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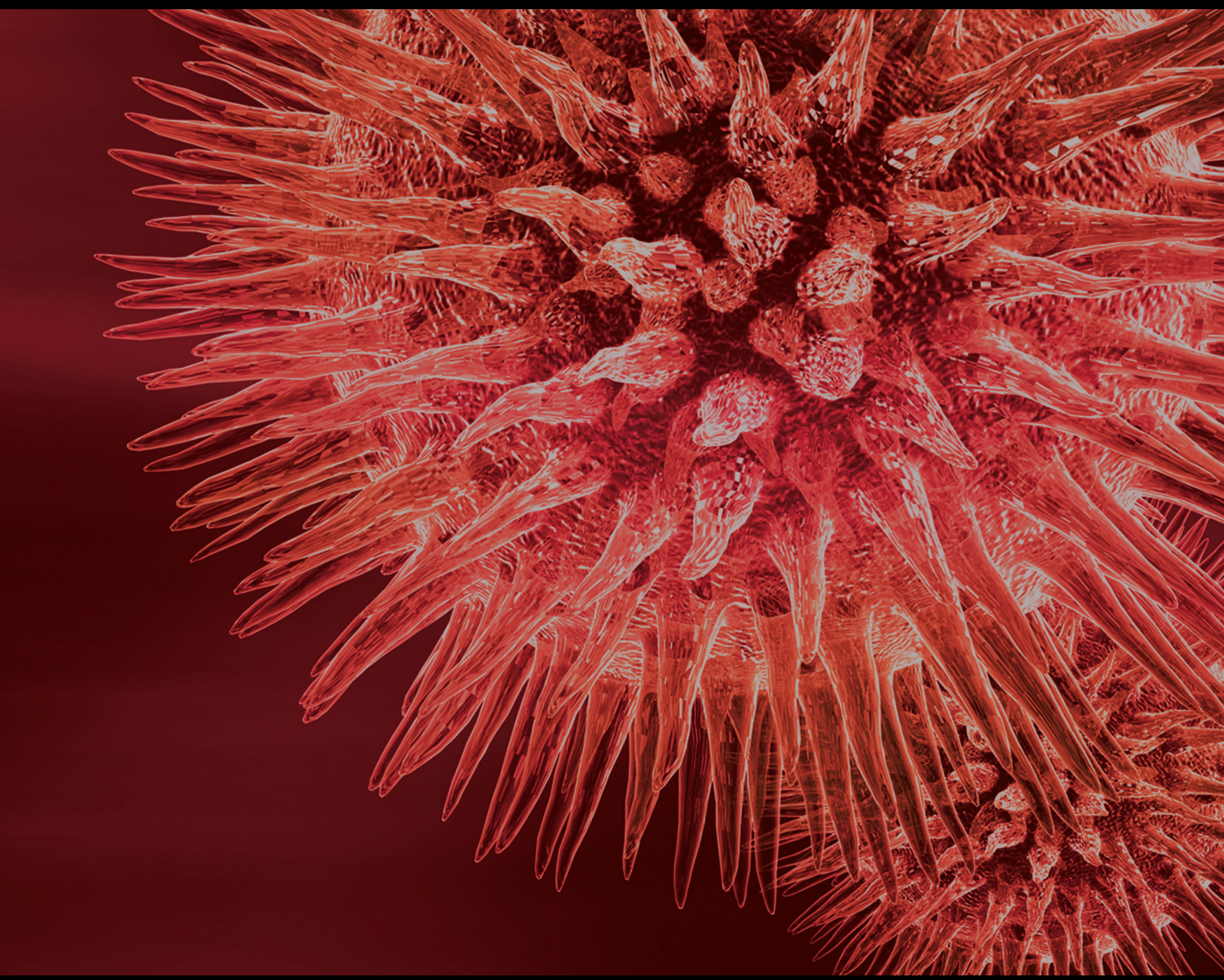

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Editorial

Alcoholic and Nonalcoholic Liver Disease: Diagnostic Assessment and Therapeutic Perspectives

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Alcoholic liver disease (ALD) and nonalcoholic fatty liver disease (NAFLD) represent main causes of chronic liver disorder in the Western countries. Both ALD and NAFLD include a wide spectrum of conditions ranging from "simple" steatosis to steatohepatitis, cirrhosis, and hepatocellular carcinoma. ALD and NAFLD, besides sharing many histological patterns, have in common a negative impact on both cardiovascular and cancer risk. Consequently, ALD and NAFLD should be considered not only liver diseases but also systemic conditions that harmfully influence both morbidity and mortality.

ALD is caused by acute and/or chronic alcohol intake while NAFLD is outlined as the presence of hepatic steatosis without other causes of secondary fat accumulation such as noteworthy alcohol consumption and hereditary disorders. NAFLD is a complex illness with genetic and environmental risk factors and is typically coupled with metabolic conditions such as obesity and diabetes.

In this special issue, we present original research as well as review articles on the following topics: relationship between gut-liver axis and NAFLD, diagnostic ability of transient elastography in NAFLD (and simultaneous hepatitis B virus), noninvasive markers of fibrosis in ALD, NAFLD, and pancreas damage, role of traditional Chinese Medicine in the treatment of NAFLD, and use of antifibrotic drug for the cure of liver disease.

Recent advances demonstrated that microbiota shows a key role in many kinds of diseases. In particular, microbiota dysbiosis seems to be involved in the pathogenesis of nonalcoholic steatohepatitis (NASH) that definitely represents the progressive form of NAFLD. Modifications of microbiota-derived mediators, alterations of gut endothelial barrier, and translocation of inflammation mediators could negatively influence the outcome of liver damage due to NASH. G. Aragonès et al., with a whole review article, discuss the role of gut microbiota-derived mediators as potential diagnostic markers of NAFLD and NASH. The article underlines the relevance of developing noninvasive tools for the screening and diagnosis of NAFLD since the current gold standard (liver biopsy) shows some well-known limits related to safety, compliance, and feasibility. In particular, liver biopsy can lead to complications such as bleeding, pain, bile peritonitis, kidney puncture, or death. Furthermore, sampling errors are common due to effort with obtaining liver specimen representative of the entire organ. In the present special issue, other data about noninvasive diagnosis of NAFLD are reported. Specifically, the concordance between transient elastography (TE) and ultrasonography (US) in assessing liver fibrosis in patients with both chronic hepatitis B (CHB) and NAFLD is evaluated. Using the liver biopsy as standard comparison, G. Zhang et al. demonstrated that TE and US

scores knowingly correlated with histologically proven liver fibrosis.

Obviously, also in patients with ALD to develop noninvasive methods of diagnosis would be important. L. Chrostek et al. analyzed the diagnostic values of the following noninvasive indirect markers of liver fibrosis: APRI, GAPRI, Forn's, FIB-4, Age-Platelet, and Hepascore in patients with ALD. Authors suggested that Hepascore showed lower diagnostic value in alcoholics than markers involving only liver enzymes, platelet count, and cholesterol. Remarkably, Forn's index emerged as the best marker among those analyzed.

The relationship between NAFLD and systemic damage is widely known particularly regarding cardiovascular risk. However, few data are available about the pathological relationship between NAFLD and pancreatitis. Abdominal obesity represents a chief element in the NAFLD pathogenesis and, at the same time, it can be a risk factor for acute pancreatitis. Interestingly, D. Wu et al. reported that NAFLD could exacerbate pancreatitis through releasing a large number of inflammatory factors. Notably, Kupffer cells (the resident macrophages of the liver) primarily mediate the NAFLD-related chronic inflammatory process.

The complexity of NAFLD and NASH pathogenesis explains the difficulties of scientific community in the development of widely approved effective treatment. The presence of parallel "hit" explains the systemic impact of NAFLD and the opportunity to develop drugs that can act on multiple levels. Y. Feng et al., conceptually in agreement with this concept, developed an animal study with the use of Jianpi Huoxue (JPHX) as treatment of NAFLD. The proposed Chinese herbal formula holds active compounds that regulate lipid metabolism and shows anti-inflammatory properties. JPHX exhibited hepatoprotective effects in animals with severe livery injuries (steatosis, inflammation, and fibrosis) decreasing lipid accumulation, inflammation, apoptosis, and fibrosis.

The main limitation of the available studies describing the treatment options for the treatment of NAFLD is the lack of a relevant effect on the fibrosis. In this direction, M. M. Arafah et al. planned a study using a rat model of liver fibrosis (obtained with intraperitoneal injections of carbon tetrachloride). A total of 45 rats were divided into 3 groups: control group, group II that received carbon tetrachloride for 8 weeks, and group III that was treated with carbon tetrachloride and nAG protein for the same time period. At the end of the experiment, serum levels of hyaluronic acid, PDGF-AB, TIMP-1, laminin, Procollagen III- N terminal peptide, and collagen IV alpha 1 chain were tested and liver biopsies were performed. NAG treatment decreased serum levels of the analyzed markers of fibrosis reducing also the histological fibrosis.

We believe that these articles may contribute to improve our knowledge in ALD and NAFLD that definitely represent the future of hepatology.

Conflicts of Interest

We declare that none of the Guest Editors have conflicts of interest.

Acknowledgments

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Research Article

Hepatoprotective Effect of Jianpi Huoxue Formula on Nonalcoholic Fatty Liver Disease Induced by Methionine-Choline-Deficient Diet in Rat

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Guest Editor: Fabio Caputo

In parallel with the prevalence metabolic syndrome, nonalcoholic fatty liver disease (NAFLD) has become the most common chronic liver disease in most countries. It features a constellation of simple steatosis, nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and even hepatocellular carcinoma. There are no approved drugs for effective management of NAFLD and NASH. Jianpi Huoxue formula (JPHX) mainly consists of Atractylodes macrocephal *(Baizhu)*, Salvia miltiorrhiza (*Danshen*), Rasux Paeonia Alba (*Baishao*), Rhizoma Alismatis (*Zexie*), and Fructus Schisandrae Chinensis (*Wuweizi*), which may have beneficial effects on NAFLD. The aim of the study was to identify the effect of JPHX on NAFLD. A NAFLD model was induced by methionine-choline-deficient food (MCD) in Wistar rats and orally administered with simultaneous JPHX, once a day for 8 weeks. Hepatocellular injury, lipid profile, inflammation, fibrosis, and apoptosis were evaluated. The results showed that JPHX significantly decreased the abnormal serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels compared with the MCD model ($P<0.05$). Furthermore, JPHX protected MCD diet-fed rats from accumulation of hepatic triglycerides (TG) and total cholesterol (TC). Histological examination demonstrated that JPHX noticeably normalized the NAFLD activity score (NAS). Moreover, JPHX ameliorated liver inflammation by decreasing TNF-α levels and reduced collagen and matrix metalloproteinases in MCD diet-fed rats. In addition, JPHX prevented rats from MCD-induced cellular apoptosis, as suggested by TUNEL staining, and suppressed the activation of caspase 3 and 7 proteins. JPHX also inhibited the phosphorylation of JNK. In conclusion, JPHX exhibited a hepatoprotective effect against NAFLD in an MCD experimental model.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in most countries and regions including the United States, Asia, the Middle East, and Europe [1]. The prevalence of NAFLD worldwide has been estimated at 25.24% and it shows an increased trend with the high prevalence of obesity, diabetes, hyperlipidemia, and metabolic syndrome [2–4]. NAFLD can be divided into two major subtypes: simple steatosis and nonalcoholic steatohepatitis (NASH). Most patients do not progress to cirrhosis, which is mainly characterized by steatosis especially macrovascular steatosis, while almost 20% patients will have a progressive liver disease called NASH [5]. Patients with NASH usually develop liver steatosis, more severe lobular and portal inflammation, and ballooning and have a higher

chance of developing fibrosis, cirrhosis, and hepatocellular carcinoma [6]. However, currently, there are no approved drugs for effective management of NAFLD and NASH, which have become a major global public health problem and place an enormous burden on health-care systems and societies. Some preventive strategies, such as lifestyle modification and an energy-restricted diet, have achieved limited success including improved steatosis and decreased aberrant aminotransferases [7]. Therefore, it is critical to develop new therapeutic targets for NAFLD treatment. Nowadays, many researches are giving new hope to patients with NAFLD and those at high risk of developing NAFLD.

In Chinese medicine philosophy, the pathogenesis of NAFLD includes spleen vacuity, liver stagnation, and phlegm-damp obstruction. Thus far, few traditional Chinese medications have been studied for treatment of patients with NAFLD [8–10]. Jianpi Huoxue (JPHX), as a Chinese herbal formula, has been used for liver disease in China for many years [11]. JPHX mainly consists of Atractylodes macrocephal *(Baishu)*, Salvia miltiorrhiza (*Danshen*), Rasux Paeonia Alba (*Baishao*), Rhizoma Alismatis (*Zexie*), and Fructus Schisandrae Chinensis (*Wuweizi*) and strengthens the spleen, emolliates the liver, dispels dampness, dissolves stasis, and promotes blood circulation [12, 13]. JPHX contains active compounds that may regulate lipid metabolism and exhibit anti-inflammatory properties. The aim of the study was to determine the role of JPHX in the development of MCD diet-induced NAFLD in rats.

We hypothesized that JPHX may have a hepatoprotective effect during progression of NAFLD. Clinically, NAFLD is characterized by elevated serum aminotransferases, accumulation of fat with more than 5% hepatocytes and no history of alcohol abuse [1, 5, 14]. Animal models of NAFLD can be induced in rodents, as with many other human diseases, by common dietary protocols, and used for developing new therapeutic approaches and investigating the disease mechanisms [15]. MCD diet has been used for over 40 years to induce NAFLD, as a classic model that showed hepatic histological features including steatosis, inflammation, and fibrosis in a short time [16]. In the present study, MCD diet-induced NAFLD in rat was used to explore JPHX as a therapeutic agent for NAFLD.

2. Materials and Methods

2.1. Animal Models. Male Wistar rats, weighing approximately 220g, were fed a methionine- and choline-deficient (MCD diet) (Trophic Animal Feed High-Tech Co., Ltd., Nantong, China) that contained amino acids, corn oil, fibers, vitamins, minerals, sodium bicarbonate, and tertiary butylhydroquinone (TBHQ), or the same diet supplemented with methionine and choline (MCS diet) for up to 8 weeks. The substrain of Wistar rats is Wistar IGS rats, which are outbred. All rats were randomly divided into five groups (6-9 rats per experimental group) including an MCS group, MCD group, and JPHX 0.60 g/kg, JPHX 1.21 g/kg, and JPHX 2.42 g/kg groups. Rats were housed in groups of 2~4 in IVC cages with comfortable bedding, and the animal room with a 12-hour dark/light cycle temperature ranged between 21 and 25°C; humidity was maintained between 55% to 65%. All rats had free access to food and water and were weighed at weekly intervals.

2.2. JPHX Formula. JPHX formula was provided by Shanghai Sunrise Traditional Chinese Medicine Co., Ltd., Shanghai, China. The NAFLD model was induced by MCD diet in rats, orally administered simultaneously with JPHX once a day until rats were sacrificed. JPHX formula has a long history of use in clinical practice in China, therefore the different JPHX doses were chosen according to previous clinical experiences. The rat dose was converted from human dose which was multiplied by 7 based on the body surface area. A dose-response study is a valid research design to evaluate the efficacy of TCM [17–19]. Three doses of JPHX formula were administrated to the rats. Rats in JPHX treatment group were administered with JPHX at three different doses of 0.60, 1.21, or 2.42 g/kg (weight of rat). Powdered formula (10.00 g) was dissolved in 50 mL distilled water and then the rats were orally administered with JPHX solution according to their body weight. The MCS group and MCD vehicle group were orally treated with distilled water. The animal study conformed to the ethical standards (UMARE-001-2017) of the Institute of Chinese Medical Science, University of Macau.

2.3. Determination of Serum and Liver Biochemical Parameters. Blood samples were collected every 2 weeks from the tale vein and 1-2 mL of blood can be obtained and centrifuged at 1000 r.c.f for 15 min at 4°C. Serum was separated to detect biomarkers, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglyceride (TG), and total cholesterol (TC) (Nanjing Jiancheng Biotech Co., Nanjing, China). Tumor necrosis factor-α (TNF-α) levels were measured by an ELISA kit (Duo-Set; R&D Systems Inc., MN, Minnesota, USA), and absorbance was read at 450 nm by a microplate reader according to the manufacturer's instructions. At the end of 8 weeks, blood samples from abdominal aorta were collected and approximately 5 mL of blood can be collected to be analyzed; hepatic TG and TC levels were determined by the same kit used for serum, as previously described [20]. Briefly, lipid was extracted using chloroform/methanol solution. The organic phase was collected and then vaporized, and the dry powder obtained was dissolved in isopropanol for the detection of hepatic TG and TC. Hepatic glutathione peroxidase (GPx) activity was detected to analyze oxidative stress by commercial kit from Nanjing Jiancheng Biotech Co. (Nanjing, China) [21].

2.4. Histopathological Analysis. At the end of 8 weeks, liver samples were collected. Livers were washed with ice PBS, dried with filter paper, and weighed. A part of the fresh liver tissue was fixed in 4% paraformaldehyde, embedded in paraffin, cut into 6-μm-thick slices, and stained with hematoxylin and eosin (H&E) and Sirius red stain for histopathology analysis. Histology was assessed in a blind manner by a pathologist. Also, a part of the fresh liver tissue was embedded in optimal cutting temperature compound (OCT) for staining with Oil red O (Sigma, St. Louis, MO, USA). Terminal

Table 1: Primers information.

Target genes	Primers	Sequence
TNFα	Forward Primer	GCCCAGACCCTCACACTC
	Reverse Primer	CCACTCCAGCTGCTCCTCT
Collagen I	Forward Primer	AGGCATAAAGGGTCATCGTG
	Reverse Primer	ACCGTTGAGTCCATCTTTGC
MMP-9	Forward Primer	GACAATCCTTGCAATGTGGATG
	Reverse Primer	CCGACCGTCCTTGAAGAAATG
β-actin	Forward Primer	AGCCATGTACGTAGCCATCC
	Reverse Primer	CTCTCAGCTGTGGTGGTGAA

deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end (TUNEL) assay was performed for apoptosis using a commercial kit according to the manufacturer's instructions (Beyotime Institute of Biotechnology, Nantong, China). Quantification of apoptosis was performed by counting the number of TUNEL-positive cells in three random fields (20 × magnification). Expression of α-SMA was determined using immunohistochemistry according to the standard method; paraffin-embedded sections were immunostained with an α-SMA antibody [22].

2.5. Quantitative Real-Time Polymerase Chain Reaction Analysis (qRT-PCR). Fresh liver samples were collected and snap-frozen in liquid nitrogen and then stored at -80°C for further analysis. Total RNA was extracted from hepatic tissue with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed into cDNA using SuperScript reverse system (Invitrogen) for amplification according to the manufacturer's protocol. Quantitative real-time polymerase chain reaction (RT-PCR) was performed by SYBR *Premix Ex Taq*™ II (Tli RNaseH Plus) (Takara, Shiga, Japan). Primers information was listed in Table 1.

2.6. Western Blotting. Liver tissue from different groups was homogenized with stainless steel beads in RIPA lysis buffer by TissueLyser II (QIAGEN, Hilden, Germany) and then centrifuged at 12,500 r.c.f for 30 min at 4°C. Supernatant was obtained and protein concentrations were determined by a Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of protein and molecular weight markers were loaded on sodium dodecyl sulfonate polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were transferred to a polyvinylidene difluoride (PVDF) membrane that was blocked with 5% nonfat milk in 1× Tris-buffered saline with 0.5% Tween-20, for 1 hour at room temperature. Membranes were incubated with primary antibodies overnight at 4°C. Horseradish peroxidase (HRP)-linked secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) were incubated at room temperature for 1 hour after washing at least three times with Tris-buffered saline Tween 20 (TBST), for 10 min each time. Signals were detected by ECL detection kit (GE Healthcare, Milwaukee, WI, USA) and quantified with Image Lab software (Bio-Rad, Hercules, CA, USA).

2.7. Data Analysis and Statistics. All data were collected from three independent experiments. Student t-test was used to compare differences between MCS group and MCD group. Differences among experimental groups were evaluated by one-way ANOVA, followed by Dunnett's multiple comparisons test. P values less than 0.05 were regarded as statistically significant. Data are described as mean ± standard deviations (SD). Alpha level was two tailed. The GraphPad prism was used for statistical analysis (version 6.0, GraphPad Inc., San Diego, CA, United States).

3. Results

3.1. JPHX Does Not Influence Body Weight and the Liver-to-Body Weight Ratio (LBW) in NAFLD. In our study, JPHX was performed on rats using an MCD diet-induced NAFLD model. The body weights of MCS diet-fed rats showed an increasing trend, while the body weights of MCD diet-fed rats gradually decreased, by approximately 20%-30%, during the 8 weeks of testing period because of a noticeably lower caloric intake (Figure 1(a)). The liver weights of the MCD vehicle group and JPHX treatment group were similar (Figure 1(b), $p = 0.1608$). In addition, the liver/body weight ratio (LBW) in MCD diet-fed rats was higher than that in MCS diet-fed rats (Figure 1(c), $p < 0.0001$). We observed that JPHX formula therapy did not significantly influence the body weights and LBW of rats.

3.2. JPHX Inhibits Hepatic Steatosis. Serum and liver TG and TC levels were detected to assess the effect of JPHX on the NAFLD model induced by MCD diet. Hepatic TG and TC levels increased noticeably in the MCD vehicle group compared to those in the MCS group. JPHX 0.60 g/kg and JPHX 1.21g/kg showed significantly lower hepatic TG (Figure 2(a), $p = 0.0007$) and TC levels (Figure 2(b), $p = 0.0003$). In addition, serum levels of TG and TC in MCD-induced rats decreased, which indicated impaired secretion of TG (Figure 2(c), for week 2, $p= 0.0004$, for week 4, $p= 0.0019$, for week 6, $p< 0.0001$, and for week 8, $p = 0.6152$) and TC from hepatocytes (Figure 2(d), for week 2, $p = 0.0002$, for week 4, $p< 0.0001$, for week 6, $p = 0.0960$, and for week 8, $p= 0.0011$). These findings correlated well with the H&E and Oil red O staining results (Figure 2(e)), where the MCD diets featured macrovesicular steatosis with large droplets of

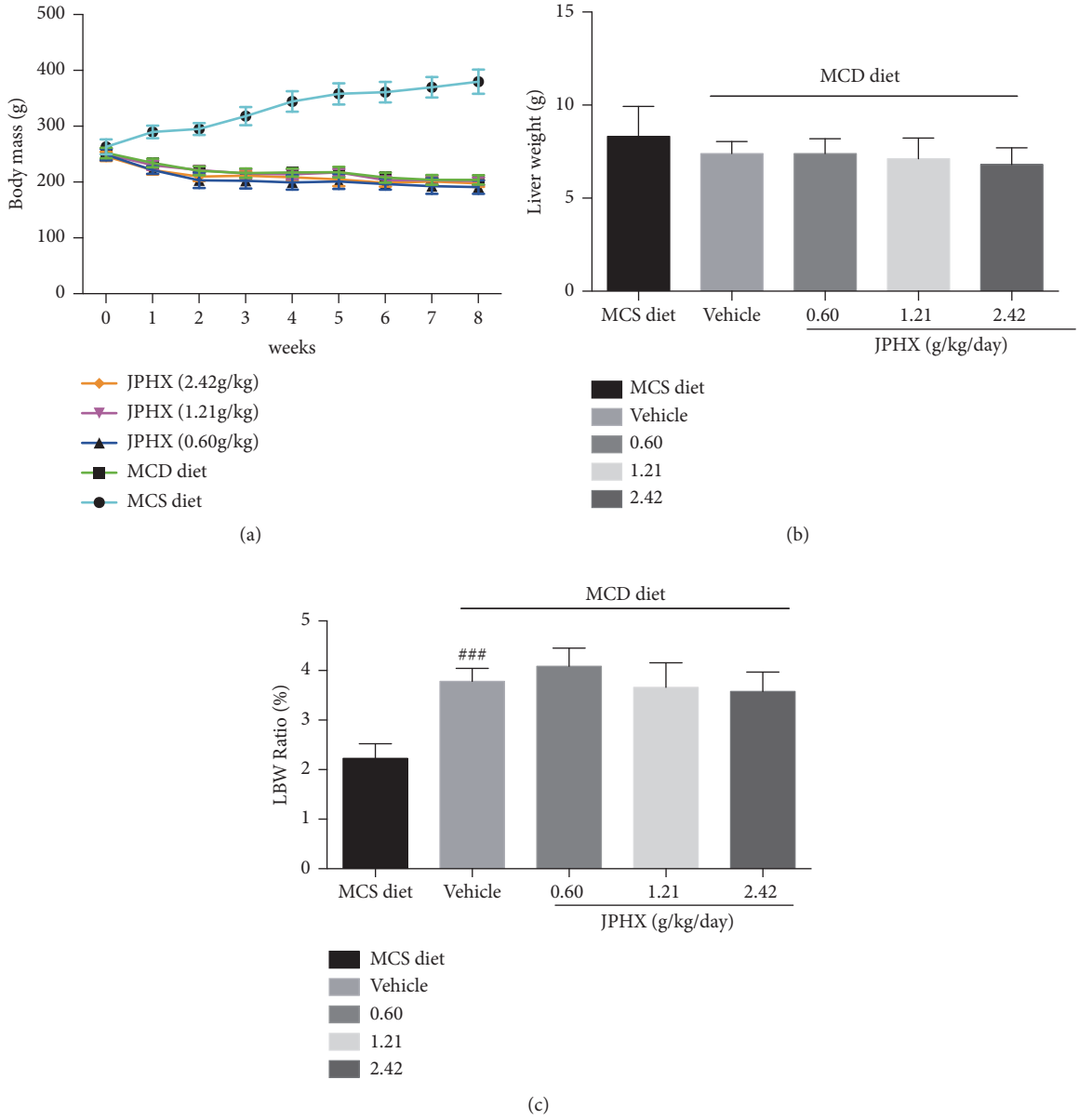

Figure 1: Effect of MCD diets on body weights, liver weights, and liver/body weight ratio in rats. (a) Body weight of rats. (b) Liver weight of rats after 8 weeks of the feeding. (c) Liver/body weight ratio of rats at 8 weeks. Values are shown as the mean ± SD. MCS n=6, MCD n=9, and JPHX treatment groups n=7. #p<0.05, ##p<0.01, and ###p<0.001, MCD diet vehicle group versus MCS diet group. $*p$<0.05, $**p$<0.01, and $***p$<0.001, MCD diet vehicle group versus JPHX treatment groups.

fat that push the nucleus aside. Animals treated with JPHX showed lower hepatic steatosis levels than those of the MCD diet vehicle rats. Similarly, lipid droplets were detected by Oil red O staining. JPHX in the different treatment groups, but especially at 1.21g/kg, noticeably attenuated the accumulation of lipid droplets.

3.3. JPHX Protects Rats from MCD Diet-Induced Liver Injury and Inflammation. Rats fed with MCD diets for 8 weeks developed NAFLD, and liver injury-related markers were detected. Serum ALT levels were dramatically increased in the MCD vehicle group due to higher ALT release from damaged liver cells. Abnormal serum ALT activities were significantly decreased by JPHX in rats (Figure 3(a), for weeks 2, 4, 6, and 8, p< 0.0001). Similarly, JPHX treatment reduced circulating AST level in rats compared to the MCD group (Figure 3(b), for weeks 2, 4, and 8, p< 0.0001; for week 6, p=0.0002). TNF-α was involved in the pathogenesis of NAFLD. MCD diets markedly increased hepati TNF-α concentrations. Treatment with JPHX (0.60g/kg and 1.21g/kg) significantly decreased this MCD diet-induced elevation in TNF-α level (Figure 3(c), p=0.0059, Figure 3(d), p = 0.0076). These findings were confirmed by H&E, which showed decreased lobular inflammation. Oxidative stress plays an

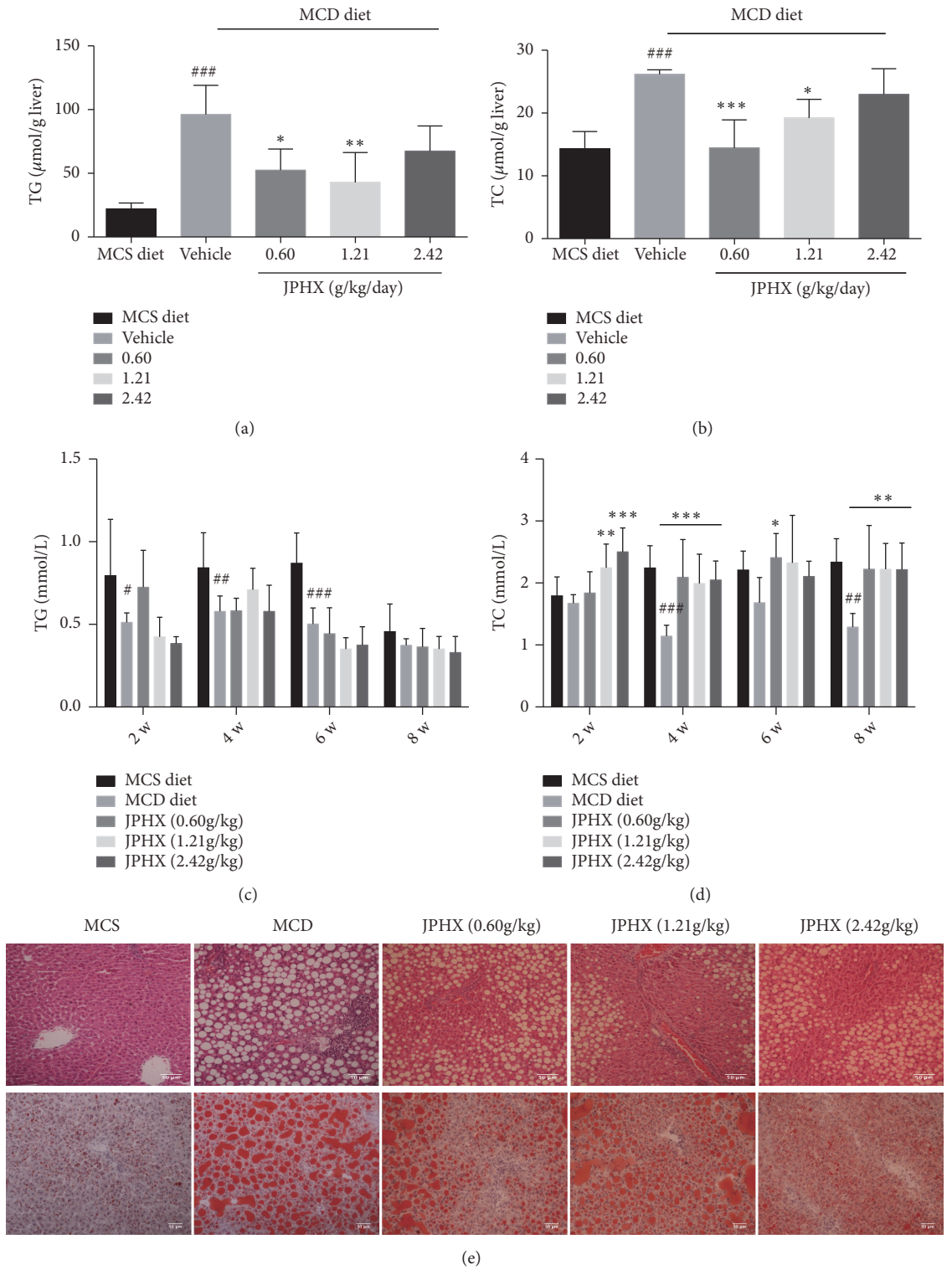

Figure 2: Effect of JPHX on accumulation of hepatic TG and TC induced by MCD diet and liver histology. (a) Liver TG levels. (b) Liver TC levels. (c) Serum TG levels. (d) Serum TC levels. (e) H&E and Oil red O staining of representative liver sections from each treatment group. Original magnification × 200. Values are shown as the mean ± SD. MCS n=6, MCD n=9, and JPHX treatment groups n=7. #$p<0.05$, ##$p<0.01$, and ###$p<0.001$, MCD diet vehicle group versus MCS diet group. *$p<0.05$, **$p<0.01$, and ***$p<0.001$, MCD diet vehicle group versus JPHX treatment groups.

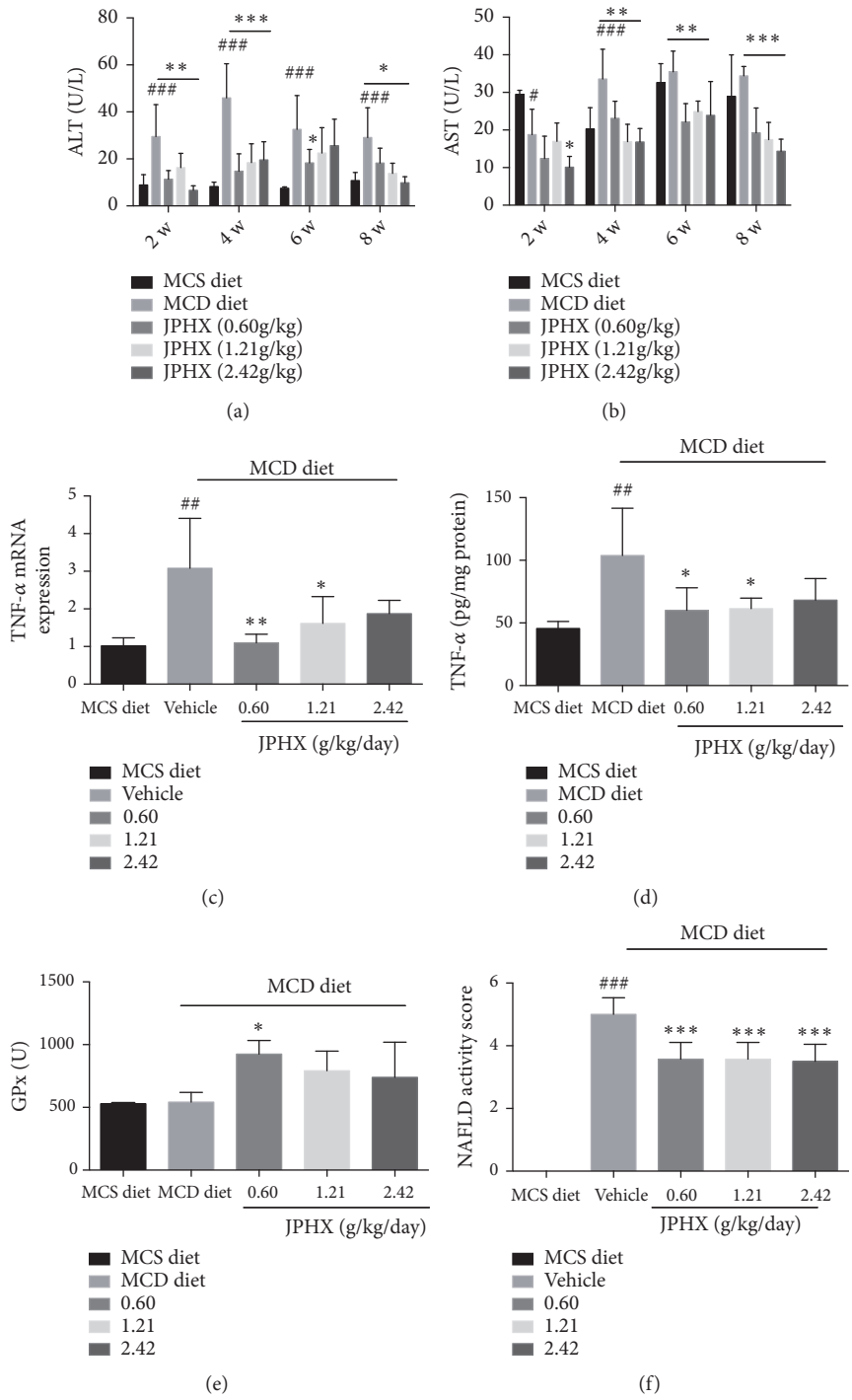

Figure 3: Liver injury-related parameters, inflammation-related parameters, and NAS score. (a) Serum ALT levels in rats fed with MCD or MCS diet at weeks 2, 4, 6, and 8. (b) Serum AST levels. (c) TNF-α mRNA expression levels were detected by qRT-PCR; data were normalized to β-actin. (d) Hepatic TNF-α concentrations were measured by an ELISA kit. (e) Effects of JPHX formula on liver GPx in rats. (f) NAFLD activity scores were analyzed by a pathologist in a blind manner. Values are shown as the mean ± SD. MCS n=6, MCD n=9, and JPHX treatment groups n=7. #p<0.05, ##p<0.01, and ###p<0.001, MCD diet vehicle group versus MCS diet group. $*p$<0.05, $**p$<0.01, and $***p$<0.001, MCD diet vehicle group versus JPHX treatment groups.

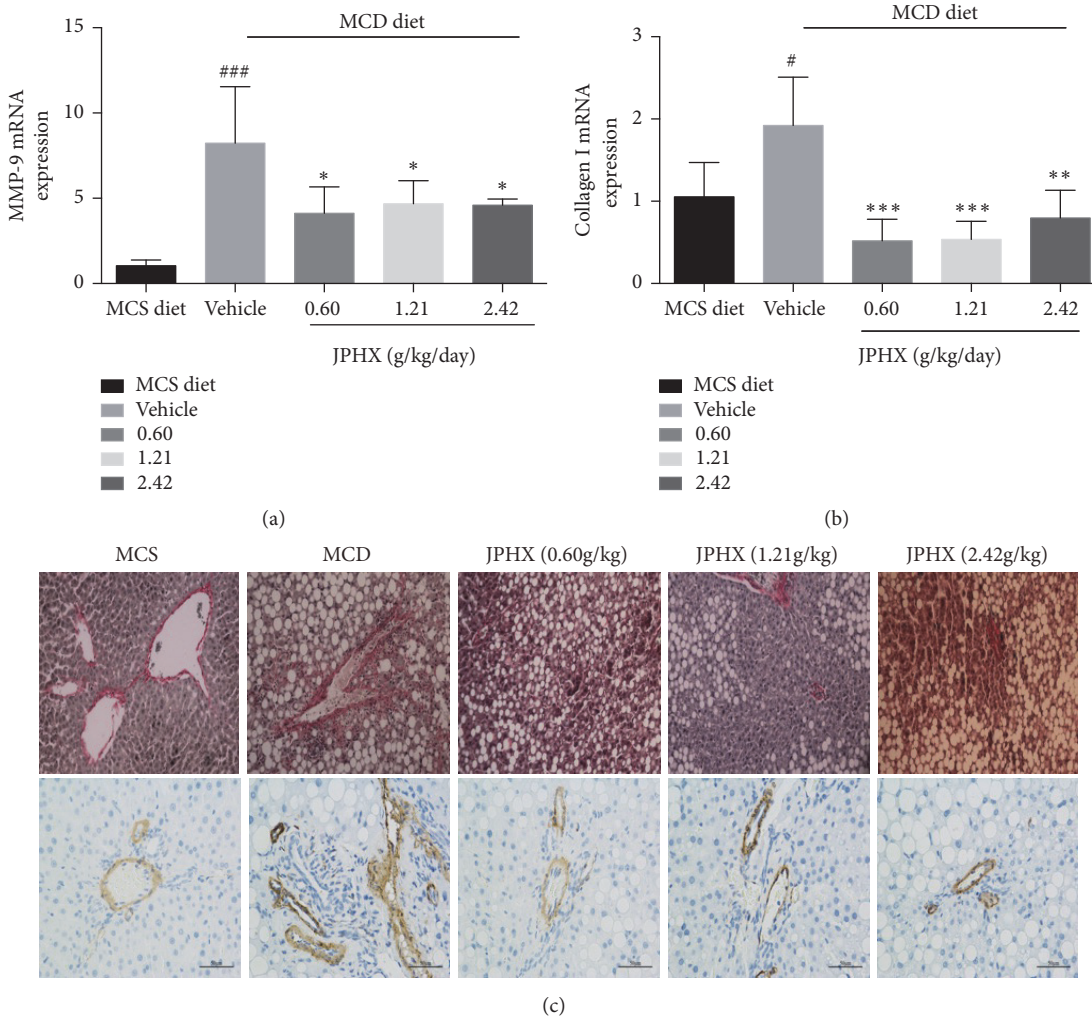

Figure 4: JPHX inhibits hepatic fibrosis in MCD diet-fed rats, as suggested by profibrotic gene expression level, Sirius red staining, and α-SMA Expressions level. (a) Collagen I and MMP-9 mRNA expression levels were detected by qRT-PCR. The relative amounts of mRNA were normalized to β-actin. (b) Sirius red staining showed noticeable pericellular fibrosis in the MCD group. Representative liver sections from different groups. Original magnification × 200. (c) Paraffin-embedded sections were immunostained with an α-SMA antibody. Representative liver sections from different groups. Original magnification × 400. Values are shown as mean ± SD. MCS n=6, MCD n=9, and JPHX treatment groups n=7. #p<0.05, ##p<0.01, and ###p<0.001, MCD diet vehicle group versus MCS diet group. *p<0.05, **p<0.01, and ***p<0.001, MCD diet vehicle group versus JPHX treatment groups.

important role in the pathogenesis of NAFLD, which causes the infiltration of Kupffer cells, cell death, and liver damage [23, 24]. Reactive oxygen species (ROS) can react with the accumulated lipids in the liver to cause lipid peroxidation. Detoxifying enzymes which scavenge ROS act as the first line of defense against ROS [15, 25]. JPHX treatment group had higher expressions of liver GPx levels compared with MCD group; however only low dose of JPHX formula show significantly higher expressions of liver GPx levels (Figure 3(e), p=0.0110). Histological examination indicated that JPHX normalized the NAS score (Figure 3(f), p< 0.0001). Our data suggested that JPHX attenuated hepatocellular injury and inflammation, as indicated by reversal of abnormal serum ALT and AST levels and reduced TNF-α expression and inflammatory infiltrates.

3.4. JPHX Prevents Fibrosis by Inhibiting Expression of Collagen I and Matrix Metalloproteinase. The hepatic mRNA levels of matrix metalloproteinase-9 (MMP-9) markedly increased in the rats fed the MCD diet alone but were downregulated by low, medium, and high doses of JPHX formula (by 50.6%, 43.0%, and 44.3%, respectively). Also, collagen I mRNA, to a significantly lesser extent in the JPHX group, showed the same trend. JPHX inhibited liver fibrosis by inhibiting aberrant collagen I and MMP-9 gene expression induced by the MCD diet (Figure 4(a), for MMP-9, p=0.0009; for collagen I, p=0.0007). These findings were consistent with the Sirius red staining results. Administration of the MCD diet was associated with pericellular fibrosis, and this response was inhibited by JPHX treatment (Figure 4(b)). Hepatic stellate as the major cell type involved in liver fibrosis follows

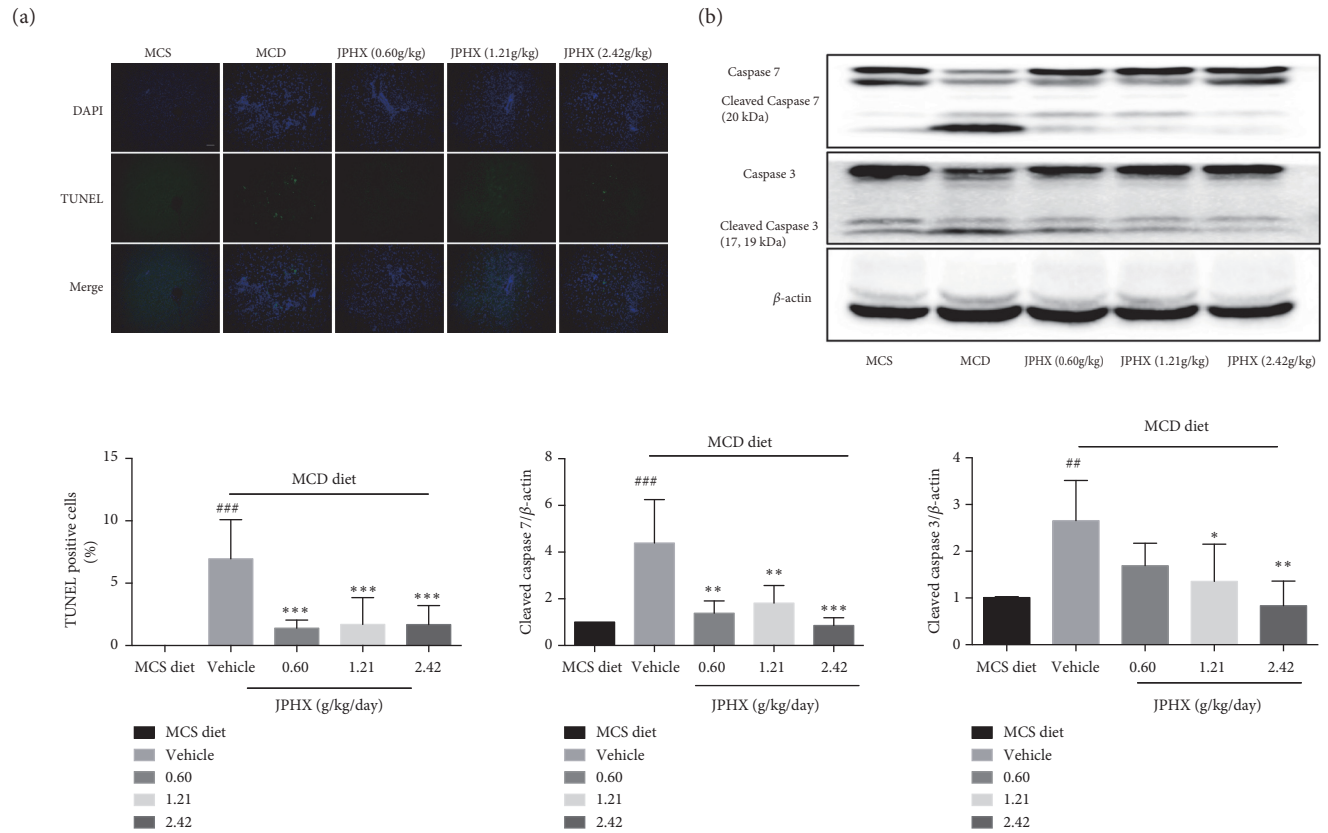

FIGURE 5: Analysis of apoptosis in liver sections and effect of JPHX on the expression levels of cleaved forms of caspase 3 and caspase 7. (a) Representative images showed apoptotic cells in the liver of different groups of rats by TUNEL staining and quantification of TUNEL-positive cells. Apoptotic nuclei stained by TUNEL (green) and counterstained with DAPI (blue) to mark nuclei; hepatocytes were imaged by confocal microscope. (b) Representative Western blotting results of total and cleaved forms of caspase 3 and caspase 7. Protein expression levels were normalized to that of β-actin. Values are shown as mean ± SD. MCS n=6, MCD n=9, and JPHX treatment groups n=7. #p<0.05, ##p<0.01, and ###p<0.001, MCD diet vehicle group versus MCS diet group. $*p$<0.05, $**p$<0.01, and $***p$<0.001, MCD diet vehicle group versus JPHX treatment groups.

its transdifferentiation into fibrogenic myofibroblasts, which cause liver fibrosis [15]. Alpha-smooth muscle actin (α-SMA) is commonly used as a marker of myofibroblast formation. Immunohistochemistry analysis revealed that the expression of α-SMA was very weak in the MCS group, whereas it was dominant in MCD group. JPHX treatment attenuated the increased expression of α-SMA in the fibrotic liver (Figure 4(c)), which is consistent with the profibrotic gene expression and Sirius red staining results.

3.5. JPHX Formula Reduces Hepatocyte Apoptosis. Hepatocyte apoptosis was measured by TUNEL staining and with reference to the expression levels of caspase 3 and 7 proteins. The slides showed few TUNEL-positive cells in the MCS group. The number of apoptotic hepatocytes obviously increased in the MCD diet-fed rats compared to the MCS diet group (Figure 5(a), p< 0.0001). Moreover, Western blotting showed that cleaved forms of caspase 3 and caspase 7 were increased in the livers of MCD diet-fed rats (Figure 5(b), for caspase 3, p=0.0062, for caspase 7, p=0.0005). We observed a significant decrease in TUNEL-positive cells, as well as cleaved caspase 3 and 7, in the JPHX treatment group compared to MCD diet feeding alone.

3.6. JPHX Decreases the Phosphorylation of JNK Signaling. It is important to note that the mitogen-activated protein kinase (MAPK) pathway plays crucial roles in inflammation and apoptosis. Our data showed that JPHX prevented the NAFLD model rats from experiencing inflammation and apoptosis. Based on our data, we measured the levels of total and phosphorylated stress-activated protein kinases (SAPK)/c-Jun N-terminal kinase (JNK), p38 MAPK, and MAPK/ERK. JPHX only inhibited the hepatic protein expression of phosphorylated JNK in the liver of NAFLD rats. Moreover, treatment with JPHX did not significantly affect the expression of p-p38 and p-ERK compared with that in rats fed the MCD diet (Figure 6, for p-JNK, p=0.0041, for p-p38, p=0.1889, and for p-ERK, p=0.5983).

4. Discussion

NAFLD and its complications contributed to high liver-related and overall mortality. Currently, controlling risk

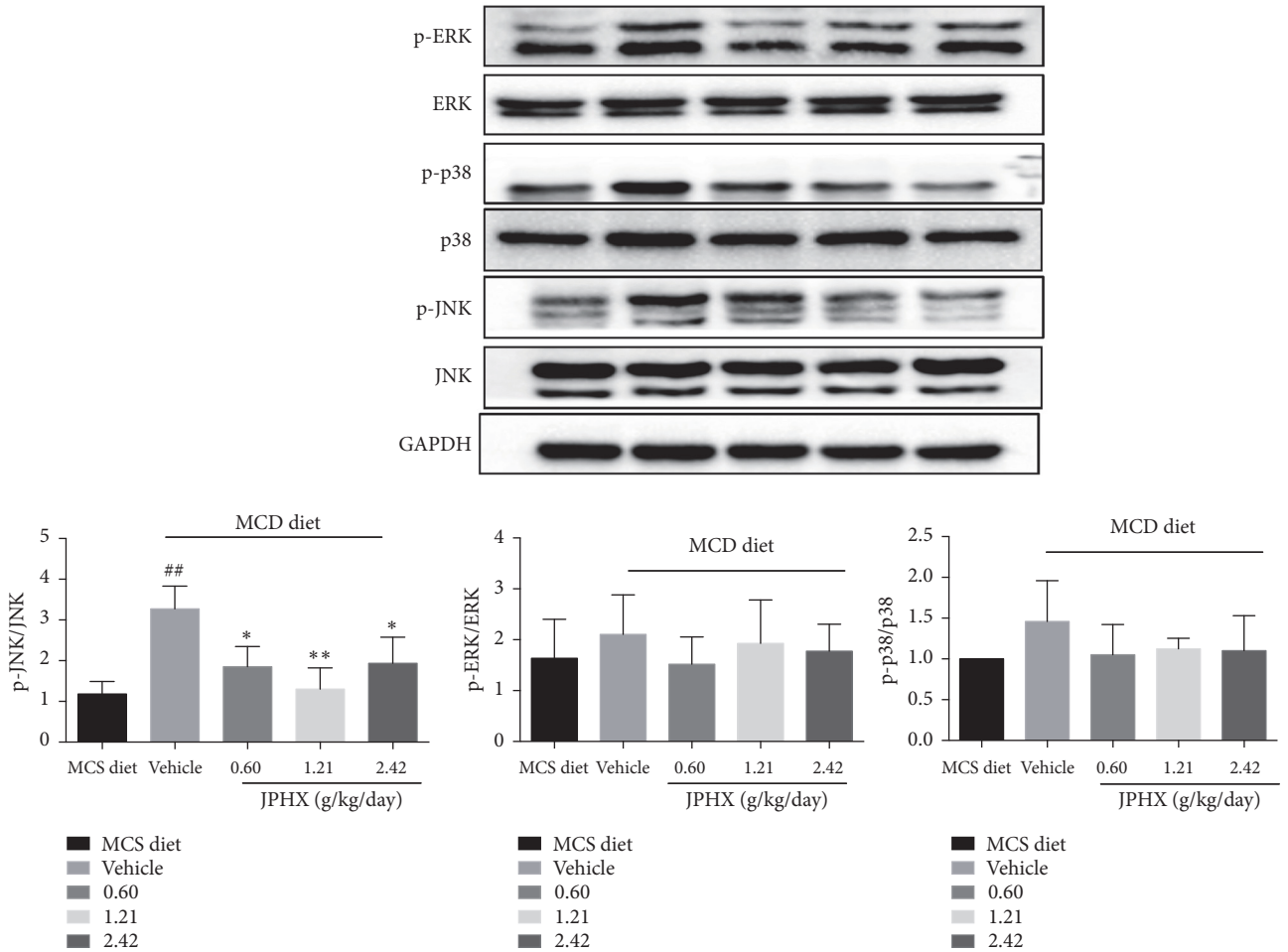

Figure 6: Effect of JPHX on MAPK pathway. JNK, p38, and ERK were measured from total and phosphorylated proteins. Protein expression levels were normalized to that of GAPDH. Values are showed as mean ± SD. MCS n=6, MCD n=9, and JPHX treatment groups n=7. #$p<0.05$, ##$p<0.01$, and ###$p<0.001$, MCD diet vehicle group versus MCS diet group. *$p<0.05$, **$p<0.01$, and ***$p<0.001$, MCD diet vehicle group versus JPHX treatment groups.

factors such as obesity, diabetes, and hyperlipidemia and weight loss through physical activity and nutritional interventions are the therapeutic options of choice for the management of NAFLD and NASH [26, 27]. At present, there are no clinically available effective drugs for the management of NAFLD and NASH, although a few compounds, such as Pioglitazone and vitamin E, show promising effects for patients with NAFLD [28]. Here, our data demonstrated that JPHX treatment prevents steatosis, liver injury, inflammation, fibrosis, and apoptosis in the MCD diet-induced NAFLD model. Based on our data, JPHX may have potential as a therapeutic formula for the alleviation of NAFLD.

Since the pathogenesis of NAFLD is complex, strategies for treating NAFLD should target multiple or prominent pathological pathways. Traditional Chinese medicine (TCM), containing a cocktail of multiple bioactive components, is an alternative solution for management of multifactorial chronic diseases, like NAFLD. The protective effect of JPHX against NAFLD probably occurs through multiple anti-inflammatory and antifibrotic candidate compounds present in JPHX formula. Some studies have demonstrated that *Atractylodes macrocephal* has an anti-inflammatory effect and could regulate lipid metabolism and improve liver and kidney function [29, 30]. *Alisol A 24-acetate (AA)*, isolated from *Rhizoma Alismatis*, also ameliorated lipid accumulation and inflammation in the NASH model [31, 32]. Our findings suggested that JPHX attenuates the accumulation of hepatic TG and TC. *Gomishi* is the dried fruit of *Fructus Schisandra chinensis*, which demonstrated anti-inflammatory properties by suppressing inducible nitric oxide synthase in rat hepatocytes [33]. In our study, liver inflammation was attenuated, as indicated by reduced infiltration of Kupffer cells and neutrophils. We also found that increased TNF-α production in MCD diet-induced NASH was decreased in JPHX-treated rats. Kupffer cells produce proinflammatory cytokines like TNF-α, which activates HSC leading to development of the disease [34]. As the main component of JPHX prescription, *Danshen* has been widely used based on its antioxidant and anti-inflammatory properties. Recent studies showed that *Danshen* protects against early stage alcoholic liver disease

in mice and its lipophilic compound, Tan IIA, inhibits lipopolysaccharide (LPS)-induced hepatic stellate cell (HSC) activation [35, 36]. The activation of HSC produces collagen, which contributes to liver fibrosis. Although fibrosis is not a component of NAS, it is strongly related to the diagnosis and prognosis of diseases and indicates a progressive liver disease [37]. Importantly, fibrosis is associated with high liver-related morbidity and mortality in humans. We demonstrated that liver fibrosis was attenuated, as shown by decreased pericellular and perisinusoidal collagen deposition and the profibrotic genes MMP-9 and Collagen I. Multiple active compounds in JPHX may interact with other compounds, resulting in the hepatoprotective effect in the experimental model of NAFLD through regulating lipid accumulation, inflammation, and fibrosis.

A variety of mechanisms contributed to the development of NAFLD. A " two-hit" model was proposed by Day and James [38]. Lipid accumulation plays a pivotal role in liver injury. Proinflammatory cytokines and oxidative stress also contribute to the progression of NAFLD [39]. JNK is involved in both of these processes [40]. It is important to note that MAPK signaling pathway is involved in physical and chemical stress, which plays a role in liver injury [41]. Some inflammatory cytokines, such as IL-6 and TNF-α, activated the phosphorylation of JNK. Sustained activation of JNK contributed to cell death. Interestingly, our data showed that JNK1 phosphorylation was significantly decreased by JPHX, whereas p38 and ERK remained unaffected. The data presented herein are in agreement with a study in which the inhibition of JNK1 was an effective treatment for NAFLD [42]. Improvement of apoptosis plays a vital role in the treatment of NAFLD [43].

Most of drugs show classical sigmoidal dose-response curves which illustrate an increase in inhibitory or stimulatory effect with rising drug concentration [44]. However, in our experiments, compared with the low dose and high dose, the effects of medium-dose (1.21 g/kg) JPHX formula were more effective. This "bell-shaped" like curves could be attributed to the characteristic of TCM that the efficacy of TCM usually comes from synergistic interactions of multiple ingredients [45]. TCM treats diseases based on a holistic rule that uses herbs or formula containing multiple compounds to rebalance the organism [46]. There are multiple active compounds in JPHX formula which targets multiple pathological pathways. Multiple herbal ingredients and their targets built a complex network with rats. As a result, the efficacy of TCM usually does not follow the typical dose-response curves [47]. In the future, with the development of systems biology and pharmacology and network pharmacology, based on our study, we can use systematic docking, herb-target network analysis, and some TCM databases to go further with the JPHX formula [46, 48, 49].

Although the MCD diet has been used for over 40 years to induce NAFLD, which is similar to hepatic histological features of human NAFLD, there are still some limitations in this model [15]. NAFLD is closely related to obesity and metabolic syndrome; however, the MCD diet model of NAFLD causes weight loss and does not exhibit insulin resistance [50, 51]. There is no ideal small animal model of NAFLD to perfectly mimic the human pathology, which reflects the imperfect understanding of human complexity pathogenesis. However, some refined animal models such as genetic models and combination models of genetic and nutritional factors have been developed to verify the hypotheses on the pathogenesis of NAFLD [52, 53]. In the future, further researches on animal model will develop novel diagnostic and therapeutic approaches to help people with NAFLD.

5. Conclusion

In conclusion, the MCD experimental model animals developed severe livery injuries, including steatosis, inflammation, and fibrosis within 8 weeks in our study. JPHX exhibited hepatoprotective effects against NAFLD by targeting lipid accumulation, fibrosis, inflammation, and apoptosis. These findings suggest that JPHX may be a promising therapeutic formula for the treatment of NAFLD.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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Research Article

The Use of Antifibrotic Recombinant nAG Protein in a Rat Liver Fibrosis Model

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Objectives. The "nAG" protein is the key protein mediating the regeneration of amputated limbs in salamanders. The senior author (MMA) developed the original hypothesis that since "nAG" is a "regenerative" protein, it must be also an "antifibrotic' protein. The antifibrotic properties were later confirmed in a rabbit skin hypertrophic scar model as well as in a rat spinal cord injury model. The aim of this study is to evaluate the potential therapeutic properties of the nAG protein in a rat liver fibrosis model. *Methodology*. Liver fibrosis was induced using intraperitoneal injections of carbon tetrachloride (CCL4). A total of 45 rats were divided equally into 3 groups: Group I (the control group) received normal saline injections for 8 weeks, Group II received CCL4 for 8 weeks, and Group III received CCL4 and nAG for 8 weeks. At the end of the experiment, the serum levels of 6 proteins (hyaluronic acid, PDGF-AB, TIMP-1, laminin, procollagen III N-terminal peptide, and collagen IV-alpha 1 chain) were measured. Liver biopsies were also taken and the stages of live fibrosis were assessed histologically. *Results*. The CCL4 treatment resulted in a significant increase in the serum levels of all 6 measured proteins. The nAG treatment significantly reduced these high levels. The degree of liver fibrosis was also significantly reduced in the CCL4/nAG group compared to the CCL4 group. *Conclusions*. nAG treatment was able to significantly reduce the serum levels of several protein markers of liver fibrosis and also significantly reduced the histological degree of liver fibrosis.

1. Introduction

Salamanders (which are lower vertebrates) are known to regenerate their amputated limbs. The "nAG" protein (nAG stands for newt Anterior Gradient) is the key protein mediating this form of regeneration [1]. The amputation stump of the salamander forms a blastema (a mound of proliferating mesenchymal cells) in which nAg is expressed. The nAG protein is expressed by Schwann cells of regenerating axons and peaks at 5–7 days postamputation. At 10–12 days, the protein is also expressed in glands in the dermis underlying the wound epithelium [1].

The senior author (MMA) developed the original hypothesis that since "nAG" is a "regenerative" protein, it must also be an "antifibrotic" protein. Hence, a new nAG gene (suitable for higher vertebrates including humans) was designed, synthesized, and cloned. The cloned vector was successfully transfected into human fibroblasts. nAG expression was found to suppress the expression of collagen in human fibroblasts regardless of the presence of Transforming Growth Factor Beta (TGFβ) [2]. The antifibrotic properties of the nAG protein were later shown in several animal models such as a rabbit model of hypertrophic scar [3], a mouse model of digital tip amputation [4], and a rat spinal cord crush injury

Table 1: Metavir histological grading of liver fibrosis.

Grade of fibrosis	Description
F0	no fibrosis
F1	Mild fibrosis: Fibrous portal expansion with mild localized fibrosis in the portal area
F2	Moderate fibrosis: Portal fibrosis with few fibrous septa.
F3	Severe fibrosis: Portal fibrosis with numerous fibrous septa.
F4	Cirrhosis: Marked portal-to-portal and portal-to central fibrosis with regenerative nodules.

model [5]. The antifibrotic effects of nAG on liver fibrosis have not been previously investigated.

Liver fibrosis may be induced by several factors such as nonalcoholic fatty infiltration of the liver, alcoholic liver disease, viral hepatitis, autoimmune hepatitis, toxin-induced hepatitis, and hereditary metabolic diseases [6, 7]. The end result of liver fibrosis is cirrhosis leading to portal hypertension, liver failure, and the increased risk of hepatocellular carcinoma. The only effective treatment of decompensated liver cirrhosis is liver transplantation [6, 7]. Fibrogenesis and organ fibrosis are mediated by different cells in different organs such as the fibroblast in the skin [3], the astrocyte in the spinal cord [5], and the stellate cell in the liver [8].

In the current study, we investigated the potential therapeutic properties of the nAG protein in liver fibrosis using a rat model.

2. Methodology

This study was approved by our institutional review board and was conducted according to the Guidelines for Animal Experiments (Project# E-13-926).

2.1. The Animal Model. We induced liver fibrosis in rats using carbon tetrachloride (CCL4) and this is a well-known model of rodent liver fibrosis [9, 10]. A total of 45 adult Sprague-Dawley rats (weighing between 240-260 gm) were divided into three groups (15 rats in each group): Group I (Control group) received twice weekly intraperitoneal injections of normal saline for 8 weeks, Group II received twice weekly intraperitoneal injections of CCL4 (0.5 ml/kg of the 25% CCL4 solution) for 8 weeks, and Group III received twice weekly intraperitoneal injections of CCL4 (0.5 ml/kg of the 25% CCL4 solution) as well as 2 ug of the recombinant nAG protein for 8 weeks.

2.2. ELISA Assessment of Protein Markers. At the end of the experiment, the serum level of six protein markers of liver fibrosis were measured in all rats using ELISA: hyaluronic acid, Platelet Derived Growth Factor-AB (PDGF-AB), Tissue Inhibitor of Metalloproteinase-1 (TIMP-1), laminin, procollagen III N-terminal peptide (PIII-NP), and collagen type IV-Alpha 1 chain (collagen 4-α1).

2.3. Histopathological Assessment. Furthermore, liver biopsies were taken from all rats at the end of the experiment (8 weeks) and the histological grading of liver fibrosis was graded using the Metavir scoring system [11] as shown in Table 1. All biopsies were stained with both Hematoxylin and Eosin (H&E) and Elastic fiber-Verhoeff's Van Gieson (EVG) stains. The grading was done by a Consultant Histopathologist who was blinded to the rat groups.

2.4. Statistical Analysis. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) version 22.0 software (SPSS Inc., Chicago, Il, USA). For the study of serum protein levels, the means and Standard Deviations (SD) were calculated for the three groups (the control and the two experimental groups). We used the one-way analysis of variance (ANOVA) to compare the three groups and the post hoc test Dunnett T3 for multiple comparisons between the groups. P-value of < 0.05 was considered significant. For the histological grading of liver fibrosis, we compared the percentages of no/mild fibrosis versus moderate/severe fibrosis in the two experimental groups using the Fisher's exact/Chi-square tests. P-value of < 0.05 was considered significant.

3. Results

3.1. ELISA Assessment of Protein Markers. The results of the serum levels of 6 proteins in three groups are shown in Table 2.

3.1.1. Hyaluronic Acid. Hyaluronic acid doubled in the CCL4 group compared to the control group ($P<0.05$ between groups I & II) and the nAG treatment was able to normalize its serum levels with no significant difference between the control and the CCL4/nAG group.

3.1.2. PDGF-AB. The serum levels of PDGF-AB increased more than 10-fold in the CCL4 group compared to the control group ($P<0.05$ between groups I & II). The nAG treatment was able to significantly reduce (but not normalize) the serum levels of PDGF-AB. Hence, there was a significant difference ($P<0.05$) between groups I & III and also between groups II & III.

3.1.3. TIMP-1. The serum levels of TIMP-1 increased more than 12-fold in the CCL4 group compared to the control group ($P<0.05$ between groups I & II). The nAG treatment was able to significantly reduce (but not normalize) the serum levels of TIMP-1. Hence, there was a significant difference ($P< 0.05$) between groups I & III and also between groups II & III.

Table 2: Results of the serum levels of various proteins in the three groups.

SERUM LEVEL OF:	Group I (Control) N=15	Group II (CCL4 treatment) N=15	Group III (CCL4/nAG treatment) N=15
Hyaluronic acid	15.133 ± 0.063 ∗	29.80 ± 6.145 ∗^	15.739 ± 3.231 ^
PDGF-AB	18.292 ± 0.023 ∗+	202.839 ± 124.73 ∗^	58.905 ± 29.198 ^+
TIMP-1	35.563 ± 0.0299 ∗+	449.25 ± 294.71 ∗^	161.919 ± 67.518 ^+
Laminin	257.27 ± 12.91 ∗+	1238.588 ± 622.419 ∗^	415.34 ± 136.934 ^+
Procollagen III N-terminal peptide	0.242 ± 0.021 ∗+	0.355 ± 0.050 ∗^	0.161 ± 0.051^+
Collagen Type IV-Alpha-1 chain	2.933 ± 2.193 ∗	21.915 ± 9.226 ∗^	3.08 ± 2.539 ^

∗There was a significant difference between the control group and the CCL4 treatment group since $P < 0.05$.
^There was a significant difference between the CCL4 treatment group and CCL4/nAG treatment group since $P < 0.05$.
+There was a significant difference between the control group and CCL4/nAG treatment group since $P < 0.05$.

Table 3: The staging of liver fibrosis in the two experimental groups.

Degree of Fibrosis	Group II: CCL4 only (n=15 rats)	Group III: CCL4 and nAG (n=15 rats)
No or mild fibrosis	2 (both rats had mild fibrosis)	9 (3 rats had no fibrosis and 6 rats had mild fibrosis)
Moderate to severe fibrosis	13 (4 rats had moderate fibrosis and 9 rats had severe fibrosis)	6 (4 rats had moderate fibrosis and 2 rats had severe fibrosis)

P= 0.008.

3.1.4. Laminin. The serum levels of laminin increased almost 5 folds in the CCL4 compared to the control group ($P<0.05$ between groups I & II). The nAG treatment was able to significantly reduce (but not normalize) the serum levels of laminin. Hence, there was a significant difference ($P<0.05$) between groups I & III and also between groups II &III.

3.1.5. PIII-NP. The serum levels of PIII-NP were significantly increased in the CCL4 group compared to the control group ($P<0.05$ between groups I & II). The nAG treatment significantly reduced the serum level of PIII-NP below levels of the control group. Hence, there was a significant difference ($P<0.05$) between groups I & III and also between groups II & III.

3.1.6. Collagen 4-α1. Finally, the serum levels of collagen 4-α1 increased more than 7 folds in the CCL4 group compared to the control group ($P<0.05$ between groups I & II). The nAG was able to normalize the serum levels of collagen 4-α1. Hence, there was no significant difference between the control and the CCL4/nAG groups.

3.2. Histopathological Assessment. As expected, all rats in the control group (n=15) had no liver fibrosis and hence, these rats were not included in the statistical analysis of the grading of liver fibrosis. Table 3 summarizes the results of the staging of liver fibrosis between two experimental groups. The nAG treatment significantly reduced the histological degree of liver fibrosis ($P= 0.008$).

4. Discussion

Our study is the first investigation of the therapeutic potential of the antifibrotic nAG protein in liver fibrosis. nAG treatment was able to significantly reduce the serum levels of several protein markers of liver fibrosis [12] and also significantly reduced the histological degree of liver fibrosis. nAG was injected intraperitoneally in our experiment. From the clinical point of view, drugs targeting organ fibrosis should consider the location of the primary profibrotic cells of the organ (the stellate cells in the liver). These cells are located in the space of Disse between the endothelial cells and hepatocytes. Hence, nAG may be more effective if combined with another carrier peptide to pass through the sinusoidal endothelial barriers and also to escape the uptake by the Kupffer cells and hepatocytes [13, 14].

The pathophysiology of liver fibrosis is well described in the literature [6]. In normal livers, the stellate cells are quiescent and they function to store vitamin A (seen histologically as intracellular retinoid droplets) and to regulate sinusoidal blood flow. After the liver insult (such as pathogens, abnormal fatty infiltration, drugs, toxins, and free radicals) the inflammatory reaction results in the release of TGFβ and several cytokines/chemokines including the interleukins IL-1B and IL-6 [6]. This results in the activation of stellate cells. Activated stellate cells lose their retinoid droplets, proliferate, transform into myofibroblasts, and release TGFβ, resulting in further cell activation. Activated stellate cells also release PDGF (inducing further stellate cell proliferation) and extracellular matrix proteins such as collagens, hyaluronic

acid, and laminin. The abnormally high level of collagen is not only due to increased collagen production, but also due to decreased collagen degradation since the production of TIMP1 is also increased [15]. The nAG treatment was able to significantly reduce the serum levels of TIMP-1 (Table 2).

Our study also showed that the nAG treatment normalized the levels of hyaluronic acid (Table 2). Hyaluronic acid is mainly synthesized in the liver and is an important component of the liver extracellular matrix. It is normally degraded in the sinusoides by the hyaluronidase enzyme. The serum level of hyaluronic acid is known to correlate with the degree of liver fibrosis in both alcoholic and nonalcoholic liver diseases [16].

The effectiveness of nAG in reducing the production of collagen production has been shown in human skin fibroblast [2]. In fibroblasts, the effect on collagen III was more pronounced than the effect on collagen I [2]. Hence, it is of no surprise that nAG treatment in the current experiment was able to significantly reduce the serum levels of PIII-NP below the normal control levels (Table 2). PIII-NP is formed during the synthesis of collagen III. The serum level of PIII-NP is a known marker for liver fibrosis [17].

The nAG treatment in our study was also able to normalize the serum levels of collagen 4-α1 which is also a marker for liver fibrosis [18]. Collagen type IV is a main component of the basement membrane. In liver fibrosis, there is excessive remodeling of the basement membrane and excessive release of its peptide fragments (such as collagen 4-α1) in the circulation [19].

Laminin in the liver is found both in the basement membrane (where it is associated with collagen type IV) as well as in the extracellular matrix (where it is associated with collagen types I & III) along the fibrous septa and within the space of Disse [20]. Hence, the serum levels of laminin correlate with the degree of liver fibrosis [20]. Our study showed that the nAG treatment is able to significantly decrease the serum levels of laminin in experimental rats (Table 2).

There are three important PDGF isoforms: PDGF-AA, PDGF-AB, and PDGF-BB. PDGF-AA selectively binds to PDGF-receptor alpha, while the latter two isoforms bind to both alpha and beta receptors of PDGF [21]. In abnormal skin fibrotic disorders, all isoforms mediate myofibroblast proliferation and excessive collagen production [22]. In the normal liver, quiescent stellate cells express only the alpha receptors of PDGF. Hence, PDGF-AA is thought to mediate the normal functions of the quiescent stellate cells. Once stellate cells get activated, they express the beta receptors of PDGF [23, 24]. Hence, several authors have studied the serum levels of PDGF-BB in patients with liver fibrosis/cirrhosis. Zhou et al. [25] found a significant negative correlation between the serum levels of PDGF-BB and the liver fibrosis stage, while a significant positive correlation was noted by Zhang et al. [26]. These contradicting results may be related to the fact that the extrahepatic concentration of PDGF-BB is known to be related to platelet count. Hence, cirrhotic patients with thrombocytopenia will have a tendency for lower levels of serum PDGF-BB [27, 28]. To our knowledge the serum levels of PDGF-AB have never been previously studied in liver fibrosis/cirrhosis in the clinical or in the experimental setting. Our study showed that the CCL4-induced liver fibrosis model resulted in more than 10-fold increase in the serum levels of PDGF-AB and that nAG treatment significantly reduced these elevated levels (Table 2).

Many drugs have been proposed for the management of liver fibrosis [29–33]. We believe that nAG will eventually be included in the list of management protocols of fibrotic liver disease.

5. Conclusions

nAG treatment was able to significantly reduce the serum levels of several protein markers of liver fibrosis and also significantly reduced the histological degree of liver fibrosis. Further studies are required to investigate the effect of nAG if combined with a carrier peptide to selectively increase its concentration in the stellate cells responsible for the fibrotic reaction in the liver.

Data Availability

Data are available at the College of Medicine Research Center, Deanship of Scientific Research, King Saud University, Riyadh, Saudi Arabia.

Conflicts of Interest

There are no conflicts of interest and no drug company is involved in funding.

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Research Article

Noninvasive Indirect Markers of Liver Fibrosis in Alcoholics

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The aim of this study was to evaluate the diagnostic values of noninvasive indirect markers of liver fibrosis: APRI, GAPRI, Forns, FIB-4, Age-Platelet, and Hepascore in alcoholics. Blood samples were collected from a randomized group of 142 alcohol-dependent patients. The diagnosis of dependency was made according to the ICD-10 WHO criteria. The values of noninvasive markers were calculated with specific algorithms. The fibrosis stage was evaluated on the basis of FibroTest. The values of APRI, Forns, FIB-4, GAPRI, AP, and Hepascore differ between various stages of liver fibrosis. Patients with fibrosis stage F0 present lower values of APRI, Forns, FIB-4, GAPRI, and Hepascore in comparison to the patients with stages F1 and F0-F1. Patients with fibrosis stages < F2 have lower values of all noninvasive markers than patients with stages ≥F2. Patients with fibrosis stages ≥F2 but <F4 have lower values of APRI, Forns, FIB-4, GAPRI, and Hepascore than patients with stage F4. The values of noninvasive markers tested here differ in various stages of liver fibrosis. To our surprise, the patented marker, Hepascore, achieves a lower diagnostic value in alcoholics than simple markers involving only liver enzymes, platelet count, and cholesterol. The best marker of liver fibrosis in alcoholic patients seems to be the Forns index.

1. Introduction

Liver diseases induced by excessive alcohol consumption are an important cause of morbidity and mortality worldwide. Alcoholic liver diseases (ALD) can manifest themselves as a one of the following disorders: alcoholic fatty liver, alcoholic hepatitis, and alcohol-related cirrhosis [1, 2]. The studies have shown that the alcoholic liver injury can develop into fibrosis or cirrhosis in up to 15% of alcoholics [3, 4]. On the other hand, alcoholic hepatitis and steatohepatitis are present in 35% of alcoholics [5]. Therefore, detection of an early stage of liver damage is the key to provide a positive outcome for therapeutic intervention. The "gold standard" for evaluating the stage of liver fibrosis—liver biopsy—is an invasive procedure which can lead to health complications in 3.0% of patients (e.g., bleeding, pain, bile peritonitis, kidney puncture, or death). Additionally, sampling errors are very common due to difficulty with obtaining liver specimen representing the whole liver [6–8]. Therefore, there is a great need for developing safer and freely available noninvasive diagnostic tools. In this study, we will follow the work of Thiele et al. who compared the accuracy of 10 liver fibrosis markers (patented or not) in patients with alcoholic liver diseases [9]. According to these results, the receiver operating characteristic curve (AUROC) of two tests—ELF and FibroTest—for advanced fibrosis (≥F3) reached and exceeded the value of 0.9. Taking into account the fact that, among others, these two tests exhibited the highest diagnostic power for identification of alcoholic patients with advanced liver fibrosis, we have treated the FibroTest as a matrix for comparing the diagnostic values of five nonpatented, noninvasive indirect markers of liver fibrosis: APRI, GAPRI, Forns, FIB-4, Age-Platelet (AP), and one patented algorithm, Hepascore, in alcoholics. Additionally, carbohydrate-deficient transferrin (CDT) as an established marker of alcohol abuse was evaluated in these patients [10]. According to our previous published work,

the relative values of CDT are affected by liver diseases and reflected the severity of liver dysfunction [11].

2. Materials and Methods

2.1. Participants. The tested group consisted of 142 alcohol-dependent patients (127 men and 15 women) from detoxification ward (Department of Detoxification, Psychiatric Hospital in Choroszcz). Patients were initially examined and interviewed regarding history of disease and their use of alcohol. The diagnosis of dependency was made on the basis of ICD-10 WHO criteria. The self-reported mean alcohol consumption was 1311 g of ethanol per week and mean time of dependency was 18 years. The patients did not undergo the liver biopsy, and fibrosis stage was established in FibroTest which was used as the reference standard. Study was in accordance with Helsinki Declaration and was approved by the Bioethical Committee at the Medical University in Bialystok.

2.2. Blood Sampling. Blood samples from a peripheral vein from each patient were collected. After centrifugation, sera were collected into 2 tubes and stored at -86°C until assayed. Besides serum, a part of each sample was collected into tubes containing 3.8% liquid sodium citrate and EDTA-2.

AST, ALT, GGT, cholesterol, α2-macroglobulin, hyaluronic acid, and bilirubin were determined on the Architect c8000 (Abbott Laboratories, Abbott Park, USA). PLT count was measured on Sysmex XS-800i (Sysmex Corporation, Singapore).

The serum biochemical markers, α2-macroglobulin, haptoglobin, apolipoprotein A1, γ-glutamyltransferase, alanine aminotransferase, and total bilirubin, were determined according to methods recommended by BioPredictive (Paris, France). FibroTest scores were computed by BioPredictive company according to the arrangement, and results were provided with security algorithms.

CDT immunoassays were carried out using the N Latex CDT test (Siemens Healthcare Diagnostics, Marburg, Germany) on BN II System (Siemens Healthcare Diagnostics, USA). CDT values were expressed as percentages of total transferrin.

2.3. Calculations

$$\text{APRI} = \left(\frac{(\text{AST [IU/L]}/50\text{IU/L})}{\text{PLT}\left[10^9/\text{L}\right]}\right) * 100$$

$$\text{GAPRI} = \left(\frac{\text{GGT [IU/L]}}{\text{PLT}\left[10^9/\text{L}\right]}\right) * 100$$

$$\text{FIB-4} = \frac{(\text{age} * \text{AST [IU/L]})}{(\text{PLT}\left[10^9/\text{L}\right] * \sqrt{(\text{ALT [IU/L]})})}$$

$$\text{Forn's index} = 7.811 - 3.131 \ln\left(\text{PLT}\left[10^9/\text{L}\right]\right) + 0.781 \ln(\text{GGT [IU/L]}) + 3.467 \ln(\text{age}) - 0.014\,(\text{cholesterol}\,[\text{mg/dL}])$$

$$\text{AP index} = \text{age} + \text{PLT}\,(\text{age: } <30 = 0;\ 30\text{-}39 = 1;\ 40\text{-}49 = 2;\ 50\text{-}59 = 3;\ 60\text{-}69 = 4;\ \geq 70 = 50, \text{PLT}\left[\times 10^9/\text{L}\right]: \geq 225 = 0;\ 200\text{-}224 = 1;\ 175\text{-}199 = 2;\ 150\text{-}174 = 3;\ 125\text{-}149 = 4;\ < 125 = 5)$$

$$\text{Hepascore} = \frac{\text{Y}}{(1 + \text{Y})}$$

$$\text{Y} = \exp(-4.185818 - (0.0249 * \text{age}) + (0.7464 * \text{sex}) + (1.0039 * \alpha\text{2-macroglobulin}) + (0.0302 * \text{hyaluronic acid}\,[\text{ng/mL}]) + (0.0691 * \text{total bilirubin}) - (0.0012 * \text{GGT [IU/L]}) \quad (1)$$

The values for sex: 1 for men and 0 for women.

2.4. Statistical Analysis. The normality of distribution was checked by means of Kolmogorov-Smirnov test with the Lilliefors correction. The analysis revealed that the distribution of APRI, FIB-4, GAPRI, and Hepascore does not follow a normal distribution ($P<0.05$), but Forns index and AP follow a normal distribution ($p>0.05$) (Figure 1). The differences between stages of liver fibrosis were evaluated by Mann-Whitney U test. To test the effect of liver diseases on the values of markers, ANOVA rank Kruskal-Wallis test was performed. We considered *P*-values <0.05 as statistically significant. The diagnostic performance of each test was calculated as sensitivity, specificity, PPV, NPV, and accuracy. To calculate the diagnostic accuracy of algorithms, the ROC curve was used.

3. Results

The average values of noninvasive markers in patients with different liver fibrosis scores are presented in Table 1. The values of APRI, Forns, FIB-4, GAPRI, AP, and Hepascore differed between the stages of liver fibrosis (ANOVA rank Kruskal-Wallis test: H=30.902, P<0.001; H=49.386, P<0.001; H=51.907, P<0.001; H=40.951, P<0.001; H=31.553, P<0.001; H=46.019, P<0.001, respectively). The levels of CDT were similar in all stages of liver fibrosis (H=13.243, P=0.066), but %CDT values were different (H=13.948, P=0.050). Patients with no or mild fibrosis (F0, F0-F1, F1, F1-F2) had lower values of all noninvasive markers than patients with moderate, advanced, or severe fibrosis (F2, F3, F3-F4, F4) (P<0.001 for all comparisons). Then, patients with fibrosis stages F2, F3, and F3-F4 had lower values of APRI, Forns, FIB-4, GAPRI, and Hepascore than patients with cirrhosis (F4) (P=0.038, P=0.017, P=0.024, P=0.012, P=0.035, respectively). We also observed that patients with no fibrosis (F0) had lower values of APRI, Forns, FIB-4, GAPRI, and Hepascore

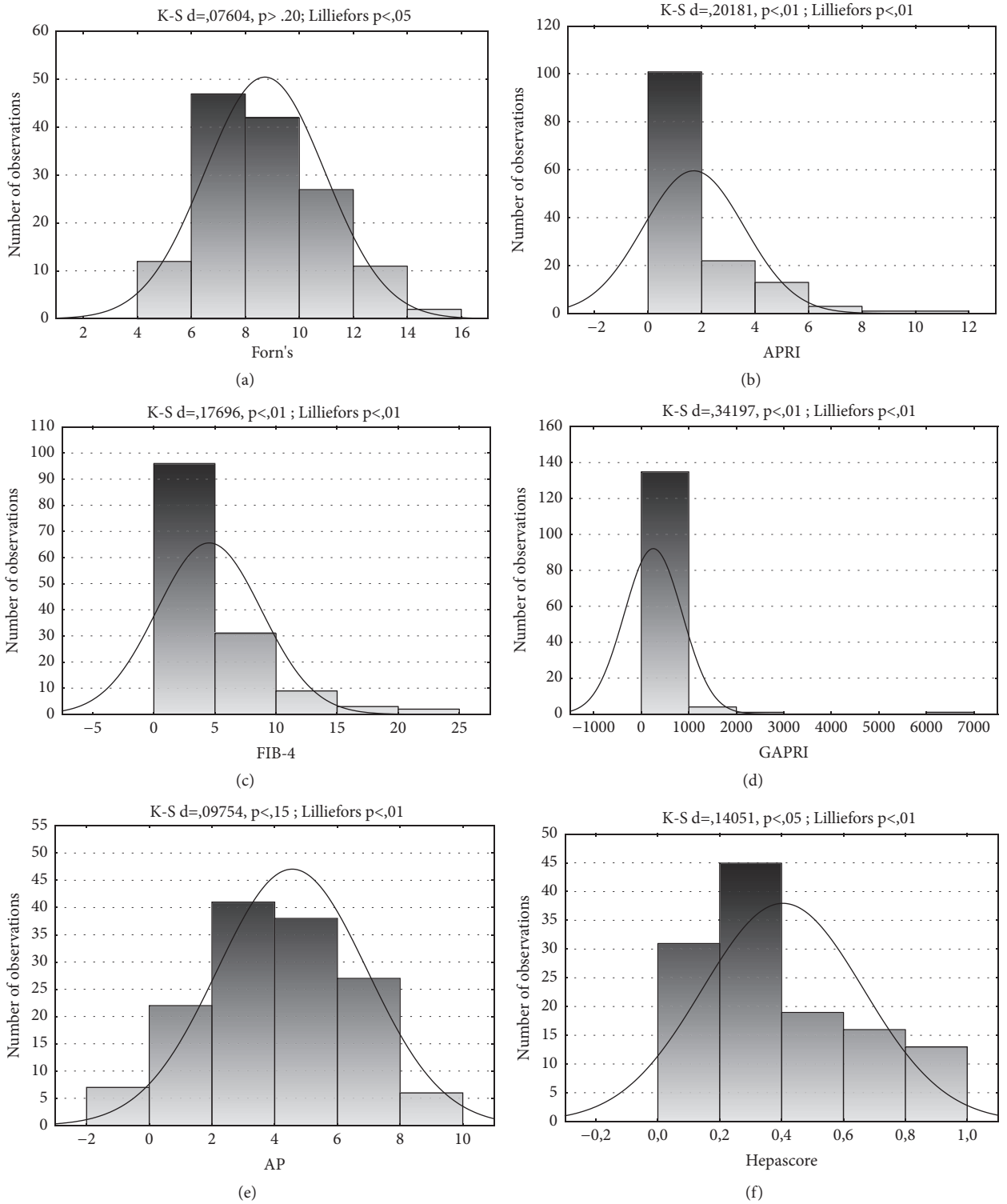

Figure 1: The distribution of noninvasive markers of liver fibrosis in alcoholics.

in comparison to patients with mild fibrosis (F0-F1 and F1) (Mann-Whitney U test: P=0.030, P=0.002, P=0.044, P=0.001, P=0.039, respectively). The values of AP index were similar in fibrosis stages F0, F0-F1, F1 (P=0.089) and F2, F3, F3-F4, F4 (P=0.173).

The average values of APRI, Forns, FIB-4, GAPRI, AP, and Hepascore were significantly higher in patients with cirrhosis (F4) in comparison with patients without fibrosis (F0) (P<0.001, P<0.001, P<0.001, P<0.001, P=0.002, P<0.001, respectively). In addition, the values of FIB-4 and GAPRI

Table 1: The values of noninvasive markers according to stages of liber fibrosis.

Marker	FibroTest score						
	F0 (n=52)	F1 (n=8)	F1-F2 (n=28)	F2 (n=11)	F3 (n=8)	F3-F4 (n=2)	F4 (n=12)
APRI	0.91±1.10	2.68±2.56	1.80±1.72	1.77±1.57	1.73±1.67	6.20±1.57	3.66±2.62
Forn's	7.31±1.68	9.38±1.44	9.10±1.92	9.89±1.66	8.96±1.32	12.97±1.14	11.50±1.79
FIB-4	2.60±2.24	5.20±3.04	4.72±3.92	6.60±6.28	3.60±1.94	17.20±7.72	9.69±4.65
GAPRI	58.90±85.34	164.67±101.30	187.55±279.82	175.29±152.20	320.66±333.47	858.87±20.00	827.54±709.34
AP	3.44±2.02	5.25±1.83	5.00±2.24	6.00±1.94	4.38±2.00	8.00±0.00	6.67±2.42
Hepascore	0.25±0.13	0.28±0.17	0.42±0.21	0.59±0.28	0.59±0.32	0.70±0.28	0.82±0.20
CDT	114.69±60.97	119.80±73.27	114.91±66.20	157.25±60.99	82.86±33.03	93.85±3.18	72.26±39.51
%CDT	4.38±2.60	5.55±3.92	5.14±3.35	7.23±2.49	3.62±1.75	4.72±0.45	2.95±1.60

Data are presented as mean ± SD.

Table 2: The values of noninvasive markers in patients with significant, no significant fibrosis, without and with cirrhosis.

Fibrosis Markers	No significant fibrosis (F0-F1, F1, F1-F2) (n=51)	Significant fibrosis (F≥2) (n=21)	Without cirrhosis (F0-F1, F1, F1-F2, F2, F3) (n=76)	Cirrhosis (F3-F4, F4) (n=14)
APRI	1.72 ± 1.73	2.18 ± 2.21	1.80 ± 1.74	4.02 ± 2.84
		P1=0.254		P2=0.001∗
Forn's	8.85 ± 1.79	9.83 ±1.83	9.12 ± 1.92	11.71 ± 1.77
		P1=0.063		P2<0.001∗
FIB-4	4.21 ± 3.37	6.47 ± 6.23	4.64 ± 4.01	10.77 ± 5.50
		P1=0.131		P2<0.001∗
GAPRI	158 ± 219	295 ± 300	276 ± 287	832 ± 652
		P1=0.020∗		P2<0.001∗
AP	4.74 ± 2.27	5.57 ± 2.13	4.91 ± 2.23	6.86 ± 2.28
		P1=-1.003		P2=0.004∗
Hepascore	0.39 ± 0.21	0.60 ± 0.28	0.43 ± 0.25	0.80 ± 0.21
		P1=0.008∗		P2<0.001∗
FibroTest	0.32 ± 0.08	0.58 ± 0.08	0.38 ± 0.14	0.87 ± 0.09
		P1<0.001∗		P2<0.001∗

Data are presented as mean ± SD. P1: P value between significant and no significant fibrosis; P2: P value between cirrhosis and no cirrhosis; ∗ means statistically significant difference.

in fibrosis stage F4 were higher than those in stage F0-F1 (P=0.005, P=0.018, respectively), and Hepascore in fibrosis stage F4 was higher compared to the stage F1 (P=0.004). The average value of GAPRI in fibrosis stage F4 was also higher than the one in the stage F1-F2 (P=0.025), and in the stage F3 it was higher than in the stage F0 (P=0.038). Hepascore, AP, and Forns scores were higher in patients with the stage F2 than in patients with the stage F0 (P=0.027, P=0.004, P=0.026, respectively). %CDT was lower in patients with advanced fibrosis/cirrhosis than in those with the stage F2 (P=0.011).

Three out of seven markers, GAPRI, Hepascore, and FibroTest, exhibited significantly higher results in patients with significant fibrosis (F≥2) in comparison to those with no significant fibrosis (F0-F1, F1, F1-F2), but all tested markers had higher values in patients with cirrhosis (F3-F4, F4) when compared to those without cirrhosis (F0-F1, F1, F1-F2, F2, F3) (Table 2).

Diagnostic power of liver fibrosis markers is presented in Table 3. Our study has shown that Forns index reached an ideal diagnostic accuracy (of 100%) and an ideal diagnostic power (AUC=1.0) for fibrosis detection in alcoholics. The remaining tests exhibited a high diagnostic power (AUCs over 0.9 for all with the exception of AP index) in the detection of fibrosis in alcoholic patients. We could observe that tested markers, except for Forns index, exhibited a high diagnostic specificity and positive predictive value (PPV). The markers APRI, GAPRI, and FIB-4 had similar diagnostic values (sensitivity: above 80%, specificity: 95% and above, accuracy: above 80%, PPV: above 99%, NPV: above 40%, and AUCs: above 0,930). Hepascore has shown a lower diagnostic

Table 3: Diagnostic value of liver fibrosis markers in alcoholics.

Marker	Cut-off	Sensitivity [%]	Specificity [%]	ACC [%]	PPV [%]	NPV [%]	AUC± SE
FIB-4	1.24	85.8	95.0	87.0	99.2	48.7	0.948±0.018
Forn's	4.11	100	100	100	100	100	1.0 ± 0.0
Hepascore	0.20	75.8	100	79.2	100	40.0	0.942±0.02
GAPRI	20.07	81.6	100	83.9	100	43.5	0.931±0.02
APRI	0.34	81.6	100	83.9	100	43.5	0.934±0.02
AP	4.00	64.5	90.0	67.7	97.8	26.5	0.867±0.036
FibroTest	0.21	61.3	93.9	69.9	96.6	46.5	0.795±0.031

accuracy (ACC<80%) than simple markers (FIB-4, Forns, GAPRI, and APRI).

Spearman's rank test demonstrated that there was a correlation between APRI, Forns, FIB-4, GAPRI, AP, Hepascore, and FibroTest in alcoholic patients (P<0.001 for all comparisons), but there was no correlation between CDT, %CDT, and FibroTest (P=0.468, P=0.556, respectively) (Figure 2).

4. Discussion

The evaluation of the severity of liver fibrosis and the associated inflammation is crucial for determination of therapeutic strategies, prognosis, and predicting potential complications in patients with alcoholic liver diseases [12]. An ideal noninvasive marker for the assessment of fibrosis in ALD should accurately and with high diagnostic sensitivity detect the presence of fibrosis as well as evaluating the stage of liver fibrosis. One of the most widely used patented liver fibrosis marker, FibroTest, has been validated in patients with hepatitis C, hepatitis B, nonalcoholic steatohepatitis, and alcoholic liver diseases [13–16]. It combines five biochemical serum markers (α2-macroglobulin, haptoglobin, γ-glutamyltransferase, bilirubin, and apolipoprotein A1) with patient's age and gender [16]. In the last large meta-analysis, FibroTest showed a good diagnostic accuracy for significant fibrosis (≥F2) and cirrhosis (F4) without discriminating between chronic liver diseases of different etiologies (mean standardized AUC for significant fibrosis was 0.84) [17]. Result of FibroTest reflects fibrosis stages according to the most used histological classification, METAVIR scoring system [18]. In our study the severity of liver damage was diagnosed on the basis of FibroTest. According to American College of Gastroenterology guidelines for alcoholic liver disease, "liver biopsy is not routinely recommended for diagnosis of alcoholic fatty liver disease. However, liver biopsy and noninvasive tools of fibrosis may be considered for diagnosis of steatohepatitis and/or liver fibrosis." [19]. The prospective study confirms the good diagnostic value of biochemical tests for fibrosis as compared with the histological analysis of liver biopsy with special caution FibroTest results with significant elevation of ALT, and/or GGT, and/or alpha-2-macroglobulin [20]. EASL Clinical Practical guidelines recommend liver biopsy for histological diagnosis of ALD [21]. According to these, liver biopsy can be done percutaneously in most patients and requires a transjugular approach in patients with a low platelet count and/or a prolonged prothrombin time. Additionally, liver biopsy is an invasive procedure with significant morbidity. Therefore, EASL guidelines do not recommend it for all patients with suspected ALD. We are aware that liver biopsy is considered as a gold standard for staging and grading of liver fibrosis but there are discordance between the degree of liver injury estimated by liver biopsy and that estimated by a panel biochemical markers. The main difficulty in this discordance analysis was the absence of a true reference standard for liver injury. The two main causes of failure for biopsy are sampling error and observer error. Poynard and coworkers stated that they "never made a management decision based on biopsy results for the following other causes: alcohol abuse, steatohepatitis, drug-induced liver disease, hemochromatosis or coinfection with hepatitis B virus or HCV." [22]. False-positive results for fibrosis based on FibroTest scores occurred in only hundredth of patients in the hospital based cohort and were attributable to various causes. It can be Gilbert syndrome (an increase in unconjugated bilirubin), hemolysis (an increase in unconjugated bilirubin and a decrease in haptoglobin), and acute inflammation (an isolated increase in α2-macroglobulin [22].

In this study, we have compared the diagnostic values of simple, noninvasive liver fibrosis markers in alcoholics. First, specificity of 100% and PPV of 100% for APRI, GAPRI, Forns index, and Hepascore have been noted. It means that there were no false-positive results in nonalcoholic patients. However, this excellent specificity was not accompanied by the highest sensitivity. It means that false negative results were present in alcoholic patients. The low negative predictive value of all markers, with the exception of Forns index, derived also from the high number of false negative results in alcoholics. It is clear that the cut-off points indicated by ROC curve are more shifted to lower values than indicated in literature data. For example, cut-off point of APRI for differentiation between no/significant/advanced fibrosis (from stage F0 to stage F3) and cirrhosis (F4) taken from literature equals 2.00, but in our study it is 0.34 [23]. The cut-off point of Hepascore for differentiation between stages F0-F2 and F3-F4 obtained the value of 0.50, but in our study it is only 0.20 [24]. The discriminative point for FIB-4 between no fibrosis and advanced fibrosis (F3-F4) was assigned to the value of 3.27, but cut-off point from ROC curve in this study reached

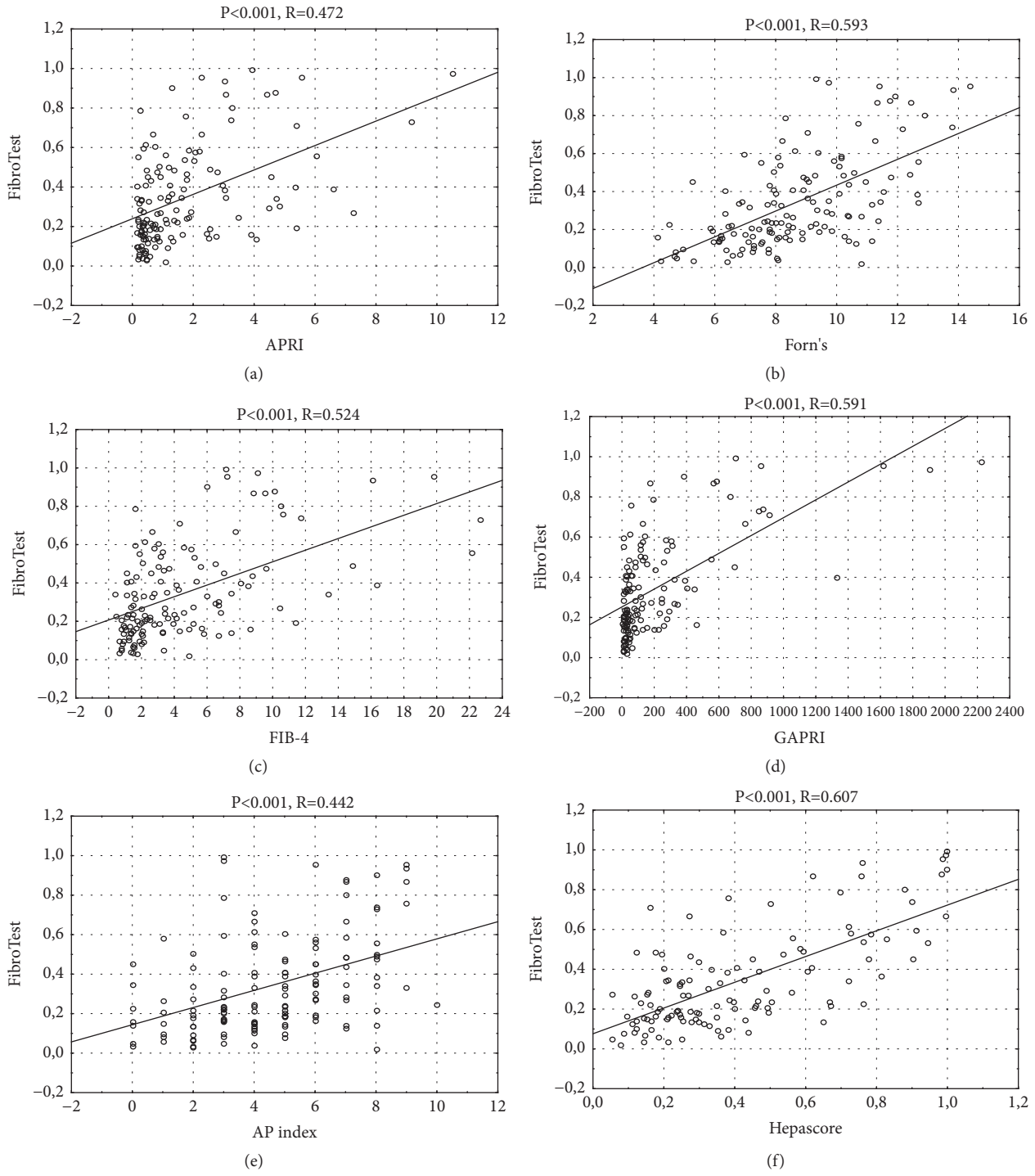

Figure 2: Spearman's rank correlation between tested noninvasive liver fibrosis markers and FibroTest.

the value of 1.24 [25]. Only the cut-off point for Forns index discriminating no fibrosis from advanced fibrosis (F≥3) equal to 4.20 was similar to the cut-off point in our study [26].

The fine specificity and PPV for APRI and GAPRI might be the result of these markers accounting only for liver enzymes (AST or GGT) and platelet count. Forns index additionally includes cholesterol, but reached an absolute diagnostic power. The level of cholesterol decreases significantly with the degree of fibrosis progression and, therefore, can reflect the degree of impairment of liver function. [27]. A score below 4.2 excluded significant fibrosis in patients with chronic hepatitis C with accuracy of 96% [26]. Forns et al. demonstrated that this model is not sufficient to detect significant fibrosis, because of its 66% positive predictive

value. Thiele et al. presenting biopsy-controlled study indicated Forns index as the best performing indirect index of advanced fibrosis [9]. On the other hand, FIB-4 based on both aminotransferases level and platelet count did not reach ideal diagnostic values. It is well established that aminotransferases (ALT and AST) constitute a part of standard laboratory panels examined in patients with liver diseases and that the most specific liver enzyme is alanine aminotransferase [28]. In turn, elevated number of platelets is a common complication in patients with chronic liver disease (PLT $<150 \times 10^9$/L) [29]. According to the published data, the value of APRI index over 1.5 accurately predicts significant fibrosis (AUC=0.88) and the value over 2.0 cirrhosis (AUC=0.94) in patients with chronic HCV [23]. APRI also correlated with the stage of fibrosis more strongly than AST or PLT alone. The next noninvasive marker of liver fibrosis tested, FIB-4, includes PLT count in addition to aminotransferases [25]. The FIB-4 score of ≥3.25 allows for a correct identification of patients who have significant fibrosis and could avoid liver biopsy. The values of FIB-4 ≥1.45 and ≥3.25 showed a good concordance with FibroTest (92.1% and 76%, respectively).

In our study, the values of all tested markers differed between stages of liver fibrosis. It is noteworthy that the majority of markers (except AP) allow differentiating between no fibrosis (F0) and mild fibrosis (F0-F1 and F1). In addition, patients with no, minimal, and mild fibrosis (F0, F0-F1, F1, F1-F2) had lower values of all markers than patients with moderate, advanced, or severe fibrosis/cirrhosis (F2, F3, F3-F4, F4). Finally, patients with the stages F2, F3, and F3-F4 fibrosis had lower values of markers (excluding AP) than patients with cirrhosis (F4). Secondly, we have shown that the markers APRI, GAPRI, and FIB-4, which incorporate liver enzymes (ALT, AST, GGT, and PLT), obtained similar diagnostic sensitivity, specificity, accuracy, PPV, NPV, and AUCs. The more complicated marker (Hepascore) had a lower diagnostic accuracy (ACC<80%) than simple markers (FIB-4, Forns, GAPRI, and APRI). This can be explained by the lower diagnostic sensitivity of this test (<80%). The imprecision of the determinations of multiple components should also be taken into consideration. However, adding cholesterol and platelet count to Forns index maximizes diagnostic values. Adding further tests to the algorithms decreases their diagnostic values, which is visible in the example of Hepascore.

Platelet counts and age are required to calculate the AP index. According to the study of Poynard and Bedossa, platelet count and age of patients are factors independently correlated with the presence of fibrosis and histological activity of liver disease [30]. With cut-off value of 6.0 or greater, the AP index diagnoses significant fibrosis with sensitivity of 52% and specificity of 93%. In our study, at the cut-off point of 4.0, we have obtained a similar sensitivity of 61.3% and specificity of 93.9%. The AP index demonstrated a lower diagnostic power to predict fibrosis in alcoholic patients.

The patented algorithm Hepascore combines the results obtained from the following biochemical tests: bilirubin, GGT, α2-macroglobulin, and hyaluronic acid [31]. Hepascore values of ≥0.50 can predict significant fibrosis with the specificity of 89%–92%. We have obtained cut-off point for Hepascore equal to 0.20, which is lower than that reported by Adams and coworkers [31]. A big advantage of Hepascore is the fact that GGT and bilirubin are measured routinely and α2-macroglobulin can be determined in any laboratory with a nephelometer using commercially available antibodies. The last parameter included in Hepascore, hyaluronic acid, is considered as a direct marker of liver fibrosis, as its synthesis is associated with the deposition of extracellular matrix [32]. It was found that serum levels of hyaluronic acid are elevated in chronic liver diseases in which the serum levels of ECM are also changed [33].

Carbohydrate-deficient transferrin (CDT) is one of the most used biomarkers of chronic alcohol abuse, mainly because of its high specificity [10]. However, the diagnostic efficiency of CDT as a marker of chronic alcohol abuse is diminished by its low diagnostic sensitivity. There are many clinical conditions that can affect the number of false-positive results in healthy controls. One of them is advanced liver diseases such as liver fibrosis and hepatocellular carcinoma [34]. According to these data the results of CDT are similar in patients with alcoholic cirrhosis and controls without liver diseases. Our alcoholic patients with cirrhosis (stage F4) have lower relative CDT values than alcoholics without fibrosis (stage F0). All tested fibrosis markers (APRI, Forns index, FIB-4, GAPRI, AP, and Hepascore) obtained higher results in stage F4 than in stage F0. It is necessary to mention here that alcoholics with score A cirrhosis (Child-Pugh scale) reached similar results of %CDT to the values in the healthy controls [11]. In this work the values of %CDT were different in the stages of liver fibrosis and were the highest in the advanced fibrosis (stage F2) and the lowest in alcoholic cirrhosis (stage F4). According to that we can conclude that the end stage of liver fibrosis diminished the sensitivity of CDT as a marker of chronic alcohol abuse, which is consistent with our previous study.

5. Conclusion

Our results suggest that simple blood tests incorporated in complex markers can be helpful in identifying a specific stages of liver fibrosis. Surprisingly, the patented algorithm, Hepascore, shows a lower diagnostic value in randomized group of alcoholics than simple markers involving only liver enzymes, platelet count, and cholesterol. According to our data the best marker of liver fibrosis in alcoholic patients is the Forns index.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Research Article

Transient Elastography and Ultrasonography: Optimal Evaluation of Liver Fibrosis and Cirrhosis in Patients with Chronic Hepatitis B Concurrent with Nonalcoholic Fatty Liver Disease

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Background and Aims. Concordance between transient elastography (TE) and ultrasonography (US) in assessing liver fibrosis in patients with chronic hepatitis B (CHB) and concurrent nonalcoholic fatty liver disease (NAFLD) has been rarely studied. This study aimed to evaluate the individual and combined performances of TE and US in assessing liver fibrosis and cirrhosis. *Patients and Methods*. Consecutive CHB patients with NAFLD were prospectively enrolled. TE and US examinations were performed, with liver biopsy as a reference standard. Receiver operating characteristic (ROC) curves were obtained to evaluate the diagnostic performance. Differences between the areas under the ROC curves (AUCs) were compared using DeLong's test. *Results*. TE and US scores correlated significantly with the histological fibrosis staging scores. TE was significantly superior to US in the diagnosis of significant fibrosis (AUC, 0.84 vs 0.73; P=0.02), advanced fibrosis (AUC, 0.95 vs 0.76; P<0.001), and cirrhosis (AUC, 0.96 vs 0.71; P<0.001). Combining TE with US did not increase the accuracy of detecting significant fibrosis, advanced cirrhosis, or cirrhosis (P=0.62, P=0.69, and P=0.38, respectively) compared to TE alone. However, TE combined with US significantly increased the positive predictive value for significant fibrosis when compared to TE alone. The optimal cut-off values of TE for predicting advanced fibrosis and cirrhosis were 8.7 kPa and 10.9 kPa, with negative predictive values of 92.4% and 98.7%, respectively. *Conclusions*. TE is useful for predicting hepatic fibrosis and excluding cirrhosis in CHB patients with NAFLD. A combination of TE and US does not improve the accuracy in assessing liver fibrosis or cirrhosis.

1. Introduction

Chronic hepatitis B (CHB) and nonalcoholic fatty liver disease (NAFLD) are chronic liver diseases with a high incidence worldwide [1, 2]. NAFLD has a spectrum comprised of fatty liver, nonalcoholic steatohepatitis (NASH), advanced fibrosis, and cirrhosis. CHB and NAFLD commonly cause cirrhosis and hepatocellular carcinoma (HCC) [3, 4]. Currently, the increasing rate of NAFLD in CHB patients is alarming [5]. A study found that NASH (a type of NAFLD) was independently correlated with liver fibrosis in patients with CHB [6]. Moreover, another cohort study found that concurrent fatty liver can independently increase hepatitis B virus (HBV)-related HCC development 7.3-fold [7]. These reports suggest that timely and accurate diagnosis of liver fibrosis in CHB patients with NAFLD is urgent. Moreover, the assessment of liver fibrosis in patients with chronic liver diseases, especially those with coetiologies, is mandatory and recommended by international practice guidelines. Liver biopsy (LB) has been the gold standard for assessing liver fibrosis [8]. However, it is invasive and may result in several complications [9]. These disadvantages make it impractical to be performed regularly

in clinical practice. Therefore, accurate and noninvasive tools that can clinically assess liver fibrosis in CHB patients with NAFLD are urgently needed.

Abdominal ultrasonography (US) is performed on CHB patients to assess structural changes and screen for HCC. Several US signs, such as an uneven or undulating liver surface, irregular echotexture of the liver parenchyma, spleen size, and changes in the diameters of vessels, have been found to be correlated with liver cirrhosis [10, 11]. Transient elastography (TE) is an ultrasound-based technology measuring liver stiffness by the difference in velocity of elastic shear wave propagation across the liver. TE has been repeatedly validated and has shown overall good accuracy in evaluating fibrosis and cirrhosis in different settings [12]. However, TE could be influenced by patient-dependent factors, including liver inflammation, liver congestion, and biliary obstruction [12, 13]. Therefore, the results should be interpreted with accurate clinical information.

The existence of NAFLD may cause morphological changes in the liver of CHB patients, which may make it more difficult to accurately evaluate the degree of fibrosis. To our knowledge, no comparison between US and TE in assessing liver fibrosis in CHB patients with NAFLD has been previously reported. Thus, the aim of this study was to evaluate the individual and combined performances of TE and US in assessing liver fibrosis and cirrhosis and to determine when TE should be added to US in CHB patients with NAFLD, using histological evaluation as the reference standard.

2. Patients and Methods

2.1. Patients. Between July 2013 and February 2018, adult CHB patients with NAFLD who were consecutively admitted to our hospital to undergo LB were prospectively enrolled. CHB patients were diagnosed as those who displayed hepatitis B surface antigen (HBsAg) positivity for more than 6 months [14, 15]. NAFLD was defined by the presence of hepatic steatosis (≥5%) and the absence of a history of significant alcohol consumption (where absence is defined as alcohol intake <20 g/day for men and intake <10 g/day for women) and the absence of other etiologies that may cause hepatic steatosis [16–18]. The exclusion criteria included the following: age <18, body mass index (BMI) ≥30 kg/m^2, aspartate aminotransferase (AST) or alanine aminotransferase (ALT) ≥5 times of the upper limit of normal (ULN), coinfection with another hepatitis virus or human immunodeficiency virus (HIV), concurrent tumors, receiving antiviral therapy, significant alcohol consumption, currently taking medications that may induce hepatic steatosis (including corticosteroids, methotrexate, and tamoxifen), and pregnancy. Blood samples were obtained on the day of LB examination. The following data were collected from all patients: age; sex; weight; height; ALT, AST, total bilirubin, albumin, alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT), fasting glucose, and lipid levels; platelet counts; and prothrombin time activity. BMI was calculated as the body weight (kg)/height^2 (m^2). The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by our hospital's ethics committee. Informed consent was obtained from each patient.

2.2. Liver Histological Analysis. A US-guided percutaneous LB (length >15 mm) of the right lobe was performed using a 16-gauge Magnum needle (Bard, Tempe, AZ, USA). Specimens were fixed in formalin and embedded in paraffin. Liver histology was assessed separately by two liver pathologists with more than 10 and 20 years of experience without knowledge of the clinical data or the TE and US results. Liver fibrosis was staged on a 0 to 4 scale according to the METAVIR scoring system [19]: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis and few septa; F3, numerous septa without cirrhosis; F4, cirrhosis. Steatosis was defined as the percentage of fat in hepatocytes and was graded as follows: 0, steatosis <5%; 1, 5-33% steatosis; 2, 34-66% steatosis; 3, steatosis >66% [17].

2.3. Liver Stiffness Measurement (LSM) by TE. TE was performed with a FibroScan system (Echosens, Paris, France) using the M probe. After an overnight fast, patients underwent TE examination within 3 days of LB by trained operators who had previously performed at least 500 scans in patients with chronic liver disease. Operators were blinded to the clinical data and pathology results. The value expressed in kilopascal (kPa) was recorded as a representation of the LSM. Up to 10 valid measurements were performed on each patient. A success rate above 60% and an interquartile range/median ratio of less than 30% were considered reliable [20].

2.4. Abdominal Ultrasound Examination. All participants underwent a B-mode liver ultrasound scan within 1 week of LB. Each scan was performed using a Supersonic Imagine Aixplorer ultrasound system (Supersonic Imagine, Aix-en-Provence, France) equipped with an SC6-1 convex array probe with a frequency of 1-6 MHz. Two sonographers (with 10 years and 15 years of US experience) reviewed the B-mode images independently to assess the interobserver agreement without knowledge of the TE results or clinical data, but the final results were obtained in consensus to assess the diagnostic performance. A US scoring system developed in previous studies [10, 11] was used to evaluate the degree of liver cirrhosis (Figure 1). The scoring system included the following signs and scores: the liver surface (1 for a smooth surface, 2 for an uneven or wavy surface, and 3 for an irregular nodular surface); the liver parenchyma (1 for homogeneous parenchyma, 2 for heterogeneous parenchyma with fine scattered hyperechoic or hypoechoic areas, and 3 for coarse liver parenchyma with an irregular pattern); hepatic vein contour (1 for a smooth vessel wall, 2 for an obscured or slightly irregular vessel wall, and 3 for an irregular vessel wall with a narrowed diameter); a spleen index, calculated as the product of the oblique and diagonal diameters (1 for product <20cm^2 and 2 for product >20cm^2). The US scores ranged from 4 for a normal liver to 11 for advanced liver cirrhosis, and the scores were recorded in a standard form.

2.5. Virological Analyses. Serum HBV markers, including HBsAg, hepatitis B e antigen (HBeAg), and hepatitis B e antibody (HBeAb), were determined using the Elecsys system

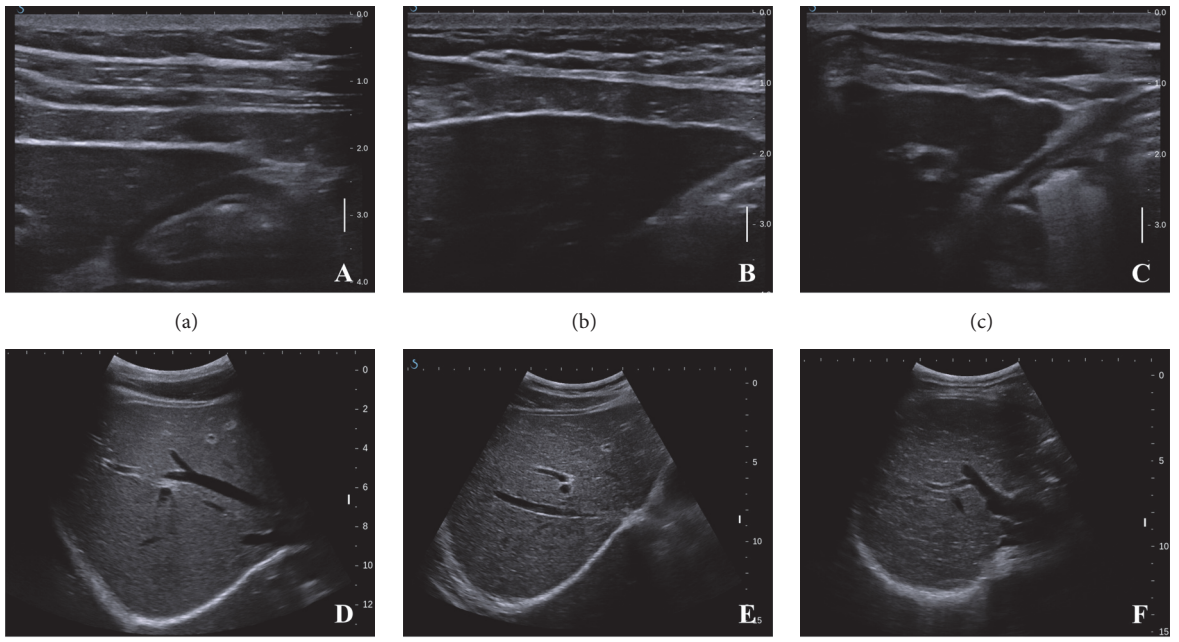

Figure 1: B-mode images of conventional ultrasonography (US) scoring system. (a) Smooth liver surface, score of 1. (b) Uneven liver surface, score of 2. (c) Irregular nodular liver surface, score of 3. (d) Homogeneous parenchyma, score of 1; and smooth hepatic vein vessel wall, score of 1. (e) Heterogeneous liver parenchyma with fine scattered hyperechoic or hypoechoic areas, score of 2. Obscured or slightly irregular hepatic vein vessel wall, score of 2. (f) Coarse liver parenchyma with an irregular pattern, score of 3.

(Hoffmann-La Roche, Basel, Switzerland). Serum HBV-DNA levels were quantified by Cobas TaqMan (Hoffmann-La Roche, Basel, Switzerland) according to the manufacturer's instructions. The limit of detection of the assay was 20 IU/ml.

2.6. Statistical Analysis. Statistical analyses were performed using SPSS version 20.0 software (Chicago, IL, USA) and MedCalc version 15.2.2 (MedCalc Software, Mariakerke, Belgium). The data are expressed as frequencies, medians and ranges or means and standard deviations, as appropriate. Differences in TE were analyzed using Student's t-tests. The interobserver reproducibility of US scores was assessed by calculating the intraclass correlation coefficient. Spearman correlation coefficients were used to analyze the correlation between TE, US scores, hepatic steatosis, and fibrosis stage, and the correlation coefficients were compared using Fisher's Z test. Receiver operating characteristic (ROC) curves were used to assess the overall accuracy and to identify optimal cut-off values. The optimal cut-off values were selected according to Youden's index. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and positive likelihood ratio (PLR) and negative likelihood ratio (NLR) were calculated with 95% confidence intervals (CIs). Differences between the areas under the ROC curves (AUCs) were compared using DeLong's test. A 2-tailed P <0.05 was considered statistically significant.

3. Results

3.1. Characteristics of CHB Patients with NAFLD. We included 429 consecutive, treatment-naive CHB patients who underwent TE, US, and LB. Forty-two patients were excluded for the following reasons: 2 patients were coinfected with hepatitis C virus (HCV), 4 patients had a history of significant alcohol consumption, 1 patient had concurrent HCC, 10 patients had a BMI ≥30 kg/m^2, and 25 patients had ALT or AST levels ≥5 times of ULN. Among the remaining patients, 293 patients did not have NAFLD (hepatic steatosis <5%), and 94CHB patients had NAFLD (hepatic steatosis ≥5%), resulting in a rate of NAFLD in CHB patients of 24.3% (94/387). A summary of patients' deposition is shown in Figure 2. Among the CHB patients with NAFLD, they had a mean age of 36.90 years; only one patient had hypertension, and none had diabetes mellitus. 29 (30.9%) patients had at least one risk factor for metabolic syndrome. And 16 (17%) patients had the diagnosis of metabolic syndrome. Overall, fibrosis stages of the enrolled patients were scored as F0 (n=19), F1 (n=28), F2 (n=16), F3 (n=17), and F4 (n=14).

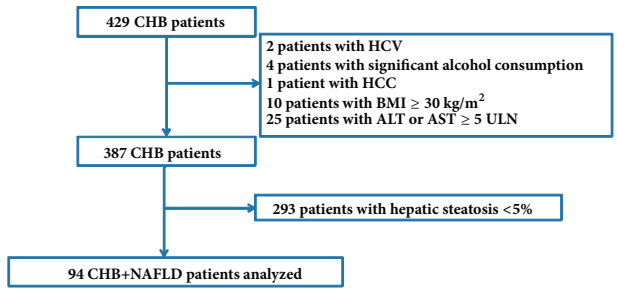

Figure 2: Selection and deposition of patients.

Table 1: Patients' characteristics.

Characteristic	Standard Value (Range)	Patients (n=94)
Mean age (years)	NA	36.90±8.17
Male gender (n, %)	NA	85 (90.4%)
Body mass index (BMI) (kg/m^2)	NA	24.20±2.68
BMI ≥ 25 (n, %)	NA	36 (37.9%)
Aspartate aminotransferase (IU/L)	15-40	29 (15-128)
Alanine aminotransferase (IU/L)	3-35	40.5 (9-173)
Total bilirubin (umol/L)	4-23.9	12.7 (5.6-32.5)
Albumin (g/L)	36-51	45.5 (36.2-51.6)
g-Glutamyltransferase (IU/L)	10-60	32.5 (12-283)
Alkaline phosphatase (IU/L)	45-125	72.5 (33-207)
Platelets count (10^3/mm^3)	100–350	200.43±54.87
Prothrombin time activity (%)	70–120	96.18±11.68
Fasting glucose (mmol/L)	3.9-6.1	4.90 (3.64-8.61)
Fasting glucose ≥5.6 mmol/L (n, %)	≥5.6	23 (24.5%)
Total cholesterol (mmol/L)	3.1-5.7	4.78±0.84
Triglyceride (mmol/L)	0.34-1.92	1.19 (0.39-4.89)
Triglyceride ≥ 1.7 mmol/L (n, %)	≥1.7	22 (23.4%)
HDL-cholesterol (mmol/L)	0.78-2.00	1.15±0.23
Reduced HDL-cholesterol (mmol/L) (n, %)	<1.03 in men <1.29 in women	29 (30.8%)
LDL-cholesterol (mmol/L)	2.07-3.10	3.17±0.83
Elevated LDL-cholesterol (n, %)	>3.10	52 (55.3%)
HBeAg positive (n, %)	>1	38 (40.4%)
HBV-DNA (log10 IU/mL)	<20 IU/mL	4.83 (1.54-8.68) *
Ultrasonography score		
4/5	NA	0/28
6/7	NA	29/18
8/≥9	NA	11/8
Fibrosis score (METAVIR)		
F0 (n, %)	NA	19 (20.2%)
F1 (n, %)	NA	28 (29.7%)
F2 (n, %)	NA	16 (17.0%)
F3 (n, %)	NA	17 (18.1%)
F4 (n, %)	NA	14 (15.0%)
Hepatic steatosis		
≥5%	NA	90 (95.7%)
34-66%	NA	3 (3.2%)
>66%	NA	1 (1.1%)

Unless otherwise indicated, data were expressed as means ± standard deviations or medians and ranges. *Eleven patients had undetectable HBV-DNA loads. HDL=high density lipoprotein; LDL= low-density lipoprotein; NA=not applicable.

The median degree of steatosis was 10% (range, 5-70%). The patients' characteristics are summarized in Table 1.

3.2. Liver Stiffness Measurement (LSM) by TE. TE examination succeeded in 94 patients with CHB and NAFLD. The success rate of TE examination was therefore 100%. As the IQR/LSM ratios were less than 30%, the results of TE measurements were considered to be reliable. Liver stiffness measured with TE ranged from 3.2 to 38.5 kPa (IQR, 5.1-9.5 kPa) (Figure 3, Table 2). The mean LSM was 5.68, 5.83, 6.34, 10.34, and 18.84 for patients with F0, F1, F2, F3, and F4, respectively. The mean LSM gradually increased from F0 to F2 (F0 vs F1, P=0.745; F1 vs F2, P=0.293). Then, the LSM increased significantly from F2 to F4 (F2 vs F3, P<0.001; F3 vs F4, P=0.002).

3.3. US Scoring System. Figure 3 shows the distributions of US scores at different fibrosis stages. The interobserver

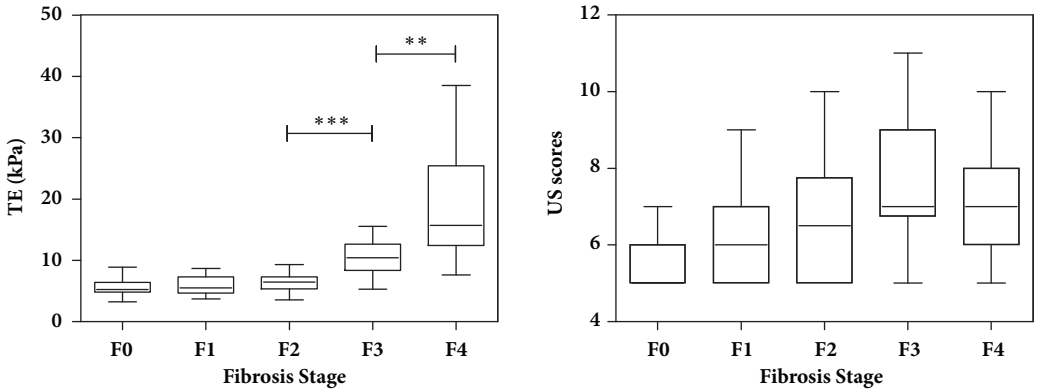

Figure 3: Box and whisker plots of TE and US scores at each fibrosis stage. The central box represents values from lower to upper quartile (25^{th} -75^{th} percentile). The line through each box represents the median. The mean liver stiffness measured with TE increased significantly from F2 to F4 (F2 vs F3, P<0.001; F3 vs F4, P=0.002). **, P <0.01. ***, P <0.001.

Table 2: Distribution of liver stiffness measured by TE at different fibrosis stages.

TE (kPa) and P value	F0 (n=19)	F1 (n=28)	F2 (n=16)	F3 (n=17)	F4 (n=14)
Mean value*	5.68±1.48	5.83±1.52	6.34±1.61	10.34±2.91	18.84±8.35
Median value†	5.2 (3.2-8.9)	5.5 (3.7-8.7)	6.5 (3.5-9.3)	10.4(5.3-15.5)	15.7 (7.6-38.5)
P value‡	Not applicable	0.745	0.293	<0.001	0.002

*Data were expressed as means ± standard deviations.
†Data were expressed as medians and ranges.
‡Mean value compared with the next lower fibrosis stage.

reproducibility of the US scores was 0.93 (95% CI: 0.89, 0.95). The comparison of AUCs revealed that the US scores had superior trends to other measures (the liver surface scores, liver parenchyma scores, hepatic vein contour scores, and the spleen index) in the diagnosis of significant liver fibrosis (AUC, 0.73 vs 0.67, 0.64, 0.67, and 0.51, respectively), in the diagnosis of advanced fibrosis (AUC, 0.76 vs 0.72, 0.63, 0.68, and 0.51, respectively), and in the diagnosis of cirrhosis (AUC, 0.71 vs 0.67, 0.64, 0.58, and 0.51, respectively). Hence, the US scores were selected for further analysis.

3.4. Correlations among TE, US, Hepatic Steatosis, and Fibrosis Stage. In the present study, LB was used as the reference standard. The correlation coefficients of TE and US scores with fibrosis stage were 0.69 (95% CI: 0.55, 0.78; P<0.001) and 0.47 (95% CI: 0.30, 0.61; P<0.001), respectively. The correlation coefficients of TE were significantly higher than that of the US scores (P=0.022). However, the degree of hepatic steatosis did not correlate with fibrosis stage (r=0.041, P=0.69), TE scores (r=0.037, P=0.72), or US scores (r=0.091, P=0.38). Next, in order to evaluate the impact of hepatic steatosis on the performance of TE in assessing liver fibrosis, patients were stratified into three groups, group 1 (5%≤hepatic steatosis<10%, n=33), group 2 (10%≤hepatic steatosis<20%, n=26), and group 3 (hepatic steatosis≥20%, n=35). The correlation coefficients of TE with fibrosis stage in different groups were 0.69 (95% CI: 0.46, 0.84; P<0.001), 0.61 (95% CI: 0.30, 0.81; P<0.001), and 0.72 (95% CI: 0.50, 0.85; P<0.001), respectively. However, no significant differences existed among the three groups in correlation coefficient (all P>0.05). Moreover, the comparison of AUCs revealed that no significant differences were found among the three groups in the assessment of liver fibrosis using TE (all P>0.05, Table 3). Taken together, these data indicate that the degree of hepatic steatosis may not influence the performance of TE in the assessment of liver fibrosis.

3.5. TE and US Scores for Significant Fibrosis Assessment (F ≥2). Figure 4 shows the diagnostic performance of TE, US, and TE combined with US, as assessed by ROC curves. Table 4 shows the AUCs and predictive values for significant fibrosis. Comparison of the AUCs revealed that both TE and TE plus US were significantly superior to US in the diagnosis of significant fibrosis (AUC, 0.84 vs 0.73, P=0.02; AUC, 0.85 vs 0.73, P=0.002). Compared with TE alone, combining TE with US did not increase the diagnostic performance of detecting significant fibrosis (AUC, 0.85 vs 0.84, P=0.62). However, their combination significantly increased the specificity (95.7% vs 76.6%, P<0.001) and PPV (94.3% vs 77.1%, P=0.002) compared to TE alone.

3.6. TE and US Scores for Advanced Fibrosis Assessment (F ≥3). The diagnostic performance of TE, US, and TE combined with US in advanced fibrosis, as assessed by ROC curves, is shown in Figure 4. Table 4 shows the AUCs and predictive values for TE and US. The comparison of AUCs revealed that TE was significantly superior to US in the diagnosis of advanced fibrosis (AUC, 0.95 vs 0.76, P<0.001). Compared with TE alone, combining TE with US did not improve the diagnostic performance of detecting advanced fibrosis

Table 3: Diagnostic performances of TE in patients with different degrees of hepatic steatosis for liver fibrosis and cirrhosis assessment.

Statistic	5% ≤HS<10% (n=33)	10%≤HS<20% (n=26)	HS ≥20% (n=35)
Significant fibrosis assessment (F≥2)			
AUC	0.78 (0.60, 0.90)	0.79 (0.59, 0.93)	0.93 (0.79, 0.99)
Standard error	0.083	0.092	0.041
Cut-off value	6.6	6.8	8.4
Sensitivity (%)	83.3 (58.6, 96.4)	66.7 (34.9, 90.1)	76.5 (50.1, 93.2)
Specificity (%)	66.7 (38.4, 88.2)	85.7 (57.2, 98.2)	100.0 (81.5, 100.0)
PPV (%)	75.0 (50.9, 91.3)	80.0 (44.4, 97.5)	100.0 (75.3, 100.0)
NPV (%)	76.9 (46.2, 95.0)	75.0 (47.6, 92.7)	81.8 (59.7, 94.8)
Comparison of AUCs			
5% ≤HS<10%	-	P=0.94	P=0.11
10%≤HS<20%	P=0.94	-	P=0.16
HS ≥20%	P=0.11	P=0.16	-
Advanced fibrosis assessment (F≥3)			
AUC	0.96 (0.83, 0.99)	0.94 (0.77, 0.99)	0.95 (0.82, 0.99)
Standard error	0.037	0.054	0.039
Cut-off value	8.6	7.2	8.4
Sensitivity (%)	90.0 (55.5, 99.7)	85.7 (42.1, 99.6)	85.7 (57.2, 98.2)
Specificity (%)	95.7 (78.1, 99.9)	89.5 (66.9, 98.7)	95.2 (76.2, 99.9)
PPV (%)	90.0 (55.5, 99.7)	75.0 (34.9, 96.8)	92.3 (64.0, 99.8)
NPV (%)	95.7 (78.1, 99.9)	94.4 (72.7, 99.9)	90.9 (70.8, 98.9)
Comparison of AUCs			
5% ≤HS<10%	-	P=0.76	P=0.85
10%≤HS<20%	P=0.76	-	P=0.88
HS ≥20%	P=0.85	P=0.88	-
Cirrhosis assessment (F=4)			
AUC	1.00 (0.89, 1.00)	0.98 (0.79, 1.00)	0.90 (0.75, 0.98)
Standard error	0.000	0.029	0.076
Cut-off value	10.7	10.0	10.9
Sensitivity (%)	100.0 (59.0, 100.0)	100.0 (15.8, 100.0)	80.0 (28.4, 99.5)
Specificity (%)	100.0 (86.8, 100.0)	95.8 (78.9, 99.9)	86.7 (69.3, 96.2)
PPV (%)	100.0 (59.0, 100.0)	66.7 (9.4, 99.2)	50.0 (15.7, 84.3)
NPV (%)	100.0 (86.8, 100.0)	100.0 (85.2, 100.0)	96.3 (81.0, 99.9)
Comparison of AUCs			
5% ≤HS<10%	-	P=0.49	P=0.19
10%≤HS<20%	P=0.49	-	P=0.33
HS ≥20%	P=0.19	P=0.33	-

Data in parentheses were 95% confidence interval. AUC=area under the ROC curve. HS=hepatic steatosis. PPV=positive predictive value. NPV=negative predictive value.

(P=0.69). When TE alone was used to predict advanced fibrosis, the PPV and NPV were 92.9% and 92.4%, respectively, when 8.7 kPa was selected as the optimal cut-off value.

3.7. TE and US Scores for the Diagnosis of Cirrhosis (F=4). TE, US, and TE combined with US for the evaluation of subclinical cirrhosis (F=4) were assessed by ROC curves, and the results are shown in Figure 4. Table 4 shows the AUCs and predictive values for TE and US. The comparison of AUCs revealed that TE was significantly superior to US in diagnosing cirrhosis (AUC, 0.96 vs 0.71, P<0.001). In addition, combining TE with US did not improve the diagnostic performance of detecting liver cirrhosis compared with TE alone (P=0.38). Compared to US, TE had a significantly higher sensitivity (92.9% vs 50.0%; P<0.001) and PPV (72.2% vs 36.8%; P<0.001) in predicting cirrhosis. Moreover, when using the cut-off value of 10.9 kPa, TE showed a high NPV of 98.7% in excluding the diagnosis.

4. Discussion

We herein report a single-center experience comparing TE, US, and their combination in the assessment of fibrosis in a cohort of CHB patients with NAFLD. In this study, comparison of AUCs revealed that TE was significantly superior to US in the diagnosis of fibrosis and subclinical cirrhosis.

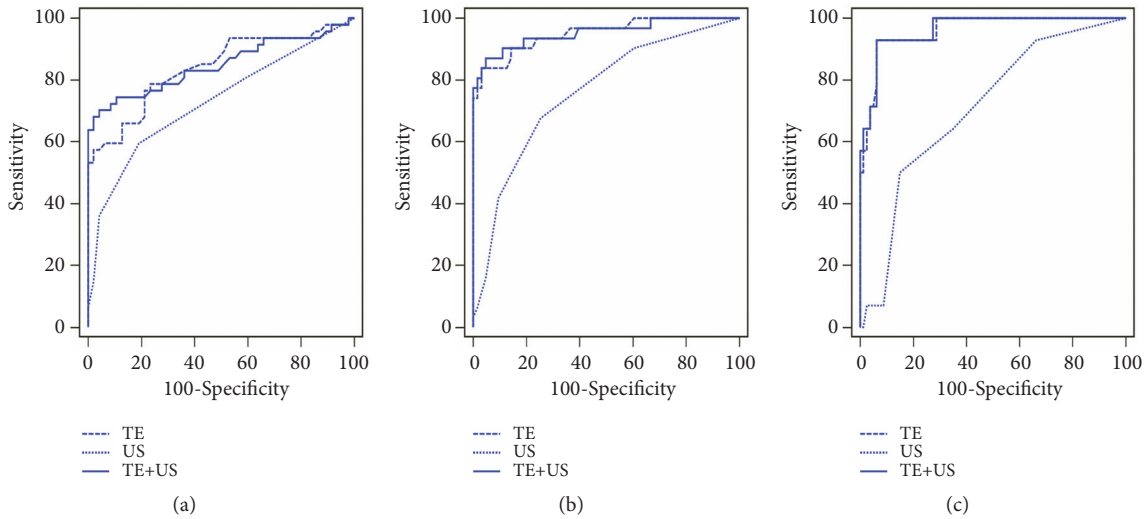

Figure 4: ROC curves of TE, US, and TE combined with US for significant fibrosis assessment (a), advanced fibrosis assessment (b), and cirrhosis assessment (c) in CHB patients concurrent with nonalcoholic fatty liver disease.

Combining TE with US did not increase the accuracy of detecting significant fibrosis, advanced cirrhosis, or cirrhosis compared to TE alone. However, combining TE with US can significantly increase the PPV in predicting significant fibrosis when compared to TE alone. The optimal cut-off values of TE for advanced fibrosis and cirrhosis were 8.7 kPa and 10.9 kPa, with NPVs of 92.4% and 98.7%, respectively.

HBV infection is a major etiology of chronic liver disease worldwide. In the past decade, NAFLD has emerged as a common liver disorder in the general population [3]. Accordingly, the number of CHB patients with concomitant NAFLD is rapidly growing. Approximately 25% of CHB patients have coexisting NAFLD [21]. In the present study, the rate of NAFLD in CHB patients was estimated to be 24.3%, which is similar to the general incidence, and approximately one-third of the current patients had at least one risk factor for metabolic syndrome. Several reports have revealed that metabolic syndrome can increase the risk of liver fibrosis progression and liver cirrhosis in CHB patients [22, 23]. Since the prognosis of patients and the treatment selection depends on fibrosis stages, accurate assessment of liver fibrosis is urgent.

Although TE may be affected by several factors, it performs well in CHB patients and may reduce the need for LB [12, 24]. The effects of hepatic steatosis on TE performance in patients with chronic HCV and NAFLD may be more definitive, resulting in overestimations of the liver fibrosis stage [25, 26]. However, the role of hepatic steatosis in CHB remains controversial [27, 28]. In the present study, the extent of hepatic steatosis did not correlate with TE, US, or the histological fibrosis stage (all $P>0.05$). Also, no significant differences existed in correlation coefficient of TE with fibrosis stage among different degrees of hepatic steatosis (all $P>0.05$). In addition, TE performs at a good to excellent level of accuracy in detecting fibrosis and subclinical cirrhosis, which is similar to the results found in a previous meta-analysis of a large sample of CHB patients [24]. These findings indicate that TE could be useful and reliable in assessing liver fibrosis in CHB patients, even in those patients concurrent with NAFLD. Nevertheless, the exact impact of hepatic steatosis on TE performance requires further evaluation.

A US scoring system consisting of four factors has been developed and has proven to be reliable in evaluating liver fibrosis and cirrhosis [10]. Since US is routinely used to assess structural changes caused by CHB, it is necessary to compare TE with US before introducing TE to evaluate fibrosis in CHB patients with NAFLD. To our knowledge, this study is the first to demonstrate the performance of TE and US in CHB patients with NAFLD. TE proved to be superior to US in the diagnostic performance of predicting significant fibrosis ($P=0.02$) and advanced fibrosis ($P<0.001$). Furthermore, a combination of TE and US was equivalent to TE in diagnostic accuracy ($P=0.62$ and $P=0.69$). However, TE and US combination increased the PPV from 77.1% to 94.3% in predicting significant fibrosis. These results indicate that TE alone was useful to assess significant fibrosis and advanced fibrosis in CHB patients with NAFLD, but TE combined with US can help predict significant fibrosis.

Cirrhosis is a high-risk factor for developing HCC and complications caused by portal hypertension. Therefore, early detection of subclinical cirrhosis can help identify high-risk patients earlier and start the optimized therapy accordingly. US is a widely available tool for diagnosing cirrhosis, despite its low sensitivity and high specificity. In the present study, the AUC of US in detecting cirrhosis was 0.71, which is relatively lower compared to the results of a previous study (AUC, 0.79) [10]. This variation could be related to differences in the enrolled patients. A previous study mainly enrolled viral patients (HBV and HCV), and only two patients had hepatic steatosis. However, this study recruited only CHB patients with NAFLD. More importantly, TE demonstrated excellent diagnostic performance in predicting subclinical cirrhosis, with high sensitivity and specificity of 92.9% and 93.8%, respectively. Furthermore, the overall diagnostic

Table 4: Diagnostic performances of TE, US, and TE combined with US in CHB patients with NAFLD for liver fibrosis and cirrhosis assessment.

Statistic	TE	US	TE plus US
Significant fibrosis assessment (F≥2)			
AUC	0.84 (0.75, 0.91)	0.73 (0.63, 0.82)	0.85 (0.76, 0.92)
Standard error	0.042	0.051	0.043
Cut-off value	6.6	6	0.61
Sensitivity (%)	78.7 (64.3, 89.3)	59.6 (44.3, 73.6)	70.2 (55.1, 82.7)
Specificity (%)	76.6 (62.0, 87.7)	80.9 (66.7, 90.9)	95.7 (85.5, 99.5)
PPV (%)	77.1 (62.7, 88.0)	75.7 (58.8, 88.2)	94.3 (80.8, 99.3)
NPV (%)	78.3 (63.6, 89.1)	66.7 (52.9, 78.6)	76.3 (63.4, 86.4)
Positive LR	3.36 (2.0, 5.8)	3.11 (1.7, 5.9)	16.50 (4.2, 64.9)
Negative LR	0.28 (0.2, 0.5)	0.50 (0.3, 0.7)	0.31 (0.2, 0.5)
Advanced fibrosis assessment (F≥3)			
AUC	0.95 (0.89, 0.98)	0.76 (0.66, 0.84)	0.95 (0.89, 0.99)
Standard error	0.024	0.052	0.026
Cut-off value	8.7	6	0.39
Sensitivity (%)	83.9 (66.3, 94.5)	67.7 (48.6, 83.3)	87.1 (70.2, 96.4)
Specificity (%)	96.8 (89.0, 99.6)	74.6 (62.1, 84.7)	95.2 (86.7, 99.0)
PPV (%)	92.9 (76.5, 99.1)	56.8 (39.5, 72.9)	90.0 (73.5, 97.9)
NPV (%)	92.4 (83.2, 97.5)	82.5 (70.1, 91.3)	93.7 (84.8, 98.3)
Positive LR	26.4 (6.7, 104.2)	2.7 (1.6, 4.3)	18.3 (6.0, 55.6)
Negative LR	0.17 (0.07, 0.4)	0.43 (0.3, 0.7)	0.14 (0.05, 0.3)
Cirrhosis assessment (F=4)			
AUC	0.96 (0.90, 0.99)	0.71 (0.61, 0.80)	0.96 (0.90, 0.99)
Standard error	0.023	0.071	0.022
Cut-off value	10.9	7	0.14
Sensitivity (%)	92.9 (66.1, 99.8)	50.0 (23.0, 77.0)	92.9 (66.1, 99.8)
Specificity (%)	93.8 (86.0, 97.9)	85.0 (75.3, 92.0)	93.8 (86.0, 97.9)
PPV (%)	72.2 (46.5, 90.3)	36.8 (16.3, 61.6)	72.2 (46.5, 90.3)
NPV (%)	98.7 (92.9, 100.0)	90.7 (81.7, 96.2)	98.7 (92.9, 100.0)
Positive LR	14.9 (6.3, 35.1)	3.3 (1.6, 7.0)	14.9 (6.3, 35.1)
Negative LR	0.08 (0.01, 0.5)	0.59 (0.3, 1.0)	0.08 (0.01, 0.5)

Data in parentheses were 95% confidence interval. AUC=area under the ROC curve. PPV=positive predictive value. NPV=negative predictive value. LR=likelihood ratio.

performance of TE was significantly different from that of US (P<0.001). Compared with TE alone, combining TE with US did not improve the diagnostic performance (P=0.38). These findings are of vital importance because TE is much simpler and safer than LB is. Given the high NPV of TE (98.7%), it is unnecessary to perform LB in patients who are suspected of cirrhosis at US examination. Patients can avoid LB and start an antiviral therapy program earlier. Thus, we suggest that TE should be performed on patients with doubtful cirrhosis at routine US.

The optimum cut-off value selected for predicting a patient's fibrosis stage differs from case to case. Etiology should be taken into account in assessing fibrosis using TE [12]. The optimum cut-off value for F ≥2 was 6.6 kPa in the present study, which was slightly lower than the values for NAFLD (6.9 kPa) and CHB patients (7.2 kPa). However, the optimum cut-off values for F ≥3 and F=4 were 8.7 kPa and 10.9 kPa, which were intermediate values between those for NAFLD (8.4 kPa and 10.3 kPa) and CHB patients (9.4 kPa and 12.2 kPa) [24, 26, 29]. This variation may be explained by the degree of hepatic steatosis. A previous study indicated that hepatic steatosis could influence the architectural structure of the liver, potentially changing the propagation time of the vibratory wave through the liver [30]. Statistical analysis indicated that cut-off values with a PLR >10.0 offer sufficient confidence to confirm the diagnosis, while an NLR <0.1 provides enough confidence to exclude the diagnosis [29]. In our study, the cut-off value of 10.9 kPa for assessing cirrhosis had a PLR of 14.9 and an NLR of 0.08. Thus, using 10.9 kPa as an optimal cut-off value in diagnosing cirrhosis in CHB patients with NAFLD may be suitable. Nevertheless, further studies are warranted to confirm the accuracy.

By definition, NAFLD indicates the absence of significant alcohol consumption. However, the optimal cut-off for significant alcohol consumption in patients with suspected NAFLD remains controversial. It was inconsistent and ranged from >10 g of alcohol per day to >40 g per day [31]. For NASH clinical trials candidate eligibility purposes, significant

alcohol consumption was defined as >30 g/day in men and >20 g/day in women [32]. Furthermore, this definition has been recommended by western guidelines, but with weak strength and relatively low quality [33, 34]. While in the Asia-Pacific region, significant alcohol consumption has been defined as >20 g/day for men and >10 g/day for women by the Asia-Pacific Working Party on NAFLD [18] and has been widely used [7, 35]. Moreover, even with consuming moderate amounts of alcohol, patients may be still predisposed to NAFLD if they have metabolic risk factors [34]. Thus, to reduce the confounding impact of alcohol, the lower cut-off of significant alcohol consumption (defined as >20 g/day for men and >10 g/day for women) was used in the present study. It should be noted that this study has several limitations. First, most patients showed mild to moderate hepatic steatosis. The impact of the entire spectrum of steatosis on TE could not be fully assessed. In addition, due to the unavailability of XL probe for TE, patients with BMI ≥30 kg/m^2 were excluded in the present study. This may represent a selection bias since steatosis is more prevalent and severe in obese patients. Third, as controlled attenuation parameter (CAP) was unavailable in our department until 2018, it was not assessed for comparison with histological or US findings. Moreover, our sample size was relatively small.

In conclusion, although TE has a better performance over US, and over the combination of TE and US, this should not discourage the use of US in CHB patients with NAFLD. TE is more reliable in the assessment of liver fibrosis and can avoid unnecessary liver biopsies. Nevertheless, US is necessary in order to exclude liver focal lesions and to assess the presence of portal hypertension.

Abbreviations

CHB:	Chronic hepatitis B
NAFLD:	Nonalcoholic fatty liver disease
LB:	Liver biopsy
TE:	Transient elastography
US:	Ultrasonography
ROC curve:	Receiver operating characteristic curve
AUCs:	The areas under the ROC curves.

Data Availability

The data used or analyzed during the current study are available from the first author (Geng-lin Zhang) on reasonable request.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Geng-lin Zhang and Qi-yi Zhao contributed equally to this work.

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Research Article

Nonalcoholic Fatty Liver Disease Aggravated the Severity of Acute Pancreatitis in Patients

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Background and Aim. The incidence of nonalcoholic fatty liver disease (NAFLD) as a metabolic disease is increasing annually. In the present study, we aimed to explore the influence of NAFLD on the severity of acute pancreatitis (AP). *Methods*. The severity of AP was diagnosed and analyzed according to the 2012 revised Atlanta Classification. Outcome variables, including the severity of AP, organ failure (all types of organ failure), and systemic inflammatory response syndrome (SIRS), were compared for patients with and without NAFLD. *Results*. Six hundred and fifty-six patients were enrolled in the study and were divided into two groups according to the presence or absence of NAFLD. The non-NAFLD group contained 278 patients and the main etiology in this group was gallstone. The NAFLD group consisted of 378 patients and the main etiology was hyperlipidemia. The incidence of mild AP, moderately severe AP, and severe AP was 77.30%, 18.3%, and 4.3% in the non-NAFLD group and 58.2%, 33.9%, and 7.9% in the NAFLD group, respectively. There were significant differences between the two groups according to the severity of AP ($P \leq 0.001$). In addition, the Ranson and BISAP scores as well as the incidence of SIRS and organ failure in the NAFLD group were higher than those in the non-NAFLD group (all $P < 0.05$). The patients were further divided into non-NAFLD, mild-NAFLD, and moderate-severe NAFLD (M+S-NAFLD) groups. The results showed that the severity of AP increased gradually from the non-NAFLD group to the M+S-NAFLD group. In addition, the incidence rates of SIRS and organ failure showed an upward trend with the aggravation of fatty liver severity. Multivariate logistic analysis showed that patients with NAFLD, especially those with M+S-NAFLD, had higher risks of SIRS and organ failure. *Conclusions*. Compared with non-NAFLD, NAFLD has a clinically relevant impact on the severity of AP and may be an early prognostic parameter for patients with AP.

1. Introduction

Acute pancreatitis (AP) is an inflammatory disease of the pancreas, with 10–20% of patients progressing to multiple organ failure coupled with a high mortality rate. The incidence of AP is increasing year by year, consistent with an increase in the number of people with metabolic syndrome. The incidence of local and systemic complications, especially mortality in patients with AP with metabolic syndrome, is noteworthy [1]. Metabolic syndrome is a clinical diagnosis based on the identification of related metabolic status. It can increase the risk of cardiovascular diseases, including diabetes, dyslipidemia, arterial hypertension, and abdominal obesity [2]. Abdominal obesity, a typical phenotype of metabolic syndrome, has been demonstrated to be an independent risk factor for AP [3]. Many clinical studies have confirmed that abdominal obesity can increase the severity of AP, prolong hospital stay, and increase the intensive care unit occupancy rate and mortality [4, 5].

Nonalcoholic fatty liver disease (NAFLD) is a phenotype of metabolic syndrome in the liver. NAFLD is related to all the components of metabolic syndrome and may be considered an additional component of the disease itself [6]. NAFLD is characterized by excessive hepatic fat accumulation, associated with insulin resistance (IR), and defined by the presence of steatosis in >5% of hepatocytes according to histological

analysis or by a proton density fat fraction (providing a rough estimation of the volume fraction of fatty material in the liver)>5.6% assessed by proton magnetic resonance spectroscopy (^{1}H-MRS) or quantitative fat/water selective magnetic resonance imaging (MRI) [7]. The incidence of NAFLD worldwide is approximately 28.01–52.34/1,000 [8], and NAFLD is increasingly recognized in the West. NAFLD is one of the main causes of chronic liver disease, which has become one of the major causes of liver disease-related morbidity and mortality in Western countries [9]. An independent epidemiological survey showed that, from 2007 to 2013, the prevalence of NAFLD in the general population increased from 23.5% to 44.3% among men and from 17.6% to 43.1% among women [10]. The prevalence of NAFLD in the average adult rose from 15% to more than 31% over a 10-year period, according to a survey in Shanghai and Beijing, China [11].

Studies have been conducted on the association between fatty liver and AP [12, 13]. Xu et al. separated 2,671 patients with pancreatitis into a fatty liver group and a non-NAFLD group. The results of the study showed that fatty liver can increase the severity of AP. However, the association between NAFLD and the severity and clinical outcomes of AP has been poorly studied. Therefore, we aimed to investigate the effect of NAFLD as a manifestation of metabolic disease on the severity of AP.

2. Methods

2.1. Inclusion and Exclusion Criteria. A retrospective analysis of 1186 patients with AP was conducted from January 2012 to December 2016. The diagnostic criteria for AP included three items: (1) typical clinical symptoms with persistent abdominal pain; (2) serum amylase and/or lipase levels three times higher than the normal upper limit; and (3) characteristic results of abdominal imaging [14]. Patients suffering from cirrhosis, hepatocellular carcinoma, alcoholic fatty liver, or chronic pancreatitis as well as those who had undergone splenectomy, were pregnant, were younger than 18 or older than 60 years, had been hospitalized repeatedly, or had incomplete medical data were excluded from the analysis. The cause of AP was considered to be biliary if gallstones or biliary sludge was observed on imaging examinations, including computed tomography (CT), magnetic resonance cholangiopancreatography, and ultrasonography [15, 16]. Hypertriglyceridemic acute pancreatitis (HTG-AP) was characterized by the presence of serum hypertriglyceridemia (≥1000 mg/dL) or by visible lactescent blood with serum hypertriglyceridemia 500–1000mg/dL without any other causes [17–19]. The exclusion criteria were age >70 or <18 years, recurrent pancreatitis, malignant tumor, ascites, pregnancy, and incomplete information. Due to the retrospective characteristics of the study from 2012 to 2016, informed consent was waived and the study was approved by the Ethics Committees of our hospital.

2.2. Diagnostic Criteria for NAFLD. Abdominal computed tomography (CT) was used to determine the presence of fatty liver based on CT values for the liver and spleen. Patients with a history of alcoholic consumption (history of drinking or equivalent alcohol consumption of more than 140 g/week for men and more than 70 g/week for women), viral hepatitis, drug-induced hepatitis, total parenteral nutrition, hepatolenticular degeneration, autoimmune liver disease, and other specific diseases that can lead to fatty liver were excluded [20].

2.3. NAFLD Classification Criteria. According to the literature, NAFLD is diagnosed based on the ratio of the CT values for the liver and spleen, which are measured in the range of 88–92 mm. Mild-NAFLD is defined as a liver/spleen CT ratio less than or equal to 1. If the ratio is higher than 0.5 and lower than or equal to 0.7, the disease is classified as moderate NAFLD. Severe NAFLD is defined as a ratio lower than or equal to 0.5 [21].

2.4. Severity Assessment of AP. According to the revised Atlanta Classification, AP severity is divided into three groups: mild, moderately severe, and severe. Mild AP (MAP) involves no organ failure and no local or systemic complications. Moderately severe AP (MSAP) is characterized by temporary organ failure and/or local or systemic complications within 48 hours without persistent organ failure. Severe AP (SAP) is defined as persistent organ failure lasting more than 48 hours [22].

2.5. Criteria for Systemic Inflammatory Response Syndrome (SIRS). SIRS is defined as the existence of two or more of the following: (1) temperature > 38°C or < 36°C; (2) heart rate > 90 beats/min or hypotension (systolic blood pressure <90 mmHg, or >40 mmHg lower than the baseline); (3) shortness of breath (>20 beats/min) or hyperventilation (PaCO2 <32 mmHg); and (4) peripheral blood leukocyte count >12 × 10^9/L or neutral rod-shaped granulocyte ratio >10%. However, other factors that may cause the above acute abnormal changes should be excluded [23].

2.6. Data Analysis. Data were analyzed with SPSS 16.0. Continuous variables were represented as the mean ± standard deviation (SD) or the median (quartile spacing) and compared using the T test. Data were evaluated based on the quantity and proportion, and descriptive statistics were used to analyze the baseline characteristics of the population; the severity was assessed using one-way analysis of variance or the Pearson chi-square test. The Kruskal–Wallis test was used for contingency table analysis. To determine whether the severity of NAFLD was related to organ failure and SIRS, the Spearman test was used. Age, gender, Body Mass Index (BMI), hypertension, diabetes, coronary heart disease (CHD), smoking, and NAFLD were set as independent variables for multivariable regression analyses, and organ failure was set as a dependent variable. Comparing the characteristics and variables among the groups, $P < 0.05$ indicated significant differences.

Table 1: Comparison of baseline and clinical characteristics between AP patients with versus without NAFLD.

Variables	Cohort N=656	non-NAFLD n=278	NAFLD n=378	P
General situation				
Age (years) (mean ± SD)	43.93 ± 9.81	45.96 ± 10.20	42.44 ± 9.24	≤ 0.078
Male (%)	413 (63.0%)	157 (61.3%)	256 (67.7%)	0.004
BMI	25.99 ± 3.61	24.71 ± 3.43	26.90 ± 3.45	≤ 0.001
Smoking	134 (20.4%)	52 (18.7%)	82 (21.7%)	0.378
Etiology				≤ 0.001
Gallstone	207 (31.6%)	129 (49.0%)	78 (20.6%)	
Hypertriglyceridemia	316 (48.1%)	69 (26.2%)	247 (65.3%)	
Others	133 (20.3%)	80 (28.8%)	53 (14.1%)	
Basic disease				
Hypertension	51 (7.8%)	22 (7.9%)	29 (7.7%)	1.000
Diabetes	104 (15.9%)	32 (11.5%)	72 (19.0%)	0.009
CHD	9 (1.4%)	6 (2.1%)	3 (0.8%)	0.179
Laboratory indicators				
WBC (10^9/L)	11.85 ± 4.97	11.17 ± 5.05	12.47 ± 4.89	0.001
ALT (IU/L)	40.00 (3, 1156)	40.45 (3, 1156)	39.50 (3, 793)	0.007
AST (IU/L)	35.00(8,850)	40.00 (8,850)	33.00 (8,850)	0.002
Cr (mmol/L)	59.00(30,1082)	57.00 (30,430)	60.00 (32,1082)	0.35
TG (mmol/L)	3.85(0.21,39.21)	1.48(0.21,30.48)	6.41 (0.24,39.21)	≤ 0.001

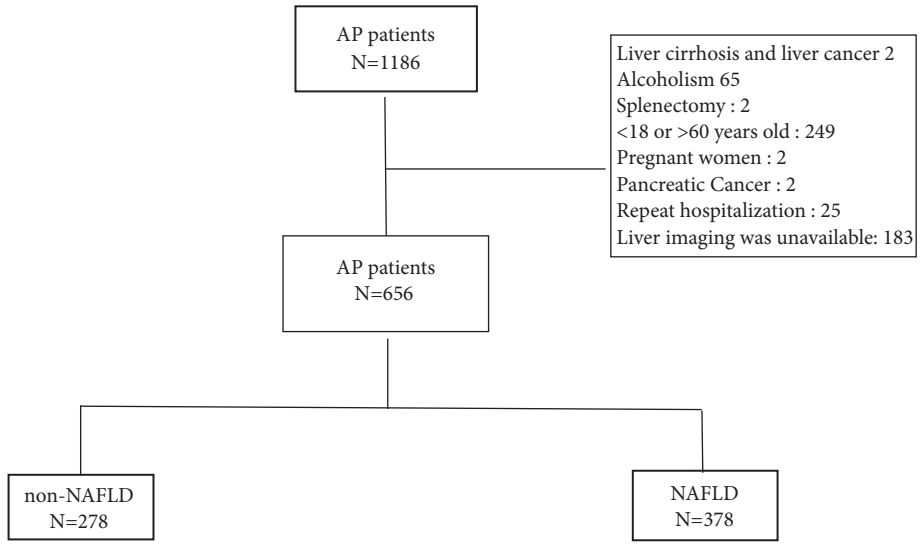

Figure 1: The distribution of AP patients.

3. Results

3.1. General Baseline Situation. A total of 1,186 patients with AP were diagnosed from January 2012 to December 2016. Five hundred and thirty were excluded according to the exclusion criteria, and 656 were enrolled in the study (as shown in the flowchart, Figure 1). Demographic characteristics are shown in Table 1. The average age of the patients was 43.93 ± 9.81 years and men accounted for 63.0%. The patient population comprised 20.4% of smokers, 7.8% of patients with hypertension, 15.9% of patients with diabetes mellitus, and 1.4% of patients with CHD. The patients were divided into non-NAFLD (278 patients) and NAFLD (378 patients) groups based on the CT results. The average age was 45.96 ± 10.20 years for the non-NAFLD group and 42.44 ± 9.24 years for the NAFLD group. The proportion of diabetes mellitus in the NAFLD group was also higher than that in the non-NAFLD group (19.0% versus 11.5%), but there was no significant difference in drinking.

The causes of AP include gallstone, hyperlipidemia, and others. As shown in Table 1, the percentage of gallstone, hyperlipidemia, and others was 31.6%, 48.1%, and 20.3% in

Table 2: Comparison of the Atlanta classification, BISAP score, Ranson score, SIRS, and organ failure with versus without NAFLD.

Variables	Cohort N=656	non-NAFLD n=278	NAFLD n=378	P
Atlanta classification				≤ 0.001
MAP	435 (66.3%)	215 (77.3%)	220 (58.2%)	
MSAP	179 (27.3%)	51(18.3%)	128 (33.9%)	
SAP	42 (6.4%)	12 (4.3%)	30 (7.9%)	
BISAP Scores				0.006
<2	545 (83.1%)	245 (88.71%)	300 (79.3%)	
>=2	111 (16.9%)	33 (12.2%)	78 (20.7%)	
Ranson Scores				0.040
<3	576 (87.8%)	253 (91.0%)	323 (85.4%)	
>=3	80 (12.2%)	25 (9.0%)	55 (14.6%)	
SIRS				≤ 0.001
NO	432 (65.9%)	214 (77.0%)	218 (57.7%)	
YES	224 (34.1%)	64 (23.0%)	160 (42.3%)	
Organ Failure				≤ 0.001
NO	468 (66.9%)	215 (77.3%)	220 (58.2%)	
YES	232 (33.1%)	63 (22.7%)	158 (41.8%)	

the cohort, respectively. Interestingly, the main etiology in the non-NAFLD group was gallstone pancreatitis (49.0%), whereas that in the NAFLD group was hyperlipidemia pancreatitis (65.3%).

3.2. Comparison of the Influence of the Presence and Severity of NAFLD in Patients with AP. We compared the laboratory indexes in the two groups and found that the white blood cell (WBC) and triglyceride concentrations, which are used to determine the severity of AP, were significantly higher in the NAFLD group. However, the serum creatinine was not significantly different in the two groups. These results showed that the severity of AP in patients with NAFLD was significantly greater than that in patients without NAFLD. In addition, compared with the non-NAFLD group, the incidence of MAP was lower (77.3% versus 58.2%) and the incidence of SAP was higher (4.3% versus 7.9%) in the NAFLD group. There was a significant difference between the two groups in the severity of AP ($P \leq 0.001$). Furthermore, the clinical scores and related complications of AP in the non-NAFLD and NAFLD groups were compared. The results showed that both the clinical scores (Ranson and BISAP scores) and related complications including SIRS and organ failure (all types of organ failure) were more serious in the NAFLD group (all $P < 0.05$ Table 2).

Based on the above results, the patients were further divided into non-NAFLD, mild-NAFLD, and moderate-severe NAFLD (M+S-NAFLD) groups according to the ratio of the CT value for the liver and spleen. As shown in Table 3, from the non-NAFLD group to the M+S-NAFLD group, the MAP ratio decreased, while the MSAP and SAP ratios increased ($P \leq 0.001$). In addition, the results showed that both the incidence of SIRS and organ failure in the M+S-NAFLD group were scientifically higher than those in the non-NAFLD group, which was consistent with the above results. Furthermore, the incidence of SIRS and organ failure showed an upward trend with the aggravation of the severity of NAFLD (P trend <0.001; Figure 2).

3.3. Logistic Regression Analysis of Organ Failure in Patients with AP. Finally, we analyzed whether organ failure correlated with the epidemiology and clinical features of NAFLD. Multivariate logistic regression analysis was performed, and the results showed that patients with mild-NAFLD had a risk of organ failure 1.771 times greater than those without NAFLD (95% confidence interval [CI] = 1.080-2.903 and P = 0.023). Furthermore, patients with M+S-NAFLD had a 3.115 times greater risk of organ failure than those without NAFLD (95% CI = 1.766-5.493 and $P \leq 0.001$). It is worth noting that patients with high TG may have a greater risk of organ failure (odds ratio = 1.026, 95% CI = 1.001-1.052, and P = 0.040 Table 4).

4. Discussion

Clinically, there are many causes of AP, including gallstone, alcohol, and hyperlipidemia. Gallstone is the primary cause globally, whereas in China, hyperlipidemia has exceeded alcohol to become the second major cause of pancreatitis [15, 19]. In the present study, patients with gallstone, hyperlipidemia pancreatitis (HTG-AP) and others accounted for 31.6%, 48.1%, and 20.3%, respectively. Further analysis of data revealed that the main cause of AP in the non-NAFLD group was gallstone, accounting for approximately 49.0%, whereas hyperlipidemia pancreatitis had the highest incidence in the NAFLD group (65.3%). The higher proportion of HTG-AP was probably because some biliary patients may have been excluded from the cohort without CT examination.

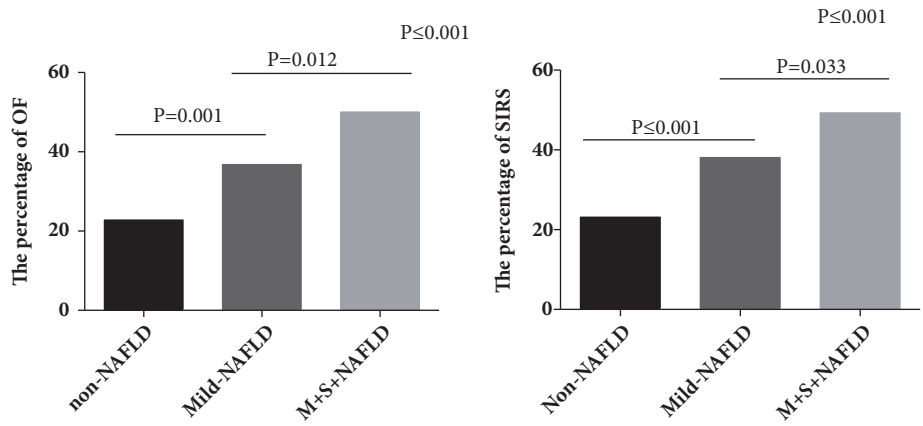

Figure 2: Comparison of SIRS and organ failure with non-NAFLD, mild-NAFLD, and M+S-NAFLD. OF: organ failure (all types organ failure); non-NAFLD: without NAFLD. Mild-NAFLD: mild nonalcoholic fatty liver disease; M+S-NAFLD: moderate-severe nonalcoholic fatty liver disease.

Table 3: Comparison of the Atlanta classification and Ranson score, with non-NAFLD, mild-NAFL, and M+S-NAFL.

Variables	non-NAFLD n=278	Mild-NAFLD n=234	M+S-NAFLD n=144	P
Atlanta classification				≤ 0.001
MAP	215 (77.4%)	149 (63.7%)	71 (49.3%)	
MSAP	51 (18.3%)	75 (32.1%)	53 (36.8%)	
SAP	12 (4.3%)	10 (4.3%)	20 (13.2%)	
Ranson Scores				0.003
<3	253 (91.0%)	208 (88.9%)	115 (79.9%)	
>=3	25 (9.0%)	26 (11.1%)	29 (20.1%)	

The results of this study showed that the severity of AP, including the clinical score, incidence of SIRS, and organ failure, in the NAFLD group was scientifically higher than that in the non-NAFLD group, which was consistent with the results reported by Xu and Mikolasevic [14, 24]. In addition, we found that the incidence of SIRS and organ failure showed an upward trend with the aggravation of the severity of NAFLD (P trend <0.001). All these findings imply that the severity of NAFLD has an impact on the course of AP. NAFLD is well known to be associated with other metabolic diseases, such as obesity, diabetes, and hyperlipidemia, and these metabolic diseases have a clear role in the severity of AP. In light of this, we further performed logistic regression analysis and determined that NAFLD was an independent risk factor for AP.

The mechanism by which NAFLD exacerbates pancreatitis remains to be elucidated. Patients with NAFLD are often associated with obesity. In our study, the BMI of NAFLD patients was 26.90 ± 3.45. The body is in a chronic inflammatory process for a long time in obesity patients, which makes the inflammatory factor response easy to expand. And NAFLD itself is an inflammatory disease that promotes chronic systemic inflammation [25–27], which may be an important reason for its exacerbation of AP. Secondly, in theory, Kupffer cells which are resident macrophages of the liver that represent approximately 70% of the liver's total macrophages play a very important role in the pathogenesis of AP by releasing a large number of inflammatory factors [28]. In the condition of NAFLD, the ability of Kupffer cells to release inflammatory factors increased greatly. In addition, NAFLD patients are often accompanied by disorders of adipokine levels, such as elevated CPR, IL-6, leptin, and reduced adiponectin levels, which make the body more prone to SIRS response [29]. Moreover there are reports that, in fatty liver mice and human, the reduction of alpha-1-antitrypsin (AAT) levels and the reduction of AAT can lead to excessive activation of inflammation [30].

Our study had the following limitations: it was retrospective, and the study population was not large enough. Second, it was a single-center study, and therefore, further research and verification are required in the future. And prospective studies are needed to demonstrate that NAFLD is a risk factor for a more severe pancreatitis. Third, liver biopsy is the gold standard for the diagnosis of NAFLD and other chronic liver diseases [31]. Previous studies showed that magnetic resonance imaging-based diagnostic methods are valuable in detecting NAFLD or determining the severity of NAFLD [32, 33]. However, in our study, an abdominal CT scan, which is our routine examination method, was used to diagnose NAFLD. This may have caused some data bias. Finally, the proportion of fatty pancreas in patients with NAFLD is higher than that in normal patients [34], and fatty pancreas may

Table 4: Logistic regression analysis of organ failure in patients with AP.

	B	P	OR	95%CI
Non-NAFLD				
Mild-NAFLD	0.572	0.023	1.771	1.080-2.903
M+S-NAFLD	1.136	≤ 0.001	3.115	1.766-5.493
Male	0.053	0.824	1.055	0.660-1.685
Age	0.009	0.465	1.009	0.986-1.032
BMI	-0.006	0.843	0.994	0.934-1.057
TG	0.026	0.040	1.026	1.001-1.052
CHD	-0.930	0.397	0.395	0.046-3.401
Diabetes	0.388	0.142	1.474	0.878-2.474
Hypertension	0.248	0.555	1.282	0.562-2.927
Smoking	0.132	0.606	1.141	0.691-1.882

have an impact on AP. However, our study was a retrospective research, so we cannot be access to the data of fatty pancreas. Moreover, because of the fat hydrolysis in pancreatic tissue after AP onset, it is difficult to assess fat content in pancreas tissue in patients with AP.

5. Conclusions

In summary, our results demonstrated that the presence of NAFLD at admission portends a higher risk of moderately severe and SAP, as well as a higher risk of SIRS and organ failure. In the clinical environment, we should pay close attention to the phenomenon of NAFLD aggravation of the severity of AP.

Abbreviations

AP: Acute pancreatitis
NAFLD: Nonalcoholic fatty liver disease
SIRS: Systemic inflammatory response syndrome
CT: Abdominal computed tomography
MAP: Mild AP
MSAP: Moderately severe AP
SAP: Severe AP
BMI: Body Mass Index
WBC: White blood cell
CI: Confidence interval
TG: Triglyceride concentrations
Cr: Creatinine
ALT: Alanine: transpeptidase
AST: Aspartate transaminase
HTG-AP: Hyperlipidemia pancreatitis
AAT: Alpha-1-antitrypsin
CHD: Coronary heart disease
M+S-NAFLD: Moderate-severe NAFLD.

Data Availability

All data generated and analyzed during this study are included in this published article. The datasets are available from the corresponding author on reasonable request.

Conflicts of Interest

All authors of this paper have no conflicts of interest to disclose.

Authors' Contributions

Yanbing Ding and Weiming Xiao contributed to study concept and design; Dacheng Wu, Songxin Xu, and Ningzhi Wang contributed to data analysis and interpretation; Dacheng Wu, Min Zhang, and Keyan Wu contributed to drafting of the manuscript; Yuanzhi Wang, Guotao Lu, Jian Wu, and Weijuan Gong contributed to critical revision of the manuscript for important intellectual content. All authors have read and approved the final version of this manuscript, including the authorship. Dacheng Wu, Min Zhang, and Songxin Xu contributed equally to this work.

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Review Article

Gut Microbiota-Derived Mediators as Potential Markers in Nonalcoholic Fatty Liver Disease

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Nonalcoholic fatty liver disease (NAFLD) is a common, multifactorial, and poorly understood liver disease whose incidence is globally rising. During the past decade, several lines of evidence suggest that dysbiosis of intestinal microbiome represents an important factor contributing to NAFLD occurrence and its progression into NASH. The mechanisms that associate dysbiosis with NAFLD include changes in microbiota-derived mediators, deregulation of the gut endothelial barrier, translocation of mediators of dysbiosis, and hepatic inflammation. Changes in short chain fatty acids, bile acids, bacterial components, choline, and ethanol are the result of altered intestinal microbiota. We perform a narrative review of the previously published evidence and discuss the use of gut microbiota-derived mediators as potential markers in NAFLD.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) comprises a variety of diseases extending from simple steatosis (SS), nonalcoholic steatohepatitis (NASH), fibrosis, and cirrhosis, with a growing prevalence worldwide, reaching around 30% of global population [1]. NASH is the severe form of the disease, and patients could develop liver cirrhosis and hepatocellular carcinoma with aging. NASH is characterized by the presence of hepatocyte ballooning and inflammation, with a worldwide prevalence of 2–3%; however almost one-third of NAFLD affected subjects progress to NASH [1].

Precise histological diagnosis, including disease stages (SS and NASH), is commonly based on liver biopsy [2]; however, biopsy comprises several potential problems such as bleeding, abdominal pain, and needs to be performed in a special clinical setting under expertise supervision [3]. Thus, there is a need for reliable and cost-effective noninvasive biomarkers, to avoid the invasiveness of biopsy. However, until date, none of the previously explored surrogate blood markers have been confirmed in large cohorts of biopsy-proven NAFLD or have proper specificity for NASH diagnosis.

Previous evidence has linked intestinal microbiota dysbiosis with obesity, insulin resistance, metabolic syndrome, and NAFLD [4–6]. Due to dysbiosis, the permeability of intestinal barrier is compromised and substances such as short-chain fatty acids, bile acids, bacterial components, choline, and endogenous ethanol reach the liver which seem to contribute to the pathogenesis of NAFLD. More recently, other metabolites or proteins as angiopoietin-like protein 4 (ANGPTL4), resistin-like molecule β (RELM β), neurotensin, glucagon-like peptide-1 (GLP-1), glucagon-like peptide-2 (GLP-2), and fibroblast growth factor 19 (FGF19) have been suggested to be involved in NAFLD pathogenesis [7, 8]. It is important to note that some of these metabolites may be employed as potential markers of NAFLD occurrence and progression.

In order to give a broad overview of primary literature published on this topic, we have used narrative review

as literature search strategy in the present article. In this sense, this narrative review will discuss NAFLD and (a) gut microbiome dysbiosis and (b) main gut microbiota-derived mediators. Their potential use as biomarkers for evaluating the status of NAFLD will also be briefly discussed.

2. Gut Microbiome Dysbiosis and NAFLD

Intestinal microbiome is composed mainly of bacteria, virus, and fungi, with several functions, such as host nutrition, bone mineralization, immune system regulation, xenobiotics metabolism, proliferation of intestinal cells, and protection against pathogens [9, 10]. The microbiome is specific to an individual and highly resilient to changes. However, it can be affected by several factors, intrinsic and extrinsic to the hosts, such as subject's genetic, dietary habits, antibiotics, and environmental changes [11–13]. Gut microbiota comprises about 1000 different species, but Firmicutes and Bacteroidetes are the most important phyla in intestinal bacteria, with a proportion of over 90% of the total [14]. A disruption in the composition—quantitative or qualitative—of the normal microbiota, is known as gut dysbiosis [15, 16]. Generally, this process includes an unfavorable change in the bacterial composition with a reduction in autochthonous (Firmicutes) bacteria and growth of other taxa (Bacteroidetes, Actinobacteria) [17].

Dysbiosis may adversely impact metabolism and immune responses, favoring NAFLD and NASH. Because of gut dysbiosis, there is an elevated production of toxic bacterial components and metabolic mediators, which consequently accumulate in the intestine. In addition, an increase in intestinal permeability and further disruption of the epithelial barrier lead to the efflux of these gut microbiota-derived mediators [16], which could reach the liver through portal circulation favoring hepatic inflammation and the development of NAFLD [18, 19]. Approximately, 70-75 % of blood that reaches the liver comes from the portal vein, which drains blood from mesenteric veins of the intestinal tract [20]; and, after the disruption of the intestinal epithelial-barrier, the liver is exposed to the microbial products and metabolites resulting from the metabolism of bacteria [21, 22]. In this sense, it has been demonstrated that patients with NAFLD have dysbiosis of intestinal microbiota, gut epithelial barrier dysfunction, and increased translocation of bacterial components to the liver [23].

Instead of the evidence relating disruption of the gut-barrier and hepatic diseases [21, 22], previous studies have demonstrated that increased intestinal permeability and endotoxin levels are not present in all patients that develop NAFLD [24, 25]. Therefore, intestinal barrier dysfunction with subsequent translocation of bacterial components because of dysbiosis is not the hallmark in the development or progression of the disease. For this reason, other mediators derived from gut microbiota dysbiosis might be also related to the pathogenesis of the disease. These mediators could be metabolites due to metabolic changes related to microbiome dysbiosis.

Several previous studies in clinical settings have associated intestinal dysbiosis with the occurrence of NAFLD [26–28] and with the progression to NASH [29, 30]. Gut microbiota-derived mediators—metabolites and bacterial components—resulting from gut dysbiosis could be representative of NAFLD progression through several mechanisms: (1) enhanced energy extraction from food nutrients by formation of short-chain fatty acids; (2) modulation of bile acid synthesis which are crucial for fat absorption and affect metabolism of glucose via farsenoid X receptor; (3) innate-immune system activation by bacterial components translocation; (4) endogenous ethanol production; and (5) reduction of choline metabolism which reduces efflux of VLDL from hepatocytes promoting inflammation [15] among others. These mechanisms involve translocation of both microbial degradation products and microbiota-derived metabolites such as short-chain fatty acids, bile acids, ethanol, and choline, which may be potentially evaluated as noninvasive blood markers of NAFLD progression.

3. Gut Microbiota-Derived Mediators in NAFLD

In the present section we will focus on the main gut microbiota-derived mediators related to NAFLD: short-chain fatty acids, bile acids, bacterial components, endogenous ethanol, and choline deficiency. Also, we have performed a summarizing table (Table 1) including the main published human studies.

3.1. Short-Chain Fatty Acids. Short-chain fatty acids (SCFAs), such as acid acetic, acid propionic, and acid butyric, are molecules with seven carbon atoms or less, mainly produced by the fermentation of indigestible carbohydrate by gut microbiota [31]. In general, these SCFAs have several effects on energy metabolism, immune response, and adipose tissue expansion and act as signaling molecules between the gut microbiota and the subject [31, 32]. Not only do SCFAs provide important sources of nutrients and energy from the intestinal epithelium but also they are precursors for lipogenesis and gluconeogenesis [32].

In general, changes in the microbiota result in increased production of SCFA in the intestine with an increased transport of monosaccharides to the liver, promoting hepatic lipogenesis and steatosis [47]. Increased acetate in the liver causes accumulation of triglyceride, because it is an important substrate for fatty acid synthesis [48], whereas raised levels of propionate promote liver gluconeogenesis [49].

Experimental studies have demonstrated that these SCFAs can remodel regulatory T cell expansion and enhance neutrophil chemotaxis, modulating inflammation in mice models [47–50]. Also, SCFAs modulate the production of several inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), IL-6, and IL-10 [51]. Recently, some studies found that high concentrations of intestinal SCFAs as result of dysbiosis and their G protein-coupled receptors play an important key role in NAFLD progression [52, 53]. SCFAs activate G-protein coupled receptors (GPCRs), specifically the subtypes GPR41 and GPR43. Activation of these GPCRs stimulates secretion

Table 1: Gut microbiota-derived mediators in human NAFLD.

Type of metabolites	Subjects	Type of sample	Alterations of gut microbiota-derived mediators	References
SHORT CHAIN FATTY ACIDS	Adults, obesity, NAFLD (SS or NASH)	Blood	Higher abundances of enzymes associated with lactate, acetate, and formate in mild/moderate NAFLD. Higher abundances of enzymes for butyrate, D-lactate, propionate, and succinate in advanced fibrosis	[33]
	Children, obesity, NAFLD	Fecal specimens	Lower acetate, formate, valerate in NAFLD	[34]
BILE ACIDS	Adults, NASH	Blood,Urine	More hydrophobic bile acid profile	[35]
	Adults NAFLD, NASH	Liver	Elevated deoxycholic, chenodeoxycholic, and cholic acids	[36]
	Adults, NAFLD, NASH	Blood	Higher glycocholate, taurocholate, glycochenodeoxycholate in NAFLD	[37]
	Adults, NASH	Fecal specimens	Higher primary to secondary BA ratio in NASH	[38]
	Children NAFLD	Blood	Higher CDCA, unconjugated primary BAs (CDCA + cholic acid), lower DCA, TDCA, GDCA, total DCA, GLCA and total lithocholic acid in NASH	[39]
TLR	Adults, NASH	Blood	Higher TLR-4/MD-2 expression on CD14 positive cells in NASH	[40]
ENDOGENOUS ETHANOL	Children, obesity, NASH	Blood	Elevated blood-ethanol concentration in NASH	[41]
	Children, NAFLD	Blood	Higher ethanol levels in NAFLD	[42]
	Children, obesity, fatty liver	Blood	Higher ethanol levels in NAFLD	[43]
CHOLINE, TMA, TMAO	Children, adolescents, adults	Blood	Decreased choline intake in postmenopausal NAFLD women with fibrosis	[44]
	Adults	Blood	Association of TMAO level and presence/ severity of NAFLD	[45]
	Adults	Blood	Higher free choline levels in NASH	[46]

of peptide-YY, inhibits gut motility, and slows intestinal transit. Therefore, nutrient absorption and energy harvest from the diet increase, promoting hepatic lipogenesis [54, 55]. Additionally, activation of GPR41 and GPR43 promotes secretion of glucagon-like-peptide-1 (GLP-1), which activates genes in hepatocytes that regulate fatty acid β-oxidation and insulin sensitivity [55, 56], promoting NAFLD occurrence and progression.

However, other previously published studies point in another direction and have reported that SCFAs could be beneficial in the progression of NAFLD; for example, butyrate activates AMP-activated protein kinase (AMPK) in the liver [57] and accelerated the assembly of tight junction proteins in the colonic epithelial cell line Caco-2 [58], improving intestinal barrier dysfunction. In addition, butyrate is able to modulate epigenetic changes decreasing the activity of histone deacetylases (HDACs), which further increase in the number of regulatory T cells, suppressing the immune response and reducing liver inflammation [59].

Furthermore, clinical studies have demonstrated SCFA enrichment in fecal samples of children and adults with NAFLD [33, 34]. These results confirm the relation between excretion of SCFA and NAFLD, although there are differences in relation to the SCFA concentrations excreted which could be related to differences in the age of subjects, diet, environmental factors, and technical issues, related to the volatility of the SCFAs [23].

The close relation between microbiota dysbiosis and SFCAs production—as part of carbohydrate bacterial fermentation—with the results of previous experimental and clinical studies provide evidence of their potential use as markers of NAFLD progression.

3.2. Bile Acids. Bile acids (BA) are steroidal molecules synthesized after cholesterol oxidation by enzymes presented in hepatocytes which are important in the regulation of glucose and lipid metabolism. They participate in the digestion and solubilization of lipids and regulate hepatic glucose and inflammation. Also, they are able to control their own synthesis through the activation of farnesoid X receptor (FXR) [60, 61]. In addition, BA function as signaling molecules that modulate several physiological processes, and gut dysbiosis can change BA pool characteristics through their effects on BA metabolism [61–63].

Gut microbiota is a critical modulator of BA pool size and composition and the process of dysbiosis could substantially alter systemic concentrations of conjugated and/or secondary bile acids, as well as increasing their synthesis. An increased level of BA causes an activation of cell death pathway mediated by inflammatory and oxidative stress cascades in

liver tissue [64, 65]. In turn, BA can have direct effects on intestinal microbiota by causing membrane disruption through their amphipathic properties, acting as a detergent for cellular membranes. This increased intestinal permeability, associated with BA modifications, has been linked to metabolic endotoxemia, insulin resistance, and inflammatory cytokine release with enhanced proinflammatory signaling cascades, common findings in patients with NAFLD [66, 67].

Previous investigations have demonstrated a BA increase in biological fluids of patients with NASH compared to subjects with healthy livers and an evident association with intestinal dysbiosis [35–37]. Kalhan et al. performed a metabolomic profile of derivates from bile acid metabolism, glutathione metabolism, lipids, carbohydrate, and amino acids, which do not differentiate patients with steatosis from those with steatohepatitis. However it revealed significant changes in certain metabolic pathways, suggesting that a metabolome study of BA and derivates could potentially be used as a noninvasive marker to evaluate the status of NAFLD and the therapeutic patient's outcome [37]. Also, levels of BA have been correlated with histopathological features, such as the degree of hepatic steatosis, the presence of cellular ballooning, and the severity of fibrosis in patients with NASH [38]. Ferslew et al. reported that NASH patients have higher total serum BA concentrations than healthy volunteers, specifically increase in taurine- and glycine-conjugated primary and secondary BA, under fasting and postprandial conditions, confirming the disruption in bile acid homeostasis in NASH physiopathology [35]. In addition, plasma levels of glycocholate, taurocholate, glycochenodeoxycholate, taurochenodeoxycholate, and ursodeoxycholic acid were increased in patients with NASH compared with patients with SS [68]. Also, levels of taurolithocholic acid, glycocholate, and taurocholate have been correlated with severity of portal inflammation, lobular inflammation, steatosis, and hepatocyte ballooning, respectively [68].

In children with NAFLD, changes in circulating BA profile have been reported too [39, 69]. The research of Jahnel et al. demonstrates that serum BA levels decrease in early NAFLD and increase during progression to fibrosis in obese children. These authors postulated that BA may have a value as a noninvasive biomarker in pediatric NAFLD progression [69].

Experimental studies have demonstrated that dysbiosis of the gut microbiota can modulate the activity of FXR in the intestine, affecting as consequence lipid metabolism in the liver [4]. Specifically, FXR not only plays an important role in maintaining bile acids but also regulates glucose and lipid metabolism via different mechanisms, such as increasing insulin sensitivity, repressing hepatic gluconeogenic genes, and increasing hepatic glycogen synthesis [70, 71].

Considering the numerous experimental and clinical published studies associating gut dysbiosis, bile acids, and NAFLD, it is expected that these molecules could be proposed as potential noninvasive markers of the disease, specifically the secondary bile acids deoxycholic acid (DCA) and lithocholic acid (LCA), which cannot be produced without bacterial fermentation [72].

3.3. Bacterial Components. The liver is exposed to potentially harmful substances derived from the gut, considered as pathogen-associated molecular patterns (PAMPs), including translocated bacteria, lipopolysaccharide (LPS), DNA, RNA, and endotoxins, which are potent inducers of tissue inflammation [15, 73]. These PAMPs might contribute to the pathogenesis of fatty liver disease by activation of the innate immune system via toll-like receptors (TLRs), which recognize these gut-derived bacterial components [73]. The translocation of these bacterial components from the gut into the portal system is facilitated by the disruption in tight junctions, which normally seal the junction between intestinal endothelial cells at their apical border, facilitated by gut microbiome dysbiosis [73].

There is evidence that dysbiosis causes permeability changes that increase portal levels of gut-derived TLR ligands (LPS or endotoxin), which further activate TLR4 on hepatic Kupffer and stellate cells [74]. During receptor activation, the adaptor molecule myeloid differentiation factor 88 (MyD88) is activated, and the downstream signaling MyD88-dependent pathway results in the activation of the nuclear factor-Kβ (NF-Kβ) leading to the expression of proinflammatory cytokines (TNF-α, IL-6, IL-8, and IL-12) and chemokines (interferon-γ [IFN-γ] and monocytes chemotactic protein-1 [MCP-1]), promoting inflammation [52, 74]. There are several intracellular cascades involved in this process which include stress-activated and mitogen-activated protein kinases, JNK (c-Jun N-terminal kinase) and p38 mitogen-activated kinases, which triggers transcription of proinflammatory genes and facilitates hepatic migration of neutrophils and monocytes, generation of oxidative stress mediators—nitrogen and oxygen reactive species—low-grade systemic inflammation, and hepatic injury [75].

In addition, TLR signaling, as a result of gut dysbiosis, can also lead to the production of inflammasomes, in peripheral and parenchymal cells, which activate a variety of processes, including cleavage of procaspase-1 to form active caspase-1, resulting in cell death dependent on caspase-1 and caspase-3 [76]. Inflammasome, which is a multimeric signaling platform that leads to the production of IL-18 and IL-1β, through NRLP3 (NOD-like receptors, pyrin domain containing 3) and NRLP6, is activated by LPS derived from dysbiosis of gut microbiota via TLR4 and TLR9 response. Reports have associated inflammasome activation with the development of liver steatosis, inflammation, and fibrosis in NAFLD patients [77, 78].

Previous studies have demonstrated that endotoxemia markers, as a result of gut dysbiosis, were associated with the pathogenesis and severity of NAFLD [79, 80]. In addition, other studies have established that the increase in endotoxin level is related to IL-1α and TNF-α production [81, 82]. In patients with NAFLD gut permeability and the prevalence of small intestinal bacterial overgrowth have been associated with the severity of steatosis [66]. In biopsy-proven human NASH, plasma IgG levels against endotoxin were found to be increased with NASH grade severity, suggesting the deleterious effect of chronic endotoxin exposure [83]. Also, enhanced expression of TLR4, the release of IL-8, and high

levels of LPS have been demonstrated in NAFLD patients [40, 67]. Furthermore, two recent studies in obese children with NAFLD showed that intestinal permeability was correlated with the degree of hepatic damage and endotoxin levels. In addition, urinary metabolome analyses identified metabolite changes associated with dietary habits, intestinal permeability, and small intestinal bacterial overgrowth (SIBO) [84, 85]. However, other reports did not reveal an association between endotoxemia and NAFLD/NASH development, suggesting that endotoxemia may not be the only driver of disease progression in all patients [41].

Multiple experimental studies have demonstrated that a high-fat diet can increase the proportion of LPS derived from gut bacteria and administration of endotoxin has been shown to induce insulin resistance and weight gain [86, 87]. On the other hand, some authors have proposed recently that the small intestine shields the liver from otherwise toxic fructose exposure, via gut microbiota [88].

There is an evident relation between gut dysbiosis, bacterial-derived components, inflammatory response, and NAFLD; therefore these bacterial mediators, especially circulating TLRs, might be used as potential noninvasive markers of disease progression.

3.4. Endogenous Ethanol Production. Dysbiosis due to changes in microbiome composition profile, specifically in *Escherichia coli* and other *Enterobacteriaceae*, increases endogenous ethanol production [41], which might contribute to liver injury by affecting intestinal permeability, with disruption of intestinal tight junctions. This allows endotoxins and ethanol trigger TLR response and inflammasome activation, which further inflammatory response in liver tissue [89]. In addition to the proinflammatory response, ethanol promotes oxidative damage and hepatocyte necrosis because of the formation of reactive oxygen and nitrogen species [90]. Endogenous ethanol inhibits the tricarboxylic acid cycle, thus increasing levels of acetate, thereby promoting triglyceride accumulation in hepatocytes [48]. Ethanol can also increase the activity of the enzyme cytochrome P450 2E1 (CYP2E1) [91] which catalyze the oxidation of ethanol but produce free radicals favoring oxidative damage, mitochondrial dysfunction and liver inflammation [90, 92].

Several studies have detected increased levels in nondietary ethanol, derived from bacteria, in obese patients [41, 93], and in patients with NASH [41, 42, 94], with a related upregulation of hepatic alcohol metabolizing capacity (alcohol dehydrogenase, aldehyde dehydrogenase, and cytochrome P450 2E1) [94]. In this sense, the group of Zhu et al. propose that microbiomes rich in ethanol-producing *Escherichia* may be a risk factor for progression from obesity to NAFLD [41]. Besides *Escherichia coli*, other gut microbial genera, including *Bacteroides*, *Bifidobacterium*, and *Clostridium*, can produce alcohol and generate a significant ethanol-mediated damage [41]. So, production of endogenous ethanol by the gut microbiota may act as a hepatotoxin, contributing to the development of NAFLD and its progression to NASH [95]. In addition, children with fatty liver showed higher levels of endogenous ethanol and LPS related to gut microbiome [43]. Moreover, children with NASH had higher serum levels of ethanol than obese and healthy children without NASH [41, 42], confirming that endogenous ethanol might contribute to the pathogenesis of NAFLD and NASH.

Gut dysbiosis with the increase in ethanol-producing bacteria (*Enterobacteriaceae*) in the microbiome is the main hypothesis to explain the differences in blood ethanol in NAFLD patients, and the importance of ethanol-derived microbiome in NASH [85]. However, other hypotheses suggest that alterations in insulin signaling followed by decreased alcohol dehydrogenase activity in the liver could be responsible for an impaired ethanol metabolism [42].

In summary, the proinflammatory and prooxidative damage as a result of endogenous ethanol in the liver, which might contribute to the pathogenesis of NAFLD, has been demonstrated; and the previous reports may support its use as a noninvasive marker of disease progression.

3.5. Reduction of Choline Metabolism. Choline is an essential nutrient obtained through both dietary intake and endogenous synthesis, being an important constituent of membrane phospholipids. The human gut microbiome actively metabolizes dietary components, including choline, and dysbiosis may alter its cellular disponibility and predispose the body to a deficiency of choline. Alterations in choline and phosphatidylcholine metabolism may have an impact on several physiological pathways, which could induce NAFLD. Choline deficiency prevents synthesis and excretion of very-low density lipoprotein (VLDL), leading to hepatic triglyceride accumulation and liver steatosis [44, 96]. In fact, the link between choline deficiency and accumulation of hepatic lipids has been recognized for more than 50 years [97], leading to the establishment of choline-deficient diets to induce models of NAFLD in animals.

In addition, choline can be metabolized to its derivate trimethylamine (TMA) by the intestinal microbiota. TMA reaches the liver via portal circulation and is subsequently oxidized by hepatic flavin-containing monooxygenases in the liver, forming trimethylamine-N-oxide (TMAO), which is then released into blood circulation [98, 99]. Previous studies have revealed that TMAO may affect lipid absorption and cholesterol homeostasis and modulate glucose and lipid metabolism by decreasing the total bile acid pool size [96]. The metabolism of choline to TMA induced by dysbiosis may result in reduced choline bioavailability and increased susceptibility to NAFLD [99]. TMAO modulates glucose metabolism and increases insulin resistance in mice on an HFD [100]. In addition, TMAO promotes inflammation in adipose tissue, which can induce insulin resistance by increasing the serum level of inflammatory cytokine C-C motif chemokine ligand 2 [100]. TMAO also affects lipid absorption and cholesterol homeostasis by reducing the conversion of cholesterol into bile acids [96].

A few studies have examined the association of choline with the fatty liver disease in animals and humans. A small number of human studies have shown that the consumption of a low-choline diet promotes fatty liver and liver damage [44, 101]. Other studies have pointed out that plasma free choline levels are positively related to the

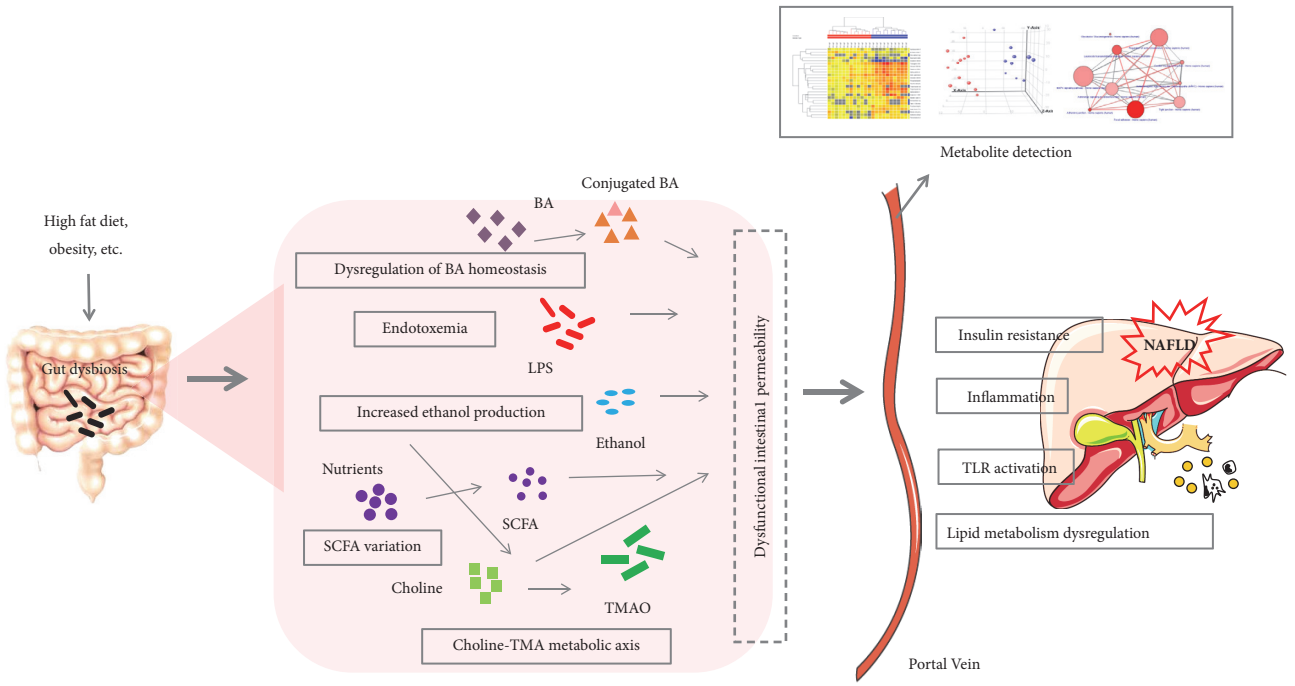

Figure 1: A schematic figure of the role of gut dysbiosis in the development and progression of nonalcoholic fatty liver disease (NAFLD) on the basis of the gut-liver axis. Environmental factors as obesity, high fat diet, or infection (among others) may induce intestinal dysbiosis and also increased intestinal permeability (malfunction of tight junctions). Substances such as short-chain fatty acids, bile acids, bacterial components, choline, and endogenous ethanol reach the liver and activation of toll-like receptors (TLRs) occurs. This activation induces insulin resistance, hepatic inflammation, lipogenesis, and oxidative stress, inducing NAFLD. BA, bile acids; LPS, lipopolysaccharides; SCFA, short chain fatty acid; TLR, toll-like receptor; TMAO, trimethylamine oxide.

severity of liver steatosis, fibrosis and NASH [45, 46]. Also, a study demonstrates the presence of a low phosphatidylcholine/phosphatidylethanolamine ratio in NASH patients, in comparison to healthy subjects [102].

On the other hand, the metabolite TMAO has been associated with the occurrence of NAFLD, and TMAO raised levels correlate with the severity of steatosis, and it has been proposed as an independent risk marker for the disease [45]. The increased risk of NAFLD might be caused by TMAO due to its effect on decreasing the total bile acid pool size via several pathophysiological mechanisms [96]: (1) by decreasing the synthesis of bile acids due to the inhibition of the key enzymes CYP7A1 and CYP27A1 and (2) by limiting the enterohepatic circulation of bile acids between the liver and intestines due to the repression of multidrug resistance protein expression.

In summary, the evidence demonstrated that choline and TMAO are associated with progression of NAFLD, indicating the potential use of these gut-derived mediators of dysbiosis as markers of disease progression.

4. Concluding Remarks

Intestinal dysbiosis can trigger intestinal inflammation and increase permeability of the gut epithelial barrier, exposing the hepatobiliary system to gut-derived mediators of dysbiosis, such as bacterial components or metabolites, which may induce NAFLD progression. Gut-derived mediators of dysbiosis contribute to steatosis activate the immune system, induce inflammatory and oxidative pathways, enhance inflammation, and promote fibrogenesis (Figure 1).

Despite the evident association between gut dysbiosis and obesity and NAFLD, derived from experimental studies, very few studies have been conducted in patients with NAFLD in order to explore the role of gut-microbiota derived mediators of dysbiosis in the occurrence and progression of the disease. Most of the previous evidence has been focused on gut microbiota as a therapeutic target to prevent or to treat NAFLD, interfering in gut dysbiosis with probiotic, prebiotic, and symbiotic supplements. Nevertheless, few studies have been focused in gut-derived mediators of dysbiosis as non-invasive markers of disease progression. The study of specific gut-derived mediators of dysbiosis—bacterial components and metabolites—may provide an opportunity to develop a specific diagnostic biomarker for NAFLD. In this sense, we propose the metabolomic study of these and other metabolites involved, in order to achieve a metabolomic profile that could be used as biomarkers for evaluating the status of NAFLD.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Gemma Aragonès and Sergio González-García contributed equally to this work.

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