Experimental Diabetes Research

Diabetes and Nonalcoholic Fatty Liver Disease

Guest Editors: Konstantinos Kantartzis, Amalia Gastaldelli, Faidon Magkos, and Jean-Marc Lavoie

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Editorial Board

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Editorial

Diabetes and Nonalcoholic Fatty Liver Disease

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With almost every third individual affected in the general population in industrialized countries and increasing prevalence among children and adolescents, nonalcoholic fatty liver disease (NAFLD) represents the most common cause of chronic liver diseases such as cirrhosis, liver failure, and hepatocellular carcinoma and is therefore the most common cause of liver transplantation. In addition, in recent years NAFLD has emerged as a key player in human metabolism. Several studies demonstrate that NAFLD is strongly associated with insulin resistance and precedes the manifestation of type 2 diabetes and cardiovascular disease.

Of particular interest, the associations of ectopic fat accumulation in the liver with insulin resistance and type 2 diabetes are stronger than the respective of visceral and intramyocellular fat, implying that liver fat is an independent factor modifying the whole-body obesity-related metabolic risk. Thus, fatty liver may be not simply another manifestation of the metabolic syndrome, but it may itself induce or worsen insulin resistance and type 2 diabetes. In other words, fatty liver may be a determinant, not merely a marker of metabolic dysfunction. Though certainly, cause-and-effect relationships are hard to establish.

It is therefore why a concerted effort of the academic disciplines is requested to study the responsible mechanisms involved in the process of hepatic fat accumulation as well as the mechanisms regulating the crosstalk between fatty liver and other tissues important for regulation of metabolism in humans. In this special issue, we have invited some papers hoping to shed light on some aspects of this very interesting field.

In the first paper of this issue "*Diagnosis and evaluation of nonalcoholic fatty liver disease,*" epidemiology and tools for diagnosing NAFLD are reviewed. Regarding epidemiology, interesting data are provided on the interaction of NAFLD and type 2 diabetes, when concurrently present. In terms of diagnostic evaluation, of particular interest is the systematic presentation of old and novel biomarkers as well as panel markers (scores) and their sensitivity, specificity, positive and negative predictive values in estimating the amount of liver fat and differentiating more progressive forms of NAFLD, such as NASH and fibrosis, from simple steatosis.

The second paper of this issue "*Role of transcription factor modifications in the pathogenesis of insulin resistance,*" evaluates the diverse types of posttranslational modification of transcription factors in insulin-sensitive tissues and their putative role in the pathogenesis of insulin resistance. The authors particularly focused on the liver, where a lot of transcription factors have key roles in metabolic pathways critical for the pathogenesis of hepatic insulin resistance and NAFLD. For instance, forkhead box protein 1 (FOXO1) and cAMP response element binding protein (CREB) are major tran-scription factors for gluconeogenic gene expression, and sterol regulatory element-binding protein-1c (SREBP-1c) and carbohydrate response element-binding protein (ChREBP) are well known to regulate the expression of genes coding for lipogenic enzymes in hyperinsulinemic and

hyperglycemic states. Thus, understanding the circumstances under which transcription factors undergo modifications will enhance our understanding of the molecular mechanisms leading to NAFLD and how it is related to insulin resistance.

The third paper of this issue "*Inhibition of aldose reductase activates hepatic peroxisome proliferators-activated receptor-α and ameliorates hepatosteatosis in diabetic db/db mice,*" examines the effect of inhibiting aldose reductase, the rate-limiting enzyme of the polyol pathway, on serum and hepatic triglyceride levels in db/db diabetic mice. The polyol-pathway is considered to be important in the development of a variety of diabetic complications but was thought not to play a significant role in liver disease, because the hepatic activity of aldose reductase is generally low. In this work, the authors inhibited aldose reductase, both pharmacologically and by a short-hairpin RNA, and found that this leads to a significant reduction in serum and hepatic triglycerides under hyperglycemia, possibly by activating PPARα and thereby lipid oxidation. These findings indicate that the polyol pathway may be upregulated under certain conditions and may contribute to the development of NAFLD.

The fourth paper of this issue "*Cholesterol synthesis is associated with hepatic lipid content and dependent on fructose/glucose intake in healthy humans,*" reports on the association of liver and visceral fat with cholesterol homeostasis. Obesity, insulin resistance, and type 2 diabetes have been shown to independently correlate with increased endogenous cholesterol synthesis. However, the associations of body fat distribution with cholesterol metabolism have not been extensively studied. The authors found that visceral and liver fat are associated with cholesterol biosynthesis but not cholesterol intestinal absorption. This suggests that people with high liver fat and hypercholesterolemia will probably profit from statin treatment. The impact of high-fructose diet, which is thought to promote liver fat accumulation and hypercholesterolemia, on cholesterol homeostasis was also studied. Of interest, high-glucose diet appeared to stimulate cholesterol synthesis more than high-fructose diet did.

In the fifth article of this issue "*Predictors of impaired glucose regulation in patients with nonalcoholic fatty liver disease*", the authors set out to find commonly measured demographic and laboratory parameters predicting an abnormal oral glucose tolerance test (OGTT) response (impaired glucose tolerance or diabetes mellitus) in patients with ultrasonography-diagnosed fatty liver. NAFLD is known to be closely associated with insulin resistance and type 2 diabetes; however, the characteristics of NAFLD patients having also hyperglycemia compared to NAFLD patients with normal glucose tolerance are not known. The authors found that if an OGTT is performed in patients with NAFLD and elevated liver enzymes, but no history of diabetes, almost half of them will display impaired glucose regulation, more likely those who are older, have higher BMI and lower HDL-cholesterol. Thus, at least those NAFLD patients with these characteristics should undergo further evaluation with an OGTT.

The final paper of this issue "*The role of metformin in the management of NAFLD*" is a review article summarizing the mechanism of action of metformin, the clinical studies performed so far on the use of metformin in patients with NAFLD, and the potential benefits of using metformin in NAFLD beyond its action in the liver. Currently, metformin is not considered to be an established treatment for NAFLD patients, probably because the existing clinical studies have been mostly small in sample size and short in duration and have provided in part controversial results. Most importantly, true randomized and controlled trials are lacking. Nevertheless, the high benefit-risk ratio of metformin, as well as its pleiotropic favourable effects (e.g., in promoting weight loss and lowering the risk of cancer), makes it an attractive treatment option for all patients with metabolic disturbances. Indeed, in this review the authors provide argumentation indicating that metformin may be of benefit in the treatment of both diabetic and nondiabetic patients with NAFLD.

The data presented and reviewed in this special issue highlight some novel or hitherto not elaborately studied aspects of NAFLD pathophysiology and treatment. Moreover, and perhaps more importantly, they underscore the complexity of factors and mechanisms regulating liver fat accumulation and depletion. We hope that they will encourage future efforts and research into understanding this very interesting and highly prevalent metabolic disorder.

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Review Article

Diagnosis and Evaluation of Nonalcoholic Fatty Liver Disease

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Nonalcoholic fatty liver disease (NAFLD) is the most common cause of elevated liver function tests results, after the commonly investigated causes have been excluded, and frequently coexists with type 2 diabetes mellitus (T2DM) because the conditions have common risk factors. As both T2DM and NAFLD are related to adverse outcomes of the other, diagnosis and valuation of fatty liver is an important part of the management of diabetes. Although noninvasive methods, such as biomarkers, panel markers, and imaging, may support a diagnostic evaluation of NAFLD patients, accurate histopathological findings cannot be achieved without a liver biopsy. As it is important to know whether steatohepatitis and liver fibrosis are present for the management of NAFLD, liver biopsy remains the gold standard for NAFLD diagnosis and evaluation. Therefore, new investigations of the pathogenesis of NAFLD are necessary to develop useful biomarkers that could provide a reliable noninvasive alternative to liver biopsy.

1. Introduction

Fatty liver, or hepatosteatosis, is characterized histologically by triglyceride accumulation within the cytoplasm of hepatocytes [1] and refers to fat accumulation in the liver exceeding 5%–10% by weight [2]. When hepatosteatosis is present in the absence of excessive alcohol consumption, it is termed non-alcoholic fatty liver disease, or NAFLD [1, 3–5], which is considered to be the hepatic manifestation of the metabolic syndrome [1, 6, 7], a constellation of frequent abnormalities involving insulin resistance, visceral obesity, dyslipidemia, diabetes, hypertension, plus additional factors. Hence, current therapeutic approaches focus on treatment of the underlying risk factors for these metabolic conditions [8]. NAFLD encompasses a spectrum of disorders ranging from simple steatosis to inflammatory steatohepatitis (NASH) and cirrhosis. Of those who develop NASH, about 20% of patients will develop cirrhosis during their lifetime [9]. Therefore, a diagnosis of NASH may result in a more aggressive therapeutic approach toward the metabolic risk factors [10]. In addition, a diagnosis of cirrhosis may suggest the need for an assessment of any associated complications, such as esophageal varices, hepatocellular carcinoma, and so on. The prevalence of NAFLD in the general adult population has been estimated to range from 10% to 24% worldwide; and is as high as 57.5% to 74% in those who are obese [11]. Currently, NAFLD is believed to account for up to 90% of all cases demonstrating elevated liver function test (LFT) results in patients after the commonly investigated causes have been excluded (e.g., viral hepatitis, alcoholism, inherited liver disease, or medications) [11].

NAFLD and Diabetes. NAFLD and type 2 diabetes mellitus (T2DM) frequently coexist because they share the risk factors of excess adiposity and insulin resistance. The prevalence of T2DM or impaired fasting glucose ranges from 18–33% in patients with NAFLD, whereas it ranges from 49–62% in T2DM patients who have NAFLD [12–15]. NASH is present in 12.2% of patients with T2DM, as compared to 4.7% in those without T2DM [16]. Moreover, T2DM increases the risk of liver-related death by up to 22-fold as well as overall death by 2.6–3.3-fold in patients with NAFLD [17]. In contrast, the presence of NAFLD among patients with

T2DM can also be a risk factor for increased mortality; a community-based study of patients with T2DM revealed that those with NAFLD had 2.2-fold increased risk of mortality compared with those without NAFLD [18]. A recent article also reported that the presence of NAFLD in T2DM patients may also be linked to increased cardiovascular disease (CVD) risk, independently of components of the metabolic syndrome [19, 20], although Ghouri et al. pointed out inconsistencies in the evidence among some articles [21]. Therefore, T2DM is a risk factor for progressive liver disease and mortality in patients with NAFLD, whereas NAFLD may be a marker of cardiovascular risk and mortality in individuals with T2DM. Both T2DM and NAFLD are related to adverse outcomes of the other. In addition, although type 1 diabetes mellitus (T1DM) is due to a relative lack of insulin, an increased prevalence of obesity and insulin resistance in this population means that NAFLD commonly coexists in patients with T1DM [22–25]. An association of NAFLD in T1DM with an increased prevalence of CVD has also been reported [25].

Hence, the diagnosis and evaluation of fatty liver is an important part of the management of diabetes. When diabetes patients are diagnosed with NAFLD, more intensive monitoring and therapeutic intervention are necessary to avoid a poor prognosis. The methods used for the diagnosis and evaluation of NAFLD can also be used to monitor the efficacy of intervention or therapy. In this review, we describe the current trends in the diagnosis and evaluation of NAFLD based on recent articles.

2. Diagnosis and Evaluation of NAFLD

The diagnosis of NAFLD needs confirmation of hepatic steatosis based on either imaging studies or liver biopsy, together with the clinical exclusion of individuals who regularly consume >20 g ethanol per day [26]. In the clinical setting, there is still no consensus about whether or not liver biopsy is required to confirm a diagnosis of NAFLD [8].

Presently, the available noninvasive markers for NAFLD include a set of clinical signs and symptoms, laboratory tests, imaging tests, and combinations of clinical and blood test results. Although several of these markers are, in general, useful for the diagnostic evaluation of a patient with suspected NAFLD, they lack the specificity and sensitivity to distinguish NAFL from NASH and to determine the presence and stage of fibrosis [10]. After a diagnosis of NAFLD, the next step is to determine the severity, and this information is necessary to understand the prognosis. Although noninvasive diagnostic methods have advanced recently, a liver biopsy is still required to determine the severity of NAFLD. When staging patients with NAFLD, there are two factors related to the severity: the level of fibrosis and the level of inflammation [27]. We will discuss both invasive and noninvasive means of assessing and staging patients with NAFLD, including information about the characteristics of each method.

2.1. Clinical Features. The majority of patients diagnosed with NAFLD are asymptomatic [8, 28]. When present, clinical symptoms and physical findings are nonspecific and unreliable for diagnosing and assessing disease severity in patients with NAFLD. Patients might have hepatomegaly, general malaise, abdominal discomfort, vague right upper quadrant abdominal pain, nausea, and other nonspecific symptoms referred to the gastrointestinal tract. Clinical examination may reveal ascites, splenomegaly, spider angiomas, palmar erythema, caput medusae, and jaundice in a small percentage of patients who present with NASH-related cirrhosis [8, 28]. The features more consistently found to be associated with disease severity include obesity, older age, diabetes, and hypertension [11].

2.2. Common Biomarkers. There is no single biochemical marker that can confirm a diagnosis of NAFLD or distinguish between steatosis, NASH, and cirrhosis [8]. Although mildly elevated serum aminotransferase levels are the primary abnormality seen in patients with NAFLD, liver enzymes may be normal in up to 78% of patients with NAFLD [12, 29]. Additionally, the entire histological spectrum of NAFLD can be observed in patients with normal alanine aminotransferase (ALT) values [30, 31]. Therefore, liver enzyme levels are not sensitive for the diagnosis of NAFLD. The elevations in ALT and aspartate aminotransferase (AST) are typically mild when present and are usually not greater than four times the upper limit of normal [29, 32]. The ratio of AST/ALT is usually less than 1 in patients who have either no or minimal fibrosis, although this ratio may be greater than 1 with the development of cirrhosis [33].

Gamma-glutamyltransferase (GGT) in the serum is frequently elevated in patients with NAFLD, and it has been reported to be associated with increased mortality [34, 35]. However, the diagnosis of NAFLD cannot be made using only GGT. Increased serum GGT levels have also been shown to be associated with advanced fibrosis in NAFLD patients, with a study of 50 NAFLD patients demonstrating an area under the receiver operating characteristic curve (AUROC) of 0.74 for the prediction of advanced fibrosis. Using a cutoff serum GGT value of 96.5 U/L, GGT predicted advanced fibrosis with 83% sensitivity and 69% specificity [36]. Alkaline phosphatase is sometimes slightly elevated, but it is rarely the only liver function test abnormality [37].

When portal hypertension and hepatic synthetic dysfunction are present with cirrhosis, hypoalbuminemia, hyperbilirubinemia, thrombocytopenia, and a prolonged prothrombin time may be seen [28]. Furthermore, an elevated ferritin level has been reported in up to 50% of NASH patients, and elevated transferrin saturation in approximately 10% [33]. However, these findings do not appear to correlate with an elevated iron concentration in the liver, and the role of hepatic iron in the pathogenesis of NASH is unclear [38].

2.3. Novel Biomarkers. Several investigators have proposed the measurement of other novel biomarkers to support the diagnosis of NAFLD, but these investigations have been limited by their lack of reproducibility or inability to accurately distinguish simple steatosis from more advanced

inflammation and fibrosis [8]. Ideally, novel biomarkers should be helpful for monitoring the progression of NAFLD over time, its response to therapeutic interventions, and for determining the prognosis of the disease. However, there is no such a biomarker available at present [10]. These novel potential biomarkers are shown herein, being classified by the key mechanisms of NASH pathogenesis that they are associated with, including "inflammation," "fibrosis," "oxidative stress," and "hepatocyte apoptosis".

2.3.1. Inflammation. A chronic low-grade inflammatory state characteristic of patients with metabolic syndrome has been extensively associated with the development of steatosis as well as liver damage in NAFLD [39, 40]. Inflammatory mediators have also been investigated as potential diagnostic tools. NAFLD is associated with an increase in tumor necrosis factor alpha (TNF-α) and decreased adiponectin, and this cytokine imbalance may play an important role in the development of NASH [41–46]. Several groups have reported the circulating levels of these cytokines in the blood from patients with NAFLD to correlate with NASH. Moreover, the TNF-α levels were also observed to correlate with the severity of inflammation and fibrosis [41, 47]. However, there are still limited data available on the accuracy and clinical usefulness of these markers for the noninvasive diagnosis of NASH [10]. Shimada et al. reported that the serum adiponectin was significantly lower in patients with early-stage NASH than in those with simple steatosis. Adiponectin had an AUROC of 0.765, sensitivity of 68%, and specificity of 79% for distinguishing early-stage NASH, using a cutoff value of $\leq 4.0\,\mu$g/mL. In this study, the combination of the serum adiponectin level with the Homeostasis Model Assessment-Insulin Resistance (HOMA-IR) level (cutoff value ≥ 3.0) and type IV collagen 7S (cutoff value ≥ 5.0 ng/mL) demonstrated a sensitivity of 94% and a specificity of 74% for diagnosing NASH [48]. In another report, using adiponectin and HOMA-IR levels, the AUROC for distinguishing between steatohepatitis and steatosis was 0.79 [43]. However, the relationship between the adiponectin levels and the severity of hepatic fibrosis still remains to be established [49, 50].

The inflammatory marker, C-reactive protein (CRP), lacks specificity for hepatic inflammation and has demonstrated mixed results for NASH. There was a significant increase in high-sensitivity CRP levels in NASH patients compared with controls in some studies [51, 52], and no significant difference in another [53]. Interleukin-6 (IL-6) has also been shown to be elevated in NASH [54]. IL-6 had an AUROC of 0.817 for distinguishing NASH from simple steatosis [55]. The IL-6 levels were also independently related to fibrosis [56].

Several authors have reported a relationship to exist between leptin, a hormone secreted from adipose tissue, and liver histology. However, the leptin levels have not been shown to correlate with the degree of steatosis or fibrosis, although some studies have previously reported patients with steatosis and NASH to demonstrate elevated levels of leptin [57–59]. In a recent study, using the combination of HOMA-IR with the adiponectin/leptin ratio, the AUROC was 0.82 for distinguishing between NASH and simple steatosis [56].

Other inflammatory markers, such as CC-chemokine ligand 2 (CCL-2) and hyaluronic acid (HA), have also been shown to be elevated in patients with NASH [60–62]. HA will be described in the next section.

2.3.2. Fibrosis. During the evaluation of NAFLD, it is important to consider the level of fibrosis. Potential fibrosis biomarkers include type IV collagen 7S domain and HA. Sakugawa et al. reported that these two biomarkers were able to exclude advanced fibrosis, with AUROCs of 0.82 and 0.80, and negative predictive values (NPV) of 84% and 78%, respectively, in a cohort of 112 NAFLD cases. These biomarkers also demonstrated positive predictive values (PPV) of 86% and 92% and AUROCs of 0.83 and 0.80 for discriminating between NASH and simple hepatosteatosis [63]. In another report, the AUROC for type IV collagen 7S domain and HA were 0.767 and 0.754, respectively, for the detection of advanced fibrosis in NASH cases, although a multiple regression analysis revealed that only the type IV collagen 7S domain was independently associated with advanced fibrosis in this study [64]. In patients with NAFLD, evaluating the HA levels was found to be useful for predicting severe fibrosis, with an AUC of 0.9, with a cutoff value of serum HA of 46.1 ug/l, yielding a sensitivity of 85% and a specificity of 80% [61]. Furthermore, the platelet count alone was demonstrated to be an independent predictor of cirrhosis, with an AUROC of 0.98 in NAFLD. In this study, HA had an AUROC of 0.97 for detecting severe fibrosis, with a lower AUROC of 0.87 shown for type IV collagen. Serum laminin, which is an extracellular matrix component, was also shown to have an accuracy of 87%, sensitivity 82%, specificity 89%, PPV 82%, and NPV 89% for prediction of fibrosis in NAFLD [65].

2.3.3. Oxidative Stress. Oxidative stress is one of the key mechanisms responsible for liver damage and disease progression in NAFLD [66, 67]. To date, many markers of oxidative stress, including lipid peroxidation products, vitamin E levels, and copper-to-zinc superoxide dismutase and glutathione peroxidase (GSH-Px) activity, have been investigated to determine whether they can be used as surrogate markers of NASH. However, mixed results were demonstrated [68–70], and there is no clear answer at present.

Thioredoxin (TRX) is induced by many oxidative stresses. A significant elevation of the serum TRX levels was demonstrated in patients with NASH in comparison to those with simple steatosis and healthy controls [71, 72].

2.3.4. Hepatocyte Apoptosis. Apoptosis plays an important role in the liver injury observed in NAFLD [73–77]. Recently, a specific byproduct of apoptosis in hepatocytes, caspase-generated cytokeratin-18 (CK-18) fragments, has been shown to be significantly elevated in patients with NASH compared with subjects with fatty liver or healthy controls, with an AUC of 0.93 for predicting NASH [78]. Additionally, Feldstein et al. demonstrated that the plasma CK-18 levels measured using ELISA were significantly higher

in patients with biopsy-proven NASH than in those with a borderline diagnosis and normal controls, with an AUROC of 0.83 for NASH diagnosis, in a US multicenter validation study. CK-18 was an independent predictor of both NASH and the severity of disease [79]. Other reported results [78, 80–85] also suggest that CK-18 can be a potentially useful biomarker for the diagnosis and differentiation of NASH from simple hepatosteatosis. CK-18 levels were observed to decrease after bariatric surgery in NASH patients [86]. Therefore, CK-18 fragments might be useful for assessing the response to therapy for NASH.

The plasma homocysteine levels [87], serum prolidase enzyme activity (SPEA) catalysis [88], plasma pentraxin 3 levels [89], and tissue polypeptide specific antigen [90] are other novel biomarkers for a diagnosis of NASH, however, additional studies are needed to determine their potential for clinical use.

2.4. Panels of Markers. Several studies have been performed to develop noninvasive diagnostic panels and scoring systems that might support the identification of liver steatosis and the diagnosis NASH and to determinate severity of fibrosis, in order to replace the invasive standard liver biopsy. Such scoring systems may potentially represent a more accurate evaluation of global liver fibrosis severity, because the distribution of fibrosis throughout the liver can be uneven in NAFLD [27].

2.4.1. Panel Markers for the Identification of Liver Steatosis. The NAFLD liver fat score includes, as variables, the presence of metabolic syndrome and T2DM, fasting serum insulin, serum AST, and the AST/ALT ratio. This score has an AUROC of 0.86-0.87 to predict liver steatosis, and addition of the genetic information to the score slightly improved the AUROC. Using the same variables, a liver fat equation was developed, from which the liver fat percentage could be estimated [91].

Bedogni et al. developed the fatty liver index (FLI), which uses the body mass index (BMI), waist circumference, triglyceride level, and GGT in the general population with low prevalence of T2DM. This index varies from 0 to 100, and the AUROC was 0.84 to detect liver steatosis [92]. They also reported that the lipid accumulation product (LAP), based on the measurement of waist circumference and the triglyceride level, proved to be a simple and reasonably accurate predictor of ultrasonographic liver steatosis, with an AUROC of 0.8 [93].

Moreover, the visceral adiposity index (VAI), which uses the BMI, waist circumference, and levels of triglycerides and high-density lipoprotein (HDL) cholesterol, is thought to be capable of indicating both the fat distribution and function. This index was reported to be associated with liver steatosis in patients with chronic hepatitis C, making it a candidate predictor of NAFLD [94].

2.4.2. Panel Markers for NASH Diagnosis. The HAIR (Hypertension, ALT, and Insulin Resistance) score was designed to predict a NASH diagnosis, and includes a combination of the presence of hypertension, elevated ALT, and insulin resistance. The presence of at least 2 parameters predicted NASH with both a high sensitivity and specificity [95].

Palekar et al. generated a clinical model to distinguish NASH from simple steatosis by combining 6 different variables including age, gender, AST, BMI, the AST/ALT ratio, and serum HA [96]. The AUROC for this model was 0.76. The presence of 3 or more of these factors had a sensitivity and specificity for a NASH diagnosis of 74% and 66%, respectively.

Moreover, a simplified model has also been proposed using a logistic regression analysis with only AST and a diagnosis of diabetes, which was able to distinguish NASH from fatty liver with or without nonspecific inflammation in bariatric surgery patients with similar accuracy as the panels described in previous studies [97]. These panels should be thus investigated to validate them in different populations.

The Nash Test combines 13 biochemical and clinical variables to predict the presence of NASH, achieving specificity, sensitivity, PPV, and NPV of 94%, 33%, 66%, and 81%, respectively [98]. Pelekar et al. combined 8-epi-PGF2$_\alpha$, TGF-β, HA, and adiponectin in a model that predicted NASH with favorable sensitivity of 73.7%, specificity of 65.7%, PPV of 68.2%, and a NPV of 68.2% [96].

2.4.3. Panel Markers for Fibrosis in NAFLD. The FibroTest is a validated set of markers for the quantitative assessment of fibrosis, and it includes α2-macroglobulin, apolipoprotein A-I, haptoglobin, total bilirubin, GGT, and ALT [99]. The mean standardized AUROC was 0.84 for advanced fibrosis in NAFLD patients after correcting for age and gender in one meta-analysis.

The NAFLD fibrosis score (NFS) is generated using a panel including six variables of age, hyperglycaemia, BMI, platelet count, albumin, and *AST/ALT* ratio (AAR), which was created using a large cohort of biopsy-proven NAFLD patients [100]. McPherson et al. reported the NFS to demonstrate an AUROC of 0.81 with NPV 92% and PPV 72% for the detection of advanced fibrosis [101]. Calès et al. demonstrated an AUROC of 0.884 for significant fibrosis, 0.932 for severe fibrosis, and 0.902 for cirrhosis [102]. In a recent meta-analysis, NFS showed AUROC, sensitivity, and specificity of 0.85, 0.90, and 0.97 for the identification of NASH with advanced fibrosis [103].

The original ELF (European Liver Fibrosis) test is a panel of automated immunoassays to detect three markers: HA, tissue inhibitor of metalloproteinase 1 (TIMP1), and aminoterminal peptide of procollagen III (P3NP), used in combination with age [104]. The simplified ELF panel excluded age, but did not change the diagnostic performance of the panel. The addition of five markers, including the BMI, presence of diabetes/impaired fasting glucose, AAR, platelet count, and albumin concentration, to the ELF test improved its diagnostic accuracy, with AUROCs of 0.98, 0.93, and 0.84 for the diagnosis of severe, moderate and no fibrosis, respectively [105].

The BARD score is a simple scoring system that can be used as a predictive tool in assessing fibrosis in patients with NAFLD. It combines three variables; the BMI, AAR, and

the presence of diabetes [106]. A study of the BARD score in 138 patients with biopsy-proven NAFLD demonstrated an AUROC of 0.67, with sensitivity, specificity, PPV, and NPV of 51%, 77%, 45%, and 81%, respectively [107].

The AST-to-platelet ratio index (APRI) was initially used as a marker of fibrosis in patients with hepatitis C [108]. Using this score, Calès et al. reported an AUROC of 0.866 for significant fibrosis, 0.861 for severe fibrosis, and 0.842 for cirrhosis in a study of NAFLD subjects [102], although significantly lower values were obtained in other studies [101, 109, 110].

The AAR [111] is easily calculated using two laboratory liver function tests. Not only is the AAR used as an individual marker, but it is also a component of several other fibrosis scoring systems, including the NFS and BARD score. Using a cut-off of 0.8, AAR alone showed an AUROC of 0.83, with a sensitivity of 74%, specificity of 78%, and a NPV of 93% for the diagnosis of advanced fibrosis in NAFLD [101]. In another study, a combination of serum AST, ALT, and the AAR had an AUROC of 0.59 for predicting steatosis, but was able to predict cirrhosis with an AUROC of 0.81. However, the addition of demographic data, comorbidities, and several other routinely measured laboratory tests increased the AUROCs to 0.79 for NASH and 0.96 for cirrhosis [112].

The components of the FIB-4 test are age with three biochemical values; the platelet count, ALT, and AST, to detect fibrosis [113]. In NAFLD patients, the FIB-4 demonstrated an AUROC of 0.86, sensitivity of 85%, specificity of 65%, and an NPV of 95% for the diagnosis of advanced fibrosis [101]. Interestingly, in a comparison of several markers of fibrosis in NAFLD subjects, FIB-4 had the highest AUROC (0.802) for the diagnosis of advanced fibrosis, followed by the AUROCs for the NAFLD fibrosis score, AAR, APRI, and AST: platelet ratio and BARD score of 0.768, 0.742, 0.73, 0.72 and 0.70, respectively [114].

The FibroMeter combines seven variables: age, weight, fasting glucose, AST, ALT, ferritin, and the platelet count [115]. In a study of 235 NAFLD patients, it had AUROCs of 0.943 for significant fibrosis, 0.937 for severe fibrosis, and 0.904 for cirrhosis, respectively. The sensitivity, specificity, PPV, and NPV of the FibroMeter for diagnosing significant fibrosis were 79%, 96%, 88%, and 92% [102].

2.5. Imaging. Although many imaging tools have been assessed in NAFLD subjects, their main focus has been the quantification of liver fat. The results of these imaging tests cannot be used to differentiate between the histological subtypes of simple steatosis or NASH, nor can they be used to stage the degree of fibrosis [116, 117]. In this section, we explain each imaging modality, while referring to the detection of hepatosteatosis, steatohepatitis, and fibrosis.

2.5.1. Ultrasonography (US). US is currently the most common method for screening asymptomatic patients with elevated liver enzymes and suspected NAFLD. US findings of fatty liver include hepatomegaly, diffuse increases in the echogenicity of the liver parenchyma, and vascular blunting. Nonsteatotic hepatic parenchyma exhibits an echotexture similar to that of renal parenchyma, but becomes "brighter" when infiltrated with fat [118]. This hepatorenal contrast can be used for detecting hepatosteatosis [118, 119]. A recent study by Palmentieri et al. of 235 patients undergoing US with liver biopsy showed a sensitivity, specificity, PPV, and NPV of 91%, 93%, 89%, and 94%, respectively, for predicting at least 30% steatosis. However, bright liver contrast was not associated with fibrosis in this study [120].

US is easily performed and has a low cost, but it also has some limitations. It is operator dependent and subject to significant intra- and interobserver variability [121]. It is impossible for US to provide quantitative information about the degree of fat accumulation. The sensitivity of US to detect steatosis decreases with a degree of fat infiltration less than 30% [122]. In obese patients, sensitivity lower than 40% has been reported to detect hepatosteatosis [123]. Finally, US has failed to prove efficacious for the detection of inflammation and fibrosis, therefore, it cannot be utilized to diagnose NASH and hepatic fibrosis [10]. In a recent study, however, Iijima et al. used an ultrasound contrast agent (Levovist; Sherling, Berlin) to distinguish between simple steatosis and NASH. Levovist contains galactose and palmitic acid and is taken up by hepatocytes [124]. These moieties participate in the sugar and fat metabolism [125]. The uptake of Levovist is observed to significantly decrease in NASH patients, thus correlating with fibrosis rather than steatosis [124]. Larger studies are needed to evaluate contrast US for use in the diagnosis of NASH and advanced fibrosis.

2.5.2. Computed Tomography (CT). CT allows for a more quantitative assessment with measurement of liver attenuation in Hounsfield units (HUs) compared to US, but the information about liver attenuation is not uniform when reported by radiologists. It appears that noncontrast CT scanning is more useful for detecting steatosis than contrast-enhanced scans [126]. Unenhanced CT is more commonly used than enhanced CT [127], and several techniques for determining the appropriate CT values include measurement of hepatic attenuation only [128, 129] and normalization of hepatic attenuation by splenic attenuation, reporting the difference in attenuation between the liver and spleen [127, 129] and the ratio of these values [129, 130]. The attenuation of the spleen is approximately 8–10 HUs less than the liver in normal subjects [127]. With unenhanced CT, an attenuation of the liver is less than 40 HUs [131], or a liver-to-spleen attenuation difference greater than −10 HUs is highly predictive of hepatosteatosis [127]. In addition, a liver-to-spleen ratio of less than 1 is sometimes used to diagnose fatty liver [127, 130]. CT has been demonstrated to be useful for diagnosing >30% steatosis by the use of liver: spleen attenuation ratios; the method has a sensitivity of 73%–100% and a specificity of 95%–100% [29, 132]. The accuracy of unenhanced CT is greatly reduced when there is a lesser degree of steatosis [129]. Other pathologies, such as hepatic siderosis, may also alter attenuation values, thus leading to a misdiagnosis [133, 134]. In longitudinal studies with young subjects, the radiation exposure associated with CT limits its use [133].

2.5.3. Magnetic Resonance Imaging (MRI) and Proton Magnetic Resonance Spectroscopy (MRS). A good correlation has been reported between MRI, US, and histology in patients with NAFLD [135]. Among these modalities, MRI has been shown to most accurately detect lower levels of steatosis than those detected by US and CT. Fatty changes are assessed by differential chemical shifts between fat and water detected by MRI. MRI was able to detect steatosis of level down to 3% [135]. A variant of MRI, MRS, has also been shown to reliably measure steatosis [136]. Szczepaniak et al. used proton MRS to measure hepatic triglyceride levels (HTGC) in 375 subjects [2]. In this study, 34.3% of the 2,287 participants studied had HTGC >5%, a level deemed diagnostic of hepatic steatosis. MRI and MRS have a higher diagnostic accuracy than US or CT, and MRS can be used to quantify hepatic steatosis. However, none of these imaging techniques have sufficient sensitivity and specificity for staging the disease, and therefore cannot distinguish between fatty liver and NASH with or without fibrosis [116]. Moreover, these tools are more expensive and less accessible than other imaging modalities.

2.5.4. Transient Elastography. Transient elastography (Fibroscan, Echosens, Paris, France) is a non-invasive method of assessing liver fibrosis which can be performed at the bedside or in an outpatient clinic. It uses ultrasound-based technology to measure liver stiffness. Although Fibroscan is less well validated in NAFLD, in a study of 97 NAFLD patients, AUROCs for the diagnosis of significant fibrosis, severe fibrosis, and cirrhosis were reported to be 0.88, 0.91, and 0.99, respectively [137]. Another study including 246 NAFLD patients showed AUROCs for the diagnosis of moderate fibrosis, bridging fibrosis, and cirrhosis of 0.84, 0.93, and 0.95, respectively [138].

The combination of transient elastography with one or more of the serum marker panels might be a potential approach for the non-invasive measurement of fibrosis in NAFLD [10].

2.6. Histology. The histological spectrum of NAFLD ranges from simple steatosis through steatohepatitis to fibrosis and cirrhosis [139]. Liver biopsy is the gold standard for diagnosis and has the additional benefit of distinguishing between NASH and simple steatosis, thus allowing for the staging of the degree of fibrosis, which also provides helpful information regarding prognosis and may influence the clinical management of NAFLD [116, 140, 141]. Liver biopsies can also exclude other liver diseases, such as drug-induced hepatotoxicity, Wilson disease and autoimmune hepatitis [11, 142]. Hence, although the diagnosis of NAFLD can usually be confirmed by only a combination of history, serological analyses, and abdominal imaging, liver tissue is needed to determine the severity of NAFLD and to rule out other possible liver diseases [8].

The histological changes in NAFLD are mainly parenchymal and appear in a perivenular location, although portal and periportal lesions may also be present [139]. Simple steatosis is usually macrovesicular, resulting from the accumulation of triglycerides within hepatocytes [143]. NASH requires evidence of lobular inflammation, which usually consists of a mixed mononuclear and neutrophil infiltration. Additionally, hepatocyte ballooning, necrosis, and Mallory's hyaline might be present [144]. Mitochondrial abnormalities may also occur in NASH, but rarely in simple steatosis [145]. As the disease progresses, the typical histological features of steatosis and inflammation frequently disappear and may become completely absent in patients with cirrhosis [146]. Thus, many cases of cryptogenic cirrhosis may be caused by NASH cirrhosis [147–149]. Hepatocellular carcinoma is a complication of NASH-related cirrhosis [117, 150] and also of precirrhotic NAFLD [151, 152]. Other histological findings characteristic of NAFLD in T1DM patients includes diabetic hepatosclerosis and glycogenic hepatopathy [25, 153].

The NAFLD Activity Score (NAS) is used for the histological assessment of NAFLD to distinguish steatosis from NASH in clinical trials [154]. NAS provides a composite score based on the degree of steatosis, lobular inflammation, and hepatocyte ballooning. A score greater than or equal to 5 is likely to represent NASH, a score of 0–2 is unlikely to represent NASH, and a score of 3 or 4 is indeterminate. The NAS does not include fibrosis, and fibrosis is reported separately, on a scale from 0 (without fibrosis) to 4 (cirrhosis) [154]. However, it should be noted that the diagnosis of definite liver steatosis based on evaluations of the patterns on liver biopsy does not always correlate with the threshold values of the semiquantitative NAS, which was developed as a tool to measure changes in NAFLD during therapeutic trials [155].

Limitations of Liver Biopsy. There are several limitations associated with liver biopsy. Liver biopsy, being an invasive procedure, has a small risk of complications including pain, bleeding, and, rarely, death. Next, since only a very small portion of the liver is obtained from the needle liver biopsy as a tissue sample (1/50,000 of the total mass of the liver), the biopsy is prone to significant sampling error [156, 157], especially regarding such features as fibrosis, which are often not uniformly distributed. Finally, another important limitation of liver biopsy is the fact that the histological analysis remains subjective, is influenced by the skill and experience of the reading pathologist, and is thus prone to intra- and interobserver variability [154, 158].

3. Conclusion

At present, NAFLD is the most common cause of elevated LFT results, after the commonly investigated causes have been excluded. NAFLD and T2DM frequently coexist because of their similar pathogenic abnormalities. As both T2DM and NAFLD are related to adverse outcomes of the other, the diagnosis and evaluation of fatty liver is an important part of the management of diabetes. Noninvasive methods are favorable ways to support a diagnosis of hepatosetaotsis, but accurate histopathological findings and staging of fibrosis cannot be achieved without a liver biopsy. It is important to determine whether NASH and liver fibrosis

are present, because close monitoring and followup are necessary for these patients. Therefore, a liver biopsy remains the gold standard for the diagnosis and evaluation of NAFLD. However, new investigations on the pathogenesis of the disease progression in NAFLD might result in the development of useful biomarkers that could provide a reliable noninvasive alternative to liver biopsy.

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Review Article

Role of Transcription Factor Modifications in the Pathogenesis of Insulin Resistance

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Non-alcoholic fatty liver disease (NAFLD) is characterized by fat accumulation in the liver not due to alcohol abuse. NAFLD is accompanied by variety of symptoms related to metabolic syndrome. Although the metabolic link between NAFLD and insulin resistance is not fully understood, it is clear that NAFLD is one of the main cause of insulin resistance. NAFLD is shown to affect the functions of other organs, including pancreas, adipose tissue, muscle and inflammatory systems. Currently efforts are being made to understand molecular mechanism of interrelationship between NAFLD and insulin resistance at the transcriptional level with specific focus on post-translational modification (PTM) of transcription factors. PTM of transcription factors plays a key role in controlling numerous biological events, including cellular energy metabolism, cell-cycle progression, and organ development. Cell type- and tissue-specific reversible modifications include lysine acetylation, methylation, ubiquitination, and SUMOylation. Moreover, phosphorylation and O-GlcNAcylation on serine and threonine residues have been shown to affect protein stability, subcellular distribution, DNA-binding affinity, and transcriptional activity. PTMs of transcription factors involved in insulin-sensitive tissues confer specific adaptive mechanisms in response to internal or external stimuli. Our understanding of the interplay between these modifications and their effects on transcriptional regulation is growing. Here, we summarize the diverse roles of PTMs in insulin-sensitive tissues and their involvement in the pathogenesis of insulin resistance.

1. Posttranslational Modifications of Transcription Factors: Relevance in the Context of Metabolic Syndrome

Transcription is the seminal event in the expression of genes and is a central point at which gene expression is regulated. Many cellular processes, including those that are tissue-specific or developmentally related, are largely controlled at the transcriptional level [1]. Transcription factors often regulate the expression of genes by binding to specific consensus sequences, or *cis* elements, within promoter regions [2]. Once bound, coregulators that either activate or repress transcription are recruited [3, 4]. Transcription factors play critical roles in regulating constitutive and inducible gene expression. In response to cellular stimuli, these proteins can be targets of modifications that affect their stability, activity, intracellular distribution, and interaction with other proteins [5]. Different external and internal signals direct distinct patterns of posttranslational modifications (PTMs), which transduce the signals for specific metabolic processes.

The number of people diagnosed with type 2 diabetes mellitus (T2DM) worldwide has been estimated to exceed 200 million [6]. Left untreated or uncontrolled, this disease can cause serious complications such as blindness, kidney damage, and vascular damage that may require the amputation of limbs or digits. T2DM is characterized by defects in both insulin sensitivity and secretion [7]. Central to this defect is insulin resistance, which reflects impaired sensitivity

of target organs—primarily liver, pancreas, adipose tissue, and muscle—to insulin [8, 9]. Although the pathogenesis of insulin resistance remains unclear, abnormal insulin signaling [10], mitochondrial dysfunction [11], endoplasmic reticulum (ER) stress [12], dysfunctional triglyceride/free fatty acid cycle intermediates [13], and inflammation [14] have been reported to be involved in mediating this disease. These abnormalities lead to alterations in the transcription of key metabolic genes accompanied by PTMs of transcription factors that may result in the suppression or activation of target genes.

Recent advances in the understanding of PTMs, including those of transcription factors, have provided greater insight into the altered gene regulation that results in insulin resistance. Interestingly, multiple PTMs—both independent and interdependent—can occur, creating the potential for diverse cellular responses through changes at the transcriptional level. In this paper, we will limit our discussion to transcription factor PTMs responsible for metabolic alterations associated with insulin resistance.

2. Types of Transcription Factor Modifications

PTMs could be considered an evolutionary solution to the limited number of transcription factors, expanding the functional repertoire of genetic regulatory elements to cover the diverse metabolic requirements that are met through regulated gene expression. Although a large number of transcription factors have been demonstrated to be modified by PTM, there are still more left to be discovered. Furthermore, the interrelationship between various types of PTM should be understood in terms of modulating the DNA binding activity, stability, localization, and protein-protein interactions. Transcription factors can undergo several different types of PTMs, including acetylation, phosphorylation, glycosylation, and ubiquitination. The transcription factors and target genes considered in this paper are listed in Table 1. In addition, the functions of PTM of transcription factors are summarized in Figure 1.

2.1. Acetylation/Deacetylation. Acetylation of histone or nonhistone proteins is critical for gene expression. This modification, which occurs on lysine residues, affects protein stability, localization, degradation, and function. Moreover, this modification can also influence protein-protein and protein-DNA interactions. Interestingly, most acetylated forms of nonhistone proteins have been shown to be involved in tumorigenesis and immune function. Our understanding of the role of acetylation of transcription factors involved in insulin resistance is incomplete, but emerging evidence indicates that acetylation influences the subcellular distribution, DNA binding ability, and proteasomal degradation of these proteins [15].

2.2. Phosphorylation/Dephosphorylation. External stimuli often lead to the activation of signal transduction pathways that result in the phosphorylation of transcription factors. Depending on the stimulus, specific amino acid residues, typically tyrosine, serine, and/or threonine, are phosphorylated by one or more protein kinases. Dephosphorylation by phosphatases can also occur in response to cellular signals. This phosphorylation/dephosphorylation dynamic can directly regulate distinct aspects of transcription factor function, including subcellular distribution, DNA binding, transacting ability, and protein stability [16, 17].

2.3. Modification by O-Linked-N-Acetylglucosamine: O-GlcNAcylation. *O*-GlcNAcylation is a dynamic, inducible, and reversible, nutrient-sensitive post-translational event in which *O*-linked-*N*-acetylglucosamine (*O*-GlcNAc) is attached to serine and/or threonine hydroxyl groups of cytosolic [18], mitochondrial [19], or nuclear proteins [18] by the concerted actions of *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase [18, 20].

UDP-GlcNAc is a major end product of the hexosamine biosynthesis pathway and functions as a cellular nutrient sensor. Sustained exposure to high concentrations of glucose and glucosamine increases UDP-GlcNAc levels, which, in turn, results in an increase in *O*-GlcNAc-glycosylated proteins and leads to glucotoxicity in various insulin-sensitive tissues [21]. Indeed, insulin-signaling molecules, including the β subunit of the insulin receptor, insulin receptor substrate (IRS)-1 and -2, the p85 and p110 subunits of phosphoinositide 3-phosphate kinase (PI3K), protein kinase B (PKB)/Akt, and 3-phosphoinositide-dependent protein kinase-1 (PDK1), are targets of OGT, and O-GlcNAcylation of these proteins causes downregulation of insulin signaling [22].

2.4. Ubiquitination and SUMOylation. The amount of intracellular protein is regulated by the rates of protein synthesis and degradation. In general, protein degradation occurs via the ubiquitin-proteasome pathway [23]. Ubiquitin, a highly conserved protein consisting of 76 amino acids, is targeted to substrate proteins and polymerized by the sequential action of three enzymes: E1, a ubiquitin-activating enzyme; E2, a ubiquitin-conjugating enzyme; E3, a ubiquitin-protein ligase [24]. The resulting protein contains multiple chains of branched ubiquitin molecules that enable recognition by the 26S proteasome, which subsequently mediates degradation of the ubiquitinated protein into small peptides [24, 25].

In addition to ubiquitination, transcription factors can also be modified by the addition of SUMO (small ubiquitin-related modifier), a protein composed of 97 amino acids. In this event, SUMO is attached to lysine residues in the substrate protein by the sequential action of three enzymes [26]. SUMOylation can affect protein stability, subcellular localization, or protein-protein interactions [27, 28]. SUMOylation often competes with ubiquitination and/or acetylation for lysine residues on target transcription factors [29, 30].

Reports have suggested that deregulated ubiquitin/proteasome-mediated degradation of insulin signaling molecules results in insulin resistance and the development of diabetes [31].

Table 1: The target genes of the transcription factors.

Transcription factor	Target gene		Reference
	Gene symbol	Description	
FOXO1	*G6PC*	Glucose-6-phosphatase	[36]
	Pck1	Phosphoenolpyruvates carboxykinase1	[181]
	Ppargc1a	Peroxisome proliferator-activated receptor-coactivator-1 alpha	[38]
	Pdx1	Pancreatic and duodenal homeobox 1	[101]
	NeuroD	Neurogenic differentiation	[107]
	MafA	V-maf (maf musculoaponeurotic fibrosarcoma) oncogene homolog A	[107]
	ADIPOQ	Adiponectin	[170]
CREB	*G6pc*	Glucose-6-phosphatase	[57]
	Pck1	Phosphoenolpyruvates carboxykinase	[57]
	Ppargc1a	Peroxisome proliferator-activated receptor-coactivator-1 alpha	[57]
SREBP-1c	*ACLY*	ATP-citrate lyase	[182, 183]
	Acaca	Acetyl-CoA carboxylase alpha	[184]
	ACACB	Acetyl-CoA carboxylase beta	[185]
	Fasn	Fatty acid synthase	[186]
	Scd1	Stearoyl-coenzyme A desaturase 1	[187]
	Elovl6	ELOVL fatty acid elongase 6	[188]
ChREBP	*Pklr*	Pyruvate kinase, liver, and RBC	[189]
	Acc1	Acetyl-CoA carboxylase 1	[190]
	Fasn	Fatty acid synthase	[191]
NF-κB	*TNF-α*	Tumor necrosis factor alpha	[192]
	IL-6	Interleukin 6	[193]
	MCP-1	Monocyte chemotactic protein 1	[194]
Sp1	*LEP*	Leptin	[195]
	LETN	Resistin	[196]

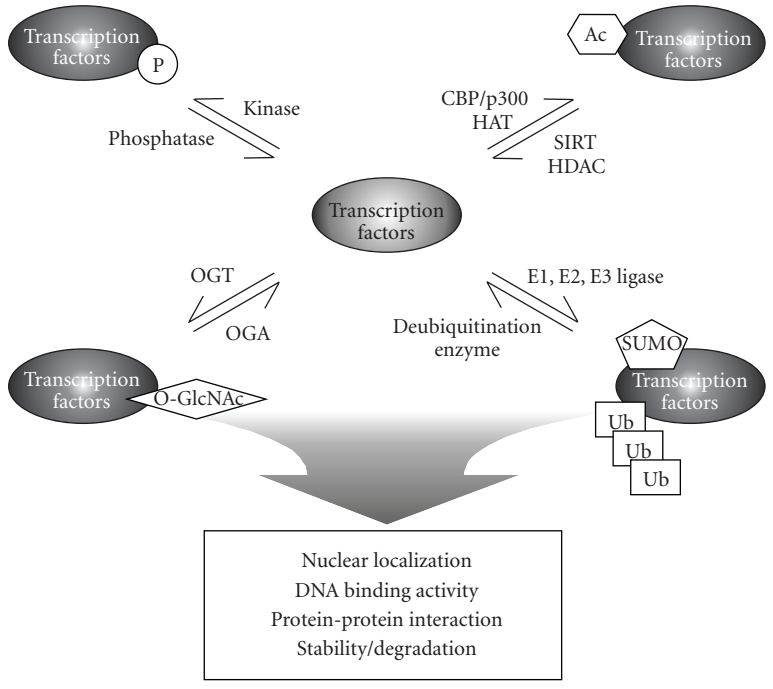


Figure 1: The types and functions of post-translational modification of transcription factors.

3. Modification of Transcription Factors in the Insulin-Sensitive Tissues

3.1. Liver Metabolism

3.1.1. Effect of Transcription Factor Modifications on Hepatic Gluconeogenesis. Hepatic gluconeogenesis is an essential process during fasting or starvation. However, activation of gluconeogenesis in patients with T2DM causes hyperglycemia. Insulin has been shown to suppress gluconeogenesis in the liver [32]. When insulin binds to its receptor, signal transduction pathways are activated that lead to the induction of Akt, which phosphorylates the Forkhead protein, FOXO1 [33, 34], a major transcription factor for gluconeogenic gene expression. The phosphorylated form of FOXO1 is translocated from the nucleus to the cytosol (Figure 2(b)).

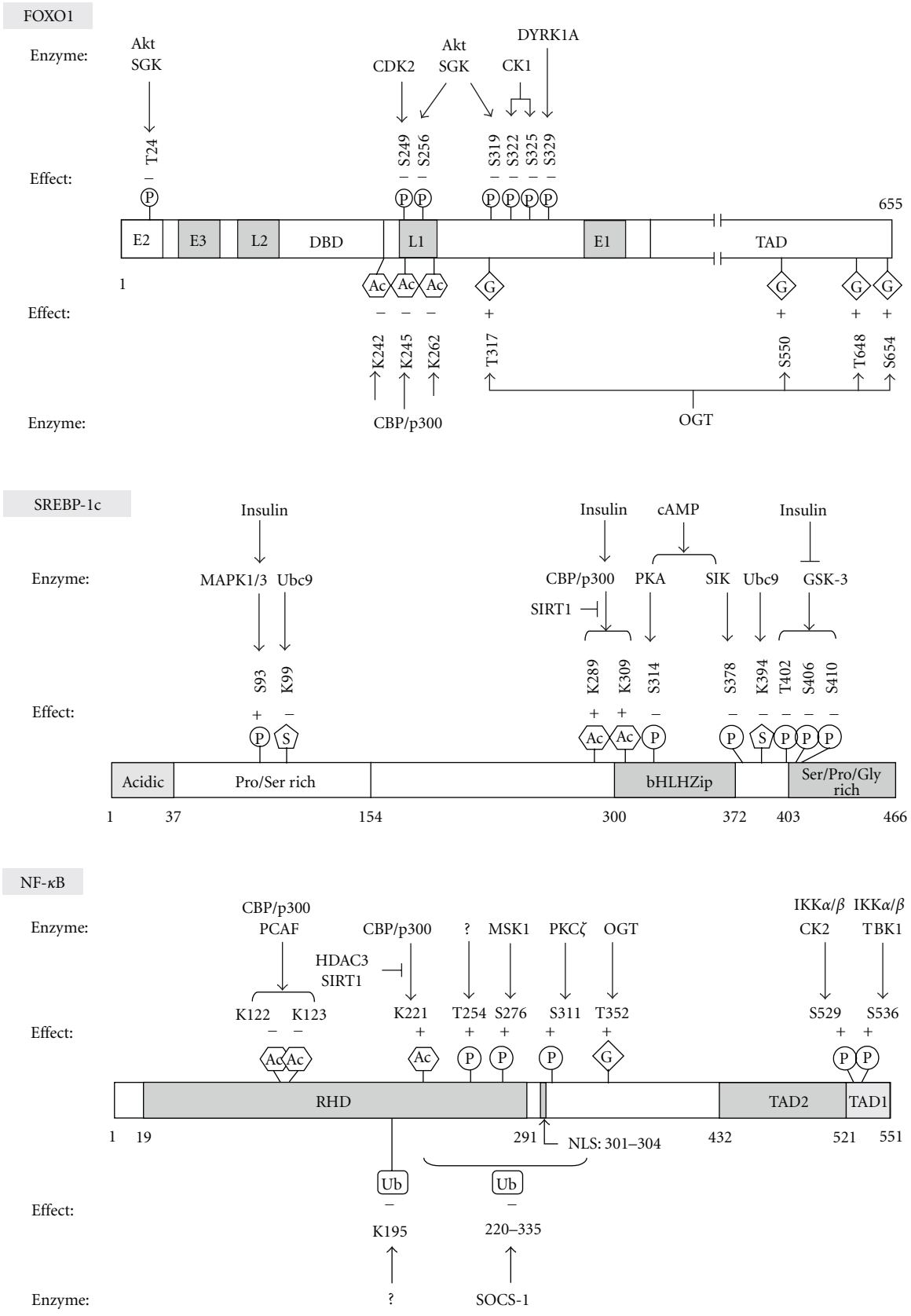
FOXO proteins have been reported to modulate a variety of cellular responses depending on the cell type [35]. Subfamilies of FOXO proteins include FOXO1 (FKHR), FOXO3a (FKHR-like1), and FOXO4/AFX (acute lymphocytic leukemia-1 fused gene from chromosome X). FOXO1 is a positive *trans* acting factor that binds to promoter regions within the glucose-6-phosphatase (*G6pc*) [36], phosphoenolpyruvate carboxykinase (*Pck1*) [37], and peroxisome proliferator-activated receptor-coactivator-1 alpha (*Ppargc1a*) genes [38]. Composed of 655 amino acids, FOXO1 contains seven phosphorylation sites, namely Thr^{24}, Ser^{249}, Ser^{256}, Ser^{319}, Ser^{322}, Ser^{325}, and Ser^{329}, which are modified by a variety of mechanisms (Figure 2(a)). Thr^{24}, Ser^{256}, and Ser^{319} are phosphorylated by protein kinase B (PKB)/Akt (v-akt murine thymoma viral oncogene homolog 1) in response to insulin/insulin growth factor-1 signaling [39]. Ser^{249} is phosphorylated by CDK2 (cyclin-dependent kinase 2) [40], whereas Ser^{322} and Ser^{325} are phosphorylated by CK1 (casein kinase 1) [41]. Lastly, Ser^{329} is phosphorylated by the dual-specificity kinase, DYRK1A (dual-specificity tyrosine-phosphorylated and regulated kinase 1A) [42].

As a result of Thr^{24}, Ser^{256}, and Ser^{319} phosphorylation [39], FOXO1 is exported from the nucleus to the cytoplasm [43] where it binds 14-3-3 proteins. Once bound, FOXO1 is retained in the cytoplasm and targeted for proteasomal degradation, preventing its reentry into the nucleus (Figure 2(b)) [44–46]. Thus, phosphorylation and ubiquitination are important post-translational modifications of FOXO1 that are critical for its degradation and, ultimately, its regulation.

The transcriptional activities of FOXO1 are also controlled by its acetylation status. Acetylation by cAMP-response element-binding protein-binding protein (CBP) attenuates FOXO1 transcriptional activity [47]. Several acetylation sites have been identified in FOXO1, namely, Lys^{242}, Lys^{245}, and Lys^{262} [48] (Figure 2(a)). Following acetylation, the positive charges associated with these lysine residues are eliminated, inhibiting FOXO1 interaction with DNA and reducing the ability of this transcription factor to recognize its own *cis* element, including the insulin-response element, in some target genes [15]. In addition, FOXO1 acetylation has been linked with increased phosphorylation at Ser^{253} by Akt [48, 49], which further decreases DNA binding. This indicates that the interplay between two types of PTMs regulates the DNA binding activity of FOXO1. On the contrary, deacetylation of FOXO1 is catalyzed by Sirtuin 1 (SIRT1), an NAD(+)-dependent deacetylase [47]. The transcriptional activity of FOXO1 is enhanced by resveratrol-activated SIRT1 resulting in the increase in the hepatic gluconeogenesis [50, 51].

A positive correlation between *O*-GlcNAcylation and insulin resistance has been demonstrated. Because *O*-GlcNAc modifications can also occur on many phosphorylation sites, it has been postulated that increased *O*-GlcNAc may hinder phosphorylation events that normally occur as a result of insulin signaling. This altered regulation can lead to insulin resistance [52]. Indeed, serine and threonine residues within FOXO1 have been shown to be modified by *O*-GlcNAcylation (Figure 2(a)), resulting in increased transcription of *G6pc* and *Ppargc1a*, as well as genes involved in the detoxification of reactive oxygen species (ROS) [53–55]. This effect is independent of FOXO1 subcellular distribution [53]. Presumably, FOXO1 glycosylation could cause a conformational change in FOXO1 and affect its affinity for DNA, which would have an impact on its intrinsic activity and interaction with other cofactors [54]. Modification of FOXO1 by *O*-GlcNAcylation has been observed in the liver of streptozotocin-induced diabetic animals, suggesting that this modification may be associated with hyperglycemia [53]. Indeed, chronic hyperglycemia can lead to hyperglycosylation of FOXO1, thus inducing *G6pc* [53], *Pck1* [54] and *Ppargc1a* genes [55], and causing further production of hepatic glucose. These observations suggest that FOXO1 *O*-GlcNAcylation is a major underlying cause of hepatic glucose overproduction in T2DM [53]. In the hyperglycemic state, *O*-GlcNAcylated PGC-1α recruits OGT to FOXO1; the associated OGT glycosylates FOXO1 and increases its transcriptional activity [56].

cAMP-response-element- (CRE-) binding protein (CREB) is another important transcription factor that stimulates gluconeogenesis. CREB directly binds to the promoters of *G6pc* and *Pck1* genes or increases gluconeogenesis by upregulating *Ppargc1a* gene expression [57]. CREB is phosphorylated at Ser^{133} in the transactivation domain by cAMP-dependent protein kinase (PKA), a modification that increases CREB transcriptional activity [58, 59]. As its name suggests, CREB is phosphorylated and activated in response to hormonal stimuli (e.g., glucagon) that activate adenylyl cyclase and thereby increase the intracellular concentration of cAMP. Binding of cAMP to PKA releases the catalytic domain of PKA from the holoenzyme, allowing it to translocate to nucleus and phosphorylate CREB [60]. In addition, phosphorylation of CREB at Ser^{133} promotes association with CBP/p300 [61] which upregulates CREB target gene expression by acetylating nucleosomal histones [62, 63] and recruiting RNA polymerase II complexes [64, 65]. By contrast, CaMKII (calcium- and calmodulin-dependent kinase II) induces phosphorylation at Ser^{142} in the transactivation domain [66], a modification that inhibits CREB activity by disrupting CREB interaction with



(a)

Figure 2: Continued.

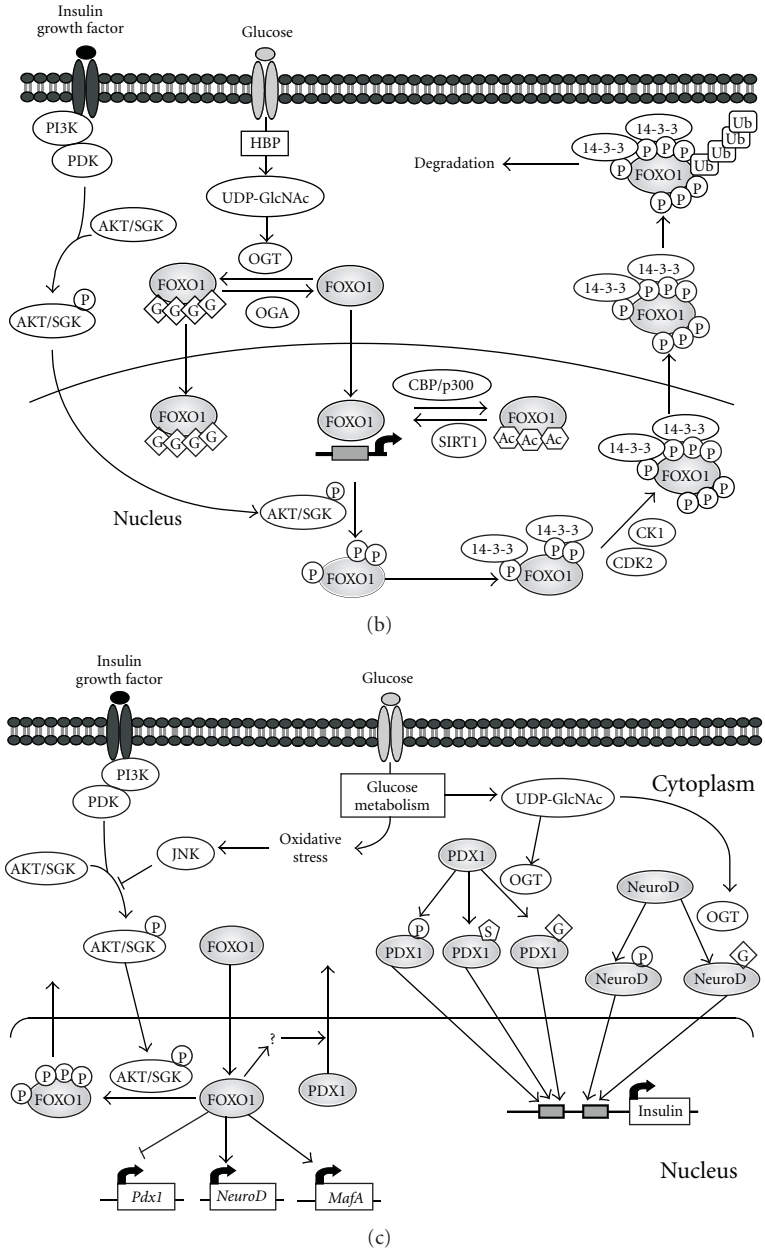


Figure 2: Post-translational modifications (PTMs) of transcription factors. (a) The positions of PTM sites in the human FOXO1, SREBP-1c, and NF-κB p65 subunit.The positions of PTM sites and the implicated modifying enzymes are shown. (+) and (–) represent activation and inhibition of the transcriptional activity of transcription factors, respectively. L1-2, nuclear localization sequences; E1-3, nuclear export sequences; DBD, DNA-binding domain; TAD, transactivation domain; RHD, Rel homology domain; NLS, nuclear localization sequence; TAD, transactivation domain. (b) Regulation of FOXO1 nucleocytoplasmic shuttling and transcriptional activity by PTMs in liver. (c) Regulation of transcription factor activities by PTMs in pancreatic β cells. P, phosphate group; Ac, acetyl group; G, *O*-linked-*N*-acetylglucosamine; Ub, ubiquitin; S, SUMO; Akt, v-akt murine thymoma viral oncogene homolog 1 (also known as protein kinase B [PKB]); SGK, serum/glucocorticoid-regulated kinase; CK1, casein kinase 1; DYRK1A, dual-specificity tyrosine-phosphorylated and regulated kinase1 A; CDK2, cyclin-dependent kinase 2. PI3K, phosphoinositide-3-kinase; PDK, phosphatidylinositol-dependent protein kinase; OGT, *O*-linked N-acetylglucosamine (GlcNAc) transferase; MAPK1/3, mitogen-activated protein kinase 1/3; Ubc9, ubiquitin conjugating enzyme 9; p300, E1A-binding protein p300; CBP, CREB-binding protein; SIRT1, sirtuin 1; PKA, protein kinase A; cAMP, cyclic adenosine monophosphate; SIK, salt-inducible kinase; GSK-3, glycogen synthase kinase-3; JNK, c-Jun N-terminal kinase; PCAF, CBP/p300-associated factor; MSK1, mitogen/stress-activated protein kinase 1; PKCζ, protein kinase Cζ; IKK, I kappa B kinase; CK2, casein kinase 2; TBK1, tank-binding kinase 1; SOCS-1, suppressor of cytokine signaling 1; HBP, hexosamine biosynthesis pathway; OGA, *O*-GlcNAcase; PDX1, pancreatic and duodenal homeobox 1; NeuroD, neurogenic differentiation; MafA, v-maf (maf musculoaponeurotic fibrosarcoma) oncogene homolog A.

CBP/p300 [67]. DNA damage-mediated phosphorylation of CREB at Ser^{111} and Ser^{121} by AMT (ataxia-telangiectasia mutated) also inhibits CREB activity by blocking CREB-CBP interaction [68, 69].

CRTC2 (CREB-regulated transcription coactivator 2) interacts with the bZIP domain of CREB and thereby induces its activity [70, 71]. The resulting CRTC2-CREB complex binds to *cis* elements in the promoters of *G6pc, Pck1*, and *Ppargc1a* genes [72, 73]. CRTC2 is also regulated by O-GlcNAcylation [74]. Further research is needed to elucidate the molecular mechanisms and site-specific roles of O-GlcNAcylation in relation to phosphorylation or other types of PTMs in terms of glucotoxicity, insulin resistance, and T2DM.

3.1.2. Modification of Transcription Factors That Regulate Lipid Metabolism Genes. NAFLD has become a common chronic disease due to western style diets. This disease manifests as a simple accumulation of triglycerides in hepatocytes (hepatic steatosis) or as steatohepatitis, which is accompanied by inflammation, fibrosis, cirrhosis, and hepatocellular carcinoma in severe cases. It has now become clear that accumulation of triglycerides in hepatocytes is correlated with T2DM, obesity, and insulin resistance. Steatosis is caused by an imbalance between lipid availability and disposal. Triglyceride accumulation in hepatocytes reflects dietary fatty acid intake, increased lipolysis in adipose tissue, or de novo lipogenesis. On the other hand, hepatic triglyceride levels are decreased by β-oxidation of fatty acid in the hepatocytes and triglyceride secretion with very low-density lipoproteins (VLDLs). In nonalcoholic fatty liver disease patients, the ratio of lipogenesis to VLDL-packaged triglyceride secretion is up to 25–30%, a substantial increase compared to the normal range of 2–5% [75, 76].

The expression of lipogenic enzymes is mainly controlled at the transcriptional level in the hyperinsulinemic and hyperglycemic state. Two major transcription factors, sterol regulatory element binding protein-1c (SREBP-1c) and carbohydrate response element binding protein (ChREBP), are well known to be involved in these states [77].

SREBP-1c is a member of the basic-helix-loop-helix-leucine zipper (bHLH-LZ) family of transcription factors. It is synthesized as an inactive form embedded in the membranes of the ER and is activated in the Golgi apparatus by proteolytic cleavage. The resulting N-terminal domain cleavage fragment (nSREBP-1c), which is the transcriptionally active form, is translocated to the nucleus. SREBP1a, which is expressed from an mRNA that overlaps that of SREBP-1c and differs from SREBP-1c only at the N-terminus, and SREBP-2, which is the product of a separate gene, regulate the expression of cholesterol synthesis genes [78]. Expression of the SREBP-1c gene and maturation and stability of SREBP-1c protein are regulated by insulin through the PI3K-PDK1-PKB/Akt pathway [79, 80]. PKB/Akt kinase phosphorylates and inhibits glycogen synthase kinase-3 (GSK3), whereas the dephosphorylated form of GSK3 phosphorylates Thr^{426}, Ser^{430}, and Ser^{434} of nSREBP-1a, causing degradation by ubiquitination through the ubiquitin ligase, FBW7 (F-box and WD repeat domain containing 7) [81]. Similarly, phosphorylation of nSREBP1c has been reported [81, 82]. Ser^{117} of SREBP-1a and Ser^{93} of SREBP-1c are phosphorylated by mitogen-activated protein kinase 1/3, and mutation of these sites abolishes insulin-induced transcriptional activity (Figure 2(a)) [83].

By contrast, cAMP might act through PKA to regulate SREBP-1c processing. Phosphorylation of Ser^{338} of SREBP-1a and Ser^{314} of SREBP-1c by PKA reduces the transcriptional activities of the corresponding transcription factors (Figure 2(a)) [84]. In addition, the nonhydrolyzable PKA activator, dibutyryl-cAMP, downregulates the proteolytic processing of SREBP-1a [85]. These results indicate that insulin and glucagon also modulate the transcriptional activity of SREBP-1c through phosphorylation. Salt-inducible kinase, a member of the AMP-activated protein kinase (AMPK) family, phosphorylates Ser^{329} of SREBP-1a and reduces lipogenic gene expression (Figure 2(a)) [86].

Modification of SREBP-1a at Lys^{123} and Lys^{418} by Ubc9, an SUMO-1-conjugating enzyme, reduces its transcriptional activity (Figure 2(a)). However, ubiquitination and SUMOylation do not compete for the same Lys residues, and SUMOylation does not affect ubiquitination-mediated SREBP degradation and stability [87].

CBP/p300-mediated acetylation of SREBP-1c increases its stability [88]. Lys^{289} and Lys^{309} residues near and within the DNA-binding domain of SREBP-1c, respectively, are acetylated by p300 and deacetylated by SIRT1 (Figure 2(a)) [89]. Levels of acetylated SREBP-1c are increased in fed mice, diet-induced obese mice, and insulin- and glucose-treated HepG2 cells. SIRT1 overexpression decreases SREBP-1c acetylation level and protein stability, causing a reduction in lipogenic gene expression [89].

ChREBP, which is also a member of the bHLH-LZ (leucine zipper) family of transcription factors, is the second of the two major transcription factors shown to induce glycolytic and lipogenic genes in hepatocytes [90]. ChREBP, also known as MLXIPL (MLX interacting proteinlike), forms a heterodimer with the bHLH-LZ protein Mlx (MAX-like protein X) that binds the carbohydrate response element of various glucose-responsive genes, including liver type pyruvate kinase (*Pklr*), fatty acid synthase (*Fasn*), and acetyl-CoA carboxylase 1 (*Acc1*) [91]. Nuclear localization of ChREBP is induced by high glucose. In starvation, glucagon increases intracellular cAMP concentrations and activates PKA. Phosphorylation of ChREBP by PKA at Ser^{196} prevents nuclear localization, whereas PKA-mediated phosphorylation at Thr^{666} inhibits DNA binding [92]. In addition, phosphorylation of Ser^{568} of ChREBP by AMPK decreases ChREBP transcriptional activity [93]. In contrast, xylulose-5-phosphate generated from glucose through the hexose monophosphate shunt activates protein phosphatase 2A delta, which dephosphorylates ChREBP and increases lipogenesis [94]. However, the regulation of ChREBP by phosphorylation and dephosphorylation remains controversial [95, 96].

A recent study has shown that by increasing the stability and transcriptional activity of ChREBP, O-GlcNAcylation of ChREBP in the hyperglycemic state is responsible for fatty acid synthesis in the mouse liver [97].

3.2. β-Cell Dysfunction and Pancreatic Failure. The pancreas maintains normal blood glucose levels by regulating insulin and glucagon secretion. Insulin, an anabolic hormone, modulates a variety of biological processes and metabolic pathways, including cell survival and proliferation, glycogen synthesis, protein synthesis, and glucose uptake into skeletal muscle and adipocytes. In an attempt to overcome the reduction in insulin activity that occurs during insulin resistance, the number of β cells increases, resulting in a compensatory hypersecretion of insulin. As the compensation fails, the β-cell phenotype is disturbed, causing a reduction in β-cell mass via apoptosis [98].

FOXO1 has been shown to modulate pancreatic β-cell development, proliferation, maintenance, expansion, and apoptosis [99, 100]. β-cell failure was observed in IRS2-deficient mice [101] and FOXO1^{S253A} transgenic mice [102] which exhibited decreased or nonfunctional FOXO1 phosphorylation, respectively. Interestingly, FOXO1 haplodeficiency partially restored β-cell proliferation in these mice and increased the expression of pancreatic and duodenal homeobox 1 (*Pdx1*) [101] (Figure 2(c)), a critical transcription factor involved in β-cell differentiation, development, and cellular function [103]. In addition, by binding the Foxa2 site within the *Pdx1* promoter, FOXO1 can inhibit the expression of this crucial transcription factor [101].

FOXO1 also regulates the subcellular distribution of PDX1 [104] (Figure 2(c)). Nucleocytoplasmic translocation of PDX1 during hyperglycemia-induced oxidative stress occurs in a Jun N-terminal-kinase- (JNK-) dependent manner, resulting in β-cell failure [105]. JNK activation during these conditions results in decreased Akt activity and subsequent FOXO1 hypophosphorylation, leading to PDX1 translocation to the cytosol [104]. In support of this, infection of HIT-T15 cells with adenovirus expressing wild-type FOXO1 led to PDX1 translocation from the nucleus to the cytosol in the absence of H_2O_2 treatment [104]. The mechanism by which nuclear FOXO1 affects PDX1 translocation remains unknown although reports have suggested that the acetylation status of the two proteins may be responsible [104].

Acetylation and deacetylation of FOXO1 are modulated by CBP/p300 and SIRT1, respectively. Transgenic mice bearing a pancreatic β-cell-specific, SIRT1-overexpressing transgene (BESTO) display improved glucose tolerance and enhanced glucose-stimulated insulin secretion [106]. In addition, oxidative stress-mediated FOXO1 deacetylation induces the expression of neurogenic differentiation (*NeuroD*) and v-maf (mafmusculoaponeurotic fibrosarcoma) oncogene homolog A (*MafA*) [107], which play roles in preserving insulin secretion in response to glucose and thereby promote β-cell compensation. However, the deacetylated form of FOXO1 is more easily degraded by ubiquitination than the acetylated form, suggesting that acetylation status regulates the stability and transcriptional activity of this protein. In contrast, deacetylation of the phosphorylation-defective ADA-FOXO1 mutant, which is constitutively nuclear by virtue of mutation of Thr^{24} and Ser^{316} to Ala(A) and Ser^{253} to Asp(D), does not affect transcriptional activity [107], indicating that the transcriptional activity of FOXO1 is independent of its phosphorylation status.

In the pancreas, glucose-induced insulin gene transcription is mediated by three β-cell-specific transcription factors: NeuroD1, PDX1, and MafA [103]. NeuroD1 and PDX1 are *O*GlcNAcylated and translocated to nucleus under high-glucose conditions, exhibiting increased DNA-binding activity and promoting insulin gene expression and insulin secretion in mouse insulinoma 6 (MIN6) cells [108, 109]. In addition, in the Gato-Kakizaki rat model of T2DM, the levels of *O*-GlcNAcylated proteins, especially those of PDX1 and *O*-GlcNAc transferase, were elevated in whole pancreas and islets of Langerhans [110].

The transcriptional activities of both PDX1 and NeuroD1 are regulated by phosphorylation upon glucose stimulation [111, 112]. In response to glucose and insulin stimulation, PDX1 is phosphorylated by stress-activated protein kinase 2 (SAPK2); phosphorylation by PI3K induces nuclear translocation and transcriptional activation [113–115]. SUMOylation causes nuclear translocation of PDX1 and increases its stability [116]. In contrast, phosphorylation of Ser^{61} and/or Ser^{66} by GSK3 during oxidative stress promotes PDX1 degradation [117].

3.3. Inflammatory Response of Macrophages. One of the risk factors for obesity-induced insulin resistance and diabetes is inflammation. Inflammatory gene expression in hepatocytes induces insulin resistance [118]. Hepatic steatosis often accompanies abdominal adiposity, and inflammation plays a pivotal role in the progression of nonalcoholic fatty liver disease. In the obese state, increased proinflammatory substances from abdominal fat might initiate hepatic inflammation and steatosis [119], highlighting the importance of understanding the role of macrophages in the initiation of obesity-induced insulin resistance in adipose tissue. Enlargement of adipose tissue as a result of excess dietary intake induces hypoxic conditions and ER stress, which are accompanied by nuclear factor-kappa B (NF-κB)- and JNK1-mediated upregulation of inflammatory genes [120, 121].

Once activated, NF-κB and JNK1 increase the production of various cytokines and chemokines from adipocytes, including tumor necrosis factor (TNF)-α, interleukin (IL)-6, monocyte chemotactic protein (MCP)-1, and plasminogen activator inhibitor-1. These molecules play key roles in the recruitment and infiltration of macrophages into adipocytes [122–125]. In fact, IL-6 has been reported to regulate the development of insulin resistance [126]. In addition, MCP-1 has been reported to increase during high-fat diet-induced obesity, thereby contributing to macrophage infiltration into adipose tissue [127]. Macrophages produce proinflammatory cytokines that amplify the inflammatory state in neighboring adipocytes, leading to the secretion of other mediators, such as adipokines and free fatty acids. Free fatty

acids enter the circulation to promote insulin resistance in hepatocytes and myocytes [128, 129].

NF-κB is a master regulator of the expression of genes involved in the inflammatory response. NF-κB is a multisubunit protein variably consisting of p50, p52, p65, c-Rel, and Rel B; p65 is the major target of protein modification [130] (Figure 2(a)). This subunit is acetylated at Lys^{221} by CBP/p300 and deacetylated by histone deacetylase 3 or SIRT1 during inflammation [131, 132]. NF-κB is also a key mediator of TNF-α-induced IL-6 gene expression [131, 133]. Notably, an SIRT1 activator was shown to attenuate the TNF-α-induced inflammatory signal. Conversely, SIRT1 knockdown in 3T3-L1 adipocytes using small inhibitory RNAs increased NF-κB acetylation and enhanced the transcription of inflammatory genes, causing insulin resistance [134, 135]. By contrast, acetylation of Lys^{122}/Lys^{123} of the p65 subunit by CBP/p300 or CBP/p300-associated factor (PCAF) decreased NF-κB DNA-binding ability and promoted NF-κB nuclear export and interaction with IκBα, ultimately, attenuating its transcriptional activity [136, 137]. Taken together, these results indicate that acetylation of specific lysine residues on p65 confers different functional consequences.

Another modification that occurs on p65 is phosphorylation. Mitogen- and stress-activated protein kinase-1 (MSK1) is a nuclear kinase that phosphorylates Ser^{276} of p65. Treatment of cells with the MSK1 inhibitor H89 has been shown to block TNF-α-induced phosphorylation of p65 *in vivo*. TNF-α promotes the interaction between p65 and MSK1, which is recruited to the IL-6 promoter [138]. P65 can also be phosphorylated by protein kinase Cζ (PKCζ) through TNF-α signaling. Phosphorylation of p65 at Ser^{311} promotes complex formation with CBP, increasing complex binding to the IL-6 promoter [139]. In addition, many inflammatory stimuli induce p65 phosphorylation at Ser^{529}/Ser^{536}, thereby increasing the transcriptional activity of NF-κB [140–142].

In response to cytokines, Thr^{254} of p65 is phosphorylated by an unknown kinase. Once phosphorylated, p65 forms a complex with Pin1, preventing binding to IκB and causing nuclear localization, resulting in greater NF-κB stability and activity [143].

The stability of p65 is also regulated by the ubiquitin-proteasome pathway. Treatment of cells with MG132 (a proteasome inhibitor) and His-Ubiquitin resulted in p65 polyubiquitination via interaction with suppressor of cytokine signaling (SOCS)-1. This ubiquitination event was negatively regulated by Pin-1 and increased the stability of p65- and NF-κB-dependent gene expression [137, 143].

TNF-α was recently reported to induce polyubiquitination of Lys^{195} in p65 and decrease the transcriptional activity of NF-κB by promoting its degradation. This effect of TNF-α on p65 appears contradictory but presumably reflects an important regulatory mechanism; that is, persistent activation of p65 by phosphorylation may be terminated by ubiquitination [144].

The expression of glycosyl transferase and NF-κB target genes is regulated by either TNF-α or hyperglycemia [145–147]. O-GlcNAcylation of p65, which occurs on Thr^{352}, decreases p65 interaction with IκBα, resulting in increased NF-κB transcriptional activity during hyperglycemia [146, 147].

3.4. Free Fatty Acids-Induced Insulin Resistance in Muscle. Skeletal muscle is one of the main target tissues which respond to insulin and other hormones [148]. Glucose uptake by muscle is stimulated by insulin. In patients with NAFLD, elevated plasma free fatty acids (FFAs) levels are responsible for insulin resistance [149, 150] causing a decrease in the insulin-stimulated glucose uptake, glycogen synthesis [151], and PI3K activity in skeletal muscle [152].

Elevated FFA in the blood causes accumulation of triacylglycerol (TG) in the muscle [153], which is shown to be associated with increased intracellular diacylglycerol (DAG), ceramides, and long-chain acyl-coenzyme A (LCA-CoA). These molecules induce insulin resistance by activating serine protein kinase C (PKC) [154]. This kinase inhibits PI3K activities by phosphorylating Ser/Thr residue of IRS-1 causing an inhibition of the insulin-stimulated translocation of the glucose transporter type 4 isoform (GLUT4) [155]. Phosphorylation of IκB by PKC dissociates IκB from NF-κB and thereby translocates NF-κB to nucleus to upregulate proinflammatory TNFα gene [154]. NF-κB is linked to fatty acid-induced impairment of insulin action in muscle [156, 157].

The increased TG in muscle may be potentially toxic to skeletal muscle presumably because of ROS overproduction which inhibits the insulin-stimulated Akt phosphorylation on Ser residue [158]. ROS also stimulates Thr phosphorylation of JNK, a kinase linked to insulin resistance [159]. An elevated TG is associated with reduced mitochondrial oxidative capacity in skeletal muscles as indicated by lower mitochondrial density, reduced capacity of electron transport, and reduced activities of oxidative enzymes [160]. Further researches are necessary to understand the contribution of PTM of transcription factor in the development of insulin resistance in muscle.

3.5. Adipokine Gene Expression and Secretion from Adipose Tissue. Contribution of adipose tissue in the maintenance of whole body insulin sensitivity is critical. Adipogenesis is a tightly regulated process that involves the complicated interrelationship of various transcription factors. One of the pivotal transcription factors is PPARγ, an essential factor of development and function [161, 162]. Hormonal stimuli to the preadipocyte trigger the expression of C/EBPβ [163] which activates the expression of two master transcription factors, C/EBPα and PPARγ [164]. PPARγ can induce adipogenesis in C/EBP$\alpha^{-/-}$ MEFs (mouse embryonic fibroblast) [165], whereas C/EBPα is unable to do the same action in PPAR$\gamma^{-/-}$ MEFs [166]. These results indicate that PPARγ plays a central role in adipogenesis.

Mitogen-activated protein (MAP) kinase induces the phosphorylation of Ser^{112} of PPARγ resulting in the reduction of transcriptional activity. This observation is supported

by a study [167] which showed that PPARγ activity was not decreased by MAP kinase when Ser^{112} was replaced by Ala. Furthermore, treatment of PD98059, an inhibitor of MAP kinase, abolished the phosphorylation of PPARγ [167].

Adipocytes store triglycerides, which are an abundant source of energy, and secrete adipokines such as adiponectin, leptin, resistin, and retinol-binding protein 4 [168]. The expression and secretion of these adipokines are regulated by PTM of various transcription factors in the context of obesity.

One such factor is FOXO1, which regulates adiponectin expression. In FOXO1 haplodeficient animals, adiponectin gene expression is significantly reduced [169]. In fact, two FOXO1 response elements have been identified in the adiponectin promoter [170]. Moreover, SIRT1 was demonstrated to increase the interaction between FOXO1 and C/EBPα and enhance subsequent binding to the adiponectin promoter [170]. These results suggest that FOXO1 deacetylation plays an important role in upregulating adiponectin expression. Adiponectin increases insulin sensitivity by promoting fatty acid oxidation in an AMPK and peroxisome proliferator-activated receptor-α-dependent manner [171].

The activity of Sp1, a ubiquitously expressed transcription factor that regulates most housekeeping genes, has been shown to be controlled by PTM [172]. In fact, Sp1 was the first transcription factor shown to be O-GlcNAcylated [173]. When O-GlcNAcylated, Sp1 is less phosphorylated and is protected from proteasomal degradation [174]. Presumably, the transcriptional activity of Sp1 may vary depending on the site of O-GlcNAcylation [21].

In 3T3-L1 and primary cultured adipocytes, glucose increases Sp1 *O*-GlcNAcylation and upregulates expression of leptin [175, 176]. Although leptin controls appetite, it is considered a proinflammatory adipokine [177].

Resistin gene expression is increased by glucosamine infusion in rats [178], whereas treatment of 3T3-L1 adipocytes with troglitazone results in decreased gene expression due to a reduction in Sp1 *O*-GlcNAcylation [179]. These experiments indicate that insulin resistance induced by chronic hyperglycemia can be modulated by *O*-GlcNAcylation of Sp1. Interestingly, *O*-GlcNAcylated Sp1 increases the expression of both leptin and resistin [180].

Perspective

The epidemics of obesity and accompanying metabolic conditions, such as T2DM, nonalcoholic fatty liver disease, and cardiovascular diseases—diseases that have been linked to insulin resistance—will pose enormous social and economic burdens in the coming decades. In these conditions, a number of transcription factors become modified and ultimately play positive or negative roles in regulating specific genes. The resulting metabolic consequences include increased hepatic gluconeogenesis, abnormal lipid metabolism and aberrant insulin biosynthesis/release from pancreatic β cells, and adipose tissue reactivity to inflammation.

Recent advances in analytic methodologies have provided additional insights into the modifications of transcription factors involved in metabolic alterations in the context of insulin resistance. Our understanding of insulin resistance is further improved by a growing appreciation of crosstalk between the different types of modification. Undoubtedly, continued research will ultimately lead to the development of novel therapeutic drugs, as evidenced by these rapid advances.

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Research Article

Inhibition of Aldose Reductase Activates Hepatic Peroxisome Proliferator-Activated Receptor-α and Ameliorates Hepatosteatosis in Diabetic db/db Mice

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We previously demonstrated in streptozotocin-induced diabetic mice that deficiency or inhibition of aldose reductase (AR) caused significant dephosphorylation of hepatic transcriptional factor PPARα, leading to its activation and significant reductions in serum lipid levels. Herein, we report that inhibition of AR by zopolrestat or by a short-hairpin RNA (shRNA) against AR caused a significant reduction in serum and hepatic triglycerides levels in 10-week old diabetic db/db mice. Meanwhile, hyperglycemia-induced phosphorylation of hepatic ERK1/2 and PPARα was significantly attenuated in db/db mice treated with zopolrestat or AR shRNA. Further, in comparison with the untreated db/db mice, the hepatic mRNA expression of *Aco* and *ApoA5*, two target genes for PPARα, was increased by 93% ($P < 0.05$) and 73% ($P < 0.05$) in zopolrestat-treated mice, respectively. Together, these data indicate that inhibition of AR might lead to significant amelioration in hyperglycemia-induced dyslipidemia and nonalcoholic fatty liver disease.

1. Introduction

The polyol pathway is a glucose metabolic shunt that is defined by two enzymatic reactions catalyzed by aldose reductase (AR, AKR1B1, EC1.1.1.21) and sorbitol dehydrogenase (SDH, EC1.1.1.14), respectively [1, 2]. Biochemically, AR catalyzes the rate-limiting reduction of glucose to sorbitol, with the aid of cofactor NADPH. SDH converts sorbitol to fructose using NAD^+. AR/the polyol pathway have been demonstrated to play important roles in the development and progression of diabetic complications in a number of tissues including kidney, retina, lens, and peripheral neuron tissues [3–5]. In the liver, however, the expression of AR is relatively low under normal physiological conditions [6, 7]. By contrast, the hepatic expression of sorbitol dehydrogenase, the second enzyme for the polyol pathway, is quite high [8]. Due to the relatively lower levels of expression of AR in the liver under normal situations, relatively little attention had been paid to its roles in the liver in the past. Recently, however, increasing evidence has suggested that hepatic AR is dynamically regulated under a variety of conditions. For instance, in rats fed with fructose, hepatic AR is significantly upregulated, which is associated with impaired activation of Stat3 and suppressed activity of PPARα in the liver [9]. In the Long Evans Cinnamon rats, induction of hepatic AR expression was shown to be associated with the development of hepatitis and hepatoma [10]. Similarly, significant upregulation of AR has also been demonstrated in other diseased liver tissues from rodents to humans [11–13].

The liver tissue plays a major role in energy metabolism, particularly glucose and lipid homeostasis. It is known that diabetes, type II diabetes mellitus (T2DM) in particular, is often associated with hepatic accumulation of triglycerides in both rodents and humans, which might eventually lead to the development of hepatic steatosis or nonalcoholic fatty liver disease (NAFLD) [14–16]. Recently, we demonstrated that deficiency or inhibition of AR caused significant dephosphorylation of hepatic PPARα, leading to the activation of this transcriptional factor as well significant reduction in serum TG levels in streptozotocin-(STZ-) diabetic mice, an experimental model for type I diabetes mellitus (T1DM) [17]. Because T2DM is clinically much predominant than T1DM, in this current study, we wanted to determine whether AR also affects PPARα in the liver of T2DM db/db mouse models. Furthermore, we wanted to determine how changes in AR activity might affect the hepatic lipid accumulation in the db/db mice. Our data suggest that inhibition of AR in the T2DM db/db mice led to significant activation in hepatic PPARα and significant reductions in serum triglycerides (TG) and hepatic TG, suggesting that under hyperglycemia, AR/the polyol pathway might be greatly upregulated to contribute significantly to the hepatic regulation of TG metabolism and the development of nonalcoholic steatohepatitis (NASH) or nonalcoholic fatty liver disease (NAFLD).

2. Materials and Methods

2.1. Antibodies and Reagents. Antibodies were obtained from the following vendors, respectively: ERK1/2 and phospho-ERK1/2 (#9100), Cell Signaling (Beverly, Mass); PPARα (sc9000) and AR (sc17735), Santa Cruz Biotechnology Inc. (Santa Cruz, Calif); phosphoserine-12 PPARα (ab3484) and phosphoserine-21 PPARα (ab3485), Abcam (Cambridge, UK); β-actin (A1978), Sigma (St. Louis, Mo). Oil-red O and other reagents were of analytical grade quality and from Sigma (St. Louis, Mo). Zopolrestat (zopol) was synthesized by the Department of Medicine Chemistry, Pfizer Global Research and Development (Groton, Conn).

2.2. Lentivirus shRNA Construct. Recombinant lentiviral vector expressing small hairpin RNA (shRNA) against mouse *AR* (pLV-shAR) and its control (pLV-shNC) were constructed by inserting double-strand shRNA oligonucleotides into plasmid pLentiLox3.7 (pLL3.7) at the *Hap*I and *Xho*I sites. Control and shRNA oligonucleotides against mouse AR were designed according to Ambion guidelines, with the sequences being 5′-ctggtcacacaacagaga-3′ and 5′-tacctaactcaggagaag-3′, respectively. Preparations of lentiviruses were performed by cotransfecting the lentiviral constructs with the packaging vectors into 293T cells using Lipofectamine 2000 (Invitrogen). Virus-containing supernatants were collected 48 h after infection. Viruses were recovered by ultracentrifugation at 110,000 ×g for 1.5 h and resuspended in PBS. Titers were determined by infecting 293T cells with serial dilutions of concentrated lentiviral preparations.

2.3. Animal Experiments. The animal experiments were conducted according to protocols and guidelines approved by the Xiamen University Institutional Animal Care and Use Committee. The db/m (BKS.Cg-m/Lepr^{db}/J) mice were obtained from the Jackson Laboratory (Bar Harbor, Maine) and bred to obtain six-week-old male db/db mice and their lean control db/m mice for this study. All animals were maintained on standard laboratory chow under a 12 : 12 h light-dark schedule. For AR inhibition by zopolrestat (zopol) treatment, six-week-old db/db mice were randomly divided into four experimental groups, namely, db/m mice, db/m mice + zopol, db/db mice, and db/db mice + zopol. For zopol treatments, the mice were administrated with 50 mg/kg body weight/day of zopol as a single daily intraperitoneal injection for 28 days. The same volumes of saline were also administrated to other control groups of mice. For *in vivo* AR knock-down experiments, six-week-old db/db mice were randomly grouped (4 mice/group). *In vivo* transduction of lentiviruses was achieved through tail vein injections of 0.1 mL of concentrated viral suspension with a viral titer of 1.0×10^9 IFU/mL in PBS. Twenty-eight days after zopol treatment or lentiviral injection, mice were sacrificed and tissues were dissected and immediately frozen in liquid N_2 and stored at −80°C until use.

2.4. Semiquantitative Analyses of mRNA Expression by RT-PCR. Total RNA was isolated from tissues using Trizol Reagent (Invitrogen) according to the manufacturer's protocol. RT-PCR was performed to determine the levels of acetyl CoA oxidase (*Aco*), carnitine palmitoyl transferase-1 (*Cpt1*), apolipoprotein C-III (*ApoC3*), and apolipoprotein A-V (*ApoA5*) mRNAs as previously described [17]. The primers used were 5′-CCGCCACCTTCAATCCAG-AGTTA-3′ and 5′-TCACAGTTGGGCTGTTGAGAATG-3′ (*Aco*), 5′-GGACGAATCGGAACAGGGATA-3′ and 5′-CCTTGTAATGTGCGAGCTGCA-3′ (*Cpt1*), 5′-CCTCTT-GGCTCTCCTGGCATCT-3′ and 5′-TGCTCCAGTAGC-CTTTCAGGG-3′ (*ApoC3*), 5′-GTGGGAGAAGACAC--CAAG-GCTC-3′ and 5′-GGTCAATGGCCTGAGTAAA-TGC-3′ (*ApoA5*), 5′-CGAGACCCCACTAA-CATCAAA-3′ and 5′-AGTCTTCTGGGTGGCA-GTGAT-3′ (GAPDH). DNA amplification was carried out using a High-Fidelity PrimeScript RT-PCR Kit (TaKaRa). The PCR products were electrophoresed on 2% agarose gels and visualized by staining with ethidium bromide. The integrated density values of the bands representing amplified products were acquired and analyzed by Image-Pro Plus software (Media Cybernetics, USA).

2.5. Western Blot Analyses. Tissues were homogenized with Polytron in ice-cold buffer (1% Triton X-100, 50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerine, 10 mM $Na_4P_2O_7$, 20 mM glycerophosphate, 10 mM NaF, 10 mM sodium orthovanadate, and proteinase inhibitor mixture). The protein concentrations of the extracts were measured using a bicinchoninic acid protein assay kit (Pierce). 40 μg protein of each sample was loaded and separated on a 12% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore).

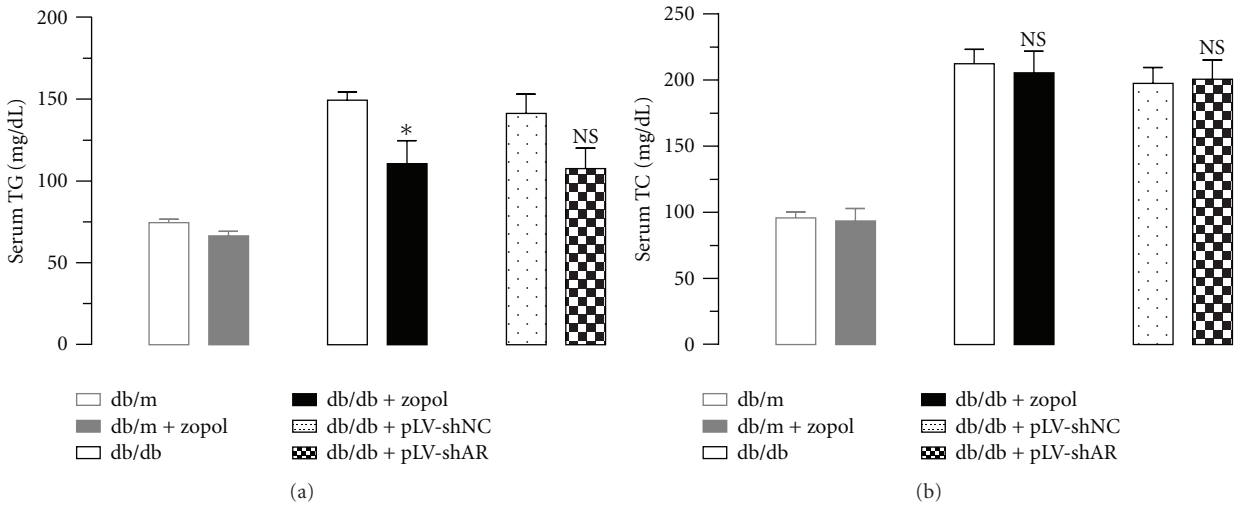

Figure 1: Effect of zopol treatment or AR knock-down on serum TG levels (a) and TC levels (b) of db/db mice. Lean control mouse groups are db/m, $n = 6$ and db/m + zopol, $n = 4$; diabetic mouse groups are db/db, $n = 6$; db/db + zopol, $n = 6$ and db/db + pLV-shNC ($n = 4$); db/db + pLV-shAR ($n = 4$). Values are expressed as the mean ± SEM. $^*P < 0.05$; NS: not significant.

Blotted membranes were then incubated either anti-ERK or anti-phospho-ERK (1 : 1000) or anti-PPARα (1 : 500) or anti-phospho-PPARα (1 : 1000) or anti-AR (1 : 500) in TBS-0.1% Tween-20 with 5% nonfat milk at 4°C overnight. After several washes, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-goat IgG (1 : 2000) in TBS-0.1% Tween-20 with 5% nonfat milk. The detection was achieved using the supersignal chemiluminescent substrate kit (Pierce).

2.6. Blood Sample Analyses. Serum TG levels were measured using a colorimetric assay (Sigma, TR0100). Total serum cholesterol was measured using a cholesterol reagent kit (Jiancheng Biotech, Nanjing, China).

2.7. Liver TG Analyses. Liver TG was extracted by chloroform/methanol. Briefly, pulverized liver was homogenized in PBS, then extracted with chloroform/methanol (2 : 1), dried overnight, and resuspended in a solution of 60% butanol 40% Triton X-114/methanol (2 : 1). Liver total TG levels were measured using a colorimetric assay (Sigma, TR0100).

2.8. Oil-Red O Staining. Frozen liver sections of 10 μm thickness were fixed in 4% paraformaldehyde and stained with 0.5% oil-red O using standard procedures.

2.9. Statistical Analyses. All data were processed and analyzed by GraphPad software (Prism 5.0) and expressed as mean ± SEM. Students' t-test was used for pair-wise comparisons and one-way ANOVA with Bonferroni's Multiple Comparison Test for multigroup analyses. Probability values less than 0.05 (*) were considered to be statistically significant; those less than 0.01 (**) more so.

3. Results

3.1. AR Inhibition-Reduced Serum TG but Not Serum TC Levels in Diabetic db/db Mice. To determine the effects of AR on systemic lipid metabolism, we measured the serum TG and TC levels in db/db mice after zopol treatment or AR knockdown (Figure 1). As shown in Figure 1(a), zopol treatment for 4 weeks caused a significant reduction in the serum TG levels in the 10-week-old male db/db mice (110.6 ± 14.17 mg/dL for db/db + zopol versus 149.3 ± 5.06 mg/dL for db/db, $P < 0.05$) but had little effects on the control db/m mice. A similar reduction in serum TG level was also observed in 10-week-old db/db mice transduced with lentiviruses carrying shRNA for AR (107.6 ± 12.38 mg/dL for db/db + pLV-shAR versus 141.6 ± 11.51 mg/dL for db/db + pLV-shNC, $P > 0.05$), although the difference was not significant statistically. In contrast to serum TG, no significant change in serum TC levels was observed in both db/db mice treated with zopol or db/db mice transduced with lentiviruses carrying AR shRNA (Figure 1(b)), which is consistent with our previous findings in the STZ-induced T1DM mouse model [17]. Together these results indicate that inhibition of AR leads to significant reductions in serum TG but not serum TC.

3.2. AR Inhibition-Reduced Hepatic TG in Diabetic db/db Mice. To determine how changes in AR expression and activity might affect hepatic lipid accumulation, we analyzed the TG contents in the liver tissues of 10-week-old male db/db mice after zopol treatment for 28 days or lentivirus-mediated AR knockdown. Oil-red O staining of liver tissues showed that substantial fat droplets were diffusely distributed in the hepatic lobules from the 10-week-old untreated db/db mice. In the liver of the db/db mice received zopol for 28 days, however, much little and smaller fat droplets were observed (Figure 2(a)). Similar reductions in hepatic lipid accumulation were observed in db/db mice treated with lentiviruses containing AR-shRNA expression cassette. The results from tissue staining were further confirmed by biochemical analyses of hepatic TG content (Figure 2(b)). Zopol treatment in db/db mice significantly reduced hepatic

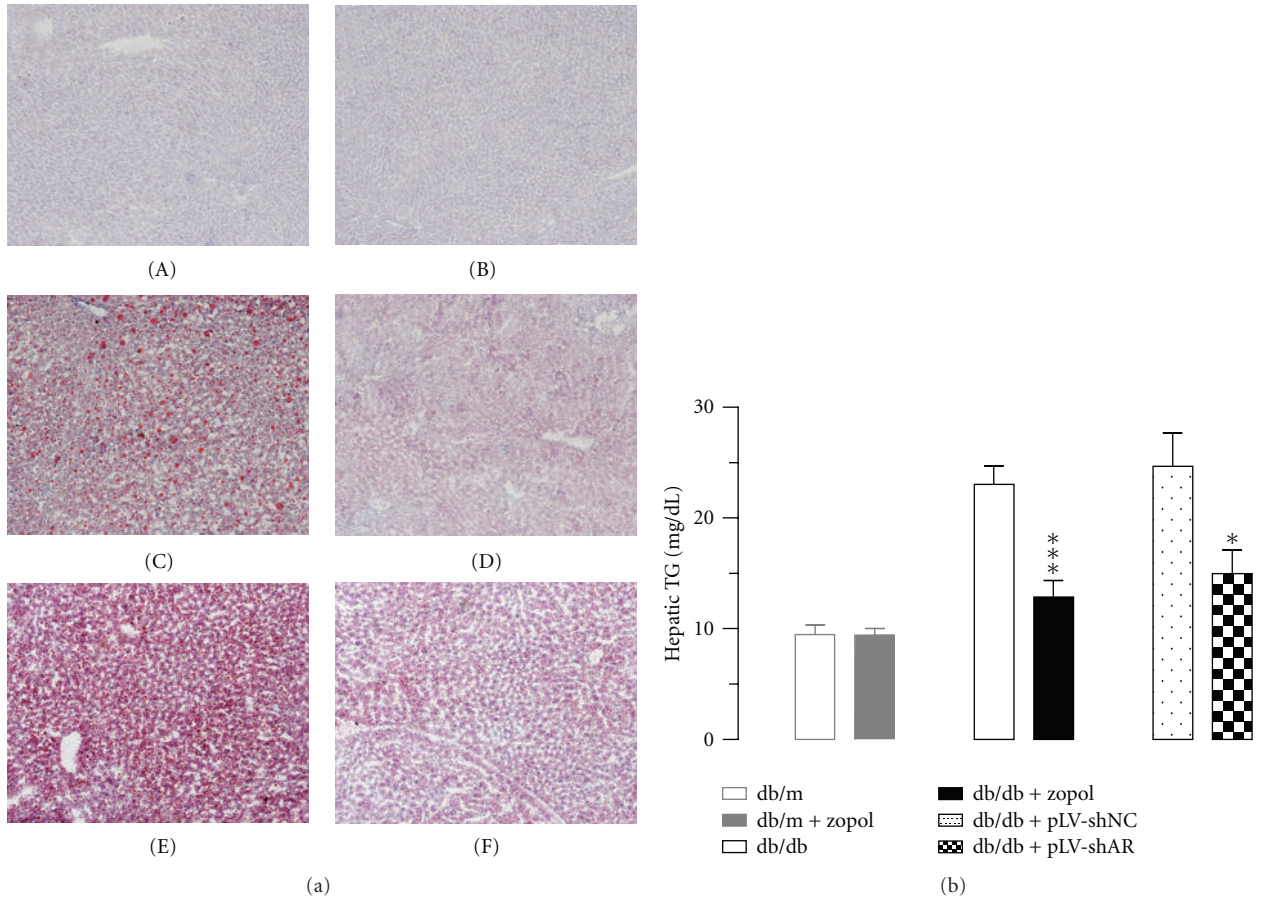

Figure 2: Effect of zopol treatment or AR knockdown on hepatic lipid in db/db mice. (a) Oil-red O staining of liver tissues of db/db mice after zopol treatment. (A) db/m; (B) db/m + zopol; (C) db/db; (D) db/db + zopol; (E) db/db + pLV-shNC; (F) db/db + pLV-shAR. Results are typical for 3 mice/group. Original magnification, ×100. (b) AR inhibition or AR knock-down reduced liver TG of db/db mice as analyzed chemically. Lean control mouse groups are db/m ($n = 6$) and db/m + zopol ($n = 4$); diabetic mouse groups are db/db ($n = 6$); db/db + zopol ($n = 6$) and db/db + pLV-shNC ($n = 4$); db/db + pLV-shAR ($n = 4$). Values are expressed as the mean ± SEM. $^{*}P < 0.05$; $^{***}P < 0.001$.

TG by about 60% (12.89 ± 1.47 mg/g tissue for db/db + zopol versus 23.06 ± 1.66 mg/g tissue for db/db, $P < 0.001$). Similarly, AR knockdown in db/db mice also significantly reduced hepatic TG by about 40% (14.99 ± 2.11 mg/g tissue for db/db + pLV-shAR versus 24.69 ± 3.02 mg/g tissue for db/db + pLV-shNC, $P < 0.05$).

3.3. AR Inhibition Led to Significant Dephosphorylation of Hepatic ERK1/2 and PPARα in the db/db Mice. In STZ-induced T1DM mice, we demonstrated previously that AR deficiency or AR inhibition led to significant dephosphorylation of hepatic ERK1/2 and PPARα [17]. To determine whether this is also the case in the T2DM db/db mice, we examined the hepatic expression and phosphorylation of these proteins in 10-week-old db/db mice and its control db/m mice. As shown in Figure 3(a), with the elevation in hepatic AR expression in the db/db mice, the phosphorylation of both ERK1/2 and PPARα (at Serine-12 and Serine-21) in the db/db mice was significantly enhanced. In db/db mice received zopol treatment for 28 days, however, the phosphorylation of both ERK1/2 and PPARα was greatly attenuated. Noteworthy is that a slight increase in phosphoserine-21 PPARα level was also observed for db/m mice following the zopol treatment. However, statistical analyses indicated the increase in pPPARα (S21) in db/m mice with zopol is not significant (data not shown). Similar attenuations in phosphorylation of both ERK1/2 and PPARα were also observed for db/db mice transduced with lentiviruses carrying shRNA for AR (Figure 3(b)). Together these results suggest that, consistent with the results from the STZ-induced T1DM mice, *in vivo* inhibition of AR in T2DM db/db mice also lead to dephosphorylation of both ERK1/2 and PPARα, which might eventually lead to the activation of hepatic PPARα to significantly affect hepatic lipid metabolism.

3.4. Dephosphorylation of PPARα Is Associated with Altered Expression of Hepatic Aco and ApoA5. To determine the effects of PPARα dephosphorylation on its target genes, we analyzed the expression of hepatic *Aco*, *ApoA5*, *ApoC3*, and *Cpt-1* mRNAs by semiquantitative RT-PCR for liver tissues from the control mice and db/db mice received

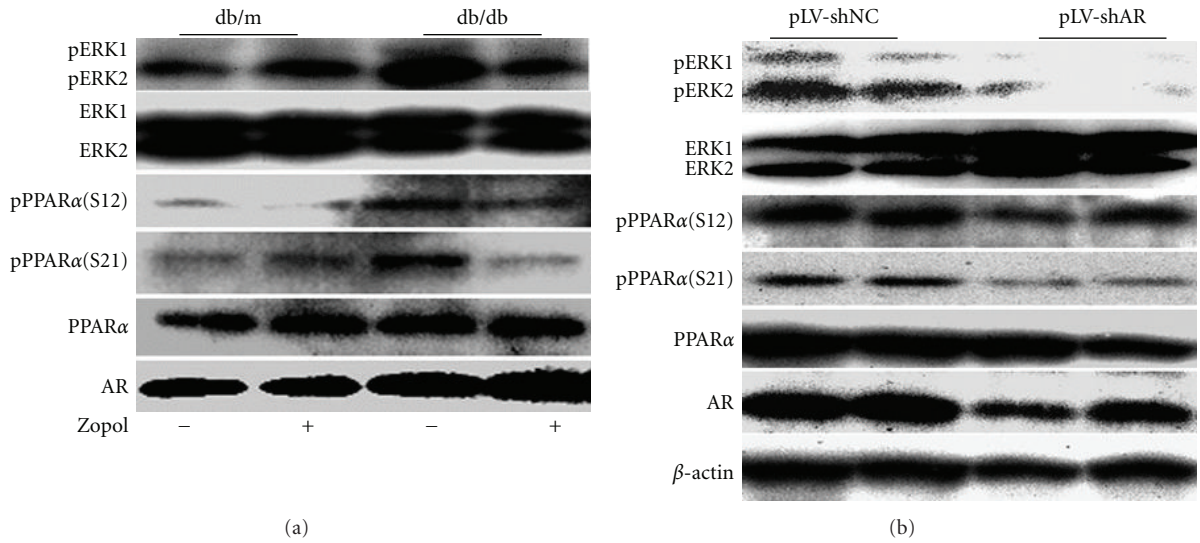

Figure 3: Effects of AR on PPARα and ERK1/2 phosphorylation in db/db mice. (a) Representative Western blot for four independent experiments. Liver tissues were dissected and analyzed 28 days after zopol treatment. (b) Representative Western blot for four independent experiments. Liver tissues were dissected and analyzed 28 days after transduction with lentiviruses containing pLV-shAR or pLV-shNC. pERK1/2, phospho-ERK1/2; pPPARα (S12), phosphoserine-12 PPARα; pPPARα (S21), phosphoserine-21 PPARα.

zopol treatment or db/db mice transduced with lentiviruses carrying AR shRNA. As shown in Figure 4(a), compared with untreated db/db mice, mRNA expression of *Aco* and *ApoA5* in zopol-treated db/db mice elevated by approximately 93% ($P < 0.05$) and 73% ($P < 0.05$), respectively. Meanwhile, zopol-treated db/db mice had a slight but not significant lower hepatic expression of *ApoC3* mRNA expression and a slight but not significant higher expression of *Cpt-1* mRNA expression than the untreated db/db mice. The upregulation of hepatic *Aco* mRNA after zopol treatment was further confirmed in db/db mice transduced with lentiviruses carrying AR shRNA (Figure 4(b)). Probably due to incomplete knockdown, however, no significant changes were observed for hepatic mRNA expression of *ApoA5*, *ApoC3*, and *Cpt-1*. Together these data indicate that inhibition of AR caused activation of hepatic PPARα to alter the expression and activity of major hepatic enzymes involved in lipid homeostasis in the T2DM db/db mice, which might have significant impact on hepatic lipid accumulation and the development or progression of NASH and NAFLD.

4. Discussion

AR/the polyol pathway is widely recognized to be involved in the pathogenesis of diabetic complications such as cataracts, nephropathy, and neuropathy [4, 18]. In contrast, relatively little attention has been paid to their potential roles in the development of diabetic lipid disorders. In spite of this, several studies have shown the possible link between activation/deactivation of AR/the polyol pathway and altered regulation in lipid metabolism. It has been reported that in diabetic patients with dyslipidemia, there are significant increases in plasma or serum and urinary sorbitol and fructose, indicating that the increased flux in the polyol pathway is concomitant with diabetic dyslipidemia [19, 20]. Moreover, pharmacological administration of several AR inhibitors including zopol were shown to reduce blood TG in rats [21], tumor bearing mice [22], and diabetic human patients [23], respectively. More recently, our group reported that in STZ-induced T1DM mouse models, genetic AR deficiency or *in vivo* inhibition by chemical inhibitors of AR significantly improved hyperglycemia-induced dyslipidemia [17]. It is therefore of interest to determine whether AR regulates PPARα and affects hepatic lipid metabolism in T2DM models. In line with our expectation, we demonstrated in this current study that inhibition of AR by zopol treatment or transduction with lentiviruses carrying shRNA for AR greatly reduced hyperglycemia-induced lipid accumulation and hepatic steatosis in T2DM db/db mice. Furthermore, we showed that AR probably regulates hepatic lipid metabolism in part by modulating the status of PPARα phosphorylation to alter its activity.

In our current study, we utilized both chemical inhibitor and mRNA knockdown as means for the inhibition of AR. The inclusion of AR knockdown as an alternative approach for the inhibition of AR was necessary because we wanted to exclude the possible side or toxic effects and nonspecific inhibition that might be associated with chemical inhibitors of AR. Although both chemical inhibition and AR knockdown resulted in similar effects on hepatic lipid metabolism and mRNA expression levels of PPARα and its target genes, AR knockdown appeared to be slightly less effective than zopol treatment. This is probably due to incomplete knockdown of AR as only a single injection was performed and that was maintained for 4 weeks before the analyses.

The exact mechanisms underlying suppression of lipid accumulation or hepatic steatosis by inhibition of AR are not completely clear at this moment and require further

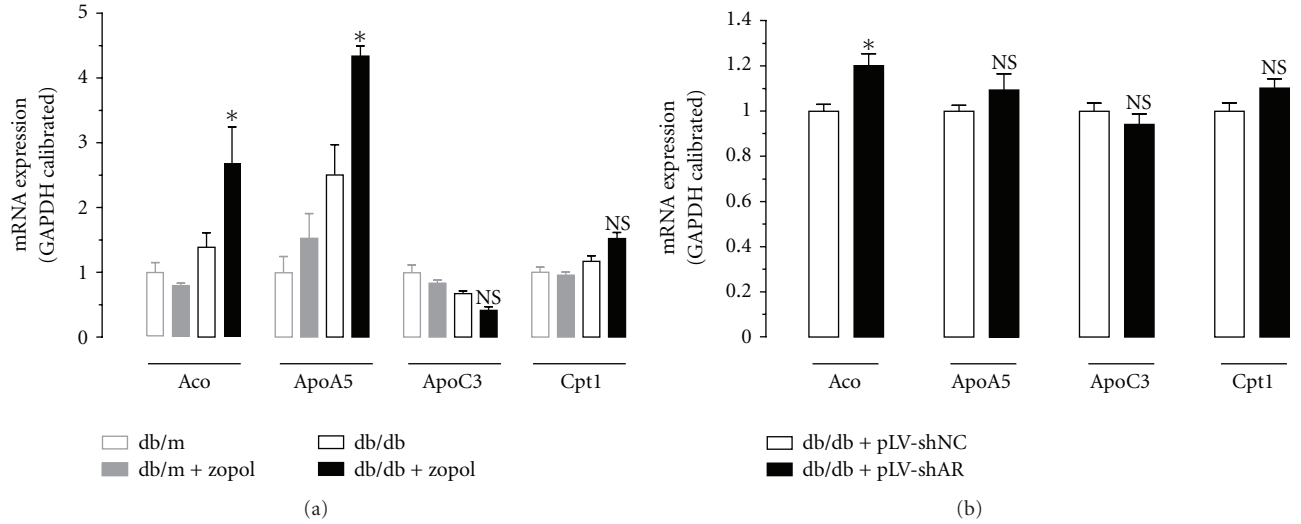

Figure 4: Hepatic mRNA expression of *Aco*, *ApoA5*, *ApoC3,* and *Cpt-1* as analyzed by semiquantitative RT-PCR in db/db mice. (a) Liver tissues were dissected and analyzed 28 days after zopol treatment. (b) Liver tissues were dissected and analyzed 28 days after transduction with lentiviruses containing pLV-shAR or pLV-shNC. Values are expressed as the mean ± SEM (n = 3-4). $^*P < 0.05$; NS: not significant.

investigations. At this moment, however, the mechanisms for increased lipid degradation and mechanisms for decreased lipid synthesis both appear to be functional. Our demonstration that inhibition of AR led to the activation of PPARα through its dephosphorylation contributes in part to increased hepatic lipid degradation following inhibition of AR. It is well established that PPARα is a central regulator for hepatic glucose and lipid metabolism as well as the development of lipid disorders including hepatic steatosis and NAFLD [24–29]. Once activated, it will tend to promote lipid catabolism by upregulating the expression of lipid catabolic enzymes such as lipoprotein lipase and *ApoA5* [30] and downregulating *ApoC3* [31]. Consistent with this, two important lipid catabolic enzymes *Aco* and *ApoA5* were significantly upregulated as a consequence of PPARα activation, although not much change in mRNA expression was observed for *Cpt1* and *ApoC3*. Inhibition of AR, on the other hand, might also result in reduced lipid synthesis. Under hyperglycemic conditions, for example, abundant glucose might be channeled into the hyperglycemia-activated AR/the polyol pathway to generate a substantial amount of fructose in the liver. Fructose has long been known to be highly lipogenic and can contribute significantly to hepatic lipogenesis, adipogenesis, insulin resistance, obesity, hypertension, metabolic syndrome, hepatic steatosis, and NAFLD [9, 32–47] in both human and rodents. When AR is inhibited or when the polyol pathway is blocked, it can therefore be expected that endogenous hepatic fructose generation will be greatly reduced such that fructose-induced lipogenesis in the liver will also be suppressed, thereby leading to the suppression of hepatic steatosis or NAFLD.

Abbreviations

Aco: Acetyl CoA oxidase
ApoA5: Apolipoprotein A-V
ApoC3: Apolipoprotein C-III
AR: Aldose reductase
Cpt1: Carnitine palmitoyl transferase-1
ERK: Extracellular signal-regulated kinase
NAFLD: Nonalcoholic fatty liver disease
NASH: Nonalcoholic steatohepatitis
PPARα: Peroxisome proliferator-activated receptor α
RT-PCR: Reverse transcription polymerase chain reaction
shRNA: Short-hairpin RNA
TG: Triglyceride
T1DM: Type I diabetes mellitus
T2DM: Type II diabetes mellitus
zopol: Zopolrestat.

Acknowledgments

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Research Article

Cholesterol Synthesis Is Associated with Hepatic Lipid Content and Dependent on Fructose/Glucose Intake in Healthy Humans

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Visceral obesity and fatty liver have been related to high synthesis and low absorption of cholesterol. This study aimed to investigate the associations of cholesterol metabolism with liver and visceral fat content in healthy humans. Another objective was to explore the effects of very-high-fructose and very-high-glucose diets on cholesterol homeostasis. We report on a cohort of 20 people (12 males, 8 females; age 30.5 ± 2.0 years; body mass index 25.9 ± 0.5 kg/m^2) who completed a four-week dietary intervention study. Between the baseline and the followup examination the study participants in addition to a balanced weight-maintaining diet received 150 g of either fructose or glucose per day. Visceral and liver fat were measured with magnetic resonance (MR) imaging and ^{1}H-MR spectroscopy, respectively. Cholesterol absorption and synthesis were estimated from the serum noncholesterol sterol concentrations. Performing cross-sectional analyses the lanosterol and desmosterol to cholesterol ratios were positively correlated with visceral and liver fat content (all $P < .03$). The lathosterol to cholesterol ratio decreased in response to high-fructose diet ($P = .006$) but not in response to high-glucose diet. To conclude, visceral and liver fat content are associated with cholesterol synthesis in healthy humans. Furthermore, cholesterol synthesis appears to be dependent on fructose/glucose intake.

1. Introduction

Serum cholesterol is either derived from intestinal absorption or from endogenous synthesis [1]. The individual balance of cholesterol absorption and synthesis is highly heritable [2]. The ATP-binding cassette transporters G5 and G8 (ABCG5/8) and the Niemann-Pick C1 Like1 protein (NPC1L1) play important roles in cholesterol homeostasis. Both genes encode proteins that are expressed in the intestine and regulate cholesterol absorption [3–5]. However, cholesterol absorption and synthesis are not only determined by genetic factors but also by the metabolic state [6–10]. For example, subjects with high body mass index display high synthesis and low absorption of cholesterol [6–8]. Furthermore, cholesterol synthesis prevails over cholesterol absorption in insulin resistance and type 2 diabetes [7–11]. In agreement, visceral obesity is associated with a high synthesis phenotype [12, 13]. Recently, fatty liver, which is thought to be involved in the pathogenesis of the metabolic syndrome [14–17], was also found to be associated with high cholesterol synthesis and low cholesterol absorption [18].

The present work aimed to investigate whether visceral and liver fat contents are correlated with cholesterol homeostasis in healthy humans. Our hypothesis was that even modest differences of liver and visceral fat content would be reflected by differences in cholesterol synthesis and absorption. To answer this question, we performed cross-sectional analyses in 20 healthy individuals who participated in a

four-week dietary intervention (either very-high-fructose or very-high-glucose diet) study [19]. Another objective of this study was to investigate the impact of very-high-fructose intake, which has been found to alter lipid metabolism [20–23], on cholesterol homeostasis.

Visceral and liver fat contents were measured with magnetic resonance (MR) imaging and ^{1}H-MR spectroscopy, respectively. To estimate cholesterol absorption and synthesis, we measured the serum concentrations of lathosterol, lanosterol, desmosterol (cholesterol precursors, indicate endogenous cholesterol synthesis), campesterol, sitosterol (plant sterols, indicate intestinal cholesterol uptake), and cholestanol (5-α saturated derivative of cholesterol indicates intestinal cholesterol uptake) [24–26].

2. Methods

2.1. Study Design and Diet. We report on an exploratory, prospective, randomized, single-blinded, outpatient, intervention study (TUbingen FRuctose Or Glucose study) [19]. Inclusion criteria were age 20–50 years, body mass index 20–35 kg/m^2, physical health, and not more than one-hour sports per week. Exclusion criteria were pregnancy, any relevant illness, fructose intolerance, medication, metal implants, regular alcohol consumption $\geq$10 g/day, and claustrophobia. The participants received 150 g (600 kcal) of either fructose or glucose per day for four weeks. They were blinded to the type of intervention. The sugar was provided in identical plastic packs of 50 g and had to be dissolved in water (50 g sugar in 250 mL water). The participants were instructed to consume the sugar in addition to a balanced weight-maintaining diet (50% carbohydrates, 35% fat, and 15% protein). Fructose or glucose was ingested three times a day (morning, midday, evening) with the main meals. Dietary counseling was provided by a trained dietitian according to the guidelines of the German Society of Nutrition. We aimed to assess compliance with the dietary prescription by close telephone contact. The participants were instructed to immediately inform the investigators in case of problems with the intake of fructose or glucose. For that, they were provided a calling card. Furthermore, compliance was evaluated by interview at visits 1 and 2. In addition, the subjects were asked to fill out food intake records on 3 days in each week of the study which were controlled and evaluated by a trained dietician using DGE PC software. Blood sampling, oral glucose tolerance testing, magnetic resonance imaging, and magnetic resonance spectroscopy were performed before and after dietary intervention. The study was approved by the local ethics committee and was conducted in accordance with the "Declaration of Helsinki." Informed written consent was obtained from all participants. Data from the 20 participants who completed the study were included in the present analyses [19].

2.2. Laboratory Analyses. Total, HDL, and LDL cholesterol concentrations were measured with a standard colorimetric method on a Bayer analyzer (Bayer Health Care, Leverkusen, Germany). The serum noncholesterol sterols were measured using gas-liquid chromatography—mass spectrometry—selected ion monitoring (Hewlett Packard 5890) with an automatic injection system (Hewlett Packard Automatic Sampler 7673A) as previously described [27]. Blood glucose was de-termined using a bedside glucose analyzer based on a glucose-oxidase method (Yellow Springs Instruments, Yellow Springs, Colo). Insulin was analyzed by microparticle enzyme immunoassay (Abbott Laboratories, Tokyo, Japan).

2.3. Oral Glucose Tolerance Test. We performed standard 75 g oral gluose tolerance tests after a 10-h overnight fast. Venous plasma samples were obtained at 0, 30, 60, 90, and 120 min for determination of plasma glucose and insulin. Insulin sensitivity was estimated from the OGTT as proposed by Matsuda and DeFronzo: $\mathrm{ISI}_{\mathrm{est}} = 10,000/\sqrt{(\mathrm{Ins}_{\mathrm{mean}} \times \mathrm{Gluc}_{\mathrm{mean}} \times \mathrm{Ins}_0 \times \mathrm{Gluc}_0)}$ [28].

2.4. Quantitative Analysis of Visceral and Liver Fat. Visceral fat mass was measured with an axial T1-weighed fast spin echo technique with a 1.5 T whole-body imager (Magnetom Sonata; Siemens Medical Solutions) in the complete abdominal region, ranging from head of femur to head of humerus [29]. Liver fat was determined by localized proton magnetic resonance spectroscopy applying a single-voxel STEAM technique with short echo time (TE) as previously described [30, 31].

2.5. Statistical Analysis. The clinical and biochemical characteristics are presented as numbers and percentages and means $\pm$ standard errors of the means for categorical and continuous data, respectively. Ratios of the noncholesterol sterols to cholesterol (measured with gas-liquid chromatography) were calculated (see Table 2). The univariate relationships of the noncholesterol sterols with cholesterol, the relationships among the noncholesterol sterol to cholesterol ratios, and the relationships of the cholesterol subfractions and the noncholesterol sterol ratios with fat depots and insulin sensitivity were analyzed with linear regression models. The results are shown as Pearson correlation coefficients. Furthermore, we performed multivariate analysis for the associations of the cholesterol subfractions and the noncholesterol sterol to cholesterol ratios with fat depots and insulin sensitivity using Analysis of Covariance (ANCOVA). Alterations in the noncholesterol sterol to cholesterol ratios in response to fructose and glucose intervention were studied with the paired samples t-test (two-sided tests). ANCOVA was used to compare the changes in the noncholesterol sterol to cholesterol ratios (e.g., change in lathosterol to cholesterol ratio between baseline and followup examination) between the fructose and glucose intervention groups, with study group as the main factor and the metabolic parameter of interest at baseline (e.g., lathosterol to cholesterol ratio at baseline examination) as covariate (two-sided tests). To estimate the treatment effect, differences in least-square means and the corresponding 95% confidence intervals were calculated based on the ANCOVA models [32]. Data that were not normally distributed (Shapiro-Wilk W test) were

Table 1: Baseline characteristics of the study participants.

	Baseline examination
Males/females, *n*	12/8
Age, years	30.5 ± 2.0
Body mass index, kg/m^2	25.9 ± 0.5
Waist, cm	85 ± 2
Visceral fat, kg	2.2 ± 0.2
Liver fat, % signal	1.5 ± 0.2
Systolic blood pressure, mmHg	117 ± 3
Diastolic blood pressure, mmHg	77 ± 2
Total cholesterol, mg/dL	175 ± 5
LDL cholesterol, mg/dL	106 ± 5
HDL cholesterol, mg/dL	54 ± 2
VLDL cholesterol, mg/dL	15 ± 2
Non-HDL cholesterol, mg/dL	122 ± 5
Fasting glucose, mmol/L	4.86 ± 0.06
Fasting insulin, pmol/L	48 ± 7
Insulin sensitivity Matsuda, arbitrary units	17.6 ± 2.1

Values are numbers and percentages and means with standard errors of the means for categorical and continuous data, respectively.

Table 2: Serum levels of the noncholesterol sterol to cholesterol ratios at baseline.

Lathosterol/cholesterol, μg/mg	1.28 ± 0.11
Desmosterol/cholesterol, μg/mg	0.49 ± 0.02
Lanosterol/cholesterol, μg/mg	0.31 ± 0.02
Campesterol/cholesterol, μg/mg	1.58 ± 0.11
Sitosterol/cholesterol, μg/mg	1.22 ± 0.09
Cholestanol/cholesterol, μg/mg	1.72 ± 0.06

Values are means with standard errors of the means.

transformed logarithmically (base-10). P values <0.05 were considered significant. The JMP statistical software package 7.0 (SAS Institute, Cary, NC, USA) was used.

3. Results

The baseline characteristics of the study participants are shown in Table 1. The mean ± standard error of the mean serum concentrations were 186 ± 5 mg/dL for cholesterol (GCMS), 0.241 ± 0.024 mg/dL for lathosterol, 0.091 ± 0.006 mg/dL for desmosterol, 0.058 ± 0.004 mg/dL for lanosterol, 0.295 ± 0.023 mg/dL for campesterol, 0.229 ± 0.018 mg/dL for sitosterol, and 0.320 ± 0.014 mg/dL for cholestanol.

The serum desmosterol and cholestanol levels were significantly related to cholesterol ($r = 0.631$, $P = .002$ and $r = 0.615$, $P = .004$, resp.). The lathosterol, desmosterol, and lanosterol to cholesterol ratios were also positively correlated (Table 3). In agreement, the ratios of campesterol and sitosterol to cholesterol showed a significant positive association (Table 3). Furthermore, the ratio of campesterol

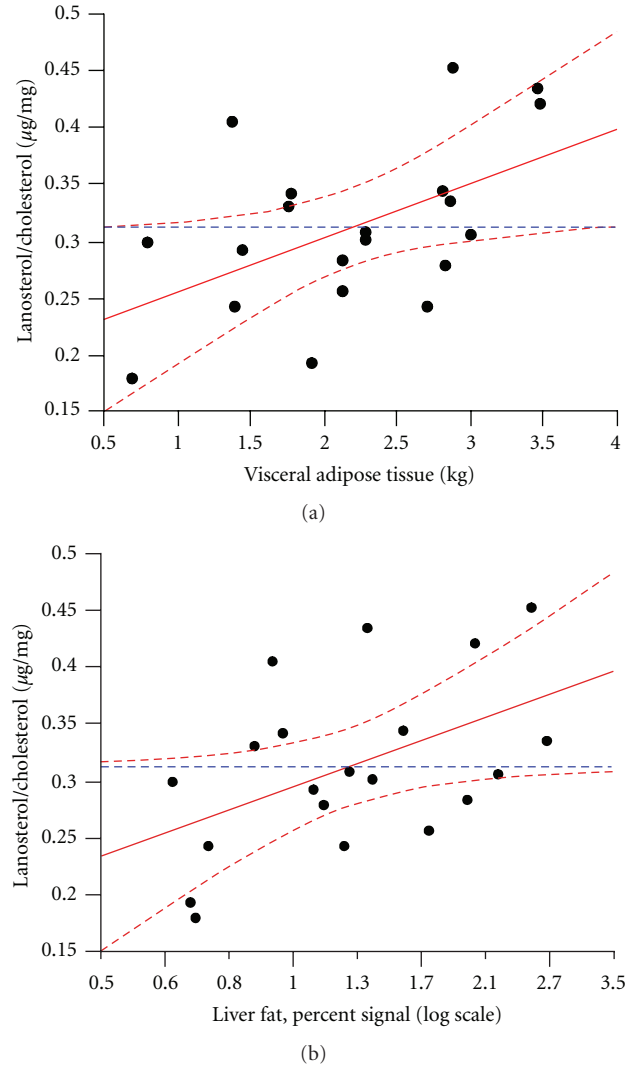

Figure 1: The associations of (a) visceral and (b) liver fat with the lanosterol to cholesterol ratio adjusted for sex, age, and body mass index.

to cholesterol was significantly related to the ratio of cholestanol to cholesterol (Table 3). The ratio of lathosterol to cholesterol was inversely related to the ratio of campesterol to cholesterol (Table 3).

High lanosterol and desmosterol to cholesterol ratios were significantly associated with increased visceral and liver fat content (Table 4). The association of the lanosterol to cholesterol ratio with visceral ($P = .033$) and liver fat ($P = .044$) was independent of sex, age, and body mass index (Figure 1). The cholesterol absorption markers were not significantly related to visceral and liver fat (Table 4). HDL cholesterol was inversely related to visceral fat and liver fat content whereas non-HDL cholesterol was positively correlated with visceral fat (Table 4). LDL cholesterol was not associated with fat depots (Table 4). The lathosterol to cholesterol ratio was inversely related to insulin sensitivity (Table 4).

The lathosterol to cholesterol ratio significantly decreased in response to very-high-fructose diet but not in

Table 3: Univariate correlations among the noncholesterol sterol to cholesterol ratios.

	Lathosterol/ cholesterol	Desmosterol/ cholesterol	Lanosterol/ cholesterol	Campesterol/ cholesterol	Sitosterol/ cholesterol	Cholestanol/ cholesterol
Lathosterol/cholesterol	—	0.572†	0.489‡	−0.493‡	−0.378	−0.378
Desmosterol/cholesterol	0.572†	—	0.607†	−0.280	−0.173	−0.357
Lanosterol/cholesterol	0.489‡	0.607†	—	−0.059	0.000	−0.036
Campesterol/cholesterol	−0.493‡	−0.280	−0.059	—	0.880*	0.486‡
Sitosterol/cholesterol	−0.378	−0.173	0.000	0.880*	—	0.375
Cholestanol/cholesterol	−0.378	−0.357	−0.036	0.486‡	0.375	—

Values are Pearson correlation coefficients calculated with linear regression; $^{*}P < 0.001$, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.05$.

Table 4: Univariate correlations of total cholesterol, cholesterol subfractions, and the noncholesterol sterol to cholesterol ratios with liver fat, visceral fat, and insulin sensitivity.

	Liver fat		Visceral fat		Insulin sensitivity	
	r	*P*	*r*	*P*	*r*	*P*
Total cholesterol	0.194	0.384	0.243	0.303	0.082	0.730
LDL cholesterol	0.386	0.145	0.363	0.116	−0.049	0.839
HDL cholesterol	−0.545	0.011	−0.593	0.006	0.045	0.852
VLDL cholesterol	0.035	0.884	0.254	0.279	0.280	0.231
Non-HDL cholesterol	0.386	0.093	0.452	0.046	0.066	0.783
Lathosterol/cholesterol	0.107	0.636	0.404	0.078	−0.500	0.025
Desmosterol/cholesterol	0.548	0.027	0.541	0.014	−0.259	0.271
Lanosterol/cholesterol	0.642	0.004	0.629	0.003	−0.364	0.114
Campesterol/cholesterol	−0.005	0.710	−0.253	0.282	0.335	0.149
Sitosterol/cholesterol	0.040	0.803	−0.279	0.234	0.200	0.397
Cholestanol/cholesterol	0.008	0.974	0.060	0.800	0.325	0.163

r Pearson correlation coefficients and *P* values calculated with linear regression (liver fat was transformed logarithmically for calculation of the *P* values).

response to very-high-glucose diet with the difference between interventions reaching statistical significance (Table 5). In agreement, there was a significant treatment effect for the alterations of the lanosterol to cholesterol ratio (Table 5). No changes or treatment effects were found for the desmosterol to cholesterol ratio and the absorption marker to cholesterol ratios (Table 5).

4. Discussion

We found that the ratios of desmosterol and lanosterol to cholesterol, which indicate endogenous cholesterol synthesis, were positively associated with visceral and liver fat content in persons at relatively low metabolic risk. The relationships of the lanosterol to cholesterol ratio with visceral and liver fat were independent of obesity. Furthermore, high ratio of lathosterol to cholesterol, also an indicator of cholesterol synthesis, was related to lower insulin sensitivity.

Our observations fit well in the context of the previously published studies in the field. High body mass index, insulin resistance, and type 2 diabetes have been independently associated with high cholesterol synthesis [6–11]. Visceral obesity and just recently fatty liver were also related to increased synthesis of cholesterol [12, 13, 18]. Our study extends these findings in the sense that we report on a cohort of healthy people. Furthermore, we have also measured the cholesterol synthesis marker lanosterol which was obviously the noncholesterol sterol most strongly related to visceral and liver fat content. We did not find significant associations of cholesterol absorption with visceral or liver fat. This may suggest that fat distribution and ectopic fat deposition in the liver primarily affect cholesterol synthesis. In this respect, it seems noteworthy that visceral fat was not significantly related to the sitosterol to cholesterol ratio in a recent study either [13].

Why is liver fat content positively correlated with cholesterol synthesis? The most important regulator of cellular cholesterol synthesis is the sterol regulatory element-binding protein 2 (SREBP2), a membrane-bound transcription factor [33, 34]. This transcription factor is highly expressed in the liver and interestingly, its activity is increased in subjects with high liver fat [35]. However, the exact mechanisms accounting for the activation of SREBP2 in subjects with high liver fat seem poorly understood.

Since cholesterol homeostasis is obviously associated with liver fat content, the following question arises: might pharmacological interventions targeting cholesterol homeostasis have an impact on hepatic lipid content? A well-performed study by Szendroedi et al. found that even high-dose simvastatin treatment has no direct effects on liver fat

Table 5: Changes in the noncholesterol sterol to cholesterol ratios in response to high-fructose or high-glucose diet.

	Fructose intervention group			Glucose intervention group			Fructose versus glucose		
	Baseline	Change	P^*	Baseline	Change	P^*	ΔLSM	95% CI	$P^\dagger$
Lathosterol/cholesterol, μg/mg	1.16 ± 0.11	−0.20 ± 0.06	0.006	1.40 ± 0.20	−0.08 ± 0.18	0.659	−0.28	−0.53 to −0.04	0.027
Desmosterol/cholesterol, μg/mg	0.45 ± 0.03	0.01 ± 0.02	0.675	0.52 ± 0.04	−0.02 ± 0.04	0.638	−0.01	−0.08 to 0.07	0.809
Lanosterol/cholesterol, μg/mg	0.30 ± 0.03	−0.03 ± 0.03	0.332	0.32 ± 0.02	0.03 ± 0.04	0.555	−0.08	−0.16 to 0.00	0.040
Campesterol/cholesterol, μg/mg	1.74 ± 0.13	−0.07 ± 0.07	0.337	1.42 ± 0.17	0.05 ± 0.15	0.746	−0.10	−0.47 to 0.27	0.569
Sitosterol/cholesterol, μg/mg	1.28 ± 0.09	−0.07 ± 0.05	0.165	1.16 ± 0.15	0.01 ± 0.09	0.889	−0.06	−0.26 to 0.14	0.562
Cholestanol/cholesterol, μg/mg	1.82 ± 0.06	−0.10 ± 0.10	0.348	1.61 ± 0.09	−0.07 ± 0.06	0.307	−0.04	−0.32 to 0.25	0.796

Values are means ± standard errors of the means; change: absolute difference between visits 1 and 2; fructose versus glucose: treatment effect of fructose intervention compared to glucose intervention; ΔLSM: difference in least squares means between fructose and glucose intervention (calculated with Analysis of Covariance with correction for baseline values); CI: confidence interval; $^*P$ value for change between visits 1 and 2 calculated with paired samples t-test (two-sided); $^\dagger P$ value for difference in change between fructose and glucose intervention (calculated with Analysis of Covariance with correction for baseline values, two-sided).

content in people with type 2 diabetes [36]. In contrast, the cholesterol absorption inhibitor ezetimibe was found to increase the reduction of liver fat in obese subjects on a weight-loss diet [37]. Whether the use of plant sterols and stanols, which similarly act as inhibitors of cholesterol absorption [38], will help to reduce hepatic steatosis remains to be investigated.

We also studied the effects of very high-fructose and very-high-glucose diets on cholesterol absorption and synthesis. High-fructose diet has been implicated in the pathogenesis of the metabolic syndrome, fatty liver, and type 2 diabetes [20–23]. Moreover, a recent study suggested that high-fructose diet was associated with increased plasma concentrations of LDL cholesterol, small dense LDL, and oxidized LDL [23]. The effect of high-fructose diet on cholesterol homostasis has not been investigated so far. According to the present findings, fructose compared with glucose appears to less strongly stimulate cholesterol synthesis. This novel observation may be explained by the fact that fructose does not provoke endogenous secretion of insulin [20], which is considered to be an important regulator of cholesterol synthesis [39]. Alternatively, the treatment effect for cholesterol synthesis may result from the significant weight gain in the glucose intervention group (+1.7 kg) which was not observed in the fructose intervention group (+0.2 kg) [19].

Consistent with earlier work [12, 13, 40, 41], the serum HDL cholesterol concentration was significantly decreased in subjects with high visceral and liver fat content in the present cohort. Hence, increased visceral and liver fat content may indicate early disturbance of lipid metabolism in healthy people. It is also in agreement with a recent trial that the serum total and LDL cholesterol concentrations were not significantly related to visceral and liver fat content in our cohort of healthy individuals [12]. Hoenig et al. even showed that low-density lipoprotein cholesterol was inversely correlated with the abdominal visceral fat area in subjects with established vascular disease [13]. The authors discussed that their finding could explain the loss of the relationship between LDL cholesterol and cardiovascular events in the obese and support the use of non-HDL cholesterol instead of LDL cholesterol as the primary therapeutic target for lipid lowering therapy [13, 42]. Our data may support this view considering that we observed a positive correlation of non-HDL cholesterol with visceral fat.

Finally, our data confirm that intestinal cholesterol absorption and endogenous cholesterol synthesis are interrelated considering the significant inverse association between the ratios of lathosterol and campesterol to cholesterol [7, 24].

The sample size of our study is relatively low. We cannot, therefore, rule out that a significant association of visceral and liver fat content with the cholesterol absorption markers could be observed in a larger cohort of healthy individuals. To compensate for this drawback, we used very precise and stringently validated analytical procedures for the quantification of the noncholesterol sterols and the fat depots. The serum concentrations of the noncholesterol sterols were measured using a highly sensitive and specific gas-liquid chromatography method. Visceral and liver fat content were quantified using magnetic resonance imaging and magnetic resonance spectroscopy, respectively. We also want to highlight that the sample size of our cohort was similar or even larger compared with previous highly recognized studies fructose intervention studies [21].

In conclusion, we found an independent association of visceral and liver fat content with cholesterol synthesis in healthy humans. Moreover, we were able to show for the first time that cholesterol synthesis is dependent on fructose/glucose intake. Studies investigating whether marked alterations of liver fat content will have an impact on cholesterol homeostasis are encouraged.

Author Contributions

A. Fritsche, G. Silbernagel, and N. Stefan designed the study. D. Lütjohann and S. Meichsner measured the noncholesterol sterols. J. Machann and F. Schick quantified the fat depots. G. Silbernagel performed the statistical analysis. A. Fritsche, D. Lütjohann, G. Silbernagel, and N. Stefan wrote the manuscript. K. Kantartzis contributed to the discussion and reviewed and edited the manuscript. All authors have read the final version and agreed to the manuscript as submitted.

Conflict of Interest

The authors have no conflict of interest to declare.

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Research Article

Predictors of Impaired Glucose Regulation in Patients with Non-Alcoholic Fatty Liver Disease

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Introduction. Many patients with non-alcoholic fatty liver disease (NAFLD) have impaired glucose regulation or type 2 diabetes mellitus (DM). We investigated characteristics of NAFLD patients associated with hyperglycemia. *Methods*. During a 2-hour oral glucose tolerance test (OGTT), serum glucose and insulin were measured in 152 NAFLD patients. *Results*. 48.7% of NAFLD patients had hyperglycemia. Age (odds ratio (OR) = 1.08, 95% confidence interval (CI): 1.03–1.13), body mass index (BMI) (OR = 1.12, 95% CI: 1.01–1.25), and lower high-density lipoprotein cholesterol (HDL-C) (OR = 0.95, 95% CI: 0.92–0.98) proved to be independent predictors of hyperglycemia. After OGTT, 30 min insulin was lower in hyperglycemic patients (74.2 ± 49.7 versus 94.5 ± 53.9 μIU/mL, $P = 0.02$), while 90 min insulin (170.1 ± 84.6 versus 122.9 ± 97.7 μU/mL, $P = 0.01$) and 120 min insulin (164.0 ± 101.2 versus 85.3 ± 61.9 μIU/mL, $P < 0.01$) were higher. *Conclusions*. NAFLD patients with higher BMI, lower HDL-C, or older age were more likely to have impaired glucose metabolism. An OGTT could be of value for early diagnosis of DM among this population.

1. Introduction

Non-alcoholic fatty liver disease is increasingly being identified in routine clinical practice [1]. Although the natural history of this disorder is variable, the presence of non-alcoholic fatty liver disease may lead to an adverse prognosis for some patients, due to liver-related disorders, as well as cardiovascular morbidity and mortality [2–4]. The pathogenesis of non-alcoholic fatty liver disease has been closely related to insulin resistance; this disorder frequently co-exists with impaired glucose tolerance (IGT) or type 2 diabetes mellitus [5–7]. The latter conditions, if present, have been associated with more severe liver disease and unfavorable prognosis [8–11].

Screening patients with non-alcoholic fatty liver disease for impaired glucose regulation or type 2 diabetes mellitus could help the earlier diagnosis and treatment of these conditions preventing their possible complications, such as cardiovascular diseases. The measurement of fasting plasma glucose is considered as the preferred initial screening test for the identification of hyperglycemia [12]. The sensitivity of this test can be variable depending on the population evaluated [13, 14]. However, it has not been clearly established, which of the patients with non-alcoholic fatty liver disease should further undergo an oral glucose tolerance test for the above-described purposes. In this regard, we sought to identify predictor characteristics for the presence of impaired glucose regulation or type 2 diabetes

in patients with non-alcoholic fatty liver disease and assess for potential differences in these patients regarding glucose metabolism.

2. Methods

We prospectively studied a cohort of patients who presented with elevated levels of serum aminotransferases at the outpatient hepatology clinic of Attikon University Hospital, in Athens, Greece, between June 2006 and September 2009, and was diagnosed with non-alcoholic fatty liver disease. The diagnosis of non-alcoholic fatty liver disease was based on the presence of hypertransaminasemia along with characteristic findings of fatty infiltration in liver ultrasonography ("bright liver" or hyperechoic appearance) and the exclusion of other possible causes of hypertransaminasemia, including alcoholic liver disease, adverse events of drugs, viral hepatitis, autoimmune disorders, and hereditary diseases affecting the liver.

The patients that were diagnosed with non-alcoholic fatty liver disease underwent an oral glucose tolerance test, unless they had a known prior history of diabetes or a fasting serum glucose ≥126 mg/dL. Specifically, 75 g of glucose were administered orally after an overnight fast. Serum glucose and insulin were measured in blood samples obtained through an indwelling peripheral vein cannula at time 0 and 30, 60, 90, and 120 min after the glucose challenge. Patients were classified as having normoglycemia (normal glucose values), impaired fasting glucose (IFG), IGT, or type 2 diabetes, according to the criteria endorsed by the American Diabetes Association [15]. Approval for the study was granted by the institutional review board.

2.1. Data Analysis. We grouped the non-alcoholic fatty liver disease patients into normoglycemic and hyperglycemic (IFG, IGT, or type 2 diabetes). We assessed for the presence of differences between the above 2 groups in the patient demographics, body mass index (BMI), and common laboratory tests. We also assessed for differences between the 2 groups in the levels of glucose and insulin obtained at the 5 time points specified above during the 75 g oral glucose tolerance test and the overall insulin response during the test. The overall insulin response was determined on the basis of the area under the curve (AUC) of insulin levels versus time, extracting the area corresponding to the baseline insulin level (net incremental insulin AUC). We used the trapezoid method to calculate the AUC. Finally, we calculated the updated homeostasis model assessment of insulin resistance (HOMA2-IR) index and assessed for differences between the 2 groups [16]. We used the independent samples t-test and the x^2-test to compare continuous and categorical variables, respectively. The variables of baseline patient characteristics with different distributions between the normoglycemic and hyperglycemic groups were entered in a stepwise, forward, binary logistic regression model to test for independent associations. P values lower than 0.05 were considered statistically significant. We used the SPSS version 15.0 (SPSS Inc., Chicago, ILL) as the software for the statistical analysis.

3. Results

A total of 152 Caucasian patients (52.0% females and 48% males) with non-alcoholic fatty liver disease, with mean ± standard deviation age of 50.1 ± 11.4 years were included in the study. According to the values of fasting serum glucose or serum glucose 2 hours after the 75 g oral glucose challenge, 78 of the 152 patients (51.3%) were normoglycemic, while 45 patients (29.6%) had IFG or IGT (21 and 41 patients, resp.), and 29 (19.1%) had type 2 diabetes (Figure 1).

In Table 1, we describe the baseline patient characteristics and common laboratory tests in non-alcoholic fatty liver disease patients with or without hyperglycemia. Age, BMI, high-density lipoprotein cholesterol (HDL-C), and serum albumin were the only variables that significantly differed between the 2 studied groups. In the multivariate analysis that included the above four variables as covariates, age (odds ratio: 1.08, 95% confidence interval: 1.03–1.13), BMI (odds ratio: 1.12, 95% confidence interval: 1.01–1.25), and HDL-C (odds ratio: 0.95, 95% confidence interval: 0.92–0.98) were found to be independently associated with the presence of hyperglycemia in patients with non-alcoholic fatty liver disease. Patients with hyperglycemia had greater age (53.0 ± 10.7 versus 47.3 ± 11.4 years, $P < 0.01$), higher BMI (30.5 ± 4.5 versus 28.5 ± 4.8 kg/m^2, $P = 0.01$), lower HDL-C (46.5 ± 13.6 versus 53.7 ± 18.8 mg/dL, $P = 0.02$), and lower serum albumin (4.1 ± 0.5 versus 4.4 ± 0.4 g/dL, $P < 0.01$) in comparison to patients with normoglycemia (Figure 1).

Table 2 depicts the associations of serum glucose and insulin values obtained at time 0 and 30, 60, 90, and 120 min after the 75 g oral glucose load between non-alcoholic fatty liver disease patients with or without hyperglycemia. Patients with hyperglycemia (IFG, IGT, or type 2 diabetes) had higher glucose values at all the above-specified time points compared with patients without hyperglycemia. Additionally, patients with hyperglycemia showed significantly higher insulin levels at time 0, 90, and 120 min compared with patients without hyperglycemia. Insulin at 30 min was lower in the patients with hyperglycemia, and insulin at 60 min did not differ between the 2 groups. The overall insulin response (net incremental insulin AUC) did not differ, as well. The HOMA2-IR index significantly differed between the two groups (Table 2).

4. Discussion

Almost half (48.7%) of the patients with non-alcoholic fatty liver disease who were evaluated in our study and had IFG, IGT, or type 2 diabetes. This group was of older age had higher BMI, lower levels of HDL-C, and lower serum albumin compared with the group of patients without hyperglycemia. Age, BMI, and HDL-C were independent predictors of the presence of IFG, IGT, or type 2 diabetes in our cohort of patients with non-alcoholic fatty liver disease. The patients with hyperglycemia seemed to be more insulin resistant compared with those without hyperglycemia. The acute phase of insulin response to the 75 g oral glucose load was less pronounced in the hyperglycemic group, as evidenced by insulin at 30 min. This was followed by more

Fasting serium glucose	2-hour serum glucose after 75-g oral glucose challenge		
	Normal glucose tolerance	IGT	DM
Normal fasting glucose	78	33	16
IFG	4	8	9
DM	0	0	4

DM: type 2 diabetes mellitus, IFG: impaired fasting glucose, IGT: impaired glucose tolerance.
Cell shaded in white indicates the 78 patients with normoglycemia.
Cells shaded in light grey indicate the 45 patients with IFG or IGT.
Cells shaded in dark grey indicate the 29 patients with DM.

Figure 1: Classification of the 152 included patients with non-alcoholic fatty liver disease on the basis of fasting serum glucose and 2-hour post-load serum glucose.

Table 1: Baseline characteristics of non-alcoholic fatty liver disease patients with and without hyperglycemia.

Patient characteristics	IFG/IGT, $n = 45$	DM, $n = 29$	Hyperglycemia (IFG/IGT or DM), $n = 74$	Normoglycemia, $n = 78$	P value (patients with hyperglycemia versus normoglycemia)
	n/N (%) OR mean ± standard deviation				
Sex, Female	21/45 (46.7%)	14/29 (48.3%)	35/74 (47.3%)	44/78 (56.4%)	0.26
Age, years	51.2 ± 10.2	55.9 ± 11.0	53.0 ± 10.7	47.3 ± 11.4	<**0.01**
Body mass index, kg/m^2	30.0 ± 4.2	31.1 ± 4.9	30.5 ± 4.5	28.5 ± 4.8	**0.01**
AST, U/L	43.7 ± 46.6	38.8 ± 23.5	41.7 ± 38.8	40.2 ± 33.4	0.81
ALT, U/L	64.6 ± 45.9	59.8 ± 39.1	62.7 ± 38.8	67.7 ± 55.6	0.55
AST/ALT ratio	0.68 ± 0.22	0.72 ± 0.30	0.70 ± 0.25	0.71 ± 0.39	0.85
GGT, U/L	86.7 ± 70.8	89.6 ± 87.1	87.9 ± 77.2	86.6 ± 88.7	0.92
Cholesterol, mg/dL	209.4 ± 40.7	209.2 ± 50.8	209.3 ± 44.6	211.9 ± 42.3	0.74
Triglycerides, mg/dL	135.1 ± 64.5	146.4 ± 76.2	139.7 ± 69.1	123.1 ± 72.3	0.18
HDL-C, mg/dL	47.6 ± 14.3	45.0 ± 12.7	46.5 ± 13.6	53.7 ± 18.8	**0.02**
LDL-C, mg/dL	128.3 ± 32.1	131.2 ± 40.3	129.6 ± 35.8	133.9 ± 34.1	0.51
Triglycerides/HDL-C ratio	3.6 ± 3.4	3.6 ± 2.2	3.6 ± 2.9	2.7 ± 2.2	0.05
Total Protein, g/dL	7.0 ± 1.5	7.0 ± 1.6	7.0 ± 1.5	7.1 ± 1.7	0.83
Albumin, g/dL	4.2 ± 0.4	4.0 ± 0.5	4.1 ± 0.5	4.4 ± 0.4	<**0.01**
Albumin/globulin ratio	3.1 ± 7.9	1.8 ± 1.7	2.5 ± 6.1	2.3 ± 3.1	0.83
Hemoglobin, g/dL	14.4 ± 1.5	13.6 ± 1.7	14.0 ± 1.6	14.1 ± 1.4	0.93
Hematocrit, %	43.4 ± 4.6	41.8 ± 4.2	42.7 ± 4.5	42.2 ± 3.8	0.45

ALT: alanine aminotransferase, AST: aspartate aminotransferase, DM: type 2 diabetes mellitus, IFG: impaired fasting glucose, IGT: impaired glucose tolerance, GGT: gamma-glutamyl transpeptidase, HDL-C: high-density lipoprotein cholesterol, IFG: impaired fasting glucose, and IGT: impaired glucose tolerance.

Table 2: Glucose and insulin regulation in response to a 75 g oral glucose tolerance test in non-alcoholic fatty liver disease patients with and without hyperglycemia.

Patient characteristics	IFG/IGT, $n = 45$	DM, $n = 29$	Hyperglycemia (IFG/IGT or DM), $n = 74$	Normoglycemia, $n = 78$	P value (patients with hyperglycemia versus normoglycemia)
	Mean ± standard deviation				
Glucose 0 min, mg/dL	89.9 ± 14.1	104.0 ± 21.9	95.4 ± 18.7	80.9 ± 10.5	<**0.01**
Insulin 0 min, μIU/mL	15.4 ± 8.3	19.5 ± 10.2	17.0 ± 9.3	13.8 ± 7.2	**0.02**
Glucose 30 min, mg/dL	160.4 ± 27.0	179.9 ± 42.1	167.7 ± 34.6	147.8 ± 32.5	<**0.01**
Insulin 30 min, μIU/mL	79.5 ± 53.4	65.3 ± 42.3	74.2 ± 49.7	94.5 ± 53.9	**0.02**
Glucose 60 min, mg/dL	204.5 ± 34.9	241.7 ± 40.3	219.1 ± 41.1	152.9 ± 39.0	<**0.01**
Insulin 60 min, μIU/mL	133.1 ± 72.0	112.4 ± 70.7	125.0 ± 71.7	136.7 ± 88.8	0.38
Glucose 90 min, mg/dL	196.9 ± 37.4	264.1 ± 38.9	219.8 ± 49.4	126.9 ± 32.9	<**0.01**
Insulin 90 min, μIU/mL	163.6 ± 80.0	183.0 ± 95.1	170.1 ± 84.6	122.9 ± 97.7	**0.01**
Glucose 120 min, mg/dL	163.2 ± 24.2	243.6 ± 38.3	194.7 ± 49.8	105.4 ± 21.7	<**0.01**
Insulin 120 min, μIU/mL	160.9 ± 96.3	168.8 ± 110.1	164.0 ± 101.2	85.3 ± 61.9	<**0.01**
Net incremental insulin AUC, μIU/mL * min	11872.9 ± 6608.2	10545.7 ± 6688.5	11352.8 ± 6626.1	10285.4 ± 6690.6	0.33
HOMA2-IR index	1.9 ± 1.0	2.5 ± 1.3	2.2 ± 1.2	1.7 ± 0.9	<**0.01**

AUC: area under the curve, HOMA2-IR: homeostasis model assessment of insulin resistance, DM: type 2 diabetes mellitus, IFG: impaired fasting glucose, IGT: impaired glucose tolerance, and LDL-C: low-density lipoprotein cholesterol.

pronounced hyperinsulinemia at 90 and 120 min after the glucose challenge.

Our study findings agree with those of other studies that have showed variability in the glucose regulation of patients with non-alcoholic fatty liver disease [10]. This could be in part attributed to the fact that non-alcoholic fatty liver disease comprises a spectrum of disorders of different severity, from simple hepatic steatosis to non-alcoholic steatohepatitis, hepatic fibrosis, and cirrhosis [6].

In our study, older age was associated with a greater likelihood for impaired glucose regulation in patients with non-alcoholic fatty liver disease. Type 2 diabetes mellitus is known to be associated with older age, a fact that reflects the long process for clinical onset of diabetes mellitus [14]. It can be assumed that metabolic derangements associated with non-alcoholic fatty liver disease and insulin resistance [17, 18] impose a stress on pancreatic β-cells that may eventually fail to compensate for the increased insulin requirements. In our study, the acute insulin response to the oral glucose load, which can be considered as a marker of β-cell function [19, 20], was found to be decreased in non-alcoholic fatty liver disease patients with hyperglycemia.

Higher BMI is also a known risk factor for type 2 diabetes mellitus [21]. In our study higher BMI independently predicted the risk for the presence of hyperglycemia among patients with non-alcoholic fatty liver disease [17]. More than half of our study population had a BMI below the threshold of 30 kg/m^2 though. Therefore, our findings should not be interpreted as if only obese patients with non-alcoholic fatty liver disease have hyperglycemia.

Serum levels of HDL-C were also lower in the subgroup of patients with hyperglycemia of the non-alcoholic fatty liver disease patients evaluated in our study. Low HDL-C is a characteristic finding in diabetic dyslipidemia and is also associated with prediabetes, insulin resistance, metabolic syndrome, and non-alcoholic fatty liver disease [20, 22–24]. The elevated levels of serum triglycerides, another feature of diabetic dyslipidemia, did not seem to differ between the non-alcoholic fatty liver disease patients who had hyperglycemia compared with those who did not, in our study. Also, comparison of the two groups on the basis of the ratio triglycerides/HDL-C, a parameter that has been associated with insulin resistance and atherogenicity [25, 26], showed only a marginal difference (Table 1).

Additionally, in our study, the non-alcoholic fatty liver disease patients with hyperglycemia had lower levels of serum albumin compared with those without hyperglycemia, although this difference was not independent of age, BMI, and HDL-C. Presumably, lower serum albumin in patients with non-alcoholic fatty liver disease could also be related to more advanced liver disease, higher degree of systemic inflammation, or albuminuria due to diabetic nephropathy [8, 27].

Screening for hyperglycemia could be considered in patients with non-alcoholic fatty liver disease given the strong association of this disorder with IGT, including diabetes mellitus. There are known risk factors for type 2 diabetes to guide the selection of patients to screen [12]. Age above 45 years and the association of a BMI above 25 kg/m^2 with HDL-C below 35 mg/dL are among the indicators for screening for type 2 diabetes proposed by the American Diabetes Association [12]. In our study, age, BMI, and HDL were independent indicators for the presence of impaired glucose regulation in patients with non-alcoholic fatty liver disease who had no known derangement in glucose metabolism.

If our patients had undergone assessment of fasting serum glucose alone, 25 of the 74 (33.8%) patients with diabetic or non-diabetic hyperglycemia and 4 of the 29 (13.8%) patients with, specifically, diabetic hyperglycemia would have been identified. In contrast, 70 of the 74 (94.6%) patients with diabetic or non-diabetic hyperglycemia and all of the 29 patients with, specifically, diabetic hyperglycemia were identified on the basis of the 2-hour serum glucose value obtained after an oral glucose tolerance test (Figure 1). Considerable discordance in the accuracy of fasting and 2-hour postload glucose for the identification of impaired glucose regulation has been observed in different populations [13, 28]. Fasting glucose may have lower sensitivity in younger individuals [13]. Additionally, IFG and IGT may more accurately reflect hepatic and muscle insulin resistance, respectively [14]. Yet, non-alcoholic fatty liver disease is associated with both hepatic and peripheral insulin resistance [29–31].

The oral glucose tolerance test is thought to be rather cumbersome to perform in everyday clinical practice [14]. Our study indicates that non-alcoholic fatty liver disease patients of older age, higher BMI, and lower HDL-C should be prioritized in this regard. In two other similar studies, older age and higher BMI have been associated with abnormal oral glucose tolerance in young male non-alcoholic fatty liver disease patients in China [32], and lower HDL-C has been associated with diabetes mellitus in non-alcoholic fatty liver disease patients in Hong Kong [33]. Besides, increased age and BMI are risk factors for more severe disease or adverse prognosis in patients with non-alcoholic fatty liver disease [2, 8, 9].

According to our findings, the value of an oral glucose tolerance test, if performed in patients with non-alcoholic fatty liver disease with appropriate risk factors, would be first to identify those with diabetic hyperglycemia. This group should be managed as for type 2 diabetes, if the diagnosis is confirmed with a second positive relevant test. An oral glucose tolerance test in selected patients with non-alcoholic fatty liver disease can also identify those with impaired glucose tolerance, which indicates an increased risk for developing type 2 diabetes. Although the risk for developing type 2 diabetes can also be determined by considering patient characteristics that are more readily available [34], the presence of impaired glucose regulation signals additionally an increased risk for the development of macrovascular complications [35]. Non-alcoholic fatty liver disease patients with impaired glucose tolerance should be encouraged to modify lifestyle factors (e.g., lose weight and increase physical activity) to prevent the development of type 2 diabetes [14]. These measures would be also important for preventing the progression of liver disease [36].

Some of the patients with impaired glucose tolerance and additional risk factors for type 2 diabetes could also be candidates to receive pharmacologic therapy, particularly with metformin [14]. Pioglitazone might also be effective in this regard [37]. These insulin-sensitizing agents have additionally shown promise for the treatment of biopsy-proven non-alcoholic steatohepatitis in small clinical trials [38, 39]. Whether non-alcoholic fatty liver disease patients with impaired glucose tolerance represent appropriate candidates to receive such medications, on top of lifestyle modification, with the aim to prevent progression into type 2 diabetes and to non-alcoholic steatohepatitis and, also, to decrease the overall cardiovascular risk, requires further study.

In conclusion, in our study of patients with non-alcoholic fatty liver disease, older age, higher BMI, and lower serum levels of HDL-C independently predicted the presence of hyperglycemia (defined as IFG, IGT, or type 2 diabetes) that was primarily identified through an oral glucose tolerance test. Conclusively, we suggest that oral glucose tolerance testing should be considered for patients with non-alcoholic fatty liver disease with one or more of the above-mentioned predictor factors for hyperglycemia to readily diagnose and manage disorders of glucose metabolism.

Conflict of interests

The authors declare that they have no conflict of interests.

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Review Article

The Role of Metformin in the Management of NAFLD

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Nonalcoholic fatty liver disease (NAFLD) is the most common liver disorder worldwide. Its prevalence ranges 10–24% in the general population, reaching 60–95% and 28–55% in obese and diabetic patients, respectively. Although the etiology of NAFLD is still unclear, several lines of evidences have indicated a pathogenetic role of insulin resistance in this disorder. This concept has stimulated several clinical studies where antidiabetic drugs, such as insulin sensitizers including metformin, have been evaluated in insulin-resistant, NAFLD patients. These studies indicate that metformin might be of benefit in the treatment of NAFLD, also in nondiabetic patients, when associated to hypocaloric diet and weight control. However, the heterogeneity of these studies still prevents us from reaching firm conclusions about treatment guidelines. Moreover, metformin could have beneficial tissue-specific effects in NAFLD patients irrespective of its effects as insulin sensitizer.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease. It includes a broad spectrum of liver alterations, ranging from pure steatosis to cirrhosis, through nonalcoholic steatohepatitis (NASH). NAFLD is characterized by liver damage and functional impairment similar to those observed in alcoholic liver disease although occurring in patients who do not drink or drink only a moderate amount of alcohol [1].

Although the pathogenesis of the disorder is not fully clarified, insulin resistance is widely considered a pivotal feature of NAFLD, which is strongly and independently associated with increased risk of type 2 diabetes mellitus (T2DM) and cardiovascular disease [2]. In the past, the diagnosis of NAFLD was mostly the result of ultrasound investigations or liver function tests performed at random. Nowadays, the great attention paid to the occurrence of metabolic syndrome and its related aspects has allowed us to put more emphasis on diagnosis and treatment of this disorder. Clinicians have now become to pay attention to early diagnosis and treatment of NAFLD in insulin-resistant obese and T2DM patients. Diet and lifestyle changes are a mainstay in the management of these patients, while specific pharmacologic treatment for NAFLD is so far lacking. Therefore, it is not surprising that several studies have evaluated the efficacy of insulin sensitizers in NAFLD patients. Among insulin sensitizers, metformin has recently acquired a central role in the treatment of T2DM and other disorders associated with insulin resistance, such as polycystic ovary syndrome (PCOS), and both experimental and clinical studies have recently supported the use of metformin as a useful adjunct in NAFLD patients.

We will review evidences concerning a possible use of metformin in the treatment of NAFLD.

2. Epidemiology and Risk Factors

Epidemiological studies on NAFLD are limited by the lack of a universal screening method used for diagnosis and by the presence of various definitions and diagnostic criteria. However, NAFLD has been reported to occur in 10–24% of the general population [3, 4], thus, representing the most common cause of elevated liver enzymes and one of the most common forms of liver disease in the world. The occurrence of NAFLD is increasing not only in Western countries, but

also in Eastern countries, where evidence of steatosis at liver ultrasounds (USs) is found in 16–30% of the general population [1]. Moreover, NAFLD is increasingly diagnosed in children and adolescents together with the concomitant increase in obesity. Small epidemiological studies have found that NAFLD occurs in 2.6–25% of obese children [5].

As already mentioned, NAFLD is strongly associated with insulin resistance and other components of the metabolic syndrome, like T2DM, central obesity, hyperlipidemia, hypertension, and with other conditions associated to insulin resistance, such as hyperuricemia, atherosclerosis, and PCOS [6, 7]. For this reason, NAFLD is now regarded as the hepatic manifestation of the metabolic syndrome, affecting up to a third of the general population [3, 4]. In fact, the occurrence of NAFLD is reported to range 28–55% in T2DM patients, 60–95% in obese patients, and 27–92% in hyperlipidemic patients [8]. Moreover, the strongest association of NAFLD is with central adiposity, which is an important risk factor even in patients with a normal body mass index (BMI). Diet, lack of exercise, and possibly small bowel bacterial overgrowth are candidate factors influencing the risk of NAFLD [3]. Finally, several studies have reported a potential role of genetic factors for the development of NAFLD. However, these studies have limitations and must be interpreted with caution [3].

3. Pathogenesis

The pathogenesis of NAFLD is still object of discussion. The prevailing hypothesis is based on the "two hits" model, proposed by Day and James in [9]. The "first hit," characterized by free fatty acid and triglyceride accumulation in liver (steatosis), is caused by insulin resistance (through lypolysis and hyperinsulinemia) and obesity (through leptin resistance). This induces a chronic inflammatory condition characterized by the release of proinflammatory cytokines and by oxidative stress, both of which are responsible of the "second hit," which induces the progression from steatosis to more advanced stages of liver damage (steatohepatitis and fibrosis). The "two hit" model has been recently challenged because an increased ratio of saturated-to-unsaturated fatty acids delivered to or stored within the liver may, in part, mediate the progression from simple steatosis to NASH. Proof of this concept has been provided by recent data showing that when triacylglycerol (TAG) precursors accumulate in the liver and the mechanisms of hepatic detoxification are overwhelmed or inactive, saturated fatty acids directly induce hepatic inflammation and insulin resistance both of which may result in steatosis progression toward more severe stages of liver disease [10]. Although the molecular mechanisms that mediate the effects of saturated fatty acids are still unclear, it has been suggested that free fatty acids and TAG metabolites (fatty acyl-CoA, diacylglycerol, ceramide) directly or via Toll-like receptors 2 and 4 (TRL2 and 4) induce endoplasmatic reticulum stress, mitochondrial disfunction, ROS production, impaired hepatic protein metabolism, inhibition of insulin signaling, and activation of inflammatory pathways (NF-κB, JNK, IKK) [11]. Moreover, as suggested by novel evidences, fatty liver releases in the circulation factors called hepatokines (i.e., fetuin A, sex hormone-binding globulin (SHBG), and selenoprotein P) that are directly involved in the pathogenesis of local and system inflammation and in peripheral and hepatic insulin resistance [12]. However, it is important to emphasize that insulin resistance remains a key player in NAFLD pathogenesis and that fatty liver may, in turn, potentiate insulin resistance. It remains controversial whether NAFLD may contribute to insulin resistance independently of the effect of age and total adiposity [13]. Studies showing that liver fat content affects insulin sensitivity in humans more strongly than visceral fat [14, 15] support a direct and important role of fatty liver in the pathogenesis of insulin resistance.

4. Diagnosis

NAFLD is essentially an asymptomatic condition. In patients with NAFLD, who do not have advanced liver disease, the most common sign on physical examination is hepatomegaly, and the diagnosis is made when a US or radiological test reveals evidence of fatty liver. Mild to moderated elevation of ALT, AST, or both are the most common findings (with the AST: ALT ratio <1) [16]. However, liver enzymes may be normal in up to 78% of patients, and; thus, enzyme elevation is insensitive for the detection of NAFLD [17].

The diagnosis of NAFLD is made after exclusion of other causes of liver disease, such as alcohol abuse, viral hepatitis, autoimmune disorders. Older age, obesity, T2DM are risk factors suggesting potential NAFLD diagnosis. Abdominal US is currently the most common method employed for qualitative assessment of hepatic steatosis, because it is noninvasive and widely available.

Both computerized tomographic (CT) scanning and, in particular, magnetic nuclear resonance (MNR) imaging seem to be more sensitive techniques for the quantification of liver steatosis. However, none of these imaging techniques have sufficient sensitivity and specificity for staging the disease, and they cannot distinguish between simple steatosis and fibrosis [18]. Liver biopsy still remains the gold standard for distinguishing between the broad range of chronic liver diseases, but it is limited by its cost, the potential risk of bleeding, and the absence of consensus regarding the histopathological criteria that firmly define NASH and differentiate between NAFLD entities [19].

Given the high prevalence of NAFLD, it would be desirable to use in clinical practice a more practicable and noninvasive diagnostic method. In a recent study, US showed high sensitivity (91.7%) and specificity (100%) in detecting fatty liver [20], and a recent review confirmed that US can accurately identify steatosis with a sensitivity and a specificity of 80–100% [19]. Moreover, the accuracy of US in detecting steatosis seems to be unaffected by obesity [21]. Finally, a recent prospective study [22] demonstrated that serial liver US is an accurate tool for noninvasive monitoring of efficacy of interventions in NAFLD patients.

4.1. Emerging Diagnostic Tools. There is mounting evidence that cytokines secreted not only from adipose tissue, namely,

adipokines, but also from hepatocytes in response to liver injury, are involved in the pathogenesis of NAFLD as well as in its progression [23]. Clinical studies suggest that serum levels of leptin, resistin, adiponectin, tumor necrosis factor α (TNF α), interleukin-6 (IL-6), visfatin, CK-18, and retinol binding protein 4 (RBP4) differ among patients with NAFLD and NASH and healthy controls [24–26].

To overcome biopsy limitations, noninvasive methods have been developed and validated for differentiating simple fatty liver from NASH and for predicting the risk of NAFLD evolution to NASH. These alternative surrogate markers of liver fibrosis include liver stiffness measurements (LSMs), using the method of transient elastography (Fibroscan) [27] and various algorithms, among which the NAFLD fibrosis score [28], the BAAT score [29], and the HAIR score [30], all based on biochemical and clinical parameters. These algorithms have all been developed as simple noninvasive scoring systems aimed at separating patients with NAFLD with and without advanced liver fibrosis by using routinely determined and easily available clinical and biochemical variables [28].

However, currently, the debate is also open on this issue, and new studies are needed.

5. Strategies in NAFLD Management

The first-line treatment of NAFLD is currently based on diet and lifestyle modifications. Most of the published studies in NAFLD population have shown that gradual weight loss (5–10%), calorie-restricted diet, and regular physical exercise lead to a decrease in the incidence of metabolic syndrome, improvement in liver enzyme profile, and resolution of hepatic steatosis [31–34]. However, most of these studies are nonrandomized and short term. Therefore, the paucity of data has limited the production of diet and exercise evidence-based guidelines for NAFLD patients. Moreover, dietary treatment is limited by the lack of compliance and the frequent regain of weight at followup [35].

A pharmacological treatment in patients with NAFLD is not universally accepted yet. Given that insulin resistance plays a key role in the pathogenesis of NAFLD, many studies have evaluated the use of insulin sensitizers as a possible treatment for this disease. Biguanides (metformin) and thiazolidinediones (TZDs), including pioglitazone and rosiglitazone, are the two classes of insulin sensitizers studied in humans [23].

Several trials have shown a beneficial effect of TZDs in patients affected by NAFLD. Three studies, two open-label and one placebo-controlled trial, have evaluated the efficacy of rosiglitazone in NAFLD patients [36–38]. All of these studies have reported an improvement in transaminases levels and hepatic inflammation. However, nowadays rosiglitazone has been removed from the market because of its significant side effects.

The second thiazolidinedione available for the time being is pioglitazone. Four small trials and a large-controlled trial have been conducted to evaluate its efficacy in the treatment of NAFLD [38–42]. In the largest controlled trial, Belfort et al. [40] compared diet plus pioglitazone to diet plus placebo in 55 patients. The pioglitazone-treated group showed an improvement in ALT (by 50%), steatosis (by 54%), insulin sensitivity (by 48%), liver inflammation, and ballooning necrosis but not fibrosis. An improvement in fibrosis was seen only in one of the small four studies mentioned above [41]. Significant amelioration of liver biochemistry, steatosis, and liver inflammation has been also reported in the multicenter placebo-controlled trial called PIVENS [43] (pioglitazone versus Vit E and versus placebo), in which NASH nondiabetic patients were treated for 96 weeks with pioglitazone (30 mg daily). Even if all of these studies have demonstrated the usefulness of pioglitazone in NAFLD, this drug has only been used in patients with biopsy-proven NASH. Furthermore, the long-term safety of this drug is not yet well established. For this reason, today the use of pioglitazone in some countries has been restricted for possible unknown long-term side effects [44]. Another insulin sensitizer that could be used in NAFLD patients is metformin. Its efficacy and usefulness will be extensively discussed in the next paragraph.

6. Role of Metformin in NAFLD

6.1. Mechanism of Action of Metformin. Metformin was introduced in clinical practise in the 1950s and is widely used as a first-line treatment for patients with type 2 diabetes mellitus [45]. The effectiveness of metformin as an antidiabetic drug is explained by its ability to lower blood glucose by decreasing gluconeogenesis in the liver, stimulating glucose uptake in the muscle, and increasing fatty acid oxidation in adipose tissue [46]. The final effect is an improvement of peripheral insulin sensitivity. At molecular level, some of the beneficial effects of this drug have been related to the phosphorylation and nuclear export of LKB1. This latter kinase activates adenosine monophosphate-activated protein kinase (AMPK), a regulator of energy metabolism, able to stimulate ATP-producing catabolic pathways (glycolysis, fatty acid oxidation, and mitochondrial biogenesis) and to inhibit ATP-consuming anabolic processes (gluconeogenesis, glycogen, fatty acid, and protein synthesis) [47]. Upon activation in response to energy stress, in the muscle, AMPK induces hexokinase II expression and GLUT4 gene upregulation and translocation to cell membrane, leading to an increase in glucose uptake. Furthermore, it phosphorylates and inhibits glycogen synthase, thereby, inducing a decrease in glycogen synthesis. In the liver, AMPK reduces hepatic gluconeogenesis by inducing the phosphorylation of CREB-binding protein (CBP) and, as consequence, the dissociation of the gluconeogenic CREB-CBP-TORC2 transcriptional complex [45]. This event, mediated by atypical protein kinase C (PKC ι/λ), triggers the disassembly of the transcription machinery and the inhibition of the expression of gluconeogenesis enzyme genes including phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase). The activation of AMPK by metformin exerts beneficial effects also on lipid metabolism. Indeed, upon metformin-induced phosphorylation, AMPK inactivates acetyl-CoA carboxylase (ACC) and 3-hydroxy-3-methylglutaryl (HMG)-CoA

reductase, decreases fatty acid synthase (FAS) expression, and activates malonyl-CoA carboxylase. The final effect is a decrease in fatty acid and cholesterol synthesis. Moreover, AMPK inhibits the sterol regulatory element-binding protein-1c (SREBP-1c), which is a transcription factor for genes involved in fatty acid synthesis [48]. SREBP-1c is induced by an excess of glucose and insulin and is inappropriately increased in NAFLD patients. Recent findings show that the effect of metformin in counteracting adipose tissue expansion occurs not only through a direct inhibition of adipogenesis but also by modulating adipokine synthesis or secretion [49]. Indeed, adiponectin, induced by metformin, directly stimulates AMPK and prevents hepatic lipid accumulation by increasing β-oxidation of free fatty acids and/or by decreasing their *de novo* synthesis. Furthermore, in *ob/ob* mice, a model of hepatic steatosis, it has been shown that metformin reversed hepatomegaly, hepatic fat accumulation, and ALT abnormalities, by reducing hepatic tumor necrosis factor-α (TNF-α) expression [50].

Although AMPK is the main player in mediating metformin effects, it is important to note that some metabolic actions of metformin occur in AMPK-independent manner and may be mediated by MAPK- and PKA-dependent mechanisms. As it has been shown by Zhang et al. [51], metformin exerts an inhibitory effect on catecholamine-stimulated lipolysis through decreasing cAMP production and reducing PKA and MAPK activities.

A schematic representation of the molecular mechanisms of metformin actions in peripheral tissues is shown in Figure 1.

6.2. Clinical Studies with Metformin. Several clinical trials have supported the beneficial role of metformin in patients with NAFLD (Table 1). Most of these studies have evaluated the effect of various doses of metformin on liver biochemistry (aminotransferase profile), histology, and metabolic syndrome features [35, 47, 52–59]. In 2001, Marchesini et al. [52] conducted the first pilot nonrandomized study using metformin (1.5 g day for 4 months) in 20 NASH nondiabetic patients. They observed a significant improvement in insulin resistance, aminotransferase levels, and liver morphology and volume in the treated group compared to the diet group. However, the study showed as limitation the fact that no follow-up biopsies were performed, and, thus, the histological improvement was not evaluated. Another small metformin versus diet trial, conducted in 17 randomized nondiabetic patients receiving metformin (850 mg twice a day), showed no differences in liver biopsies between treated and untreated groups. By contrast, ALT, AST, body mass index, and insulin resistance markers improved significantly in the metformin-treated group in comparison to controls [54].

Six open-label trials [47, 53, 55, 57, 58, 60] have evaluated the liver histology modification together with serum aminotransferase levels and insulin resistance markers' amelioration in NAFLD patients treated with metformin (dose ranging from 1.4 g/day to 2.0 g/day and treatment duration varying from 24 to 48 weeks) alone or in association with other drugs. All these studies reported an improvement in the indices of insulin resistance: five studies reported a reduction in liver function test values and one reported a non-significant increase of these values [53]. In terms of histological improvement, only three trials [47, 55, 57] showed significant differences in inflammation, steatosis, and fibrosis after treatment with metformin. In contrast to these promising results, some recent open-label studies have found no benefit of metformin treatment (dose ranging from 1.5 g to 1.7 g/day and for a period of 6–12 months) on liver steatosis, aminotransferase levels, and insulin resistance markers compared to lifestyle changes or control untreated group [59, 61, 62]. However, these latter studies were conducted in small series of patients. Furthermore, these controversial results could be due to the different duration and dose of the treatment and to the variable time periods between the first and the second biopsy.

Recently, we have conducted a prospective randomized study in which we evaluated the efficacy of the addiction of low dose of metformin (500 mg twice a day) to dietary treatment (1300 kcal) in 50 obese and nondiabetic patients. We found that metformin plus dietary therapy was associated with an improvement or even disappearance of hepatic steatosis similar to what observed with diet treatment alone. Metformin treatment was also associated with a significantly greater amelioration of several metabolic parameters (increased insulin sensitivity and reduced fasting glucose) than diet alone. Fasting glucose, basal serum insulin, and HOMA-IR index values decreased in both groups. However, differences both in fasting glucose (from 92.4 ± 9.9 to 89.1 ± 9.3 mg/dL, $P = 0.04$) and HOMA-IR index (from 3.3 ± 1.6 to 2.4 ± 1.2, $P = 0.003$) reached statistical significance only in the metformin group.

At baseline, impaired fasting glucose (IFG) was found in approximately 35% NAFLD patients in both groups. At the end of the study, IFG disappeared in 86% of metformin treated patients and in 62% of patients receiving only diet treatment. Metformin treatment was also significantly more effective than diet alone in reducing the proportion of patients who met the diagnostic criteria of metabolic syndrome (20% reduction in the metformin group, $P = 0.0008$ versus 4% reduction in the diet group, $P = 0.9$). Given the high proportion of NAFLD patients with metabolic syndrome and the association between metabolic syndrome and type 2 diabetes mellitus (T2DM) and cardiovascular diseases [1, 41], our results suggest that a low dose of metformin might be proposed to NAFLD patients, especially if they meet the diagnostic criteria of metabolic syndrome [35].

The potential role of metformin has also been examined in pediatric patients with NAFLD. Results in pediatric population were similar to those of adults and supported the beneficial effects of metformin on biochemistry liver profile and metabolic parameters, but not on histological features.

The first study was conducted by Schwimmer et al. [63], who tested metformin (1 g/day) in ten insulin-resistant children with biopsy-proven steatohepatitis, for a period of 24 weeks. More recently, Naideau et al. [64] randomized fifty obese and insulin-resistant adolescents to receive lifestyle recommendations plus metformin (850 mg twice a day for 6 months) or placebo. In both studies the treatment

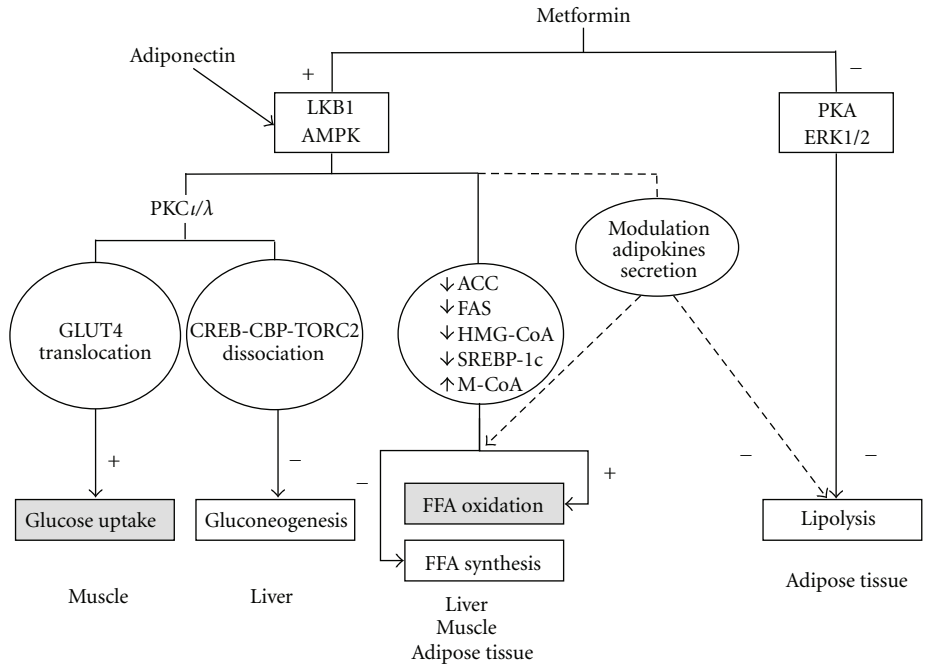

Figure 1: Metformin action in peripheral tissues. Metabolic effects of metformin are mainly mediated through the activation of adenosine monophosphate-activated protein kinase (AMPK), a master regulator of glucose and lipid metabolism. In skeletal muscle, metformin increases glucose uptake by enhancing the atypical protein kinase C (PKC) ι/λ-dependent glucose transporter (GLUT4) translocation to the cell membrane, while, in liver, metformin-dependent activation of PKC ι/λ reduces gluconeogenic enzyme gene expression through the dissociation of the CREB-CBP-TORC2 complex via CREB binding protein phosphorylation. In liver, muscle, and adipose tissues, AMPK decreases cholesterol and fatty acid synthesis and increases fatty acid oxidation by inhibiting the enzymes acetyl-CoA carboxylase (ACC), 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase and fatty acid synthase (FAS) and activating the malonyl-CoA carboxylase (M-CoA). Moreover, it downregulates the sterol regulatory element-binding protein-1c (SREBP-1c), which is a transcription factor for lipogenetic genes. In adipose tissue, metformin inhibits lipolysis through attenuation of PKA and ERK1/2 signaling. It may also impact on the endocrine function of adipose tissue, through modulation of adipokines synthesis or secretion, probably in an AMPK-dependent manner. Adiponectin also activates AMPK, thereby, enhancing metformin action.

with metformin resulted in serum aminotransferases, liver fat, and insulin sensitivity improvements as compared with untreated or placebo-treated group.

In another small observational trial conducted for 24 months in ten obese-overweight children with NAFLD [65], metformin (1.5 g/day) did not appear more effective than lifestyle intervention in ameliorating levels of aminotransferases and liver histology, but it significantly improved metabolic parameters and HOMA index. Similar results were obtained in a larger randomized multicenter placebo-controlled trial called the TONIC (Treatment of Nonalcoholic Liver Disease in Children) in which 57 children with NAFLD were treated with metformin (1 g/day) for 96 weeks. The study demonstrated that metformin is not superior to placebo in attaining a sustained reduction in ALT levels and significant improvements in histological features [66].

Although these clinical trials have sometimes shown controversial results, probably because they have generally been small, short term, and with often inconsistent outcomes, it is undoubted that metformin, by improving metabolic features of NAFLD, does show promise in the management of this liver disease. However, further larger randomized controlled trials of sufficient duration and using histological endpoints are needed to assess the effectiveness of this drug in modifying the natural history of NAFLD.

7. A Rational for the Use of Metformin in NAFLD

7.1. Effects on Metabolic Abnormalities and Cardiovascular Risk. As previously mentioned, NAFLD is now considered a hepatic manifestation of the metabolic syndrome. Patients with NAFLD frequently have many clinically significant comorbidities, such as obesity, impaired glucose tolerance, type 2 diabetes, hypertension, and hyperlipidemia (high fasting serum triglyceride and LDL levels and low HDL values). The complex of these pathological conditions leads to an increased cardiovascular risk [23] and may contribute to the progression of hepatic damage. The therapeutical approach to NAFLD, therefore, aims at ameliorating these metabolic derangements, all linked to insulin resistance.

Treatment with an insulin-sensitizing agent, such as metformin, may correct several of these components of the metabolic syndrome. Moreover, in diabetic patients, metformin provides cardiovascular protection that cannot be attributed

Table 1: Summary of metformin trials in NAFLD/NASH patients.

References	Study design	Patients	Therapy	Outcomes
Marchesini et al. [52]	OL, SA	20 patients, (OB); NASH, elevated AMTs	Metformin 1.5 g/d; 4 months	↓ ALT ↓ IR ↓ liver volume
Nair et al. [53]	OL, SA	28 patients, (OW/OB/T2DM); NAFLD	Metformin 20 mg/kg/d; 12 months	↓ ALT and AST ↓ IR Histology improved
Uygun et al. [54]	OL, RAND	36 patients, (OW/OB); NASH, elevated AMTs	Metformin 1.7 g/d + diet versus diet; 6 months	↓ IR ↓ ALT and AST Histology not improved
Bugianesi et al. [55]	OL, RAND (MC)	110 patients, (OW/OB/T2DM); NAFLD, elevated AMTs	Metformin 2 g/d + diet versus vit E + diet versus die; 12 months	↓ AST and ALT Histology improved
Schwimmer et al. [63]	SA	10 patients (OB/NT2DM children); NASH, elevated AMTs	Metformin 1 g/d; 6 months	↓ AST and ALT ↓ Liver fat ↓ IR
Duseja et al. [56]	OL, NRAND	50 patients, (OW/OB); NAFLD, elevated AMTs	Metformin 1.5 g/d; 6 months versus diet	↓ ALT and AST ↓ IR
Loomba et al. [47]	OL, SA	28 patients, (OW/OB/T2DM); NASH, elevated AMTs	Metformin 2 g/d; 12 months	Histology improved ↓ ALT and AST ↓ IR
de Oliveira et al. [57]	OL, SA	20 patients, (OW/OB/T2DM); NASH, elevated AMTs	Metformin 1 g/d; 12 months	↓ ALT Histology improved ↓ IR
Idilman et al. [58]	OL, RAND	74 patients, (OW/OB/T2DM); NASH, elevated AMTs	Metformin 1.7 g/d; 12 months	↓ ALT Histology not improved ↓ IR
Nobili et al. [65]	OL	57 patients (OW/OB children); NASH/NAFLD	Metformin 1.5 g/d versus diet; 24 months	↓ ALT and AST ↓ IR Histology improved
Haukeland et al. [59]	PLAC, RAND	48 patients (OW/OB/T2DM); NAFLD, elevated AMTs	Metformin versus placebo; 6 months	↓ ALT and AST ↓ IR Histology not improved
Nadeau et al. [64]	RAND	50 patients (OB children); NAFLD/elevated AMTs	Metformin 1.7 g/d + diet versus diet; 6 months	↓ ALT and AST ↓ IR Ultrasound pattern improved
Garinis et al. [35]	OL, RAND	50 patients, (OW/OB); NAFLD, normal AMTs	Metformin 1 g/d + diet versus diet; 6 months	↓ ALT and AST, ↓ IR Ultrasound pattern improved ↑ Adiponectin

Abbreviations: ALT, alanine transaminase; AMTs, aminotransferases; AST, aspartate aminotransferase; IR, insulin resistance; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NRAND, non randomized; NT2DM, non type 2 diabetes mellitus; OB, obese; OL, open label; OW, overweight; PLAC, placebo controlled; RAND, randomized; SA, single arm; T2DM, type 2 diabetes mellitus.

only to its antihyperglycemic effects. These additional cardioprotective effects may be related to the favorable actions of metformin on lipid metabolism, vascular smooth-muscle and cardiomyocyte intracellular calcium handling, endothelial function, hypercoagulation, and platelet hyperactivity [67].

Metformin therapy may result in a significant antihypertensive effects [68], which include both insulin-dependent and insulin-independent vasodilatory actions and probably also central antihypertensive effects [69]. Several authors have shown that metformin improves lipoprotein profiles with a decrease in low-density lipoprotein (LDL) cholesterol levels, triglycerides, and high-density lipoprotein (HDL) cholesterol levels [3]. Metformin has been reported to reduce markers of inflammation and to lessen hypercoagulation and increase fibrinolysis by decreasing levels of plasminogen activator inhibitor-1 and increasing tissue plasminogen activator activity. Metformin also improves functional and

biochemical markers of endothelial reactivity as well as surrogate indexes of coronary atherosclerosis [67]. Another relevant mechanism is the reduction of circulating advanced glycated end products (AGEs), which are oxidative mediators of endothelial dysfunction. Moreover, metformin is able to stimulate intracellular AMPK and to activate the endothelial isoform of NOSs in human aortic endothelial cells [70].

7.2. Effects on Weight Loss. The reported prevalence of obesity in patients with NAFLD varies from 30 to 100% [8] and increases with increasing BMI. An analysis of liver histology suggests that the prevalence rates of steatosis and steatohepatitis are approximately 15% and 3%, respectively, in nonobese persons, while they increase to 65% and 20%, respectively, in patients with class I-II obesity (BMI: 30.0–39.9 kg/m^2) and to 85% and 40%, respectively, in extremely obese patients (BMI: $\geq 40\,kg/m^2$) [71].

These data support the rationale of using metformin, which help reducing body weight in obese patients with and without diabetes [72–75] and induces a significant reduction in total body fat and visceral fat [74]. Weight loss during metformin treatment has been attributed to decreased net caloric intake [76], probably through appetite suppression, an effect largely independent of gastrointestinal side effects of metformin [73]. Reduction in hyperinsulinemia related to reduced insulin resistance may have an additive effect on weight reduction in obese, insulin-resistant patients [77–79].

It has been reported that even a modest weight loss can produce improvements in markers for NAFLD, namely, ALT and imaging markers of liver fat [80, 81].

In the Diabetes Prevention Program, metformin use was associated with a small improvement in ALT levels over time. Weight loss appeared to be the dominant mediator of this effect, and the 4-year cumulative incidence for development of abnormal ALT values was lowest in patients who lost the most weight [82].

7.3. Effects on Glucose Disorders. The prevalence of T2DM in NAFLD varies from 10 and 75% [8], and this condition is the only independent variable associated with advanced-stage NAFLD [13]. Indeed, the great frequency of impaired fasting glucose and impaired glucose tolerance in NAFLD requires a therapeutic approach which delays and reduces the onset of diabetes. Metformin therapy contributes to protect pancreatic β-cell reserve and delay diabetes by lowering blood glucose and reducing peripheral insulin resistance.

In a large randomized placebo-controlled trial, the Diabetes Prevention Program (DPP) recently showed that improvement in insulin sensitivity, through either intensive lifestyle modification or metformin, reduces the risk of developing T2DM in high-risk individuals [83]. Data obtained in the same population demonstrated that metformin does not mask the development of diabetes but provides a curative effect on glucose derangement [78].

Recently, we compared the efficacy of a treatment with low-dose metformin and dietary measures alone in obese, nondiabetic patients with NAFLD in a 6-month, prospective, randomized study. After therapy, the proportion of patients with impaired fasting glucose declined from 35 to 5% ($P = 0.04$) in the metformin group, a proportion significantly higher in respect to the control group [35].

7.4. Effects on Polycystic Ovary Syndrome. PCOS represent a very common condition affecting 6-7% of reproductive aged women [84]. Insulin resistance has a pivotal role in ovulatory disfunction and androgen excess and represents a strong pathogenetic link with metabolic abnormalities associated with metabolic syndrome and NAFLD. Moreover, the prevalence of NAFLD in PCOS women ranges from 30 to 60% [85].

The pleiotropic action of metformin makes it a first-line medical therapy in PCOS women. Metformin decreases ovary production of total and free testosterone levels and improves follicular growth with both an indirect action, through the reduction of hyperinsulinemia, and a direct action on ovarian tissue, through the increase in AMPK and a reduction in CYP17 activity [84]. Clinically, metformin therapy improves hirsutism and normalizes menstrual cycles and induces ovulation in PCOS patients [67]. In PCOS, this approach with metformin introduced a pharmaceutical option targeting various aspects of this syndrome, which were previously neglected but that may contribute to adverse cardiometabolic outcomes.

7.5. Effects on Cancer Risk. T2DM and obesity are associated with an increased risk of a variety of cancers [86–91], while weight control is associated with a decreased cancer risk [92]. Recent data have elucidated some molecular mechanisms by which insulin resistance is involved in cancer [93]. Moreover, metabolic syndrome is associated with a worsen cancer outcome [94].

Hepatocellular carcinoma (HCC) is a complication of NAFLD-associated cirrhosis, and the majority of "cryptogenic" HCC in the United States is attributed to NAFLD [94]. Both T2DM and metabolic syndrome are also associated with HCC. However, it is unclear whether NAFLD predisposes patients to HCC in the absence of cirrhosis. Studies supported evidence that HCC may develop in NAFLD unaccompanied by cirrhosis [95].

These observations have several implications in NAFLD prevention and treatment: first because early treatment of NAFLD plays a role in primary prevention of HCC and second because metformin, itself may have an antitumor effect both in vitro and in vivo [91, 96]. In fact, T2DM patients, who are prescribed metformin, have a lower risk of cancer compared to patients that are not treated with metformin [97, 98].

The upstream regulator of AMPK is a protein kinase known as LKB1, a well-recognized tumor suppressor. Activation of AMPK by metformin and exercise requires LKB1, and this would also explain why exercise is beneficial in the primary and secondary prevention of certain cancers [97].

Metformin is known to activate AMPK and to inhibit cyclin D1 expression and proliferation of cultured cancer

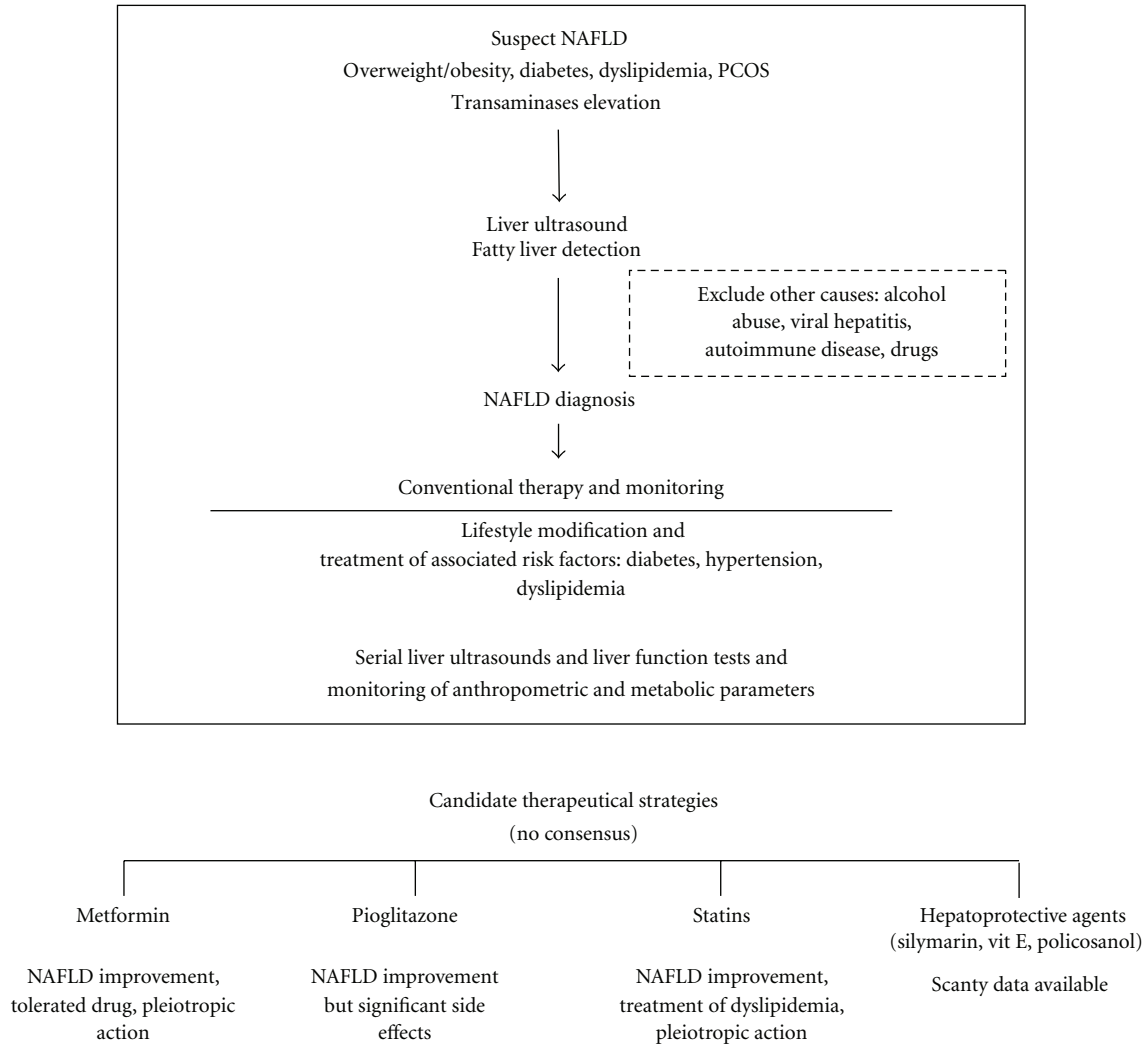

Figure 2: Schematic representation of NAFDL diagnosis and management. No consensus is available for the use of insulin sensitizers (metformin and pioglitazone), although studies have generally shown beneficial effects. The US Food and Drug Administration (FDA) has recently released a note to inform that the use of pioglitazone for more than one year may be associated with an increased risk of bladder cancer. In France the use of pioglitazone has been suspended while in Germany it is recommended not to start pioglitazone in new patients. Scanty data are available with regard to the efficacy of statins and hepatoprotective agents. Liver ultrasonography, the most practicable method for NAFLD detection is actually used to monitor response to treatment although not yet validated. Liver function tests may be useful despite a poor sensibility for NAFLD diagnosis and disease monitoring. Metabolic parameters still represent viable indexes of response to therapy.

cells. However, the mechanisms of action by which metformin mediates cell cycle arrest are not completely understood [99].

In a retrospective case-control study performed in 465 HCC patients, it has been found that T2DM is an independent risk factor for HCC and pre-exists in the majority of HCC patients. Moreover, in patients with T2DM, there was a direct association of HCC with insulin and sulphonylureas treatment and an inverse relationship with metformin therapy [100]. In addition, the predictive value of hyperinsulinemia in total cancer mortality [101] and fatal liver tumor incidence [102] has been demonstrated in nondiabetic subjects by two recent prospective studies.

8. Monitoring the Efficacy of Metformin Therapy in NAFLD

As we previously mentioned, most studies with metformin show an improvement in liver aminotransferases and liver histology. However, it is still unclear what is the best way to monitor the response to therapy in NAFLD patients. In fact, despite an increase in transaminases is common in NAFLD, liver enzymes may be normal in up to 78% of patients and, thus, are insensitive not only for NAFLD diagnosis but also for disease monitoring. On the other hand, although liver biopsy is still considered the gold standard for distinguishing between the broad range of chronic liver diseases [19],

it has several limitation, including cost, the potential risk of bleeding, and the absence of consensus regarding the histopathological criteria that firmly define NASH and differentiate between NAFLD entities [8, 103]. Accordingly, a repeated liver biopsy after a short term therapeutic trial is considered too invasive and impracticable to be applied in clinical practice, especially when considering the high prevalence of the disease [5].

Liver ultrasonography, the most practicable method for NAFLD detection, is not yet validated to monitor response to treatment. Thus, new sensitive and noninvasive markers of response are needed. As already mentioned, several markers have been proposed, such as fibrosis score and cytokines, but remain yet to be validated. Therefore, metabolic parameters still represent viable indexes of response to therapy.

9. Emerging Concepts and Candidate Future Therapies in NAFLD

Since the etiology and pathogenesis of NAFLD are not entirely clarified, new therapies await future developments in our understanding of key pathogenetic mechanisms of NAFLD. Ongoing research is exploring novel approaches that look promising in preclinical models.

MicroRNAs (miRNAs) have been recently studied in a rat model of NAFLD [104] and in cultured human hepatocytes [105] and seem to have an important role in hepatic energy metabolism and in the pathophysiological process of NAFLD. Several miRNAs are dysregulated in NAFLD, and this contributes to dysregulation of genes involved in hepatocyte proliferation, apoptosis, inflammation, and glucose and lipid metabolism [106]. The development of an effective and safe approach for correcting miRNA dysregulation is a new challenge for NAFLD therapy.

Novel chemically engineered oligonucleotides, termed "antagomirs," have been shown to be efficient and specific silencers of endogenous miRNAs [107]. Particularly, both in monkeys [108] and in mice [107], antagomir against miRNA-122 resulted in a reduction of hepatic steatosis. A phase I study in humans is ongoing [106].

The regulation of AMPK activity is another emerging molecular target for the treatment of NAFLD. Pharmacological AMPK activators are being developed for the treatment of multiple metabolic disorders including NAFLD. Beside glucose homeostasis, AMPK also regulates hepatic lipid metabolism [106]. AMPK activation by AICAR or alpha-lipoic acid has been shown to decrease liver fat content in lean and obese rodents [109, 110]. These preclinical data need confirmation in human trials.

Decreased levels of glucagon-like peptide 1 (GLP-1) are common in obese patients [111], and reduced incretin action has been demonstrated in nondiabetic, nonobese patients with NASH, thus, prompting evaluation of GLP-1 analogs in fatty liver [106]. In obese mice, exendin, a GLP-1 agonist, may ameliorate insulin resistance and decrease histologic steatosis [112]. Only one case report illustrates a similar effect in humans [113]. Appropriate trials are, therefore, needed to asses the potential use of GLP-1 agonists in NAFLD [111].

In conclusion results of preclinical studies on emerging therapy of NAFLD are encouraging, but further work is needed to evaluate the efficacy and safety of these new agents in humans.

10. Conclusive Remarks

No drug is currently available as specific treatment for NAFLD, and no drug can substitute for lifestyle modification. However, available evidences clearly show a pivotal role of metformin in improving metabolic alterations associated with NAFLD. Therefore, metformin, because of its metabolic effects and its safety profile, remains a promising drug in NAFLD therapy, especially in patients that meet the diagnostic criteria of metabolic syndrome [35]. A schematic representation of NAFLD diagnosis and management is shown in Figure 2.

Conflict of Interests

The authors declare no conflict of interests.

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