, asymptomatic to severe cholestasis, sinusoidal obstruction syndrome, acute liver failure requiring transplantation as well as progression to cirrhosis and portal hypertension. It is essential that physicians and specialists caring for patients with acute and chronic liver disease understand the small, but central role of herb-induced liver injury in a subset of patients who follow complementary and alternative medical practices and in whom an etiology of liver disease cannot be ascertained after extensive and conventional evaluation. The type of herb-induced liver injury and knowledge of its natural course is also important for treatment decisions and prognostication. Further studies that focus on the identification of beneficial and toxic components in Ayurvedic herbs, regulated curbing on the use of polyherbal formulations and educating the masses through public-industry partnerships, on the potential severe toxicity of certain herbs remain an unmet need.

Table 1 A summary of all Ayurvedic herbs implicated in liver injury with potential mechanisms of toxicity, liver injury pattern, histopathology patterns and clinical outcomes

Ayurvedic herb	Author/study /country	Pattern of liver injury (hepatocellular, cholestatic or mixed type)	Liver biopsy features	Suspected potential toxic component (s)	Clinical outcomes and comments
Withania somnifera (Ashwagandha)	Inagaki *et al*[10]/report/Japan	Cholestatic type	Intrahepatic cholestasis, canalicular bile plugs	Unclassified triterpenoids	Resolved, survived
	Björnsson *et al*[11]/series (*n* = 5)/Iceland and United States	Cholestatic and mixed type	Cholestatic hepatitis		Resolved, survived
Bacopa monnieri, Centella asiatica (Brahmi or Gotu-kola)	Teschke *et al*[14] /report/Germany	Hepatocellular type	Not performed	Saponin triterpenoids. phytoglycosides, autoantibody or immune-mediated	Resolved, survived. However, multiple other associated herbals used
	Jorge *et al*[17]/series (*n* = 3)/Argentina	Mixed type	Granulomatous cholestatic hepatitis		One patient progressed to cirrhosis and acute decompensation on repeat herb intake; in another, complete resolution noted
	Dantuluri *et al*[18] /report/United Kingdom	Hepatocellular type	Not performed		Acute liver failure, spontaneous resolution, survived
Curcuma longa (Turmeric)	Lukefahr *et al*[22]/ report/United States	Hepatocellular type	Autoimmune hepatitis	Immunomodulatory polyphenolic compounds, drug triggered autoantibodies related liver injury	Complete resolution
	Suhail *et al*[23]/report/United States	Hepatocellular type	Acute panlobular hepatitis with early parenchymal collapse		Complete resolution in 3 wk
	Lee *et al*[24]/report/United States	Hepatocellular type	Autoimmune hepatitis with additional eosinophilic and neutrophilic interface hepatitis		Complete resolution
	Imam *et al*[25]/report/United States	Hepatocellular type	Not performed		Complete resolution
	Luber *et al*[26]/two patient report/Australia	Mixed type in first case and hepatocellular type in second patient	Biopsy performed in first case showed mixed lobular inflammation and severe interface hepatitis; biopsy not performed in case two		Complete resolution in both patients, autoantibodies positive in second patient
	Chand *et al*[27]/report/Australia	Hepatocellular type	Acute hepatitis with mixed inflammatory infiltrate of lobules and interface hepatitis with focal necrosis and mild cholestasis		Complete resolution, no autoantibodies noted, but high immunoglobulin G was remarkable
Guggul / Guggulipids (as part of polyherbal formulations and weight	Grieco *et al*[29]/report/Italy	Hepatocellular type	Necroinflammation with lobular eosinophilic inflammation	Guggul sterols associated herb-herb and herb-drug interaction; no direct	Complete resolution

loss supplements)				hepatotoxicity described	
	Yellapu *et al*[30]/report/United States	Hepatocellular type	Massive hepatic necrosis		Acute liver failure necessitating cadaveric liver transplantation
	Polavarappu *et al*[31] /report/United States	Hepatocellular type	Not performed		Spontaneous resolution on drug withdrawal
	Dalal *et al*[32]/report/United States	Hepatocellular type	Mixed portal inflammation, interface hepatitis, eosinophilic lobular inflammation with ceroid laden macrophages		Spontaneous resolution on drug withdrawal
Psoralea corylifolia (Bakuchi, Babchi seeds)	Nam *et al*[34]/report/South Korea	Mixed type	Zone 3 necrosis, cholestasis and severe mixed inflammatory infiltration of lobules	Terpenoids like bakuchiol and coumarinoids	Complete clinical resolution
	Cheung *et al*[35]/series (n = 3)/	Hepatocellular type	Not performed		All three patients had uneventful recovery after drug withdrawal
	Smith *et al*[36]/report/United Kingdom	Hepatocellular type	Cholestatic acute hepatitis		Clinical resolution on drug withdrawal
Cassia angustifolio (Indian Senna)	Beuers *et al*[41]/report/Germany	Mixed type	Perivenular necrosis, lymphohistiocytic portal and lobular inflammation	Anthracoid sennoside and rhein anthron metabolites	Complete resolution, recurrence on rechallenge
	Sonmez *et al*[42]/report/Turkey	Mixed type	Bridging hepatocellular necrosis and canalicular cholestasis		Complete resolution
	Seybold *et al*[43] /report/Germany	Hepatocellular type	Not performed		Complete resolution
	Vanderperren *et al*[44]/Belgium	Hepatocellular type	Not performed	High amounts of cadmium on toxicology evaluation	Acute liver failure with renal failure, resolved on aggressive supportive care
Aloe vera	Rabe *et al*[52]/report/Germany	Cholestatic type	Portal and lobular lymphoplasmacytic inflammation, eosinophilic granulomas, bridging necrosis and bilirubinostasis	Aloe alkaloids, anthraquinones	Complete resolution on herbal drug withdrawal
	Parlati *et al*[53]/report and review series (n = 9)/France	Hepatocellular type	All patients had portal and lobular inflammation with neutrophils and lymphoplasmacytes along with granulomas, acidophil bodies, ballooning of hepatocytes, extensive bridging necrosis and bilirubinostasis		All cases had clinical resolution of symptoms, no acute liver failure and no chronicity noted on follow up
	Manso *et al*[56]/series on Herbalife® products/Spain	Hepatocellular type	Cholestatic hepatitis		Complete resolution after supplement withdrawal
	Vázquez-Fernández *et al*[57] /report/Spain	Cholestatic type	Cholestatic hepatitis with lymphocyte predominant portal inflammation		Acute decompensation of cirrhosis (underlying HCV related infection); outcome not reported
Morinda citrifolia (Noni juice)	Millonig *et al*[59]/report/Austria	Hepatocellular type	Severe mixed inflammatory infiltration of portal tracts with lobular eosinophilic inflammation and hepatocellular cholestasis in zone 3	Suspected noni anthraquinones	Complete resolution on herbal medicine withdrawal
	Stadlbauer *et al*[60] / two cases report/Austria	Both cases hepatocellular type	First case, confluent necrosis, second case centrilobular necrosis and mild inflammatory infiltration in both		First patient progressed to acute liver failure and underwent successful liver transplantation; second patient had complete clinical recovery on conservative care
	Yu *et al*[61]/report/United States	Hepatocellular type	Acute hepatitis with portal inflammation and periportal necrosis,		Paediatric case (14-year-old boy) with

			hepatocellular cholestasis and numerous eosinophils in lobules		complete recovery on supportive care
Heliotropium sp., Trichodesma sp., Eupatorium sp., Senecia sp., Crotalaria sp. Chelidonium majus sp., Castilleja sp. Holarrhena antidysenterica	Neuman *et al*[66]/review series > 30 cases/Canada	Hepatocellular and mixed type	Early pathologic changes include the deposition of fibrinogen and factor VIII within the venular walls and liver sinusoids. In acute stage, haemorrhage into markedly dilated sinusoids with hepatocyte atrophy is noted, the sinusoids become denuded, parenchymal collapse is evident followed by the constriction and obliteration of small central veins by subendothelial swelling or fibrosis. The sinusoidal fibrosis and nodular regeneration may occur leading to cirrhosis and portal hypertension at later stages	Pyrrolizidine alkaloids	The acute form is rapidly fatal in 20% to 40% of patients. Adults have worse prognosis that the paediatric age group. Approximately 15% with acute disease will progress to subacute or chronic injury, and develop end-stage liver disease in a few years.
Garcinia cambogia (Malabar Tamarind)	Crescoli *et al*[71] and Kothadia *et al*[72]/case series review, *n* = 66/	Hepatocellular type	Acute hepatitis with necroinflammation and parenchymal collapse is commonly noted on histopathology	Hydroxycitric acid and adulteration with heavy metals and other toxic ingredients	Commonly self-limiting even after a prolonged course; acute liver failure and acute on chronic liver failure leading to death described; in liver failure, transplantation has been performed to increase survival; chronic and prolonged DILI has been described leading to cirrhosis and portal hypertension
Gymnema sylvestre (Gurmar or sugar destroyer)	Shiyovich *et al*[74] and Dara *et al*[75]/series of patients including those consuming herbal and dietary supplements such as Hydroxycut® containing *Gymnema*	Mixed type	Necroinflammation of the lobules and portal regions with marked ductular proliferation and neutrophilic infiltration of the portal areas	Triterpene saponins, gymnemic acids and polyalkaloids	Usually self-limiting with complete resolution after herbal drug withdrawal
Tribulus Terrestis (Gokshura or Puncture vine)	Talasaz *et al*[77] and Ryan *et al*[78] /reports / Iran and United States respectively	Hepatocellular type	Not performed	Phylloerythrin and beta-carboline alkaloids are suspected toxins	Associated with seizures and acute kidney injury (mostly bile cast nephropathy); usually self-limiting and responsive to conservative care
Valeriana officinalis (Valerian)	MacGregor *et al*[81] series (*n* = 4) /Edinburgh	Hepatocellular type	Not performed	Suspected toxins include valerian alkaloids and sesquiterpenes	All patients had uneventful recovery after drug withdrawal and supportive care
	Cohen *et al*[82]/report/United States	Cholestatic type	Not performed		
	Vassiliadis *et al*[83] /report/Greece	Hepatocellular type	Lymphocytic and eosinophilic portal inflammation with perivenular necrosis and small bile duct damage		
	Duoros *et al*[84]/series (*n* = 5)/Berlin	Hepatocellular and mixed type	Extensive necrosis, lobular and portal severe necroinflammation with neutrophils, lymphocytes and eosinophils		

HCV: Hepatitis C virus; DILI: Drug-induced liver injury.

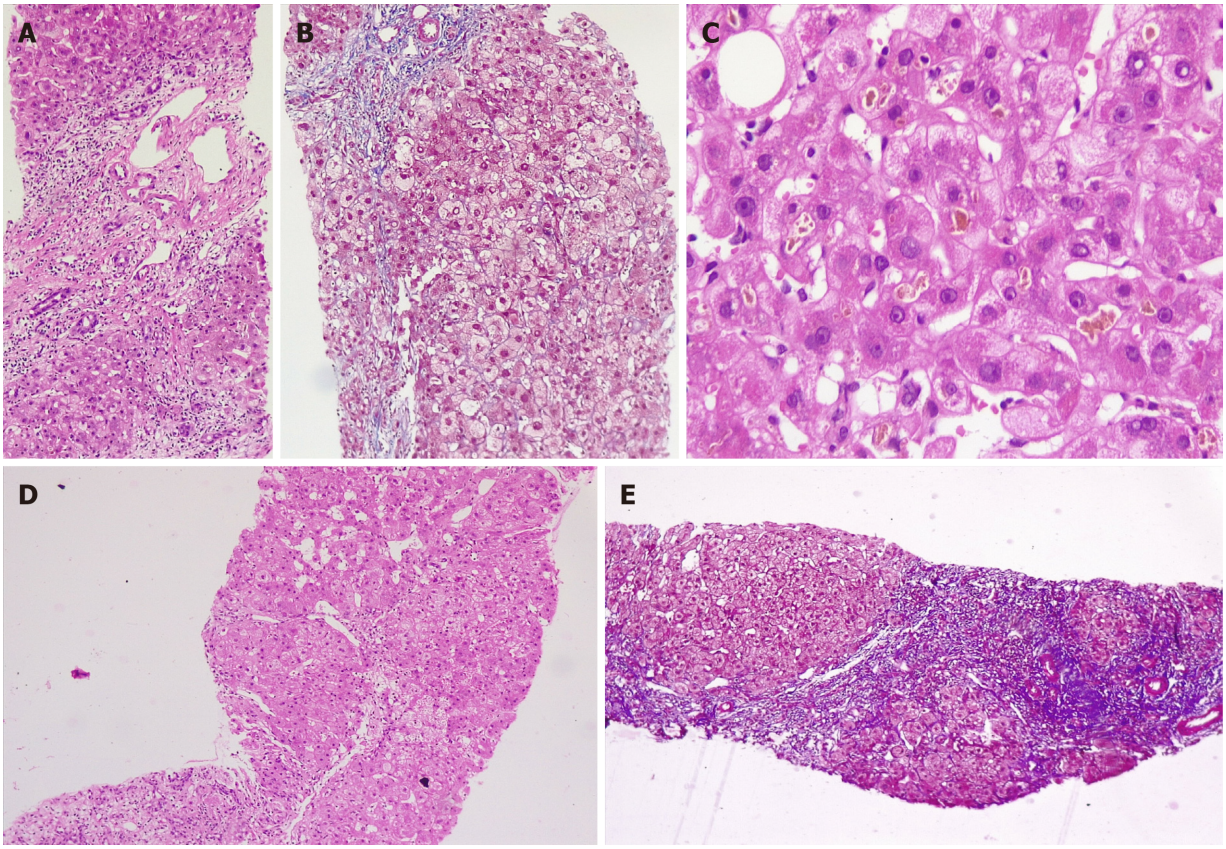

Figure 8 Development of acute severe cholestatic hepatitis leading to chronic herb-induced liver injury resulting in cirrhosis in a young female, after ingestion of Valerian (Tagara®). Percutaneous liver biopsy performed in August 2019 (A-C) revealed portal and periportal necrosis with mixed lobular inflammation [A, hematoxylin and eosin (H&E) stain, 100 ×], pale blue staining in the regions of necrosis and hepatocyte loss is notable on Masson-trichrome staining (B, 200 ×); hepatocellular and canalicular cholestasis is notable (C, H&E, 400 ×); on follow-up, liver biopsy showed progression of fibrosis with vague hepatocyte nodule formation (D, H&E, 200 ×) with central to central and central to portal bridging fibrosis suggestive of cirrhosis and nodule formation on Masson-trichrome stain (E, 100 ×).

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ORIGINAL ARTICLE

Basic Study

N-acetylcysteine and glycyrrhizin combination: Benefit outcome in a murine model of acetaminophen-induced liver failure

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Abstract

BACKGROUND

Acetaminophen overdose is the most frequent cause of drug-induced liver failure in developed countries. Substantial progress has been made in understanding the mechanism of hepatocellular injury, but N-acetylcysteine remains the only effective treatment despite its short therapeutic window. Thus, other hepatoprotective drugs are needed for the delayed treatment of acetaminophen-induced hepatotoxicity. Our interest focused on glycyrrhizin for its role as an inhibitor of high mobility group box 1 (HMGB1) protein, a member of the family of damage-associated molecular pattern, known to play an important pathological role in various diseases.

AIM

To investigate the efficacy of the N-acetylcysteine/glycyrrhizin combination compared to N-acetylcysteine alone in the prevention of liver toxicity.

METHODS

Eight-week-old C57BL/6J wild-type female mice were used for all our experiments. Mice fasted for 15 h were treated with acetaminophen (500 mg/kg) or vehicle (phosphate-buffered saline) by intraperitoneal injection and separated into the following groups: Glycyrrhizin (200 mg/kg); N-acetylcysteine (150 mg/kg); and N-acetylcysteine/glycyrrhizin. In all groups, mice were sacrificed 12 h following acetaminophen administration. The assessment of hepatotoxicity was performed by measuring plasma levels of alanine aminotransferase, aspartate aminotransferase and lactate dehydrogenase. Hepatotoxicity was also evaluated by histological examination of hematoxylin and eosin-stained tissues sections. Survival rates were compared between various groups using Kaplan-Meier curves.

RESULTS

Consistent with data published in the literature, we confirmed that intraperitoneal administration of acetaminophen (500 mg/kg) in mice induced severe liver injury as evidenced by increases in alanine aminotransferase, aspartate aminotransferase and lactate dehydrogenase but also by liver necrosis score. Glycyrrhizin administration was shown to reduce the release of HMGB1 and significantly decreased the severity of liver injury. Thus, the co-administration of glycyrrhizin and N-acetylcysteine was investigated. Administered concomitantly with acetaminophen, the combination significantly reduced the severity of liver injury. Delayed administration of the combination of drugs, 2 h or 6 h after acetaminophen, also induced a significant decrease in hepatocyte necrosis compared to mice treated with N-acetylcysteine alone. In addition, administration of N-acetylcysteine/glycyrrhizin combination was associated with an improved survival rate compared to mice treated with only N-acetylcysteine.

CONCLUSION

We demonstrate that, compared to N-acetylcysteine alone, co-administration of glycyrrhizin decreases the liver necrosis score and improves survival in a murine model of acetaminophen-induced liver injury. Our study opens a potential new therapeutic pathway in the prevention of acetaminophen hepatotoxicity.

Key Words: Acetaminophen; Acute liver injury; Glycyrrhizin; N-acetylcysteine; N-acetylcysteine/glycyrrhizin combination; Murine model; High mobility group box 1

Core Tip: Acetaminophen overdose is the most common cause of drug-induced liver failure in the developed countries. Substantial progress has been made in understanding the mechanism of hepatocellular injury, but N-acetylcysteine remains the only effective treatment despite its short therapeutic window. We present here our first results on the combination of N-acetylcysteine and glycyrrhizin in a murine model of acetaminophen-induced liver injury. Acetaminophen toxicity was induced by an intraperitoneal dose of 500 mg/kg. Hepatotoxicity was assessed by biochemical and histopathological analyses. Survival rates were also compared. Our results suggest, for the first time, that the combination of N-acetylcysteine and glycyrrhizin may be effective in preventing acetaminophen-induced liver injury in mice.

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INTRODUCTION

Acetaminophen, also known as N-acetyl-p-aminophenol (APAP), is one of the most widely used drugs for its analgesic and antipyretic properties. Although

for the care and use of laboratory animals. Animal protocols were approved by the local Ethic Committee of the Université Libre de Bruxelles (License No. 1230406, Animalerie de la Faculté de Médecine du Campus Erasme, Brussels, Belgium; Protocol Nos. 488N and 734N).

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acetaminophen is a safe and effective drug at recommended doses, it can cause hepatotoxicity and acute liver failure in the event of overdose[1,2]. The hepatotoxicity of acetaminophen remains the leading cause of acute liver failure in the United States and Europe but the mechanism of hepatotoxicity is still incompletely understood and therapeutic options are limited[3,4]. After ingestion, a majority (> 90%) of acetaminophen is metabolized by glucuronidation and sulfation reactions to produce non-toxic metabolites. A small fraction (< 10%), undergoing oxidation, is metabolized by CYP450 isoforms, mainly CYP2E1, to N-acetyl-p-benzoquinone imine (NAPQI), a toxic metabolite. Under normal conditions NAPQI, which binds covalently to cysteine groups on proteins (APAP adducts), is rapidly detoxified by glutathione (GSH)[5]. There is strong evidence that depletion of hepatic GSH and the covalent binding of NAPQI to cellular macromolecule contribute to protein modification and mitochondrial dysfunction with ATP depletion, leading to massive centrilobular necrosis[6].

N-acetylcysteine (NAC) is the standard therapy for treatment of APAP overdose. This drug counters acetaminophen toxicity by increasing the detoxification of NAPQI by direct conjugation with GSH or by increasing GSH synthesis[7]. In this way, NAC acts to prevent the accumulation of the toxic metabolites of APAP in hepatocytes and thereby prevents hepatocytes necrosis. However, to ensure effective treatment, NAC should be administered within 8-10 h after ingestion of acetaminophen[8,9]. Since the symptoms of APAP overdosage are often overlooked, the administration of NAC is often insufficient or ineffective due to its short therapeutic window. In addition, restoration of the GSH store is not sufficient to stop the progression of APAP-induced hepatotoxicity[10-12]. Thus, in case of acute liver failure, the only alternative remains liver transplantation, a rare resource associated with significant consequences (long-term immunosuppression, frequent medical follow-up, cost). New therapies are clearly needed.

As a medicinal resource, traditional Chinese herbs have attracted attention as food with health benefits and as herbal medicines. Glycyrrhizin (GL), an aqueous extract of licorice root, is composed of glycyrrhetinic acid and two molecules of glucuronic acid. In patients with chronic hepatitis, it is already commonly used in Japan and has been evaluated in therapeutic trials in Europe[13,14]. GL has various pharmacological actions, including anti-inflammatory, anti-viral, antioxidative, anti-liver cancer, immunomodulatory and cardioprotective activities. GL is also known for its hepatoprotective effects[15]. The different mechanisms of action of GL are not yet all known. However, GL has been described as an inhibitor of the high mobility group box 1 (HMGB1) protein that binds directly to both HMGB boxes and inhibits its cytokine activities[16]. In our previous *in vitro* experiments, we observed that APAP induced release of HMGB1 from damaged hepatocytes and we demonstrated the released HMGB1 contributed to the death of neighboring hepatocytes[17].

In the present study, we focused on the effects of GL, NAC, or co-administration of these two drugs in a murine model of APAP hepatotoxicity. The aim was to explore the efficacy of the combination of two drugs that act at different stages of the acetaminophen metabolism process and to evaluate the potential protective role of this combination in acute liver injury induced by APAP overdose.

MATERIALS AND METHODS

Animal model of APAP-induced liver injury and treatments.

Eight-week-old C57BL/6J wild-type female mice were obtained from The Jackson Laboratory (Bar Harbor, ME, United States). Upon arrival, the mice were acclimatized to laboratory conditions (21 °C, humidity 50%) for 1 wk prior to experimentation. Mice were maintained on 12-h light-dark cycle with free access to food and water in accordance with the Guide for the Care and Use of Laboratory Animals. Animal protocols were approved by the local Ethic Committee of the Université Libre de Bruxelles (Protocol Identifiers: 488N).

In all experiments, after 15 h fasting with free access to water, mice received an intraperitoneal injection of APAP at the dose of 500 mg/kg body weight. In some experiments, GL (200 mg/kg), NAC (150 mg/kg) or phosphate-buffered saline, as vehicle, was administered to the animals at various times after APAP injection. Mice were sacrificed at different time points after APAP challenge by cervical dislocation under anesthesia; blood was collected, and the liver was removed. Blood samples were centrifuged at 13523 × *g* for 5 min and supernatants were stored at -20 °C. Upon removal, the biggest lobe of each liver was fixed in 4% formaldehyde and three other

lobes were snap-frozen and stored at -80 °C for RNA isolation. For survival experiments, animals were followed for 172 h. Mice were euthanized when they became moribund per the criteria of lack of response to stimuli or lack of righting reflex.

Assessment of hepatotoxicity

Liver injury was determined by measuring plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) but also by histological examination of hematoxylin and eosin (H&E)-stained tissues sections.

ALT, AST and LDH measurements were performed using commercially available kits (Roche/Hitachi, Brussels, Belgium), based on methods recommended by the International Federation of Clinical Chemistry. Briefly, blood was collected by retroorbital puncture and centrifuged in a cold room (13523 × *g*; 5 min) before storage at -20 °C for 24 h. ALT and AST concentrations were then measured in plasma samples at 37 °C using a photometric method based on the rate of decrease of NADH, which is directly proportional to the rate of pyruvate formation and therefore to the activity of ALT or AST. The latter, directly proportional to the quantity of ALT or AST present in the cells, is expressed in International Units per liter (IU/L). The reading of the kinetics was conducted during 4 min at 340 nm. LDH concentration was measured by ultraviolet assay. Briefly, lactate dehydrogenase catalyzes the conversion of lactate to pyruvate; NAD is reduced to NADH in the process. The initial rate of the NADH formation is directly proportional to the catalytic LDH activity. It is determined by photometrically measuring the increase in absorbance.

Necrosis score was determined by histological examination of H&E-stained tissue sections. Briefly, the large lobes of the liver, taken after each sacrifice, were fixed in a buffered isotonic solution of pH 7.4 of 4% formaldehyde for at least 24 h. Then, they were cut in half widthwise and placed in cassettes which were placed directly into the formalin. After that, pieces of liver were dehydrated by successive baths of isopropanol and toluene and impregnated with paraffin to form blocks. Sections 5 µm thick were made using the microtome and deposited on glass slides, which were then soaked in gelatinous water. Finally, slides were incubated for at least 30 min in an oven at 35-40 °C before being stained with H&E to reveal the cell structures, respectively the nucleus and the cytoplasm.

H&E-stained slides obtained were then analyzed under an optical microscope in a blinded manner. Centrilobular necrosis following treatment with APAP was scored by a grading system as described previously[18].

Assessment of hepatic GSH

Assessment of hepatic GSH levels was performed using Bioxytech GSH-400 colorimetric assay kit and following the manufacturer's protocol (OxisResearch™, Foster City, CA, United States). Briefly, the lobe of the liver was washed with 0.9% NaCl before being blotted on paper and weighed. Then, tissue was homogenized in 5% ice cold metaphosphoric acid and centrifuged at 3000 × *g* for 10 min at 4 °C. Finally, the clear upper aqueous layer was collected for the assay. The enzyme concentration obtained is expressed as nmol of enzyme per milligram of protein using bovine serum as a standard. The protein concentration was evaluated in liver homogenates using Quick Start Bradford Protein Assay (Bio-Rad, Hercules, CA, United States).

Assessment of ratio GSH/the oxidized state (GSSG)

Assessment of the GSH/GSSG ratio was performed using GSH/GSSG Ratio Detection Assay Kit (Fluorometric - Green) and following the manufacturer's protocol (Abcam, Cambridge, United Kingdom). Briefly, the liver lobe was washed with 0.9% NaCl before being blotted on paper and weighed (20 mg of tissue was required by the protocol). Then, the tissue was homogenized in 5% ice cold metaphosphoric acid and centrifuged at 14000 × *g* for 10 min at 4 °C. The clear upper aqueous layer was collected and sample deproteinization was performed using trichloroacetic acid and sodium bicarbonate. After this step, thiol green indicator reaction mix was added to the deproteinized samples and the fluorescence measurement was performed (Ex/Em = 490/520 nm). In two separate assay reactions, GSH (reduced) was measured directly with a GSH standard and total GSH (GSH + GSSG) was measured by using a GSSG standard.

Enzyme-linked immunosorbent assay

HMGB1 concentrations in the plasma of mice were measured by a sandwich-enzyme

immunoassay (IBL International GmbH, Hamburg, Germany) following the manufacturer's protocol. Briefly, with the wells of the plate being coated with purified anti-HMGB1 antibody, the protein of interest binds specifically to the immobilized antibody during the first incubation (24 h, 37 °C). After the washing step to remove all unbound components of the starting sample, a second peroxidase-labelled antibody was distributed to the wells. After incubation (2 h, room temperature), the enzyme substrate (solution containing TMB and buffer with 0.005 M hydrogen peroxide) was added. The enzyme reaction took place for 30 min and was stopped by addition of a 0.35 M hydrogen sulfate solution. The intensity of the light produced, directly proportional to the amount of HMGB1 present in our sample, was measured using a spectrophotometer (Multiskan Ascent) at a wavelength of 450 nm. Concentration of HMGB1 is expressed as ng/mL.

Immunohistochemical staining of HMGB1 in the liver

Serial sections (5 μm thickness) of formalin-fixed and paraffin-embedded liver were immunostained for HMGB1 (1:1000) by indirect immuno-peroxidase method using Discovery Ventana (Roche Diagnostics GmbH, Mannheim, Germany).

Quantification of HMGB1 in the liver

Immunohistochemical expression of nuclear HMGB1 was quantified as previously described[19,20]. The immunostained sections were acquired at 20 × using a Hamamatsu NanoZoomer HT2.0 whole slide scanner (Hamamatsu Photonics, Hamamatsu City, Japan). Finally, semi-quantitative image analysis software (Tissue Map 3.0; Definiens, Munich, Germany) was independently applied to all corresponding digitalized slides. An average of 695146.9 ± 238143.2 nuclei was analyzed per liver and HMGB1 staining intensity, expressed as the labelling index, which represented the percentage of stained pixels in the nuclear area, was quantified.

RNA extraction and RT-qPCR

Frozen liver samples were homogenized in lysis buffer by MagNa Lyser (Roche Diagnostics, Brussels, Belgium). mRNA was extracted by High Pure RNA Tissue kit (Roche Diagnostics). Briefly, the homogenates were first centrifuged (15871 × *g*) for 10 min. Chloroform was added to the supernatant recovered and the mixture was centrifuged for 15 min at 4 °C. Ethanol 70% was added before transfer to a column (high pure spin filter tubes) and centrifuged for 30 s at 13000 × *g*. DNAse was added to the column and incubated for 15 min at room temperature. Then, three successive washes of the column were performed. Finally, the column was washed with elution buffer to remove all the RNA retained in the filter and recover it in a clean Eppendorf. The mRNA quality/purity of each sample was evaluated before RT-qPCR using the NanoDrop™ 1000 Spectrophotometer (ThermoFisher Scientific, Waltham, MA, United States). We evaluated the concentration of RNA in each sample as well as the ratio 260/280 (to exclude the presence of protein, phenol and other contaminants) and the ratio 260/230 (to exclude the presence of co-purified contaminants). None of the samples used had a ratio less than 1.8.

Retro-transcription of the mRNA into cDNA was performed as follows: 4 μL of oligo-dT primer (0.1 μg/μL; Eurogentec, Liege, Belgium) was joined to 9 μL of H_2O containing 1 μg of RNA. This mixture was incubated at 65 °C for 5 min and then cooled on ice. After that, 7 μL of RT mix, consisting of 5 × buffer, deoxyribonucleotide triphosphates (10 mmol/L), porcine RNAse inhibitor (50 U/μL) and reverse transcriptase (20 U/μL), were added. Finally, the mixture was incubated at 42 °C for 1 h, and then at 70 °C for 15 min.

Quantification of cDNA was performed by real time PCR using the LightCycler (Roche Diagnostics). Detection of the amplified product was carried out using a fluorescent probe (*Taq*Man; Roche) and the relative expression of the gene of interest was calculated against β-actin and GAPDH gene (housekeeping gene) following the Pfaffi method[21]. The sequences of primers used are listed in Table 1.

Reagents

GL was purchased from Sigma-Aldrich (Darmstadt, Germany). NAC (Lysomucil®) was provided by Zambon (Brussels, Belgium). Acetaminophen (Paracetamol Fresenius Kabi) was purchased from Fresenius Kabi (Homburg, Germany).

Statistical analysis

Statistical analyses were performed using SPSS Statistics 18.0 (Chicago, IL, United States). Difference testing between groups was performed using the Mann-Whitney *U*

Table 1 List and sequence of primers used for qPCR analysis

Real time PCR			
CYP2E1	Mouse	Forward	5′-AAGCGCTTCGGGCCAG-3′
		Reverse	5′ TAGCCATGCAGGACCACGA-3′
		Sonde	5′TCACACTGCACCTGGGTCAGAGGC-3′
GAPDH	Mouse	Forward	Confidential Roche diagnostics
		Reverse	
		Sonde	
β-actin	Mouse	Forward	5′-TCCTGAGCGCAAGTACTCTGT-3′
		Reverse	5′-CTGATCCACATCTGCTGGAAG-3′
		Sonde	5′-ATCGGTGGCTCCATCCTGGC-3′

or Student's *t* tests, as appropriate. We assessed mice rates of survival using the Kaplan-Meier method and compared survival between groups using the log-rank test. A P value of < 0.05 was considered statistically significant.

RESULTS

Administration of GL, at the same time as APAP, reduced the severity of liver injury

Female C57BL/6J mice treated with overdose of APAP (500 mg/kg) showed evidence of severe hepatic injury as indicated by significantly increased ALT values (Figure 1A), necrosis of centrilobular hepatocytes (Figure 1B and F) and GSH depletion (Figure 1C). Moreover, increased HMGB1 concentrations were observed with a peak 6 h after APAP administration (Figure 1D). Parallel to this phenomenon, a decrease in the nuclear staining of HMGB1 in hepatocytes was observed from 6 h after APAP injection (Figure 1E and F).

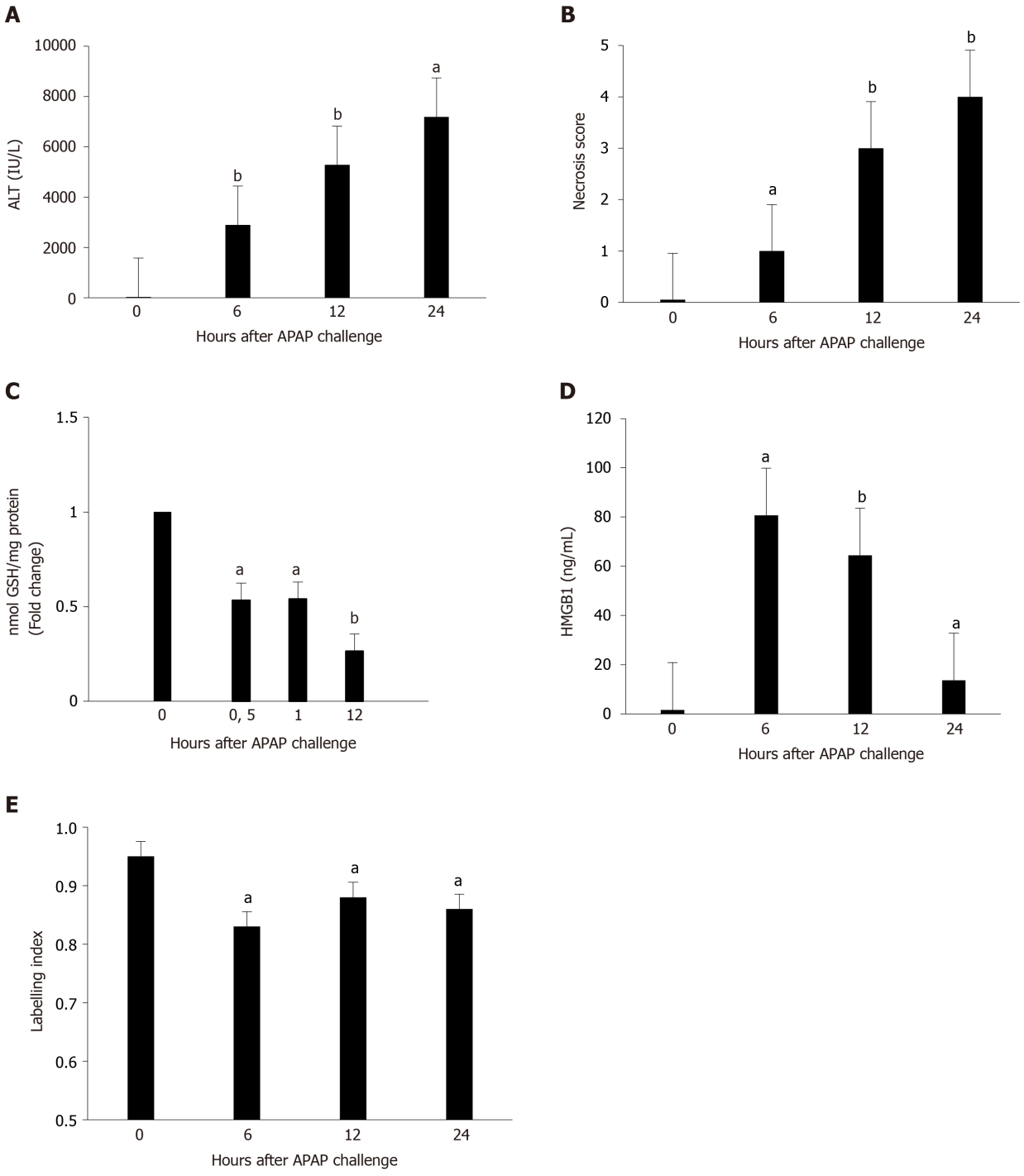
Concomitant administration of GL (200 mg/kg) and APAP (500 mg/kg) reduced the severity of the liver injury, as shown by ALT levels (Figure 2A), AST levels (Figure 2B), LDH levels (Figure 2C), and hepatocyte necrosis (Figure 2D and E) in mice sacrificed after 12 hours. In addition, a reduction in HMGB1 concentration was observed (Figure 2F) as well as maintenance of nuclear HMGB1 immunostaining in hepatocytes (Figure 2E and G).

Administration of NAC/GL combination, at the same time as APAP, reduced the severity of liver injury as well as GL or NAC alone

The efficacy of NAC treatment after APAP overdose is well documented in the literature and we have confirmed these results in our murine model. Indeed, after co-administration of NAC (150 mg/kg) and APAP (500 mg/kg), a significant decrease in ALT levels (Figure 3A), AST levels (Figure 3B), LDH levels (Figure 3C), and necrosis score (Figure 3D and E) was observed in mice sacrificed after 12 h. In addition, a reduction in HMGB1 concentration was observed (Figure 2F) as well as the maintenance of nuclear HMGB1 immunostaining in hepatocytes (Figure 2E and G).

Co-administration of NAC/GL and APAP was then investigated in the same murine model (APAP 500 mg/kg; sacrificed after 12 h). As shown in Figure 4, significant decreases in ALT levels (Figure 4A), AST levels (Figure 4B), LDH levels (Figure 4C), and centrilobular hepatocytes necrosis (Figure 4D and E) were observed. In addition, a reduction in HMGB1 concentration was observed (Figure 4F) as well as the maintenance of nuclear HMGB1 immunostaining in hepatocytes (Figure 4E and G). The latter results demonstrated that the NAC/GL combination is as effective as NAC alone when treatment is administered at the same time of APAP.

GSH levels (Figure 5A) and GSH/GSSG ratio (Figure 5B) were also assessed. As expected, administration of GL did not influence GSH levels, while administration of NAC restored GSH stores. When mice were given NAC/GL combination, partial restoration of GSH stores was observed. GSH is known to reduce NAPQI and protect against oxidative damage. As expected, after an overdose of APAP, we observed an increase in oxidative stress resulting in a drastic decrease in the GSH/GSSG ratio. This situation returned to normal after administration of NAC and NAC/GL, as shown by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Hours after APAP challenge
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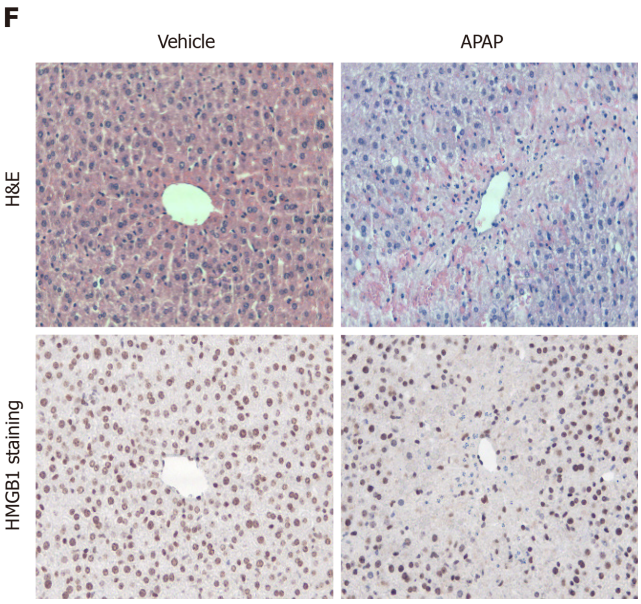


Figure 1 Murine model of acetaminophen-induced liver injury: Hepatotoxicity assessment. A: Alanine aminotransferase (ALT) levels were measured in sera of vehicle-treated mice (0 h) and in sera of mice sacrificed 2, 6, 12 or 24 h after acetaminophen [APAP; 500 mg/kg] administration (5 mice in each group); B: Liver necrosis was scored in the same groups of mice; C: Hepatic glutathione (GSH) levels were measured at 30 min, 1 and 12 h after APAP challenge. The enzyme concentration obtained is expressed as nanomoles of enzyme per milligram of protein using bovine serum as a standard; D: High mobility group box 1 (HMGB1) levels were measured in the same groups of mice; E: Quantification of nuclear expression of HMGB1 in the same groups of mice; F: Representative hematoxylin and eosin (magnification × 200) and HMGB1-stained images (magnification × 200) of murine liver 24 h after vehicle or APAP challenge. Results are expressed as mean ± standard error. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* 0. Experiments were reproduced three times. H&E: Hematoxylin and eosin.

the high ratio, whereas GL offered less protection.

Delayed administration of NAC/GL combination reduced APAP-induced hepatocytes necrosis compare to GL or NAC alone

We explored the effect of delayed administration of GL, NAC or NAC/GL combination under the same conditions (APAP 500 mg/kg; mice sacrificed after 12 h). As shown in Figure 6A and B, all treatments (GL, NAC and NAC/GL) administered 2 h or 6 h after APAP administration, resulted in significant decreases in ALT and AST at similar levels. Regarding the plasma concentration of LDH, NAC and the NAC/GL combination remained effective at 2 h and 6 h, in contrast to GL which no longer showed a protective effect at 6 h after APAP administration. Furthermore, in Figure 6D and E, we observed a decrease in the necrosis score in all treatment groups. It is interesting to note that when treatment was administered 2 h and 6 h after APAP, NAC/GL combination was associated with a lower necrosis score than NAC or GL alone.

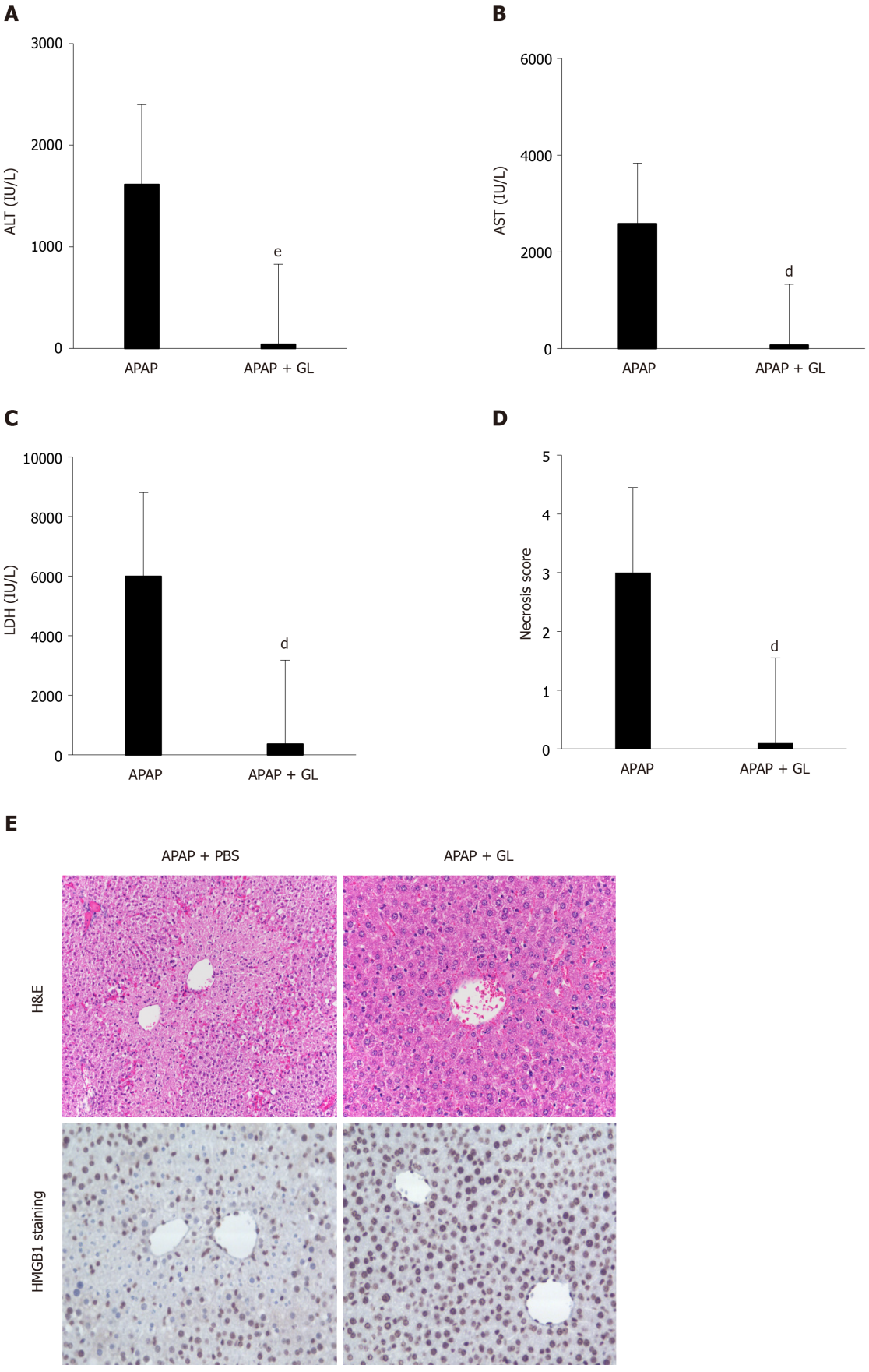
Regarding the HMGB1 protein, all treatments (GL, NAC and NAC/GL) given at 2 h or 6 h after APAP administration decreased HMGB1 concentration (Figure 7A). GL and NAC/GL combination continued to maintain the nuclear localization of HMGB1 immunostaining in hepatocytes (Figure 7B and C).

The administration of NAC and NAC/GL was still effective in restoring GSH stores and protecting against oxidative stress, as shown in Figure 8A and B, respectively. On the other hand, delayed administration of GL, 2 h and 6 h after acetaminophen, was no longer effective in restoring GSH stores and protecting against oxidative stress.

Delayed administration of NAC/GL combination increased mice survival following APAP-induced liver injury compared to GL or NAC alone

Survival rates were then analyzed for each treatment using Kaplan-Meier curves. As shown in Figure 9, administration of NAC/GL combination was associated with improved survival rates. Indeed, we observed that when treatment (GL, NAC or NAC/GL) was administered at the same time as APAP, the survival of mice was significantly increased regardless of the treatment. However, if treatment (GL, NAC or NAC/GL) was given 2 h after APAP administration, both NAC and NAC/GL significantly improved survival in mice, but the GL lost its protective effect.

Thereafter, if treatment (GL, NAC or NAC/GL) was administered 6 h after APAP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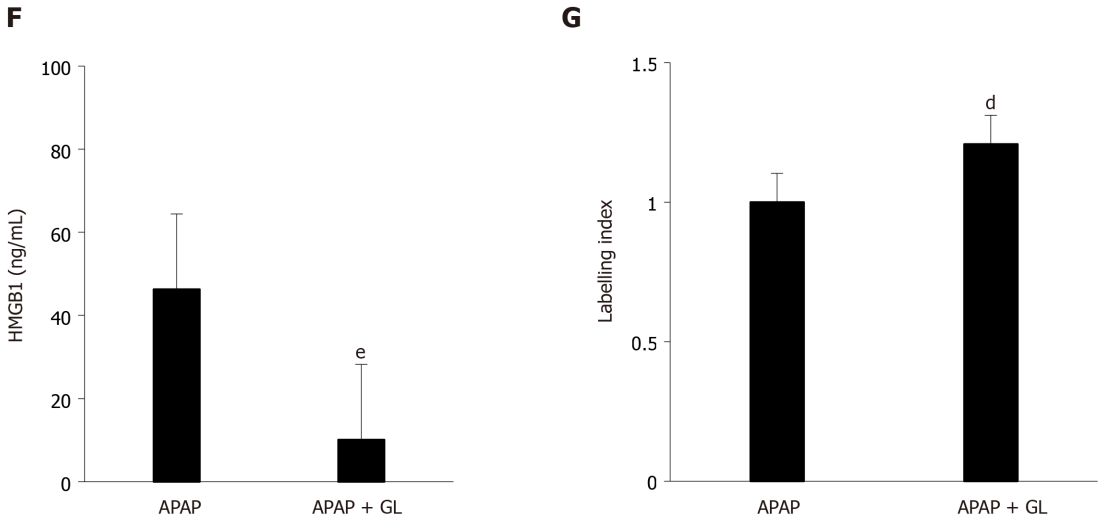


Figure 2 Administration of glycyrrhizin at the same time as acetaminophen, reduced the severity of liver injury. A: Alanine aminotransferase (ALT) levels were measured after 12 h in the plasma of mice treated by vehicle or glycyrrhizin (GL) at the time of acetaminophen (APAP) injection (10 mice in each group); B: Aspartate aminotransferase (AST) levels were measured after 12 h in the plasma of mice treated by vehicle or GL at the time of APAP injection (10 mice in each group) C: Lactate dehydrogenase (LDH) levels were measured after 12 h in the plasma of mice treated by vehicle or GL at the time of APAP injection (10 mice in each group); D: Liver necrosis was scored in the same group of mice; E: Representative hematoxylin and eosin (H&E; magnification × 200) and high mobility group box 1 (HMGB1)-stained images (magnification × 400) of murine liver 12 h after APAP challenge in the same group of mice; F: HMGB1 levels were measured in the same group of mice; G: Quantification of nuclear expression of HMGB1 in the same groups of mice. Results are expressed as mean ± standard error. $^{c}P < 0.05$, $^{d}P < 0.01$, $^{e}P < 0.001$ *vs* APAP. Experiments were reproduced three times.

administration, only the NAC/GL combination showed significant survival efficacy.

Protective effects of the NAC/GL combination do not result from inhibition of hepatic expression of CYP2E1

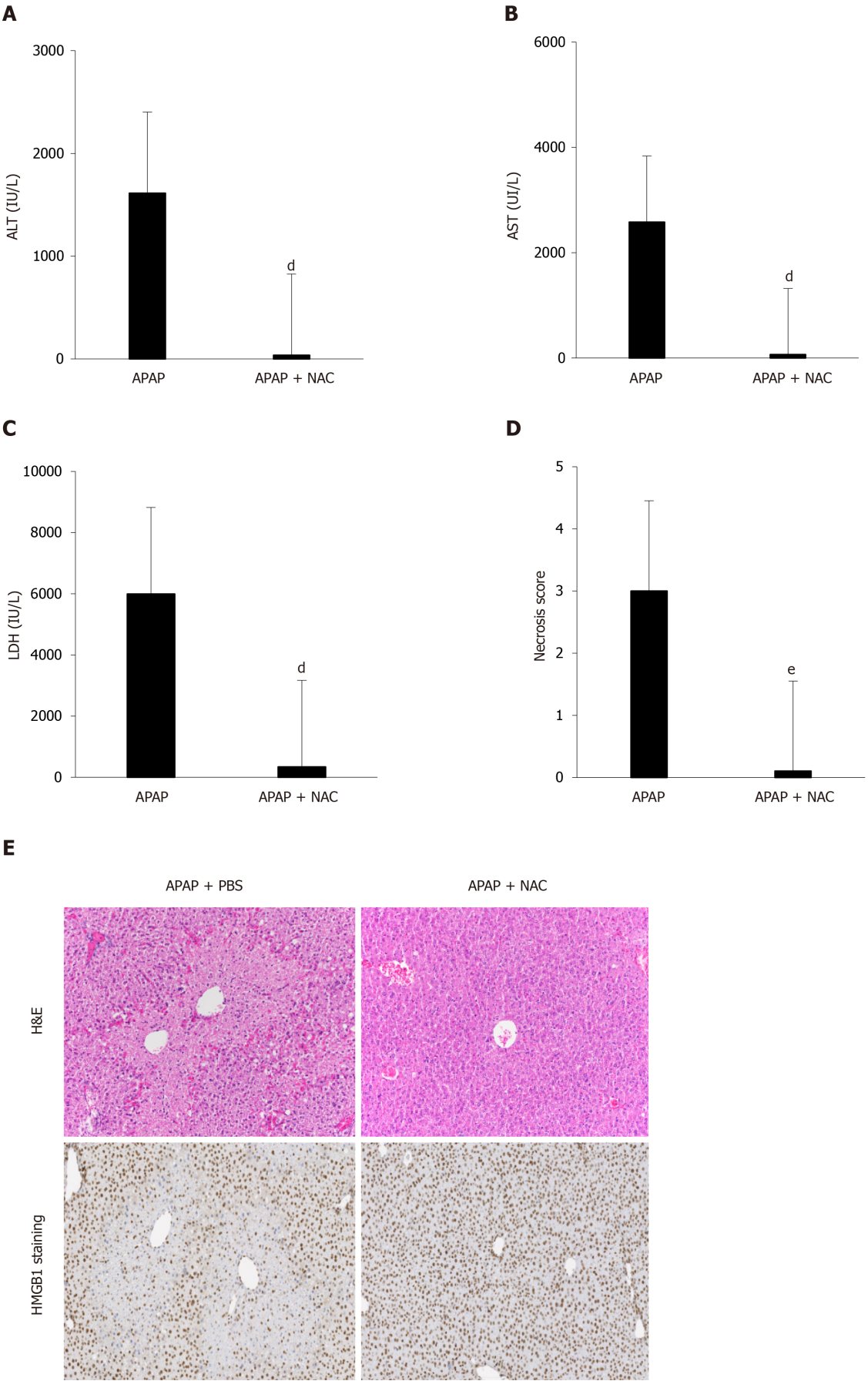
Yang *et al*[22] observed a decrease in CYP2E1 mRNA expression after glycyrrhetinic acid administration in a murine model of APAP-induced liver injury; these results suggested the influence of glycyrrhetinic acid, a metabolite of GL, on APAP metabolism. To exclude this possibility in our murine model, RT-qPCR was performed on liver extracts. Decreased expression of CYP2E1 was observed over time in mice after APAP administration, as shown in Figure 10A. However, this decrease is no longer observed in mice treated with GL, NAC or NAC/GL combination. These results, consistent with others[23], demonstrated the lack of inhibition of CYP2E1 mRNA expression by the treatments used in our murine model.

DISCUSSION

Since NAC is less efficient for delayed treatment of acetaminophen-induced liver injury, other therapies need to be explored. One such a drug is GL, the main biologically active component of licorice. This drug has already shown, in other diseases, a variety of pharmacological effect resulting from anti-inflammatory and antioxidant activities. We have previously confirmed the hepatoprotective effect of GL in a murine model of APAP-induced liver injury and these results have been replicated in a human hepatocyte cell line[17].

The aim of this study was to compare the potential efficacy of the NAC/GL combination *vs* GL or NAC alone. This study is based on the desire to combine an antioxidant drug that acts on the early phase of acetaminophen toxicity and an anti-inflammatory drug that acts on the late phase, after hepatocyte necrosis induced by the accumulation of the acetaminophen toxic metabolite.

We compared these three additional treatments on the liver injury by analysis of biochemical and histopathological parameters. At first, we studied the efficacy of each treatment when administered at the same time of APAP. Regardless of the treatment administered, an improvement in liver injury was observed. Next, we investigated the delayed administration of these three treatments. We observed a similar improvement in ALT, AST and LDH levels. Interestingly, the administration of NAC/GL, 2 h and 6 h after APAP, decreased centrilobular hepatocyte necrosis, in contrast to NAC and GL.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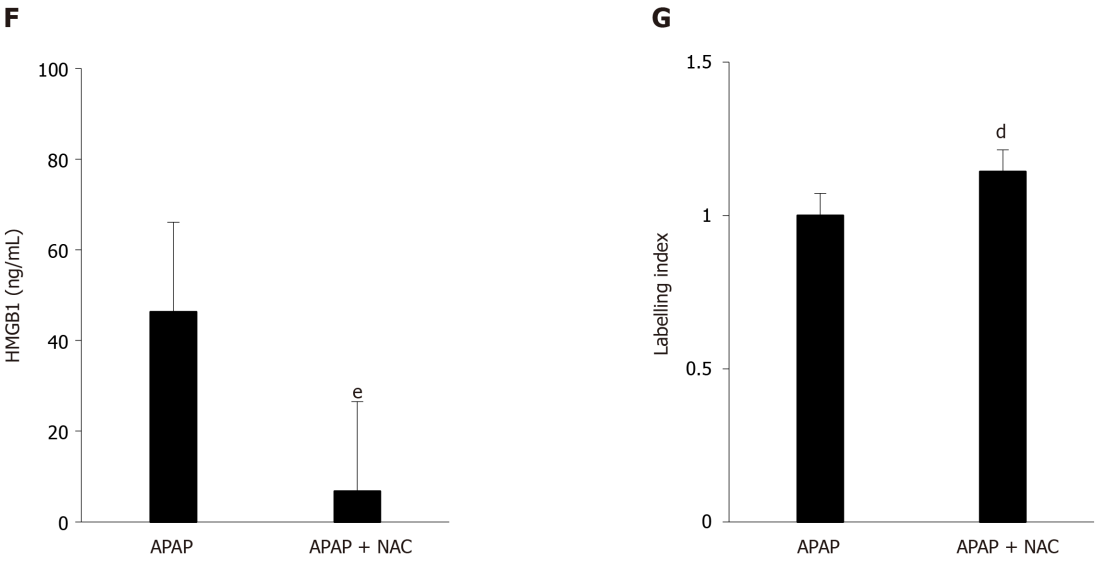


Figure 3 Administration of N-acetylcysteine, at the same time as acetaminophen, reduced the severity of liver injury. A: Alanine aminotransferase (ALT) levels were measured after 12 h in the plasma of mice treated by vehicle (phosphate-buffered saline; PBS) or N-acetylcysteine (NAC, 150 mg/kg) at the time of acetaminophen (APAP, 500 mg/kg) injection (n = 10); B: Aspartate aminotransferase (AST) levels were measured after 12 h in the plasma of mice treated by vehicle (PBS) or NAC (150 mg/kg) at the time of APAP (500 mg/kg) injection (n = 10); C: Lactate dehydrogenase (LDH) levels were measured after 12 h in the plasma of mice treated by vehicle (PBS) or NAC (150 mg/kg) at the time of APAP (500 mg/kg) injection (n = 10); D: Liver necrosis was scored in the same group of mice; E: Representative hematoxylin and eosin (H&E) and high mobility group box 1 (HMGB1)-stained images (magnification × 200) of murine liver 12 h after APAP challenge in the same group of mice; F: HMGB1 levels were measured in the same group of mice; G: Quantification of nuclear expression of HMGB1 in the same groups of mice; Results are expressed as mean ± standard error. $^{c}P < 0.05$, $^{d}P < 0.01$, $^{e}P < 0.001$ *vs* APAP. Experiments were reproduced three times.

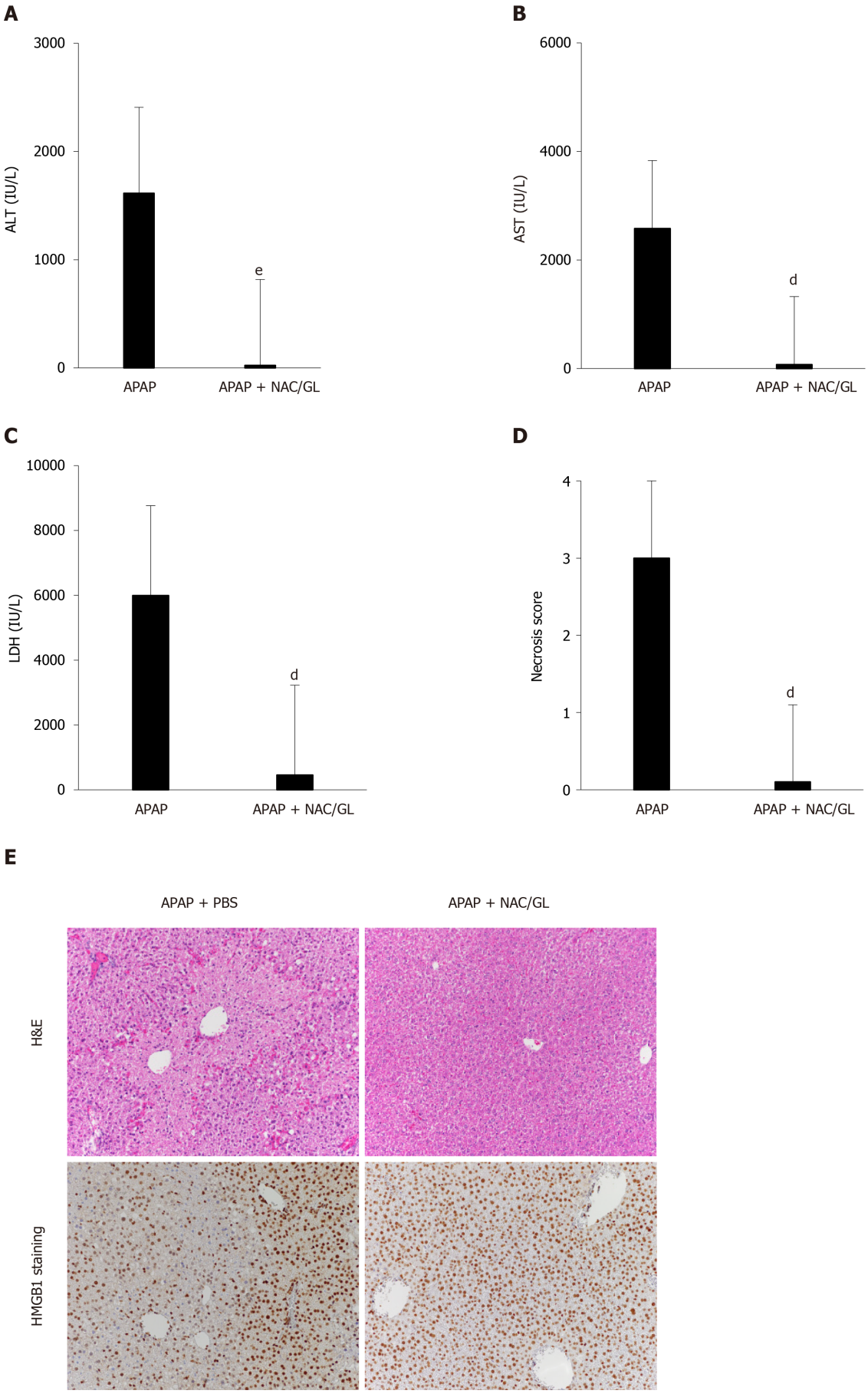
Third, we assessed treatment-dependent survival rates. We observed that GL already lost its efficacy when administered 2 h after APAP and NAC when administered 6 h after APAP. It is important to note that the survival benefit was only observed in mice receiving NAC/GL. These observations suggest potential alternative mechanisms for this survival benefit.

In view of the encouraging results obtained with the NAC/GL combination in the prevention of APAP-induced liver damage, it was important to examine whether the properties of each drug were maintained. Therefore, in each experiment we focused on the HMGB1 protein and the level of GSH. Regardless of when the NAC/GL combination was injected, the concentration of HMGB1 in the plasma of our mice was significantly reduced. In addition, we observed the maintenance of the immunohistochemical staining of the protein in hepatocytes. Knowing the properties of HMGB1 as a "damage associated molecular pattern" protein, these two observations confirm in us the idea of a protective effect. The main mechanism of action of NAC is to promote hepatic GSH synthesis which supports the detoxification of NAPQI and reduces protein binding[24]. In our model, a complete recovery of hepatic GSH content was observed when treatment, NAC or NAC/GL, was administered at the same time of APAP. In addition, hepatic GSSG levels are decreased compared to APAP alone, as shown by the GSH/GSSG ratio, suggesting the absence of increasing levels of oxidative stress. When treatment was administered later than APAP, partial recovery of hepatic GSH levels was observed while the GSH/GSSG ratio remained similar. Thus, it appears that NAC loses its efficacy in the synthesis of GSH when administered at later times, when oxidative stress does not appear to be higher. These results need further explorations.

To our knowledge, this combination was not already tested in mice. Xu *et al*[25] had investigated this association in rats, however. They showed no benefit of NAC/GL combination *vs* the use of NAC alone in the APAP-induced liver injury. However, these results are to be interpreted with caution. Indeed, as described in the literature, rats are defined as resistant to the liver-damaging effects of APAP due to low mitochondrial dysfunction which prevented oxidative stress[26-28]. This could explain why this combination works in our murine model.

By browsing the literature, we observed that female mice are described as resistant to acetaminophen. In order to rule out the possibility of impact of sex on the efficacy of the NAC/GL combination on paracetamol-induced liver lesions, we confirmed our results on male mice (Supplementary data).

This study opens a potential new therapeutic pathway in the prevention of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H&E
HMGB1 staining

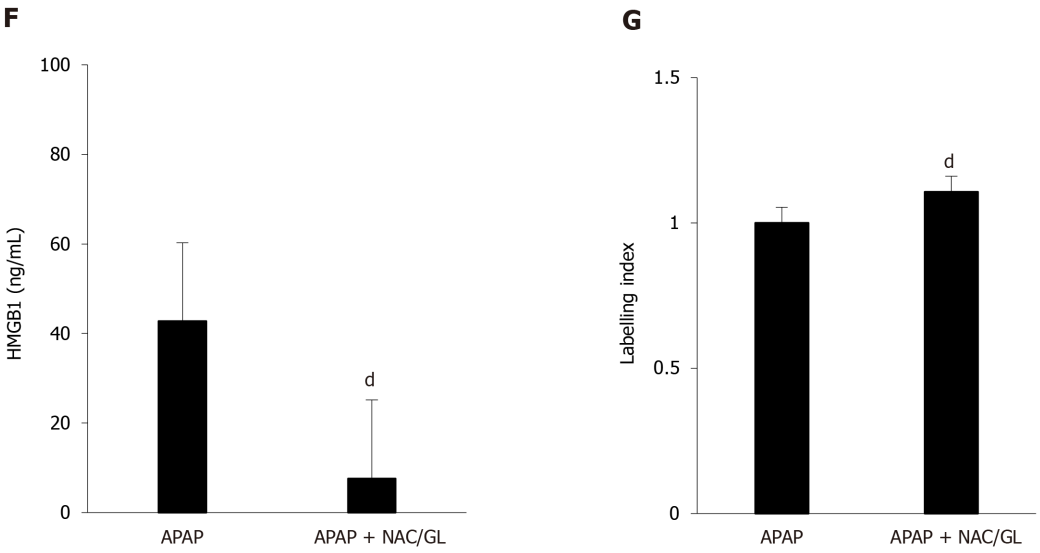


Figure 4 Administration of N-acetylcysteine/glycyrrhizin combination, at the same time as acetaminophen, reduced the severity of liver injury as well as glycyrrhizin or N-acetylcysteine alone. A: Alanine aminotransferase (ALT) levels were measured in the plasma of vehicle-treated mice and mice treated with N-acetylcysteine (NAC, 150 mg/kg), glycyrrhizin (GL, 200 mg/kg) or NAC/GL, 2 h or 6 h after acetaminophen (APAP) injection (10 mice in each group); B: Aspartate aminotransferase (AST) levels were measured in the plasma of vehicle-treated mice and mice treated with NAC (150 mg/kg), GL (200 mg/kg) or NAC/GL, 2 h or 6 h after APAP injection (10 mice in each group); C: Lactate dehydrogenase (LDH) levels were measured in the plasma of vehicle-treated mice and mice treated with NAC (150 mg/kg), GL (200 mg/kg) or NAC/GL, 2 h or 6 h after APAP injection (10 mice in each group); D: Liver necrosis at 12 h after APAP challenge was scored in the same group of mice; E: Representative hematoxylin and eosin (H&E) and high mobility group box 1 (HMGB1)-stained images (magnification × 200) of murine liver 12 h after of murine liver 12 h after vehicle or APAP challenge; F: HMGB1 levels were measured in the same group of mice; G: Quantification of nuclear expression of HMGB1 in the same groups of mice. Mice were scarified 12 h after APAP administration. Results are expressed as mean ± standard error. $^{c}P < 0.05$, $^{d}P < 0.01$, $^{e}P < 0.001$ *vs* APAP. Experiments were reproduced three times.

acetaminophen hepatotoxicity.

CONCLUSION

In conclusion, compared to NAC alone, concomitant administration of GL decreased the liver necrosis score and improved the survival during acetaminophen-induced liver injury in mice. These results suggest, for the first time, that the combination of an antioxidant like NAC and an anti-inflammatory drug like GL prevents the liver damage induced by acetaminophen intoxication.

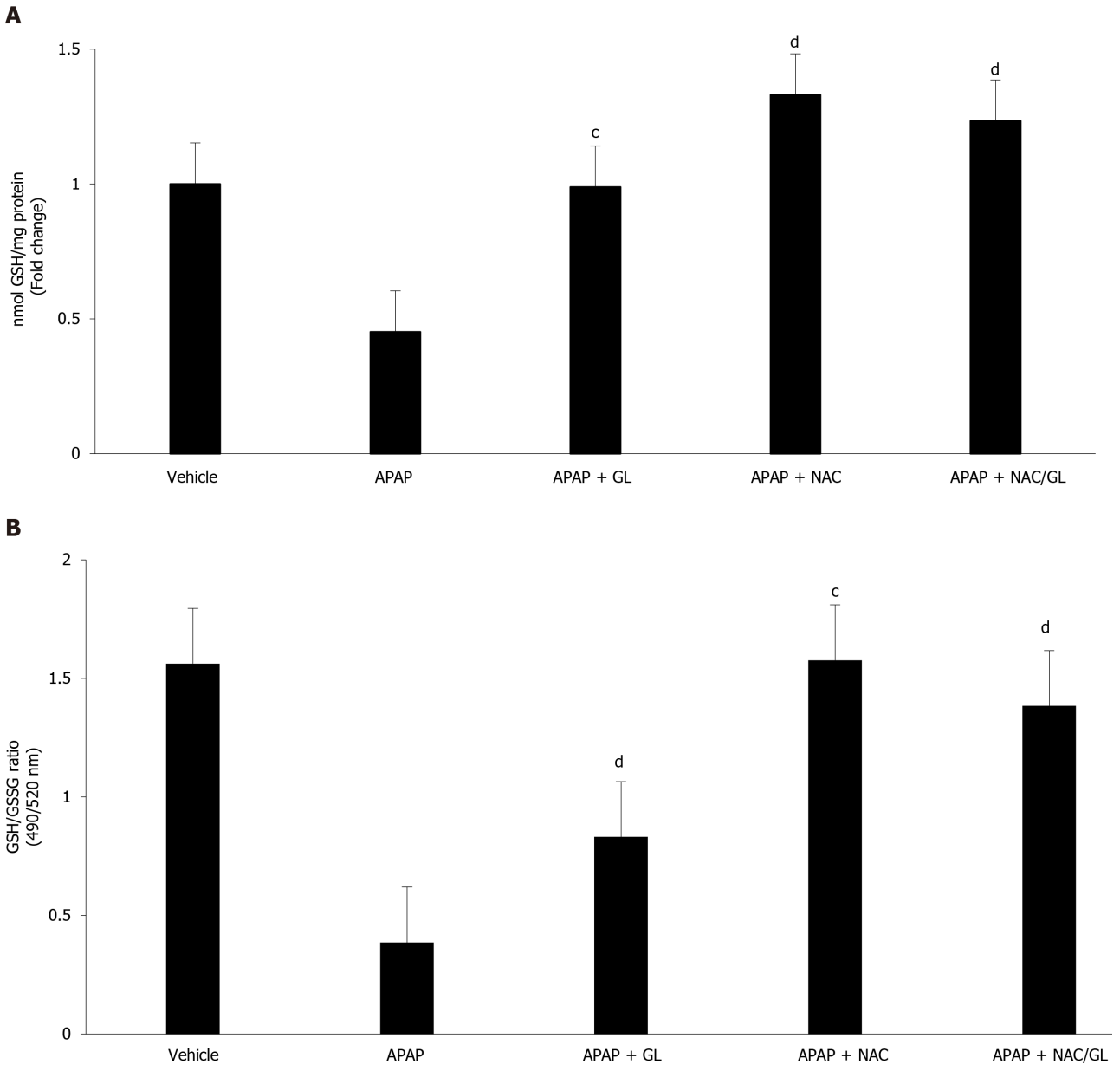
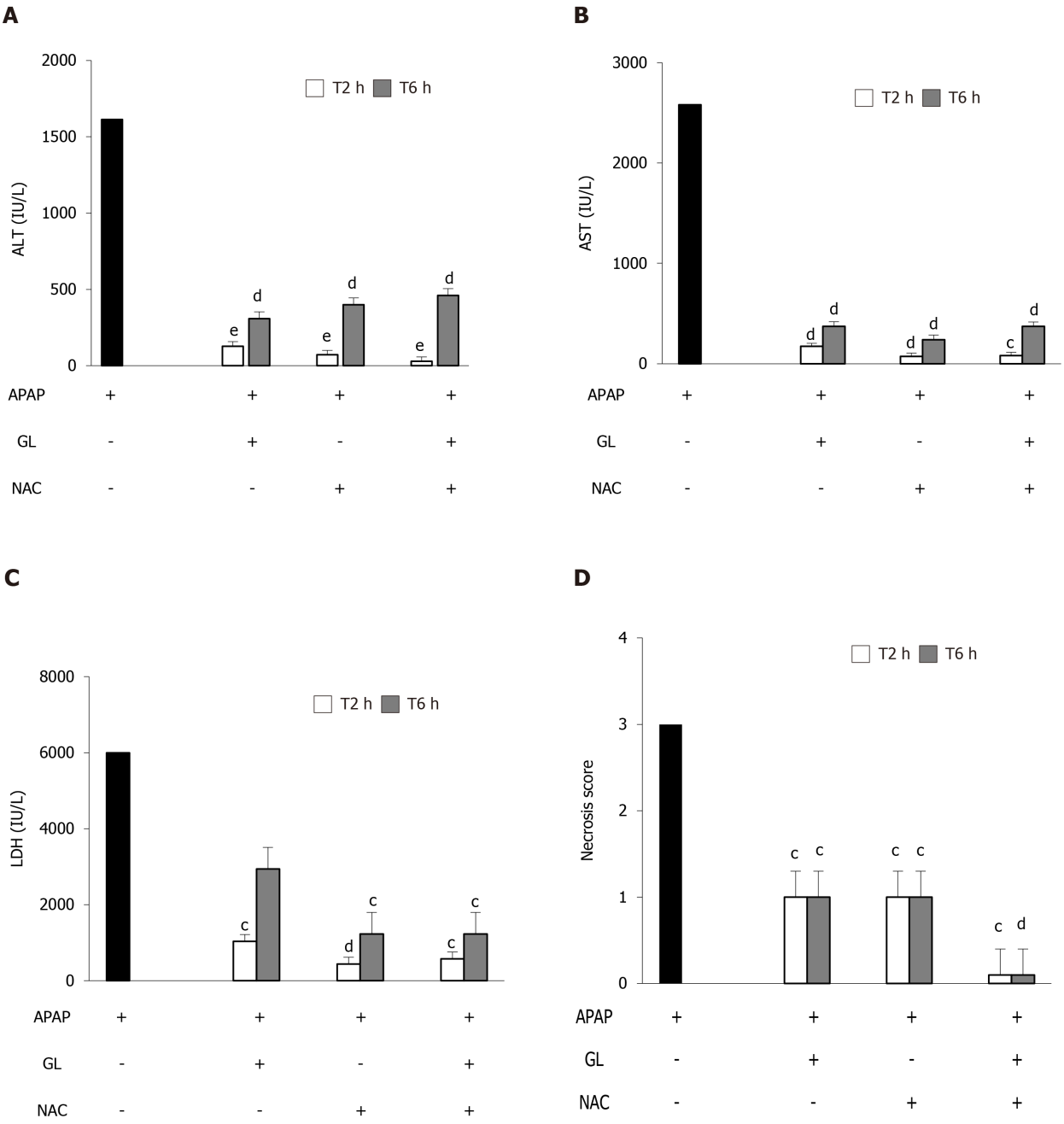


Figure 5 Acetaminophen overdose induced hepatic glutathione depletion and oxidative stress. A: Hepatic glutathione (GSH) levels were measured in mice treated with N-acetylcysteine (NAC, 150 mg/kg), glycyrrhizin (GL, 200 mg/kg) or NAC/GL, at the same time of acetaminophen (APAP) injection (10 mice in each group). Mice were scarified 12 h after APAP administration. Assessment was performed using colorimetric assay kit. The enzyme concentration obtained is expressed as nmol of enzyme per milligram of protein using bovine serum as a standard; B: Glutathione (GSH)/the oxidized state (GSSG) ratio was evaluated in the same group of mice using fluorometric assay kit. In two separate assay reactions, GSH (reduced) was measured directly with a GSH standard and total GSH (GSH + GSSG) was measured by using a GSSG standard. Fluorescence measurement was performed at Ex/Em = 490/520 nm. Results are expressed as mean ± standard deviation. $^{c}P < 0.05$, $^{d}P < 0.01$, $^{e}P < 0.001$ *vs* APAP.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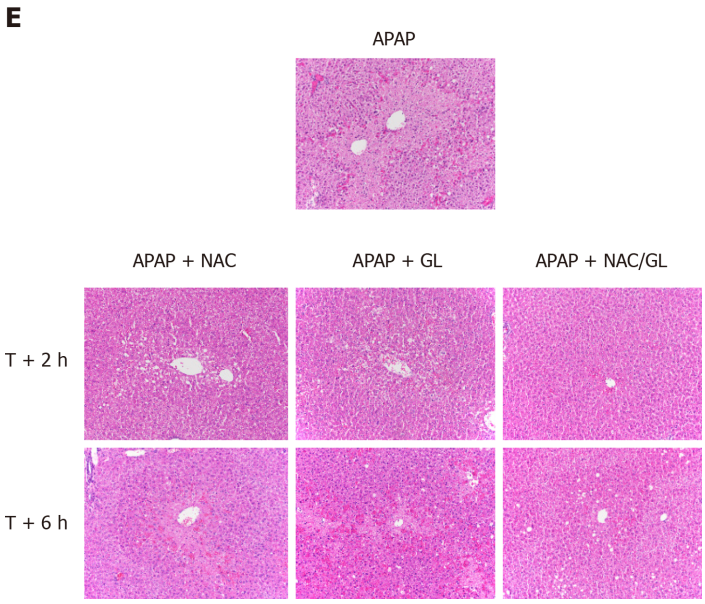

Figure 6 Delayed administration of N-acetylcysteine/glycyrrhizin combination reduced acetaminophen-induced hepatocytes necrosis compare to glycyrrhizin or N-acetylcysteine alone. A: Alanine aminotransferase (ALT) levels were measured in the plasma of vehicle-treated mice and mice treated with N-acetylcysteine (NAC, 150 mg/kg), glycyrrhizin (GL, 200 mg/kg) or NAC/GL, 2 h or 6 h after acetaminophen (APAP) injection (10 mice in each group). Mice were scarified 12 h after APAP administration; B: Aspartate aminotransferase (AST) levels were measured in the plasma of vehicle-treated mice and mice treated with NAC (150 mg/kg), GL (200 mg/kg) or NAC/GL, 2 h or 6 h after APAP injection (10 mice in each group). Mice were scarified 12 h after APAP administration; C: Lactate dehydrogenase (LDH) levels were measured in the plasma of vehicle-treated mice and mice treated with NAC (150 mg/kg), GL (200 mg/kg) or NAC/GL, 2 h or 6 h after APAP injection (10 mice in each group). Mice were scarified 12 h after APAP administration; D: Liver necrosis at 12 h after APAP challenge was scored in the same group of mice; E: Representative hematoxylin and eosin (H&E)–stained images (magnification × 200) of murine liver 12 h after vehicle or APAP challenge. Results are expressed as mean ± standard deviation. $^{c}P < 0.05$, $^{d}P < 0.01$, $^{e}P < 0.001$ *vs* APAP. Experiments were reproduced three times.

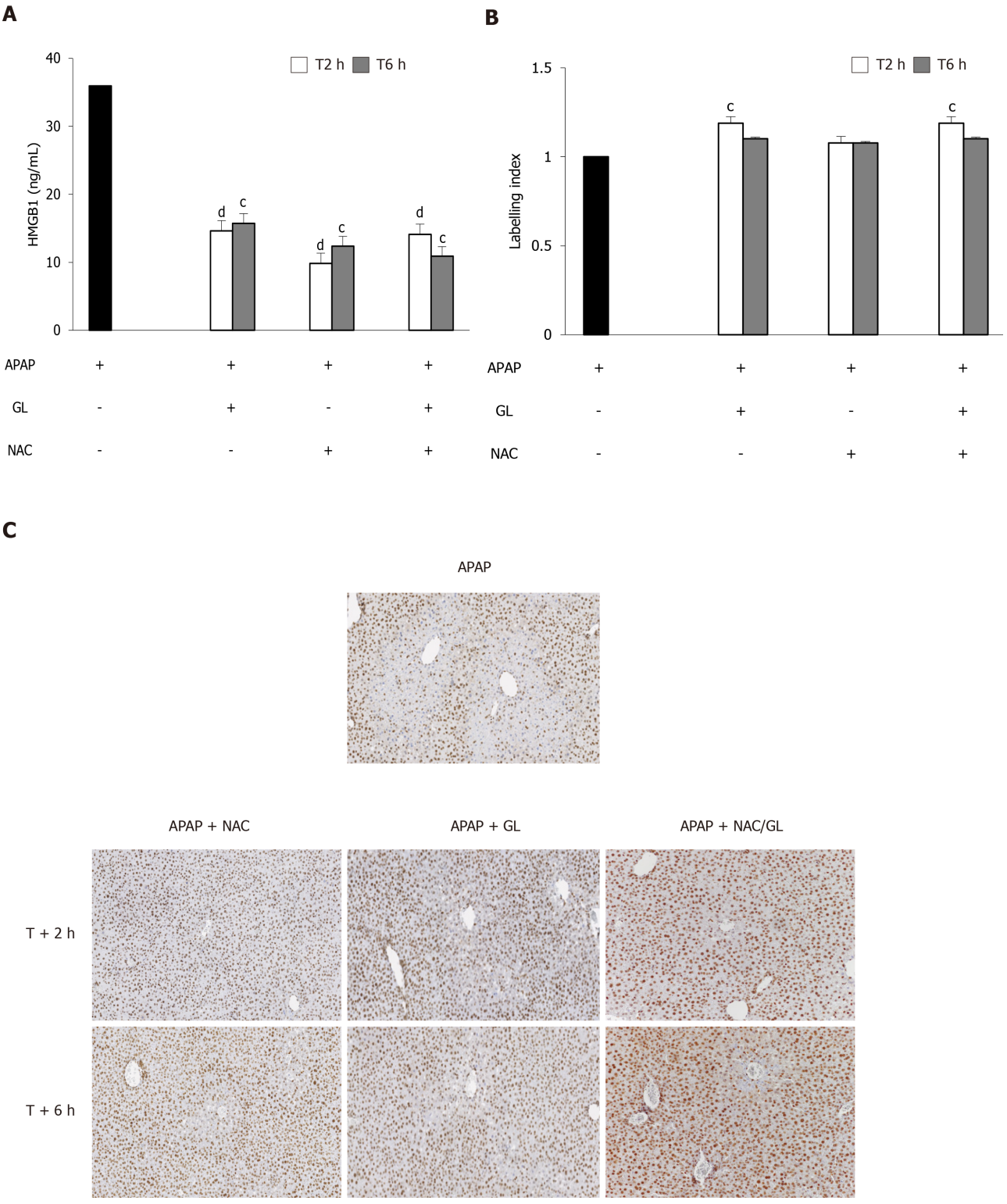

Figure 7 Delayed administration of N-acetylcysteine/glycyrrhizin combination reduced high mobility group box 1 release as well as glycyrrhizin alone. A: High mobility group box 1 (HMGB1) levels were measured in the plasma of vehicle-treated mice and mice treated with N-acetylcysteine (NAC, 150 mg/kg), glycyrrhizin (GL, 200 mg/kg) or NAC/GL, 2 h or 6 h after acetaminophen (APAP) injection (10 mice in each group). Mice were scarified 12 h after APAP administration; B: Quantification of nuclear expression of HMGB1 in the same groups of mice; C: Representative hematoxylin and eosin (H&E) and HMGB1-stained images (magnification × 200) of murine liver 12 h after APAP challenge in the same group of mice. Results are expressed as mean ± standard error. $^{c}P < 0.05$, $^{d}P < 0.01$, $^{e}P < 0.001$ *vs* APAP. Experiments were reproduced three times.

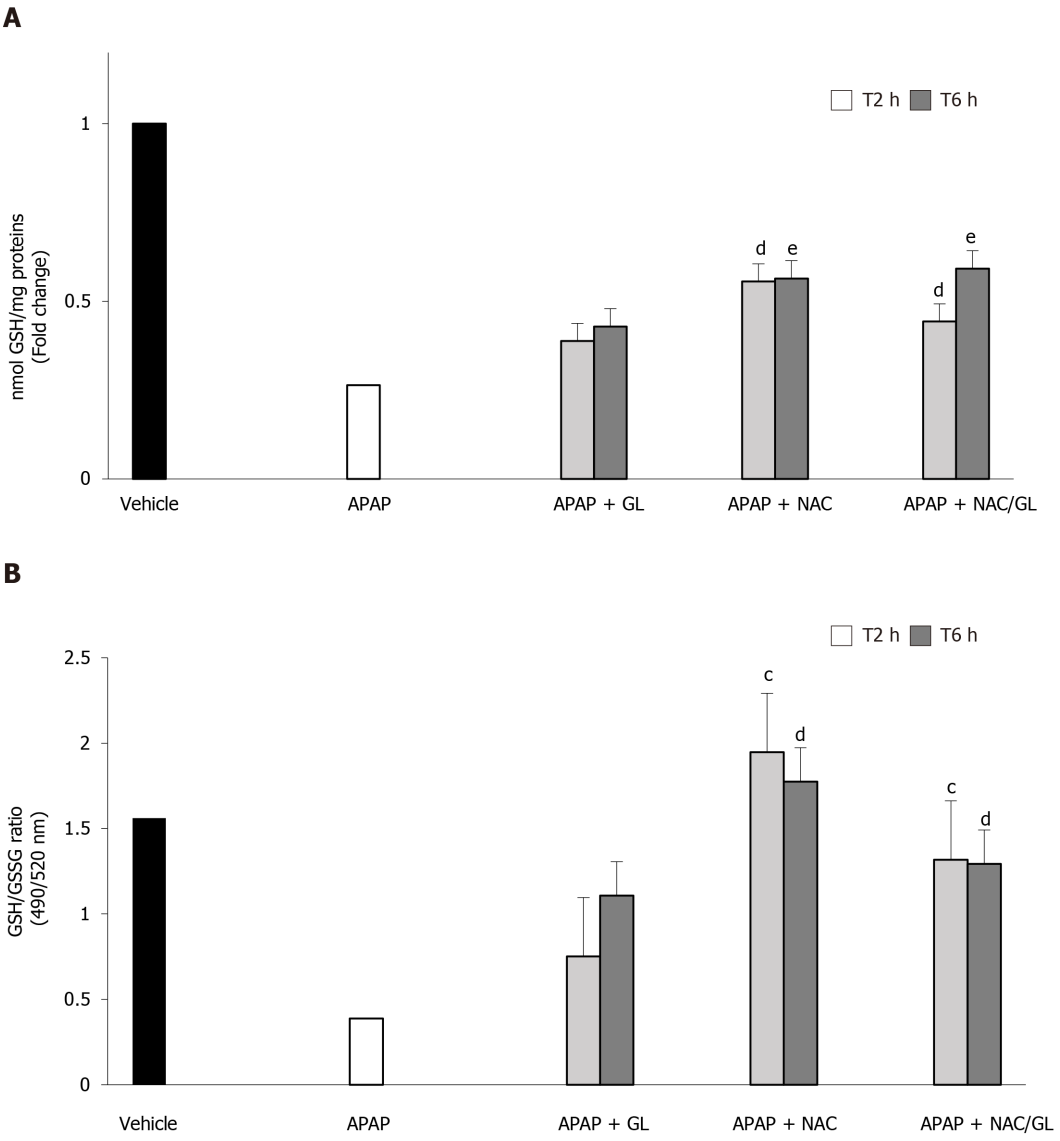

Figure 8 Delayed administration of N-acetylcysteine/glycyrrhizin combination restores partially glutathione stores. A: Hepatic glutathione (GSH) levels were measured in mice treated with N-acetylcysteine (NAC, 150 mg/kg), glycyrrhizin (GL, 200 mg/kg) or NAC/GL, 2 h or 6 h after acetaminophen (APAP) injection (10 mice in each group). Mice were scarified 12 h after APAP administration. Assessment was performed using colorimetric assay kit. The enzyme concentration obtained is expressed as nmol of enzyme per milligram of protein using bovine serum as a standard; B: Glutathione (GSH)/the oxidized state (GSSG) ratio was evaluated in the same group of mice using fluorometric assay kit. In two separate assay reactions, GSH (reduced) was measured directly with a GSH standard and total GSH (GSH + GSSG) was measured by using a GSSG standard. Fluorescence measurement was performed at Ex/Em = 490/520 nm. Results are expressed as mean ± standard error. $^{c}P < 0.05$, $^{d}P < 0.01$, $^{e}P < 0.001$ *vs* APAP.

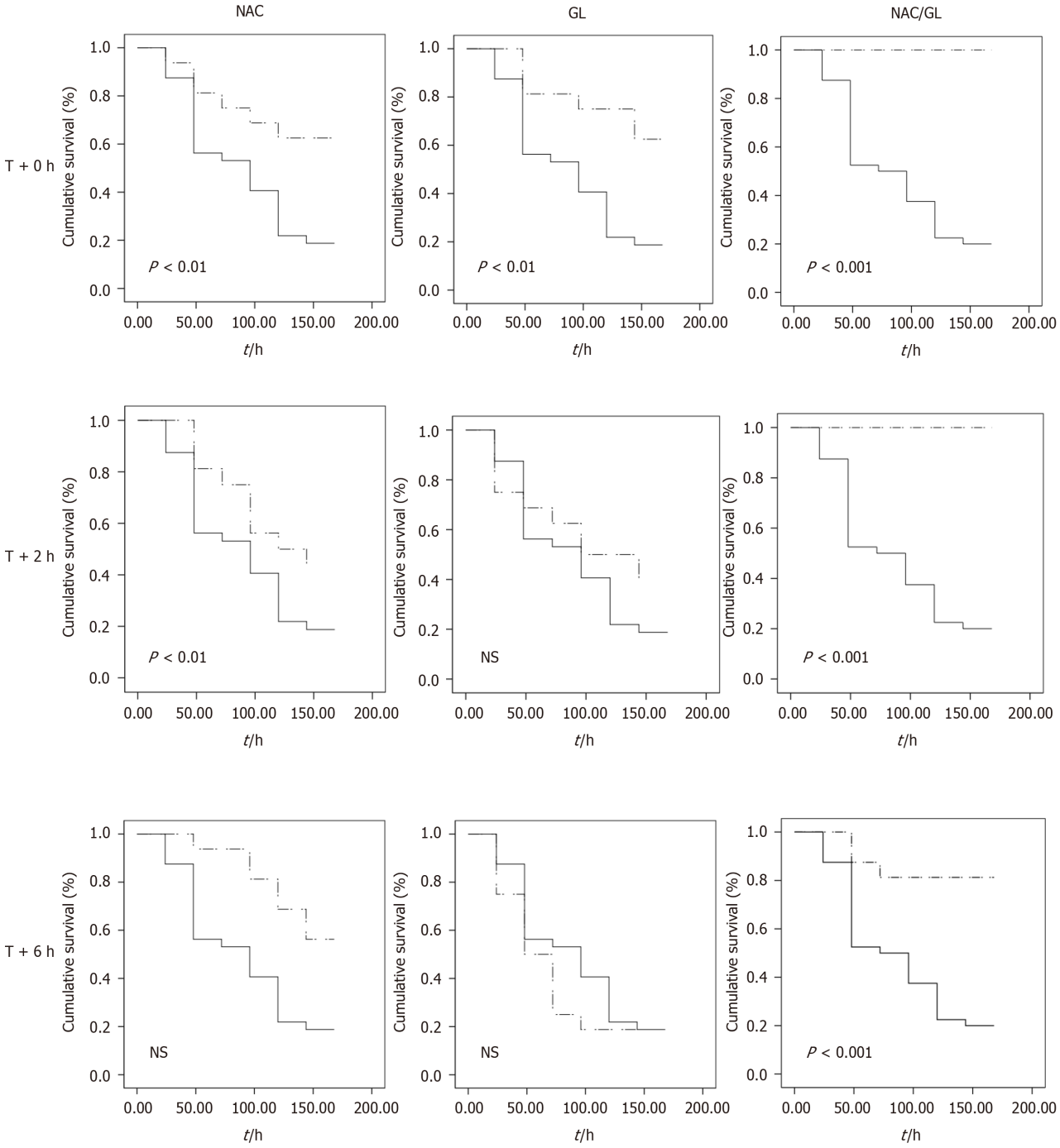

Figure 9 Delayed administration of N-acetylcysteine/glycyrrhizin combination increased mice survival following acetaminophen-induced liver injury compared to glycyrrhizin or N-acetylcysteine alone. Comparison of cumulative probability mice survival after acetaminophen (APAP) challenge (10 mice in each group). Mice were treated with glycyrrhizin (GL, 200 mg/kg), N-acetylcysteine (NAC, 150 mg/kg) or by a NAC/GL combination. Treatment was co-administered with APAP or administered 2 h or 6 h after APAP injection. Mice were followed for 172 h. Mice were euthanized when they became moribund per the criteria of lack of response to stimuli or lack of righting reflex. Kaplan-Meier survival curves were compared using log-rank test. $^{c}P < 0.01$, $^{d}P < 0.001$ *vs* APAP. Experiments were reproduced three times.

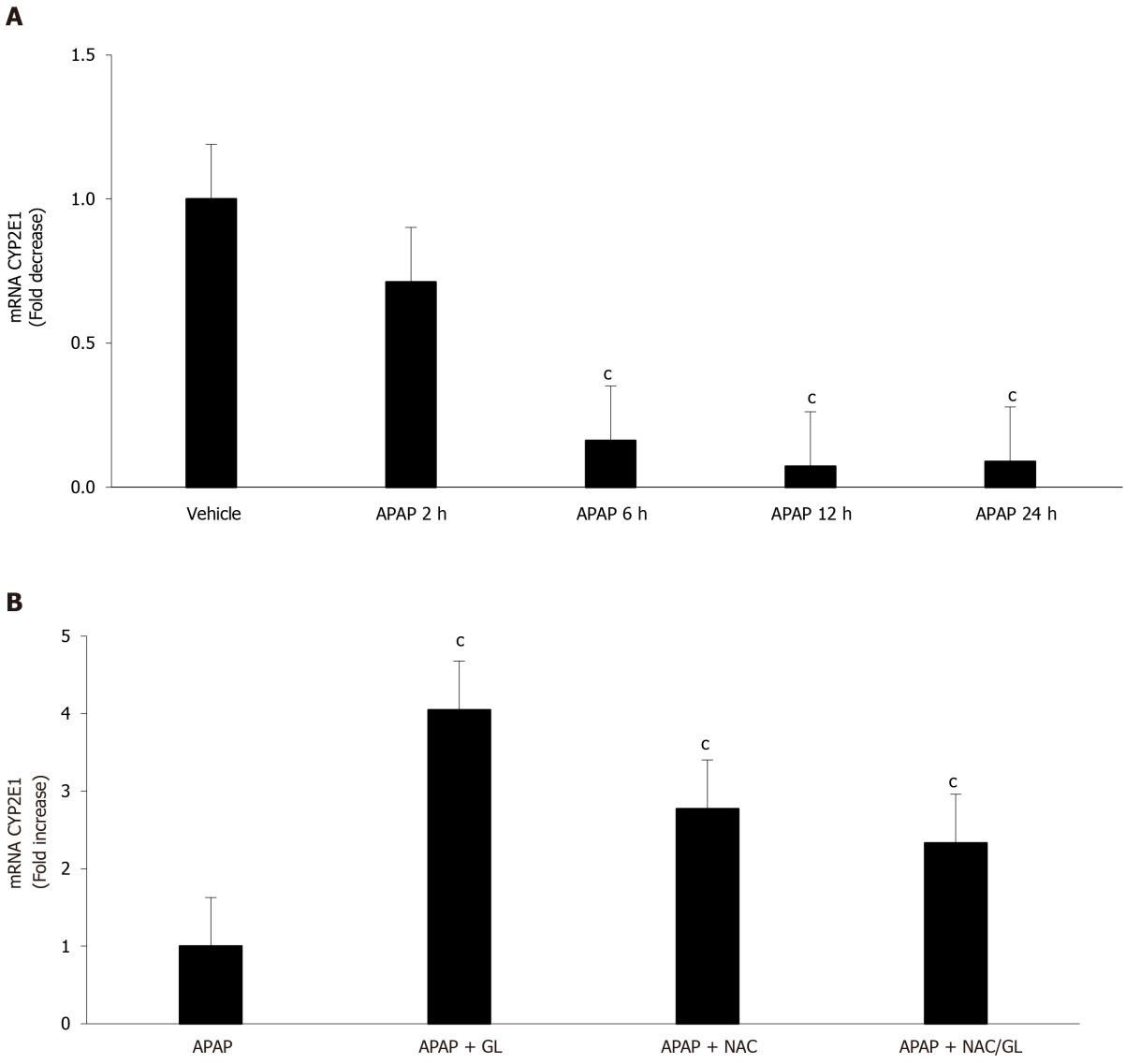

Figure 10 Protective effects of the N-acetylcysteine/glycyrrhizin combination do not result from inhibition of hepatic expression of CYP2E1. A: CYP2E1 mRNA expression was assessed by RT-qPCR in liver extract of vehicle-treated mice and mice sacrificed 2, 6, 12 or 24 h after acetaminophen (APAP, 500 mg/kg) injection (5 mice in each group); B: CYP2E1 mRNA expression was assessed by RT-qPCR in liver extract of mice treated with glycyrrhizin (GL, 200 mg/kg), N-acetylcysteine (NAC, 150 mg/kg), or NAC/GL, at the same time of APAP (10 mice in each group). Mice were scarified 12 h after APAP administration. The relative expression of the gene of interest was calculated using the Pfaffi method. $^{c}P < 0.05$, $^{d}P < 0.01$, $^{e}P < 0.001$ *vs* APAP. Experiments were reproduced three times.

ARTICLE HIGHLIGHTS

Research background

Acetaminophen overdose is the most frequent cause of drug-induced liver failure in the developed countries. Despite substantial progress in the understanding of the mechanism of hepatocellular injury, N-acetylcysteine remains the only effective treatment if administered within 8 h to 10 h of acetaminophen ingestion. Thus, other hepatoprotective drugs are needed for the delayed treatment of acetaminophen-induced hepatotoxicity.

Research motivation

Our interest focused on glycyrrhizin for its role as an inhibitor of high mobility group box 1 protein, a member of the family of damage associated molecular pattern, known to play important pathological roles in different diseases.

Research objectives

The present study aimed to investigate the efficacy of the N-acetylcysteine/

glycyrrhizin combination compared to N-acetylcysteine alone in the prevention of liver toxicity.

Research methods

Eight-week-old C57BL/6J wild-type female mice were used for all our experiments. Mice fasted for 15 h were treated with acetaminophen (500 mg/kg) or vehicle (phosphate-buffered saline) by intraperitoneal injection and separated into the following groups: Glycyrrhizin (200 mg/kg); N-acetylcysteine (150 mg/kg); and N-acetylcysteine/glycyrrhizin. Hepatotoxicity was assessed by biochemical and histopathological analyses. Survival rates were also compared.

Research results

In C57BL/6J mice, glycyrrhizin administration was shown to reduce the release of HMGB1 and to significantly decrease the severity of acetaminophen-induced liver injury. Thus, the co-administration of glycyrrhizin and N-acetylcysteine was investigated. Administered concomitantly with acetaminophen, the combination significantly reduced the severity of liver injury. Delayed administration of the combination of drugs, 2 h or 6 h after acetaminophen, also induced a significant decrease in hepatocyte necrosis compared to mice treated with N-acetylcysteine alone. In addition, administration of N-acetylcysteine/glycyrrhizin combination was associated with an improved survival rate compared to mice treated with only N-acetylcysteine.

Research conclusions

Compared to N-acetylcysteine alone, co-administration of glycyrrhizin decreases the liver necrosis score and improves survival in our murine model of acetaminophen-induced liver injury.

Research perspectives

Further experiments are needed to better investigate the efficacy of the N-acetylcysteine/glycyrrhizin combination, but these results suggest for the first time that the combination of an antioxidant like N-acetylcysteine and an anti-inflammatory drug like glycyrrhizin prevents the liver damage induced by acetaminophen intoxication.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Transaminitis is an indicator of mortality in patients with COVID-19: A retrospective cohort study

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Abstract

BACKGROUND

Since its discovery in Wuhan, China in December of 2019, the novel coronavirus has progressed to become one of the worst pandemics seen in the last 100 years. Recently, there has been an increased interest in the hepatic manifestations of coronavirus disease 19 (COVID-19).

AIM

To describe the demographic and clinical characteristics of COVID-19 positive patients and study the association between transaminitis and all-cause mortality.

METHODS

This is a descriptive retrospective cohort study of 130 consecutive patients with a positive COVID PCR test admitted between March 16, 2020 to May 14, 2020 at a tertiary care University-based medical center. The Wilcoxon-rank sum test and paired *t*-test were used for comparing non-parametric and parametric continuous variables respectively and a multivariable logistic regression models to study the association between transaminitis and mortality using SAS version 9.4 (SAS Institute, Cary, NC, United States).

formal analysis; Gupta K, Dhamoon SA and Sapkota B contributed to the validation; Sharma A, Lamichhane J, Dhamoon SA and Sapkota B supervised the manuscript.

Institutional review board statement: This study was approved by SUNY Upstate IRB.

Informed consent statement: This study was approved by the SUNY Upstate IRB board and an informed consent document was not needed. Please refer to the IRB document for further details.

Conflict-of-interest statement: None of the authors have any affiliations with or involvement in any organization or entity with any financial interest or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Data sharing statement: Not available.

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RESULTS

Out of the 130 patients, 73 (56%) patients were found to have transaminitis and 57 (44%) did not. When compared to patients without transaminitis, the transaminitis group was found to have a higher median body mass index (30.2 kg/m^2 *vs* 27.3 kg/m^2, $P = 0.04$). In the multivariate analysis those with transaminitis were found to have 3.4 times higher odds of dying as compared to those without transaminitis adjusting for gender, the Age-adjusted Charlson Comorbidity Index and admission to the intensive care unit ($P = 0.03$).

CONCLUSION

Our study showed that transaminitis on admission was associated with severe clinical outcomes such as admission to the intensive care unit, need for mechanical ventilation, and mortality.

Key Words: COVID-19; Liver; Mortality; Transaminitis; Liver enzymes; Aspartate aminotransferase; Alanine aminotransferase

Core Tip: Gastrointestinal symptoms have been well described in coronavirus disease 19 (COVID-19). In recent studies, transaminitis has been seen in patients with COVID-19. Our study has compared the characteristics between patients with transaminitis and patients without transaminitis. Transaminitis on presentation is an indicator of higher mortality in patients with COVID-19. This study shows the importance of identifying transaminitis in patients with COVID-19. It will help clinicians prognosticate based on the presence or absence of transaminitis on initial presentation.

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INTRODUCTION

Since its first description in Wuhan, China in December of 2019, the novel Coronavirus has progressed to become arguably the worst pandemic seen in the last 100 years[1,2]. The ease of transmissibility of the virus coupled with its penchant for resulting in life-threatening acute respiratory distress syndrome in certain groups of the population[3] has resulted in international lockdowns. As of June 20, 2020, 8.3 million cases have been reported in 213 countries, of which 2 million are present in the United States alone. While it took two and half months to reach 100000 coronavirus disease 19 (COVID-19) cases in the United States, it only took another 2 mo to reach 100000 COVID19 associated death[4,5].

While it is evident that manifestations are primarily respiratory in nature[6], the gastrointestinal manifestations of COVID-19 too, have been described as an important finding and have sparked a great deal of interest among clinicians and researchers alike. There currently exists a great deal of literature describing the GI manifestations of COVID-19 and its importance in diagnosis, prognosis and mode of transmission[7-9]. The most commonly described gastrointestinal symptom that has been reported is diarrhea, which has been reported to be present in 3%-30% of patients testing positive with COVID-19[7-10]. The presence of viral shedding in stool samples of patients with evidence that gastrointestinal symptoms may manifest devoid of respiratory complaints lays the basis for a potential fecal-oral mode of transmission[9,11]. Other symptoms, such as nausea, vomiting and abdominal pain have also been studied in great detail and have implications for patient prognosis[12].

More recently, there has been an increased interest in the hepatic manifestations of COVID-19[13-15]. Studies suggest that transaminases may act as a surrogate marker for disease severity and a predictor of mortality[15,16]. This may be in part due to the similarity of the genome between COVID-19 [severe acute respiratory syndrome

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coronavirus 2 (SARS-CoV-2)] and SARS-CoV[17] or due to the ability of the virus to bind to the ACE2 receptor[7,18,19] which has now been established to be present in not only the alimentary canal[9,20], but also in hepatic cholangiocytes[12,21]. This binding would allow for viral entry and replication within the hepatocytes during the initial phases.

The clinical impact of the hepatic manifestations of COVID-19 infection has led to our attempt to describe the association of transaminitis with patient morbidity and mortality in the Central New York population. This would be the first study in the United States, outside of New York City to study the effect of the disease on liver function. The aims of our study are to describe the demographic and clinic characteristics of hospitalized COVID-19 positive patients and study the association between transaminitis and all-cause mortality.

MATERIALS AND METHODS

Study design

This is a retrospective cohort study of 130 consecutive patients with a positive COVID PCR test admitted between March 16, 2020 to May 14, 2020 at a tertiary level University medical center. The study was approved by SUNY Upstate Institutional Review Board (IRB). Adult patients ≥ 18 years with a COVID positive PCR test, admitted to either of the hospitals, were included in the study. Patients who were 90 years and older or those that tested positive but did not get admitted to the hospital (including patients who only had ED visits) were excluded from the study. All study participants were followed until discharge or death until May 25, 2020.

A manual review of electronic medical records was done by three authors (Suresh Kumar VC, Mukherjee S and Harne PS). Data was abstracted into an IRB approved data collection sheet. An initial screen yielded 340 eligible medical records (Figure 1). Upon viewing the charts individually using the inclusion and exclusion criteria, a total of 210 records were excluded (154 patients were not admitted in either hospital, 40 patients did not possess an aspartate aminotransferase/alanine aminotransferase (AST/ALT) on admission, 16 patients were above the age of 89). A total of 130 patients were included in the study. Information on co-morbidities was examined and extracted based on ICD-9 and ICD-10 codes attached to the medical records and was manually verified for accuracy by one of the authors. Only laboratory and imaging findings on the initial presentation to the hospital were included in our study. Peak values or trends were not examined due to the potential for several confounders. All data were assessed based on the reference ranges of our institution's laboratory and was represented in standard units. We defined elevated liver enzymes or transaminitis as an elevation in ALT and/or AST. In women, the upper limit of normal (ULN) was > 32 IU/L for AST and > 33 IU/L for ALT. In men, the ULN was > 40 IU/L for AST, > 41 IU/L for ALT. Comorbidity was measured using the age-adjusted Charlson Comorbidity Index (AACI) score which predicts a 10-year survival based on the total score. The AACI score was based on the weighted sum of a patient's pre-existing comorbid conditions and a point was added for every decade of life after age 50[22,23] (Table 1).

Statistical analysis

We included 130 patients based on the inclusion and exclusion criteria. The Wilcoxon-rank sum test and paired t-test were used for comparing non-parametric and parametric continuous variables respectively. The Chi-square test was used for comparing categorical variables. We used multivariable logistic regression models to study the association between transaminitis and death. Further, we used the median AACI to categorize individuals as having a high or low comorbidity score. A two-sided α of 0.05 was used to establish significance. All analyses were performed by a biomedical statistician (Gupta K) in SAS version 9.4 (SAS Institute, Cary, NC, United States).

RESULTS

Patient characteristics

A total of 130 adult patients were included in the final analysis. 59 (45.4%) were females and 71 (54.6%) were males with a median age of 62 years. On average, the

Table 1 Charlson Co-morbidity index

Co-morbid condition	cCCI weights	uCCI weights
Myocardial infarction	1	0
Congestive heart failure	1	2
Peripheral vascular disease	1	0
Cerebrovascular disease	1	0
Dementia	1	2
Chronic obstructive pulmonary disease	1	1
Connective tissue disorder	1	1
Peptic ulcer disease	1	0
Mild liver disease	1	2
Diabetes without complication	1	0
Diabetes with complication	2	1
Hemiplegia or paraplegia	2	2
Chronic kidney disease stage III	2	1
Any malignancy without metastasis	2	2
Leukemia	2	
Lymphoma	2	
Moderate or severe liver disease	3	4
Metastatic tumor	6	6
AIDS	6	4
Maximum score	33	24

Adapted from Ternavasio-de la Vega *et al*[27] (2018). AIDS: Acquired Immune Deficiency Syndrome; cCCI: Classical Charlson Comorbidity Index; uCCI: Updated Charlson Comorbidity Index.

patients were found to be overweight with a median body mass index of 28.7 kg/m^2. The median AACI was found to be 3.5 indicating that the average 10-year survival was at least greater than 53% based on the score. 58 patients (44.6%) were admitted to the intensive care unit and 43 (33.1%) required ventilator support. Of 102 (78.5%) patients were discharged from the hospital, 28 (21.5%) died with a follow-up rate of 100%.

The median enzymes were ALT-29 IU/L, AST-39 IU/L, alkaline phosphatase (ALP)-77 IU/L, total bilirubin-0.4 mg/dL and direct bilirubin-0.2 mg/dL (n = 92). Although 46 (35.4%) patients were on medications such as that could impact liver function such as acetaminophen, statins etc., all 130 patients had baseline AST, ALT and ALP values that were within the ULN prior to this hospitalization indicating that the medications were not a confounding factor. For gastrointestinal (GI) symptoms, 34 (26.2%) had diarrhea, 34 (26.2%) had anorexia, 32 (24.6%) had nausea, 21 (16.2%) had abdominal pain and 37 (28.5%) had other GI symptoms such as loss of taste, vomiting, constipation, dysphagia, and reflux. The baseline clinical characteristics of the study population are summarized in Table 2.

Of 73 (56%) patients were found to have transaminitis and 57 (44%) did not have transaminitis. There was no significant difference in age or gender between the groups. When compared to patients without transaminitis, the transaminitis group was found to have a higher median body mass index (BMI) (30.2 kg/m^2 *vs* 27.3 kg/m^2, P = 0.04). They had a higher median ALT (48 IU/L *vs* 15 IU/L, P < 0.001), AST (66 IU/L *vs* 20 IU/L, P < 0.001), ALP (97 IU/L *vs* 70 IU/L, P < 0.001). The patients with transaminitis also had significantly lower albumin (3.0 g/dL *vs* 3.4 g/dL, P = 0.01). Patients with transaminitis had more chest X-ray findings such as ground glass opacities and bilateral infiltrates (75.3% *vs* 56.1%, P = 0.02). They had a higher rate of intensive care unit (ICU) admission (53.4% *vs* 33.3%, P = 0.02) and higher rate of need for ventilator support (42.5% *vs* 21.1%, P = 0.02). The difference between the two groups are shown in Table 3.

Table 2 Demographic and baseline characteristics of the study population

Variable	Study population (*n* = 130)
Age, yr, median (IQR)	62 (48, 73)
Females	59 (45.4)
Body mass index, kg/m^2, median (IQR)	28.7 (26, 36)
Smoking	38 (29.2)
Alcohol	30 (23.1)
Diarrhea	34 (26.2)
Nausea	32 (24.6)
Abdominal pain	21 (16.2)
Anorexia	34 (26.2)
Other gastro-intestinal symptoms[1]	37 (28.5)
X-ray findings[2]	87 (66.9)
Hypertension	69 (53.1)
Admitted to ICU	58 (44.6)
On ventilator	43 (33.1)
Age-adjusted Charlson Index, median (IQR)	3.5 (1, 6)
On medications with gastro-intestinal side-effects	46 (35.4)
Death	28 (21.5)
Lactic Acid, mean (SD), *n* = 97	1.7 (1.4)
Lactic acid dehydrogenase, mean (SD), *n* = 92	424 (182.6)
D-dimer, median (IQR), *n* = 105	1.7 (0.9, 3.7)
Alanine aminotransferase, IU/L, median (IQR)	29 (16, 53)
Aspartate aminotransferase, IU/L, median (IQR)	39 (23, 68)
Alkaline phosphatase, IU/L, median (IQR)	77 (60, 115)
Total bilirubin, mean (SD)	0.4 (0.3)
Direct bilirubin, mean (SD), *n* = 92	0.2 (0.1)
Albumin, g/dL, mean (SD)	3.2 (0.8)
Prothrombin time, seconds, mean (SD), *n* = 81	16.9 (8.1)

[1]Other gastro-intestinal symptoms include loss of taste or smell, constipation, dysphagia, reflux and vomiting.
[2]X-ray findings including ground glass opacities and bilateral infiltrate. All values are reported as *n* (%), mean (SD) or median (25th, 75th). *P* value: Chi-square test for categorical variables, two-sided *t*-test for parametric continuous variables and Wilcoxon rank-sum test for nonparametric continuous variables. ICU: Intensive care unit; IQR: Interquartile range.

In the univariate analysis to study the association between death and presence of transaminitis, those with transaminitis had 2.9 times higher odds of dying as compared to those without transaminitis (P = 0.03). This association remained significant after adjusting for confounders (Table 4). In the multivariate analysis those with transaminitis were found to have 3.4 times higher odds of dying as compared to those without transaminitis adjusting for gender, the AACI and admission to the ICU. An AACI score above the median value of 3.5 (OR = 2.9, 95%CI: 3.5-48.4) and admission to the ICU (OR = 3.6, 95%CI: 1.2-10.4) were significantly associated with the outcome in the final model.

DISCUSSION

Hepatic manifestations (such as transaminitis, bilirubin elevations, hypoalbuminemia,

Table 3 Characteristics of those with and without transaminitis

	Transaminitis (*n* = 73)	No transaminitis (*n* = 57)	*P* value
Age, yr, median (IQR)	63 (48, 72)	62 (48, 76)	0.9
Females	31 (42.5)	28 (49.1)	0.4
Body mass index, kg/m^2, median (IQR)	30.2 (26.5, 36.8)	27.3 (24.5, 33.2)	0.04
Smoking	19 (26)	19 (33.3)	0.4
Alcohol	20 (27.4)	10 (17.5)	0.2
Diarrhea	24 (32.9)	10 (17.5)	0.05
Nausea	18 (24.7)	14 (24.6)	1.0
Abdominal pain	11 (15.1)	10 (17.5)	0.7
Anorexia	19 (26)	15 (26.3)	1.0
Other gastro-intestinal symptoms[1]	19 (26)	18 (31.6)	0.5
X-ray findings[2]	55 (75.3)	32 (56.1)	0.02
Hypertension	38 (52.1)	31 (54.4)	0.8
Age-adjusted Charlson Index, median (IQR)	3 (1, 6)	4 (1, 6)	0.7
On ventilator	31 (42.5)	12 (21.1)	0.01
Admitted to intensive care unit	39 (53.4)	19 (33.3)	0.02
On medications with GI side-effects	26 (35.6)	20 (35.1)	1.0
Alanine aminotransferase, IU/L, median (IQR)	48 (34, 84)	15 (10, 20)	< 0.001
Aspartate aminotransferase, IU/L, median (IQR)	66 (42, 100)	20 (14, 26)	< 0.001
Alkaline phosphatase, IU/L, median (IQR)	97 (66, 124)	70 (56, 92)	< 0.001
Total bilirubin, mean (SD)	0.6 (0.3)	0.4 (0.2)	< 0.001
Albumin, g/dL, mean (SD)	3.0 (0.8)	3.4 (0.7)	0.01

[1]Other gastro-intestinal symptoms include loss of taste or smell, constipation, dysphagia, reflux and vomiting.
[2]X-ray findings including ground glass opacities and bilateral infiltrate. All values are reported as *n* (%), mean (SD) or median (25^{th}, 75^{th}). *P* value: Chi-square test for categorical variables, two-sided *t*-test for parametric continuous variables and Wilcoxon rank-sum test for nonparametric continuous variables. IQR: Interquartile range; GI: Gastrointestinal.

Table 4 Multivariable logistic model for death in patients with and without transaminitis

Variable	Model 1		Model 2		Model 3	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Transaminitis	2.9 (1.1-7.4)	0.03	3.1 (1.2-8.0)	0.02	3.4 (1.2-10.1)	0.03
Gender, reference females	-		0.5 (0.2-1.2)		0.5 (0.2-1.4)	
AACI above median score of 3.5	-		-		12.9 (3.5-48.4)	
Admission to intensive care unit	-		-		3.6 (1.2-10.4)	

Model 1 is unadjusted. Model 2 is adjusted for gender. Model 3 is adjusted for gender, age adjusted-Charlson Index and admission to the intensive care unit. AACI: Age adjusted-Charlson Index; OR: Odds ratio; CI: Confidence interval.

etc.) resulting in an overall disturbance in homeostasis is garnering attention as a clinically significant consequence of COVID-19 infection. We defined transaminitis as elevations of either AST or ALT more than the ULN. In our retrospective cohort study of 130 patients, we found that patients with transaminase elevation had an overall 2.9-times higher odds of dying as compared to those who did not. Additionally, it was seen that transaminase elevation was more likely to be seen in patients who were admitted to the ICU and required mechanical ventilation. This potentially reveals the

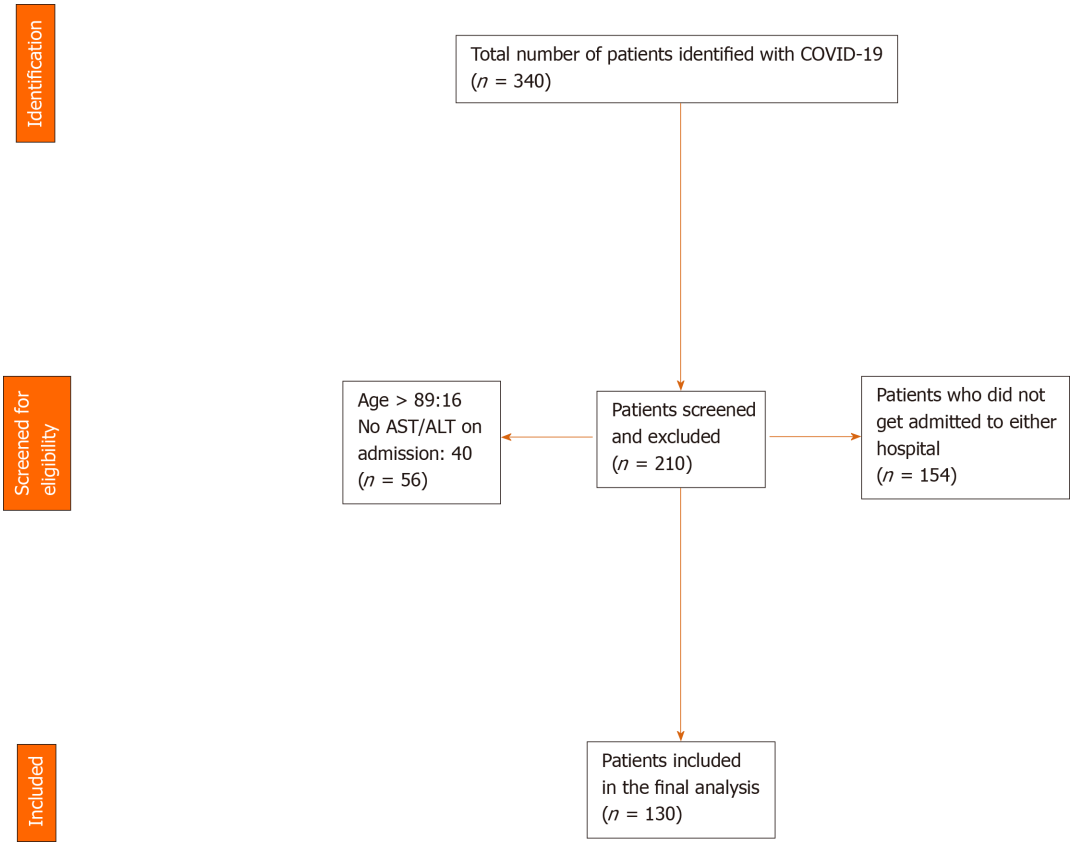

Figure 1 Patient selection flow diagram. COVID: Coronavirus 19; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

role of transaminase elevation as a prognostic marker for patients infected with the SARS-CoV-2 virus. This finding was in concert with the studies suggesting a higher risk of ICU admission, mechanical ventilation, and deaths in patients with evidence of transaminitis on admission[8,15].

The mechanism of liver injury in COVID-19 infection is largely unknown. The expression of the ACE2 receptor not only on the alimentary canal but also on the hepatic cholangiocytes and biliary epithelial cells seems to be implicated in viral entry and replication. The consequent liver injury is thought to be due to the insults mediated by direct viral effects and the host's immune response[7,12,18,19,21,24]. A mixed hepatocellular pattern of liver injury was observed in our patients with a statistically significant elevation of total bilirubin levels in the patients with transaminitis, consistent with impaired clearance and cholestasis which would be expected with the expression of ACE2 receptor on biliary epithelia.

Emerging data on hypoalbuminemia and thrombosis points towards the impaired synthetic function of the liver in patients with COVID-19 infection[25]. In our study, patients with transaminitis had a greater degree of hypoalbuminemia, which was similar to the study conducted by Phipps *et al*[15].

Obese patients have a higher level of free fatty acids which puts them at risk of transaminitis. The BMI of a patient has been an independent predictor of hospitalization and severity of COVID-19 infection as outlined by Stefan *et al*[26] in their review. Our study showed that patients with transaminitis (with normal baseline transaminases) were more likely to have a higher BMI than those without transaminitis. Our patients in the transaminitis arm had a larger incidence of ground-glass opacities on chest X-rays as compared to the non-transaminitis arm, which was another indicator of the severity of this disease.

The existing literature points towards diarrhea as the most common GI complaint in COVID-19 patients[6,7,9,10]. Although not statistically significant, diarrhea seemed to be more common in patients with transaminitis than those without (P = 0.05), however, further studies are required to substantiate an association.

Strengths and limitations

Our study is the first study to our knowledge outside of New York City in the United States focusing on establishing an association between transaminitis and various

patient demographic and clinical characteristics as well as morbidity and mortality parameters in patients with COVID-19 infection. We had a follow-up rate of 98.5% which helped minimize attrition. We attempted to minimize confounding by using initial lab values on presentation rather than trends or peaks.

However, the study was not without limitations. The sample size was relatively small as compared to already published data on the subject. A comparison group of non-COVID19 patients would have helped minimize bias further. Finally, since the data is from admitted patients in two hospitals, it might not be generalizable to the general population especially with asymptomatic or mild disease.

CONCLUSION

In this study, transaminitis on admission was associated with severe clinical outcomes. Its potential role as a prognostic marker for hospitalized patients with COVID-19 infection is highlighted here.

ARTICLE HIGHLIGHTS

Research background

Since its discovery in Wuhan, China in December of 2019, the novel coronavirus has progressed to become one of the worst pandemics seen in the last 100 years. Recently, there has been an increased interest in the hepatic manifestations of coronavirus disease 19 (COVID-19).

Research motivation

To understand if transaminitis was an indicator of severity of the disease in patients with COVID-19.

Research objectives

Describe the demographic and clinical characteristics of COVID-19 positive patients and study the association between transaminitis and all-cause mortality.

Research methods

This is a retrospective cohort study of 130 consecutive patients with a positive COVID PCR test admitted between March 16, 2020 to May 14, 2020 at a tertiary care University-based medical center.

Research results

Transaminitis on admission was associated with severe clinical outcomes such as admission to the intensive care unit, need for mechanical ventilation, and mortality.

Research conclusions

There is a potential role of transaminase elevation as a prognostic marker for hospitalized patients with COVID-19 infection.

Research perspectives

This brings into perspective the need for careful assessment for transaminitis on presentation in a patient with COVID-19 as this is shown to be an indicator for mortality in this study.

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ORIGINAL ARTICLE

Retrospective Study

Clinical efficacy of direct-acting antiviral therapy for recurrent hepatitis C virus infection after liver transplantation in patients with hepatocellular carcinoma

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Abstract

BACKGROUND

Recurrent hepatitis C virus (HCV) infection of transplanted liver allografts is universal in patients with detectable HCV viremia at the time of transplantation. Direct-acting antiviral (DAA) therapy has been adopted as the standard of care for recurrent HCV infection in the post-transplant setting. However, there are insufficient data regarding its efficacy in liver transplant (LT) recipients with a history of hepatocellular carcinoma (HCC), and the risk of HCC recurrence after DAA therapy is unknown.

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AIM
To demonstrate predictors of DAA treatment failure and HCC recurrence in LT recipients.

METHODS
A total of 106 LT recipients given DAAs for recurrent HCV infection from 2015 to 2019 were identified (68 with and 38 without HCC). Descriptive statistics and logistic regression models were used to estimate the multivariate odds ratios and respective 95% confidence intervals for predictors of treatment failure and HCC recurrence.

RESULTS
Six patients (6%) experienced DAA therapy failure post-LT and 100 (94%) had a sustained virologic response at follow-up week 12. A high alanine transaminase level > 35 U/L at treatment week 4 was a significant predictor of treatment failure. Relapse to pre-LT DAA therapy is a predictor of post-LT HCC recurrence, $P = 0.04$. DAA relapse post-LT was also associated with post-transplantation HCC recurrence, $P = 0.05$.

CONCLUSION
DAAs are effective and safe in the treatment of recurrent HCV infection in LT recipients with history of HCC. Relapse to pre- and post-LT DAA therapy is associated with post-transplantation HCC recurrence.

Key Words: Direct-acting antiviral; Liver transplant; Hepatocellular carcinoma; Recurrence

Core Tip: Our study is the first to find an association between direct-acting antiviral relapse and hepatocellular carcinoma (HCC) recurrence in patients with past history of HCC pre-transplant. Also, our study is the first to highlight high sustained virologic response in patients with past history of HCC after liver transplantation which is similar to patients without past history of HCC as we removed the tumor-harboring liver.

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INTRODUCTION

Hepatitis C virus (HCV) infection is the leading indication for liver transplantation[1]. Recurrent HCV infection of transplanted liver allografts is universal in patients with detectable HCV viremia at the time of transplantation[2]. Evidence of recurrent HCV infection of an allograft occurs as early as 4 wk after transplantation, with development of hepatitis within 6-12 mo in 70%-90% of HCV-infected liver transplant (LT) recipients[3,4]. Furthermore, allograft cirrhosis with a poor outcome occurs within 5 years after transplantation in 20%-30% of these patients[5].

Direct-acting antiviral (DAA) therapy has been adopted as the standard of care for recurrent HCV infection after transplantation[6]. Patients with sustained virologic responses to antiviral therapy may experience fibrosis regression and reduced mortality rates[7,8]. In pre-transplant studies, there are conflicting data regarding the impact of DAA treatment on de-novo or recurrent hepatocellular carcinoma (HCC) in HCV-infected patients[9-12].

Researchers have investigated the safety and efficacy of DAA treatment in LT recipients[13-15]. However, the efficacy in LT recipients with a history of HCC has not been investigated. Moreover, there are no data regarding HCC recurrence in LT recipients following DAA therapy, especially patients who experienced treatment

failure. Little is known about predictors of DAA treatment response after LT for HCC. Therefore, we performed this study to (1) Highlight the efficacy and safety of DAA therapy in LT recipients with HCV infections and history of HCC; and (2) Investigate the impact of DAA use on post-transplantation HCC recurrence.

MATERIALS AND METHODS

Study design

A total of 106 LT recipients given DAAs for recurrent HCV infection from January 1, 2015, to March 1, 2019, at Baylor College of Medicine were retrospectively identified.

Criteria for study inclusion were (1) Age of 18 years or older; (2) History of liver transplantation; (3) Positive anti-HCV and HCV ribonucleic acid prior to and after transplantation; and (4) Treatment with an oral DAA regimen with or without ribavirin. We excluded (1) Patients with active hepatitis B virus (HBV) infection evident by positive hepatitis B surface antigens or HBV deoxyribonucleic acid; (2) Patients with human immunodeficiency virus infection; and (3) Any other metabolic, viral or genetic causes of chronic liver disease. Patients were classified into two groups: Those with pre-transplantation HCC (PHCC; n = 68) and those without pre-transplantation HCC (PnHCC; n = 38). The initiation of DAA treatment and the regimen type and duration were determined by the treating transplant hepatologists. Demographic and clinical information for each participant were documented and stored in a secure database under a Baylor College of Medicine Institutional Review Board-approved protocol. All patients gave written informed consent for data collection prior to their LTs.

Patient information

Clinical data: The collected information included the following baseline characteristics: Age, sex, ethnicity, body mass index (BMI), medical history (comorbid conditions, stage of liver disease), adverse events, baseline (at time of DAA initiation) and on-treatment laboratory values obtained every 4 wk, including HCV ribonucleic acid levels, until the end of treatment (EOT) and at follow-up visits. BMI was calculated at treatment initiation, the EOT, and the last follow-up visit (normal BMI, < 25 kg/m^2; overweight, 25-30 kg/m^2; obese, > 30 kg/m^2).

Laboratory data: The following laboratory test results were analyzed: Anti-HCV and HCV ribonucleic acid levels; HCV genotypes; Hepatitis B surface antigens; Anti-hepatitis B surface antibodies; Hepatitis B core antibodies; Anti-human immunodeficiency virus antibodies; Epstein-Barr virus, cyotmegalovirus, iron studies, ceruloplasmin, auto-antibodies, serum albumin, alkaline phosphatase, creatinine, sodium, alanine aminotransferase, and total bilirubin levels; Prothrombin time and international normalized ratio; Hemoglobin levels; and Platelet counts. Test results were analyzed to exclude other viral, metabolic, autoimmune causes of liver disease. Estimated glomerular filtration rates were calculated using the Modification of Diet in Renal Disease equation. The model for end-stage liver disease-sodium score was calculated using serum bilirubin, creatinine, and sodium levels and the international normalized ratio.

Clinical efficacy: Treatment efficacy was demonstrated by sustained virologic response at follow-up week 12, defined as an undetectable HCV ribonucleic acid level in the blood at week 12 after the EOT. Accordingly, patients were classified as having DAA responses or failures.

Clinical safety: Adverse events were defined as any events that required an HCV medication dose reduction or treatment discontinuation or the addition of a concomitant medication for management. Events of special interest in this study were HCC recurrence, HBV reactivation and acute allograft rejection.

Statistical Analysis

Stata software (Stata Corp, College Station, TX, United States) was used for statistical analysis. Descriptive statistics were employed, with Student t-test and Mann-Whitney-Wilcoxon test statistics used to assess the significance of mean and median differences in continuous variables between study groups. The Pearson χ^2 or Fisher exact test was used to test for differences in the distribution of categorical data between groups. To adjust for the small sample size, multivariate exact unconditional logistic regression

analyses were performed. For each risk factor, the adjusted odds ratios and respective 95% confidence intervals were calculated. All odds ratios were adjusted for age, sex, and significant factors from univariate analysis. *P* value less than 0.05 was defined as significant.

RESULTS

Baseline patient characteristics

Most patients in the PHCC and PnHCC groups were male and white (Table 1). The patients with PHCC were significantly older ($P = 0.01$), with a significantly more extensive past history of smoking than the patients with PnHCC ($P < 0.01$). Baseline comorbidities and cirrhosis were similar in both groups. However, diabetes mellitus was significantly higher in the PHCC group ($P < 0.01$).

HCV genotypes 1a and 1b were present in 50%, and 21% of the PHCC patients, respectively, and 66% and 21% of the PnHCC patients, respectively ($P = 0.1$). Although not statistically significant, more PHCC patients had HCV genotype 3a than PnHCC patients (22% *vs* 11%; $P = 0.1$). The mean ± SD BMIs at DAA treatment initiation were comparable in the PHCC and PnHCC patients (28.5 ± 4.5 kg/m^2 *vs* 27.9 ± 5.5 kg/m^2; $P = 0.6$).

Pre-transplant relapse rate to antiviral treatment was higher in the PHCC patients than in the PnHCC patients (34% *vs* 16%; $P = 0.06$). Although there was no statistically significant difference, more PHCC patients received HCV- or anti-HBc-positive allografts than PnHCC patients [6% *vs* 0% ($P = 0.3$) and 15% *vs* 3% ($P = 0.09$), respectively].

Table 1 shows significant variations in the mean and median laboratory and clinical values between the two groups. Baseline (at time of DAA initiation) laboratory values were similar in the PHCC and PnHCC patients except for the serum creatinine level, estimated glomerular filtration rate, and the model for end-stage liver disease-sodium score, which were markedly lower in the PHCC patients (Table 1).

Clinical efficacy

The median time from liver transplantation to DAA treatment initiation in the entire cohort was 34 mo (range, 1-331 mo), and the median follow-up duration after DAA treatment completion was 20 mo (range, 3-46 mo). The median time from liver transplantation to DAA treatment initiation in the PHCC patients was 28 mo (range, 1-171 mo) which is significantly shorter than in the PnHCC patients, 91.5 mo (range, 2.0-331.0 mo) ($P < 0.0001$). One hundred patients (94%) experienced sustained virologic response at follow-up week 12 (SVR12) (DAA response), whereas 6 patients (6%) did not (DAA failure). The median times from transplantation to DAA therapy initiation were 32.5 mo (range, 1.0-331.0 mo) in the DAA response group and 54.5 mo (range, 8.0-148.0 mo) in those who had DAA failure ($P = 0.7$).

All patients who did not experience SVR12 were non-cirrhotic. Two patients had relapses at follow-up week 12, three patients did so at follow-up week 4, and one patient suffered viral breakthrough at treatment week 12.

In the DAA response group, the rates of concordance between SVR12 and SVR at follow-up week 24, and between SVR12 and quantitative HCV polymerase chain reaction at the last follow-up visit were both 100%, as no patients had relapses after experiencing SVR12.

Figure 1 shows the numbers of patients who had responses to and failures of the 16 DAA regimens used in this study. We observed no significant variations in the treatment distribution between the PHCC and PnHCC groups ($P = 0.3$). The six patients who had DAA failures received five DAA regimens: (1) Sofosbuvir (SOF)/velpatasvir /voxilaprevir plus ribavirin (RBV) for 12 wk ($n = 2$); (2) SOF, daclatasvir, and RBV for 24 wk ($n = 1$); (3) SOF/ledipasvir for 24 wk ($n = 1$); (4) SOF/simeprevir for 12 wk ($n = 1$); and (5) Glecaprevir /pibrentasvir for 12 wk ($n = 1$).

Table 2 lists the univariate and multivariate odds ratios (ORs) and 95% confidence interval (CI) for potential predictors of post-LT DAA therapy failure. Univariate analysis showed a significant risk of failure ($P < 0.05$) in patients with HCV-positive allografts; HCV genotype 3a; high alanine aminotransferase (ALT) level > 35 U/L and or quantitative HCV polymerase chain reaction > 15 IU/mL at treatment week 4. However, multivariate analysis after adjustment for these predictors demonstrated a high ALT level > 35 U/L at treatment week 4 was significantly associated with DAA therapy failure ($P = 0.04$) after adjustment for the confounding effect of the significant factors described above. Pre-transplant relapse to DAA treatment and history of pre-

Table 1 Characteristics of the study population according to pre-transplantation hepatocellular carcinoma status

Characteristic	Number (%)		*P* value
	Non-HCC (*n* = 38)	HCC (*n* = 68)	
Demographics			
mean (± SD) age at treatment initiation, years	58.2 ± 11.1	62.2 ± 5.9	0.01
Median age at treatment initiation, yr (range)	62 (28-74)	62.5 (47.0-78.0)	0.2
Male	25 (66)	50 (74)	0.5
Female	13 (34)	18 (26)	
White	19 (50)	37 (54)	0.4
Black	11 (29)	11 (16)	
Hispanic	6 (16)	17 (25)	
Asian	2 (5)	3 (4)	
Epidemiologic characteristics			
mean (± SD) baseline BMI	27.9 ± 5.5	28.5 ± 4.5	0.6
Median baseline BMI, (range)	27.0 (21.0-48.5)	28.5 (17.0-46.0)	0.3
Alcohol drinking	11 (29)	32 (47)	0.08
Cigarette smoking	18 (47)	51 (75)	0.01
Baseline co-morbidities			
Diabetes Mellitus	20 (53)	53 (78)	0.01
CKD	20 (53)	23 (34)	0.06
eGFR < 30 mL/min/1.73 m^2	7 (18)	2 (3)	0.01
Cirrhosis	5 (15)	4 (8)	0.5
HCV-positive allograft	0	4 (6)	0.3
Anti-HBc-positive allograft	1 (3)	10 (15)	0.09
Pre-transplant relapse to antiviral treatment	6 (16)	23 (34)	0.06
mean (± SD) time from LT to DAA initiation, mo	99.3 ± 79.7	39.4 ± 40.5	0.0001
Median time from LT to DAA initiation, months (range)	91.5 (2.0-331.0)	28 (1-171)	0.0001
Baseline laboratory values			
mean (± SD) creatinine level, mg/dL	1.9 ± 1.9	1.2 ± 0.8	0.08
Median creatinine level, mg/dL (range)	1.2 (0.7-8.7)	1.0 (0.7-7.0)	0.01
mean (± SD) AFP level, ng/mL	10.2 ± 30.5	6.8 ± 12.8	0.5
Median AFP level, ng/mL (range)	3.2 (2.0-149.0)	3.1 (1.3-88.0)	0.5
mean (± SD) MELD-Na score	13 ± 5	10 ± 4	0.01
Median MELD-Na score (range)	13 (6-24)	9 (6-24)	0.01
Clinical outcomes			
Relapse at follow-up week 12	1 (3)	5 (7)	0.4
SVR12	37 (97)	63 (93)	0.4
HCC recurrence after DAA treatment	0	6 (9)	0.08

HCC: Hepatocellular carcinoma; SD: Standard deviation; BMI: Body mass index; CKD: Chronic kidney disease; HCV: Hepatitis C virus; HBc: Hepatitis B core; LT: Liver transplant; DAA: Direct-acting antiviral; AFP: Alpha-fetoprotein; SVR: Sustained virologic response.

transplant HCC did not impact SVR12 rates in patients treated with DAA post-LT.

We observed normalization of ALT levels ≤ 35 U/L at treatment week 4 in 85% of the patients (Figure 2 and Table 3). Moreover, the mean platelet count in all patients improved significantly over the follow-up period with a mean difference of -36 (-47.9 to -6), $P < 0.001$ (Figure 3 and Table 4).

HCC recurrence after DAA treatment

In the PHCC group, six patients (9%) experienced HCC recurrence after DAA treatment initiation. The median time from DAA therapy initiation to HCC recurrence was 8.5 mo (range, 1.0-29.0 mo).

The demographic characteristics, HCC risk factors, and clinical features were similar in the patients who had HCC recurrences after DAA treatment ($n = 6$) and those who did not ($n = 62$), demonstrating a lack of a significant impact of these factors on prediction of recurrence after the treatment (Table 5). Of the patients who experienced HCC recurrence after DAA treatment, 5 patients (83%) had relapses to pre-transplant DAA treatment, suggesting pre-transplant relapse is a predictor of post-transplant HCC recurrence. The estimated adjusted OR was 14.9 (95%CI: 1.1-219.3).

In addition, we identified relapse to the post-transplantation DAA therapy as a potential predictor of HCC recurrence ($P = 0.05$), with a 10-fold greater risk of recurrence in patients who had treatment failures than in responders. The estimated adjusted OR was 10.6 (95%CI: 1.0-121.6) (Table 5).

RBV-containing regimens

We also categorized patients into two groups according to use of RBV (Table 6). Demographic characteristics and baseline laboratory values were similar for patients given DAA therapy with and without RBV except for the serum creatinine and hemoglobin levels, the model for end-stage liver disease-sodium score, estimated glomerular filtration rate, and presence of chronic kidney disease.

Patients who received DAA regimens with and without RBV had similar SVR12 rates (95.5% *vs* 92.1%; $P = 0.7$).

BMI and DAA therapy

A high baseline BMI (≥ 25 kg/m^2) did not have a more significant impact on SVR12 or HCC recurrence after DAA treatment than a low BMI (< 25 kg/m^2) (Tables 2 and 5). However, among those with PHCC, weight loss by the end of DAA treatment was a significant predictor of HCC recurrence ($P = 0.03$).

Safety and tolerability

Only one patient in the PnHCC group had to discontinue DAA treatment SOF/ledipasvir at 16 weeks due to adverse events, which consisted of significant fatigue and headache, but achieved SVR12. However, of the patients receiving RBV-containing DAA regimens, 26 (38%) needed RBV discontinuation or dose modification due to adverse events, including anemia, fatigue, nausea, and elevated serum creatinine level.

No patients experienced decompensation during or after DAA treatment until the last follow-up visit. Also, none of anti-HBc-positive patients (30%) experienced HBV reactivation during or after DAA therapy until the last follow-up visit.

Physicians administered tacrolimus-containing regimens to 83% of the patients during DAA therapy. We found no significant changes in immunosuppressive drug dosages or levels during DAA treatment. Five patients (5%) experienced mild acute rejection episodes within a median time of 11 mo (range, 5-28 mo) after DAA treatment completion, all of whom experienced SVR12 and rejection episodes were controlled.

DISCUSSION

The present study is the first to demonstrate that pre-transplant relapse to DAA therapy is a significant predictor of post-transplant HCC recurrence. Post-LT DAA therapy relapse is also associated with HCC recurrence in LT recipients. High ALT level > 35 U/L at treatment week 4 is a predictor of DAA treatment failure.

All oral DAA treatments were recently adopted as the standard of care for treatment of recurrent HCV infection in LT recipients. However, contradictory data regarding the increased risk of HCC recurrence in pre-transplant patients after DAA therapy, and HCC influence on DAA SVR rates may affect the decision to treat unique

Table 2 Risk factors for post-liver transplant direct-acting antiviral therapy response (Logistic regression analysis)

Variable	Number of DAA therapy responses/failures (*n* = 100/6)	Univariate OR (95%CI)	*P* value	Multivariate OR (95%CI)	*P* value
Age > 60 yr	60/4	1.3 (0.2-15.4)	0.8		
Male	70/5	2.1 (0.2-104.7)	0.9		
Baseline BMI (≥ 25 kg/m^2)	76/5	1.6 (0.2-77.7)	0.9		
Alcohol drinking	39/4	3.1 (0.4-35.7)	0.4		
Cigarette smoking	64/5	2.8 (0.3-136.6)	0.6		
Diabetes Mellitus	70/3	0.4 (0.1-3.4)	0.5		
CKD	42/1	0.30 (0.01-2.60)	0.4		
Cirrhosis	9/0	-			
Pre-LT relapse to DAA	25/4	5.9 (0.8-68.7)	0.09		
Anti-HBc-positive allograft	9/2	4.9 (0.4-40.4)	0.2		
HCV-positive allograft	2/2	22.0 (1.3-381.3)	0.02	3.2 (0.1-856.8)	0.8
HCV genotype 3a	15/4	10.9 (1.4-130.9)	0.03	1.40 (0.02-89.10)	0.9
Presence of HCC before LT	63/5	2.9 (0.3-142.6)	0.6		
Rejection during/after DAA therapy	5/0	-	-		
ALT level at treatment week 4 (> 35 IU/L)	16/4	1.02 (1.00-1.05)	0.001	1.04 (1.00-1.10)	0.04
Quantitative HCV PCR at treatment week 4 (> 15 IU/mL)	8/3	10.9 (1.3-96.4)	0.01	3.6 (0.1-172.3)	0.5

HCC: Hepatocellular carcinoma; BMI: Body mass index; CKD: Chronic kidney disease; DAA: Direct-acting antiviral; HCV: Hepatitis C virus; OR: Odds ratio; CI: Confidence interval; ALT: Alanine aminotransferase; LT: Liver transplant; HBc: Hepatitis B core; PCR: Polymerase chain reaction.

Table 3 mean (± SD) alanine aminotransferase levels in patients who had direct-acting antiviral therapy responses and failure (baseline, week 4, week 8, and week 12)

SVR12 Achievement	Baseline ALT, mean (± SD)	ALT at treatment week 4, mean (± SD)	ALT at treatment week 8, mean (± SD)	ALT at treatment week 12, mean (± SD)
Responders	80.67 (± 102.447)	25.21 (± 20.416)	19.78 (± 11.651)	20.21 (± 10.994)
Failures	133.00 (±103.057)	61.17 (± 71.337)	54.50 (± 63.385)	56.83 (± 43.938)
P values	0.2	0.001	0.0001	0.0001

ALT: Alanine aminotransferase.

populations of patients as LT recipients with past histories of HCC.

There is increasing evidence of interaction between DAA therapy and HCC in the recent literature. Patients with HCC tend to have low rates of SVR12, whereas those who do not experience SVR12 have increased risk of HCC[16-18]. In an editorial, Hollande and Pol[19] recommended intensive surveillance for HCC in patients who had DAA therapy failure. Yet researchers did not investigate these findings in LT recipients with PHCC[17]. Consistently, we observed high risk of HCC recurrence in LT recipients who had DAA treatment failure. However, history of pre-transplant HCC did not impact SVR12 rates in patients treated with DAA post-LT as we removed tumor-affected livers.

LT recipients may be a reliable population to study the interaction between DAAs and HCC, especially in those with a failed DAA therapy after transplantation. This is driven by two factors. First, LT recipients have the benefit of receiving non-cirrhotic liver allografts with low prevalence of non-characterized nodules, which investigators have pointed to as the cause of *de-novo* and recurrent HCC in patients undergoing

Table 4 mean (± SD) platelet counts in patients who had direct-acting antiviral therapy responses and failure (baseline, week 4, week 8, and week 12)

SVR12 Achievement	Baseline PLT, mean (± SD)	PLT at treatment week 4, mean (± SD)	PLT at treatment week 8, mean (± SD)	PLT at treatment week 12, mean (± SD)
Responders	152.54 (± 55.880)	172.33 (± 72.156)	164.94 (± 53.580)	169.42 (± 59.499)
Failures	150.00 (± 35.157)	153.00 (± 58.258)	167.25 (± 57.058)	160.67 (± 60.902)
P values	0.9	0.5	0.9	0.7

PLT: Platelet.

Table 5 Risk factors for hepatocellular carcinoma recurrence post-liver transplant (Logistic regression analysis)

Variable	No HCC recurrence/HCC recurrence (*n* = 62/6)	Univariate OR(95%CI)	*P* value	Multivariate OR (95%CI)	*P* value
Age > 60 yr	39/3	0.6 (0.1-4.8)	0.8		
Male	45/5	1.9 (0.2-94.5)	0.9		
White	34/3	0.8 (0.1-6.7)	0.9		
Baseline BMI (≥ 25 kg/m^2)	50/4	0.5 (0.1-5.9)	0.7		
EOT BMI (< 25 kg/m^2)	11/4	8.8 (1.1-109.3)	0.03	12.3 (1.2-131.0)	0.03
Alcohol drinking	28/4	2.4 (0.3-28.3)	0.6		
Cigarette smoking	48/3	0.3 (0.1-2.5)	0.3		
Cirrhosis	4/0	-	-		
Pre-LT relapse to DAA	18/5	11.7 (1.2-590.7)	0.006	14.9 (1.1-219.3)	0.04
Anti-HBc-positive allograft	8/2	3.3 (0.3-27.8)	0.4		
HCV-positive allograft	4/0	-	-		
HCV genotype 3a	12/3	4.1 (0.5-34.2)	0.2		
Post-LT relapse to DAA	2/3	26.2 (2.2-432.9)	0.002	10.6 (1.0-121.6)	0.05

HCC: Hepatocellular carcinoma; BMI: Body mass index; DAA: Direct-acting antiviral; HCV: Hepatitis C virus; OR: Odds ratio; CI: Confidence interval; LT: Liver transplant; HBc: Hepatitis B core; EOT: End of treatment.

DAA treatment[20]. Moreover, LT recipients are not decompensated, as some authors attributed increased HCC risk in the DAA era to less selection bias when compared with that in the interferon era, as they excluded interferon-based treatment from decompensated patients with Child-Pugh class B or C[21]. Second, the use of explant pathology to confirm the diagnosis of HCC rather than relying on imaging modalities that can misdiagnose HCC supports the use of LT recipients in investigating the interaction between DAA therapy and HCC.

None of the patients in the present study who had DAA therapy failure or recurrence of HCC were cirrhotic. This contradicts the suggestion that HCC recurrence or DAA failure risk is greater in cirrhotic than non-cirrhotic populations.

Whereas a specific explanation for the interaction between HCC and DAA therapy has yet to be established, some studies suggested that cross-talk among inflammatory, immune response, and angiogenesis pathways modulates the impact of DAAs on progression of HCC and the risk of its recurrence[22,23]. Additionally, HCC may interact biologically with DAA therapy, which may influence pre-transplant SVR12 achievement and increases the risk of HCC recurrence in patients who failed DAA therapy with a prior history of HCC irrespective of HCC treatment response or liver

Table 6 Characteristics of the study population according to use of ribavirin

Characteristic	Number (%)		*P* value
	RBV-containing DAA regimens (*n* = 68)	Non-RBV containing DAA regimens (*n* = 38)	
Age ≥ 60 yr	38 (58)	26 (70)	0.2
Male	51 (75)	24 (63)	0.3
Female	17 (25)	14 (37)	
White	37 (54)	19 (50)	0.8
Black	15 (22)	7 (18)	
Hispanic	13 (19)	10 (26)	
Asian	3 (4)	2 (5)	
Diabetes Mellitus	45 (66)	28 (74)	0.5
CKD	22 (32)	21 (55)	0.02
eGFR < 30 mL/min/1.73 m^2	1 (2)	8 (21)	0.001
Cirrhosis	4 (8)	5 (16)	0.3
mean (± SD) creatinine level, mg/dL	1.2 ± 1.0	1.9 ± 1.8	0.02
mean (± SD) hemoglobin level, g/dL	14.0 ± 1.7	12.0 ± 2.0	0.0001
mean (± SD) MELD-Na score	11 ± 4	13 ± 6	0.01
SVR12	65 (95.5)	35 (92.1)	0.7

RBV: Ribavirin; CKD: Chronic kidney disease; DAA: Direct-acting antiviral; SVR: Sustained virologic response.

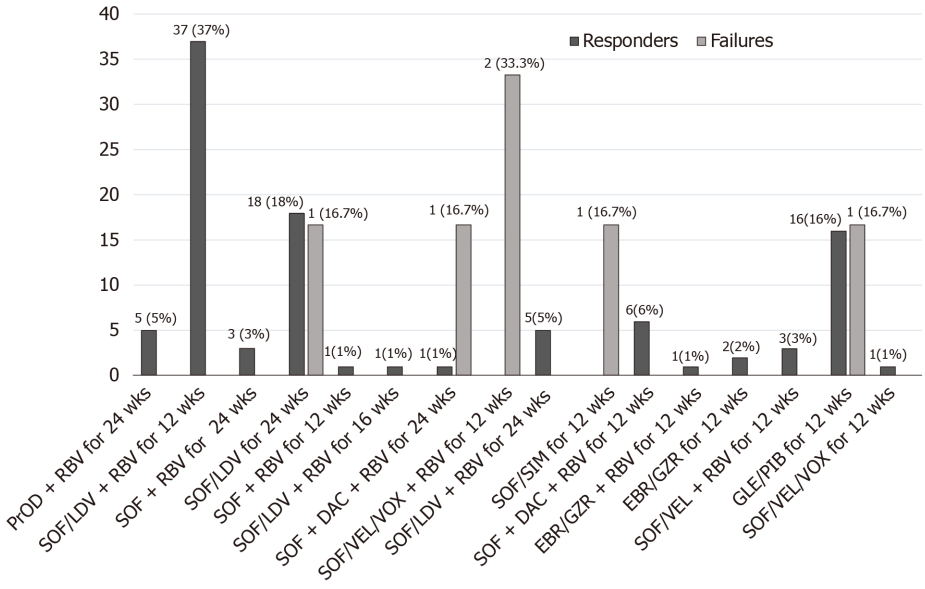

Figure 1 Numbers and percentages of the study patients who had responses to (*n* = 100) and failure of (*n* = 100) the 16 direct-acting antiviral regimens. EBR: Elbasvir; GZR: Grazoprevir; PrOD: Paritaprevir-ritonavir-ombitasvir-dasabuvir; SIM: Simeprevir.

transplantation[17,19]. Genetic susceptibility and variability in the genes involved in HCC risk or DAA metabolism may also play a role in the interaction between DAAs and HCC. Integration of the use of molecular markers with DAA therapy should be explored in future studies.

There is no consensus regarding the optimum time to start DAA treatment after an LT. We found no difference in the SVR12 rate between patients who received early (< 1 year) and late (≥ 1 year) DAA therapy after LTs. Nevertheless, five of six patients who did not experience SVR12 received DAA therapy more than 1 year after their LTs.

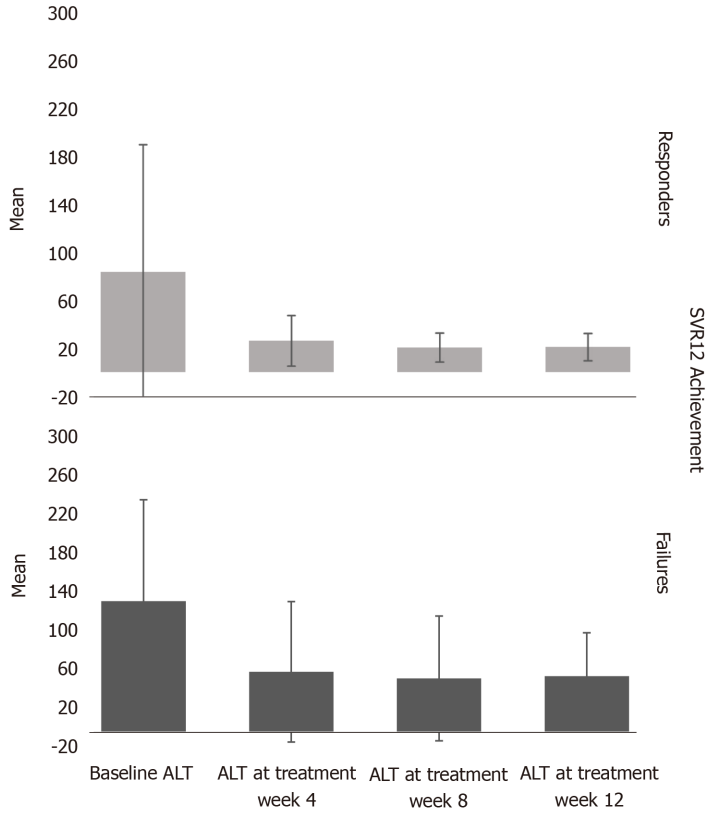

Figure 2 mean (± SD) alanine aminotransferase levels in patients who had direct-acting antiviral therapy responses and failure (baseline, week 4, week 8, and week 12).

We have no explanation why patients with PHCC received DAA treatment earlier than patients with PnHCC in our study. This may be attributed to more rigorous follow-up, better compliance, and fear of decompensation or HCC recurrence after liver transplantation in patients with PHCC.

During post-treatment follow-up, no patients had relapses after SVR12, with 100% concordance between SVR12 rates and SVR rates at follow-up week 24 and between SVR12 rates and quantitative HCV polymerase chain reaction at the last follow-up visit. This establishes SVR12 as an appropriate endpoint for demonstrating the clinical efficacy of all-oral DAA treatment similar to that in previous trials of sofosbuvir/RBV with and without interferon[24].

Consistent with a previous study[25], we showed that failure of the ALT level to normalize at treatment week 4 was a strong predictor of DAA treatment failure. This may result in enhancement of patient management *via* (1) Intensification of DAA treatment, either with RBV or extension of the treatment period, and (2) Thorough evaluation for other causes of a persistently elevated ALT level at treatment week 4, such as alcoholism, high body mass index, and type 2 diabetes mellitus.

Multivariate analysis showed no impact of HCV genotype 3 or an HCV-positive allograft on the risk of failure or HCC recurrence after DAA treatment.

Similar to other studies[13,14], we did not observe a significant difference in SVR12 rates between LT recipients who received DAA regimens with and without RBV. However, such an observation should be interpreted conservatively, as these patients were not randomized to receive RBV. Moreover, patients who received RBV were either cirrhotic or had a history of failure to antiviral therapy or quantitative HCV polymerase chain reaction > 15 IU/mL at treatment week 4, with RBV added to their regimens to intensify them and achieve SVR12. Use of RBV therefore may have helped this population to have an SVR12 rate similar to those in other favorable populations.

Overweight or obesity at baseline did not influence SVR12 achievement or HCC recurrence after DAA treatment. We observed no significant changes in body mass index from baseline to the end of DAA treatment. However, among those with PHCC, weight loss by the end of DAA treatment was a significant predictor of HCC recurrence, which may be attributed to release of inflammatory cytokines[26].

Tacrolimus-containing regimens were the most commonly used

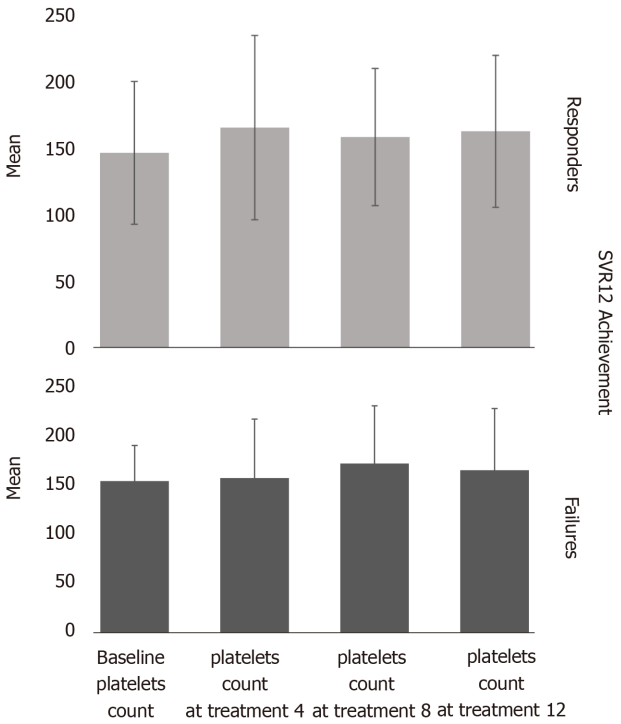

Figure 3 mean (± SD) platelet counts in patients who had direct-acting antiviral therapy responses and failure (baseline, week 4, week 8, and week 12).

immunosuppressive medications in DAA therapy. Acute cellular rejection occurred in 5% of the patients after DAA treatment (median time of 11 mo after therapy), which is comparable with rejection rates in the literature[13].

HBV reactivation with DAA treatment is rare, with an estimated risk of 1.4% in patients with resolved HBV infections[27]. In our study, none of anti-HBc-positive patients experienced HBV reactivation during or after DAA therapy until the last follow-up visit.

This study has several strengths. It is the first to highlight predictors of HCC recurrence in patients in whom DAA treatment failed after an LT while adjusting for potential confounding factors. Furthermore, pre-transplant relapse to DAA therapy may predict post-transplant HCC recurrence. Given the uniqueness of LT recipients with recurrent HCV infection and HCC, we acknowledge the limitation of a single-center, retrospective study with a small sample size. Accordingly, we applied a rigid exact method of logistic regression analysis to overcome this limitation.

CONCLUSION

In conclusion, our results support the safety and efficacy of DAA treatment in LT recipients with history of HCC. Relapse to pre-transplant DAA therapy is a significant predictor of HCC recurrence following transplantation. Furthermore, relapse to post-transplant DAA therapy is associated with HCC recurrence. ALT level > 35 U/L at treatment week 4 is a significant predictor of treatment failure. Future validation of our findings in a multicenter study of a larger sample is warranted.

ARTICLE HIGHLIGHTS

Research background

Recent studies have shown lower sustained virologic response in patients with hepatocellular carcinoma (HCC) pre-transplant. Moreover, there are conflicting data regarding HCC recurrence in patients treated with direct-acting antivirals (DAAs). However, there are insufficient data regarding the efficacy of DAA in liver transplant

(LT) recipients with past history of HCC.

Research motivation

To identify risk factors for DAA relapse and HCC recurrence after LT.

Research objectives

To highlight the efficacy and safety of DAA therapy in LT recipients with hepatitis C virus infections and history of HCC and to investigate the impact of DAA use on post-transplantation HCC recurrence.

Research methods

We retrospectively analyzed the data of our center of LT recipients who received DAA therapy for hepatitis C virus recurrence and their relapse and HCC recurrence outcomes.

Research results

Six patients (6%) experienced DAA therapy failure post-LT and 100 (94%) had a sustained virologic response at follow-up week 12. DAA relapse post-LT was associated with post-transplantation HCC recurrence, $P = 0.05$. Pre-LT DAA relapse is a predictor of post-LT HCC recurrence, $P = 0.04$.

Research conclusions

DAA is safe and effective in LT recipients with history of HCC. High alanine aminotransferase > 35 U/L at treatment week 4 and pre-LT DAA relapse are predictors of HCC recurrence after transplant. Relapse to DAA therapy post-LT is a potential risk factor for HCC recurrence.

Research perspectives

Performing a multicenter study of a larger sample to validate our findings.

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ORIGINAL ARTICLE

Retrospective Study

Surgical treatment of gallbladder cancer: An eight-year experience in a single center

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Author contributions: Kamada Y collected the data; Kamada Y and Hori T analyzed the data, reviewed the published literature and wrote the manuscript; Kamada Y and Hori T contributed equally to this work; all authors discussed therapeutic options, reviewed previous papers, and provided important opinions; Zaima M and Hori T supervised this report.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the Shiga General Hospital.

Informed consent statement: The patients involved in this study

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Abstract

BACKGROUND

Gallbladder cancer (GBC) is the most common biliary malignancy and has the worst prognosis, but aggressive surgeries [*e.g.*, resection of the extrahepatic bile duct (EHBD), major hepatectomy and lymph node (LN) dissection] may improve long-term survival. GBC may be suspected preoperatively, identified intraoperatively, or discovered incidentally on histopathology.

AIM

To present our data together with a discussion of the therapeutic strategies for GBC.

METHODS

We retrospectively investigated nineteen GBC patients who underwent surgical treatment.

RESULTS

Nearly all symptomatic patients had poor outcomes, while suspicious or incidental GBCs at early stages showed excellent outcomes without the need for two-stage surgery. Lymph nodes around the cystic duct were reliable sentinel nodes in suspicious/incidental GBCs. Intentional LN dissection and EHBD resection prevented metastases or recurrence in early-stage GBCs but not in advanced GBCs with metastatic LNs or invasion of the nerve plexus. All patients with positive surgical margins (*e.g.*, the biliary cut surface) showed poor outcomes. Hepatectomies were performed in sixteen patients, nearly all of which were minor hepatectomies. Metastases were observed in the left-sided liver but

provided written informed consent authorizing the use and disclosure of their protected health information.

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not in the caudate lobe. We may need to reconsider the indications for major hepatectomy, minimizing its use except when it is required to accomplish negative bile duct margins. Only a few patients received neoadjuvant or adjuvant chemoradiation. There were significant differences in overall and disease-free survival between patients with stages ≤ IIB and ≥ IIIA disease. The median overall survival and disease-free survival were 1.66 and 0.79 years, respectively.

CONCLUSION
Outcomes for GBC patients remain unacceptable, and improved therapeutic strategies, including neoadjuvant chemotherapy, optimal surgery and adjuvant chemotherapy, should be considered for patients with advanced GBCs.

Key Words: Gallbladder cancer; Surgery; Prognosis; Outcome; Metastasis; Lymph node; Extrahepatic bile duct

Core Tip: Gallbladder cancer (GBC) has a poor prognosis. Our GBC patients who underwent surgeries were retrospectively evaluated. Lymphadenectomy and resection of the extrahepatic bile duct prevented metastases or recurrence in early-stage GBCs but not in advanced GBCs with metastatic lymph nodes or invasion of the nerve plexus. We should reconsider the indications for major hepatectomy, when it is required to achieve negative bile duct margins. There were significant differences in overall and disease-free survival between patients with stages ≤ IIB and ≥ IIIA disease. The median overall survival and disease-free survival were 1.66 and 0.79 years, respectively. Postoperative outcome remains unacceptable.

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INTRODUCTION

Gallbladder cancer (GBC) remains a relatively rare malignancy with a variable presentation[1-7], but it is the most common biliary malignancy and has the worst prognosis[3,4,7]. Although GBC generally carries a poor prognosis[1-4,7-15], complete surgical resection is associated with improved outcomes in GBC patients[1,2,11,14,16-23]. Some surgeons have suggested that aggressive surgeries [*e.g.*, resection of the extrahepatic bile duct (EHBD), extended hepatectomy and intentional lymph node dissection (lymphadenectomy) of the para-aortic lymph nodes (LNs)] may improve long-term survival in patients with advanced GBC[1,2,9,11,13,14,16,18-25]. Metastatic LNs, invasion into the peribiliary nerve plexuses and positive surgical margins of the biliary tract are important prognostic factors[1,7,11,15,18,21].

Although radical resection is considered by many to be the ideal management strategy for GBC[1,2,11,14,16-23], selecting the appropriate surgical procedure based on the depth of the primary tumor and the clinical stage of GBC is still controversial[1-4,10,11,14,16,17,19,20,23,24,26]. Optimal treatment for advanced GBC is unclear[1,2,24], especially in patients with T2 disease according to the tumor-node-metastasis (TNM) classification[27]. The use of radical cholecystectomy and extended procedures with EHBD resection are a matter of debate[1,2,10,14,16,20,24,25]. Radical cholecystectomy involves an extended cholecystectomy with a full-thickness resection and a wedge resection with partial hepatectomy of the gallbladder bed[28-31]. The impact of routine resection of the EHBD, extended LN dissection, and major hepatectomy on outcomes still lacks consensus[1,11,16,20,24].

We retrospectively investigated our patients who underwent surgical treatment for incidentally or non-incidentally diagnosed GBC; their data are presented together with a discussion of the therapeutic strategies for GBC and a literature review.

MATERIALS AND METHODS

Patients

A total of nineteen GBC patients who underwent surgical treatment at our institution from January 2011 to March 2019 were enrolled in this study. The patients comprised five men and fourteen women, with a mean age of 72.5 ± 11.5 years. Two patients had a past history of other cancers, and one patient had cholelithiasis as a comorbidity. None had viral hepatitis, alcoholic hepatitis or non-alcoholic steatohepatitis. The GBCs were staged according to the TNM classification[27].

This retrospective study was approved by the ethics review committee for clinical studies of our institution. This study was performed in accordance with the ethical guidelines of the Declaration of Helsinki. Informed consent was obtained from all patients before enrollment.

Statistical analysis

All results are shown as mean ± SD or median (range). Survival rates were calculated using the Kaplan–Meier method, and the log-rank test was used for between-group comparisons. All calculations were performed using SPSS Software (SPSS Inc., Chicago, IL, United States). Values of $P < 0.05$ were considered statistically significant.

RESULTS

Preoperative management

Preoperative profiles are summarized in Table 1. Six patients presented with fever and abdominal pain, and two patients had obstructive jaundice; the remaining eleven patients (57.9%) were asymptomatic (Table 1). The eight symptomatic patients were categorized as stage ≥ IIB (Table 1). Biliary drainage was required in two symptomatic patients with stage IVB disease (Cases 14 and 17) due to obstructive jaundice and acute cholangitis (Table 1).

Preoperative evaluation

Pancreaticobiliary maljunction was observed in two patients (Cases 2 and 16; Table 1). No patients had occupational risk factors for GBC. Six patients were initially diagnosed with benign diseases (two stage IIB patients and one each with stage IA, IB, IIA and IIIA disease) (Table 1), and these patients were classified as having suspicious or incidental GBCs. The preoperative stages of patients who received radical surgery were stage IVB (five patients); stage IIA (three patients); and stages IIB, IIIA, IIIB and IVA (two of each stage; Table 1).

Preoperative chemotherapy and radiation

None of the patients received neoadjuvant chemotherapy or radiation. One patient received chemotherapy [four courses with gemcitabine (GEM) + cisplatin (CDDP)] and radiation (60 Gy) for unresectable GBC; this patient underwent conversion surgery (Case 10).

Surgical treatment

Operative factors are summarized in Table 2. Eleven patients underwent primary extended cholecystectomy with full-thickness resection and partial hepatectomy of the gallbladder bed, and three patients underwent wedge resection of the gallbladder bed as a two-stage surgery (Table 2). Although partial hepatectomy and/or wedge resection of the gallbladder bed were performed in fourteen patients, only one patient received a systemic hepatectomy (right-lobe hepatectomy accompanied by partial caudate lobectomy; Case 17).

A total of eight patients (three with stage IIB disease and one each with stage IIA, IIB, IIIA, IVA and IVB disease) underwent intentional resections of the EHBD, including five who underwent pancreaticoduodenectomy (Table 2). Lymphadenectomy of the para-biliary, para-arterial and peri-portal venous LNs was performed in eleven patients (57.9%; three with stage IIIB disease; two each with stage IIB, IIIA, IIIB and IVB disease; and one each with stage IIA and IVA disease). The para-aortic LNs were dissected in one stage IVB patient (Case 19).

Laparoscopic cholecystectomy was initially performed in seven patients with a preoperative diagnosis of benign disease, and three of these patients (two with stage IIB disease and one with stage IIIA disease) received two-stage surgery based on

Table 1 Preoperative evaluation, chemotherapy and/or radiation, and the final stage

Case	Symptomatic (Yes or No)	Preoperative biliary drainage (Yes or No)	PBM (Yes or No)	Preoperative diagnosis (GBC or benign)	Preoperative stage[1]	Neoadjuvant chemotherapy (Yes or No)	Preoperative radiation (Yes or No)	Conversion to surgery (Yes or No)	The final stage[1]	Adjuvant chemotherapy (Yes or No)	Chemotherapy for metastases/recurrences (Yes or No)
1	No	No	No	Benign	-	No	No	No	IA	No	No
2	No	No	Yes	Benign	-	No	No	No	IB	No	No
3	No	No	No	GBC	IIIB	No	No	No	IIA	No	No
4	No	No	No	Benign	-	No	No	No	IIA	No	No
5	No	No	No	GBC	IIA	No	No	No	IIA	No	No
6	Yes	No	No	Benign	IIB	No	No	No	IIB	No	No
7	Yes	No	No	Benign	IIB	No	No	No	IIB	No	Yes
8	No	No	No	Benign	IIIA	No	No	No	IIIA	No	No
9	Yes	No	No	GBC	IVB	No	No	No	IIIA	No	No
10	Yes	No	No	GBC	IVA	No	No	Yes	IIIA	No	Yes
11	No	No	No	GBC	IIA	No	No	No	IIIA	Yes	No
12	Yes	No	No	GBC	IIIA	No	No	No	IIIA	No	No
13	No	No	No	GBC	IVA	No	No	No	IIIB	No	No
14	Yes	Yes	No	GBC	IVB	No	No	No	IIIB	No	No
15	No	No	No	GBC	IIA	No	No	No	IIIB	No	No
16	No	No	Yes	GBC	IVB	No	No	No	IIIB	No	No
17	Yes	Yes	No	GBC	IVB	No	No	No	IVA	No	No
18	Yes	No	No	GBC	IIIB	No	No	No	IVB	No	No
19	No	No	No	GBC	IVB	No	No	No	IVB	No	Yes

[1]Staging according to the tumor-node-metastasis classification. GBC: Gallbladder cancer; PBM: Pancreaticobiliary maljunction.

pathological findings. All three underwent wedge resection, and one patient received resection of the EHBD (Cases 6, 7 and 8; Table 2). Three patients (stages IA, IB and IIA) did not receive two-stage surgery (Cases 1, 2 and 4).

Curative resections, evaluated as graphic and surgical R0 according to the Japanese guidelines (General rules for clinical and pathological studies on cancer of the biliary

Table 2 Operative factors

Case	Surgical approach (Primary or two-stage)	Surgical procedures			Caudate Lobectomy (Yes or No)	Graphic/Surgical R0[3] (Curative resection) (Yes or No)	Postoperative complications	
		Resected area[1]	Intentional LN dissection[2] (Yes or No)	EHBD resection (Yes or No)			POD	Grade[4]
1	Primary	LC	No	No	No	Yes	-	-
2	Primary	LC	No	No	No	Yes	-	-
3	Primary	Extended cholecystectomy	Yes	Yes	No	Yes	Intraperitoneal abscess (8)	3a
4	Primary	LC	No	No	No	Yes	-	-
5	Primary	Extended cholecystectomy	No	No	No	Yes	-	-
6	Two-stage	Wedge resection	Yes	No	No	Yes	-	-
7	Two-stage	Wedge resection	Yes	Yes	No	Yes	-	-
8	Two-stage	Wedge resection	Yes	No	No	Yes	Intraperitoneal abscess (8)	2
9	Primary	Extended cholecystectomy	No	No	No	No	Intraperitoneal abscess (20)	3a
10	Primary	Extended cholecystectomy	No	No	No	No	-	-
11	Primary	Extended cholecystectomy	Yes	Yes	No	No	-	-
12	Primary	Extended cholecystectomy	No	No	No	Yes	-	-
13	Primary	Extended cholecystectomy + SSpPD	Yes	Yes	No	Yes	Intraperitoneal abscess (14)	2
14	Primary	Extended cholecystectomy + SSpPD + partial resection of the portal vein	Yes	Yes	No	Yes	-	-
15	Primary	LC	No	No	No	Yes	-	-
16	Primary	Extended cholecystectomy + SSpPD	Yes	Yes	No	Yes	Pancreatitis (21)	2
17	Primary	Right hepatectomy + SSpPD	Yes	Yes	Yes	No	Pancreatic fistula (14)	3b
18	Primary	Extended cholecystectomy+partial hepatectomy	Yes	No	No	Yes	-	-
19	Primary	Extended cholecystectomy + SSpPD	Yes	Yes	No	Yes	-	-

[1]Extended cholecystectomy was the primary whole-layer resection with partial hepatectomy, and the wedge resection was partial hepatectomy of the gallbladder bed at two-stage surgery.
[2]Intentional dissection of parabiliary, paraarterial and periportal venous lymph nodes.
[3]R0 means no remnant of gallbladder cancer by curative resection, according to the Japanese guideline (General rules for clinical and pathological studies on cancer of the biliary tract).
[4]Grading according to the Clavien-Dindo classification. EHBD: Extrahepatic bile duct; LC: Laparoscopic cholecystectomy; LN: Lymph node; POD: Postoperative day; SSpPD: Subtotal stomach-preserving pancreaticoduodenectomy.

tract[32]), were accomplished in fifteen patients. A total of four patients (three with stage IIIA and one with stage IVA disease; Cases 9, 10, 11 and 17) received non-curative resections; the biliary cut surface or surgical surface margins were each positive in two patients (Table 3).

Intraoperative factors

The median operative time was 269 min (range, 32-775 min). Median blood loss was 430 mL (range, 0-3700 mL), and blood transfusions were needed in four patients. Intraoperative histopathological examination was performed in eight patients to assess the primary tumors, cut surface of the biliary tract, nerve plexus and LNs. Although carcinomas were correctly identified in all cases, the extension (*i.e.*, oncological depth) of the primary tumor was misdiagnosed by intra-operative examination in one patient (Case 6).

Postoperative course during the early postoperative period

Postoperative complications were observed in six patients (four cases of intraperitoneal abscess and one each of pancreatitis and pancreatic fistula), and these complications were categorized as grade 2 (n = 3), grade 3a (n = 2) and grade 3b (n = 1) according to the Clavien–Dindo classification[33] (Table 2).

Pathological assessment

The pathological findings are summarized in Table 3. Diagnoses of tubular, papillary, and poorly-differentiated adenocarcinomas were made in 12, 3 and 2 cases, respectively. Additionally, one adenosquamous cell carcinoma and one neuroendocrine carcinoma were diagnosed. No satellite lesions of dysplasia and/or neoplasia were observed. Only one patient (Case 1) was diagnosed with mucosal cancer (a so-called "m cancer"), and neither a second-stage surgery after laparoscopic cholecystectomy nor an extended cholecystectomy was chosen for this patient (Table 2).

The primary tumor was located in the liver in 10 patients and the ventral side in nine (Table 3). Invasions into the lymphoid duct, vessels and peribiliary nerve plexus were pathologically assessed, respectively. These invasions occurred in six, ten and five cases, respectively (Table 3). Notably, nine GBCs invaded into the GB neck or cystic duct (Table 3).

Positive margins at the biliary cut surface were observed in two patients (Cases 11 and 17), and positive margins at the ventral, dorsal and/or hepatic surfaces were

Table 3 Pathological findings

Case	Tumor occupation[1]		Depth[2] (invaded organ)	Invasions into the neck and/or cystic duct (Yes or No)	Invasions			Positive margin at the cut end (Yes or No)	Metastatic LNs (Positive LNs/total LNs)	Metastatic LN #12c[3] (The LN around the cystic duct) (Positive LNs/sampling LNs)
		Ventral or liver side			Lymphoid duct (Yes or No)	Vessels (Yes or No)	Nerve plexus (Yes or No)			
1	Gb	Ventral side	m	No	No	No	No	No	-	-
2	Gf	Ventral side	mp	No	No	No	No	No	0 / 1	0 / 1
3	Gf	Ventral side	ss	No	No	No	No	No	0 / 13	-
4	Gf	Ventral side	ss	No	No	No	No	No	-	-
5	Gf	Ventral side	ss	No	No	Yes	No	No	-	-
6	Gfbn	Liver side	ss	Yes	No	No	No	No	0 / 20	-
7	Gfbn	Liver side	ss	Yes	No	No	No	No	0 / 19	0 / 1
8	Gfb	Liver side	ss	No	No	Yes	No	No	0 / 16	-
9	Gfbn	Liver side	si (liver)	Yes	No	Yes	No	Yes	-	-
10	Gfb	Liver side	ss	No	No	No	No	No	-	-
11	Gfbn	Ventral side	si (liver)	Yes	Yes	Yes	Yes	Yes	0 / 6	-
12	Gfbn	Liver side	si (liver, EHBD)	Yes	Yes	No	No	Yes	-	-
13	Gfb	Liver side	si (liver)	No	Yes	Yes	Yes	No	2 / 35	-
14	Gnb	Liver side	si (liver, EHBD)	Yes	No	Yes	Yes	No	3 / 13	-
15	Gf	Ventral side	ss	No	No	No	No	No	1 / 1	1 / 1
16	Gfbn	Ventral side	ss	Yes	Yes	Yes	No	No	2 / 6	1 / 1
17	Gfbn	Ventral side	si (EHBD)	Yes	Yes	Yes	Yes	Yes	0 / 4	-
18	Gfbn	Liver side	si (liver)	Yes	No	Yes	No	No	0 / 4	-
19	Gfbn (b)	Liver side	si (liver)	No	Yes	Yes	Yes	No	18 / 29	-

[1]Tumor location according to the Japanese guideline (General rules for clinical and pathological studies on cancer of the biliary tract).
[2]Tumor depth according to the Japanese guideline (General rules for clinical and pathological studies on cancer of the biliary tract).
[3]The lymph node around the cystic duct, according to the Japanese guideline (General rules for clinical and pathological studies on cancer of the biliary tract). Gf: Fundus; Gb: Body; Gn: Neck. EHBD: Extrahepatic bile duct; LN: Lymph node.

identified in two patients (Cases 10 and 12; Table 3).

The final TNM stage based on pathology[27] was stage IA, IB or IVA in one patient each; stage IIB or IVB in two patients each; stage IIA in three patients; stage IIIB in four patients and stage IIIA in five patients (Table 1).

Lymphoid metastasis

The median number of harvested LNs was 13 (range, 1–35). Actual LN metastases were observed on pathology in five patients, with a median of two metastatic LNs per patient (range, 1–18) (Table 3). In one patient, six of the seven para-aortic LNs were metastatic (Case 19).

Histopathological assessment of the LNs around the cystic duct (*i.e.*, the 12c LNs according to the Japanese guideline[32]) was performed in four patients with suspicious or incidental GBC, and metastasis was detected in two patients. These two cases had other LN metastases (Cases 15 and 16; Table 3).

Nerve plexus metastasis

Invasion of the nerve plexus was pathologically observed in five patients, and three of these cases (60.0%) were GBCs with invasion into the GB neck and/or cystic duct (Table 3). Although these five patients also underwent resection of the EHBD (Tables 2 and 3), all five patients died from metastases and/or recurrences (Table 4). In two patients with advanced GBC, positive margins were observed at the cut surface of the biliary tract in spite of EHBD resection (Cases 11 and 17; Table 3). Only one of eight patients who received EHBD resection (12.5%) survived without any metastases and/or recurrences (Case 3; Tables 2 and 4), and this patient was categorized as stage IIA (Table 1).

Liver metastasis

A total of fifteen patients received hepatectomies (Table 2). Pathological examination of the resected liver specimens revealed direct invasion into the liver in seven patients (Table 3). Liver metastases occurred postoperatively in six patients (three in segment 4, one each in segments 5 and 7, and one in the majority of the liver); four of the targeted segments were located in the left side of the liver (66.7%) (Table 4). Four of these six patients' primary tumors showed vessel invasion (Table 3). Four metastatic tumors occurred in the left lobe (Table 4), and no metastases were observed in the caudate lobe.

Adjuvant and postoperative chemotherapy

Only one patient (stage IIIA) received adjuvant chemotherapy: Six courses of S-1 (Case 11). Three patients received chemotherapy after detection of metastases and/or recurrences. The regimens employed were GEM + CDDP, GEM + CDDP followed by GEM + S1, and CDDP + vinblastine, respectively (Cases 7, 10 and 19).

Outcomes

The clinical courses of all patients were followed for a median of 2.03 years (0.15–5.05 years). Metastasis and/or recurrence was observed in fourteen patients (74.7%); the five patients without metastasis/recurrence had stage IIA disease (two patients) and stage IA, IB or IIB disease (one of each) (Tables 1 and 4). Metastasis occurred in the liver (six patients), para-aortic LNs (four patients) intraperitoneal dissemination (four patients), and local recurrence (four patients) (Table 4).

The overall survival curves by stage are shown in Figure 1A; there was a significant difference in overall survival between patients with stage ≤ IIB and stage ≥ IIIA disease (P = 0.0080; Figure 1B). The overall median survival in the eleven patients with oncological death was 1.66 years (range, 0.16-3.36 years; Figure 2A).

The disease-free survival curves by stage are shown in Figure 1C; there was a significant difference in disease-free survival between patients with stage ≤ IIB and ≥ IIIA disease (P = 0.0054; Figure 1D). The disease-free interval of the fourteen patients with recurrences was 0.79 years (0.12–4.01 years; Figure 2B).

Comprehensive flowchart for important points in our patients

The specific characteristics of each patient may be difficult to understand. Characteristic findings and important points (*e.g.*, preoperative diagnosis, surgical treatment, histopathological assessments, stage according to the TNM classification and prognosis) are summarized in Figure 3.

Table 4 Metastases, recurrences and outcome

Case	Metastasis and/or recurrence (Yes or No)	The target sites of metastases/recurrences[1]	Postoperative liver metastases (Right and/or left side)	Disease-free survival (yr)	Overall survival (yr)	Follow-up term (yr)	Prognosis (Dead or alive)
1	No	-	-	2.4	2.4	2.4	Alive
2	No	-	-	2.3	5.0	5.0	Alive
3	No	-	-	3.6	3.6	3.6	Alive
4	No	-	-	3.6	3.6	3.6	Alive
5	Yes	Liver	Right side	0.4	0.8	0.8	Alive
6	No	-	-	8.0	8.0	8.0	Alive
7	Yes	Peritoneal cavity	-	2.0	2.8	2.8	Dead
8	Yes	Liver	Right side	1.4	2.0	2.0	Dead
9	Yes	Peritoneal cavity	-	0.1	0.2	0.2	Dead
10	Yes	Local site, hepatic hilum	-	1.2	2.6	2.6	Dead
11	Yes	Perineal cavity	-	0.8	1.0	1.0	Dead
12	Yes	Local site, liver	Left side	0.1	0.2	0.2	Dead
13	Yes	Peritoneal cavity, paraaortic LN	-	0.4	1.7	1.7	Dead
14	Yes	Local site	-	0.9	3.4	3.4	Dead
15	Yes	Paraaortic LN	-	4.0	5.4	5.4	Alive
16	Yes	Bone, paraaortic LN	-	2.0	7.5	7.5	Alive
17	Yes	Liver remnant	Left side	0.6	0.7	0.7	Dead
18	Yes	Liver	Right and left sides	0.2	0.3	0.3	Dead
19	Yes	Liver, local site, paraaortic LN	Left side	0.6	1.0	1.0	Dead

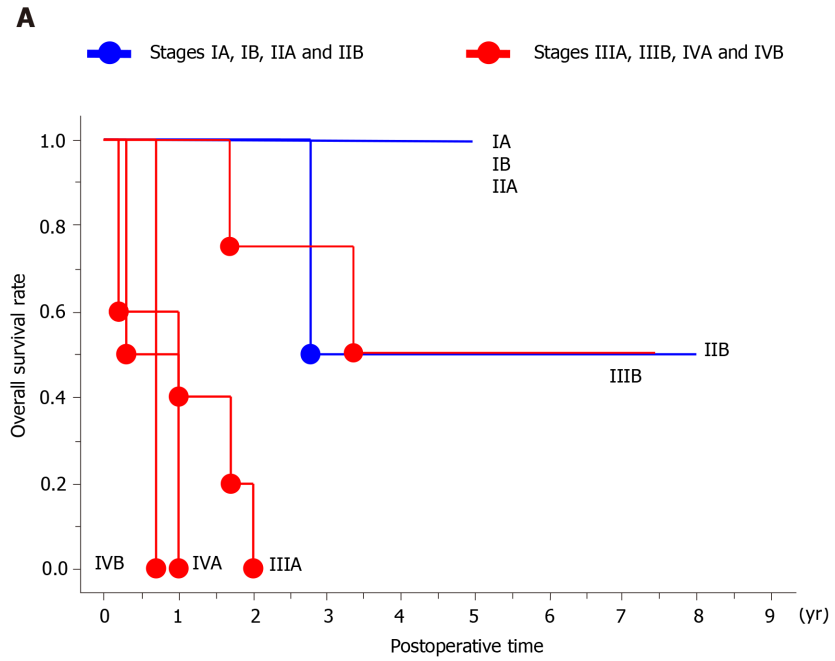
[1]Perineal cavity involved dissemination and carcinomatosis. LN: Lymph node.

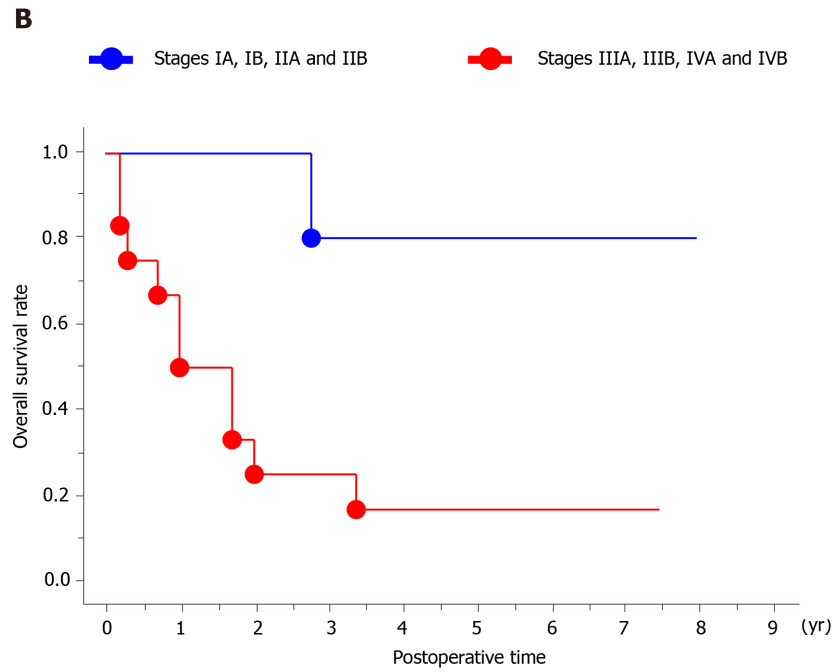
DISCUSSION

In GBC patients, the presence of associated symptoms is considered a relative contraindication to radical resection as these patients have a poor prognosis and high postoperative morbidity[1-3,9]. Of our eight symptomatic patients (Table 1), all were categorized as stage ≥ IIB, and seven (87.5%) showed poor outcomes (Table 4). Although jaundiced patients with advanced GBC should be considered as candidates for surgical resection, careful evaluation is important before undertaking aggressive surgery in this population[2,9].

Certain factors (*e.g.*, liver injury and occupational history) are associated with an increased risk of developing GBC[12]. In particular, data support a relationship between pancreaticobiliary maljunction and GBC[34]. Among our patient population, only two cases (10.5%) had pancreaticobiliary maljunction.

Gallbladder cancer can be suspected preoperatively, identified intraoperatively, or discovered incidentally on final pathology[2-4,26,35]. In cases with suspicious or incidental GBCs, lesions tend to be stage T2 or T3 by the TNM classification[26,27]. Once GBC is diagnosed, a two-stage surgery should be considered[26], although simple or extended cholecystectomy produces comparable survival outcomes in GBC patients with T1 lesions[17]. Among our patients, the three with suspicious/incidental GBC of lower stages showed excellent outcomes without two-stage surgery (Cases 1, 2 and 4). Perforation during the initial surgery carries a higher risk of dissemination[35], although extended resection of adjacent organs may not be necessary in order to achieve radicality even in this instance[25]. Therefore, radical cholecystectomy (*e.g.*, full-thickness resection and extended cholecystectomy) should be considered in the absence of unexpected rupture among patients with suspicious and incidental GBCs. In this study, we assessed the importance of metastasis to the 12c LNs[32] in suspicious and incidental GBCs. Of the four patients with suspicious/incidental GBCs in which the 12c LNs were histologically assessed, the two patients with 12c LN metastasis


A
Stages IA, IB, IIA and IIB
Stages IIIA, IIIB, IVA and IVB
Overall survival rate
1.0
0.8
0.6
0.4
0.2
0.0
IA
IB
IIA
IIB
IIIB
IVB
IVA
IIIA
0
1
2
3
4
5
6
7
8
9
(yr)
Postoperative time


B
Stages IA, IB, IIA and IIB
Stages IIIA, IIIB, IVA and IVB
Overall survival rate
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
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7
8
9
(yr)
Postoperative time

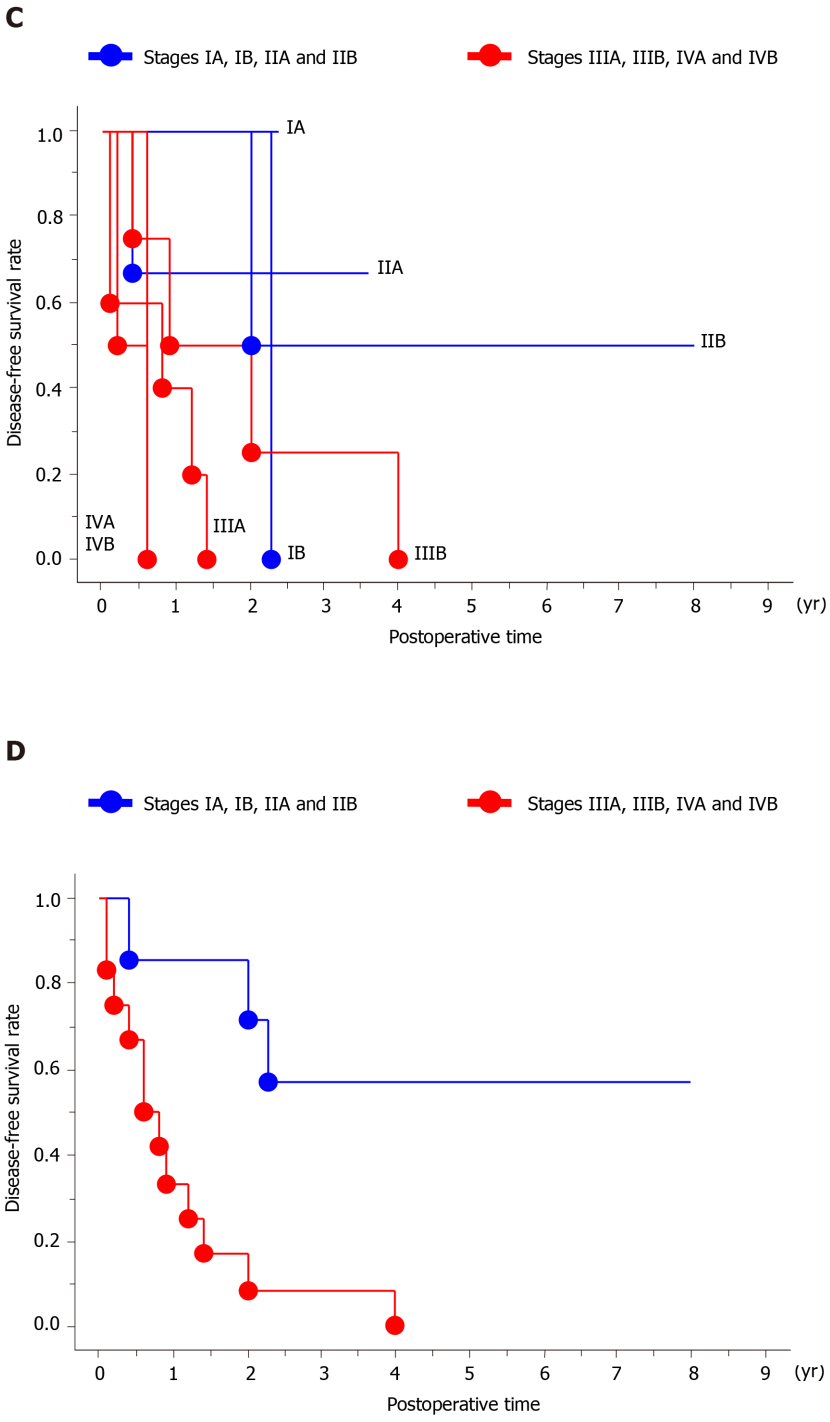

Figure 1 Overall and disease-free survival. A: The overall survival curves for each stage are shown; B: The overall survival significantly differed between patients with stage ≤ IIB (blue line) and those with stage ≥ IIIA (red line) disease (P = 0.0080); C: Disease-free survival curves for each stage are shown; D: There was a significant difference in the disease-free survival of patients with stage ≤ IIB (blue line) and patients with stage ≥ IIIA (red line) disease (P = 0.0054).

(Cases 15 and 16) had other metastatic LNs, while the two patients without 12c LN metastasis did not have other LN metastases (Cases 2 and 7). Even though the number of sampled LNs was small (1 or 2), the 12c LNs seem to be useful sentinel nodes in patients with suspicious and incidental GBCs. For patients with suspicious/incidental GBCs, full-thickness cholecystectomy and sampling of the 12c LNs may be suitable for the initial surgery, although two-stage surgery may be required based on histological findings.

GBCs tend to invade adjacent structures, including the liver parenchyma, bile duct, major vessels, nerve plexuses and regional LNs[1,11,14,20]. Metastatic LNs and bile duct margins are important prognostic factors[1,7,11,15,18-21], and invasion into the nerve plexuses is also a significant independent prognostic factor[11]. Intentional dissection of the LNs and nerve plexuses is still controversial[1,2,11,15,16,19,20,22,24,25]. Extended dissection of

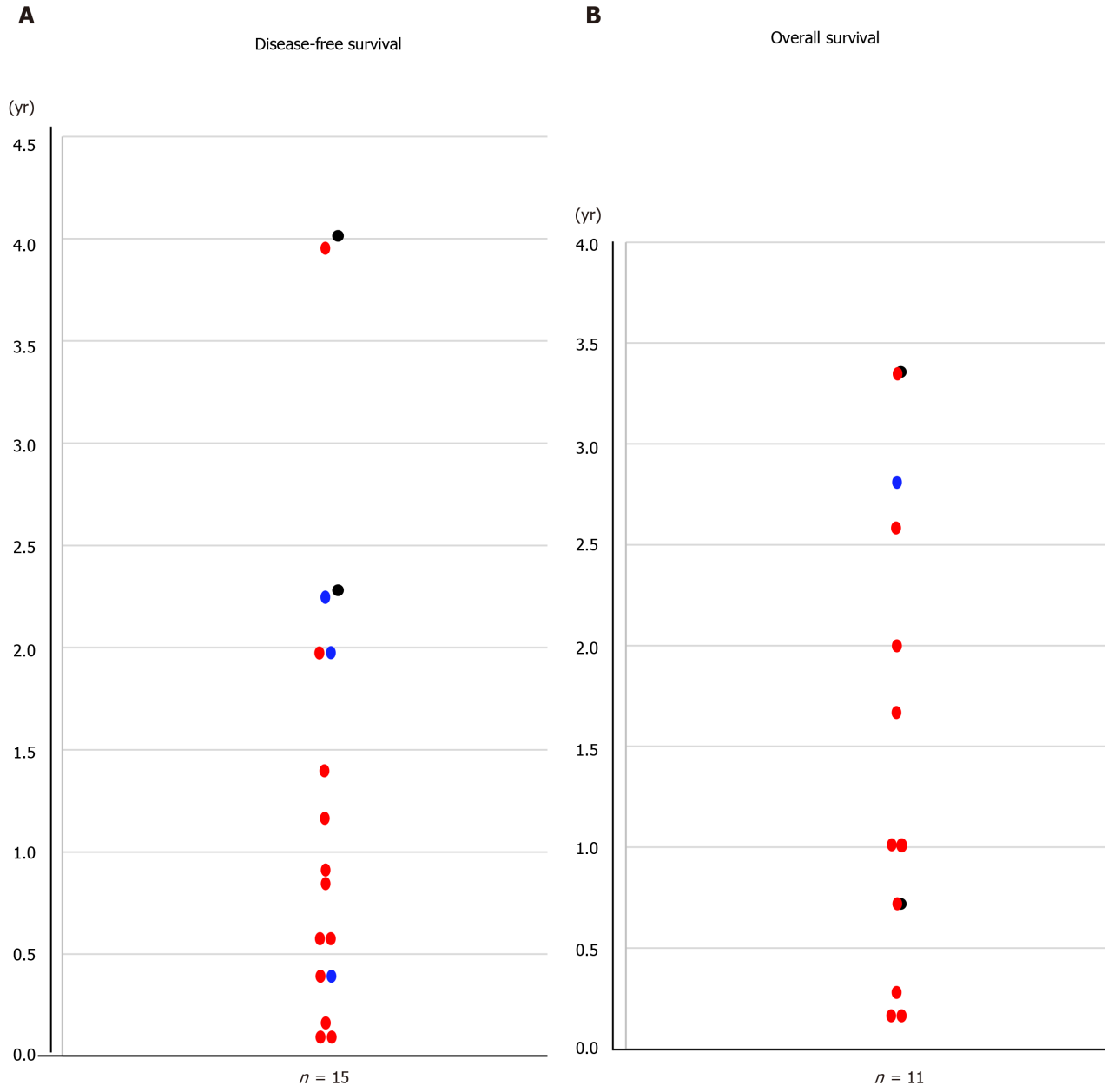

Figure 2 The median overall and disease-free survival. A: The median overall survival of the 11 deceased patients was 1.66 years (0.16–3.36 years); B: The median disease-free interval of the 15 recurrent patients was 0.79 years (0.12–4.01 years).

LNs and/or nerve plexuses should involve *en bloc* resection of the EHBD[1,11,16,20,24,36]. From the viewpoint of achieving curative resection, EHBD resection may have some advantages[1,11,16,20]. However, although routine EHBD resection in GBCs without bile duct invasion is associated with improvements in harvested LNs and local recurrence rate, this procedure does not improve the survival rate and is associated with a higher morbidity rate[14,16,24]. Among our patients, four of the five patients with metastatic LNs received intentional extended LN dissection, but all these patients developed metastases and/or recurrences (Cases 13, 14, 16 and 19). Extended LN dissection worked well in only one patient with early stage disease (Case 3). Moreover, nerve plexus invasion was observed in five patients, and all five of these patients succumbed to oncological death despite EHBD resection (Cases 11, 13, 14, 17 and 19). Of patients undergoing EHBD resection, only one patient with early stage disease survived without any metastases and/or recurrences (Case 3). Unfortunately, extended dissection of the nerve plexuses was not beneficial for advanced GBC patients with invasion into the GB neck and/or cystic duct in our study. Similar to previous reports[1,9,11,15,19,20,24], our advanced GBC patients with metastatic LNs and invasion into the nerve plexus experienced poor outcomes even after aggressive surgery including removal of the EHBD.

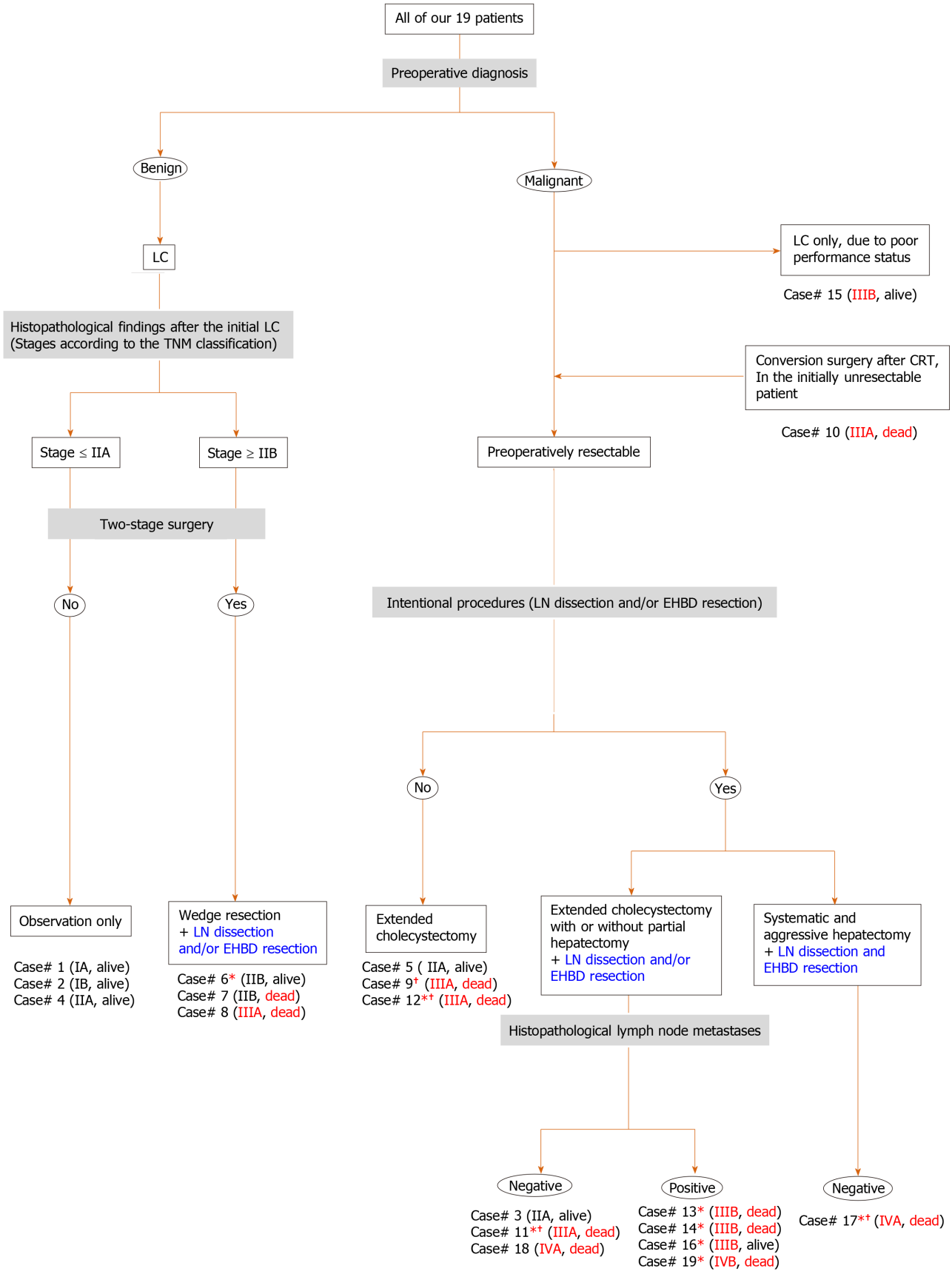

Figure 3 Comprehensive flowchart of important points in our patients. Important points (*e.g.*, preoperative diagnosis, surgical treatment, histopathological assessments, stage according to the tumor-node-metastasis classification and prognosis) are summarized. The final stages in each case are shown in parentheses. The outcomes in each case are also shown in parentheses (dead or alive). *Cases with invasive findings of the lymphoid duct and/or nerve plexuses on the histopathological assessment. †Cases with a positive margin at the surgical cut end of the biliary tract. CRT: Chemoradiotherapy; ENBD: Extrahepatic bile duct resection; LC: Laparoscopic cholecystectomy; LN: Lymph node.

Major hepatectomy for biliary hilar malignancy may involve intraoperative risks and/or postoperative mortality[14,37]. Extended resection with partial hepatectomy and dissection of the regional LNs may be an option for GBC patients with T2 lesions[1,2,21], but major hepatectomy should only be performed in select cases[1,10]. Minimal hepatectomy should be performed to achieve curative resection whenever possible[1]. Drainage from the gallbladder is an important metastatic pathway[6,36], especially for liver metastases[6,36]. Previous publications have suggested that biliary drainage into the left-sided liver, including the caudate lobe, has an impact on metastasis[6,21,23,36,38,39]. Some physicians have documented that the caudate lobe is an important target site for metastases[21,23,26,38,39] and recommend complete resection of the caudate lobe, including Spiegel's lobe[21,36,38,39]. Among our patient population, liver resections were performed in fifteen patients, but a systematic hepatectomy was only performed in one patient (Case 17). Despite hepatic resection, the liver was preserved as a target site of metastases in our patients. We observed metastases in the left-sided liver (Cases 12, 17, 18 and 19) but did not detect metastases in the caudate lobe in any of the eighteen patients with caudate lobe remnants. Accordingly, we suggest that complete resection of the caudate lobe may not be necessary in all cases. Positive surgical bile duct margins should be considered a strong negative prognostic factor[1], and our two patients with positive biliary tract margins showed very poor outcomes (Cases 11 and 17). Therefore, although minimal hepatectomy should be considered for some GBC patients if complete resection can be accomplished by this method[1,10], aggressive hepatectomy is mandatory if needed to accomplish negative biliary margins[1,20].

Overall survival and disease-free survival are shown in Figure 1, both of which clearly differ for patients with stage ≤ IIB *vs* stage ≥ IIIA disease. The median overall and disease-free survival times for patients were 1.66 and 0.79 years, respectively. Aggressive treatment, including extended surgeries, may be beneficial for early-stage GBC[1,25], especially in populations with disease stages ≤ IIB. However, the poor prognosis of advanced GBC has been documented even after extended and/or aggressive procedures[1,9,15,20,24]. Despite this poor prognosis, the role of chemotherapy and radiotherapy for GBCs remains controversial[4]. Among our patients, only one patient (who became resectable) received chemotherapy and radiation before conversion surgery (Case 10), and adjuvant chemotherapy was only performed in one patient with a non-curative resection (Case 11). Some chemotherapy regimens have been developed for GBC[1,10], and certain regimens (*e.g.*, S-1 + cisplatin + GM, S-1 + GM, GM + cisplatin, nanoparticle albumin-bound-paclitaxel + cisplatin + GM and capecitabine) are considered to be useful[1,10,40-42]. Considering the frequency of early recurrence and/or metastases, the timing of surgery and use of neoadjuvant chemotherapy may need to be re-evaluated. Aggressive adjuvant chemotherapy should be considered[10], and surgical resection of metastases and/or recurrences may offer a better chance of long-term survival in select patients[18]. As other physicians have previously documented[1,2,4,7,8,10,21], standard therapeutic strategies need to be established for advanced GBC.

Here, we reviewed the main literature in this field[20,43-71], and crucial remarks in each are summarized in Table 5. The highest volume center in Japan is Nagoya University (Nagoya, Japan), and they continuously documented their decennial results[14,18,20,22,51,72-75]. Also, we analyzed the existing guidelines in Europe (in 2017)[76,77], United States (in 2019)[78] and Japan (in 2019)[79] in detail. Especially for a comparison purpose, important comments on therapeutic strategies for GBCs in each guideline are summarized in Table 5. Central inferior bisegmentectomy (*i.e.*, hepatectomy of segments 4b and 5) is suitable for GBC[80-82], although we employed radical or extended cholecystectomy. Skillful surgeons conversed on the topic of minimally invasive approaches to GBC[83-87], and we all may have to focus on these advanced manipulations in the near future.

This study was as a comparative, observational and retrospective study performed at a single institution, and our sample size was small. Also, this study was not a randomized controlled trial. Accordingly, we cannot rule out bias and other potential limitations. Of course, we understood that our study's conclusions must be interpreted with extreme caution. However, we hope this report will inform the management of GBCs in future patients.

In conclusion, even in the current era, the survival of GBC patients remains unacceptable. Improved therapeutic strategies, including neoadjuvant chemotherapy, optimal surgery, and adjuvant chemotherapy, should be developed to better treat patients with advanced GBC.

Table 5 Important literature and guidelines

Ref.	Country	Year	Remarks	Comments in each guideline
[43]	Germany	2013	Prognosis of incidental GBC was not influenced by the primary access technique.	Conventional open surgery is recommended for suspicious GBCs.
[44]	Japan	2003	Preoperative information indicated strategies for surgical treatment of GBCs.	Conventional open surgery is recommended for suspicious GBCs.
[45]	Japan	1996	Outcome of radical surgery for GBCs was evaluated according to the TNM classification.	Intentional LN dissection and prophylactic EHBD resection are considered for potential pathological invasions.
[46]	Japan	2004	Strong consideration should be given to intentional LN dissection and EHBD resection.	Intentional LN dissection and prophylactic EHBD resection are considered for potential pathological invasions.
[47]	Japan	2013	Hepatectomy of segments 4a and 5 was not superior to extended cholecystectomy in patients with pathological T2.	The EHBD resection does not improve the prognosis in patients with T2N0.
[48]	Korea	2013	Radical resection (R0 surgery) including EHBD resection should be considered in patients with T2 and T3 (A single-centre retrospective study).	The EHBD resection does not improve the prognosis in patients with T2N0.
[49]	Japan	2014	Surgery might not be indicated for patients with advanced invasion to the EHBD and visible paraaortic LN metastasis (A single-centre retrospective study).	The EHBD resection does not improve the prognosis in patients with T2N0.
[50]	Korea	2015	Two-stage surgery was highly recommended for patients with pathological T2 (A single-centre retrospective study).	The EHBD resection does not improve the prognosis in patients with T2N0.
[51]	Japan	2015	Combined treatment of intentional LN dissection and prophylactic EHBD resection had no survival impact for patients without the EHBD invasion (A single-centre retrospective study).	The EHBD resection does not improve the prognosis in patients with T2N0.
[52]	Japan	2012	Hepatectomy procedures (*e.g.*, systematic, segmental and partial resections) did not significantly affect surgical outcomes	Radical resection (R0 surgery) is the most important prognostic factor
[53]	United States	2008	GBC was commonly diagnosed incidentally, and two-stage surgery revealed a high incidence of residual disease.	Overall prognosis is poor.
[54]	United States	2004	Surgeries were not routinely indicated for advanced GBCs with jaundice.	Jaundice is common in patients with advanced GBC.
[55]	France	2011	EHBD resection increased postoperative morbidity but did not improve survival.	Partial hepatectomy without EHBD resection indicates incidental GBC.
[56]	Korea	2011	Extended cholecystectomy was not advantageous over simple cholecystectomy for patients with T1b.	Simple cholecystectomy is adequate therapy for patients with T1a.
[57]	United States	2007	Radical resection for patients with T2 and T3 resulted in a significant survival advantage compared with simple cholecystectomy.	Advantages of radical resection including extended hepatectomy for incidental GBC and patients with T1b are controversial.
[58]	Canada	2008	Intentional LN dissection and EHBD resection may have stage-specific effects on survival.	Radical resection improves survivals in patients with T1b and T2 (not in patients with T3).
[59]	Korea	2008	Cholecystectomy with intentional LN dissection without EHBD resection was recommended for patients with T1b.	Advantages of radical resection including extended hepatectomy for incidental GBC and patients with T3b are controversial.
[60]	United States	2009	Radical resection had survival advantage for patients with T1b and T2.	Radical resection improves survival in patients with T1b and T2 (not in patients with T3).
[61]	United States	2011	Extended surgery including intentional LN dissection improved survival for incidental GBC	Aggressive surgeries including hepatectomy, LN dissection and EHBD resection are indicated for patients with T3, localized hepatic invasion and regional LN metastases.
[62]	Japan	2012	Extended cholecystectomy was adequate for patients with T2, and more aggressive surgeries were indicated for patients with T3, localized hepatic invasion and regional LN metastases.	Aggressive surgeries including hepatectomy, LN dissection and EHBD resection are indicated for patients with T3, localized hepatic invasion and regional LN metastases.
[63]	United States	2009	Major hepatectomy and EHBD resection were significantly associated with perioperative morbidity, and were not mandatory in all cases.	Independent prognostic factors associated with survival are T factor, N factor, pathological poor differentiation and EHBD involvement.
[64]	United States	2007	EHBD resection did not yield a greater count of LNs. Over one-third had residual disease in the EHBD at two-stage surgery.	During two-stage surgery, EHBD resection is indicated for negative cystic duct margins.
[65]	United	2000	Radical resection can provide long-term survival, even for large	Aggressive surgeries including hepatectomy, LN

	States		tumors with extensive liver invasion.	dissection and EHBD resection are indicated for patients with T3, localized hepatic invasion and regional LN metastases.
[66]	United States	2007	Incidental GBCs during laparoscopic cholecystectomy did not indicate immediate conversion to open surgery, and these patients should be referred to a tertiary care center for further surgery.	There was no difference in surgical deficit between immediate resection at the initial hospital and delayed resection at tertiary care center.
[67]	France	2011	Jaundice was a poor prognostic factor, but radical resection had survival benefit especially in highly selected patients with N0.	Radical resection improves survival in patients with N0.
[20]	Japan	2011	Patients with advanced GBCs were candidates for EHBD resection, if radical resection (R0) was achievable.	Radical resection improves survival in patients with EHBD invasion.
[68]	India	2016	Chemoradiotherapy in unresectable GBCs resulted in the resectability, and subsequent radical surgery (R0) had survival benefit. LN regression could serve as a predictor of response to radiochemotherapy.	Chemoradiotherapy in unresectable GBCs may result in the resectability, and conversion surgery (R0) has survival benefit.
[69]	United States	2017	Radical surgeries after favorable responses to neoadjuvant chemotherapies were associated with long-term survival in selected patients.	Chemoradiotherapy in unresectable GBCs may result in the resectability, and conversion surgery (R0) has survival benefit.
[70]	United States	2011	Pathological assessment of at least 6 LNs was important.	Patients with incidental GBC and T2 associated with residual tumor, and should undergo surgery to reflect the adverse outcome.
[71]	Canada	2012	Adjuvant radiochemotherapy had the greatest benefit in patients with positive LNs and R1 disease.	Adjuvant radiochemotherapy is beneficial.

EHBD: Extrahepatic bile duct; GBC: Gallbladder cancer; LN: Lymph node.

ARTICLE HIGHLIGHTS

Research background

Gallbladder cancer (GBC) is the most common biliary malignancy with the worst prognosis, but aggressive surgeries may improve long-term survival.

Research motivation

We evaluated our own data along with a discussion of therapeutic strategies for GBC.

Research objectives

Nineteen GBC patients who underwent surgical treatment were enrolled in this study.

Research methods

We retrospectively investigated our patients with incidentally or non-incidentally diagnosed GBC.

Research results

Suspicious or incidental GBCs at earlier stages showed excellent outcomes without the need for two-stage surgery. Lymph nodes (LNs) around the cystic duct were reliable sentinel nodes in suspicious/incidental GBCs. Extended lymphadenectomy and resection of the extrahepatic bile duct (EHBD) prevented metastases or recurrence in early-stage GBCs but not in advanced GBCs with metastatic LNs or invasion of the nerve plexus. All patients with positive surgical margins showed poor outcomes, and we may need to reconsider the indications for major hepatectomy, minimizing its use except when it is required to accomplish negative bile duct margins. There were significant differences in overall and disease-free survival between patients with stages ≤ IIB and ≥ IIIA disease. The median overall survival and disease-free survival were 1.66 and 0.79 years, respectively.

Research conclusions

Outcomes for GBC patients remain unacceptable.

Research perspectives

Improved therapeutic strategies should be considered for patients with advanced GBCs.

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ORIGINAL ARTICLE

Prospective Study

Can gadoxetic acid–enhanced magnetic resonance imaging be used to avoid liver biopsy in patients with nonalcoholic fatty liver disease?

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Abstract

BACKGROUND

Nonalcoholic fatty liver disease (NAFLD) is a major cause of liver disease worldwide. The diagnosis of nonalcoholic steatohepatitis (NASH), the most severe form of NAFLD, is crucial and has prognostic and therapeutic implications. However, currently this diagnosis is based on liver biopsy and has several limitations.

AIM

To evaluate the performance of gadoxetic acid–enhanced magnetic resonance imaging (GA-MRI) in differentiating isolated steatosis from NASH in patients with NAFLD.

METHODS

In this prospective study, 56 patients with NAFLD (18 with isolated steatosis and 38 with NASH) underwent GA-MRI. The contrast enhancement index (CEI) was calculated as the rate of increase of the liver-to-muscle signal intensity ratio from before and 20 min after intravenous GA administration. Between-group differences in mean CEI were examined using Student's *t* test. The area under the receiver operator characteristic curve and the diagnostic performance of gadoxetic acid–enhanced magnetic resonance imaging were evaluated.

RESULTS

The mean CEI for all subjects was 1.82 ± 0.19. The mean CEI was significantly lower in patients with NASH than in those with isolated steatosis (P = 0.008). Two CEI cut-off points were used: < 1.66 (94% specificity) to characterize NASH and > 2.00 (89% sensitivity) to characterize isolated steatosis. CEI values between 1.66 and 2.00 indicated liver biopsy, and the procedure could be avoided in 40% of patients with NAFLD.

CONCLUSION

GA-MRI is an effective noninvasive method that may be useful for the differentiation of NASH from isolated steatosis, and could help to avoid liver biopsy in patients with NAFLD.

Key Words: Fatty liver; Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Gadoxetic acid

Core Tip: Nonalcoholic fatty liver disease (NAFLD) is a major cause of liver disease worldwide. The diagnosis of nonalcoholic steatohepatitis, the most severe form of NAFLD, is crucial and has prognostic and therapeutic implications. However, currently this diagnosis is based on liver biopsy. In this study, we demonstrated that gadoxetic acid–enhanced magnetic resonance imaging could be useful for the evaluation of patients with NAFLD, as it allows stratification according to the probabilities of nonalcoholic steatohepatitis and isolated steatosis, thereby avoiding liver biopsy in many patients.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide, affecting individuals in all age groups and of all ethnicities. It affects 20%-40% of the population in Western countries and 5%-40% of the population in the Asia-Pacific region[1,2]. The prevalence of NAFLD is increasing in parallel with the epidemics of overweight, obesity, type 2 diabetes mellitus, and metabolic syndrome[1,3-5]. It is characterized by fatty liver infiltration in the absence of significant alcohol consumption or other known liver disease, and encompasses a spectrum of conditions that range from isolated steatosis to nonalcoholic steatohepatitis (NASH), fibrosis, and cirrhosis[1,3,6-8]. Isolated steatosis is a reversible state of excessive fat accumulation in hepatocytes 9. NASH progresses to liver fibrosis and cirrhosis in up to 30% of cases within 10 years, and 4%-27% of these patients are at risk of hepatocellular carcinoma and cirrhosis-induced liver failure[1-3]. NASH-induced cirrhosis is the second most common indication for liver transplantation in the United States 10, and it will become the most common indication in the near future[4].

The differentiation of relatively benign isolated steatosis from potentially progressive NASH is crucial and has prognostic and therapeutic implications[2,3,5,7,8]. The reference standard for differentiation of these two entities is based on liver histopathological findings[2,4,7-9]. However, liver biopsy has several limitations, including its invasive nature, with rare but potentially life-threatening complications, poor patient acceptance, sampling error, and intra-and interobserver variability in findings[3,4,7]. In addition, liver biopsy is a high-cost procedure, requiring a day hospitalization, a specialist physician to perform the procedure and a pathologist with expertise in hepatobiliary disease for sample analysis. Given the high prevalence of NAFLD, liver biopsy is not a good option for routine clinical practice[10].

Abundant research has been performed to develop noninvasive diagnostic methods for the early detection of NASH and its accurate differentiation from isolated steatosis, due to the utmost clinical importance of this diagnosis. Plasma biomarkers of parenchymal liver disease, alone or combined with clinical parameters, have shown a high degree of accuracy for the differentiation of advanced fibrosis from mild or no fibrosis, but not for NASH diagnosis[2-4]. Traditional imaging modalities [*i.e.*, ultrasonography, computed tomography, and magnetic resonance imaging (MRI)] are routinely used to detect and stage diffuse liver disorders based on morphological features. These methods can be used to detect and quantify liver steatosis. However, they cannot differentiate NASH from isolated steatosis[2-5]. Magnetic resonance elastography has shown promise for the detection of liver fibrosis, but it is not sensitive for the identification of steatohepatitis without fibrosis[4,8]. In addition, MR elastography has limited availability due to the need for specialized equipment[11].

The introduction of gadoxetic acid (GA), a paramagnetic hepatobiliary MRI contrast agent, has expanded the role of MRI, particularly for functional imaging of chronic liver diseases such as NAFLD[3,9,12]. GA is taken up by hepatocytes and excreted into the biliary system by hepatocyte membrane transporters[13]. The expression of membrane transporters determines enhancement in the hepatobiliary phase[9,12]. Two proof of concept studies yielded favorable results in terms of relative liver enhancement after GA administration for the differentiation of isolated steatosis from NASH[7,9]. However, these studies were retrospective, and further validation with prospective studies is required. The purpose of this study was to evaluate gadoxetic acid–enhanced magnetic resonance imaging (GA-MRI) with analysis of the contrast enhancement index (CEI) based on muscle-corrected liver signal intensity (SI) as a noninvasive method for the diagnosis of NASH in patients with NAFLD, using histopathology as the reference standard.

MATERIALS AND METHODS

This study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the local research ethics committees. Written informed consent was obtained from the study participants.

Patients

Patients aged ≥ 18 years with NAFLD diagnosed by liver biopsy were included in this study. The patients underwent MRI within 6 mo of liver biopsy, provided that the clinical conditions observed at the time of biopsy had been maintained. Exclusion criteria were: Secondary cause of chronic liver disease (viral hepatitis, autoimmune

liver disorder, Wilson disease, hemochromatosis, alpha-1-antitripsin deficiency), use of drugs that may induce liver steatosis in the past 6 mo, hepatotoxin exposure, history of bariatric surgery, significant alcohol consumption (≥ 10 g alcohol/d in women and ≥ 20 g alcohol/d in men), and GA-MRI contraindication. Data on patients' age, sex, weight, height, and body mass index (BMI) were collected.

Histological evaluation

All patients underwent subcostal biopsy of the right liver lobe with ultrasonography guidance and a 16-gauge Menghini biopsy needle. Specimens were fixed in 10% formaldehyde solution and embedded in paraffin. The sections were then stained with hematoxylin and eosin, Masson's trichrome, and Perls' stains. Only biopsy samples with lengths ≥ 10 mm and six complete portal tracts were included in the analysis.

An expert liver pathologist (C.F.F.C, with 15 years of experience in liver pathology) blinded to the imaging results evaluated the liver biopsy specimens. The extent of steatosis was evaluated semi-quantitatively by assessing the percentage of involvement by steatotic hepatocytes in the liver parenchyma: 0%-33% = mild, 34%-66% = moderate, and > 66% = severe[14]. Steatohepatitis was defined and graded according to the NASH Clinical Research Network Scoring System, by assessment of variable degrees of steatosis accompanied by mixed-cell inflammatory infiltrates in the hepatic lobules and ballooning of hepatocytes, with or without apoptosis and necrosis[6]. Fibrosis was scored from F0 to F4 (F0, absent; F1, perisinusoidal or portal/periportal fibrosis; F2, zone 3 perisinusoidal fibrosis and periportal fibrosis; F3, bridging fibrosis; and F4, cirrhosis)[6].

MRI protocol

All MRI examinations were performed on a 1.5-T Philips Achieva unit (Philips Healthcare, Best, The Netherlands) with a 16-channel SENSE XL Torso coil. Each patient received a bolus injection of GA (Primovist, 0.025 mmol/kg body weight; Bayer-Schering, Berlin, Germany) into a cubital or antecubital vein at a rate of 1 mL/s, followed by a 20-mL saline flush. A fat-suppressed three-dimensional T1-weighted gradient echo sequence was obtained without enhancement and in the hepatobiliary phase (20 min after contrast administration) with the following parameters: TR/TE = 4.0/2.0 ms, flip angle = 10°, field of view = 38 cm, matrix = 192 × 190, interpolation to 224 × 224, slice thickness = 4.0 mm, overlap = 2.0 mm, and anteroposterior phase direction. A multi-echo two-dimensional gradient echo sequence with a 120-ms TR, 20° flip angle, and 12 multiple echoes of 1.2 ms duration was also obtained.

Image analysis

A radiologist with 10 years of experience in abdominal MRI (V.B.A.) who was blinded to the patients' clinical history, laboratory data, and histopathological findings measured SI using commercially available software (Horos version 2.0.1; https://www.horosproject.org/). SI was measured in regions of interest (ROIs) in the liver and paravertebral muscles on unenhanced and hepatobiliary-phase images. Four ROIs were drawn in the liver [three in the right lobe (one in segment V) and one in the left lobe, 2.5 cm^2 each], with avoidance of blood vessels, biliary structures, motion artifacts, and partial volume effects. ROIs (1.0 cm^2 each) were also drawn in the paravertebral muscles (Figure 1). The ROIs were first defined on post-contrast images and then copied onto the corresponding pre-contrast images using the software's copy and paste function, to ensure the same ROI positions for CEI calculation. Average pre- and post-contrast values for liver and muscle ROIs were obtained for each subject.

The liver signal intensity ratio (SIR) was calculated as the ratio of the liver SI to the paraspinal muscle SI separately for unenhanced (SIR_{pre}) and hepatobiliary-phase (SIR_{post}) images. The CEI was calculated as SIR_{post} / SIR_{pre}. The Youden index was used to determine the optimal CEI value for NASH prediction, and two alternative cut-off points were established.

The MRQuantif software (available at https://imagemed.univ-rennes1.fr/en/mrquantif/download.php from the imaging department of the University Hospital of Rennes) was used to detect and quantify hepatic iron overload using a multi-echo two-dimensional gradient echo sequence.

Statistical analysis

Statistical analyses were performed with SPSS software (version 23; SPSS Inc., Chicago, IL, United States). Categorical variables are presented as numbers and percentages. The Shapiro–Wilk test was used to assess the distribution of continuous data. Differences between patients with NASH and those with isolated steatosis were

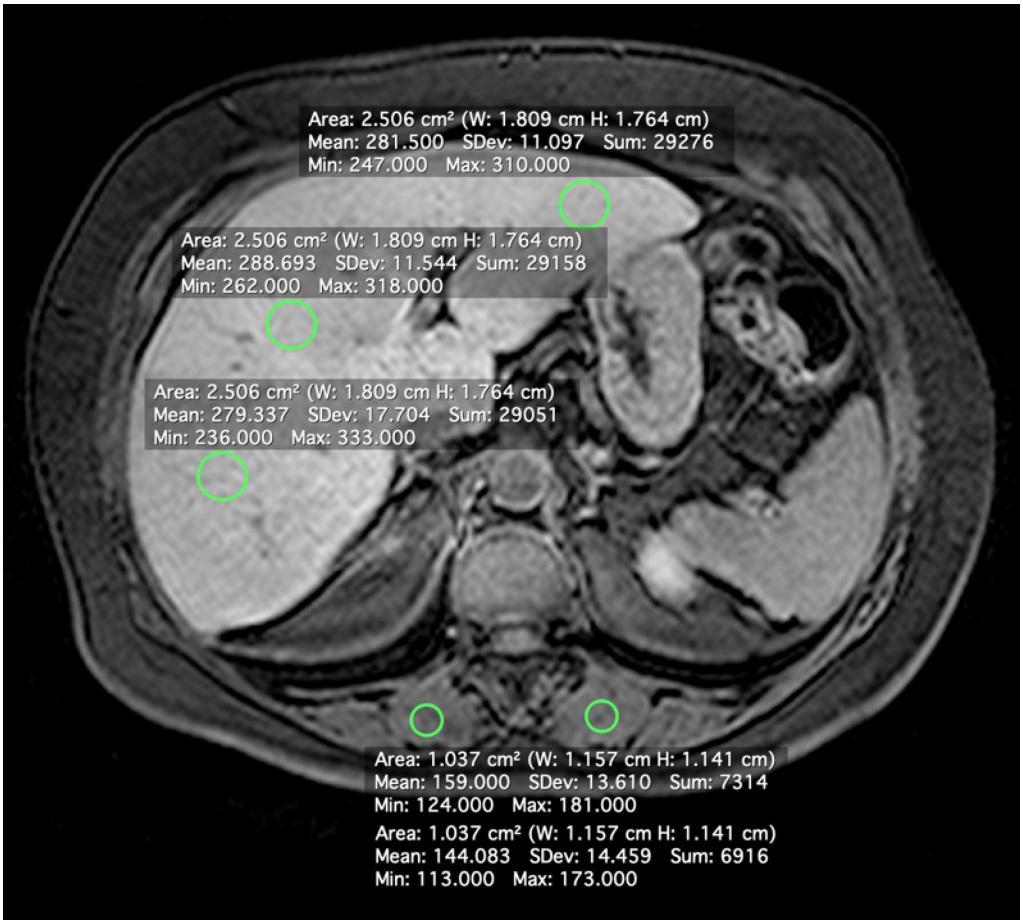

Figure 1 Axial magnetic resonance image showing the positions of regions of interest for the measurement of signal intensity of the liver parenchyma and paravertebral muscles.

examined using mean values and Student's *t* test for normally distributed continuous variables, median values and the Mann–Whitney test for non - normally distributed continuous variables, and the chi-squared test for categorical variables. To evaluate the performance of GA-MRI in differentiating between NASH and isolated steatosis, receiver operating characteristic curve analysis was performed to calculate the area under the curve, sensitivity, and specificity. Cut-off points with 80%-90% sensitivity and specificity were identified. P value < 0.05 were considered to be significant.

RESULTS

Demographic and clinical characteristics

During the study period, 59 consecutive patients diagnosed with NAFLD by histopathology were prospectively invited to undergo MRI. Three patients were excluded due to severe respiratory imaging artifacts. Of the 56 subjects included in the analysis, 18 patients had isolated steatosis (33% male, 67% female; mean age, 57 ± 8 years) and 38 patients had NASH (24% male, 76% female; mean age, 57 ± 9 years). The mean body weight of all patients was 84.2 ± 14.9 kg, and the mean BMI was 32.5 ± 5.1 kg/m^2. The prevalences of diabetes, dyslipidemia (elevated triglyceride and decreased high-density lipoprotein cholesterol concentrations), and hypertension were 73%, 86%, and 88%, respectively, with no difference between the NASH and isolated steatosis groups. Sex, age, BMI, and most laboratory values were also similar in the two groups. The mean plasma level of alanine aminotransferase was significantly higher in patients with NASH than in those with isolated steatosis. Baseline characteristics of the overall study population are summarized in Table 1.

Histological characteristics

The biopsies were performed at a mean interval of 2.7 ± 1.5 mo before MRI. The mean

Table 1 Baseline features of the study population and comparison of the isolated steatosis and nonalcoholic steatohepatitis groups

Characteristic	Total (*n* = 56)	Isolated steatosis (*n* = 18)	NASH (*n* = 38)	*P* value
Age, yr	57.5 ± 8.4	57.5 ± 8.0	57.5 ± 8.7	> 0.93
Sex, female	73.2	66.7	76.3	0.52
Body mass index, kg/m^2	32.5 ± 5.1	31.6 ± 5.6	32.9 ± 4.9	0.37
Systemic arterial hypertension	87.5	94.4	84.2	0.40
Diabetes mellitus	72.7	83.3	67.6	0.33
Dyslipidemia	85.7	72.2	92.1	0.09
Triglycerides (mg/dL)	167.2 ± 7.6	167.9 ± 91.4	166.8 ± 70.2	0.99
Total cholesterol (mg/dL)	192.4 ± 41.2	195.9 ± 40.5	190.7 ± 41.9	0.66
Aspartate aminotransferase (U/L)	31.5 ± 16.0	24.8 ± 5.5	34.6 ± 18.3	0.07
Alanine aminotransferase (U/L)	48.8 ± 30.3	34.2 ± 11.5	55.7 ± 33.9	0.01
Gama-glutamyl transpeptidase (U/L)	77.7 ± 66.2	62.5 ± 43.6	84.8 ± 74.0	0.26
Total bilirubin (mg/dL)	0.56 ± 0.21	0.52 ± 0.15	0.57 ± 0.24	0.52
Albumin (g/dL)	4.1 ± 0.4	4.0 ± 0.3	4.1 ± .47	0.32

Data are reported as means with standard deviations or percentages. NASH: Nonalcoholic steatohepatitis.

length of the biopsy specimens was 17.8 ± 4.1 mm. Among the 18 patients with isolated steatosis, 5.6% had severe steatosis, 22.2% had moderate steatosis, and 72.2% had mild steatosis. No patient with isolated steatosis showed fibrosis. In the NASH group, 23.7% of the 38 patients had severe steatosis, 65.8% had moderate steatosis, and 10.5% had mild steatosis. The proportion of moderate or severe steatosis was higher among patients with NASH ($P < 0.001$). The distribution of fibrosis in the NASH group was: F4, 7.9%; F3, 10.5%; F2, 5.3%; F1, 65.8%; and F0, 10.5%. The histological characteristics of the liver biopsy samples are summarized in Table 2.

MRI characteristics

The mean CEI in the study population was 1.82 ± 0.19. The mean CEI was significantly lower in the NASH group (1.78 ± 0.16) than in the isolated steatosis group (1.92 ± 0.22; $P = 0.008$; Figure 2). The area under the receiver operating characteristic curve for the distinction of the two groups was 0.68. The optimal CEI cut-off value was 1.76, which resulted in a specificity of 83% and sensitivity of 47%. The CEI cut-off value of 1.66 showed 94% specificity and that of 2.00 showed 92% sensitivity for the diagnosis of NASH (Table 3). The CEI did not differ according to the steatosis degree, hepatocellular ballooning, lobular inflammation, or fibrosis stage.

The majority (89%) of patients did not present liver iron overload, with an estimated liver iron concentration of 22.8 ± 10.4 μmol/g. The remaining patients (11%) had mild iron overload, with no significant difference between the isolated steatosis and NASH groups.

DISCUSSION

NAFLD is a major cause of liver disease worldwide (1) NASH is the most severe form of NAFLD and is the leading cause of end-stage liver disease in many countries; (2) The diagnosis of NASH is based on liver biopsy. Given its high prevalence (affecting about 1/3 of the global population) and the potential for cirrhosis, noninvasive methods for the early diagnosis of NASH are needed. In this study, we demonstrated that GA-MRI could be useful for the evaluation of patients with NAFLD, as it allows stratification according to the probabilities of NASH and isolated steatosis, thereby avoiding liver biopsy in many patients.

We found that the CEI was significantly lower in patients with NASH than in those with isolated steatosis, which facilitates differentiation between these entities. A main factor that may explain this difference is the expression of liver cell transporters. Multidrug resistance protein (MRP) transporters are responsible for GA excretion into

Table 2 Histological characteristics by study group

Characteristic	Total (*n* = 56)	Isolated steatosis (*n* = 18)	NASH (*n* = 38)
Fibrosis stage			
0	-	100	10.5
1	-	-	65.8
2	-	-	5.3
3	-	-	10.5
4	-	-	7.9
Steatosis grade			
1	30.4	72.2	10.5
2	51.8	22.2	65.8
3	17.8	5.6	23.7

Values are percentages. NASH: Nonalcoholic steatohepatitis.

Table 3 Differentiation of nonalcoholic steatohepatitis from isolated steatosis using different contrast enhancement index cut-offs

CEI cut-off	Sensitivity (%)	Specificity (%)
1.66	32	94
1.76	47	83
2.00	92	28

CEI: Contrast enhancement index.

the biliary system and sinusoidal backflow[3,15]. Some rodent studies have shown greater MRP transporter excretion expression in NASH models compared with steatosis models[16,17]. Other rodent studies have demonstrated the ability to differentiate isolated steatosis from NASH by comparing liver SI on images obtained by GA-MRI[18,19]. We believe that expression of the MRP transporter is also increased in patients with NASH, causing an increase in GA excretion by hepatocytes with a consequent decrease in hepatobiliary-phase liver SI relative to that of patients with isolated steatosis, reflected by the difference in the CEI between these groups.

Although the CEI differed significantly between the study groups, the cut-off point identified by the Youden index showed low sensitivity, despite good specificity. Therefore, we suggest two more extreme CEI cut-offs, one with greater sensitivity and the other with greater specificity, which appear be more clinically useful for the identification of patients with greater probability of having NASH or isolated steatosis. The combined use of these cut-offs can avoid liver biopsy in 40% of patients who undergo MRI, as it enables the identification of patients who should undergo more aggressive treatment (*i.e.*, those with steatohepatitis) and those who could be managed mainly with lifestyle changes (*i.e.*, those with isolated steatosis; Figure 3). Considering the high prevalence of NAFLD in the global population, biopsy avoidance in 40% of cases would be of great benefit, as liver biopsy is a procedure with high cost, risk of complications, sampling error, and inter- and intra-rater variability. In addition, the frequency of patients' refusal to undergo biopsy exceeds 50% in some centers, and the frequency of physician reluctance is as high as 30%[4]. The results of this study suggest that the use of these two extreme CEI cut-off values allows the detection and exclusion of NASH while maintaining high compliance among physicians and patients, as liver biopsy would be indicated only for patients with CEIs falling between the suggested cut-offs.

Only two human studies have examined the ability to differentiate isolated steatosis from steatohepatitis using the liver SI after GA injection, with promising results[7,9]. However, these studies have limitations, including their retrospective designs. In addition, the liver SI measurements were defined by an arbitrary scale, without internal correction, in both studies, which prevents comparison of repeated

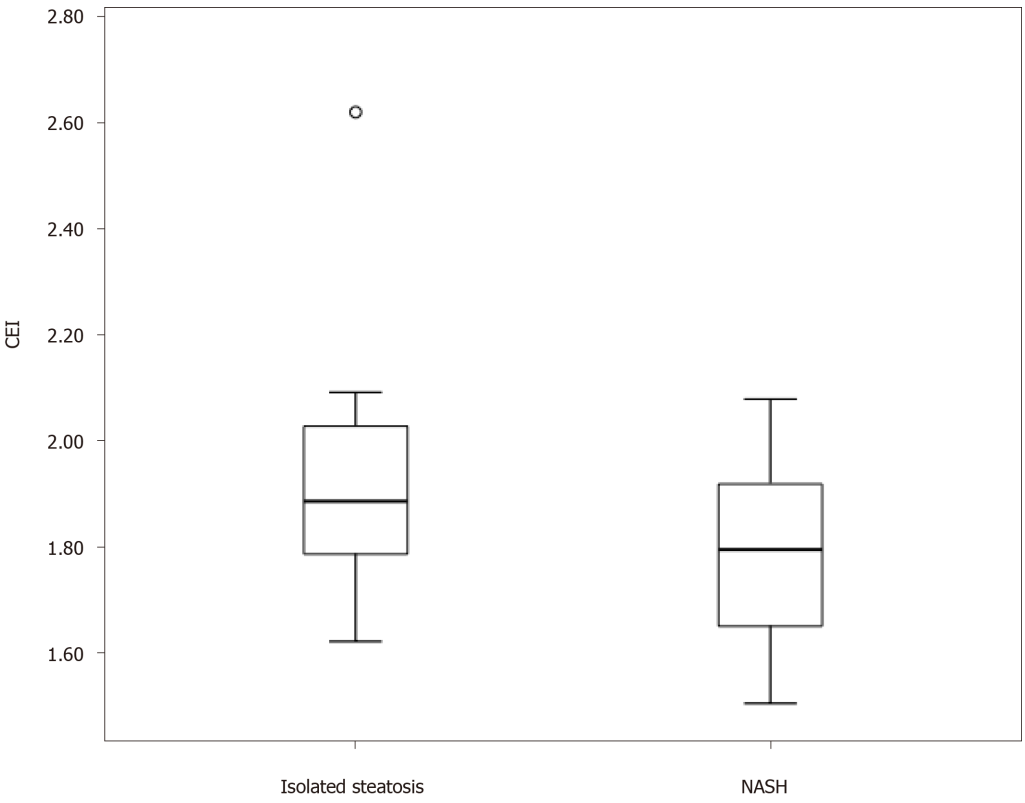

Figure 2 Boxplot of contrast enhancement indices in the isolated steatosis and nonalcoholic steatohepatitis groups. CEI: Contrast enhancement index.

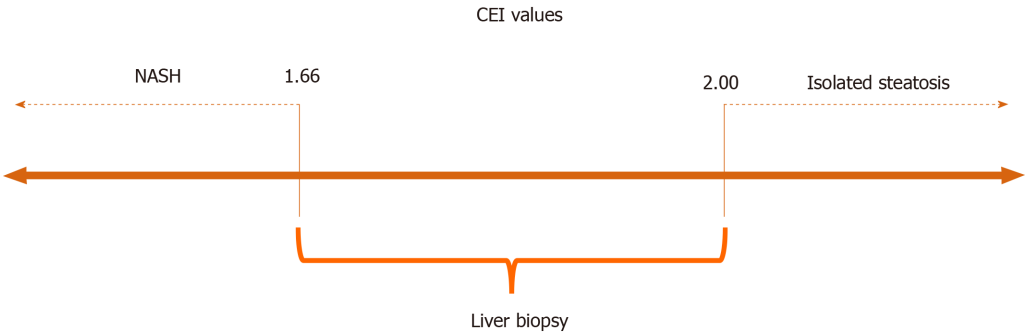

Figure 3 Contrast enhancement index cut-offs values suggested. NASH: Nonalcoholic steatohepatitis; CEI: Contrast enhancement index.

examinations in the same patient or between different patients. In our study, we used internal muscle correction, which increases reproducibility. In addition, neither of the previous studies involved evaluation of the hepatic iron concentration, which could lead to misclassification. The sample examined in one of the studies was small (25 patients)[9]. In the other study, heterogeneity of the groups impaired the analysis, as the isolated steatosis group included patients in whom fibrosis was detected by histopathology[7]. In addition, they did not propose cut-off points.

Given their average size, the biopsy samples examined in this study were representative[20-22]. CEI values did not differ according to the steatosis degree (P = 0.45), hepatocellular ballooning (P = 0.33), or lobular inflammation (P = 0.84); the proportion of each of these histopathological variables varied greatly in each NAFLD patient, presumably influencing the outcomes of their separate analysis. In addition, the CEI did not differ according to liver fibrosis (P = 0.18), probably due to the low prevalence of fibrosis scores ≥ F2, and especially F4, which was observed in only three patients; in previous studies, the degree of fibrosis has correlated inversely with liver enhancement in patients with chronic liver disease of various etiologies[7,9,12,23,24]. As the aim of the present study was to evaluate the ability of the CEI to differentiate simple steatosis from NASH, we consider the inclusion of few cases of advanced fibrosis in

our group to be an advantage.

The presence and level of liver iron concentration have been reported to influence liver enhancement by GA in the hepatobiliary phase[25]. In our sample, only a few patients presented mild hepatic iron overload (11%), with no significant difference between groups. Liver iron deposition does not appear to have influenced our results.

This work has a few potential limitations. The major limitation is the relatively small sample size. The sample was small due to the strict inclusion and exclusion criteria, especially the selection only of patients who had undergone biopsy. However, our sample was larger than the optimal sample size (n = 51), calculated with a high significance level (0.01) and statistical power (0.99). In addition, most (68%) patients in our sample had NASH, which is not typical for an NAFLD population. This distribution occurred because patients selected for biopsy had more severe NAFLD. We believe that this factor did not interfere with the achievement of our objective, which was to assess the diagnostic ability of the CEI, derived from GA-MRI examination, to differentiate NASH from isolated steatosis, rather than to examine the prevalence of either disease. Similar to the situation in other studies[7-9], the interval between liver biopsy and MRI was longer than ideal in this study.

To our knowledge, this work was the first prospective study to examine the ability to differentiate isolated steatosis from NASH in patients with NAFLD using GA-MRI. In addition to the prospective design, our methodology had other advantages; all histological samples were of sufficient size and were analyzed by a single pathologist with experience in hepatobiliary diseases, and all patients underwent MRI with the same device and examination protocol, avoiding bias due to differences in histological analysis, examination protocol, and MRI field strength. Furthermore, we used liver SI values corrected by paravertebral muscle SI to increase reproducibility and allow comparison of examinations in the same patient and between patients. In addition, we used a simple method that is highly reproducible and broadly accessible for all patients undergoing GA-MRI. Also, this is the first study that proposed cut-off points of CEI to identify and exclude NASH, easily performed by radiologists.

Whereas patients with NAFLD usually undergo imaging examinations for overall assessment of the hepatic parenchyma, the possibility of using GA-MRI as a noninvasive and comprehensive diagnostic modality holds great promise. Furthermore, the MRI scan has lower risk of complications, is cheaper, easier to perform and more widely available when compared to liver biopsy. Although the costs of medical procedures are highly variable in different countries, MRI scan seems to result in a more favorable cost-benefit ratio considering all the complexity of liver biopsy procedure.

In conclusion, patients with NASH have significantly lower CEIs in the hepatobiliary phase of GA-MRI than do patients with isolated steatosis. Thus, this preliminary study suggests that GA-MRI may be an effective noninvasive method for the identification of patients for whom early intervention and more aggressive therapy should be implemented and those with low probability of having NASH, avoiding liver biopsy in up to 40% of the NAFLD population. Liver biopsy, the gold standard method, would still be required for cases in which GA-MRI findings are inconclusive. However, further studies with a larger sample size are warranted to validate the proposed cut-off points.

ARTICLE HIGHLIGHTS

Research background

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide, affecting up to 40% of the world population. It is characterized by fatty liver infiltration, and encompasses a wide clinical spectrum, ranging from a relatively benign isolated steatosis from potentially progressive nonalcoholic steatohepatitis (NASH), liver fibrosis and cirrhosis.

Research motivation

The diagnosis of NASH is crucial and has prognostic and therapeutic implications. Liver biopsy is currently the gold standard for diagnosing progressive NASH and has several limitations, such as sampling error, cost, and risk of complications.

Abundant research has been performed to develop noninvasive diagnostic methods for the early detection of NASH and its accurate differentiation from isolated steatosis, due to the utmost clinical importance of this diagnosis.

Research objectives

To evaluate the performance of gadoxetic acid–enhanced magnetic resonance imaging (GA-MRI) to differentiate NASH in patients with NAFLD using histopathology as the reference standard.

Research methods

In this prospective study, 56 patients with NAFLD (18 with isolated steatosis and 38 with NASH) underwent GA-MRI. Contrast enhancement index (CEI) was calculated as the rate of increase of the liver-to-muscle signal intensity ratio before and 20 min after intravenous GA administration. Between-group differences in mean CEI were tested with the Student's *t*-test. Area under the receiver operator characteristic curve, and the diagnostic performance of GA-MRI were evaluated.

Research results

The mean CEI for all subjects was 1.82 ± 0.19. The mean CEI was significantly lower in patients with NASH than in those with isolated steatosis (P = 0.008). Two CEI cut-off points were used: < 1.66 (94% specificity) to characterize NASH and > 2.00 (89% sensitivity) to characterize isolated steatosis. CEI values between 1.66 and 2.00 indicated liver biopsy, and the procedure could be avoided in 40% of patients with NAFLD.

Research conclusions

Patients with NASH have significantly lower CEIs in the hepatobiliary phase of GA-MRI than do patients with isolated steatosis. This study suggests that GA-MRI may be an effective noninvasive method for the identification of patients for whom early intervention and more aggressive therapy should be implemented, avoiding liver biopsy in up to 40% of the NAFLD population.

Research perspectives

The possibility of using GA-MRI as a noninvasive and comprehensive diagnostic modality holds great promise. As it is a preliminary study, further prospective studies with a larger sample size are warranted.

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ORIGINAL ARTICLE

Prospective Study

Sofosbuvir plus ribavirin is tolerable and effective even in elderly patients 75-years-old and over

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Institutional review board statement: All study protocols were approved by the ethics committees of the participating hospitals.

Informed consent statement: Written informed consent was obtained from all patients included in this study.

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Abstract

BACKGROUND

Although clinical use of sofosbuvir plus ribavirin has been approved for patients infected with genotype 2 hepatitis C virus, patients ≥ 75-years-old have not been included in previous clinical trials.

AIM

To evaluate the real-world safety and efficacy of sofosbuvir plus ribavirin for elderly patients (≥ 75-years-old) compared to nonelderly patients, we conducted a post-marketing prospective cohort study.

METHODS

We treated 265 patients with genotype 2 hepatitis C virus using standard approved doses of sofosbuvir (400 mg/d) plus ribavirin adjusted by body weight, administered orally for 12 wk.

RESULTS

Sustained virological response rates for the overall cohort, patients < 65-years-old, ≥ 65-years-old but < 75-years-old, and ≥ 75-years-old were 97% (258/265), 98%

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(93/95), 97% (84/87), and 98% (81/83), respectively (P = 0.842). Logistic regression analyses identified history of hepatocellular carcinoma treatment and alpha-fetoprotein as factors significantly associated with sustained virological response. Alpha-fetoprotein was the only independent factor identified. Sustained virological response rate was significantly lower for patients with hepatocellular carcinoma treatment (91%) than for patients without history of hepatocellular carcinoma treatment (98%, P = 0.004). One patient (0.4%) discontinued treatment due to drug-induced pneumonia. Dose reduction or interruption of ribavirin was required for 12.1% (32/265) of patients because of anemia, including 7.7% (14/182) of patients < 75-years-old and 21.7% (18/83) of patients ≥ 75-years-old (P = 0.002).

CONCLUSION
Although ribavirin dose reduction or interruption was required with advanced age, sofosbuvir plus ribavirin appears tolerable and highly effective even in patients ≥ 75-years-old.

Key Words: Hepatitis C virus; Genotype 2; Sofosbuvir; Ribavirin; Elderly patients; Cirrhosis

Core Tip: This was a multicenter post-marketing prospective cohort study of sofosbuvir plus ribavirin therapy for patients with genotype 2 hepatitis C virus in a real-world clinical setting. Combination therapy using sofosbuvir plus ribavirin was tolerable and highly effective even in elderly patients ≥ 75-years-old.

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INTRODUCTION

Although interferon (IFN)-based therapy was standard for hepatitis C virus (HCV) infection for many years, clinical trials of IFN-free direct-acting antiviral agent (DAA) regimens using sofosbuvir and ribavirin for patients with genotype 2 HCV have been reported from Western countries since 2013[1-3]. Those clinical trials demonstrated higher viral eradication rates and lower discontinuation rates than IFN-based therapies, and treatment-related health-related quality of life impairment during treatment was reportedly mild in these clinical trials[4]. However, patients ≥ 75-years-old were not included in the sofosbuvir plus ribavirin regimen of those clinical trials. In May 2015, clinical use of combination therapy comprising sofosbuvir and ribavirin was approved as the first IFN-free therapy for patients infected with genotype 2 HCV in Japan[5]. This therapy was well tolerated and achieved a high sustained virological response (SVR) rate of 96% in a Japanese Phase III trial. However, patients ≥ 75-years-old were again not included in that trial. The safety and effectiveness of sofosbuvir plus ribavirin for elderly patients ≥ 75-years-old has thus remained unclear.

In real-world settings, elderly patients ≥ 75-years-old represent a substantial and growing population and carry a high risk of advanced liver diseases such as cirrhosis and hepatocellular carcinoma (HCC). These patients should be treated as soon as possible. However, ribavirin has various specific side effects that affect tolerability, such as hemolytic anemia, fatigue, cough, depression, and chest pain[6,7], whereas sofosbuvir is tolerable with minimal adverse effects[8]. In our previous study of low-dose pegylated IFN plus ribavirin therapy for elderly and/or cirrhotic patients infected with HCV genotype 2, drug dose reduction or interruption rates among nonelderly cirrhotic patients, elderly noncirrhotic patients, and elderly cirrhotic patients were all relatively high (65%, 63%, and 77%, respectively)[9]. A high risk of

ribavirin dose reduction or interruption and lower effectiveness in elderly patients can thus be expected even with sofosbuvir plus ribavirin therapy. To evaluate the real-world safety and efficacy of sofosbuvir plus ribavirin for elderly patients ≥ 75-years-old compared to nonelderly patients, we conducted a post-marketing prospective cohort study.

MATERIALS AND METHODS

Patients

This was a multicenter prospective cohort study. Between June 2015 and June 2017, all patients at Wakayama Medical University Hospital, Naga Municipal Hospital, Hidaka General Hospital, and Wakayama Rosai Hospital who were eligible were enrolled in the present study. The inclusion criterion was infection with HCV genotype 2. Exclusion criteria were any of following: (1) Infection with genotypes other than genotype 2; (2) Hemoglobin concentration < 10 g/dL; (3) Estimated glomerular filtration rate < 30 mL/min/1.73 m^2; (4) Decompensated cirrhosis (Child-Pugh class B or C); and (5) Any form of cancer. However, patients who had received curative cancer treatments were not excluded from this study. Therefore, patients with HCC who had undergone surgical resection or ablation therapy were included in this study.

Liver cirrhosis was diagnosed clinically from liver biopsy or imaging studies such as ultrasonography, contrast-enhanced computed tomography, and/or magnetic resonance imaging using morphological signs of cirrhosis from portal hypertension, such as portosystemic shunt or hypersplenism. Sample size for the present study was determined by practicability but was planned to exceed the number of patients analyzed in the Japanese phase III trial[5]. All study protocols were approved by the ethics committees of the participating hospitals. Written informed consent was obtained from all patients included in this study. The present study was registered on the University Hospital Medical Information Network (trial ID: 000023269).

Treatment regimens

Standard approved doses of 400 mg sofosbuvir (Sovardi, Gilead, TKY, Japan) plus ribavirin (Copegus, Chugai Pharmaceutical, TKY, Japan or Rebetol, MSD, TKY, Japan) adjusted by body weight (1000 mg/d for patients weighing > 80 kg, 800 mg/d for patients weighing ≤ 80 but ≥ 60 kg, and 600 mg/d for patients weighing < 60 kg) were orally administered for 12 wk. If hemoglobin level fell to < 10 g/dL, ribavirin dose was reduced to 200 mg/d, and if hemoglobin level fell to < 8.5 g/dL, ribavirin was discontinued. If the attending physician judged this treatment as difficult to continue due to adverse events, both sofosbuvir and ribavirin were discontinued.

Laboratory test

HCV-RNA load was measured using quantitative reverse transcription polymerase chain reaction (COBAS TaqMan PCR assay version 2; Roche Diagnostics, Branchburg, NJ, United States). HCV genotype was determined using the antibody serotyping method (SRL, TKY, Japan). HCV serotypes 1 and 2 correspond to genotypes 1a/1b and 2a/2b, respectively. When HCV serotype could not be determined, genotype was examined using real-time polymerase chain reaction assay (BML, TKY, Japan). HCV-RNA was checked on the day of therapy initiation and every 4 wk during treatment. Biochemical analyses including blood cell counts, C-reactive protein level, blood sugar level, and liver and renal function tests were performed every 2 wk during treatment.

Assessment of effectiveness

Rapid virological response (RVR) was defined as serum HCV-RNA negativity in week 4 after therapy initiation. End-of-treatment response was defined as serum HCV-RNA negativity in week 12 after therapy initiation. SVR was defined as HCV-RNA negativity at 24 wk after the end of therapy. The primary end point of this study was SVR at 24 wk after the end of therapy. Treatment failure was defined as non-SVR.

Assessment of safety and tolerability

Patients were assessed for the safety and tolerability of treatment by attending physicians who monitored adverse events and laboratory parameters such as blood cell counts and liver and renal function tests every 2 wk. Adverse events were assessed according to Common Terminology Criteria for Adverse Events version 4.0. The incidence of and reasons for therapy discontinuation or interruption due to adverse

events were analyzed.

Statistical analysis

Therapeutic efficacy was evaluated using an intention-to-treat analysis. The Mann-Whitney *U* test or the *t*-test was used to analyze continuous variables. Fisher's exact test or the chi-square test was used to analyze categorical variables. Logistic regression analysis for univariate comparisons was performed to investigate factors contributing to SVR. When multiple factors were significant from univariate analyses, multivariate analysis was also performed to identify independent factors. Values of $P < 0.05$ were considered statistically significant. SPSS for Windows version 24J statistical software (SPSS, TKY, Japan) was used for all data analyses.

RESULTS

Baseline characteristics of patients

A total of 265 patients met the inclusion and exclusion criteria and were enrolled in the present study. Although one patient discontinued treatment due to an adverse event, all enrolled patients completed follow-up for evaluation of safety and effectiveness. Patient characteristics are summarized in Table 1. Median age was 68-years-old (range, 17-86 years), and the cohort was comprised of 150 male and 115 female patients. Patients ≥ 75-years-old, cirrhotic patients, patients with moderate chronic kidney disease (defined as estimated glomerular filtration rate < 60 mL/min/1.73 m^2), patients with a history of HCC treatment, and patients with a history of pegylated IFN plus ribavirin therapy accounted for 31%, 34%, 20%, 12%, and 15%, respectively. Median baseline hemoglobin level was 13.6 g/dL (range, 10.2-20.1 g/dL).

Comparison of pre-treatment factors between patients ≥ 75-years-old and < 75-years-old

A comparison of pre-treatment factors between patients ≥ 75-years-old and < 75-years-old is shown in Table 2. Significant differences were seen in height, weight, body mass index, cirrhosis, chronic kidney disease, history of HCC treatment, white blood cells, hemoglobin, platelets, alanine aminotransferase, γ-glutamyl transferase, alpha-fetoprotein (AFP) levels, and estimated glomerular filtration rate.

Treatment response

SVR rates overall and according to age groups are shown in Figure 1. SVR rates for the overall cohort, patients < 65-years-old, patients ≥ 65-years-old but < 75-years-old, and patients ≥ 75-years-old were 97% (258/265), 98% (93/95), 97% (84/87), and 98% (81/83), respectively. No significant differences were observed among age groups ($P = 0.842$).

A comparison of the viral negativity rate between patients ≥ 75-years-old and < 75-years-old during treatment is shown in Figure 2. RVR rates for patients ≥ 75-years-old and < 75-years-old groups were 84% and 89%, respectively. Although RVR rate tended to be higher in patients ≥ 75-years-old than in patients < 75-years-old, the difference was not significant ($P = 0.266$). End-of-treatment response rates of patients ≥ 75-years-old and < 75-years-old were 99% and 100%, respectively. No significant difference was apparent between groups ($P = 1.000$).

SVR rates according to background factors are summarized in Figure 3. Although no significant difference was seen in comparisons of SVR rates according to sex, cirrhosis, elderly cirrhosis, IFN plus ribavirin therapy, anemia (hemoglobin < 12 g/dL), or chronic kidney disease, a significant difference was seen between patients with and without a history of HCC treatment ($P = 0.004$). In patients ≥ 75-years-old with a history of IFN plus ribavirin therapy, all patients achieved SVR (100%; 14/14). In patients ≥ 75-years-old with cirrhosis, the SVR rate was 98% (40/41).

Factors contributing to SVR

Results of logistic regression analysis to investigate factors contributing to SVR are shown in Table 3. On univariate analyses, a history of HCC treatment and AFP were factors significantly contributing to SVR. Multivariate analyses revealed AFP as the only factor independently associated with SVR.

Table 1 Patient characteristics

	***n* = 265**
Age in yr	68 (17-86)
≥ 75 yr	83 (31%)
Sex as male/female	150/115 (57%/43%)
Cirrhosis	91 (34%)
Moderate chronic kidney disease, eGFR < 60 mL/min/1.73 m^2	53 (20%)
History of HCC treatment	33 (12%)
History of pegylated IFN + ribavirin therapy	39 (15%)
Height in cm	160.2 (134.0-182.0)
Weight in kg	58.0 (32.3-99.3)
BMI in kg/m^2	22.6 (15.5-35.0)
Baseline HCV-RNA in logIU/mL	6.0 (1.4-7.4)
WBC as /mm^3	5000 (1810-13260)
Hb in g/dL	13.6 (10.2-20.1)
Platelets as × 10^4/mm^3	16.2 (4.6-38.9)
AST in IU/L	43 (12-257)
ALT in IU/L	39 (6-394)
γ-GT in IU/L	32 (5-898)
eGFR	73 (31-156)
AFP in ng/mL	4.3 (1.0-88.3)

Values are expressed as median (range) or numbers (percentage). γ-GT: γ-glutamyl transferase; AFP: Alpha-fetoprotein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; eGFR: Estimated glomerular filtration rate; Hb: Hemoglobin; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HCV-RNA: Hepatitis C virus-ribonucleic acid; IFN: Interferon; WBC: White blood cells.

Treatment failure

Non-SVR was shown in seven patients (3%). Factors between patients with and without SVR are compared in Table 4. When drug adherence was defined as a percentage of the actual administered dose to the planned dose, RVR adherence was not identified as significantly related to non-SVR. History of HCC treatment was the only factor significantly related to non-SVR.

Safety and tolerability

The discontinuation rate due to adverse events was 0.4% (1/256). The reason for discontinuation was drug-induced pneumonitis with positive results for sofosbuvir on drug-induced lymphocyte stimulation testing. Adverse event profiles for the overall cohort, patients ≥ 75-years-old, and patients < 75-years-old are shown in Table 5. The most frequent adverse event other than anemia was elevated uric acid level (Grade 1). No severe liver injury or exacerbation of renal dysfunction was seen. A similar safety profile was observed between patients ≥ 75-years-old and < 75-years-old, except for ribavirin dose reduction or interruption due to anemia. Median ribavirin adherence was significantly lower for patients ≥ 75-years-old (96.8%) than for patients < 75-years-old (100%, P = 0.001). Ribavirin dose reduction or interruption was required in 12.1% (32/265) of patients because of anemia, and anemia appeared in 7.7% (14/182) of patients < 75-years-old, and 21.7% (18/83) of patients ≥ 75-years-old. A significant difference in ribavirin dose reduction or interruption rate was also seen between groups (P = 0.002).

Table 2 Comparison of pre-treatment factors between patients ≥ 75-years-old and < 75-years-old

Factors	Patients ≥ 75-yr-old, *n* = 83	Patients < 75-yr-old, *n* = 182	*P* value
Age in yr	79 (75-86)	64 (17-74)	< 0.001
Sex as male/female	42/41 (51%/49%)	108/74 (59%/41%)	0.183
Height in cm	156.7 (134.0-170.0)	162.0 (140.0-182.0)	< 0.001
Weight in kg	53.5 (32.3-81.4)	60.0 (37.6-99.3)	< 0.001
BMI in kg/m^2	22.2 (15.5-29.5)	23.1 (16.5-35.0)	0.009
Cirrhosis	41 (49%)	50 (28%)	< 0.001
CKD, eGFR < 60 mL/min/1.73 m^2	28 (34%)	25 (14%)	< 0.001
History of HCC treatment	18 (22%)	15 (8%)	0.002
History of IFN-based therapy	21 (25%)	42 (23%)	0.693
HCV-RNA as logIU/mL	6.1 (2.3-7.3)	5.9 (1.4-7.4)	0.894
WBC in mm^3	4540 (1810-13260)	5200 (2200-11400)	0.004
Hb in g/dL	12.9 (10.2-20.1)	14.1 (10.6-17.6)	< 0.001
Platelets as × 10^4/mm^3	14.2 (4.9-32.8)	17.4 (4.6-38.9)	< 0.001
AST in IU/L	40 (14-183)	42 (12-252)	0.589
ALT in IU/L	30 (6-139)	44 (6-394)	< 0.001
γ-GT in IU/L	25 (7-361)	37 (5-888)	0.001
AFP in ng/mL	3.9 (1-32.9)	4.8 (1.1-88.3)	0.014
eGFR	66.0 (33.0-106.9)	77.6 (30.9-156.0)	< 0.001

Values are expressed as median (range) or number (percentage). γ-GT: γ-glutamyl transferase; AFP: Alpha-fetoprotein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; CKD: Chronic kidney disease; eGFR: Estimated glomerular filtration rate; Hb: Hemoglobin; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HCV-RNA: Hepatitis C virus-ribonucleic acid; IFN: Interferon; WBC: White blood cells.

DISCUSSION

This was a multicenter post-marketing prospective cohort study of sofosbuvir plus ribavirin therapy for patients infected with genotype 2 HCV in a real-world clinical setting. In the present study, 31% of patients were ≥ 75-years-old, and 12% had a history of HCC treatment. Furthermore, 34% of enrolled patients had cirrhosis, and 20% had moderate chronic kidney disease.

Although some real-world data based on post-marketing cohort studies of sofosbuvir plus ribavirin have been reported, few reports have evaluated safety and efficacy for patients ≥ 75-years-old. Regarding safety, Ogawa *et al*[10] indicated that the frequency of adverse effects was higher for a ≥ 65-year-old group (18.9%) than for the < 65-year-old group (4.3%, $P < 0.001$). However, discontinuation of all drugs was required for only 3 of the 446 patients (0.7%)[10]. Atsukawa *et al*[11] indicated that the incidence of anemia increased significantly with age, and ribavirin dose reduction rate increased sharply in patients > 70-years-old[11]. Anemia during treatment occurred in 10.6% (23/218) of patients < 75-years-old, and in 48.1% (25/52) of patients ≥ 75-years-old[11]. However, none of those 270 patients discontinued use of either ribavirin or sofosbuvir[11]. In the present study, although dose reduction or interruption of ribavirin due to anemia was required in 21.7% of patients ≥ 75-years-old and 7.7% of patients < 75-years-old, treatment discontinuation was required for only one patient (0.4%). Therefore, although careful monitoring of anemia and ribavirin dose adjustment is necessary to avoid discontinuation of therapy, this treatment appears tolerable even in patients ≥ 75-years-old.

Regarding efficacy, Nishida *et al*[12] reported that although the difference was not significant, patients ≥ 75-years-old tended to show a lower SVR rate than patients < 75-years-old (81.3%, 13/16 for patients ≥ 75-years-old; 96.0%, 24/25 for patients < 75-years-old)[12]. Atsukawa *et al*[11] showed SVR rates of 98.1% (51/51) for patients ≥ 75-years-old and 96.8% (211/218) for patients < 75-years-old ($P = 0.999$)[11]. Ogawa *et al*[10] reported that although SVR12 was achieved by 95% (69/73) of patients > 75-years-old,

Table 3 Uni- and multivariate analyses of pre-treatment factors contributing to sustained virological response

	Univariate			Multivariate		
Factors	***P* value**	**OR**	**95%CI**	***P* value**	**OR**	**95%CI**
Age, per 1-yr increase	0.782	0.991	0.931-1.055			
≥ 75 yr	0.874	1.144	0.217-6.022			
Sex, female	0.977	1.023	0.224-4.663			
Height, per 1-cm increase	0.316	0.957	0.879-1.043			
Weight, per 1-kg increase	0.568	0.982	0.924-1.044			
BMI, per 1-kg/m^2 increase	0.929	0.990	0.792-1.238			
Cirrhosis	0.214	0.382	0.084-1.743			
CKD, eGFR > 60 mL/min/1.73 m^2	0.569	0.616	0.116-3.266			
History of HCC treatment	0.027	0.175	0.037-0.822	0.072	0.225	0.044-1.145
History of IFN-based therapy	0.763	0.774	0.146-4.091			
HCV-RNA, per 1-logIU/mL increase	0.644	0.831	0.380-1.821			
WBC in mm^3	0.703	1.009	0.963-1.057			
Hb, per 1-g/dL increase	0.196	1.411	0.837-2.380			
Platelets, per 1 × 10^4/mm^3 increase	0.790	0.984	0.873-1.109			
AST, per 1-IU/L increase	0.552	1.007	0.983-1.033			
ALT, per 1-IU/L increase	0.608	1.005	0.987-1.023			
γ-GT, per 1-IU/L increase	0.490	1.007	0.987-1.027			
AFP, per 1-ng/mL increase	0.004	0.955	0.926-0.985	0.015	0.959	0.926-0.992
eGFR, per 1-mL/min/1.73 m^2 increase	0.124	1.036	0.990-1.083			

γ-GT: γ-glutamyl transferase; AFP: Alpha-fetoprotein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; CI: Confidence interval; CKD: Chronic kidney disease; eGFR: Estimated glomerular filtration rate; Hb: Hemoglobin; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HCV-RNA: Hepatitis C virus-ribonucleic acid; IFN: Interferon; OR: Odds ratio; WBC: White blood cells.

the SVR12 rate was significantly lower in cirrhotic patients > 75-years-old with a history of IFN treatment (73.3%, 11/15) than in noncirrhotic patients > 75-years-old (100%, 17/17; $P < 0.01$)[10]. In the present study, the SVR rate of patients ≥ 75-years-old was 98% (81/83). Among patients ≥ 75-years-old with a history of IFN plus ribavirin therapy, the SVR rate was 100% (14/14). Furthermore, the SVR rate of cirrhotic patients ≥ 75-years-old was also extremely high (98%, 40/41). From these results, a high SVR rate (> 95%) would be expected even in patients ≥ 75-years-old, irrespective of cirrhosis or history of IFN treatment.

Discontinuation of pharmacotherapy[13], history of HCC[10,13], cirrhosis (advanced fibrosis)[10,14,15], renal dysfunction[16], history of IFN-based treatment[10,15], lower serum albumin, and ribavirin dose at baseline[14] have all been reported as factors associated with non-SVR of sofosbuvir plus ribavirin. Hirosawa *et al*[13] indicated that the risk factor most strongly associated with non-SVR was a history of HCC treatment (odds ratio: 9.29)[13]. In the present study, a history of HCC treatment and AFP were factors significantly associated with SVR on univariate analysis, and AFP was the only independent factor on multivariate analyses. High serum AFP levels in patients without HCC have been associated with advanced liver fibrosis and a risk of HCC occurrence[17,18]. Sofosbuvir plus ribavirin might thus be less effective in cases showing advanced liver fibrosis. Patients with a history of HCC treatment or high AFP level should probably be treated using some other ribavirin-free DAA therapy.

In recent HCV treatment guidelines from Western countries, sofosbuvir plus ribavirin therapy is no longer recommended because of the adverse effects of ribavirin and the relatively lower SVR rate compared to other DAA therapies[14,19,20]. In fact, the

Table 4 Comparison of factors between patients with and without sustained virological response

Factors	SVR, *n* = 258	Non-SVR, *n* = 7	*P* value
Age in yr, *n* (range)	68 (17-86)	74 (39-79)	0.682
Sex as male/female	146/112	4/3	1.000
Height in cm	160.1 (134.0-182.0)	161.5 (155.0-177.0)	0.350
Body weight in kg	58.0 (32.3-99.3)	63.2 (54.0-66.2)	0.267
BMI in kg/m^2	22.6 (15.5-38.0)	22.6 (20.2-25.9)	0.778
Cirrhosis	87	4	0.237
CKD	51	2	0.630
History of HCC treatment	30	3	0.044
History of IFN-based therapy	61	2	0.672
HCV-RNA as logIU/mL	6.1 (2.7-7.6)	6.3 (3.3-7.0)	0.713
White blood cells as /mm^3	5005 (1810-13260)	4100 (2260-8700)	0.581
Hemoglobin in g/dL	13.6 (10.2-20.1)	12.5 (11.6-14.7)	0.793
Platelets as × 10^4/mm^3	16.2 (4.6-38.9)	14.9 (9.9-30.3)	0.930
AST in IU/L	42 (12-252)	37 (19-90)	0.789
ALT in IU/L	39 (6-394)	57 (19-79)	0.843
γ-GTP in IU/L	32 (5-888)	36 (13-461)	0.942
AFP in ng/mL	5.2 (1.0-445.0)	10.6 (1.3-29.7)	0.521
eGFR in mL/min/1.73 m^2	73.1 (30-240.2)	72.2 (50.5-105.8)	0.166
Ribavirin adherence in %	100 (28-100)	100 (100-100)	0.323

Values are expressed as median (range) or number of patients. γ-GTP: γ-glutamyl transferase; AFP: Alpha-fetoprotein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; CKD: Chronic renal disease; eGFR: Estimated glomerular filtration rate; HCC: hepatocellular carcinoma; HCV: Hepatitis C virus; HCV-RNA: Hepatitis C virus-ribonucleic acid; IFN: Interferon; SVR: Sustained virological response.

real-life SVR rate from nationwide German data was lower compared to SVR rates of clinical trials (83% in intention-to-treat analysis)[21]. Recently, ribavirin-free DAA therapies such as glecaprevir plus pibrentasvir, and sofosbuvir plus ledipasvir have also been approved for use in patients with HCV genotype 2 in Japan[22,23]. These therapies have shown no adverse effects due to ribavirin and have thus become first-line treatments. These therapies also represent rescue treatments for patients with sofosbuvir plus ribavirin failure. However, in consensus statements and recommendations on the treatment of hepatitis C from the Asian-Pacific Association for the Study of the Liver, sofosbuvir plus weight-based ribavirin for 12 wk is recommended as a first-line treatment, and ledipasvir and sofosbuvir for 12 wk is recommended for treatment-naïve HCV genotype 2 patients who cannot tolerate ribavirin[24]. In addition, SVR rates from Asian real-world data were similar to those of the phase III trial[25]. Sofosbuvir plus ribavirin therapy offers advantages in terms of both cost and real-world evidence.

Some limitations need to be considered for the present study. First, some selection biases would be present. Second, the number of patients may not have been sufficient to reach definitive conclusions regarding safety and effectiveness in patients ≥ 75-years-old. Third, reasons for failure of this therapy could not be clarified by our analysis because the number of patients who did not achieve SVR was too small. Furthermore, sofosbuvir-specific resistance-associated substitutions (RASs) were not tested for in this study. The prevalence of the naturally occurring RAS S282T, as the only known variant conferring sofosbuvir resistance *in vitro*, is reportedly rare in genotype 2 (0.22%)[26]. However, RAS A150V has recently been found to be associated with reduced response to treatment with sofosbuvir and ribavirin and has appeared in genotype 2a (13.8%) and genotype 2b (1.03%)[26]. In addition, the naturally occurring nucleoside inhibitor-specific RASs (E237G, M289I/L, L320 F, and V321A/I) are also found in genotype 2[26]. The influence of these preexisting RASs on SVR should be analyzed using a larger number of cases with treatment failure.

Table 5 Adverse events during treatment

	Total, *n* = 265	Patients ≥ 75-yr-old, *n* = 83	Patients < 75-yr-old, *n* = 182	*P* value
Treatment discontinuation due to adverse events[1]	1 (0.4%)	1 (1.2%)	0	0.313
Dose reduction or interruption of ribavirin due to anemia[2]	32 (13.2%)	18 (21.7%)	14 (7.7%)	0.002
Dermatitis	9 (3.4%)	1 (1.2%)	8 (4.4%)	0.281
Depression	3 (1.1%)	1 (1.2%)	2 (0.5%)	1.000
Headache	4 (1.5%)	0	4 (2.2%)	0.313
Infection	4 (1.5%)	1 (1.2%)	3 (1.6%)	1.000
Other adverse events	11 (4.2%)	5 (6.0%)	6 (3.3%)	0.328
Elevated bilirubin level	2 (0.8%)	1 (1.2%)	1 (0.5%)	0.529
Elevated transaminase level	4 (1.5%)	2 (2.4%)	2 (1.1%)	0.592
Elevated serum ammonia level	5 (1.9%)	2 (2.4%)	3 (1.6%)	0.650
Elevated uric acid level	26 (9.8%)	10 (12.0%)	16 (8.8%)	0.504

[1]Drug-induced pneumonitis with positivity to sofosbuvir on drug-induced lymphocyte stimulation test.
[2]Significant difference between patients ≥ 75-years-old and < 75-years-old ($P < 0.01$).

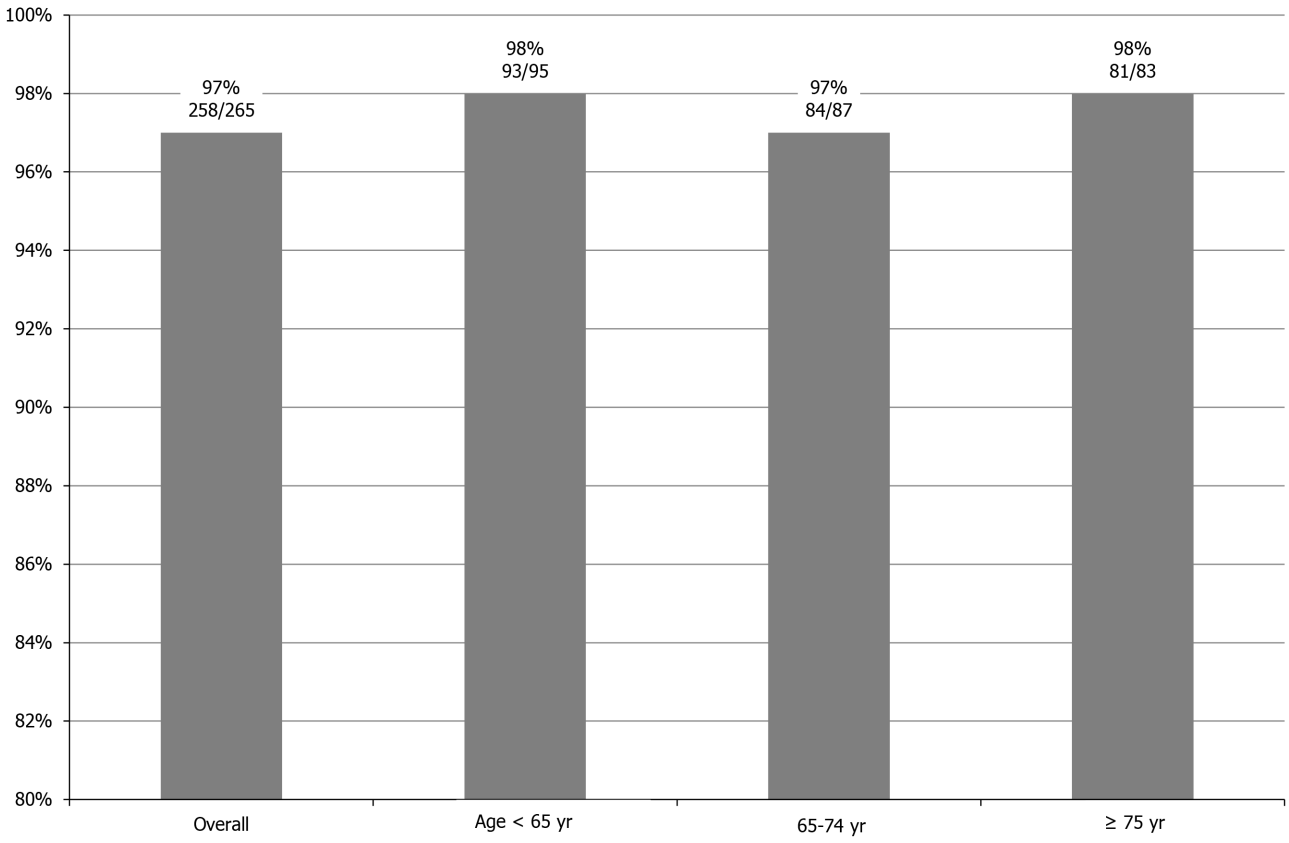

Figure 1 Sustained virological response rates according to age groups. The sustained virological response rates for the overall cohort, patients < 65-years-old, patients ≥ 65-years-old but < 75-years-old, and patients ≥ 75-years-old are 97% (258/265), 98% (93/95), 97% (84/87), and 98% (81/83), respectively. No significant differences are apparent among age groups ($P = 0.842$).

Sofosbuvir and ribavirin represent an acceptable and effective treatment even for patients ≥ 75-years-old in a real-world setting. An extremely high SVR rate can be achieved when adequate management for adverse effects is performed to avoid discontinuation of treatment, irrespective of age.

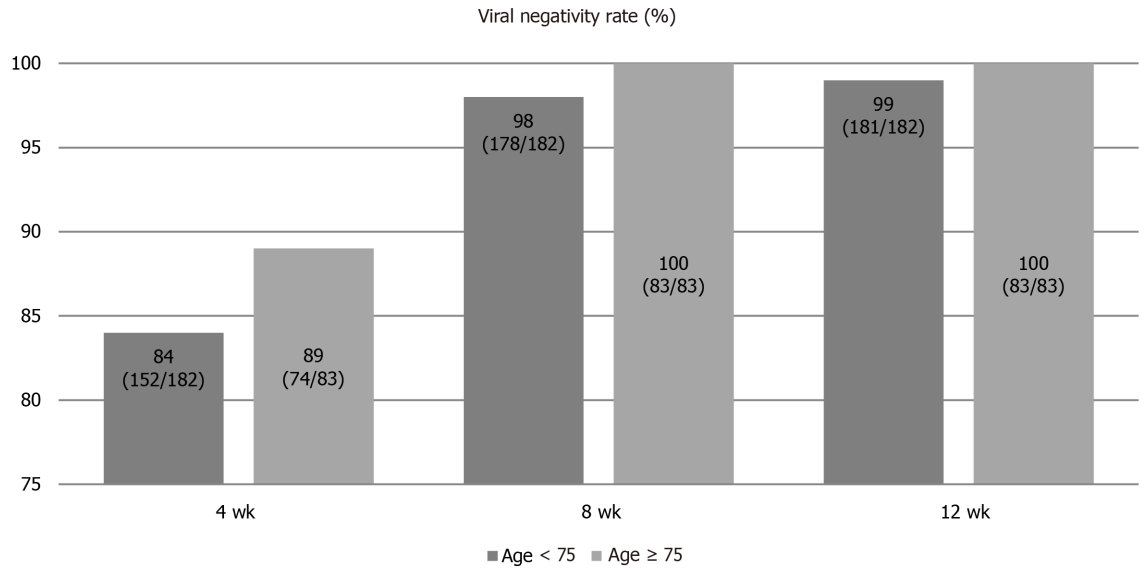

Figure 2 Comparison of viral negativity rates between patients ≥ 75-years-old and < 75-years-old during treatment. Rapid virological response rates of patients ≥ 75-years-old and < 75-years-old are 84% and 89%, respectively. Although rapid virological response rates tend to be higher in patients ≥ 75-years-old than in patients < 75-years-old, the difference is not significant (P = 0.266). End-of-treatment response rates for patients ≥ 75-years-old and < 75-years-old are 99% and 100%, respectively. No significant difference is seen between groups (P = 1.000).

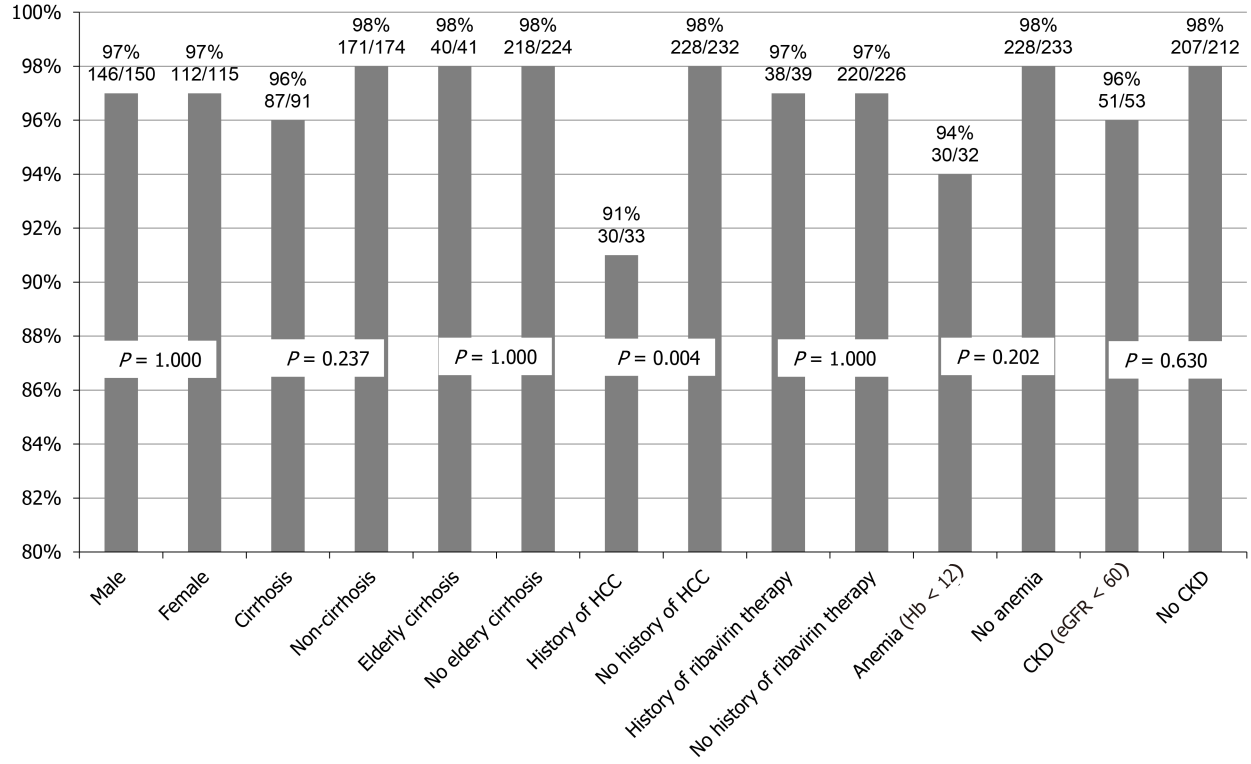

Figure 3 Sustained virological response rates according to background factors. Although no significant differences are seen in comparisons of sustained virological response rates according to sex, cirrhosis, elderly cirrhosis, interferon plus ribavirin therapy, anemia (hemoglobin < 12 g/dL), or chronic kidney disease (estimated glomerular filtration rate < 60 mL/min/1.73 m^2), a significant difference is evident between patients with and without a history of hepatocellular carcinoma treatment (P = 0.004). CKD: Chronic kidney disease; eGFR: Estimated glomerular filtration rate; Hb: Hemoglobin; HCC: Hepatocellular carcinoma.

ARTICLE HIGHLIGHTS

Research background

In real-world settings, elderly patients infected with hepatitis C virus (HCV) represent a substantial and growing population and carry a high risk of advanced liver diseases such as cirrhosis and hepatocellular carcinoma. Therefore, these patients should be treated as soon as possible. Clinical trials of interferon-free direct-acting antiviral agent regimens using sofosbuvir and ribavirin for patients with genotype 2 HCV have been reported since 2013. However, patients ≥ 75-years-old were not included in the sofosbuvir plus ribavirin regimen of those clinical trials. The safety and effectiveness of sofosbuvir plus ribavirin for elderly patients ≥ 75-years-old has thus remained unclear.

Research motivation

In recent HCV treatment guidelines from Western countries, sofosbuvir plus ribavirin therapy is no longer recommended because of the adverse effects of ribavirin and the relatively lower sustained viral response (SVR) rate compared to other direct-acting antiviral agent therapies. However, in consensus statements and recommendations on the treatment of hepatitis C from the Asian-Pacific Association for the Study of the Liver, sofosbuvir plus weight-based ribavirin for 12 wk is recommended as a first-line treatment, and SVR rates from Asian real-world data were similar to those of the phase III trial. Sofosbuvir plus ribavirin therapy also offers advantages in terms of both cost and real-world evidence. The real-world safety and efficacy of sofosbuvir plus ribavirin for elderly patients ≥ 75-years-old can provide useful information regarding treatment strategy for elderly patients with HCV in the Asia-Pacific region.

Research objectives

The aim of the present study is to evaluate the real-world safety and efficacy of sofosbuvir plus ribavirin for elderly patients ≥ 75-years-old compared to non-elderly patients

Research methods

This is a multicenter post-marketing prospective cohort study of sofosbuvir plus ribavirin therapy for patients infected with genotype 2 HCV in a real-world clinical setting. We treated 265 patients with genotype 2 HCV using standard approved doses of sofosbuvir (400 mg/d) plus ribavirin adjusted by body weight, administered orally for 12 wk. In the present study, 31% of patients were ≥ 75-years-old, and 12% had a history of hepatocellular carcinoma (HCC) treatment. Furthermore, 34% of enrolled patients had cirrhosis, and 20% had moderate chronic kidney disease. The primary end point of the present study was SVR at 24 wk after the end of therapy.

Research results

Regarding efficacy, SVR rates for the overall cohort, patients < 65-years-old, ≥ 65-years-old but < 75-years-old, and ≥ 75-years-old were 97% (258/265), 98% (93/95), 97% (84/87), and 98% (81/83), respectively (P = 0.842). Among patients ≥ 75-years-old with a history of interferon plus ribavirin therapy, the SVR rate was 100% (14/14). Furthermore, the SVR rate of cirrhotic patients ≥ 75-years-old was also extremely high (98%, 40/41). From these results, a high SVR rate (> 95%) would be expected even in patients ≥ 75-years-old, irrespective of cirrhosis or history of interferon treatment. Logistic regression analyses identified history of HCC treatment and alpha-fetoprotein as factors significantly associated with SVR. SVR rate was significantly lower for patients with HCC treatment (91%) than for patients without history of HCC treatment (98%, P = 0.004). Regarding safety, although dose reduction or interruption of ribavirin due to anemia was required in 21.7% of patients ≥ 75-years-old and 7.7% of patients < 75-years-old, treatment discontinuation was required for only one patient (0.4%). Therefore, this treatment appears tolerable even in patients ≥ 75-years-old.

Research conclusions

Sofosbuvir and ribavirin represent an acceptable and effective treatment even for patients ≥ 75-years-old in a real-world setting. An extremely high SVR rate can be achieved when adequate management for adverse effects is performed to avoid discontinuation of treatment, irrespective of age.

Research perspectives

Sofosbuvir plus ribavirin might be less effective in patients with a history of HCC treatment or high alpha-fetoprotein level, irrespective of age. These patients should probably be treated using some other ribavirin-free direct-acting antiviral agent therapy.

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CASE REPORT

Non-alcoholic fatty liver disease later diagnosed as myotonic dystrophy

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Abstract

BACKGROUND

Myotonic dystrophy (MD) is sometimes accompanied by metabolic/endocrine disorders, including dyslipidemia, central obesity, and hypogonadism. Due to considerable individual differences in the severity and progression of myopathy, MD patients with minimal-to-mild muscle symptoms might be followed as having other diseases, such as non-alcoholic fatty liver disease (NAFLD).

CASE SUMMARY

A 40-year-old non-obese man without a history of regular ethanol consumption was referred to our hospital due to persistent liver dysfunction and hyperlipidemia. His body mass index was 23.4 kg/m^2. Liver histology demonstrated macrovesicular steatosis, ballooned hepatocytes with eosinophilic inclusion bodies, and perisinusoidal fibrosis, leading to the diagnosis of non-alcoholic steatohepatitis (NASH). Although he had no discernable muscle pain or weakness, persistently high serum creatine kinase (CK) and myoglobin levels as well as the presence of frontal baldness, a hatched face, history of cataract surgery, and grip myotonia indicated the possibility of MD. Southern blotting of the patient's DNA revealed the presence of CTG repeats, confirming the diagnosis.

CONCLUSION

When gastroenterologists encounter NAFLD/NASH patients, serum CK should be verified. If hyperCKemia, frontal baldness, a hatched face, history of cataract surgery, and grip myotonia are noted, the possibility of MD may be considered.

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Core Tip: We describe a patient with non-alcoholic steatohepatitis (NASH) who was later diagnosed as having myotonic dystrophy (MD). Some MD patients with minimal-to-mild muscle symptoms may be misdiagnosed as having non-alcoholic fatty liver disease (NAFLD). Therefore, when gastroenterologists encounter patients with NAFLD/NASH, serum creatine kinase (CK) should be verified. If high serum CK levels persist in the presence of frontal baldness, a hatched face, history of cataract surgery, and grip myotonia, the possibility of MD may be considered.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a common cause of persistent liver dysfunction and is defined as the presence of hepatic steatosis without regular consumption of ethanol or drugs[1,2]. For the diagnosis of NAFLD, other causes of chronic liver injury, such as hepatitis virus infection, bacterial and parasitic infection, autoimmunity, drugs and toxicants, hemochromatosis, Wilson disease, and citrin deficiency should be excluded. Additionally, the possibility of secondary NAFLD, including gastrointestinal/pancreatic surgery, hypothyroidism, hyperadrenalism, and pancreatic exocrine insufficiency, needs to be carefully surveyed[3,4].

Although it is accepted that endocrine/metabolic disorders are closely associated with the development of NAFLD, the relationship between myopathy and NAFLD has not been fully addressed. Among several types of myopathy, myotonic dystrophy (MD) is often accompanied by metabolic/endocrine disorders, such as dyslipidemia, central obesity, insulin resistance, and hypogonadism[5]. Due to considerable variation in the severity and progression of myopathy, MD patients with minimal-to-mild muscle symptoms may be followed as having other diseases. We herein describe a patient with non-alcoholic steatohepatitis (NASH), a severe phenotype of NAFLD, later diagnosed as having MD.

CASE PRESENTATION

Chief complaints

A 40-year-old non-obese man without a history of regular ethanol consumption was referred to our hospital due to persistent hypertransaminasemia and hyperlipidemia.

History of present illness

It was pointed out that he had liver dysfunction and hyperlipidemia at annual health checkups from 35 years of age. He had noticed easy fatigability, but presumed that it was caused by overwork and insufficient sleep. He had no history of regular ethanol, drugs, or supplements consumption, or smoking.

History of past illness

He had no history of past blood transfusion, surgical treatment, or acupuncture.

Physical examination

His body mass index was 23.4 kg/m^2. He was asymptomatic with no signs of hepatomegaly, xanthoma, or Achilles tendon thickening.

Laboratory examinations

Laboratory findings revealed significant increases in serum aspartate aminotransferase (65 U/L, normal 13-30), alanine aminotransferase (103 U/L, normal 7-23), total cholesterol (298 mg/dL, normal 142-220), and triglycerides (TG; 318 mg/dL, normal 30-150). Hyperinsulinemia and greater index of homeostasis model assessment for insulin resistance indicated the presence of insulin resistance. Hepatitis virus markers and autoantibodies were all negative and immunoglobulins, ferritin and ceruloplasmin were within normal ranges. Laboratory data at the time of liver biopsy are shown in Table 1.

Imaging examinations

Abdominal ultrasonography revealed hyperechogenic liver parenchyma with deep attenuation, indicating the presence of steatosis.

Further work-up

A percutaneous liver biopsy was conducted to evaluate his persistent liver dysfunction. Liver histology showed macrovesicular steatosis, ballooned hepatocytes with eosinophilic inclusion bodies (Figure 1A, arrows), and perisinusoidal fibrosis (Figure 1B), which led to the diagnosis of NASH. According to the criteria proposed by Kleiner *et al*[6], the score for steatosis, lobular inflammation, and hepatocyte ballooning were 2 (moderate), 1 (few), and 1 (few), respectively, for a total NAFLD activity score of 4. Bezafibrate (400 mg/d) was commenced for his hypertriglyceridemia.

A blood examination to assess the effects of fibrate treatment 1 mo later revealed elevated levels of creatine kinase (CK; 757 U/L, normal 62-287) and myoglobin. The patient denied any symptoms of muscle pain, weakness, or dark urine. The abnormalities in serum CK and myoglobin persisted despite immediate cessation of the fibrate. Thyroid function was normal.

The patient's face was closely inspected again (Figure 2). He had frontal baldness at the age of 40 and a hatchet face. Manual muscle testing demonstrated that he was very slow opening his hands after grasping forcefully (*i.e.*, grip myotonia). Careful history taking revealed a prior cataract operation. These findings indicated the possibility of MD.

MD is an autosomal dominant disease that affects the dystrophia myotonica protein kinase (DMPK) gene on chromosome 19[5]. The expansion of CTG trinucleotide repeats in the 3′ untranslated region of this gene results in the retention of an expanded RNA repeat that is directly toxic to myocytes. Southern blotting of the patient's DNA after BamHI digestion detected an additional band caused by CTG repeats (Figure 3), confirming the diagnosis of MD.

FINAL DIAGNOSIS

The final diagnosis in this patient case was NASH with underlying MD.

TREATMENT

The patient is being treated with eicosapentaenoic acid (2700 mg/d) for dyslipidemia and is under calorie restriction by medical dieticians.

OUTCOME AND FOLLOW-UP

Since the diagnosis of MD, the patient has been followed for 14 years. His hepatosteatosis and mild elevation of serum aminotransferases have persisted, with no findings indicating liver cirrhosis or cancer. However, he has suffered from progressive muscle weakness and reduced physical activity.

DISCUSSION

MD is a relatively common myopathy with almost 100% penetrance that is estimated to afflict approximately 5-7 per 100000 persons worldwide[5]. The condition is

Table 1 Laboratory findings at the time of liver biopsy

Item	Value	Item	Value	Item	Value
WBC	4450 /μL	BUN	10 mg/dL	IgG	1512 mg/dL
RBC	544 × 10^4 /μL	Cr	0.8 mg/dL	IgA	235 mg/dL
Hb	17.0 g/dL	UA	7.2 mg/dL	IgM	131 mg/dL
Plt	19.9 × 10^4/μL	Na	146 mEq/L	ANA	(-)
		K	4.3 mEq/L	HBsAg	(-)
TP	7.4 g/dL	Cl	109 mEq/L	Anti-HCV	(-)
Alb	4.2 g/dL				
T-Bil	0.5 mg/dL	**T-Chol**	**257 mg/dL**	TSH	2.1 μIU/mL
AST	**74 U/L**	**TG**	**274 mg/dL**	FT3	3.2 pg/mL
ALT	**102 U/L**	HDL-C	76 mg/dL	FT4	1.0 ng/dL
LDH	260 U/L				
ALP	202 U/L	FBS	87 mg/dL	HA	27 ng/mL
GGT	**121 U/L**	HbA1c	5.0 %	4C7S	4.2 ng/mL
ChE	390 IU/L	**Insulin**	**30 μU/mL**		
		HOMA-IR	**6.4**	Fe	91 μg/dL
				Ferritin	77 ng/mL
				Cu	89 μg/dL
				Ceruloplasmin	26 mg/dL

Bold underlined parameters indicate abnormally elevated values. WBC: White blood cell; RBC: Red blood cell; Hb: Hemoglobin; Plt: Platelet count; TP: Total protein; Alb: Albumin; T-Bil: Total bilirubin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; LDH: Lactate dehydrogenase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyl transferase; ChE: Choline esterase; BUN: Blood urea nitrogen; Cr: Creatinine; UA: Uric acid; T-Chol: Total cholesterol; TG: Triglycerides; HDL-C: High-density-lipoprotein cholesterol; Fe: Iron; FBS: Fasting blood sugar; HbA1c: Hemoglobin A1c; HOMA-IR: Homeostasis model assessment for insulin resistance; Ig: Immunoglobulin; ANA: Anti-nuclear antibody; HBsAg: Hepatitis B virus surface antigen; HCV: Hepatitis C virus; TSH: Thyroid stimulating hormone; FT3: Free triiodothyronine; FT4: Free thyroxine; HA: Hyaluronic acid; 4C7s: Type 4 collagen 7S.

characterized by two different mutations: An expansion of CTG repeats in the 3′ untranslated region of the DMPK gene on chromosome 19 and an expansion of CCTG repeats in intron 1 on zinc finger protein 9 on chromosome 3, which are classified as MD type 1 and type 2, respectively. Southern blotting of the present patient led to the diagnosis of MD type 1.

MD is a multisystemic disease exhibiting diverse clinical manifestations, including muscle weakness, myotonia, early cataracts/baldness, arrhythmia, neuropsychiatric symptoms, and gonadal atrophy. The common features of MD type 1 are myotonia and insulin resistance, which are caused by aberrant splicing of chloride channel 1 and insulin receptor RNA in skeletal muscle due to the toxic effect of retained CUG-expanded repeats. Whole-body glucose disposal is reduced by 15%-25% after insulin infusion in MD type 1 patients, and insulin sensitivity, as well as insulin receptor RNA and protein, in the skeletal muscle is significantly decreased. Since peripheral insulin resistance is commonly observed in MD patients, metabolic disturbances, such as dyslipidemia, diabetes, and NAFLD/NASH may co-exist.

Indeed, it was reported that liver test abnormalities were frequent in MD patients[7-10]. Shieh *et al*[11] prospectively evaluated the abnormalities in liver enzymes and metabolic parameters of 31 MD type 1 patients. The prevalence of diabetes, impaired fasting glucose, and metabolic syndrome (MetS) was approximately 12%, 21%, and 41%, respectively. Furthermore, 44% of MD patients had liver test abnormalities and 42% had NAFLD, as defined by abnormal liver chemistry tests and ultrasonography findings.

We reviewed the clinical data of 7 MD type 1 patients with NAFLD who underwent liver biopsy, including this case, and summarized the pathological features in Table 2[11-14]. Ballooned hepatocytes were detected in 5 patients, who were all histologically diagnosed as having NASH. Our case did not receive a repeated liver biopsy. However, he did not show gradual decreases in platelet count or serum albumin, nor

Table 2 Summary of previously reported liver pathology in 7 myotonic dystrophy type 1 patients with non-alcoholic fatty liver disease

Item	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Steatosis (0-3)	3	1	1	2	1	3	2
Lobular inflammation (0-3)	0	0	0	1	1	3	1
Ballooning (0-2)	1	1	0	ND	1	2	1
NAFLD activity score (0-8)	4	2	1	ND	3	8	4
Diagnosis	NASH	NASH	SS	ND	NASH	NASH	NASH
Fibrosis (0-4)	2	1a	1a	1	1a	4	1a
Ref.	Shieh *et al*[11]	Shieh *et al*[11]	Shieh *et al*[11]	Bhardwaj *et al*[12]	Yamada *et al*[13]	Ariake *et al*[14]	Our case

Pathological findings were scored according to the criteria proposed by Kleiner *et al*[6]. ND: Not determined; NASH: Non-alcoholic steatohepatitis; SS: Simple steatosis (non-alcoholic fatty liver).

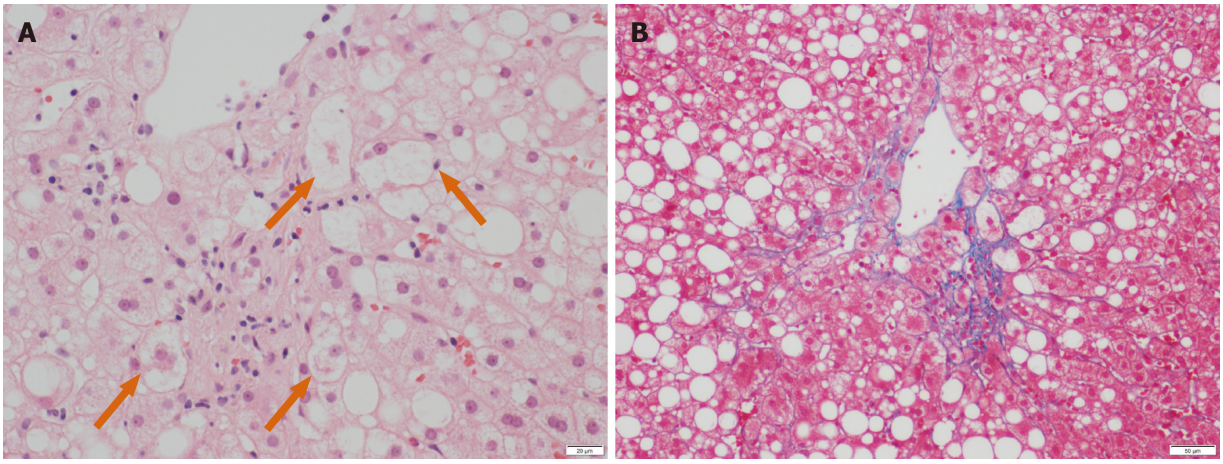

Figure 1 Pathological findings of liver biopsy. A: Liver specimen stained by hematoxylin and eosin exhibited moderate macrovesicular steatosis mainly around the central vein with occasional focal lobular inflammation. Some ballooned hepatocytes were detected (orange arrows) According to the criteria proposed by Brunt *et al*, the score for steatosis, lobular inflammation, and hepatocyte ballooning were 2 (moderate), 1 (few), and 1 (few), respectively. The calculated NAFLD activity score was 4. Scale bar represents 20 μm; B: Liver specimen stained by the Azan-Mallory method showed pericentral/perisinusoidal fibrosis (score 1a). Scale bar represents 50 μm.

did he exhibit remarkable values for indicators of liver fibrosis progression or imaging findings suggestive of liver cirrhosis, such as splenomegaly, enlargement of the left lobe, or liver surface irregularity, during his 14-year follow-up. One report described a case of MD type 1 progressing to NASH-derived liver cirrhosis (Case #6 in Table 2)[14]. Additionally, a case of MD type 2 manifested as myopathy, mild myotonia, cataract, diabetes, erectile dysfunction, gastrointestinal dysmotility, dysarthria, mild myocardial thickening and non-alcoholic and non-hepatitic liver cirrhosis was documented[15]. Therefore, regular monitoring of laboratory indicators of liver synthesis ability (*e.g.*, serum albumin and prothrombin time) and liver fibrosis (*e.g.*, platelet count and serum type 4 collagen 7S) as well as liver imaging is advised for MD patients with NAFLD. In patients with cryptogenic non-alcoholic cirrhosis, the possibility of MD may be considered.

Vujnic *et al*[16] examined the prevalence of MetS components in 66 MD type 1 patients and found dyslipidemia to be the most frequent [hypertriglyceridemia (67%) and low HDL cholesterol (35%)]. On the other hand, the prevalence of hypertension, central obesity, and hyperglycemia was relatively low (18%, 13%, and 9%, respectively). This observation indicated that hypertriglyceridemia often co-existed in MD type 1 patients and that some MD patients with minimal-to-mild muscle symptoms might be mistaken as having hypertriglyceridemia and/or NAFLD. Insulin resistance enhances hepatic TG synthesis and the secretion of very-low-density lipoprotein particles, but reduces the activity of lipoprotein lipase, consequently leading to raised circulating TG levels. Fibrates are commonly available for treating hypertriglyceridemia, but myotoxicity and rhabdomyolysis are possible adverse

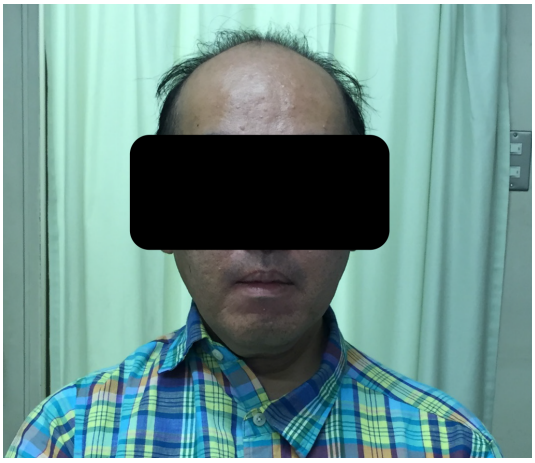

Figure 2 Photograph of the patient. Frontal baldness and a hatchet face were noted.

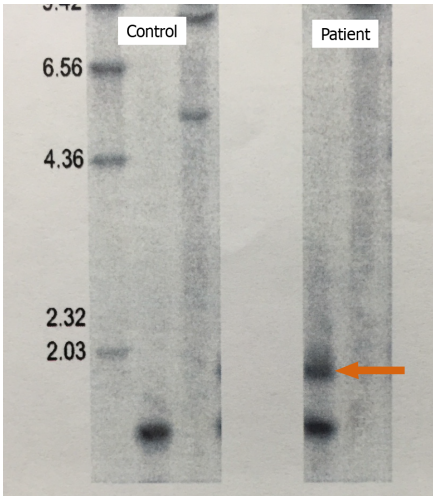

Figure 3 Southern blotting of patient DNA. DNA samples were digested with BamHI. Compared with the control sample, an additional band caused by CTG repeats was detected (arrow), confirming the diagnosis of myotonic dystrophy.

effects. Before commencing fibrate administration for NAFLD/NASH patients with hypertriglyceridemia, serum CK levels should be checked to rule out the possibility of latent MD.

Recent studies documented the contribution of small intestinal bacterial overgrowth and disrupted bile acid metabolism to the pathogenesis of NAFLD/NASH[1,2,17,18]. Tarnopolsky *et al*[19] documented that 65% of MD type 1 patients with gastrointestinal symptoms exhibited small intestinal bacterial overgrowth using glucose breath hydrogen testing. Additionally, it was reported that specific bile acids, such as dihydroxymono-oxocholanic acid and dihydroxycholanic acid, were detected in the serum of MD type 1 patients and biliary ursodeoxycholic acid was reduced[20], indicating bile acid abnormality accompanied by MD. Although the pathogenesis of NAFLD/NASH is multifactorial, these factors might be associated with NAFLD/NASH in MD.

CONCLUSION

MD is a relatively common congenital myopathy accompanied by peripheral insulin resistance. However, some MD patients with minimal-to-mild muscle symptoms might be followed as having other diseases associated with insulin resistance, such as hypertriglyceridemia, postprandial hyperglycemia, and NAFLD/NASH. In patients with abnormal liver function tests, muscular diseases might be hidden at all ages[21-24]. When gastroenterologists encounter NAFLD/NASH patients, serum CK should be

verified. If hyperCKemia is detected, the possibility of MD may be considered and careful observation of the patient's face, and history taking for cataracts may help uncover subclinical MD.

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