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# Diet and Lifestyle in Nonalcoholic Fatty Liver Disease

Lead Guest Editor: Roberto Martínez-Beamonte
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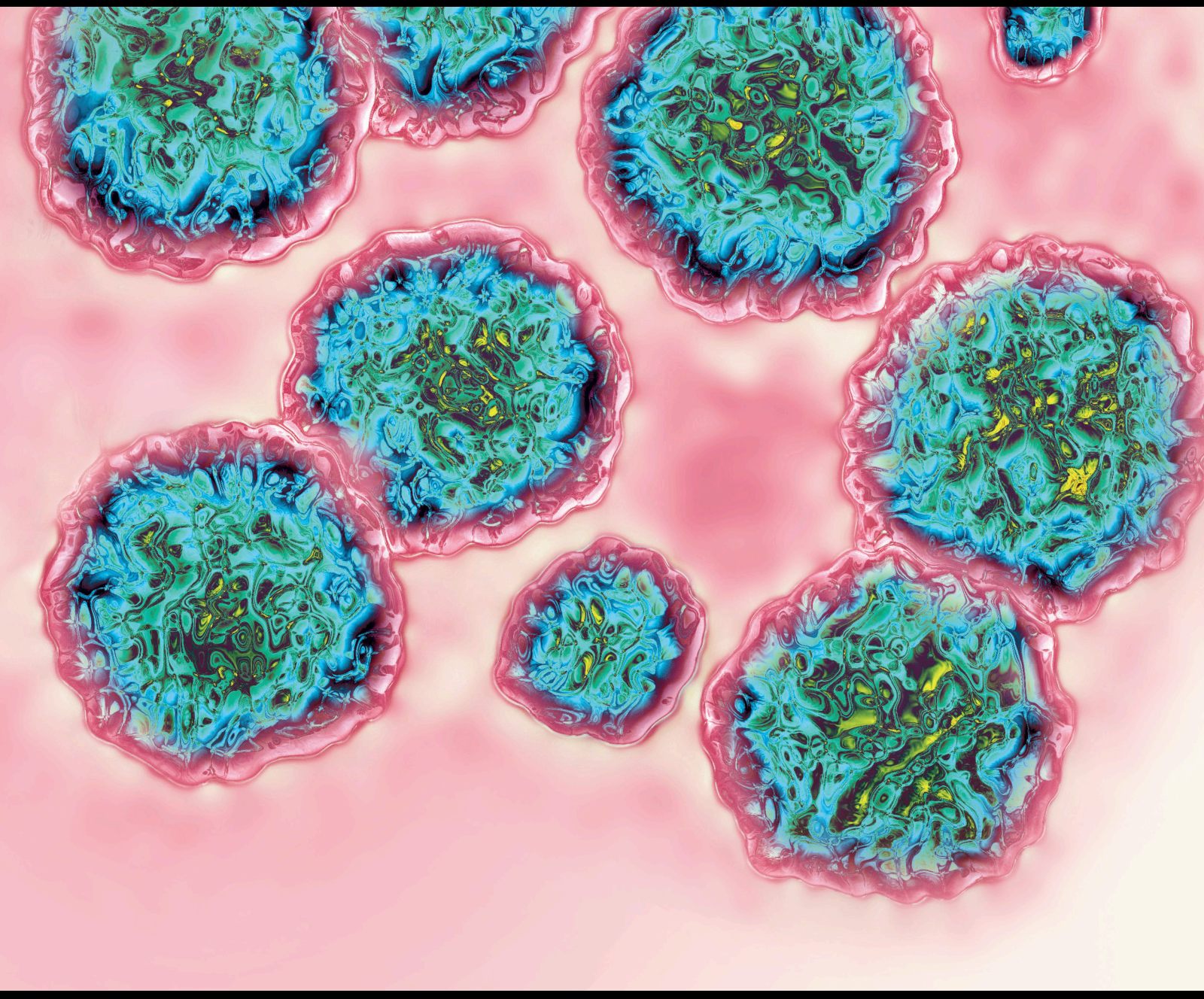

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# Editorial Board

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*Editorial*

# Diet and Lifestyle in Nonalcoholic Fatty Liver Disease

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The liver is an essential metabolic organ which governs body energy metabolism connected with adipose tissue and skeletal muscle among other tissues. The prevalence of obesity has reached epidemic proportions in many countries around the world and continues to grow every year which is caused by multiple factors, with diet and lifestyle being the most researched and therefore most important.

Nonalcoholic fatty liver disease (NAFLD) is one of the several metabolic complications associated with obesity. The pathology of NAFLD is difficult to recognize or diagnose especially in early stages without a biopsy and therefore can remain undetected for significant time allowing the disease to progress.

The diagnosis of NAFLD is crucial to be able to start adequate treatment including changes in diet and lifestyle in the first stage of the disease when the pathology is reversible and prevent the development of severe forms of the disease such as nonalcoholic steatohepatitis (NASH) or the irreversible cirrhosis stage.

When the liver becomes damaged, this can lead to some metabolic alterations that have a severe and multifaceted impact in type 2 diabetes mellitus (T2DM), visceral obesity, and cardiovascular disease related to elevated plasmatic cholesterol, triglycerides, transaminases, and others that indicate hepatic disorders and oxidative stress.

This special issue aims to bring articles to assemble the latest progress to combat NAFLD, NASH, and cirrhosis mainly related to overweight and obesity which are increasingly prevalent in today's society. The manuscripts received by the journal were carefully selected pursuing this objective.

I. Grgurevic et al.'s review paper "Natural History of Nonalcoholic Fatty Liver Disease: Implications for Clinical Practice and an Individualized Approach" summarizes the current knowledge on the natural history of NAFLD and suggests there is not still strong evidence of significant association between the inflammatory component of NAFLD and the adverse clinical outcomes. The authors propose older age (>50 years) and presence of T2DM and some genetic variants as indicators of increased risk of having liver fibrosis.

The review made by Z. Miao et al. showed new insights and basis for medical therapy on adipose metabolic diseases, related to diet, and more concrete with the consumption of vitamin D. This review summarized the relationship between vitamin D and the adipose tissue that represent the major storage of vitamin D and classified its biological roles and functionalities with the regulation and development on adipogenesis and other metabolic diseases, calcium homeostasis, and energy metabolism.

H. Ou et al. have written a cross-sectional study with 225 patients with NAFLD, smokers, and nonsmokers, to evaluate a lifestyle parameter over NAFLD. Liver-significant fibrosis was diagnosed by liver stiffness using a FibroScan. The results indicate that the risk of fibrosis in smokers with NAFLD is significantly higher, showing higher changes in significant liver fibrosis and advanced liver fibrosis in these patients, with influence of age and AST levels too.

T. Himoto et al. made a cross-sectional study with diet intervention during 6 months in patients with NAFLD to determine the nutritional and dietary factors associated with muscle volume loss in these patients with presarcopenia, sarcopenia, and sarcopenic obesity. The authors indicate that there are different factors that regulate muscle volume loss in male and female NAFLD patients.

Another interesting paper written by B. Martínez et al. used a special diet during 2 months in a porcine animal model to induce fatty liver. After the dietary fatty liver induction, the authors used 10 mg/kg/day of melatonin to reverse the fatty liver, feeding the animals with the same steatosic special diet. The results showed that the melatonin group does not increase steatosis, with a decrease of MDA levels.

A. M. Akinnuga et al. presented the first work using bredemolic acid in a diet-induced prediabetic rat model during 12 weeks. The prediabetes model showed an increase in AST and ALT, liver glycogen and triglycerides, and lipid peroxidation and a decrease in SREBP1 expression. The bredemolic acid administration decreased liver enzyme concentration and hepatic oxidative stress and improved antioxidant enzymes such as SOD and GPx, thus improving liver function.

## Conflicts of Interest

The editors declare no conflicts of interest.

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*Research Article*

# Verification of the Nutritional and Dietary Factors Associated with Skeletal Muscle Index in Japanese Patients with Nonalcoholic Fatty Liver Disease

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We sought to identify the frequencies of presarcopenia, sarcopenia, and sarcopenic obesity in patients with nonalcoholic fatty liver disease (NAFLD) and to cross-sectionally determine the nutritional and dietary factors associated with loss of skeletal muscle mass in such patients. Dietary and body component changes produced by a diet intervention were longitudinally investigated. Forty-six NAFLD patients (24 males and 22 females) were enrolled. A second diet treatment was performed at 6 months after entry in 19 of the enrolled patients (6 males and 13 females). Body compositions and dietary nutrients at six months later were compared with those at entry. Three of the 24 (13%) males and four of the 22 (18%) females fulfilled the criteria for presarcopenia and one (5%) female NAFLD patient was in the criteria for sarcopenia at baseline. None of the patients were in the criteria for sarcopenic obesity. The factors associated with skeletal muscle index in the males were body mass index (BMI), insulin-like growth factor-1, total energy intake, and lipid intake, but only BMI and bone mineral density in females at baseline. The diet intervention decreased the skeletal muscle mass in the 6 males by decreasing the total energy intake via lower protein and lipid intakes and improved their liver dysfunction. In the 13 females, a decrease in total energy intake via lower carbohydrate and lipid intake did not change the skeletal muscle mass. These results suggest that loss of skeletal muscle mass is frequently observed in nonobese NAFLD patients and that the frequency of sarcopenic obesity seems to be rare in NAFLD patients. The nutritional and dietary factors that regulate loss of skeletal muscle mass were distinct between our male and female NAFLD patients. Thus, the skeletal muscle mass of such patients as well as their body weight and liver function should be monitored during diet interventions.

## 1. Introduction

Sarcopenia, a concept proposed by Rosenberg [1] in 1989, characterized by loss of skeletal muscle mass and low muscle strength, is widely recognized as a primary factor responsible for frailty [2] and is regulated by muscle mass, muscle strength, and physical performance [3]. The clinical outcomes of sarcopenia in Asian countries appears to be more serious than those in Western countries, because in Asian countries, the aging of the population is in rapid progress and the proportion of older individuals continues to increase. The physical status, ethnicity, and lifestyle in Asian countries differ in many ways from those in Western countries. Therefore, original diagnostic criteria for sarcopenia that are appropriate for Asian people were required [4]. However, outcome-based data were not available in Asian Working Group for Sarcopenia (AWGS). Therefore, the working group aimed to standardize the cutoff values for evaluating sarcopenia in Asian people.

Sarcopenia is largely classified into two categories: primary sarcopenia and secondary sarcopenia [3]. Primary sarcopenia is called "age-related sarcopenia." Secondary

sarcopenia includes activity-related sarcopenia, disease-related sarcopenia (e.g., type 2 diabetes mellitus (T2DM), liver cirrhosis, osteoporosis, and varieties of malignant diseases), and nutrition-related sarcopenia such as malnutrition [3]. Sarcopenia is associated with not only numerous hormonal factors, including insulin-like growth factor-1 (IGF-1) [5], insulin [6], testosterone [7], and oxytocin [8] but also nutritional factors such as trace elements [9, 10], vitamin D, protein, leucine [11], polyunsaturated fatty acid (PUFA) [12], and body mass index (BMI) [13]. Inflammatory cytokines and myokines, including interleukin-6 (IL-6) and myostatin, are also thought to affect the skeletal muscle mass [14].

In the field of liver disease, many investigators primarily have focused on patients with liver cirrhosis as a target of the researches on sarcopenia [15]. The quality of life among cirrhotic patients associated with sarcopenia is severely impaired, and the prognosis of these patients is quite unfavorable. Several studies following the report by Hong and colleagues [16] in 2014 have indicated that sarcopenia was present even in patients with nonalcoholic fatty liver disease (NAFLD), implying that NAFLD might be one of the risk factors for sarcopenia [17–21].

NAFLD, characterized by a spectrum ranging from simple steatosis to steatohepatitis without a habit of excessive alcohol consumption, is one of the most prevalent chronic liver diseases worldwide [22]. NAFLD is frequently associated with metabolic abnormalities, including obesity, impaired glucose tolerance, and dyslipidemia [23]. Accordingly, diet and/or exercise treatment should be recommended as a first choice of therapies for NAFLD patients [24]. However, the effects of diet treatment for NAFLD patients on the patients' skeletal muscle mass have not been fully established.

"Sarcopenic obesity," a comorbid disorder of sarcopenia and obesity [25], is estimated to be present in approximately 3%–20% of older populations [26]. The prognosis of patients with sarcopenic obesity appears to be much more unfavorable than that of patients with obesity or sarcopenia alone [27]. We have speculated that some patients with sarcopenic obesity are hidden among the NAFLD patients who are obese, but there are few published data regarding sarcopenic obesity in patients with NAFLD.

The primary purposes of this study were to identify the frequencies of sarcopenia and sarcopenic obesity in Japanese patients with NAFLD and to determine which factors are the most responsible for loss of skeletal muscle mass in these patients. We also sought to establish whether a diet treatment for patients with NAFLD would affect their skeletal muscle mass as well as the body fat mass and liver function.

## 2. Materials and Methods

*2.1. Study Design.* This study was designed by a cross-sectional and longitudinal trial. Nutritional and dietary factors were analyzed in each participant at baseline. Dietary intervention by a dietitian was also performed without any medications. In addition, the participants were urged to perform the physical activities, including aerobics and resistance exercise training, for 30 minutes three times a week. Changes in body compositions and nutrients were also analyzed 6 months later among the participants who could follow up.

*2.2. Study Population.* We enrolled 46 patients with NAFLD (24 males and 22 females) from the outpatients of Ritsurin Hospital (Takamatsu, Japan) treated between 2016 and 2018. None of the enrolled NAFLD patients had a habit of excessive daily alcohol consumption, and all patients showed radiological findings by abdominal ultrasound and/or computed tomography (CT) test that are compatible with fatty liver. NAFLD patients who took medication for hypertension, dyslipidemia, and/or T2DM were excluded in this study.

The study protocol complied with all of the provisions of the Declaration of Helsinki. The design of this study was approved by the Ethical Committee of Ritsurin Hospital, and informed consent was obtained from each patient before his or her entry in this study.

*2.3. Laboratory Assessments.* Varieties of clinical parameters in the 46 patients were measured to verify the relation to sarcopenia at baseline. Fasting blood samples were taken in the morning for the measurements of plasma glucose and serum immunoreactive insulin (IRI), alanine aminotransferase (ALT), total cholesterol (T-Cho), LDL-cholesterol (LDL-C), triglyceride (TG), and free testosterone levels. The plasma glucose and serum IRI, AST, ALT, T-Cho, and TG levels , HbA1c, and peripheral platelet counts were measured using standard laboratory techniques. The serum free testosterone levels were determined by an immune radio metric assay (IRMA). Insulin resistance was evaluated based on the homeostasis model for the assessment of insulin resistance (HOMA-IR) value using the following equation: HOMA-IR value = fasting insulin ($\mu$U/ml) × fasting glucose (mg/dl)/405. Insulin resistance was defined as a HOMA-IR value exceeding 2.5. The measurement of serum IGF-1 concentrations performed using a commercially available kit (TFB Inc, Tokyo, Japan). The assay of IGF-1 was canceled from the enrolled patients who were younger than 40 years old because aging affects the release of the hormone. The serum zinc levels and 25-OH vitamin $D_3$ levels were determined by the atomic absorption method and a double-antibody radioimmunoassay, respectively. The clinical diagnosis of zinc deficiency (less than 60 $\mu$g/dl) and that of 25-OH vitamin $D_3$ deficiency (less than 20 ng/ml) were based on the previous reports [28, 29], respectively. Plasma branched-chain amino acids (BCAA: valine, leucine, and isoleucine) and tyrosine levels were determined by the high-performance liquid chromatography (HPLC) method, and the BCAA to tyrosine ratio (BTR) was calculated. The patients' bone mineral density (BMD) was determined at the lumbar spine (L2-L4) using a DEXA system (GE Health Care, Madison, WI). Osteoporosis was defined as a BMD value less than 70% of the young adult mean (YAM).

Body mass index (BMI) was estimated as a hallmark of obesity. Obesity was defined as a BMI over 25.0 kg/m$^2$, because the reported proportion of the Japanese population with a BMI higher than 30 kg/m$^2$ is 2%-3% [30], whereas the

proportion of obesity in Western countries ranges from 10% to 20% [31]. Dyslipidemia was defined as a serum LDL-C level exceeding 140 mg/dl and/or TG level exceeding 150 mg/dl [32]. The diagnosis of T2DM was made on the basis of the exclusion of insulin depletion and a fasting plasma glucose level exceeding 126 mg/dl, an oral glucose tolerance test-2 hr plasma glucose level ≥200 mg/dl, a casual plasma glucose level ≥200 mg/dl, and/or a HbA1c level ≥6.5% [33]. Hypertension was designated as blood pressure exceeding 140/90 mmHg [34]. The severity of hepatic fibrosis was estimated by calculating the FIB-4 index as follows: age (years) × AST (IU/L)/platelet count ($\times 10^9$/L) $\times \sqrt{\text{ALT}}$ (IU/L) [35].

*2.4. Criteria for Sarcopenia.* For the analysis of body compositions, the skeletal muscle mass of each patient's entire body and that of the limbs, the amount of body fat, and the body fat percentage were measured by a direct segmental multifrequency bioelectric impedance analysis (BIA) using an InBody 470 system (InBody Japan, Osaka, Japan).

Sarcopenia was defined as both loss of skeletal muscle mass and a decline in hand grip strength on the basis of the Sarcopenia Assessment Criteria issued by the Japan Society of Hepatology [36]. We calculated each patient's skeletal muscle index (SMI) [37], an indicator for loss of skeletal muscle mass by using the following formula: SMI = the appendicular skeletal muscle mass (kg)/height $(m)^2$. We set 7.0 kg/$m^2$ or less for the males and 5.7 kg/$m^2$ or less for the females as the values that indicate loss of skeletal muscle mass.

Hand grip strength was measured in each patient in a standing position using a hand dynamometer (TKK5401, Takei, Niigata, Japan). We designated 26 kg or less for the males and 18 kg or less for the females as decreased muscle strength.

Sarcopenic obesity was defined in this study as both sarcopenia and obesity, because a precise definition of sarcopenic obesity has not been established. Presarcopenia was defined herein as loss of skeletal muscle mass alone. The definition of hidden obesity was based on a BMI less than 25 and a body fat percentage exceeding 25% in the males and 30% in the females, respectively.

*2.5. Dietary Assessments.* The patients' dietary intake was assessed with the use of three day diet diaries at baseline and 6 month later. The total energy, protein, leucine, carbohydrate, and lipid intakes were estimated in each patient by the Standard Tables of Food Composition in Japan-2015 [38].

*2.6. Statistical Analyses.* Data are represented as the mean ± standard deviation (SD). The Mann–Whitney *U* test was applied for two groups. Fisher's exact probability test was used to compare the differences in frequencies. The relationships among quantitative variables were analyzed by Spearman's correlation coefficient. The paired *t* test was used to compare the valuables at baseline and 6 months later. $p$ values less than 0.05 were considered significant.

## 3. Results

*3.1. Patients' Characteristics.* The clinical characteristics of the 46 NAFLD patients (24 males, 22 females) enrolled in this study at baseline are shown in Table 1. The age was lower in male patients than in female patients but not significant. Concurrent obesity (BMI ≥25) was present in 15 (63%) of the 24 male and 11 (50%) of the 22 female patients, respectively. There was no significant difference in BMI between the groups.

The values of HOMA-IR ranged from 0.82 to 5.2 among ten male and nine female patients. Four of these 10 (40%) males and four of the nine (44.4%) females met the category for insulin resistance, respectively. The values of HOMA-IR were almost equal between the males and the females.

Zinc deficiency was identified in three of the 23 males (13.0%) and four of the 22 females (18.2%), respectively. The frequency of vitamin $D_3$ deficiency was higher in female than in male patients (81.8% vs. 70.0%, $p > 0.05$).

The FIB-4 index ranged from 0.4 to 2.25 in the males and from 0.64 to 3.49 in the females, respectively. The FIB-4 index in two of the females exceeded 2.67 [39], which corresponded to liver cirrhosis. The BMD analysis revealed that another female patient had osteoporosis.

The frequencies of concurrent life style-related diseases, including T2DM, dyslipidemia, and hypertension, were approximately equivalent between the males and females.

*3.2. Frequencies of Presarcopenia, Sarcopenia, and Sarcopenic Obesity.* As shown in Table 2, three of the 24 (13%) male NAFLD patients fulfilled the criteria for presarcopenia, and none of the males met the criteria for sarcopenia or sarcopenic obesity at entry. Among the 22 female NAFLD patients, four (18%) met the criteria for presarcopenia and one (5%) met the criteria for sarcopenia, respectively. None of female patients fulfilled the criteria for sarcopenic obesity, either.

*3.3. Profiles of NAFLD Patients Associated with Loss of Skeletal Muscle Mass.* The details of the eight NAFLD patients with presarcopenia or sarcopenia at entry are listed in Table 3. Except for one male, these patients were not in the criteria for obesity. The body fat percentages in the 7 nonobese patients were higher than 25% in the males and 30% in the females, respectively, indicating that these nonobese patients with muscle volume loss fulfilled the category for "hidden obesity."

*3.4. Nutritional and Dietary Factors Associated with SMI.* Next, the nutritional and dietary factors associated with the SMI were analyzed at baseline in the male and female NAFLD patients, respectively. As shown in Table 4, the males showed a significantly close correlation between the SMI and the BMI and between the SMI and the IGF-1 level. However, there were no significant differences in the males between SMI and serum ALT, 25-OH vitamin $D_3$, zinc, or free testosterone levels. The values of HOMA-IR, FIB4-

Table 1: Clinical characteristics of the enrolled patients.

| Parameter | Male ($n = 24$) | Female ($n = 22$) | $p$ value |
|---|---|---|---|
| Age (years) | 50.4 ± 14.0 | 56.3 ± 9.4 | 0.1037 |
| BMI (kg/m$^2$) | 26.2 ± 3.0 | 26.0 ± 3.7 | 0.7585 |
| SMI (kg/m$^2$) | 7.80 ± 0.72 | 6.44 ± 0.82 | < 0.0001 |
| Hand grip strength (kg) | 37.7 ± 6.5 | 23.2 ± 3.8 | < 0.0001 |
| BMD (g/cm$^2$) | 1.26 ± 0.31 | 1.03 ± 0.17 | 0.1184 |
| ALT (IU/L) | 46.0 ± 31.0 | 31.9 ± 21.6 | 0.0765 |
| FIB-4 index | 1.10 ± 0.50 | 1.40 ± 0.77 | 0.2060 |
| LDL-C (mg/dl) | 123.9 ± 26.9 | 138.2 ± 37.9 | 0.1263 |
| TG (mg/dl) | 142.1 ± 84.8 | 148.2 ± 81.0 | 0.6521 |
| HbA1c (%) | 5.73 ± 0.42 | 5.88 ± 0.53 | 0.6139 |
| HOMA-IR | 2.04 ± 1.04 ($n = 10$) | 2.89 ± 1.70 ($n = 9$) | 0.2701 |
| BTR | 7.48 ± 1.34 ($n = 23$) | 6.66 ± 1.12 | 0.0495 |
| 25-OH-vitamin D3 (ng/ml) | 18.4 ± 6.1 ($n = 23$) | 16.1 ± 4.1 | 0.3622 |
| Zinc ($\mu$g/dl) | 76.7 ± 12.9 ($n = 23$) | 72.8 ± 11.3 | 0.3754 |
| Free testosterone (pg/ml) | 9.3 ± 3.2 | ND | ND |
| Concurrent T2DM | 5 (17%) | 6 (27%) | 0.4336 |
| Concurrent dyslipidemia | 10 (42%) | 13 (59%) | 0.1881 |
| Concurrent hypertension | 4 (17%) | 3 (14%) | 0.551 |

ND, not determined.

Table 2: Frequencies of presarcopenia, sarcopenia, and sarcopenic obesity in the male and female NAFLD patients.

| Criteria | Male ($n = 24$) | Female ($n = 22$) | $p$ value |
|---|---|---|---|
| Presarcopenia | 3 (13%) | 4 (18%) | 0.449 |
| Sarcopenia | 0 (0%) | 1 (5%) | 0.4783 |
| Sarcopenic obesity | 0 (0%) | 0 (0%) | 0.9999 |

index, BTR, and BMD in the males were not significantly associated with the SMI, either.

Fifteen of the 24 male NAFLD patients began a diet therapy at baseline. Among the nutritional factors, the estimated total energy and lipid intakes were significantly correlated with SMI in the male patients. Estimated protein intake, leucine intake, and carbohydrate intake were not associated with SMI in the male group.

Among the female patients, the BMI and BMD values were significantly correlated with the SMI at entry (Table 5). Eighteen of 22 female patients received a diet treatment at entry. None of the nutritional factors were revealed to be significantly associated with SMI in the female group.

*3.5. Changes in Body Compositions and Nutritional Components by the Diet Therapy.* Six males and 13 females received a second diet therapy at 6 months after their entry in this study. We determined the changes in these patients' body compositions, nutrients, serum ALT levels, and hand grip strength.

In the six male patients, the diet therapy tended to decrease the average total energy intake (baseline: 1.957 ± 401 kcal to 6 months: 1.814 ± 349 kcal, $p = 0.0787$, Figure 1). The decrease in energy intake by produced by the diet therapy was responsible for the reduction of protein intake (baseline: 75.2 ± 16.9 g to 6 months: 67.1 ± 11.5 g, $p = 0.1318$) and lipid intake (baseline: 64.0 ± 26.0 g to 6 months: 52.3 ± 17.7 g, $p = 0.0721$), whereas the average carbohydrate intake was approximately equivalent between that at entry and 6 months later (baseline: 249.4 ± 25.8 g to 6 months: 247.4 ± 26.2 g, $p = 0.4127$). The serum ALT level decreased significantly from the baseline to the 6 months measurement (baseline: 46.2 ± 19.9 IU/L to 6 months: 31.0 ± 7.5 IU/L, $p < 0.05$), and the SMI was decreased (baseline: 7.55 ± 0.70 kg/m$^2$ to 6 months: 7.37 ± 0.85 kg/m$^2$, $p = 0.1238$), although there was no significant difference in SMI between baseline and 6 months later. No significant changes were observed in body weight (baseline: 66.0 ± 6.1 kg to 6 months: 65.2 ± 6.6 kg, $p = 0.2388$), skeletal muscle mass (baseline: 44.9 ± 6.1 kg to 6 months: 44.7 ± 6.7 kg, $p = 0.3839$), body fat mass (baseline: 18.6 ± 2.6 kg to 6 months: 18.4 ± 2.0 kg, $p = 0.3949$), and hand grip strength (baseline: 35.4 ± 8.5 kg to 6 months: 34.8 ± 8.5 kg, $p = 0.2412$) (Figure 2). Four male patients whose energy intake was decreased had lower SMI or approximately equivalent SMI at 6 months, compared with the baseline (Figure 1).

Among the 13 females, who received the diet therapy, the average total energy decreased significantly from the baseline to 6 months later (baseline: 1.855 ± 331 kcal to 6 months: 1.673 ± 325 kcal, $p < 0.01$, Figure 3). The decrease in total energy intake was derived from lower carbohydrate intake (baseline: 254.4 ± 44.4 g to 6 months: 226.4 ± 38.8 g, $p < 0.001$) and lipid intake (baseline: 61.6 ± 15.6 g to 6 months: 56.0 ± 15.3 g, $p = 0.0956$). The mean protein intake was almost equal between that in the baseline and after 6 months (baseline: 63.6 ± 13.5 g to 6 months: 60.7 ± 15.7 g, $p = 0.2054$). The diet therapy significantly decreased these female patients' serum ALT level (baseline: 38.3 ± 25.0 IU/L to 6 months: 27.2 ± 12.8 IU/L, $p < 0.05$) and body fat mass (baseline: 24.8 ± 8.7 kg to 6 months: 23.3 ± 8.1 kg, $p < 0.05$), and it tended to decrease their body weight (baseline: 62.6 ± 12.7 kg to 6 months: 61.2 ± 11.4 kg, $p = 0.0607$), although no significant changes occurred in the SMI (baseline: 6.44 ± 0.87 kg/m$^2$ to 6 months: 6.41 ± 0.86 kg/m$^2$, $p = 0.3191$), skeletal muscle mass (baseline: 35.7 ± 4.7 kg to 6 months: 35.7 ± 4.1 kg, $p = 0.5278$), or hand grip strength (baseline: 22.4 ± 4.0 kg to 6 months: 22.1 ± 4.5 kg, $p = 0.2804$) (Figure 4).

Table 3: Profile of the NAFLD patients with loss of skeletal muscle mass.

| Gender | Age | Criteria | SMI | Hand grip strength | BMI | Body fat (%) |
|---|---|---|---|---|---|---|
| Female | 75 | Sarcopenia | 4.68 | rt: 15.2 kg<br>lt: 13.5 kg | 22.7 | 44.1 |
| Female | 52 | Presarcopenia | 5.45 | rt: 19.2 kg<br>lt: 19.1 kg | 20.3 | 31.7 |
| Female | 54 | Presarcopenia | 5.58 | rt: 26.1 kg<br>lt: 21.5 kg | 19.5 | 28.9 |
| Female | 62 | Presarcopenia | 5.27 | rt: 20.0 kg<br>lt: 22.1 kg | 21.1 | 35.5 |
| Female | 75 | Presarcopenia | 5.73 | rt: 20.5 kg<br>lt: 23.2 kg | 23.8 | 35.2 |
| Male | 67 | Presarcopenia | 6.33 | rt: 31.6 kg<br>lt: 28.6 kg | 23.6 | 32.6 |
| Male | 34 | Presarcopenia | 6.99 | rt: 37.9 kg<br>lt: 34.0 kg | 24.4 | 32.6 |
| Male | 63 | Presarcopenia | 6.6 | rt: 26.2 kg<br>lt: 26.5 kg | 26.1 | 37.4 |

rt, right; lt, left.

Table 4: Nutritional and dietary factors associated with SMI in male patients at baseline.

| Parameter | $r$ | $p$ value |
|---|---|---|
| ALT ($n = 24$) | 0.0596 | 0.7749 |
| IGF-1 ($n = 19$) | 0.5426 | 0.0212 |
| HOMA-IR ($n = 10$) | −0.0182 | 0.9565 |
| 25-OH-vitamin D3 ($n = 23$) | 0.1269 | 0.5518 |
| Zinc ($n = 23$) | −0.2825 | 0.1851 |
| BTR ($n = 23$) | 0.083 | 0.697 |
| FIB-4 index ($n = 24$) | −0.0509 | 0.8072 |
| BMD ($n = 7$) | 0.6785 | 0.0965 |
| Free testosterone ($n = 13$) | 0.2418 | 0.4023 |
| BMI ($n = 24$) | 0.657 | 0.0016 |
| Total energy intake ($n = 15$) | 0.6429 | 0.0162 |
| Protein intake ($n = 15$) | 0.1036 | 0.6984 |
| Leucine intake ($n = 15$) | 0.1464 | 0.5838 |
| Lipid intake ($n = 15$) | 0.5429 | 0.0422 |
| Carbohydrate intake ($n = 15$) | 0.0643 | 0.8099 |

Table 5: Nutritional and dietary factors associated with SMI in female patients at baseline.

| Parameter | $r$ | $p$ value |
|---|---|---|
| ALT ($n = 22$) | 0.1352 | 0.5355 |
| IGF-1 ($n = 18$) | 0.1839 | 0.4483 |
| HOMA-IR ($n = 9$) | 0.0333 | 0.9249 |
| 25-OH-vitamin D3 ($n = 22$) | 0.0877 | 0.6879 |
| Zinc ($n = 22$) | 0.0724 | 0.7401 |
| BTR ($n = 22$) | −0.1621 | 0.4577 |
| FIB-4 index ($n = 22$) | −0.3845 | 0.078 |
| BMD ($n = 10$) | 0.697 | 0.0366 |
| BMI ($n = 22$) | 0.8944 | <0.0001 |
| Total energy intake ($n = 18$) | −0.1847 | 0.4463 |
| Protein intake ($n = 18$) | −0.129 | 0.5948 |
| Leucine intake ($n = 18$) | −0.2817 | 0.2454 |
| Lipid intake ($n = 18$) | −0.0258 | 0.9152 |
| Carbohydrate intake ($n = 18$) | −0.2425 | 0.3173 |

## 4. Discussion

Our analyses revealed that sarcopenia and presarcopenia were present in even NAFLD patients. However, the frequencies of presarcopenia and sarcopenia were lower than those in the previous studies [17–19]. One of the reasons for the lower frequency of loss of skeletal muscle mass might be derived from the milder hepatic fibrosis in our patients. There were only two patients among the 46 patients whose livers were assessed as showing advanced hepatic fibrosis. Petta and colleagues reported that sarcopenia was significantly correlated with the severity of hepatic fibrosis in patients with NAFLD [19]. The number of NAFLD patients with more severe hepatic fibrosis was not sufficient. Thus, we may have a bias to evaluate the correlation between the skeletal muscle mass and the severity of hepatic fibrosis in this study.

Contrary to the expectation, none of the NAFLD patients fulfilled the criteria for sarcopenic obesity. Except for one male patient with presarcopenia, all of the patients who met the criteria for sarcopenia and presarcopenia were in the category of nonobese, which is called "hidden obesity." Our findings established that the SMI was significantly associated with the BMI, implying that loss of skeletal muscle mass was frequently observed in nonobese NAFLD patients rather than obese patients. Our results were quite different from the previous reports that sarcopenic obesity was observed even in patients with NAFLD [18]. Nonobese NAFLD patients tended to have less severe disease at presentation and have a more favorable prognosis than obese patients [40]. To the best of our knowledge, this is the first report of nonobese NAFLD patients being susceptible to loss of skeletal muscle mass. Unfortunately, the clinical definition of "sarcopenic obesity" has not been determined yet. Hence, urgent establishment of the definition is earnestly desired.

Our results demonstrated that the hormonal factors associated with SMI were the IGF-1 level as well as the BMI

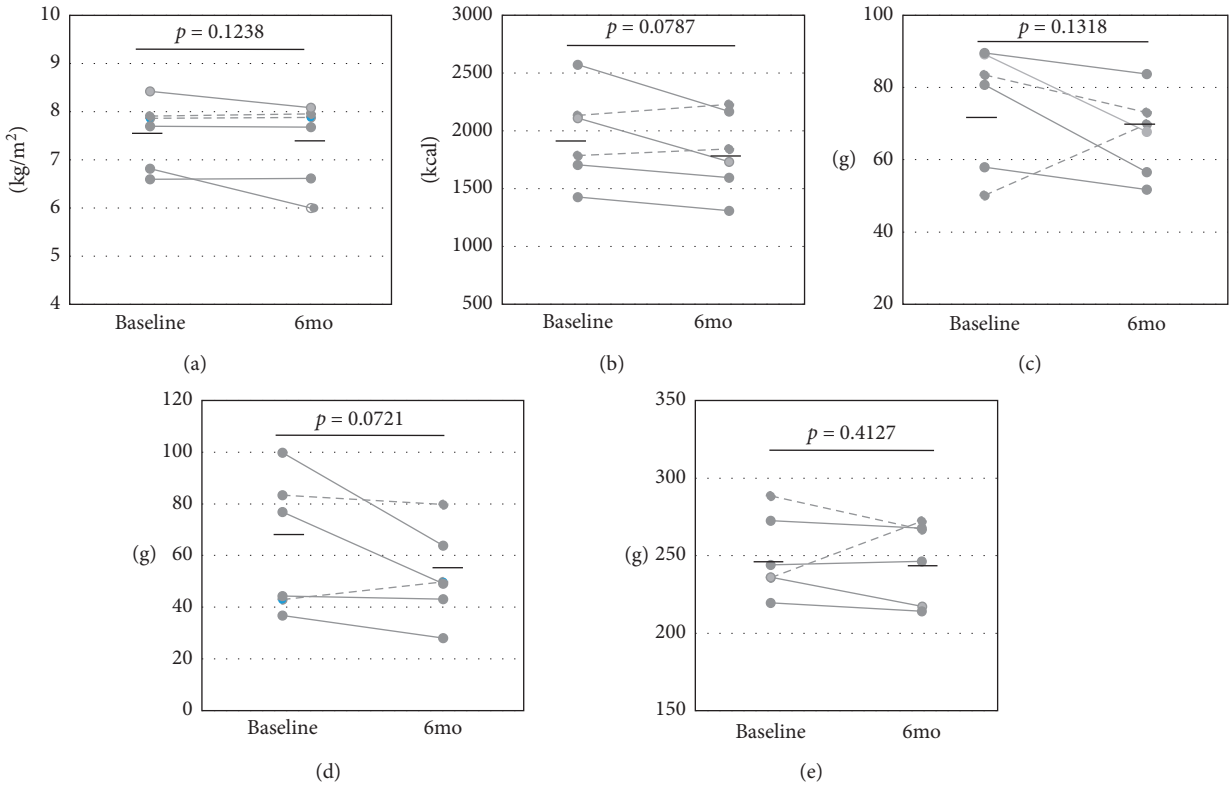


Figure 1: Changes in nutritional components by the diet treatment in the male patients. Horizontal bars represent the average value of each parameter. Broken lines correspond to patients whose total energy intake was increased by the diet treatment. (a) SMI. (b) Total energy intake. (c) Protein intake. (d) Lipid intake. (e) Carbohydrate intake.

in the male NAFLD patients. It has been well established that IGF-1 signaling plays an essential role in the formation, maintenance, and regeneration of skeletal muscles via insulin receptor substrate-1 (IRS-1) and mammalian target of rapamycin complex 1 (mTORC1) [5]. It was of interest that a decrease in the serum IGF-1 level was confirmed in an experimental animal model of NAFLD [41]. Therefore, a decrease in IGF-1 production eventually evokes loss of skeletal muscle mass in male [42]. The decrease in growth hormone (GH) release is likely to cause the decline of IGF-1 production, but it does not affect muscle strength [43]. Unfortunately, we did not determine serum GH level in each patient. Further examination will be required to clarify that.

In contrast, our findings elucidated that the factors linked to the SMI were the BMI and the BMD in female patients with NAFLD. Yilmaz noted that concurrent osteoporosis was frequently observed in patients with NAFLD [44]. The concept of biochemical communications between bones and muscles has been proposed. Several types of growth factors, myokines, and osteokines are thought to affect both bones and muscles. Therefore, sarcopenia may develop more severely in proportion to the severity of osteoporosis [45]. It has been well documented that the deterioration of BMD frequently causes sarcopenia in patients with NAFLD [46]. It is conceivable that vitamin $D_3$ deficiency was commonly observed in our NAFLD patients [47].

Estrogen is also a crucial factor for skeletal growth and the maintenance of skeletal integrity in women. A decrease in estrogen secretion in females is likely to ameliorate the skeleton's responsiveness to exercise more than in males, leading to loss of skeletal muscle mass [42]. These results described above may indicate that the hormonal factors that contribute to the regulation of the skeletal muscle mass were quite distinct between male and female NAFLD patients.

The results of our analyses did not demonstrate that the patients' muscle mass was associated with insulin resistance, the free testosterone, vitamin $D_3$, or zinc levels. Lee and colleagues previously revealed that loss of skeletal muscle mass was unrelated to insulin resistance and correlated only with the severity of hepatic fibrosis in such patients [17], which is consistent with our present findings. Higher frequency of vitamin $D_3$ deficiency in female patients may account for higher frequency of skeletal muscle mass loss than in male patients in this study. Direct evidence that zinc deficiency evoked loss of skeletal muscle mass was not obtained. An indirect correlation may thus be present between the zinc level and skeletal muscle mass. We did not determine the patients' serum selenium level, total testosterone, or estradiol concentrations. These should be examined in a future study.

Our results demonstrated that the dietary factors that contribute to the regulation of the skeletal muscle mass in the male patients were the total energy intake and the lipid intake. Hayashi and colleagues revealed that insufficient energy intake was significantly associated with sarcopenia in patients with compensated liver cirrhosis [48], supporting

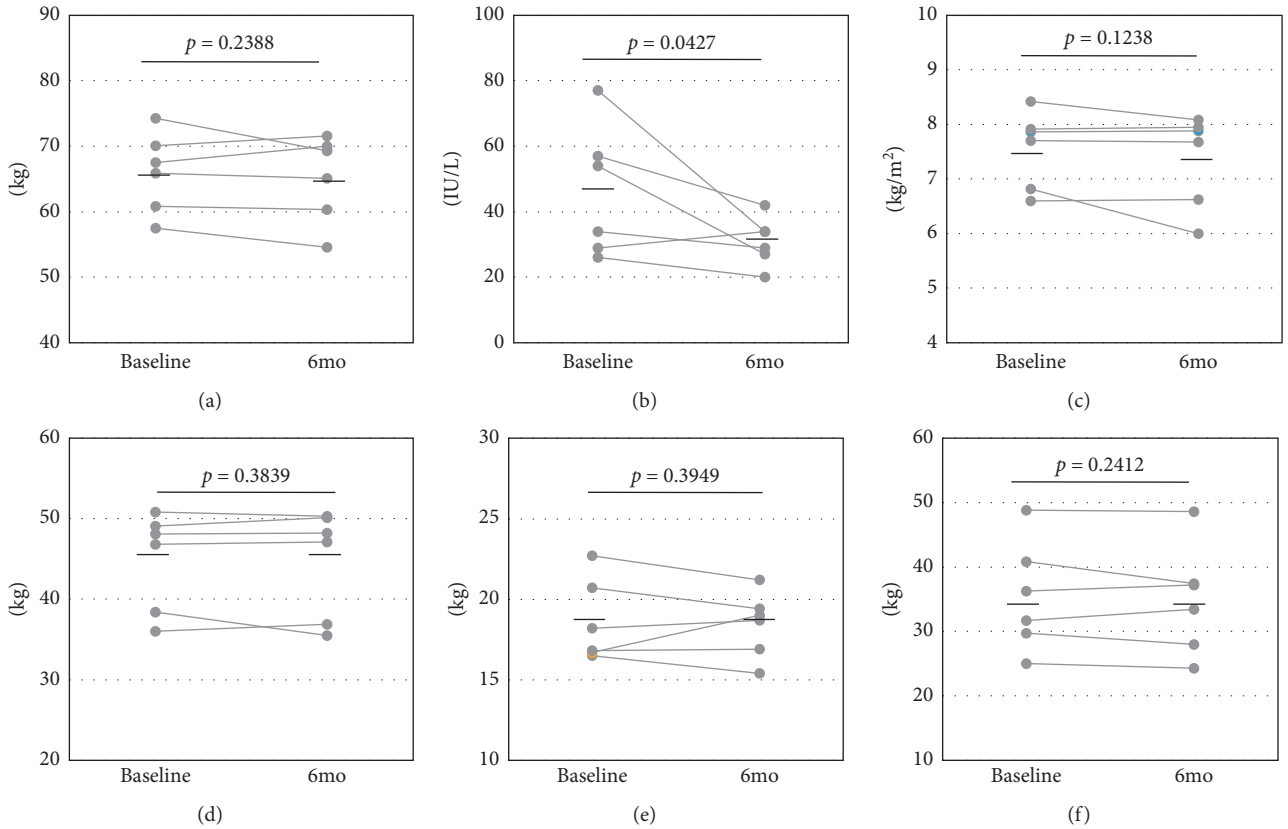


Figure 2: Changes in body compositions by the diet treatment in the male patients. Horizontal bars represent the average value of each parameter. (a) Body weight. (b) ALT. (c) SMI. (d) Skeletal muscle mass. (e) Body fat mass. (f) Hand grip strength.

our results in the present study. However, no significant dietary factors associated with SMI were revealed in our female NAFLD patients. These results may suggest that male patients are likely to affect the dietary factors more strongly than female patients. Surprisingly, we did not obtain evidence that the protein intake was associated with the skeletal muscle mass, which was the opposite of a previous study's results [49]. It has been well recognized that leucine directly activates mTORC1 that stimulates protein synthesis and decreases autophagy [50]. We expected that the leucine intake was associated with the skeletal muscle mass in the enrolled patients. However, the leucine intake did not particularly contribute any regulation of the skeletal muscle mass in such patients at all.

The effects of the dietary intervention on body composition were also different between our male and female groups. The total energy intake was decreased by way of a decline in their protein and lipid intakes due to the diet treatment in male patients. Consequently, the SMI value was decreased 6 months later, although the improvement of fatty liver was observed in such patients. This finding may support our finding that the muscle mass in the male patients was associated with the total energy intake and the lipid intake. In the female patients, a decreased total energy intake was accomplished by the diet intervention via reduction in their lipid intake and carbohydrate intake. The change in their protein intake was not observed in such patients. Then, fatty liver was improved and the SMI was maintained in the female patients. Taking these results into consideration, it appears that sufficient protein intake is necessary to maintain the muscle mass in NAFLD patients, although our study confirmed that the protein intake was not associated with the skeletal muscle mass in such patients. Further examinations are necessary to clarify this discrepancy. Moreover, the effects of diet treatment for NAFLD appeared to be entirely distinct between male and female patients.

There are several limitations to address. The sample size was small ($n = 46$) in our study, although many useful results were obtained. A larger-scale cohort study is required for confirmation of our results. Second, we did not perform histological examinations of the livers of the enrolled patients. We were unable to investigate the correlations between skeletal muscle mass and hepatic fibrosis or hepatic steatosis in the enrolled patients. Third, most of the enrolled patients were predicted to show mild hepatic fibrosis, because most of FIB-4 index values in the enrolled patients were under 2.67. We therefore did not sufficiently evaluate the skeletal muscle mass in NAFLD patients with advanced hepatic fibrosis in this study. Fourth, a qualitative evaluation of skeletal muscle was not performed in this study. The severity of myosteatosis, characterized by excessive fat deposition into the skeletal muscle, was not evaluated at all. We examined the skeletal muscle mass alone in this study. Fifth, a period for the dietary assessments was only three days. In

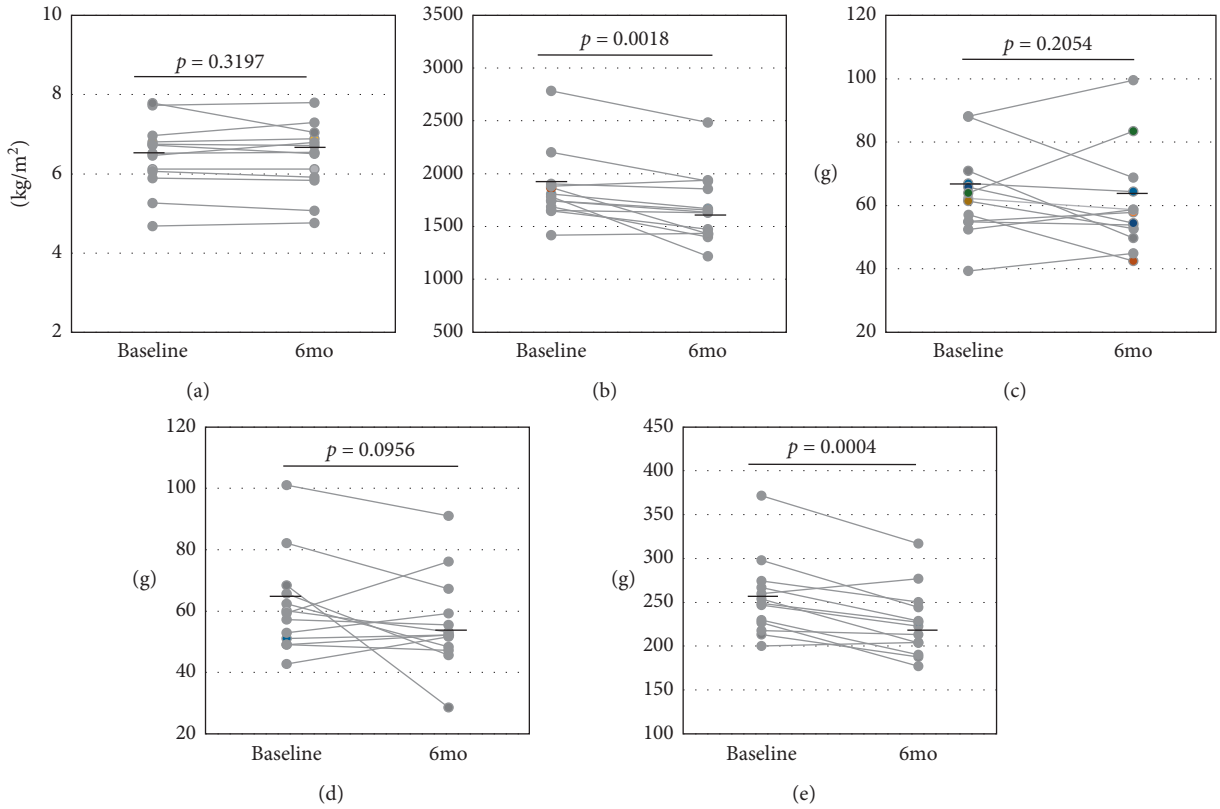


Figure 3: Changes in nutritional components by the diet treatment in the female patients. Horizontal bars represent the average value of each parameter. (a) SMI. (b) Total energy intake. (c) Protein intake. (d) Lipid intake. (e) Carbohydrate intake.

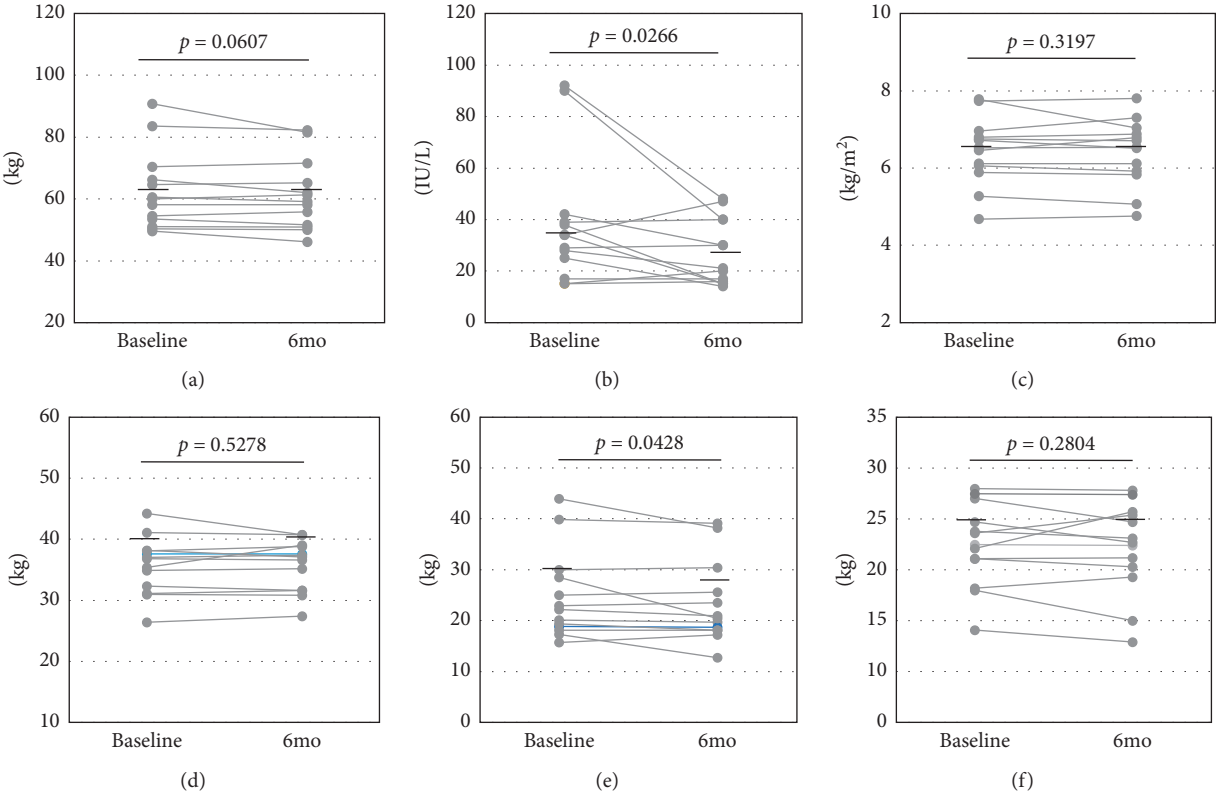


Figure 4: Changes in body compositions by the diet treatment in the female patients. Horizontal bars represent the average value of each parameter. (a) Body weight. (b) ALT. (c) SMI. (d) Skeletal muscle mass. (e) Body fat mass. (f) Hand grip strength.

addition, the dietary assessments were based largely on the patients' declarations. Thus, we have limitations to more detailed and objective evaluation for the dietary assessments. Finally, we did not evaluate the effect of the physical activity in each patient, although we confirmed the frequency of the physical training in a week. Then, the data obtained from this study might have a bias in the nutritional and dietary assessment.

## 5. Conclusions

Our results reveal that loss of skeletal muscle was frequently present in nonobese patients with NAFLD and that the frequency of sarcopenic obesity appears to be low among NAFLD patients. The nutritional and dietary factors associated with skeletal muscle index were distinct between the male and female NAFLD patients. The dietary intervention was effective for fatty liver but was harmful for skeletal muscle mass in some presarcopenic or sarcopenic patients. Therefore, the skeletal muscle mass as well as the body weight and liver function should be monitored during dietary intervention for NAFLD patients.

## Data Availability

Patients' data included within this article are also available from the corresponding author upon request.

## Conflicts of Interest

The authors have no conflicts of interest to declare.

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*Research Article*

# Effect of Melatonin as an Antioxidant Drug to Reverse Hepatic Steatosis: Experimental Model

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*Introduction*. The hepatic steatosis of the nonalcoholic origin or NAFLD is increasing at present, particularly in Western countries, parallel to the increase in obesity, constituting one of the most prevalent hepatic processes in the Western society. Melatonin has been successfully tested in experimental models in mice as a drug capable of reversing steatosis. The effect of melatonin on fat metabolism can be summarized as a decrease in lipid peroxidation and a decrease in oxidative stress, biochemical phenomena intimately related to fat deposition in the hepatocyte. There are hardly any studies in large animals. *Objective*. In this study, we investigate the effects of melatonin administered orally at a dose of 10 mg/kg/day to reverse established hepatic steatosis induced by a special diet in a porcine animal model. *Materials and Methods*. We analyze the parameters of oxidative stress: malondialdehyde (MDA), 4-hydroxyalkenals (4-HDA), and carbonyls, degree of fat infiltration (analyzed by direct vision by a pathologist and by means of a computer program of image treatment), and serological parameters of lipid metabolism and hepatic damage. These parameters were analyzed in animals to which hepatic steatosis was induced by means of dietary modifications. *Results*. We have not been able to demonstrate globally a beneficial effect of melatonin in the improvement or reversal of liver steatosis once established, induced by diet in a porcine animal model. However, we have found several signs of improvement at the histological level, at the level of lipid metabolism, and at the level of oxidative stress parameters. We have verified in our study that, in the histological analysis of the liver sample by means of the program image treatment (free of subjectivity) of the animals that continue with the diet, those that consume melatonin do not increase steatosis as much as those that do not consume it significantly ($p = 0.002$). Regarding the parameters of oxidative stress, MDA modifies in a significant manner within the group of animals that continue with the diet and take melatonin ($p = 0.004$). As for lipid metabolism, animals that maintain the steatotic diet and take melatonin lower total and LDL cholesterol levels and increase HDL levels, although these results do not acquire statistical significance. *Conclusions*. In this study, it has not been possible to demonstrate a beneficial effect of melatonin in the improvement or reversal of liver steatosis once established and induced by diet in the porcine model. It is true that signs of improvement have been found at the histological level, at the level of lipid metabolism, and at the level of oxidative stress phenomena, when comparing animals with established steatosis that are treated with melatonin with those who do not take it. This work is the first study conducted in a large animal model in which the effect of melatonin is studied as a treatment in the reversal of established hepatic steatosis.

## 1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is characterized by the accumulation of fatty acids, triglycerides, and cholesterol in the cytoplasm of the hepatocyte. It occurs in subjects who do not drink alcohol or drink moderately (<20 g/day) and is considered as the expression in the liver of a complex syndrome called "metabolic syndrome." NAFLD includes two clinical entities: nonalcoholic fatty liver (NAFL), which refers to the presence of hepatic steatosis without evidence of hepatocellular damage or fibrosis, and nonalcoholic steatohepatitis (NASH), which is the presence of hepatic steatosis that is associated with inflammation and liver damage with or without fibrosis. NASH may progress to cirrhosis, liver failure, and hepatocarcinoma [1].

The prevalence of NAFLD is not well known and is probably underestimated. This is because most patients remain asymptomatic or have discrete biological alterations, the absence of precise serological markers, and the need for liver biopsy for definitive diagnosis. However, we know that steatosis is one of the most prevalent liver diseases in the Western world, linked to the increase in obesity and metabolic syndrome, and varies widely depending on the population studied. Two Japanese studies [2, 3] published an incidence in the general population of 31 and 86 cases, respectively, of suspected NAFLD per 1000 persons/year. In severely obese patients undergoing bariatric surgery, the prevalence of NAFLD may exceed 90% and up to 5% may have cirrhosis.

The treatment of hepatic steatosis once established is achieved primarily through dietary restrictions and lifestyle changes [4–7]. Drugs used in the treatment of metabolic syndrome (antidiabetics, statins, etc.) have also been used [8]. Recently, the efficacy of various antioxidant agents, such as vitamin E and melatonin [9–11], has been demonstrated in the treatment of hepatic steatosis. There is currently no specific harmless and effective treatment for hepatic steatosis.

Melatonin is a natural hormone synthesized by the pineal gland in animals. Melatonin synthesis is not found exclusively in the pineal gland, as its secretion has been described in numerous peripheral organs such as the retina, bone marrow, skin, and gastrointestinal tract and in some cells such as lymphocytes and platelets [12, 13].

One of the main properties of melatonin is its powerful antioxidant effect [12], which is attributed to its ability to neutralize free radicals and its indirect detoxifying action by stimulating antioxidant enzymes [14]. It has been proven that melatonin supplements could protect against some diseases such as atherosclerosis, cancer, and Alzheimer's disease. At the level of hepatic metabolism, melatonin has been tested as a drug to prevent the process of ischemia-reperfusion [15] and also as a treatment of liver damage by toxins such as alcohol, carbon tetrachloride, aflatoxin, or chemotherapy agents [16–19].

Melatonin has been shown to be beneficial in the treatment and prevention of NAFLD in murine experimental models. Experimental studies have been carried out in rodents, the majority with the intention of preventing diet-induced hepatic steatosis, observing a decrease in blood lipids, an improvement in hepatic enzymes, and a decrease in oxidative stress parameters, as well as an improvement in histology [20–25]. There are hardly any studies in large or small animals that prove this effect. In humans, studies have been carried out that demonstrate the lipid lowering and cytoprotective capacity of melatonin in hepatic steatosis once established, although it is true that there are hardly any studies that demonstrate histological improvement [26–29].

Our working hypothesis is based on the fact that melatonin is an effective treatment to reverse established and induced hepatic steatosis by means of a special diet in a porcine animal model, as well as in the murine model. The aim of our study is to assess the effect of melatonin administered orally on lipid metabolism, hepatic histology, and oxidative stress parameters.

## 2. Material and Methods

*2.1. Model and Sample: An Experimental Animal.* We use the great English white pig as an experimental animal. This model was chosen as such because it is consistent with the hepatic physiology of the human. The animals were obtained from a breeding farm for animal experimentation, which were free of diseases or parasites and with an approximate weight of 45 kg and an approximate age of 6 months. All animals that did not meet a minimum weight (30 kg) or that showed signs of diseases (diarrhoea, adynamia, dermatosis, and abnormal behaviour) were excluded from the experiment. The animals were kept in cages, shared by 3 or 4 animals, and provided with feeders and water. The study was approved by the Advisory Ethics Commission for Animal Experimentation of the University of Zaragoza.

*2.2. Experimental Design and Study Groups.* The induction of steatosis was carried out by means of a special diet, without using any pharmacological agent. Its main characteristics were as follows: high content in saturated fat (25%), deficiency in methionine and choline, and supplemented with 2% cholesterol plus 0.5% sodium cholate. The animals belonging to study groups 1 and 3 received melatonin doses of 10 mg/kg/day orally for 4 weeks. The drug was acquired in powder form, which was encapsulated in a sucrose excipient. It was administered in a single dose by means of capsules that were ingested at the same time as the diet described above, mixing the drug with part of the diet in such a way as to ensure complete intake.

A control group consisted of 6 animals; healthy animals without steatosis underwent a normal nonsteatosis diet and underwent a single surgical intervention for the purpose of obtaining samples, whose values served as a reference. 31 animals were part of the study. For 3 months, they were on an original diet of our group specially designed to produce steatosis, at the end of which, they underwent an open biopsy (to obtain sufficient samples). After the surgical intervention, the animals were assigned to 4 study groups: group 1 ($N = 12$): animals that maintained a steatotic diet for 1 more month and received concomitant treatment during

this month with melatonin (10 mg/kg/day); group 2 ($N = 9$): animals that maintained a steatotic diet for one more month and received no drug; group 3 ($N = 5$): animals that did not continue with the steatotic diet but received melatonin treatment for one month at the same dose; group 4 ($N = 5$): animals that did not continue with the steatotic diet and did not receive any drug. After this time, the animals underwent a second surgical intervention to obtain the same samples of liver tissue, blood, and serum. After this second operation, the animals were slaughtered.

In each surgical intervention, a minilaparotomy was performed to observe the macroscopic characteristics of the liver and to take a sample of hepatic tissue from segment IV. This sample was divided into two parts: one of them was kept in formaldehyde for anatomopathological study. Another sample was frozen and preserved in liquid nitrogen to obtain oxidative stress parameters: MDA and carbonyls. During the intervention, a sample of 20 ml of venous blood extracted from the portal vein or the vena cava was also obtained. The blood was centrifuged at 3000 rpm for 3 min to obtain serum. Biochemical parameters were obtained from the blood sample.

*2.3. Induction of Hepatic Steatosis.* In the experimental porcine model, hepatic steatosis can be induced by means of a specially developed and proven diet that resembles the induction of steatosis in humans. This is without the need to use drugs or toxic products. The induction of steatosis was carried out through a special diet whose main characteristics were as follows:

(i) High saturated fat content (25%)

(ii) Deficient in methionine (<1/4 of the needs) and choline (1/6 of the needs)

(iii) Supplemented with 2% cholesterol and 0.5% sodium cholate.The following table shows its composition

*2.4. Histological Analysis of Liver Biopsy.* To determine the degree of hepatic steatosis, we used two methods. Method 1 is a conventional semiquantitative analysis in which the pathologist estimates the percentage of steatosis under direct vision (HEX ×1000) of each sample expressed in %. Method 2 is a method of quantification via computer-assisted analysis consisting of an estimate of the area of computer-assisted steatosis of samples obtained and previously selected.

The program measures the degree of steatosis by analyzing tonalities from photographs taken of each sample of hepatic tissue, without areas occupied by vessels and other structures. We use a specific software assisted by Matlab®, created by the research group itself to perform a digital analysis of the biopsy samples. It has three fundamental functions:

(i) Cal-estea.m: it is the main function that performs the calculation of steatosis

(ii) Filter-m: it is the function of the gray filter, which allows the program to be adapted to the wide range of shades that can be presented to us in the sample

(iii) Region-m: it is the function that eliminates regions that do not correspond to fat (for example, veins with white tones)

*2.5. Measurement of Oxidative Stress Parameters: MDA and Carbonyls.* The concentration of MDA + 4-hydroxyalkenals (4-HDA) was determined by a colourimetric method based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA or with 4-HDA, at a temperature of 45°C. The condensation of one MDA or 4-HDA molecule with two molecules of N-methyl-2-phenylindole produces a stable chromophore which, in the presence of methanesulfonic acid, has a maximum absorbance at 586 nm [30].

In order to evaluate the oxidative damage to the proteins of the homogenized ones, the determination of the carbonyl remains of the proteins was used. This method is based on the reaction of the carbonyl remains of proteins with dinitrophenylhydrazine (DNPH), forming a derivative that is quantified by measuring its absorbance in the range 360–390 nm. During the procedure, trichloroacetic acid (TCA) is used for the precipitation of proteins, washes for the removal of excess DNPH that has not reacted with carbonyl residues, and guanidine to redissolve the proteins in order to facilitate spectrophotometer readings.

From the absorbance obtained, the concentration of carbonyl moieties was calculated using the Beer–Lambert law and the molar absorption coefficient of DNPH ($\varepsilon = 22{,}000\ \text{M-1} \times \text{cm}^{-1}$). Finally, after the determination of the total proteins, the results were expressed in nmol of carbonyl moieties/mg of total proteins.

The laboratory equipment included a spectrophotometer with disposable plastic cuvettes to measure the visible range and quartz cuvettes to measure in the ultraviolet (UV) range.

*2.6. Measurement of Biochemical Parameters.* The measurement of serological parameters of hepatic function (aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), alkaline phosphatase (FA), and bilirubin (B)) and plasma lipid concentration (total cholesterol, HDL cholesterol, and LDL cholesterol) was performed by automatic processors for automatic reading of concentrations of liver lipids and enzymes.

*2.7. Variables to Be Studied.* The following variables were studied: the weight of the animal, steatosis reached, blood lipids (triglycerides (TG), total cholesterol, HDL cholesterol, and LDL cholesterol), hepatic enzymes (aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), alkaline-FA phosphatase, and bilirubin), parameters of oxidative stress (MDA and carbonyls), and histology (degree of fat infiltration).

*2.8. Statistic Analysis.* To analyze the relationship between the variables of a study, a bivariate analysis is carried out. To study the linear relationship between two quantitative variables, the Pearson or Spearman correlation coefficient is

used. The correlation coefficient can take values between −1 and 1, indicating the zero correlation between the variables under study. To evaluate the differences between the first biopsy or pre and the second biopsy or post, in relation to the quantitative variables of the study, means comparison methods are used for related samples. Wilcoxon is used when the variable does not follow the normal distribution, and Student's $t$-test is used when there is normality.

To quantify the difference between both time periods, we calculate the percentage of change. Positive results will indicate increased values in the postbiopsy and negative results will indicate decreased values.

## 3. Results

*3.1. Steatosis Reached.* The total of 31 (100%) animals in the study group presented significant hepatic steatosis in the first biopsy, moderate (steatosis 30–59%) and severe (steatosis >60%) after being on a special diet for 3 months, both in the measurement made by the pathologist and in the measurement made by the computer program.

Figure 1 shows the average degree of steatosis of the animals for each of the animals according to pathologist analysis and digital image analysis. There are no statistically significant differences in the average steatosis reached per group.

It is observed that the degree of steatosis according to the digital analysis provides in all cases average values lower than those provided according to an analysis by a pathologist. On average, the degree of steatosis according to a pathologist scores 40.68 (DE = 14.66) more than that according to the digital analysis at the premoment.

Figure 2 shows the optical microscopy image of the animal with the highest percentage of steatosis being seen by a pathologist (95% of steatosis) and the optical microscopy image of the animal with the lowest degree of steatosis being seen under direct vision by a pathologist (40% of steatosis).

*3.2. Weight.* In all groups, there was an increase in average weight between the two time periods evaluated, as shown in Figure 3, this increase being statistically significant in groups 1, 3, and 4 ($p = 0.03$, $p = 0.043$, and $p = 0.043$, respectively).

In all groups, the percentage change (% difference in weight at the end of the study with respect to the initial weight) was studied, calculated as follows:

$$\%\ \text{weight change} = \frac{\text{postweight} - \text{preweight}}{\text{preweight}}. \quad (1)$$

*3.3. Biochemical Variables: Triglycerides, Total Cholesterol, HDL, LDL, AST, ALT, GGT, and FA.* Figures 4–7 show the mean prevalue and postvalue of each of the parameters evaluated for each of the study groups.

*3.3.1. Group 1.* In group 1 (the group that continued with the steatotic diet and was treated with melatonin), no parameter shows statistically significant differences ($p > 0.05$) before and after treatment.

*3.3.2. Group 2.* In group 2 (the group that continued with the steatotic diet and was not treated with melatonin), the average AST decreased significantly ($p = 0.008$), going from 75.11 UI/L (DE = 47.29) to 32.67 UI/L (DE = 13.93); there is also a significant decrease ($p = 0.017$) of the average B going from 0.73 UI/L (DE = 0.83) to 0.22 (DE = 0.26).

*3.3.3. Group 3.* In group 3 (the group that discontinued the diet and was treated with melatonin), the average values of total cholesterol, HDL, LDL, GGT, and FA decreased at the end of the study period in a statistically significant way, compared with the values at the beginning ($p = 0.043$ in all cases). However, we observe a significant increase in ALT and AST ($p = 0.043$ and $p = 0.042$ respectively).

*3.3.4. Group 4.* In group 4 (the group that discontinued the steatotic diet and was not treated with melatonin), the average values of TG, total cholesterol, HDL, LDL, AST, GGT, and FA also decreased at the end of the study period, presenting significant differences between the two study time periods (pre and post) ($p = 0.043$ in all cases). However there is a significant increase in ALT ($p = 0.042$).

*3.4. Analysis of Hepatic Fat Infiltration.* Figure 8 shows graphically the degree of average steatosis by groups in the two study time periods analyzed by the direct vision of the pathologist.

A decrease in steatosis was observed in groups 3 and 4, and an increase in steatosis in groups 1 and 2, with a significant decrease in group 4 ($p = 0.042$), the animals that discontinued their diet and were not treated with melatonin.

Figure 9 shows graphically the degree of average steatosis by groups in the two study time periods digitally analyzed by the image processing software.

There is a decrease in steatosis in groups 3 and 4, and there is an increase in steatosis in groups 1 and 2. This increase in group 2 is statistically significant ($p = 0.08$), as is the decrease in group 4 ($p = 0.043$).

The degree of steatosis measured by the program provides lower average values in relation to the degree of steatosis measured by the pathologist. On average, the degree of steatosis measured by the direct vision of the pathologist [31] scores 40.68% more in the premoment and 36.87% more in the postmoment. In Figure 10, it is possible to observe, in a graphical way, the average values of the degree of steatosis of both measurements by groups for both periods of study (pre and post) and how the same tendency is observed for the two methods of measurement employed.

Figure 11 shows the relationship between the percentage of change obtained with the program and the percentage of change obtained with the pathologist.

The percentage of change of steatosis between the pretime and posttime of the follow-up of the subjects shows a strong linear relationship between both methods, obtaining

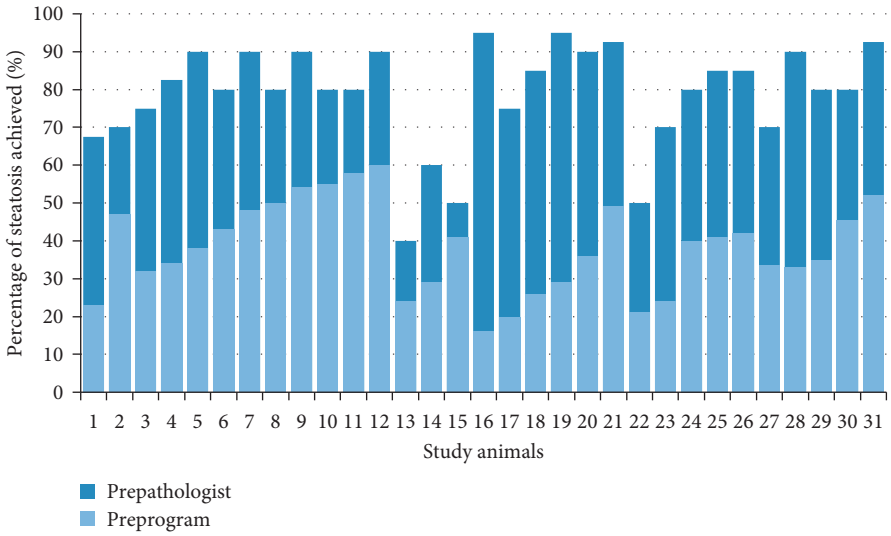
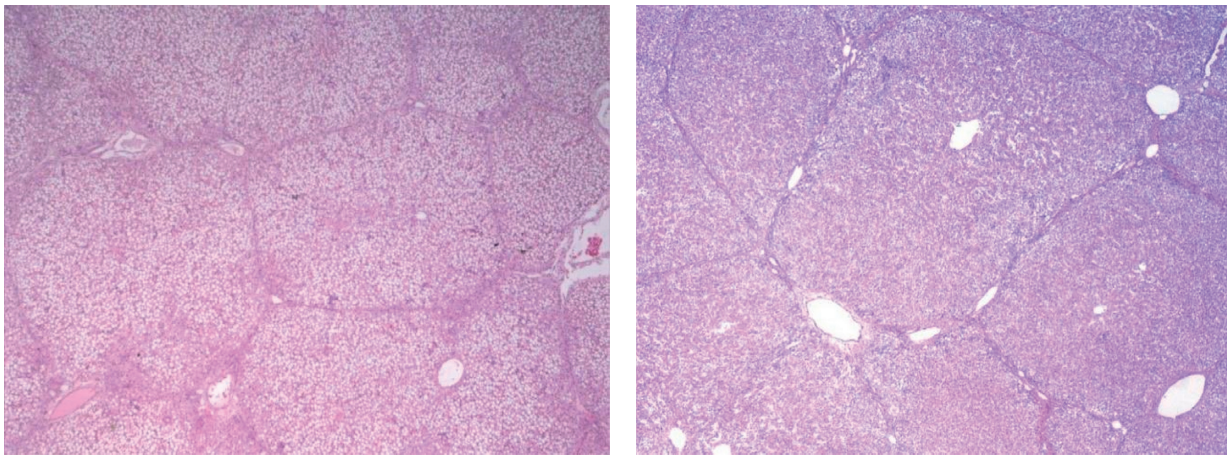



Figure 1: Average steatosis reached by each of the animals, according to the pathologist and program.



(a) (b)

Figure 2: Optical microscopy image, HE 2x dyeing with 95% steatosis (a) and optical microscopy image, HE 2x dyeing with 40% steatosis (b).

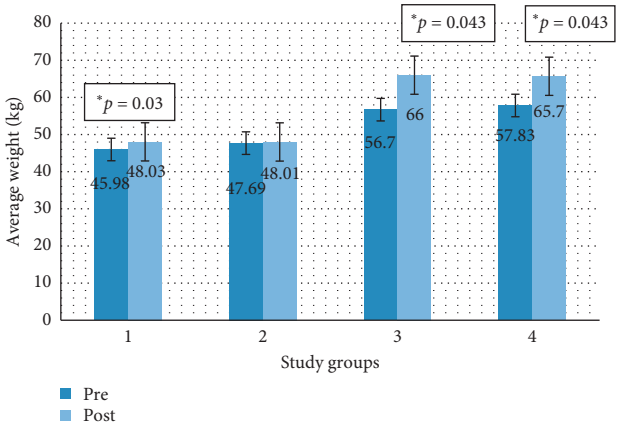



Figure 3: Average pre-post weight (kg) by study groups. Group 1: animals that maintained a steatotic diet and received concomitant treatment. Group 2: animals that maintained a steatotic diet and received no drug. Group 3: animals that did not continue with the steatotic diet but received melatonin treatment. Group 4: animals that did not continue with the steatotic diet and did not receive any drug.

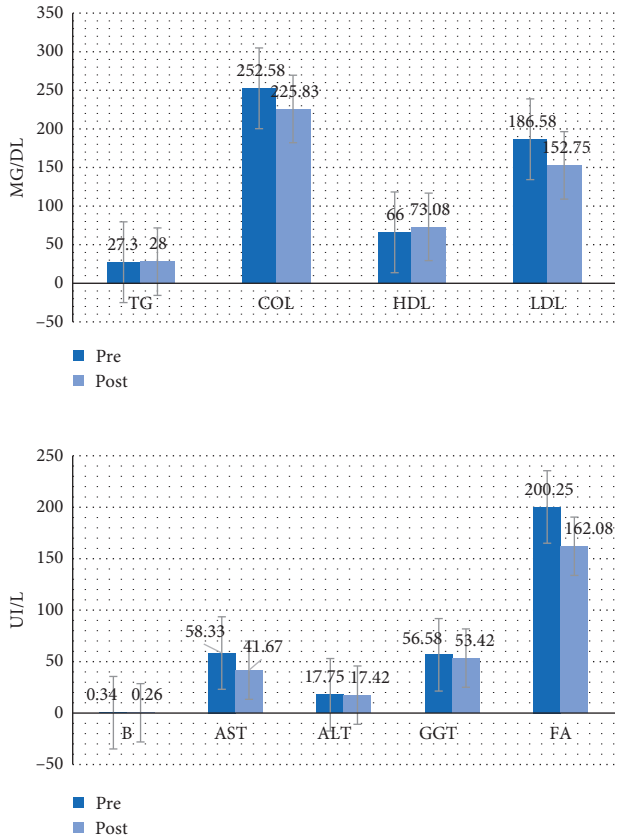



Figure 4: Average values of the pre-post parameters for group 1. TG = triglycerides, COL = total cholesterol, HDL = HDL cholesterol, LDL = LDL cholesterol, B = bilirubin, AST = aspartate aminotransferase, ALT = alanine aminotransferase, GGT = gamma-glutamyl transferase, FA = alkaline phosphatase.

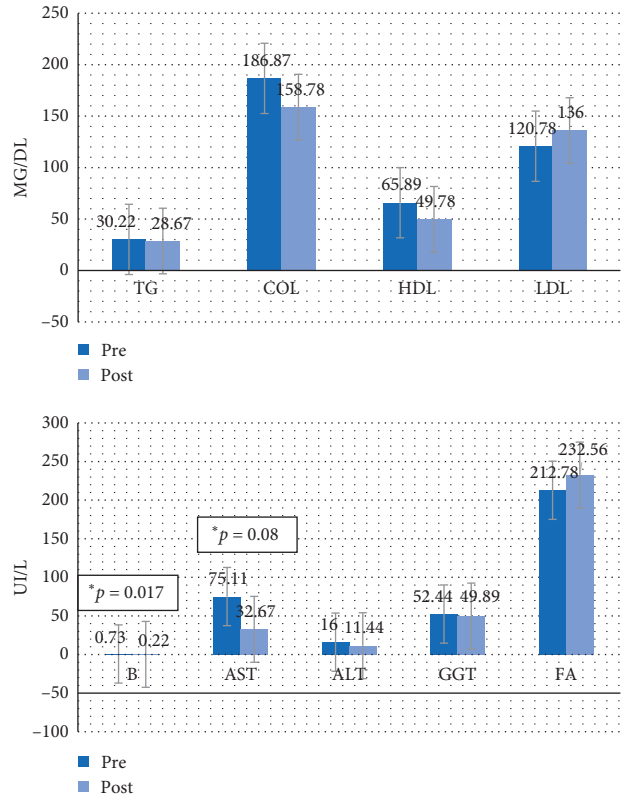



Figure 5: Average values of the pre-post parameters for group 2. TG = triglycerides, COL = total cholesterol, HDL = HDL cholesterol, LDL = LDL cholesterol, B = bilirubin, AST = aspartate aminotransferase, ALT = alanine aminotransferase, GGT = gamma-glutamyl transferase, FA = alkaline phosphatase.

a correlation coefficient of 0.767 with a level of significance being lower than 0.001.

*3.5. MDA.* Figure 12 shows graphically the degree of average MDA by groups in the two study periods.

In all study groups, there was a decrease in the average value of MDA, being statistically significant in group 1 ($p = 0.04$), the subjects that continued the steatotic diet and were given melatonin.

*3.6. Carbonyls.* Figure 13 shows graphically the average carbonyl degree by groups in the two study periods.

In groups 2, 3, and 4, there is an average increase of carbonyls, being only significant in group 4 ($p = 0.028$). The small average decrease in group 1 (subjects that continue on a diet and are treated with melatonin) does not have statistical significance, as in the case of MDA.

## 4. Discussion

NAFLD is one of the most prevalent hepatic processes in the Western society and one of the major causes of liver failure in the Western world. Usually associated with, but not exclusively to, obesity and the metabolic syndrome, it could lead to a real epidemic in the future. Numerous authors have shown that the reduction of excess weight and the consequent resistance to insulin can restore liver physiology and histology [8,9]. But at the present time, there is no effective pharmacological treatment of NAFLD that is not linked to a lifestyle change, including weight loss. Many drugs, antioxidants, hepatoprotectors, vitamin complexes, free radical scavengers, etc. have been tested with the idea of either avoiding the accumulation of lipids in the hepatocyte or extracting lipids from cells, showing beneficial effects.

Although more studies are needed to define their usefulness in NAFLD [32–37], the discovery of a drug with these properties could change the spectrum and impact of the disease.

Melatonin, the universal hormone that is produced in different concentrations, in almost all our anatomy, has been studied as one of the possible solutions to the alteration of lipid metabolism and fat deposition in the hepatocyte [12]. Experimental studies have shown an action of melatonin on fat metabolism, which can be summarized as a decrease in lipid peroxidation and a decrease in oxidative stress [13], biochemical phenomena intimately related to the accumulation of lipids in the hepatocyte. Other effects that have been attributed to melatonin are as follows: stimulation of antioxidant enzymes in hepatocytes, regulation of antioxidant enzyme transcription genes, scavenging of oxygen free

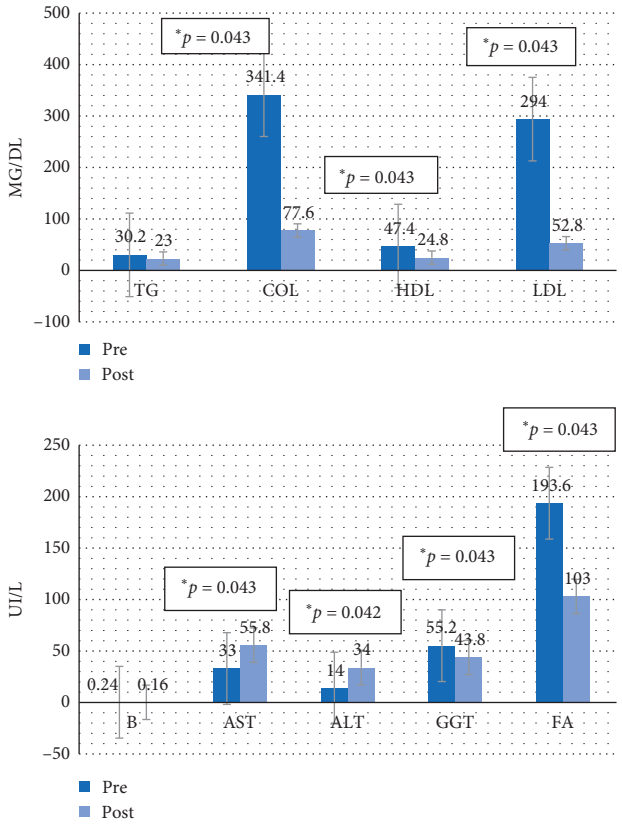



Figure 6: Average values of the pre-post parameters for group 3. TG = triglycerides, COL = total cholesterol, HDL = HDL cholesterol, LDL = LDL cholesterol, B = bilirubin, AST = aspartate aminotransferase, ALT = alanine aminotransferase, GGT = gamma-glutamyl transferase, FA = alkaline phosphatase.

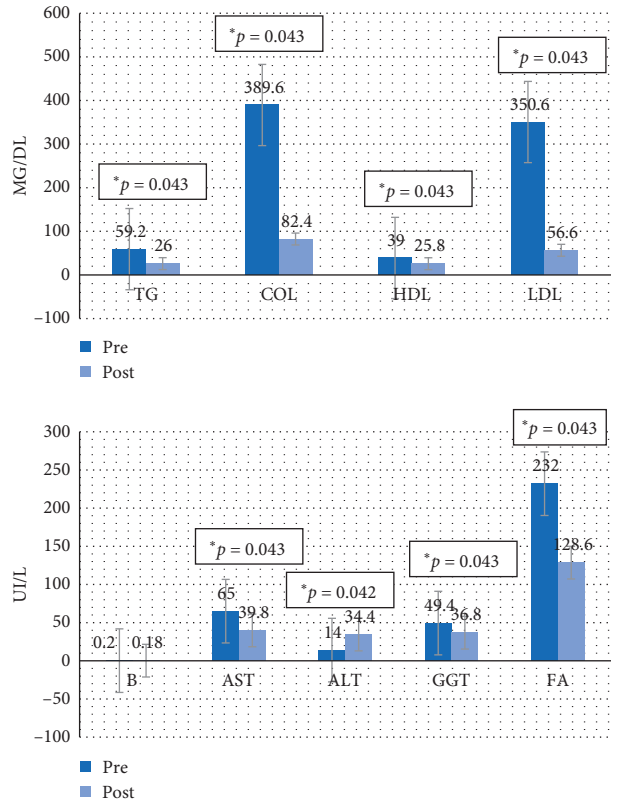



Figure 7: Average values of the pre-post parameters for group 4. TG = triglycerides, COL = total cholesterol, HDL = HDL cholesterol, LDL = LDL cholesterol, B = bilirubin, AST = aspartate aminotransferase, ALT = alanine aminotransferase, GGT = gamma-glutamyl transferase, FA = alkaline phosphatase.

radicals, stimulation of glutathione synthesis, increase in the activity of other antioxidant molecules, decrease in the generation of free radicals in mitochondria, decrease in the expression of proteins that have an effect on the accumulation of lipids in the hepatocyte cytoplasm, protection of cell membranes against lipid peroxidation, and reduction of proinflammatory factors responsible for the progression of NAFLD to NASH.

Several experimental models have been described to resemble human NAFLD. The ideal model would be one that reflects both the histology and the physiopathology of the disease in its different stages. Of course, this model should be reproducible, reliable, simple, predictable, and economical. Most of these models have been described in rodents. There are few models of hepatic steatosis in large animals, and in most of them, liver damage has been induced by the administration of toxins such as alcohol [38,39].

In 2009, Lee et al. described, for the first time, a NASH model in large animals produced by dietary manipulation using Ossabaw miniature pigs [40]. This model is not very useful due to the restriction of the use of these animals. In our work, we used an experimental model, the pig White-Landrace, in which we reproduced steatosis. This model is original and sufficiently proven in previous studies of our group and that has the particularity that only by means of dietetic manipulation, without using drugs or toxics, controlled and very high degrees of steatosis are achieved. We have not found any model published thus far that induces, without the use of substances harmful to the liver or an aggressive diet (excess of polyunsaturated, fructose fats, or the deficit of elements necessary for beta-oxidation such as choline and methionine), a degree of macrovesicular steatosis similar to ours, in such a short time and with minimal repercussions on the physiology of the animal. Another strength of our experimental model is that it allows us to check liver damage and quantify steatosis by performing liver biopsies before and after the development of steatosis; in many murine models, this is not possible, and in humans, no author performs them.

All steatogenic diets described induce an increase in the weight of the animal. In our study, all animals increased in weight after the introduction of the steatogenic diet. The data is consistent with the data found in the literature (Ossabaw pigs). The effects of melatonin were null on the weight of the animal.

In order to explore the effect on the lipid metabolism of melatonin, we studied lipid peroxidation (LPO) on the polyunsaturated fatty acids of the hepatocyte (during the process of accumulation of lipids in the hepatocyte, the action of free radicals on the lipids takes place mainly on the polyunsaturated fatty acids of cellular samples, causing their

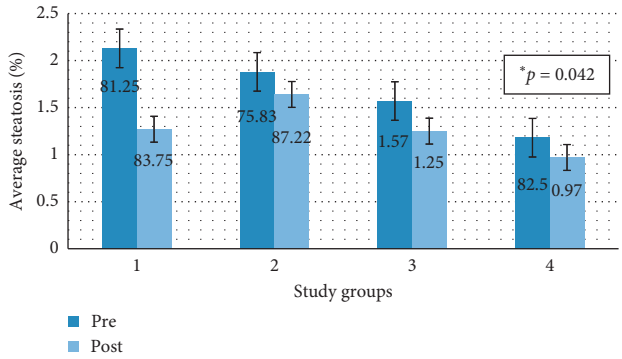



Figure 8: Average pre-post steatosis by study groups. Group 1: animals that maintained a steatotic diet and received concomitant treatment. Group 2: animals that maintained a steatotic diet and received no drug. Group 3: animals that did not continue with the steatotic diet but received melatonin treatment. Group 4: animals that did not continue with the steatotic diet and did not receive any drug.

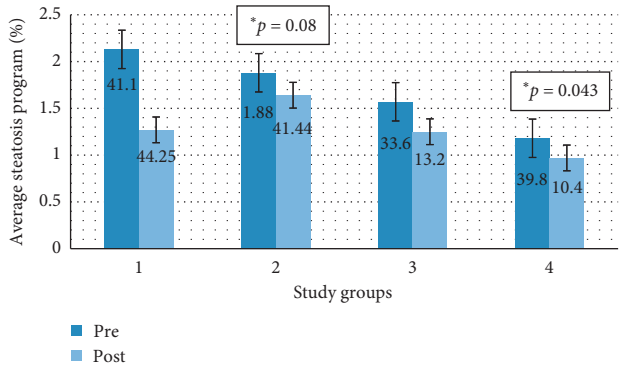



Figure 9: Percentage of average pre-post steatosis (program) by study groups. Group 1: animals that maintained a steatotic diet and received concomitant treatment. Group 2: animals that maintained a steatotic diet and received no drug. Group 3: animals that did not continue with the steatotic diet but received melatonin treatment. Group 4: animals that did not continue with the steatotic diet and did not receive any drug.

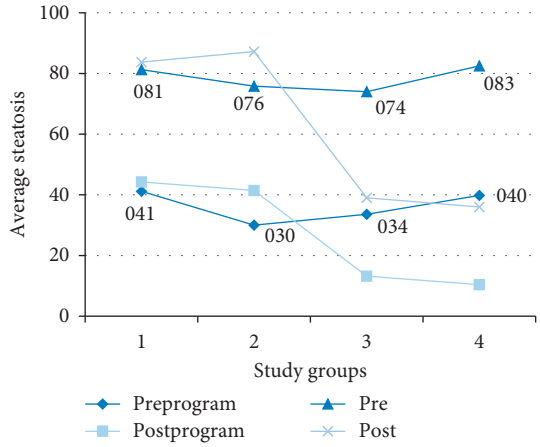



Figure 10: Percentage of average pre-post steatosis (program) by study groups. Group 1: animals that maintained a steatotic diet and received concomitant treatment. Group 2: animals that maintained a steatotic diet and received no drug. Group 3: animals that did not continue with the steatotic diet but received melatonin treatment. Group 4: animals that did not continue with the steatotic diet and did not receive any drug.

peroxidation). The final products of this process of LPO are aldehydes, hydrocarbon gases, and various chemical residues, with MDA and 4-HDA being the majority. Therefore, the concentration of MDA+4-HDA is an indicator of the degree of peroxidation of the lipids of the biological membranes [41].

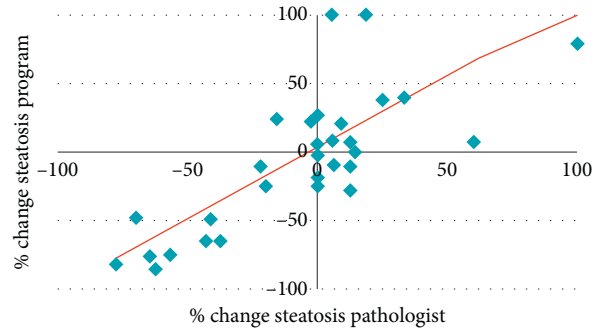



Figure 11: Scatter diagram. % change steatosis program–% change steatosis pathologist.

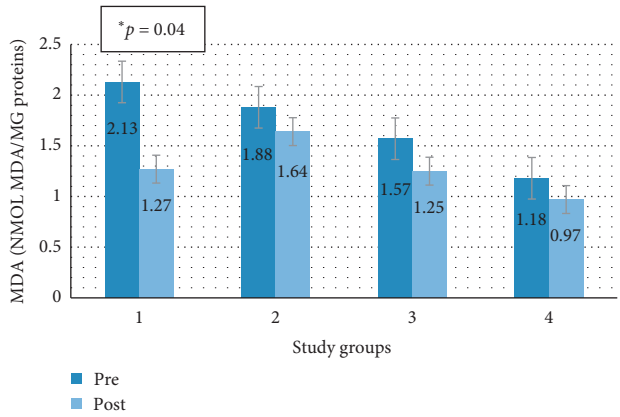



Figure 12: Average pre-post MDA by study groups. Group 1: animals that maintained a steatotic diet and received concomitant treatment. Group 2: animals that maintained a steatotic diet and received no drug. Group 3: animals that did not continue with the steatotic diet but received melatonin treatment. Group 4: animals that did not continue with the steatotic diet and did not receive any drug.

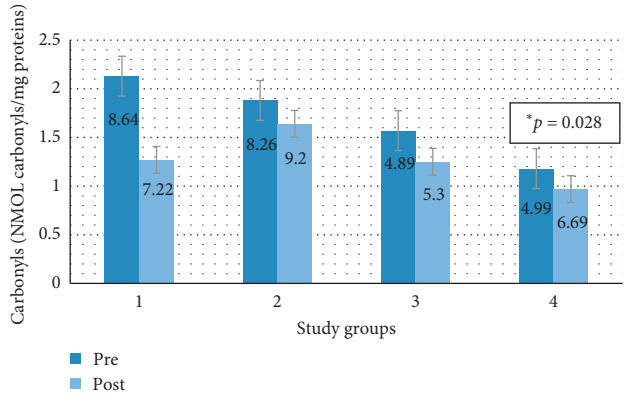



Figure 13: Medium pre-post carbonyls by study groups. Group 1: animals that maintained a steatotic diet and received concomitant treatment. Group 2: animals that maintained a steatotic diet and received no drug. Group 3: animals that did not continue with the steatotic diet but received melatonin treatment. Group 4: animals that did not continue with the steatotic diet and did not receive any drug.

In our study, we found that both MDA and carbonyls decreased in the groups that were administered melatonin with respect to those who did not receive it; therefore, this indicates an effect of the hormone on lipid metabolism at the intracellular level.

Plasma levels of cholesterol and triglycerides increased in animals subjected to steatogenic diet but were not modified by the effect of melatonin with statistical significance. However, we can observe that animals that continue with the diet and take melatonin (group 1) exhibit lowered LDL and

cholesterol levels and increased HDL levels. This could indicate some protective effect of melatonin in this group of animals, although it lacks statistical significance. In the literature, we find works that ratify the lipid-lowering effect of melatonin [22, 24, 28, 42, 43]. One reason that might argue this result is that, in our experimental model, we start from established hepatic steatosis, with chronically elevated blood lipid levels, a situation that does not occur in the other experimental studies, since melatonin is administered concomitantly with the administration of the high-fat diet as prevention.

With respect to the levels of liver enzymes (ALT and GGT), bilirubin, and FA, in our study, their levels have not been altered by the action of melatonin. In other experimental studies on murine models, Pan et al. [20] were able to demonstrate that the administration of intraperitoneal melatonin at doses of 5 to 10 mg/kg/day for 12 days was effective in reducing serum ALT and AST. The difference with our work is that the previous authors gave jointly the steatotic diet and melatonin to the experimental animals and our work starts from animals with an already established NAFLD; that is to say, perhaps melatonin could not have a preventive effect of the hepatic lesion when it is administered early (before the accumulation of fat in the hepatocyte), being its effect null when NAFLD is already established.

The work carried out by Hatzis et al. [21] gives us some light in this respect. The authors were able to demonstrate that hepatic cell necrosis was significantly lower in rats that had received a diet rich in fats and melatonin at doses of 5 mg or 10 mg/kg/day via intraperitoneal for 4–8 weeks, finding that the levels of AST and ALT were lower after receiving the drug. However, in their work, a group of animals underwent induced steatosis (through diet) before being treated with melatonin, because this last group of animals did not obtain any hepatic cytoprotective effect, like the animals in our study. It can be concluded that melatonin is ineffective as a treatment once NAFLD is established. In our work, the degrees of NAFLD reached after the diet were very high, much more than those obtained in similar studies with other experimental models; perhaps with more moderate degrees of fat infiltration, melatonin could have some effect, and this extreme should be investigated.

The traditional method of evaluation of the degree of hepatic steatosis, used by most authors in the world, is performed by evaluating the histological sample stained with Hematoxylin-Eosin evaluation performed more or less subjectively by a pathologist. To avoid subjectivity and improve accuracy, we also use a method of evaluation by digital image analysis through a program created by the team itself, to contrast this subjectivity. In our study, it was observed that the measurement of steatosis by digital analysis provides in all cases some average values lower than those obtained by the direct vision of the pathologist. Studies published in the literature corroborate this overestimation [44]. An explanation of this overestimation, as several authors explain, is that the measured area of hepatocyte vacuoles is better measured by computer methods than the purely visual way, since the computer is capable of eliminating areas of the sample with a similar visual aspect (in the design of our computer program, we took this fact into account). In our study, the percentage change of steatosis between the pretime and posttime of subject tracking shows a strong linear relationship between the two methods. In conclusion, in our study, we obtained a high correlation between both methods, but with an overestimation of the degree of steatosis by the pathologist analysis.

In our study, melatonin was unable to restore histology, not even to the low steatosis figures at baseline, although we observed the phenomenon that the degree of fat infiltration, which increased gradually if the animals continued to take the steatotic diet, remained stable if they were given melatonin, with significant differences. These data corroborate experimental data in murine models. Hatzis et al. [21] demonstrated a protective effect of melatonin when administered synchronously with a high-fat diet; however, this effect could not be demonstrated in the group of rats that received melatonin at a late stage of the experiment, i.e., as a treatment once hepatic steatosis was established. Pan et al. [20] also showed that a moderate or high dose of melatonin (5–10 mg/kg/day) improved the degree of hepatic steatosis when administered concomitantly to the high-fat diet.

Human studies have the handicap that they do not perform biopsies to corroborate the improvement of hepatic steatosis after the administration of melatonin, probably because of the not inconsiderable percentage of complications that this diagnostic method entails.

With this work, we can demonstrate a protective effect of melatonin in terms of the lower progression of steatosis in the histological sample; however, we cannot show improvement, as in other studies. We also discovered a hypolipidemic effect of melatonin, since animals that maintain the steatotic diet and take melatonin reduce their levels of total and LDL cholesterol whilst increasing their levels of HDL cholesterol, although these results are not statistically significant. As previously stated, in our work, we start from very high levels of steatosis. Rodents in the experimental studies were administered melatonin concomitantly with the fat-rich diet, so these studies aim to find a protective effect of melatonin in the establishment of hepatic steatosis, not the reversal of hepatic steatosis, which is the aim of our study. On the other hand, in studies carried out in humans, the degree of steatosis from which we start does not reach the very high levels of steatosis from which we start. It is, therefore, possible that melatonin has a preventive effect on liver injury or even serves as a treatment for hepatic steatosis when the degree of fat involvement of hepatocytes is lower, being difficult to reverse when the hepatocyte involvement is the majority and does not cease the stimulus that produced it.

Another aspect to keep in mind is the melatonin administration time. We have seen that in our study positive results are obtained at the molecular level, in the intimate mechanism of production of NAFLD; that is to say, we observe that melatonin attenuates oxidative stress and lipid peroxidation in groups of animals that continue with the diet; however, the changes at a histological level have only been demonstrated at the time of preventing progression. This could be because the period of administration of

melatonin (4 weeks) or the dose administered (10 mg/kg/day) is insufficient to observe changes in the accumulation of hepatocyte lipids at the histological level. Studies with a longer administration time of melatonin or with a higher dose would be needed to demonstrate this effect.

We believe that one of the greatest strengths of our study was the experimental model used. It is original from our group who are experienced in another work [45]. In addition, we tried to individualize the effect of melatonin from the effect of the intake of the steatotic diet. Many of the papers do not include groups with discontinuation of the diet or seek purely the protective effect of the drug administered in conjunction with the diet. In our work, we preferred to use 4 different groups of animals, which forced us to use a considerable number of experimental animals but allowed us to simulate the different scenarios in which melatonin can be administered as a drug to treat NAFLD.

## Data Availability

The data used to support this study are provided in the Supplementary Materials. The complementary data can be obtained from the corresponding author upon request.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

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*Research Article*

# Bredemolic Acid Ameliorates Selected Liver Function Biomarkers in a Diet-Induced Prediabetic Rat Model

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*Background*. Prediabetes is an intermediary hyperglycaemic state that precedes type 2 diabetes mellitus (T2DM) in which abnormal metabolism of glucose and lipids occurs in organs such as the liver. Evidence has shown that, about 70% of T2DM patients develop hepatic dysfunction which is found to begin during the prediabetic stage. Bredemolic acid, a pentacyclic triterpene, has been found to improve insulin sensitivity in diet-induced prediabetic rats. The effects of this compound on liver function, however, are unknown. This study was therefore designed to investigate the effects of BA on liver function in high fat-high carbohydrate (HFHC) diet-induced prediabetic rats. *Methods*. Thirty-six (36) male rats that weigh 150 g–180 g were divided into two groups, the non-prediabetic ($n = 6$) and the prediabetic groups ($n = 30$) that were fed normal diet (ND) and HFHC diet, respectively. The prediabetic rats were further subdivided into five groups ($n = 6$) and treated with either BA (80 mg/kg) or metformin (MET, 500 mg/kg) every third day for 12 weeks. After 12 weeks, blood samples and the liver were collected for biochemical analysis. *Results*. The induction of prediabetes resulted in increased release of liver enzymes (AST and ALT), increased liver glycogen and triglyceride, lipid peroxidation, and decreased sterol regulatory element-binding protein (SREBP1c) and antioxidant enzymes. However, the administration of BA decreased liver enzyme concentrations, decreased hepatic oxidative stress, and improved antioxidant enzymes such as SOD and GPx. *Conclusion*. BA administration improved liver function in diet-induced prediabetic rats in the presence or absence of dietary intervention.

## 1. Introduction

Prediabetes is a state of intermediate hyperglycaemia that causes abnormal changes in intracellular metabolism of most body tissues including the liver [1]. Presently, the observed increase in the prevalence of prediabetes and type 2 diabetes mellitus (T2DM) in developed and developing countries is reported to be due to sedentary lifestyles coupled with high-caloric diets [1–3]. However, studies have shown that excessive intake of high-caloric diets induces skeletal muscle insulin resistance which results into the shunting of glucose from the skeletal muscle to the liver thereby leading to increased hepatic glycogen production and storage [4–6]. Several studies have shown that continuous intake of high quantities of fats and carbohydrates alters liver function by accumulation of ectopic fats as a result of *de novo* lipogenesis which is mediated by transcription factors such as sterol regulatory element-binding protein (SREBP1c) under insulin action [7, 8]. Moreover, excessive hepatic accumulation of free fatty acid or triglyceride leads to hepatic insulin resistance, hepatic dysfunction, and nonalcoholic fatty liver disease (NAFLD) that is characterized by fat infiltration into the hepatocytes [9–14]. Consequently, the infiltration of fat into the hepatocytes triggers oxidative stress, and reduces antioxidant enzymes production and caused an inflammatory cascade of reactions that produce progressive fibrotic

hepatic damage known as nonalcoholic steatohepatitis (NASH). Cross-sectional studies have demonstrated that liver function markers such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are altered due to oxidative stress and hepatic dysfunction [15–18]. However, it has been established that approximately 70% of T2DM patients have liver dysfunction and complications [19–21]. There is also evidence from other studies that suggested that liver dysfunction and complications can also begin during the prediabetic stage [21–23].

Current treatment focuses on a combination of dietary and pharmacological interventions, but there has been reports of low compliance as patients merely use pharmacological intervention without diet modification thus reducing the efficacy of the pharmacological intervention [24–27]. Therefore, novel compounds that can ameliorate liver dysfunction in the prediabetic condition even in the absence of dietary intervention are necessary. Oleanolic acid and maslinic acid are pentacyclic triterpenes that have been found to have antidiabetic and antioxidant properties [28–30]. In our laboratory, we have shown that chronic ingestion of a high fat-high carbohydrate diet leads to the development of prediabetes which is accompanied by liver complications. We have further shown that bredemolic acid (BA), a structural isomer of maslinic acid, is able to restore glucose homeostasis in diet-induced prediabetes by improving insulin sensitivity both in presence and absence of dietary intervention [31]. However, the effects of BA on liver function in diet-induced prediabetes have not been established. Hence, the aim of this study is to investigate the effects of bredemolic acid on selected biomarkers of liver function in a diet-induced prediabetic rat model.

## 2. Materials and Methods

*2.1. Animals.* Thirty-six (36) male Sprague Dawley rats (150–180 g) obtained from Biomedical Research Unit, University of KwaZulu-Natal (UKZN), were kept under standard environmental conditions i.e., constant humidity (55 ± 5%), temperature (22 ± 2°C), 12 h day : 12 h night cycle. The animals were acclimatized for 2 weeks and consumed standard rat chow (Meadow Feeds, South Africa) and water *ad libitum* before being fed on the experimental high fat-high carbohydrate (HFHC) diet (AVI Products (Pty) Ltd., Waterfall, South Africa) to induce prediabetes. The HFHC diet consists of carbohydrate (55% kcal/g), fats (30% kcal/g), and proteins (15% kcal/g) as described in our previous study [27, 31]. All the experimental designs and procedures were carried out according to the ethics and guidelines of the Animal Research Ethics Committee (AREC) of the UKZN, Durban, South Africa.

*2.2. Experimental Design.* After acclimatization, the animals were divided into two groups, the normal diet (ND) non-prediabetic control ($n = 6$) and the HFHC diet prediabetic groups ($n = 30$). All the animals in the prediabetic group consumed HFHC diet and drinking water that was supplemented with 15% fructose for 20 weeks to induce prediabetes while the non-prediabetic control group (NPD, Group 1) fed on ND and water *ad libitum* for 20 weeks as well. At the 20$^{th}$ week, prediabetes was confirmed by fasting blood glucose and oral glucose tolerance test which have been described in the previous research study [31].

*2.3. Treatment of Prediabetic Animals.* After 20 weeks of prediabetes induction, the non-prediabetic control (NPD, Group 1) animals were continuously fed on standard rat chow for 12 weeks. Thirty (30) prediabetic animals were randomly assigned into 5 different groups (Group 2 to Group 6, $n = 6$). Group 2 (PD) served as the untreated prediabetic control group and continuously consumed the HFHC diet for 12 weeks; Group 3 (ND + MET) were prediabetic animals that switched to standard rat chow and received metformin (MET) for 12 weeks; Group 4 (HFHC + MET) were prediabetic animals that continuously consumed HFHC diet with MET treatment; Group 5 (ND + BA) were prediabetic animals that switched to standard rat chow and received BA for 12 weeks; and Group 6 (HFHC + BA) were prediabetic animals that continuously consumed HFHC diet and received BA as treatment for 12 weeks. Treatment via oral administration of MET (7.2 mg/kg, extrapolated from 500 mg/70 kg human dose) or BA (80 mg/kg) was carried out every third day for 12 weeks as described in our previous study [31].

*2.4. Blood Collection and Tissue Harvesting.* After the 12$^{th}$ week treatment period, the animals were sacrificed. The animals were placed in a gas anaesthetic chamber (Biomedical Research Unit, UKZN, Durban, South Africa) and anaesthetised with Isofor (100 mg/kg, Safeline Pharmaceuticals, Roodepoort, South Africa) for 3 minutes. Blood samples were collected from the animals using cardiac puncture and put into different precooled EDTA containers. The blood samples were centrifuged (Eppendorf centrifuge 5403, Germany) at 4°C, 503$g$ for 15 minutes to obtain plasma. Each of the plasma was aspirated into plain sample bottles and stored at −80°C in a BioUltra freezer (Snijers Scientific, Tilburg, Holland) until ready for biochemical analysis. Also, the liver tissue samples were excised, weighed, and rinsed in cold normal saline solution and snapped frozen in liquid nitrogen before storage in the BioUltra freezer for biochemical analysis of selected metabolic parameters.

*2.5. Relative Liver Weight.* The relative liver weights of all the animals in each experimental group were determined from the percentage of the ratio of liver weight to the body weight i.e.,

$$\text{relative liver weight} = \frac{\text{liver weight}}{\text{body weight}} \times 100. \quad (1)$$

*2.6. Biochemical Analysis.* Liver enzymes (AST and ALT) in the plasma were analysed with IDEXX Catalyst One Chemistry Analyzer (IDEXX Laboratories Inc., Westbrook,

USA) while SREBP1c in the liver homogenate was analysed by following specific ELISA kit procedures using manufacturer's instructions (Elabscience Biotechnology Co., Ltd., Houston, TX, USA). Fasting blood insulin (FBI) was also analysed and determined as reported in our previous study [31].

*2.7. Liver Triglycerides.* The preparation of liver tissue samples and the homogenate medium used for determination of hepatic triglyceride were according to the manufacturer instruction in the triglyceride assay kit (Elabscience Biotechnology Co., Ltd., Houston, TX, USA). 50 mg of liver tissue was homogenized on ice in 500 $\mu$l phosphate buffer saline (PBS) and centrifuged at 8000 rpm for 10 minutes, 4°C. The supernatant was then aspirated into Eppendorf tubes, and triglycerides were determined using the triglyceride assay kit as instructed in the manufacturer's manual. The absorbance of the samples was measured at 510 nm by using Spectrostar Nanospectrophotometer (BMG Labtech, Ortenburg, LGBW Germany).

*2.8. Liver Glycogen Assay.* Glycogen assay was determined in the liver by following previous established protocol [27, 28, 32]. The absorbance was determined by using the Spectrostar Nanospectrophotometer at 620 nm.

*2.9. Lipid Peroxidation and Antioxidant Profile.* The concentration of malondialdehyde (MDA) in the liver was determined to estimate the amount of lipid peroxidation according to previously described protocol [29, 32]. Furthermore, the antioxidant profile of the liver was determined by measuring the activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) according to the manufacturer's instructions (Elabscience Biotechnology Co., Ltd., Houston, TX, USA).

*2.10. Statistical Analysis.* The statistical data were presented in mean ± SEM. The data were analysed by two-way analysis of variance (ANOVA) with Bonferroni test (post hoc test) via GraphPad Prism 5 software. Also, Pearson's correlation was used to calculate the correlation coefficient between FBI and hepatic SREBP1c through the GraphPad Prism 5. The level of statistical significance was determined at $p < 0.05$.

## 3. Results

*3.1. Relative Liver Weight.* The effects of BA treatment on relative liver weights in non-prediabetic and prediabetic rats with or without diet intervention were determined. The relative liver weight of untreated prediabetic (PD) rats was significantly increased by comparison with the non-prediabetic control (NPD) rats ($p < 0.05$). However, the relative liver weight of the animals is dependent on the type of diet administered. Therefore, the administration of BA or MET and diet intervention significantly decreased the relative liver weight when compared with PD ($p < 0.05$), see Figure 1.

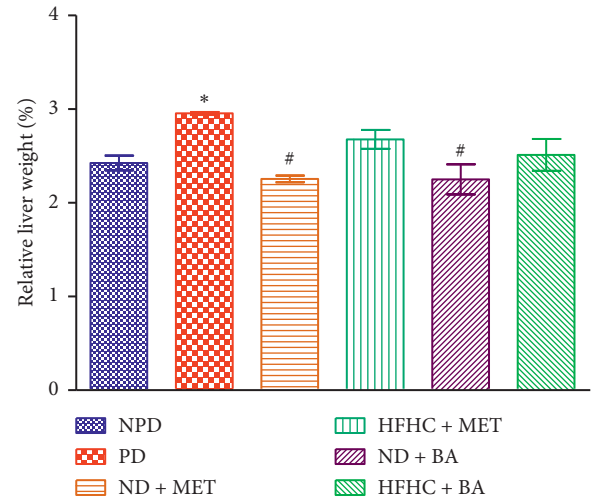


Figure 1: Effects of BA with the presence or absence of dietary intervention on the relative liver weight in prediabetic rats. $^{*}p < 0.05$ in comparison with NPD; $^{\#}p < 0.001$ in comparison with PD.

*3.2. Liver Enzymes.* Plasma AST and ALT concentrations in the PD group were significantly increased ($p < 0.01$) compared with the NPD group. However, the administration of BA with or without diet intervention significantly decreased the plasma AST and ALT concentrations when compared with PD. The plasma ALT levels of metformin-treated rats with diet intervention (ND + MET) were significantly decreased when compared with PD while the plasma AST of ND + MET was insignificantly different when compared with PD ($p < 0.05$), see Figure 2.

*3.3. SREBP1c.* The liver SREBP1c concentration was determined in non-prediabetic and prediabetic rats. The liver SREBP1c levels were significantly decreased in PD groups when compared with the NPD group ($p < 0.001$). The administration of BA with or without diet intervention significantly increased the liver SREBP1c concentration in comparison with the PD group ($p < 0.001$). Interestingly, the administration of metformin with diet intervention (ND + MET) significantly increased the SREBP1c concentration when compared with the PD group ($p < 0.05$). The administration of metformin in the absence of dietary intervention did not have any significant effects when compared with the PD control, see Figure 3.

*3.4. FBI and Hepatic SREBP1c Correlation.* The correlation between FBI and hepatic SREBP1c was determined in all the groups under different experimental conditions as indicated in Table 1. There was a significant negative correlation between FBI and hepatic SREBP1c in PD, HFHC + MET, and ND + MET groups ($r = -0.9144$, $-0.8869$, and $-0.8691$, respectively) at $p < 0.05$. Therefore, as FBI increased significantly in impaired insulin signaling, there was significant decrease in hepatic SREBP1c concentration. However, there was insignificant correlation between the FBI and hepatic SREBP1c in non-prediabetic (NPD) and prediabetic rats

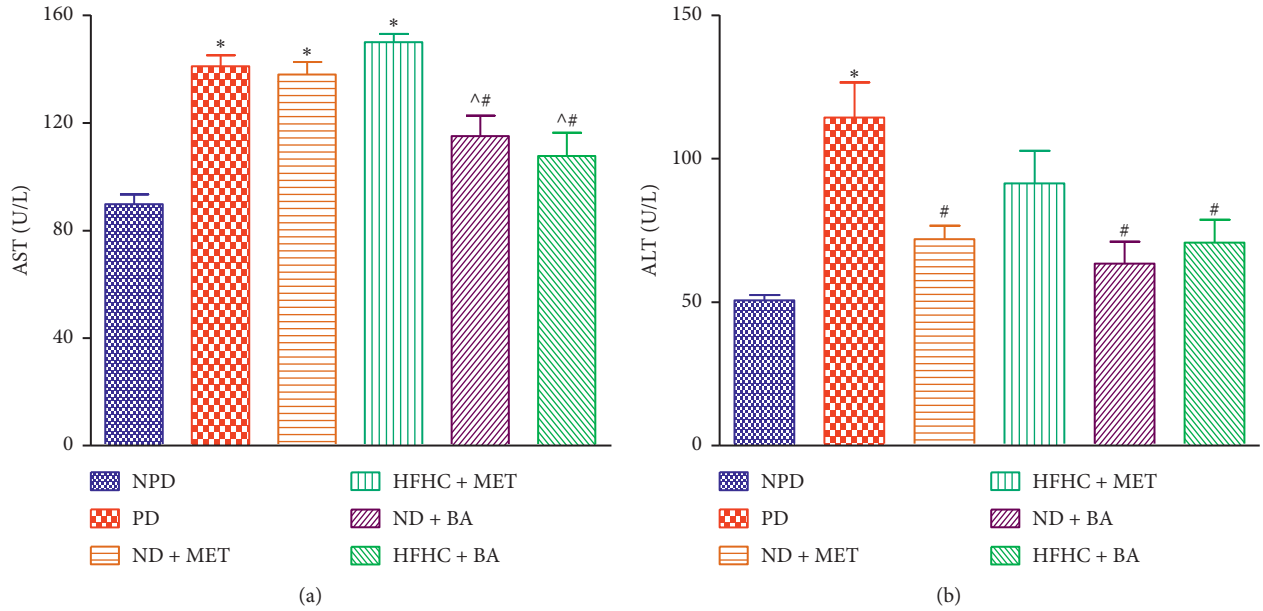


Figure 2: Effects of BA with the presence or absence of dietary intervention on the plasma AST and ALT in prediabetic rats. $^{*}p < 0.001$ in comparison with NPD; $^{\#}p < 0.05$ in comparison with PD.

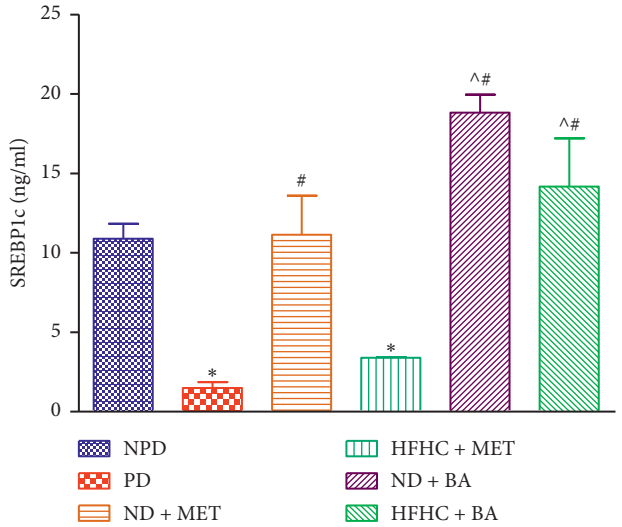


Figure 3: Effects of BA with the presence or absence of dietary intervention on the liver SREBP1c in prediabetic rats. $^{*}p < 0.001$ in comparison with NPD, $^{\#}p < 0.001$ in comparison with PD, and $\hat{p} < 0.01$ in comparison with HFHC + MET.

treated with BA in the absence or presence of dietary intervention.

*3.5. Liver Triglycerides.* Liver triglyceride concentrations were significantly increased in the PD group by comparison with the NPD group ($p < 0.001$). The liver triglyceride concentration of BA-treated rats with or without diet intervention significantly decreased when compared with the PD group ($p < 0.001$). Similar results were observed with the use of metformin, see Figure 4.

*3.6. Liver Glycogen.* Liver glycogen concentrations of the PD group were significantly increased by comparison with the NPD group ($p < 0.001$). The administration of BA with or without diet intervention significantly decreased liver glycogen concentrations by comparison with PD ($p < 0.001$). Similarly, the administration of metformin treated with or without diet intervention significantly decreased the liver glycogen concentration when compared with PD, see Figure 5.

*3.7. Lipid Peroxidation and Antioxidant Enzyme Activity.* As shown in Table 2, liver MDA concentration in the untreated PD group was significantly increased by comparison with the NPD group ($p < 0.001$). The administration of BA and metformin with or without diet intervention significantly decreased the liver MDA concentration when compared with the PD group ($p < 0.05$). Liver SOD and GPx activities of the untreated PD group were significantly decreased when compared with the NPD group ($p < 0.05$). The SOD and GPx activities in the liver of BA-treated rats with or without diet intervention were significantly increased in comparison with those in PD group ($p < 0.05$).

## 4. Discussion

This study examined the effects of BA on selected markers of liver function in diet-induced prediabetic rats. Triterpenes such as maslinic acid and oleanolic acid have been reported to ameliorate oxidative stress in the liver via increased release of antioxidant enzymes and improved liver function via increased activity of glycogenic enzymes to decrease hepatic glucose production in diabetic rats [29, 32]. In a previous study, BA was shown to improve insulin sensitivity in the skeletal muscle by increasing the expression of GLUT 4;

Table 1: Correlation between fasting blood insulin (FBI) and hepatic sterol regulatory element-binding protein (SREBP1c) in non-prediabetic (NPD) rats, prediabetic control (PD), and prediabetic rats treated with BA in the presence or absence of dietary intervention. $r$ = Pearson's correlation coefficient, $R^2$ = coefficient of determination, and $n$ = sample size.

| Groups | Correlation analysis | Independent variable: FBI | Dependent variable: hepatic SREBP1c |
|---|---|---|---|
| NPD | $r$ | 0.8068 | 0.8068 |
| | $R^2$ | 0.6510 | 0.6510 |
| | $n$ | 6 | 6 |
| | $p$ value | $0.0524^{NS}$ | $0.0524^{NS}$ |
| PD | $r$ | −0.9144 | −0.9144 |
| | $R^2$ | 0.8361 | 0.8361 |
| | $n$ | 6 | 6 |
| | $p$ value | $0.0107^{*}$ | $0.0107^{*}$ |
| ND + MET | $r$ | −0.8691 | −0.8691 |
| | $R^2$ | 0.7552 | 0.7552 |
| | $n$ | 6 | 6 |
| | $p$ value | $0.0246^{*}$ | $0.0246^{*}$ |
| HFHC + MET | $r$ | −0.8869 | −0.8869 |
| | $R^2$ | 0.7866 | 0.7866 |
| | $n$ | 6 | 6 |
| | $p$ value | $0.0185^{*}$ | $0.0185^{*}$ |
| ND + BA | $r$ | 0.4651 | 0.4651 |
| | $R^2$ | 0.2164 | 0.2164 |
| | $n$ | 6 | 6 |
| | $p$ value | $0.3526^{NS}$ | $0.3526^{NS}$ |
| HFHC + BA | $r$ | −0.7381 | 0.7381 |
| | $R^2$ | 0.5448 | 0.5448 |
| | $n$ | 6 | 6 |
| | $p$ value | $0.0939^{NS}$ | $0.0939^{NS}$ |

$^{NS}$Not significant; $^{*}p < 0.05$.

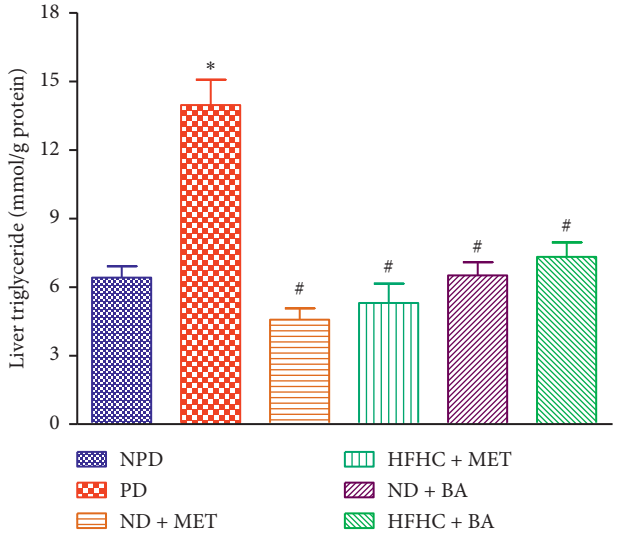


Figure 4: Effects of BA with the presence or absence of dietary intervention on the liver triglyceride in prediabetic rats. $^{*}p < 0.001$ in comparison with NPD; $^{\#}p < 0.001$ in comparison with PD.

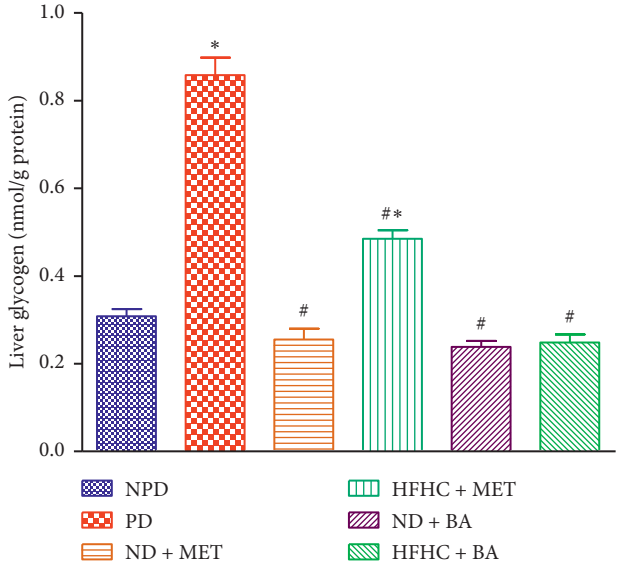


Figure 5: Effects of BA with the presence or absence of dietary intervention on the liver glycogen in prediabetic rats. $^{*}p < 0.001$ in comparison with NPD; $^{\#}p < 0.001$ in comparison with PD.

however, the effects of this triterpene on liver function in the prediabetic state were not determined [31]. Hence, this study is a continuation of the previous study [31] and sought to evaluate the effects of BA on selected markers of liver function in a diet-induced prediabetic rat model. The liver plays a key role in maintaining glucose homeostasis as it balances the production of glucose and the conversion of glucose to glycogen [33]. In a postprandial state, blood glucose increases, and insulin is secreted to enhance glycogenesis and inhibit glycogenolysis [34]. However, studies have shown that chronic consumption of high fat-high carbohydrate diet results in the induction of prediabetes which is characterized by hyperinsulinaemia, impaired

Table 2: Effects of BA with the presence or absence of dietary intervention on the liver lipid peroxidation and antioxidant enzyme activities in prediabetic rats. Values are presented as mean ± SEM ($n = 6$).

| Groups | Malondialdehyde (MDA) (nmol/g protein) | Superoxide dismutase (SOD) (nmol·min$^{-1}$·mL·mg$^{-1}$protein) | Glutathione peroxidase (GPx) (nmol·min$^{-1}$·mL·mg$^{-1}$protein) |
|---|---|---|---|
| NPD | $4.11 \pm 0.51$ | $2.99 \pm 0.06$ | $1.67 \pm 0.09$ |
| PD | $12.34 \pm 1.31^{*}$ | $1.66 \pm 0.22^{*}$ | $1.08 \pm 0.06^{*}$ |
| ND + MET | $5.00 \pm 0.26^{\#}$ | $2.14 \pm 0.02^{\#}$ | $1.79 \pm 0.07^{\#\wedge}$ |
| HFHC + MET | $6.41 \pm 0.27^{\#}$ | $1.83 \pm 0.13^{*}$ | $1.05 \pm 0.05^{*}$ |
| ND + BA | $4.89 \pm 0.44^{\#}$ | $2.47 \pm 0.06^{\#}$ | $1.87 \pm 0.10^{\#\wedge}$ |
| HFHC + BA | $6.68 \pm 0.65^{\#}$ | $2.59 \pm 0.02^{\#}$ | $1.89 \pm 0.04^{\#\wedge}$ |

$^{*}p < 0.05$ in comparison with the non-prediabetic (NPD) control, $^{\#}p < 0.05$ in comparison with the prediabetic (PD) control, and $\wedge p < 0.05$ in comparison with the HFHC + MET group.

glucose tolerance, and peripheral and hepatic insulin resistance, as well as liver damage [1, 35, 36]. In the prediabetic state, due to hyperinsulinaemia and selective muscle insulin resistance, most ingested glucose is shunted to the liver leading to increased hepatic glycogenesis [6, 37]. In addition, since the liver is insulin-independent, excess glucose in the blood can diffuse into the hepatic cells through facilitated diffusion which is mediated by glucose transporter 2 (GLUT (2)) [14, 34, 38]. Similarly, the elevated liver glycogen concentration observed in untreated prediabetic rats in this study can be attributed to the increased diversion of excess glucose to the liver. This showed that consumption of high fat-high carbohydrate diet can result into diversion of glucose to the liver as a compensatory mechanism in the presence of selective muscle resistance in the prediabetic state [34]. However, the administration of BA with or without diet intervention significantly reduced liver glycogen concentrations. Previous studies have shown that administration of BA in the prediabetic state improves insulin sensitivity in the skeletal muscle through increased GLUT 4 expression [31]. We suggest that this improved insulin sensitivity in the periphery leads to decreased amounts of glucose being shunted to the liver thus resulting in the observed decrease in liver glycogen concentrations.

In nondiabetic subjects, metabolism of glucose is largely carried out in the skeletal muscle [39, 40]. In the prediabetic state, as glucose delivery to the liver increases, *de novo* lipogenesis and hepatic lipid accumulation increase under the influence of transcription factors such as SREBP1c [6, 14, 37, 40]. SREBP1c is a major transcription factor which regulates *de novo* lipogenesis through direct activation from AKT (protein kinase B) in the insulin signaling pathway [8, 41, 42]. In the prediabetic state, when insulin signaling is impaired, the direct activation of SREBP1c by AKT is altered, and the SREBP1c expression decreases [6–8]. On the contrary, the hepatic *de novo* lipogenesis is not solely dependent on insulin signaling through activation of SREBP1c, but the activation of SREBP1c to stimulate *de novo* lipogenesis depends on insulin signaling [6, 43]. However, when the insulin signaling pathway is impaired in prediabetes, *de novo* lipogenesis is still elevated due to the substrate push mechanism in which there is increased substrate delivery to the liver followed by increased esterification of fatty acids into triglycerides [6]. In this study, we observed that the concentration of SREBP1c in the liver was significantly lowered in untreated prediabetic rats by comparison with the non-prediabetic rats. According to our correlation analysis between fasting blood insulin and hepatic SREBP1c, the decreased hepatic SREBP1c in untreated prediabetic rats may be due to the alteration of insulin signaling in the prediabetic state since SREBP1c expression is insulin-dependent. In addition, the correlation analysis showed that there was an inverse relationship between the increased fasting blood insulin and the hepatic SREBP1c concentration under the insulin-resistant condition. This observation is in correlation with previous studies which reported that insulin signaling is not totally required for hepatic lipogenesis, and that availability of the substrate can facilitate delivery of substrates into the liver for lipogenesis [6, 44]. Of note, the BA-treated rats had a significantly increased SREBP1c thus suggesting that BA ameliorated insulin signaling which may have resulted into the increased SREBP1c concentration in the liver. Furthermore, high fructose consumption has been reported to increase hepatic lipogenesis and glycogenesis [1]. Fructose, unlike glucose, is solely metabolized in the liver thereby providing additional substrates for *de novo* lipogenesis and ectopic fat accumulation in the liver, thus leading to NAFLD [1, 10]. In this study, we observed that the liver triglyceride in untreated prediabetic rats significantly increased when compared with non-prediabetic rats. The increased liver triglyceride in untreated prediabetic rats can be attributed to increased substrate delivery to the liver or decreased hepatocellular triglyceride disposal, as well as decreased fatty acid oxidation [45]. However, the administration of BA significantly decreased hepatic triglycerides, and this suggests that BA may decrease substrate delivery to the liver by divergence of the substrates to other organs for metabolism, increased $\beta$ oxidation of fat, or increased triglyceride disposal via very low-density lipoprotein (VLDL) exportation from the liver.

Moreover, due to the increased hepatic lipogenesis and glycogenesis, the production of free radicals is elevated, and this results into oxidative stress [46]. Oxidative stress is due to an imbalance between oxidant and antioxidant enzymes [46]. Antioxidants are stable molecules that donate electrons to rampaging free radicals in order to neutralise the free radical capacity to damage tissues or organs [47, 48]. In this study, we observed that lipid peroxidation (MDA) in the liver was significantly increased, and antioxidant enzyme (SOD and GPx) production in the liver was significantly decreased in the

untreated prediabetic rats when compared with non-prediabetic rats. The increased lipid peroxidation was due to increased production of free radicals while the decreased antioxidant capacity of the liver was as a result of decreased production of antioxidant enzymes (SOD and GPx) in the mitochondria of hepatocytes during prediabetes. On the contrary, BA administration with or without diet intervention significantly lowered lipid peroxidation and significantly increased the liver antioxidant enzymes. This may be due to the fact that BA neutralises the free radicals in the mitochondria of hepatocytes by donation of electron through hydroxyl radical scavenging activity which has been reported in other triterpenes [49]. This is in line with similar observations made on earlier studies using other triterpenes [28, 32, 49].

Furthermore, studies have shown that elevated liver enzymes (AST and ALT) in the plasma can be due to necrosis of the hepatocyte during liver damage [18]. AST and ALT are released into the blood stream whenever hepatocytes are damaged, and this has been reported to occur during prediabetes [18]. In this study, these enzymes were significantly elevated in untreated prediabetic rats by comparison with non-prediabetic rats. The increased liver enzymes in the plasma suggested that liver cells are damaged through oxidative stress and increased hepatic lipogenesis or glycogenesis. However, BA administration caused a decrease in the concentration of liver enzymes suggesting that BA may improve hepatic function via its antioxidant and antilipidemic effects in the liver as observed in this study. Of note, triterpenes are nontoxic antioxidants and have low pharmacokinetics of three days; therefore, the ameliorative effects of BA in the absence of dietary intervention on liver function markers compared with metformin in this study may be attributed to this low pharmacokinetic feature. In conclusion, the administration of BA in both the presence and absence of dietary modification can potentially be one of the therapeutic approaches to attenuate hepatic dysfunction or improve hepatic functions in the prediabetic state.

## Data Availability

The data used to support the findings of this study are available upon request from the corresponding author. However, the data on body weight, fasting blood insulin (FBI), fasting blood glucose, and oral glucose tolerance test which are relevant for this study have been reported in our previous study.

## Conflicts of Interest

The authors declare no conflicts of interest.

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*Review Article*

# A Potential Linking between Vitamin D and Adipose Metabolic Disorders

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Vitamin D has been discovered centuries ago, and current studies have focused on the biological effects of vitamin D on adipogenesis. Besides its role in calcium homeostasis and energy metabolism, vitamin D is also involved in the regulation of development and process of metabolic disorders. Adipose tissue is a major storage depot of vitamin D. This review summarized studies on the relationship between vitamin D and adipogenesis and furthermore focuses on adipose metabolic disorders. We reviewed the biological roles and functionalities of vitamin D, the correlation between vitamin D and adipose tissue, the effect of vitamin D on adipogenesis, and adipose metabolic diseases. Vitamin D is associated with adipogenesis, and vitamin D supplements can reduce the burden caused by metabolic diseases. The review provides new insights and basis for medical therapy on adipose metabolic diseases.

## 1. Introduction

Vitamin D is an essential nutrient for the prevention of rickets and is responsible for the intestinal absorption of calcium, phosphate, and magnesium [1, 2]. Vitamin D can be obtained from food, but most of it is synthesized from 7-dehydrocholesterol in the skin via ultraviolet irradiation [3, 4]. The mechanism of vitamin D action is through its active form, 1$\alpha$, 25-dihydroxyvitamin $D_3$ [1$\alpha$, 25$(OH)_2D_3$], which regulates the transcription of target genes and thus plays an important role in calcium homeostasis and metabolism [5–7]. Vitamin D deficiency or insufficiency is still a common issue in developing countries [8, 9]. Among 734 adolescents ranging from 12 to 18 years, 87.6% of participants had vitamin D deficiency [10]. Aside from its involvement in calcium and bone mineralization, vitamin D has multiple functions in adipose tissue, adipogenesis, glucose-insulin homeostasis, cell growth, and so on [11–13].

Adipose tissue is a vital organ in energy homeostasis and glucose metabolism [14–16]. Adipose tissue is also an endocrine organ secreting proteins and releasing fatty acids [17, 18]. It is composed of various cell types, including mature adipocytes, preadipocytes, fibroblasts, macrophages, and immune cells. The predominant cell types existing in adipose tissue are mature adipocytes. Preadipocytes differentiate into mature adipocytes in adipose tissue, and this process requires the regulation of transcription factors (peroxisome proliferator-activated receptor (PPAR), CCAAT enhancer-binding protein (C/EBP), and Kruppel-like factor proteins) [19, 20]. Vitamin D is mainly stored in adipose tissue, while vitamin D receptor (VDR) is expressed in adipose tissue [21, 22]. VDR is an activated transcription factor of active vitamin D. Previous studies investigated the effect of vitamin D on adipogenesis in animal models [23, 24], and results suggested that vitamin D exerts anti-adipogenic activity in 3T3-L1 preadipocytes [25–27]. Meanwhile, vitamin D deficiency or insufficiency is involved in the regulation of insulin secretion, glucose levels, and inflammation causing adipose metabolic diseases, such as obesity, multiple sclerosis, diabetes, and fatty liver [28–32].

Hence, we reviewed the correlation between vitamin D and adipose tissue, along with related metabolic disorders

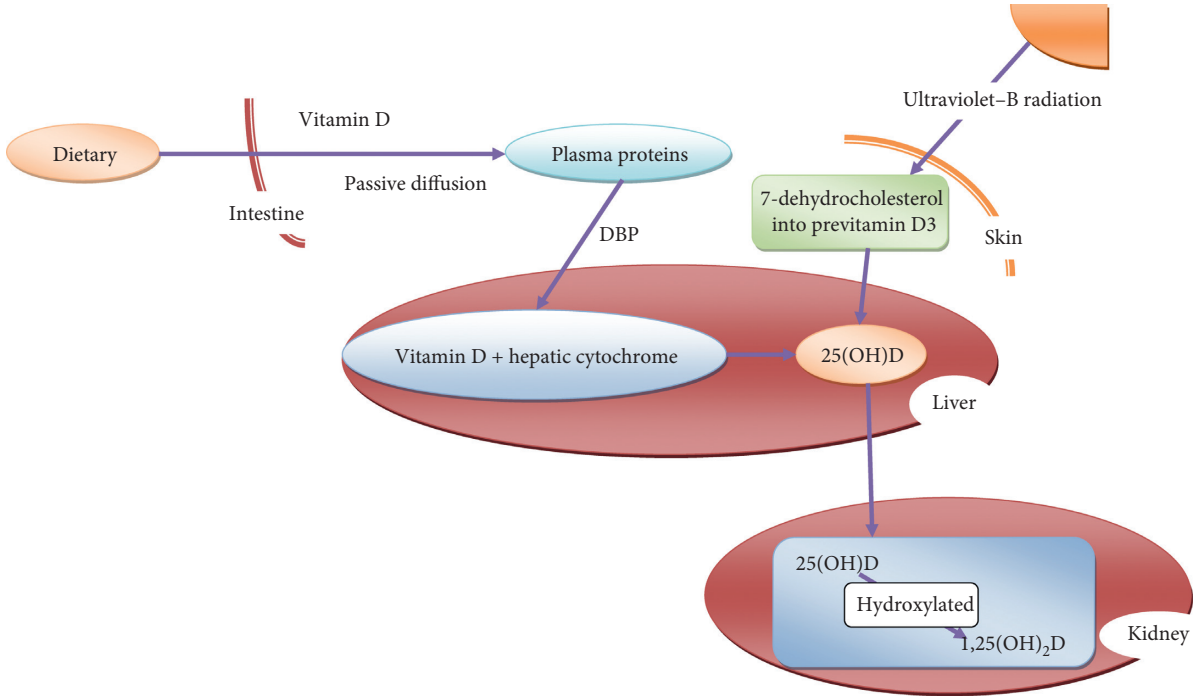


Figure 1: Bioactivation of vitamin D.

(diabetes, nonalcohol fatty liver, and cardiovascular diseases). This study aimed to establish the relationship between vitamin D and metabolic disorders and furthermore to determine whether such disorders are affected by vitamin D supplementation, adipose vitamin D metabolism, and increased or reduced vitamin D activation. The vitamin D status, regulation of vitamin D by adipose tissue, effect of vitamin D on adipogenesis, and related metabolic diseases were also discussed.

## 2. Bioactivation of Vitamin D

Only a small amount of fat-soluble vitamin D can be obtained from the diet and from supplements [33]. The major source of vitamin D is produced in the skin through a sunlight-dependent chemical reaction [4, 34]. Exposure of the skin to ultraviolet-B radiation from the sun converts 7-dehydrocholesterol into previtamin $D_3$, which can be isomerized to vitamin $D_3$ [35]. Vitamin $D_3$ is then converted into calcifediol (25-(OH)D) in the liver and further hydroxylated into $1,25(OH)_2D$ in the kidney (Figure 1) [36]. Normally, the serum concentration of 25-(OH)D is measured to determine an individual's vitamin D status in serum, whereas $1,25(OH)_2D$ is the biological active form of vitamin D [37–39].

The biological activity of $1,25(OH)_2D$ is mediated through binding to VDR [40]. VDR is also well-documented as calcitriol receptor and is a member of the steroid hormone nuclear receptor family [41]. In humans, VDR is encoded by the VDR gene [42]. VDR widely exists in tissues and cells, such as skeleton, kidney, renal, skin, and immunocytes [4]. In nuclear, $1\alpha,25\text{-}(OH)_2D_3$, which is the active form of vitamin D, is capable of binding to VDR and form a heterodimer with retinoid X receptor (RXR); the complex binds to RNA polymerase and VDR interacting protein, resulting in the regulation of DNA transcription [43, 44]. Downstream targets of VDR are involved in calcium homeostasis, immune response, and cancer development [45]. Hindered VDR expression can impact diverse diseases, including cardiovascular disease, diabetes, tumors, tuberculosis, and multiple sclerosis [46].

After dietary intake, vitamin D needs to be absorbed by meal of fat through passive diffusion in the intestine. The absorbed vitamin D is transported to the liver by binding to diverse plasma proteins, such as vitamin D-binding protein (DBP), $\beta$-lipoprotein, and albumin [47–49]. DBP is an important carrier protein that can attenuate the toxicity of vitamin D by limiting its bound metabolites to target cells [50]. In the liver, vitamin D is converted into 25(OH)D catalyzed by the several hepatic cytochrome P-450s [51–53]. Of note, the metabolite is released into plasma and transported to the kidney, where it is converted into 1,25(OH)D, and is finally transported throughout the body (Figure 1) [54]. After synthesis, absorption, and transport, active vitamin D is distributed to hydrophobic parts of tissues [55]. Unlike other fat-soluble vitamin, vitamin D is not stored in the liver (except in some fish livers) [56]. Vitamin D is mostly stored in adipose tissue, and a large amount of vitamin D is combined with lipid, resulting in release and metabolic difficulties [57, 58].

## 3. Vitamin D and Lipid Metabolism

*3.1. Regulation of Activation of Vitamin D by Adipose Tissue.* Aside from the important roles of vitamin D in intestinal calcium, phosphate uptake, and bone mass regulation, it is

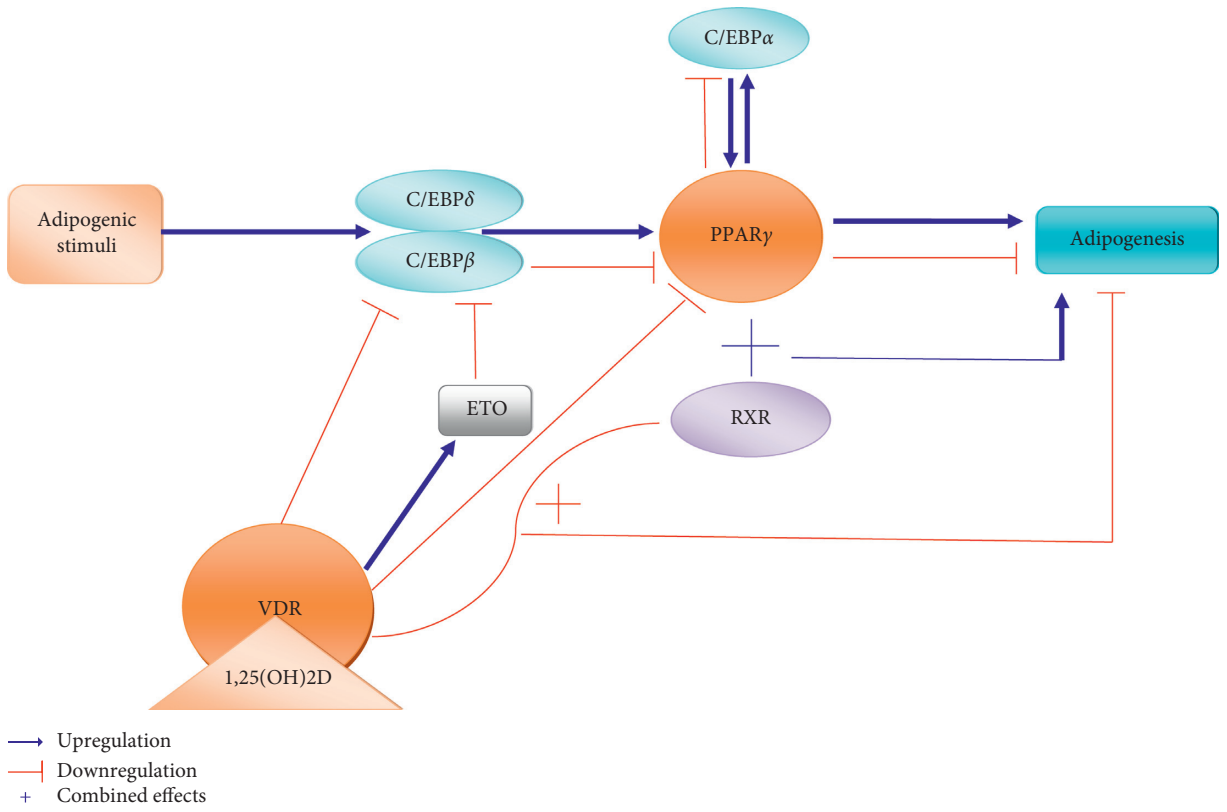


Figure 2: The relationship between vitamin D and adipogenesis. ETO: eight twenty-one. PPARγ: peroxisome proliferator-activated receptor γ. C/EBPα: CCAAT enhancer binding protein α. C/EBPβ: CCAAT enhancer binding protein β. C/EBPδ: CCAAT enhancer binding protein δ. VDR: vitamin D receptor. RXR: retinoid X receptor.

also involved in other processes, including cell growth, immune functions, inflammation regulation, and neuromuscular functions [59–63]. Vitamin D could be regulated by hydroxylation that includes two-step enzymatic processes. Hydroxylation is performed by enzymes CYP27B1, CYP2J2, CYP27A1, and CYP3A4 in the liver and CYP27B1 in the kidney [64]. These enzymes are consecutively expressed in subcutaneous adipose tissue (SAT) and visceral adipose tissue [65, 66]. The CYP27B1 gene is expressed in the SAT of lean individuals and in 3T3-L1 preadipocytes, and it is regulated by calcitonin, hormones, calcium, and phosphorus [53]. Thus, the location of these enzymes in adipose tissue could implicate the production of active vitamin D.

Previous reports showed that the concentrations of vitamin D in blood below 50 nmol/L indicated vitamin D deficiency, and the reference values of vitamin D levels in blood should exceed 75 nmol/L; lower serum vitamin D levels correlated with higher frequency of obesity and excessive body weight [67–69]. Circulating 25(OH)D level depends on the storage of vitamin D in adipose tissue, indicating that adipose tissue probably affects the activation, regulation, or action of obesity via regulation of vitamin D [57, 70, 71]. In the presence of calcitriol, adipogenesis is blocked by VDR via downregulating both C/EBPβ nuclear protein levels and mRNA expression. In addition, 1,25(OH)2D3 allows for the upregulation of eight twenty-one (ETO), which is the core-repressor of C/EBPβ, and finally leads to C/EBPβ deficiency in adipogenesis [72]. VDR expression in 3T3-L1 cells inhabited PPARγ mRNA levels, which decreased adipogenesis [73]. These data indicated that VDR reduced adipogenesis through decreasing the expression of C/EBPβ and PPARγ and increasing ETO expression. The molecular mechanism of inhibitory effect of VDR on adipogenesis maybe due to the fact that RXR is a heterodimeric partner for both PPARγ and VDR, respectively, and that VDR leads to competition between RXR and PPARγ to decrease adipogenesis (Figure 2). Previous studies confirmed the competitive relationship between VDR and PPARγ for RXR [73–75]. Certainly, the mechanism underlying still needs to be proved by further investigation. Taken together, these investigations suggest that vitamin D plays a complex role through VDR and the transcription pathways in regulating adipogenesis.

*3.2. Effect of Active Vitamin D on Adipogenesis.* Adipogenesis is a cascade of differentiation that leads to adipocyte maturation. Adipocytes can affect many adipogenesis-related functions, such as adipokine secretion, lipid synthesis, fatty acid transfection, and insulin signaling response [76]. A vast amount of molecular interactions is involved during adipogenesis, and the main component is the expression of C/EBPβ and PPARγ [77]. C/EBPβ and C/EBPδ are expressed in the early stage of adipogenesis. The adipogenesis is promoted under the regulation of C/EBPα, β, and δ [78]. 1,25(OH)2D3 can inhibit 3T3-L1 preadipocyte

differentiation by downregulating C/EBP$\beta$ and PPAR$\gamma$ (Figure 2) [79, 80]. When combined with genistein, 1,25(OH)2D3 inhibits adipocyte lipid-binding protein 2 expression and fat accumulation in 3T3-L1 preadipocytes [81].

VDR plays a vital role in adipogenesis. The activity of vitamin D is performed through 1,25(OH)2D3-VDR actions, and the target organs of VDR are the liver, kidney, genitourinary tract, intestine, bone, brain, and various immune cells [5, 82]. VDR is expressed at the early stage of adipose differentiation [83]. Macrophage inflammation can induce the expression of VDR [84]. Knockdown of VDR in mice could lead to low-fat mass, high rates of $\beta$-oxidation, and adipogenesis inhibition; in $VDR^{+/+}$ cells, 1,25(OH)2D3 treatment can block adipogenesis [72, 83]. In the absence of 1,25(OH)2D3, unliganded VDR also inhibits 3T3-L1 preadipocyte differentiation [73]. These data suggest a potential correlation between VDR and adipogenesis. In different phases of adipogenesis, 1,25(OH)2D3 could exert anti-adipogenic activity through the WNT/$\beta$-catenin pathway, the expression of mRNA modulation, and phosphorylation of extracellular regulated kinase via the mitogen-activated protein signaling pathway [80, 85].

Adiponectin is a hormone produced in adipose tissue and the brain. It is involved in the regulation of fatty acid oxidation [86, 87]. Adiponectin is abundant in plasma and is inversely related to body mass index [88]. Therefore, the biological effect of adiponectin can be related to serum concentration. Increased adiponectin in transgenic mice showed that the differentiation of 3T3-F442A cells is reduced through suppression of the expression of preadipocyte factor-1 mRNA and CCAAT enhancer-binding protein [89]. Adiponectin also plays a role in the suppression of metabolic disorders that may cause obesity, nonalcoholic fatty liver disease (NAFLD), or type-2 diabetes mellitus (T2DM) [87, 90, 91]. Moreover, administration of leptin and adiponectin can reverse insulin resistance in mice [92]. 1,25(OH)2D3 treatment can upregulate adiponectin in vitro and inhibit anti-inflammatory cytokine expression, and daily intake of fortified vitamin D can improve inflammation in T2DM [88, 93, 94]. However, data on the effect of $1,25(OH)_2D_3$ on adiponectin in human adipocytes are lacking. Therefore, active vitamin D probably acts in adipogenesis by affecting insulin resistance, VDR, leptin expression, or inflammatory response [95–100].

## 4. Vitamin D Deficiency with Lipid Metabolism Diseases

*4.1. Type-2 Diabetes.* Diabetes mellitus (DM) is a consequence of metabolic disorders that contributes to the morbidity of obesity [101]. This disease has four types: type-1 DM (T1DM), type-2 DM (T2DM), gestational diabetes, and specific diabetes types with known causes [102]. T1DM, referred to as insulin-dependent DM, is caused by failure to produce enough insulin; T2DM is caused by the inability of the body to respond properly to insulin; gestational diabetes often occurs in pregnant women and could be overcome after pregnancy; and the fourth kind of DM is diabetes with known causes [102, 103]. About 90% of diabetes cases are T2DM. Therefore, insulin resistance is a major factor for T2DM development. The morbidity of T2DM can be affected by environment, obesity, and age [104].

Low concentration of vitamin D is associated with T2DM patients. The serum concentration of 1,25(OH)D in 69.9% of 103 patients is lower than 20 ng/ml and negatively correlated with hemoglobin A1C and insulin resistance [105]. In adults aged over 45 years, vitamin D deficiency is significantly associated with occurrence of T2DM [106]. Substantial evidence shows a link between vitamin D and T2DM [107].

Vitamin D deficiency can inhibit pancreatic insulin secretion; vitamin D can protect $\beta$-cells through cytokine regulation, promote depolarization by regulating the function of calcium-binding protein on pancreatic $\beta$-cells, and regulate the concentration of calcium ions and the flow of calcium through the cell membrane [108–111]. Therefore, the potential role of vitamin D is to induce the expression of insulin receptor, promote the expression of PPAR$\gamma$, or affect glucose transporter activity by regulating intracellular calcium levels [112–114].

Inflammation also participates in contributing insulin resistance. In T2DM patients, 1,25(OH)D can improve insulin resistance through negative regulation of the expression of inflammatory cytokines, such as interleukin-1, interleukin-6, interleukin-8, and tumor necrosis factor $\alpha$ [115]. Vitamin D deficiency can affect insulin secretion and resistance; thus, it plays a role in the occurrence and development of T2DM [116–118]. However, these meta-analyses still need to be improved because of the limited concentration used in the study (at least 2000 IU/day) and the short investigation period on the patients.

*4.2. Nonalcoholic Fatty Liver Disease.* NAFLD is a stress-induced liver injury that is associated with insulin resistance and metabolic syndrome [119]. The causes of NAFLD are diabetes, obesity, age, and diet [120]. NAFLD has two types, namely, nonalcoholic fatty liver and nonalcoholic steatohepatitis [121, 122]. This disease is usually treated through weight loss and exercise. NAFLD is the most common chronic liver disorder in western countries [123]. It is a continuous process of liver injury, which may lead to steatohepatitis, cirrhosis, and liver cancer [124]. Vitamin D plays an important role in NAFLD development [125]. About 75% of 5847 insulin resistance and metabolic syndrome patients have vitamin D deficiency [126]. The serum concentration of 1,25(OH)D is lower in patients with NAFLD than in normal patients, and the fatty liver index is negatively correlated with 1,25(OH)D level [127]. Treatment with vitamin D can improve insulin resistance in patients with glucose intolerance [128]. In vivo studies found that vitamin D deficiency and VDR knockdown reduce the secretion of insulin from pancreatic $\beta$-cells [129]. These results support that low levels of serum 25(OH)D are related to NAFLD.

Vitamin D is associated with insulin resistance phenotypic markers, such as HOMA-IR, ISI, adiponectin, triglyceride, and high-density lipoprotein cholesterol [130, 131]. A prospective study on 524 nondiabetic patients

aged 40–69 years has reported that serum 25(OH)D level is negatively correlated with blood glucose level and insulin resistance level [132]. The decrease in insulin sensitivity, pancreatic $\beta$-cell function, and insulin synthesis and secretion caused by low vitamin D level is related to insulin resistance. Vitamin D deficiency can promote the progress of impaired glucose tolerance, increase the expression of renin angiotensin system components, and damage the transcriptional function of pancreatic genes [133]. Vitamin D also decreases insulin resistance by downregulating the expression of PPAR$\gamma$2, suppressing the differentiation of 3T3-L1 preadipocytes, and inhibiting adipogenesis [134]. Studies in vivo and in vitro showed that vitamin D is related to the pathogenesis and progress of NAFLD.

*4.3. Cardiovascular Risk.* Previous study indicated that endothelial dysfunction represents an early event in cardiovascular diseases, and there is an association between vitamin D levels and endothelial dysfunction. In addition, vitamin D levels negatively correlated with flow-mediated dilatation (FMD) in many patients affected by type 2 diabetes, whereas current data are still insufficient to confirm vitamin D deficiency or insufficiency lead to an increased cardiovascular risk [135]. The relationship between vitamin D deficiency and cardiovascular risk, as well as mechanism underlying also still needs to be proved by further investigation.

## 5. Conclusions

This review was dedicated to reveal the correlation between vitamin D and adipogenesis, with emphasis on the diseases related to adipose metabolic disorders. Vitamin D has several influences on adipogenesis. Active vitamin D is mainly produced, stored, and degraded in adipose tissue, and VDR is expressed in adipose tissue. Vitamin D affects adipogenesis by regulating the expression of adipocyte transcription factors, such as PPAR$\gamma$, C/EBP$\alpha$, and LPL, and through affecting insulin resistance, VDR and unliganded VDR, and adipokine secretion.

Adipose metabolic disorders, such as obesity, diabetes, and NAFLD, were specifically chosen in this review. Obesity is a common occurrence worldwide, and it can lead to diabetes and NAFLD. Many studies have indicated that vitamin D deficiency or insufficiency plays an important role in the development and process of obesity, diabetes, and NAFLD. Aside from the diseases discussed in the review, other diseases are also associated with vitamin deficiency, such as hyperlipidemia, ketosis, ketonuria, and atherosclerosis. However, the effect of vitamin D on adipogenesis in lean individuals and the function of calcium in adipogenesis are still keeping elusive. Vitamin D supplements are a promising way to alleviate the burden caused by these diseases. In conclusion, vitamin D administration can provide a new basis for medical therapy.

## Conflicts of Interest

We declare that we have no conflicts of interest.

## Authors' Contributions

All authors participated in the search, writing, and revising of the manuscript. Zhiguo Miao and Shan Wang contributed equally to this work.

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*Review Article*

# Natural History of Nonalcoholic Fatty Liver Disease: Implications for Clinical Practice and an Individualized Approach

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Nonalcoholic fatty liver disease (NAFLD) is becoming the most prevalent liver disease worldwide, associated with epidemics of overweight and resulting metabolic syndrome (MetS). Around 20–30% of patients with NAFLD develop progressive liver fibrosis, which is the most important predictor of liver-related and overall morbidity and mortality. In contrast to classical understanding, no significant association has been demonstrated between the inflammatory component of NAFLD, i.e., nonalcoholic steatohepatitis (NASH), and the adverse clinical outcomes. Older age (>50 years) and presence of type 2 diabetes mellitus, in addition to some genetic variants, are most consistently reported indicators of increased risk of having liver fibrosis. However, critical driving force for the progression of fibrosis and risk factors for this have still not been fully elucidated. Apart from the genetic profile, gut dysbiosis, weight gain, worsening of insulin resistance, and worsening of liver steatosis represent candidate factors associated with unfavourable development of liver disease. Cardiovascular events, extrahepatic malignancies, and liver-related deaths are the leading causes of mortality in NAFLD. As patients with advanced fibrosis are under highest risk of adverse clinical outcomes, efforts should be made to recognize individuals under risk and rule out the presence of this stage of fibrosis, preferably by using simple noninvasive tools. This process should start at the primary care level by using validated biochemical tests, followed by direct serum tests for fibrosis or elastography in the remaining patients. Patients with advanced fibrosis should be referred to hepatologists for aggressive lifestyle modification and correction of the components of MetS, and cirrhotic patients should be screened for hepatocellular carcinoma and oesophageal varices.

## 1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is now considered the most prevalent chronic liver disease (CLD) worldwide [1, 2]. It is expected to become the most common cause of end-stage liver disease (i.e., cirrhosis and hepatocellular carcinoma) in the near future and, consequently, the most common indication for liver transplantation (LT) [3, 4]. The prevalence of NAFLD goes hand by hand with the prevalence of overweight and obesity, as well as metabolic syndrome (MetS), and due to its multisystemic effect, this combination represents the most serious health threat responsible for increasing number of cardiovascular, oncologic, and liver-related morbidity and mortality [1, 2, 5–7]. Over the last two decades, significant improvements have been achieved in understanding the natural history of

NAFLD, which was important to understand the clinical behaviour of the disease [2]. This in turn enabled more precise risk stratification and the development of rational diagnostic pathways with the final aim of preventing liver-related and other complications [8]. In this review, we intended to present the current knowledge on the natural history of NAFLD and its implications for a rational and more individualized approach to this condition, harbouring features of global epidemics.

## 2. Epidemiology of NAFLD

The prevalence of NAFLD in the general population varies from 13.48% in Africa to 30.45% in South America and 31.79% in the Middle East [1, 2]. The prevalence in Europe is 23.71% and in the United States 24.1% [2]. The prevalence in the United States differs among ethnic groups, the highest being in Hispanic Americans (29%), and even by the country of origin (Mexican Americans 33% and Dominicans 16%) [2]. NAFLD is defined as the presence of >5% of liver steatosis in the absence of other causes of steatosis and CLD (chronic viral hepatitis, autoimmune and other metabolic liver diseases, and the use of medications that can induce steatosis) in the absence of significant alcohol consumption (>21 drinks/week in men and >14 drinks/week in women) [2]. The presence of simple steatosis is defined as nonalcoholic fatty liver (NAFL), whereas nonalcoholic steatohepatitis (NASH) is a more aggressive form of NAFLD that includes a histological presentation of steatosis, ballooning, and lobular inflammation that leads to fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) [2, 3]. Recent data revealed that almost 15–20% of HCCs occur in NAFLD patients without cirrhosis [2, 3].

NAFLD is closely related to metabolic syndrome (MetS) and its individual components: diabetes mellitus type 2 (T2DM), arterial hypertension, dyslipidaemia, and obesity [2, 5]. Actually, NAFLD has been recognized as the liver manifestation of MetS [5–7]. Therefore, the majority of NAFLD patients have metabolic comorbidities, and a small proportion of NAFLD patients have "lean" NAFLD [2]. The prevalence of lean NAFLD is 7% in the US and up to 25% in some Asian countries [2, 4]. However, the majority of NAFLD cases are related to MetS and its individual components [4, 5]. According to a meta-analysis, the prevalence of NAFLD, NASH, and advanced fibrosis ($F \geq 3$ according to the METAVIR scoring system) in T2DM patients was 57.8%, 65.26%, and 15.05%, respectively [2, 4]. In addition, the overall prevalence of dyslipidaemia among NAFLD and NASH patients was 69.16% and 72.13%, while the overall prevalence of hypertension was 39.34% and 67.97%, respectively [2]. Finally, the prevalence of MetS among NAFLD and NASH patients was 41% and 71%, respectively [2, 5]. Regarding the association between NAFLD and obesity, the majority of morbidly obese patients undergoing bariatric surgery have NAFLD, 20% to 30% of them have NASH, and 10% have advanced fibrosis [9]. The high prevalence of MetS and its individual components in NAFLD patients suggests a high risk for CVD in addition to liver-related morbidity, while an increased risk of other extrahepatic chronic diseases (chronic kidney disease, T2DM, colorectal cancer, etc.) has been demonstrated as well [2, 6, 7, 10].

## 3. Natural History and Predictors of Mortality in NAFLD

The accumulation of new scientific knowledge has provided better insights into the natural course of NAFLD. Two European studies demonstrated the higher mortality of NAFLD patients relative to the general population, whereas mortality was lower in comparison to patients with alcoholic liver disease (ALD) and hepatitis B and C [11, 12]. With an average follow-up of 13.7 and 28 years, the mortality of NAFLD patients reached 22% and 40%, respectively, which was 37.5% and 69% higher than in the general population [12]. The three most common causes of mortality for patients with NAFLD were cardiovascular diseases (30% to 61.5% of cases), followed by extrahepatic malignancies (19% to 28%) and liver-related deaths (7.7% to 19%) [11, 12]. In both studies, when compared to the general population, mortality was significantly higher only in patients with NASH and not in those with isolated steatosis [11, 12]. In the cohort from the US, 131 NAFLD patients were followed for an average of 18.5 years: overall mortality was 59.5%, irrespective of the presence of NASH. Likewise, the main causes of death were coronary disease (28.2%), extrahepatic malignancies (17.9%), and liver-related complications (15.4%). However, liver-related mortality was significantly higher in patients with NASH compared to non-NASH counterparts (17.5% vs. 2.7%; $P = 0.0048$), as well as in diabetics, elderly patients, and those with reduced albumin at baseline [13].

It should be noted that, in these earlier studies, all patients with NASH were analysed as a cohort with no specific distinction with regard to the presence of fibrosis versus isolated steatosis; hence, most of them had some degree of fibrosis. It is therefore not surprising that NASH was considered as a risk factor for a worse outcome. Nevertheless, even at that time, Ekstedt and colleagues demonstrated that the absence of periportal fibrosis at baseline liver biopsy had a 100% negative predictive value (NPV) for the development of liver-related complications [11]. Subsequent studies have separately analysed the individual histological categories within NAFLD, thus enabling a more detailed risk stratification. In a multicentre study involving 619 patients with histologically verified NAFLD who were followed for an average of 12.6 years, the overall mortality was 33.2%, of which 38.3% was due to cardiovascular diseases, 18.7% was due to extrahepatic malignancies, and 8.8% was due to liver-related complications [14]. Among the analysed histological categories, only fibrosis and not steatosis, nor the presence of liver inflammation (i.e., NASH), was associated with overall and liver-related mortality [14]. Independent predictors of overall mortality were higher stages of fibrosis, starting already from stage 1 relative to stage 0, older age, diabetes, and smoking, while statin administration had a protective effect [14]. The single independent predictor of liver-related

mortality was the stage of fibrosis, starting from stage F2 [14]. The effect and interaction of the stage of fibrosis and the presence of NASH on survival without liver-related complications have been analysed separately [14]. Survival was significantly higher in patients without fibrosis than in the patients with fibrosis, regardless of the presence of NASH [14]. Similar conclusions were reached by the authors of another study that analysed the survival of a cohort of 229 patients with NAFLD, followed for an average of 26.4 years. In this cohort, significantly higher mortality relative to the general population was observed only in patients with F3 and F4 stages of liver fibrosis regardless of the presence of NASH, whereas mortality was not increased in patients with NASH who had lower stages (F0 to F1) of fibrosis [15]. In another large Swedish study, 646 patients with NAFLD were followed for an average of 20 years [16]. The presence of NASH was not associated with liver-related or overall mortality. Following the adjustments for age, sex, and the presence of diabetes, the risk of overall mortality was only increased in patients with F3 and F4 stages of fibrosis relative to NAFLD patients without fibrosis [16]. The presence of NASH did not influence the prognostic impact of fibrosis, whereas the stage of fibrosis significantly affected the prognosis in patients with NASH [16]. The same results were obtained for liver-related outcomes defined as the decompensation of cirrhosis or the development of HCC. The time within which the first 10% of patients from each stage of fibrosis would have developed severe liver diseases was estimated to be 2.3 years for patients with stage F3, 9.3 years for stage F2, and 22 to 26 years for stage F0-1 [16]. According to the meta-analysis, which included five studies with a total of 1,495 NAFLD patients and 17,452 patient-years of follow-up, an exponential increase in overall mortality and especially liver-related mortality was observed with worsening stages of fibrosis [17]. The overall mortality increased significantly already at stage F1 relative to F0, while liver-related mortality increased only starting at stage F2 [17]. The main limitation of this meta-analysis is the lack of data on cofounders and as such, it was not possible to adjust the results according to age, sex, and comorbidities (e.g., T2DM) [17]. It is interesting to note that, in the large multinational cohort study of 458 NAFLD patients with advanced fibrosis (F3 and F4) at baseline biopsy, those with F3 had more vascular events and nonliver cancers during the mean follow-up period of 5.5 years, whereas patients with compensated cirrhosis more frequently developed liver decompensation, HCC, and liver-related death. Liver-related events were associated with the presence of cirrhosis and mild steatosis (<33% fatty transformed hepatocytes), and history of moderate alcohol consumption contributed to these outcomes only in patients with cirrhosis and not F3 fibrosis [18].

The relationship between T2DM and NAFLD is of particular interest, as it shows a bidirectional interaction. T2DM is found in about a quarter of patients with NAFLD, while NAFLD is found in about three quarters of patients with T2DM [2, 19]. As previously mentioned, mortality was significantly increased in patients with NAFLD and T2DM [19]. The same was observed in T2DM patients with NAFLD, for whom the risk of mortality was about twofold higher than in those without NAFLD (two studies with a total of 2,350 patients with T2DM monitored for an average of 6.5 and 11 years) [20, 21]. However, in one abstract by an American group of authors, the mortality in patients with T2DM who had NAFLD was not higher relative to those without NAFLD [22]. Although it seems intuitive, the screening for NAFLD in patients with T2DM has not proven to be effective in terms of cost-benefit analysis, primarily due to the limited potential and the side effects of available medications for NAFLD, as well as the lack of reliability of noninvasive diagnostic methods that still need to be examined in the population with T2DM, which should also be taken into account [23–25].

*3.1. Fibrosis Progression in NAFLD.* Fibrosis progression does not occur in all patients with NAFLD and not at the same rate. Data on the fibrosis progression in NAFLD are based on a small number of studies in which a paired biopsy was performed at follow-up. In a meta-analysis involving 11 studies that included 411 patients with an average 14-year interval between two liver biopsies, interesting data were obtained [26]. Fibrosis progression was observed in 36% of patients, with no difference in the proportion of progressors between patients with isolated steatosis and those with NASH [26]. Accordingly, fibrosis progresses regardless of NASH, and isolated steatosis does not exclude the possibility of progressive fibrosis development [26]. However, the rate of progression of fibrosis was higher in patients with NASH (about seven years for one histological stage of fibrosis) relative to isolated steatosis (14 years for one stage of fibrosis) [26]. In the group of progressors, about 20% developed severe fibrosis (F3 to F4) very quickly (six years on average) regardless of the presence of NASH [26]. In the multivariate analysis, factors associated with rapid progression were hypertension and a low baseline AST/ALT ratio [26]. Limitation to this study should be acknowledged due to selection bias as follow-up biopsies were not performed per protocol but were performed as a response to clinical need or suspicion of the disease progression. Similar results were presented in a recent study conducted with 60 NAFLD patients with an interval of 8.4 years between paired liver biopsies [27]. The progression of fibrosis was observed in 43% of patients, regardless of the presence of NASH or isolated steatosis at the baseline biopsy, and there was also no difference in the rate of progression between the groups (about seven years for one stage of fibrosis in NASH and about 10 years in patients with isolated steatosis) [27].

Recently, data from the prospective phase 2b placebo-controlled trial of simtuzumab for the treatment of NAFLD patients ($N = 475$) with F3 and F4 fibrosis who had baseline and follow-up biopsies were presented. Since the investigated compound failed to show, the efficacy trial was terminated after 96 weeks, and patients from all treatment groups were then analysed together. Progression to cirrhosis was observed in 20% of F3 patients, and liver-related events in 19% of patients with baseline cirrhosis. Both outcomes

were related to the amount of fibrosis at baseline and at follow-up (quantified by histology and serum markers of fibrosis), but not to necroinflamatory activity as expressed by the NAFLD Activity Score [28].

The risk of fibrosis is to some extent genetically determined, and several genetic variants (single nucleotide polymorphisms, SNPs) associated with the progression of fibrosis have been identified [29]. Patatin-like phospholipase domain-containing protein 3 (PNPLA3, also known as adiponutrin) and transmembrane 6 superfamily member 2 (TM6SF2) have been most thoroughly evaluated [29]. The replacement of the isoleucine with methionine at the 148 codon (I148M) of PNPLA3 results in decreased hydrolysis of triglycerides in hepatocytes, while the E167K variant of TM6SF2 (change of glutamate to lysine at codon 167) leads to decreased VLDL secretion from the liver [29]. Both polymorphisms are associated with a higher risk of developing steatosis, NASH, cirrhosis, and HCC [29]. According to the results of a meta-analysis, carriers of the I148M PNPLA3 polymorphism (rs738409C/G), i.e., GG homozygotes, have 73% more fat in the liver and a 3.2-fold higher risk of developing fibrosis than CC homozygotes [29]. Recently, the genetic variant (*rs72613567:TA*) in hydroxysteroid 17-beta dehydrogenase 13 (HSD17B13) was shown to be associated with decreased risk of NASH and liver fibrosis. This polymorphism leads to decreased expression of the HSD17B13 protein in hepatocytes and reduced enzymatic activity against some biological compounds such as leukotriene B4, which is involved in lipid-mediated inflammation, as well as against oestradiol [30]. In addition to genetics and the indicators at the beginning of the clinical evaluation of a patient, features indicating a higher risk of fibrosis progression during the monitoring period have been identified. They comprise elevated ALT and AST, the worsening of insulin resistance, weight gain of at least 5 kg, decreased platelets, and the worsening of liver steatosis relative to the initial biopsy [11]. In another study, the development of diabetes and a higher FIB-4 index during the follow-up period were also identified as significant [31].

*3.2. Fibrogenesis in NAFLD: NASH vs. Other Mechanisms.* Since NAFLD/NASH is a complex disorder of multifactorial aetiology, based on multiple parallel hits, the critical pathophysiological mechanisms responsible for fibrosis development and progression are still not fully explained [32]. The main pathways involved in fibrogenesis are associated with insulin resistance, lipotoxicity resulting from free fatty acid (FFA) overload and their derivatives, environmental factors such as diet, obesity, and microbiota, genetics, endoplasmic reticulum stress, mitochondrial dysfunction, hypoxia, apoptosis, and ultimately, hepatic stellate cell (HSC) activation [32–35]. It is interesting to note that, in some cases, fibrosis is not NASH dependent and inflammation is not a key mechanism of fibrosis development [27].

Adipose tissue lipolysis, de novo lipogenesis from glucose and fructose and dietary fat are the main sources of FFAs stored in the liver. The overflow of FFAs results in the depletion of hepatocytes' ability to produce triglycerides (TGs) [32]. Excessive FFAs are transformed into lipotoxic agents, which impair the endoplasmic reticulum and mitochondria, evoke oxidative stress, activate inflammasomes, and potentiate apoptosis [32, 33]. Microsomal FFA metabolism induces ROS production in the liver [34]. Oxidized FFAs can also catalyse lipid peroxidation reactions that are directly cytotoxic [35]. Pointing to the abovementioned mechanisms, the impairment of FFA disposal or increased inflow to the liver alongside the depletion of their detoxification results in fibrosis progression independent of NASH [32].

Gut dysbiosis has been suggested as an additional factor for the development and progression of NAFLD [36, 37]. An elevated ratio of Gram-negative to Gram-positive bacteria and *Firmicutes* to *Bacteroidetes* with an upregulated number of mucous-degrading bacteria impairs the gut barrier NAFLD [38]. This in turn leads to the increased translocation of bacterial fragments and endotoxin absorption to the portal blood flow that finally enters the liver [39]. These compounds activate signalling pathways in the liver depending on nuclear factor $\kappa$B (NF-$\kappa$B) and pattern recognition receptors (PRRs) either localized on cell membrane surface Toll-like receptors (TLRs) or localized in cytosol NOD-like receptors (NLRs). NLRs are linked to inflammasomes, protein complexes which when activated stimulate cell apoptosis, and the release of proinflammatory cytokines from inflammatory cells. Very high inflammasome activity was found in NAFLD patients and was associated with insulin resistance, high levels of FFAs, the overproduction of leptin, and the downregulation of adiponectin synthesis [40]. Inflammasomes and absorbed lipopolysaccharides (LPSs) may directly affect HSCs and macrophages (Kupffer cells) by stimulating the production of smooth muscle actin, transforming growth factor $\beta$1 (TGF $\beta$1), and collagen fibres, which promotes the development and progression of fibrosis [41]. Even a small amount of intestinal endotoxins may have an effect on increased sensitivity to leptin, exacerbating fibrosis progression [41]. Gut microbiota exerts significant interaction with bile acids (BA) influencing their chemical structure and composition. This in turn leads to altered BA signalling, potentially contributing to the development of liver fibrosis [42].

Oxidative stress, lipid overload of hepatocytes, and the impaired function of mitochondria lead to an energy imbalance and hypoxia [33, 34]. Hypoxia stimulates neoangiogenesis, which might be observed even in bland steatosis and seems to be independent of NASH [43]. The development of fibrosis is preceded by angiogenesis, but in later stages, angiogenesis strictly correlates with the severity of fibrosis. NASH in severely obese patients had no influence on the hepatic expression of angiogenic factors [43, 44].

NAFLD is associated with an improper adipokine profile with increased levels of leptin and a decreased concentration of adiponectin [45–47]. Leptin promotes fibrogenesis indirectly through the activation of Kupffer cells and sinusoidal endothelial cells (SECs) through the upregulation of TGF$\beta$1 production and directly by the activation of HSCs [48–51]. Additionally, leptin induces proliferation and inhibits the apoptosis of HSCs [52].

Adiponectin has a hepatoprotective and antifibrogenic effect in cases of liver injury and protects against liver steatosis [48, 51]. This effect is independent of its metabolic action and is associated with the modulation of HSCs, which express both adiponectin receptors [53]. Adiponectin activation of AMPK disrupts leptin-mediated hepatic fibrosis by upregulating suppressors of cytokine signalling 3 (SOCS-3) [54].

Hyperinsulinaemia promotes profibrogenic signals in HSCs, either directly or as a cofactor of TGF-$\beta$1 [55]. In addition, hyperglycaemia, which is commonly observed in NAFLD patients, determines the occurrence of the non-enzymatic glycation and oxidation of proteins and lipids, resulting in the formation of advanced glycation end products (AGEs). HSCs express a receptor for AGEs and undergo activation when exposed in vitro to glyceraldehyde-derived AGEs. Moreover, AGEs may activate fibrogenesis through the modulation of TNF$\alpha$-converting enzyme activity [56, 57]. These results explain why the degree of insulin resistance IR is related to disease severity and the fact that NAFLD patients with coexisting T2DM exhibit more progressive disease and faster fibrosis progression.

## 4. How to Recognize the Risk of Liver Fibrosis in NAFLD

The question is how to identify patients with NAFLD who have a higher risk of fibrosis on the basis of simple clinical features. Studies that examined clinical risk (mostly cross-sectional studies with only baseline liver biopsy) identified older age (usually >50 years), higher BMI (>28 to 30 kg/m$^2$), T2DM, NAFLD Fibrosis score (NFS), and FIB-4 score as the indicators for the presence of fibrosis in NAFLD patients [14, 30, 58]. The prevalence of liver fibrosis in the general population has been investigated using noninvasive methods [59]. The Rotterdam study evaluated 3,041 people ≥45 years of age from the general population without a history of chronic liver disease [60]. All of them underwent transient elastography (TE) of the liver. Liver stiffness of ≥8 kPa was considered clinically significant, and liver steatosis was determined by ultrasound [60]. Clinically significant fibrosis was found in 5.6% of subjects, with a significant association with liver steatosis and T2DM [60]. Off note, given the modest predictive value of TE for advanced fibrosis (with cutoff values generated at tertiary care centres), the true prevalence of clinically significant fibrosis in general population is probably even lower [59]. Accordingly, the simple clinical risk profile for fibrosis is constituted by persons >50 years of age, overweight, and diabetic. By using simple noninvasive diagnostic tools, additional risk indicators including fatty liver was determined by ultrasound and elevated values of biochemical indicators such as FIB-4 and NFS [9, 24, 60, 61]. The prevalence of advanced fibrosis ($F \geq 3$) was reported to be 15.05% in T2DM patients with NAFLD [2] and 10% in obese patients undergoing bariatric surgery [9]. Since central obesity and diabetes are both components of MetS, it is plausible to expect an incremental rise of risk for liver fibrosis in patients with more components of MetS. Indeed, T2DM increases the adjusted risk for significant to severe fibrosis by 25.41%, while NAFLD patients with T2DM and hypertension have a 26.32% risk of significant to advanced fibrosis [5, 62]. More recently, Younossi et al. have demonstrated that MetS in NAFLD patients is strongly associated with increased cardiovascular, liver-related, and all-cause mortality [63]. According to these data and expert consensus, NAFLD patients with T2DM and other MetS components who have increased liver enzyme levels are at the highest risk for significant and advanced fibrosis and should be considered for liver biopsy [4, 63].

## 5. Implications for Clinical Practice

*5.1. Should We Search for NAFLD?* The risk of having fatty liver obviously goes hand in hand with the epidemiology of overweight/obesity, and it is frequently observed in patients with one or more components of MetS [1, 5]. Fatty liver can be easily diagnosed by ultrasound, and it is a widely available, noninvasive, and cheap procedure. In areas where ultrasound is not available, simple biochemical indices might be used instead (for example, the fatty liver index) [61, 64]. However, to actively search for fatty liver is a very contentious issue. As already pointed out from the results of the Rotterdam study, significant fibrosis was associated with the presence of fatty liver especially in combination with T2DM. On the other hand, most guidelines do not recommend screening for NAFLD. It might be worth exploring if people at risk for NAFLD should be screened for fibrosis, but this needs to be proved by further data.

*5.2. What to Search for in a Patient Diagnosed with NAFLD?* The results of the quoted studies have important implications for clinical practice. Firstly, the most important prognostic category in patients with NAFLD is the stage of liver fibrosis, as it determines the risk of developing liver-related complications and overall mortality [15]. In that regard, patients with an established diagnosis should be tested for the presence of advanced fibrosis as they are associated with the risk of overall and liver-related mortality [15, 16, 64, 65]. Patients with advanced fibrosis should be monitored by a hepatologist [8, 15]. Patients who have been diagnosed with stage F2 fibrosis should also be monitored by a hepatologist particularly in the presence of metabolic comorbidities such as obesity and diabetes [8, 16]. Patients in stages F0 to F1 are not expected to develop complications of liver diseases over a period of about 20 years and may continue to be monitored at the primary care level [16, 65]. Secondly, diabetes worsens the prognosis of NAFLD, whereas some disagreement exists about the effect of NAFLD on mortality in patients with diabetes [20–22]. Therefore, an important clinical goal is the adequate regulation of diabetes. Thirdly, NASH without the presence of fibrosis does not significantly affect prognosis, so diagnostic tests to determine its presence are not of critical clinical importance (with the exception of biopsy, all noninvasive NASH tests have not shown sufficient diagnostic reliability) [26, 27, 57, 62]. The regression of fibrosis, rather than the curing of NASH, appears to be a key goal in treating patients with NAFLD [32]. Fourthly, the degree of steatosis also does

not indicate the risk of fibrosis, nor is it associated with clinical outcomes in terms of overall mortality or liver-related mortality, and as such, it does not constitute relevant prognostic data. However, since the worsening of steatosis during follow-up may be associated with the progression of fibrosis [11] and the reduction of steatosis may be a measurable parameter for the success of dietary measures, it seems that the quantification of steatosis at baseline and during follow-up may be a useful parameter, although additional studies are required to support this claim.

Finally, since NAFLD is closely related to insulin resistance, MetS, and its individual components (T2DM dyslipidaemia, obesity, and arterial hypertension), all NAFLD patients should be evaluated for these conditions by performing simple anthropometric measurements and simple laboratory tests [8, 66]. In addition, it has become clear that NAFLD is not only a "liver disease" but also a risk factor for many other extrahepatic diseases, including cardiovascular diseases (CVD), chronic kidney disease (CKD), T2DM, and colorectal cancer [7]. According to these data, physicians who manage NAFLD patients should recognize and evaluate extrahepatic manifestations of NAFLD and should not only focus on liver disease [6, 7, 67, 68].

*5.3. How Should Liver Disease and Associated Extrahepatic Conditions in NAFLD Be Diagnosed?* Given the epidemiological characteristics, i.e., the prevalence of NAFLD and the fact that the vast majority of patients will never develop severe liver disease, it is neither realistic nor necessary to perform liver biopsy in all patients. A diagnostic algorithm for NAFLD should be simple, using preferably noninvasive tools that are widely available and provide reliable results [8, 61, 64]. The goal of noninvasive methods should be to reliably quantify the amount of liver fibrosis [15, 17]. Advanced fibrosis, i.e., stages F3 and F4, should be ruled out first by a simple biochemical test (e.g., FIB-4 or NFS) at the primary care level [64, 65]. These tests proved better in terms of diagnostic performance for advanced fibrosis as compared to other simple biochemical tests such as APRI or BARD [69, 70]. Such patients may continue to be treated by a family physician with the correction of components of MetS and a reevaluation of the stage of liver fibrosis in three to five years [64, 65]. Patients with a high or intermediate risk of severe fibrosis based on the results of noninvasive biochemical tests should be referred to a second line of testing, which may include another biochemical test (preferably direct tests measuring extracellular matrix components, such as the ELF test of FibroMeter) or liver elastography (TE being the best validated; LSM values <8 kPa exclude significant fibrosis, LSM >9.5 to 10 kPa indicate severe fibrosis or compensated advanced chronic liver disease, while values of >15 kPa suggest cirrhosis [61, 71, 72]). Patients in whom the second line of tests confirms advanced fibrosis should be referred to a hepatologist in order to reliably assess the presence of cirrhosis and portal hypertension, as these require further specific investigations that include screening for the presence of oesophageal varices and HCC [73]. Since noninvasive tests have a modest positive predictive value for cirrhosis (about 50 to 70%), patients who have been diagnosed with cirrhosis based on noninvasive tests usually need a liver biopsy to reliably determine the stage of fibrosis, as well as to assess the presence of other histological components [8, 74]. As explained in the previous paragraph, it may be useful to quantify steatosis at baseline and during the follow-up period, although solid scientific evidence is still lacking. The best validated noninvasive measure for this purpose is the controlled attenuation parameter (CAP) coupled with TE [61, 64]. Proposed algorithm for risk stratification in patients with NAFLD is depicted in Figure 1. Patients with NAFLD have a two- to fivefold higher risk for the development of T2DM, and accordingly, screening for T2DM in NAFLD patients should be implemented by periodically performing simple laboratory tests (fasting or random blood glucose, HbA1c, and standardized 75 g OGTT in high-risk groups) [6, 8, 63]. NAFLD is associated with an enhanced risk of CVD and its progression [10, 67]. The age and the presence of MetS and its individual components as well as the presence of significant fibrosis are risk factors for CVD in NAFLD patients; these patients should therefore be referred to a cardiologist [75]. The CVD risk in this subgroup of NAFLD patients can be assessed by dobutamine stress ECHO, CT coronary angiography, and/or coronary angiography, which is the gold standard [75]. On the other hand, there are insufficient data to recommend the screening of NAFLD patients with advanced liver disease who are not candidates for LT, as well as those without significant fibrosis. Asymptomatic, low-risk patients should be evaluated for traditional CVD risk factors (i.e., MetS components). Methods used to estimate CVD risk in the general population, such as the Framingham Risk Score, need to be validated in patients with NAFLD [10, 75]. Consequently, the current data are insufficient regarding the optimal screening strategy for asymptomatic NAFLD patients, and further studies are needed [75]. Since the association between NAFLD and the development of chronic kidney disease (CKD) has been demonstrated as well, NAFLD patients could benefit from annual screening for CKD [68]. This may include the analysis of simple laboratory parameters that are available in every day clinical practice (such as the determination of serum creatinine and albuminuria) [65, 68].

*5.4. Who Should Be Screened for HCC?* NAFLD is increasingly becoming an aetiological factor for HCC, and it is likely that, in the near future, NAFLD will become the leading cause of HCC [76]. Although HCC predominantly develops against a background of liver cirrhosis, 15 to 20% of HCC in NAFLD occur in the noncirrhotic liver [3, 76]. Given the high prevalence of NAFLD in the general population, this observation calls for increased attention. Effective screening programmes are currently lacking in part because current knowledge does not allow for the precise stratification of cancer risk in NAFLD patients [76]. However, according to a large American study, NAFLD, age >65 years, T2DM, and Hispano race have been identified as independent predictors of HCC risk in the general population [77]. In NAFLD

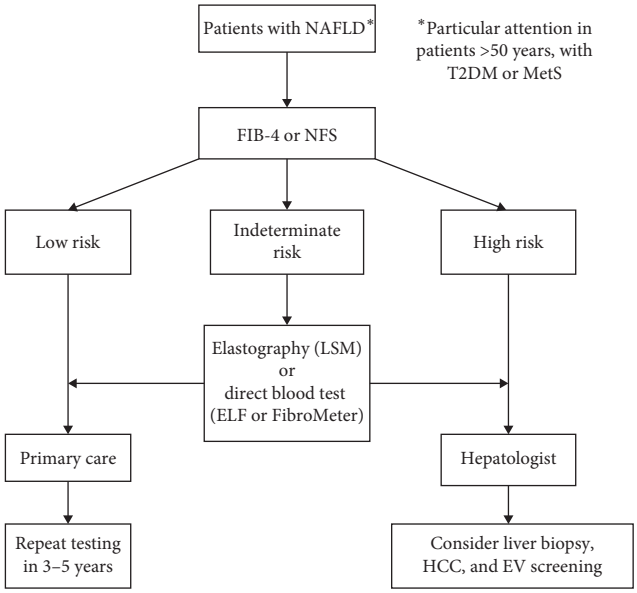


Figure 1: Proposed diagnostic algorithm to stratify patients with nonalcoholic fatty liver disease (NAFLD) according to the risk of having advanced ($F \geq 3$) liver fibrosis. Diagnostic process begins by using first tier testing-nonproprietary algorithm (FIB4 or NFS) from available simple blood tests and demographic data. Those with low risk need no further testing and might be followed in primary care (general practitioners) with repeated risk assessment at 3–5 years. Those with high risk require immediate referral to secondary care (hepatologist). Patients with indeterminate risk should proceed to second-tier testing, liver stiffness measurement (LSM) by elastography, or direct blood tests for fibrosis by patented algorithms (such as ELF or FibroMeter). ELF = enhanced liver fibrosis score; EV = oesophageal varices; HCC = hepatocellular carcinoma; MetS = metabolic syndrome; NFS = nonalcoholic fatty liver disease fibrosis score; T2DM = type 2 diabetes mellitus.

patients, cirrhosis, age >65 years, male sex, and Hispano race were associated with a higher incidence of HCC. Nevertheless, cirrhosis was demonstrated to be the main risk factor because the incidence of HCC in patients with NAFLD cirrhosis was 13.55 per 1,000 patient-years, compared to only 0.04 per 1000 patient-years in patients without cirrhosis [77]. This was the basis for the current recommendation for HCC screening only in patients with NAFLD cirrhosis since the incidence of HCC is too low to justify the introduction of a screening programme to all patients with NAFLD [77, 78]. Ultrasound is a preferable method for HCC screening due to its availability and noninvasiveness, and it should be performed every six months. It has a sensitivity of 84% and a specificity of 91% for the detection of HCC. Since the majority of HCCs appear in cirrhotic livers, with a coarse echostructure and nodular appearance, the detection of early stage HCC has a lower sensitivity of only 47% [79]. Therefore, screening for HCC should be performed by experienced operators with high-quality ultrasound machines.

## 6. Conclusions

Nonalcoholic fatty liver disease is becoming the most prevalent liver disease worldwide, mostly due to its association with epidemics of overweight/obesity, T2DM, and MetS. Around 20–30% of patients with NAFLD are under risk of developing progressive liver fibrosis, which is the most important predictor of both liver-related and overall morbidity and mortality. Contrary to previous beliefs, no significant association has been demonstrated between the inflammatory component of NAFLD, i.e., NASH, and the adverse clinical outcomes. Older age (>50 years) and presence of T2DM, in addition to some genetic variants, are indicators of increased risk of having liver fibrosis. However, critical driving force for the progression of fibrosis and risk factors for this have still not been fully elucidated. Apart from the genetic profile, gut dysbiosis, weight gain, worsening of insulin resistance, and worsening of liver steatosis represent candidate factors associated with unfavourable development of liver disease. Cardiovascular events and extrahepatic malignancies, followed by liver-related deaths, represent the leading causes of mortality in NAFLD. As patients with advanced fibrosis are under highest risk of adverse clinical outcomes, efforts should be made to recognize individuals under risk and rule out the presence of this stage of fibrosis, preferably by using simple noninvasive tools. This process should start at the primary care level by using validated biochemical tests, followed by direct serum tests for fibrosis or TE in the remaining patients. Patients with advanced fibrosis should be referred to hepatologists for aggressive lifestyle modification and correction of the components of MetS, and cirrhotic patients should be screened for HCC and oesophageal varices.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

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*Research Article*

# Association between Smoking and Liver Fibrosis among Patients with Nonalcoholic Fatty Liver Disease

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*Objective*. We aimed at analyzing the role of smoking in hepatic fibrosis in patients with nonalcoholic fatty liver disease (NAFLD) and at exploring the related risk factors. *Methods*. This was a cross-sectional study that included a total of 225 patients with NAFLD. Among them, 127 were nonsmokers and 98 were smokers. Liver significant fibrosis was diagnosed when the liver stiffness (LS) value was higher than 7.4 kPa. The diagnostic criterion for NAFLD was a controlled attenuation parameter (CAP) value of >238 dB/m. The CAP and LS values were measured using FibroScan. *Results*. FibroScan showed that the LS value in the smokers was significantly higher than that in the nonsmokers (10.12 ± 10.38 kPa vs. 7.26 ± 6.42 kPa, $P = 0.013$). The proportions of patients with liver significant fibrosis and advanced liver fibrosis among the smokers were significantly higher than those among the nonsmokers ($P = 0.046$). Univariate analysis showed that age, weight, high AST level, low PLT level, and smoking were the risk factors associated with liver fibrosis in the smokers with NAFLD while multivariate analysis showed that age (OR = 1.029, $P = 0.021$), high AST level (OR = 1.0121, $P = 0.025$), and smoking (OR = 1.294, $P = 0.015$) were the independent risk factors associated with liver fibrosis in the patients with NAFLD. Moreover, high AST level (OR = 1.040, $P = 0.029$), smoking index (OR = 1.220, $P = 0.019$), and diabetes mellitus (OR = 1.054, $P = 0.032$) were the independent risk factors for liver fibrosis among the smokers with NAFLD. *Conclusion*. This study showed that smoking was closely associated with liver fibrosis among the patients with NAFLD. For patients with NAFLD who smoke, priority screening and timely intervention should be provided if they are at risk of liver fibrosis.

## 1. Introduction

With the increase in the incidence of obesity and the associated metabolic syndrome, nonalcoholic fatty liver disease (NAFLD) has become an important cause of chronic liver disease [1]. Epidemiological studies have shown that 20% to 30% of individuals in Western countries develop NAFLD [1–3]. According to the definition of the American Association for the Study of Liver Diseases, NAFLD is a disease characterized by hepatic steatosis and lipid storage without excessive drinking history [2]. According to the change in the pathological degree, NAFLD can be divided into three stages: simple fatty liver (nonalcoholic fatty liver), nonalcoholic steatohepatitis, and cirrhosis.

NAFLD is closely related to metabolic syndrome [4]. Obesity, type 2 diabetes mellitus, and dyslipidemia are considered to be important risk factors for NAFLD [1, 4–6]. NAFLD is closely related not only to metabolic abnormalities but also to poor living behaviors [7–9]. All three abovementioned important risk factors for NAFLD are associated with unhealthy lifestyles. Therefore, NAFLD is generally considered to be a disease associated with an unhealthy lifestyle. Many studies have revealed that changes in unhealthy lifestyles can reduce the transaminase levels and improve NAFLD [10–13].

Smoking is a common poor living behavior in daily life. It can damage the antioxidant system [14, 15]. Although smoking can increase the risk for liver fibrosis and cirrhosis in patients with chronic hepatitis B (CHB) infection [16, 17], only a few studies have investigated the relationship between smoking and NAFLD. Suzuki et al. [18] reported that smoking is associated with high levels of alanine aminotransferase (ALT) in patients with NAFLD. Another study reported that smoking is an independent risk factor for NAFLD [19]. Although these authors have confirmed that smoking is associated with the occurrence of NAFLD, there is no research report on whether smoking promotes liver fibrosis in patients with NAFLD.

Hence, we enrolled smokers and nonsmokers with NAFLD and analyzed liver fibrosis among the smokers in this study. The risk factors for liver fibrosis were explored to provide medical evidence for screening and early diagnosis of liver fibrosis in smokers with NAFLD.

## 2. Methods

*2.1. Subjects.* This was a cross-sectional study that included a total of 225 patients with NAFLD. Among them, 127 were nonsmokers and 98 were smokers. All patients were recruited from the First Affiliated Hospital of Xiamen University from May 2015 to April 2018. Patients were included when they met the following criteria: diagnosis of NAFLD according to the diagnostic criterion of a controlled attenuation parameter (CAP) value of >238 dB/m according to previous recommendations and confirmation with ultrasonography [4, 20–22]. Conversely, patients were excluded when they met the following criteria: (1) use of medications that can induce hepatic steatosis (e.g., corticosteroids, estrogen, methotrexate, or amiodarone) within 6 months of study inclusion, (2) evidence of coinfection with hepatitis C, hepatitis D, or human immunodeficiency virus, (3) autoimmune liver disease, and (4) heavy alcohol consumption or alcohol abuse, defined as alcohol consumption of >10 g/day. The Institutional Review Board of the First Affiliated Hospital of Xiamen University approved the study. Each enrolled patient provided informed consent.

*2.2. FibroScan Test.* Liver fibrosis and steatosis were diagnosed on the basis of the liver stiffness (LS) and CAP values [23, 24]. These values were assessed by a professionally trained technician using FibroScan (Echosens, Paris, France) according to the manufacturer's instructions. The LS values were expressed in kilopascals and CAP values in decibels per meter. The ratio of the interquartile range (IQR) of the LS value to the median (IQR/M) was calculated as an indicator of variability. Only procedures with at least 10 valid measurements, a success rate of at least 60%, and an IQR/M ratio of <0.3 were considered reliable and then used for the analysis. The CAP value was measured only using validated measurement tools according to the same criteria used for the LS value and on the same signals, ensuring obtainment of a liver ultrasonic attenuation simultaneously and in the same volume of liver parenchyma as in the LS value. The median of the individual measurements was considered the final CAP value.

Among the patients with NAFLD, hepatic steatosis was diagnosed at the CAP values of >238 dB/m, according to previous recommendations [4, 20–22].

*2.3. Patient Information Collection.* Patient information, including demographic characteristics, physical examination, and laboratory test results, was collected. The demographic characteristics assessed included age, sex, and smoking history. Physical examination results, including height and weight, were recorded. Blood pressure was also measured after the FibroScan test. Laboratory test results, including the levels of platelet (PLT), serum aspartate aminotransferase (AST), and ALT, were collected in accordance with standard procedures. These laboratory test results were obtained using standard automated techniques within 14 days of the FibroScan test. Smoking index = daily tobacco intake $*$ duration of smoking.

Blood pressure was measured using a standard mercury sphygmomanometer. All patients were asked to rest for at least 5 minutes before measurement. Each patient required at least three blood pressure measurements, with an interval of 1 minute each. The average value of the three measurements was used for the analysis.

*2.4. Statistical Analysis.* Continuous variables were expressed as means ± standard deviations and categorical variables as percentages. The chi-square test and *t*-test were used to detect whether differences between the two groups were statistically significant. Univariate and multivariate logistic regression analyses were used to explore the risk factors associated with liver fibrosis and advanced liver fibrosis in the patients with NAFLD. The Data Analysis and Quality Control Program for SPSS for Windows version 13.0 was applied for the statistical analysis.

## 3. Results

*3.1. Demographic and Clinical Characteristics of the Patients.* A total of 225 patients with NAFLD were enrolled in this study. Among these patients, 98 were smokers (smoking group) and 127 were nonsmokers (nonsmoking group). The proportion of male patients in the smoking group was significantly higher than that in the nonsmoking group ($P < 0.001$). The weight of the patients in the smoking group was higher than that of the patients in the nonsmoking group ($P = 0.037$). The proportion of patients with diabetes mellitus in the nonsmoking group was significantly lower than that in the smoking group ($P = 0.012$). The serum levels of ALT, AST, and PLT were comparable between the two groups, as shown in Table 1.

*3.2. Comparison of Liver Fibrosis between the Two Groups.* The liver conditions of fibrosis were compared (Table 2). The LS value of the smoking group was significantly higher than that of the nonsmoking group (10.12 ± 10.38 kPa vs.

Table 1: Baseline demographic and clinical characteristics by groups.

| Variables | Nonalcoholic fatty liver disease | | $P$ |
|---|---|---|---|
| | Smoking | Nonsmoking | |
| Sample size | 98 | 127 | |
| Sex (F/M) | 3/95 | 49/78 | <0.001 |
| Age (years) | 45.77 ± 13.12 | 41.01 ± 11.55 | 0.004 |
| Height (cm) | 168.36 ± 6.47 | 166.96 ± 7.48 | 0.142 |
| Weight (kg) | 67.53 ± 12.50 | 63.98 ± 12.76 | 0.037 |
| SBP (mm/Hg) | 127.03 ± 12.52 | 129.09 ± 14.15 | 0.260 |
| DBP (mm/Hg) | 82.07 ± 7.85 | 83.66 ± 9.28 | 0.177 |
| ALT | 90.74 ± 72.34 | 80.49 ± 81.94 | 0.324 |
| AST | 67.84 ± 45.29 | 63.41 ± 40.67 | 0.444 |
| PLT | 216.11 ± 63.96 | 210.11 ± 60.91 | 0.476 |
| T2DM (Y/N) | 21/77 | 12/115 | 0.012 |

Table 2: Proportion of liver fibrosis and advanced fibrosis by groups.

| Variables | Nonalcoholic fatty liver disease | | $P$ |
|---|---|---|---|
| | Smoking | Nonsmoking | |
| Sample size | 98 | 127 | |
| Liver stiffness value | | | 0.046 |
| <7.4 (kPa) | 52 | 88 | |
| 7.4–9.8 (kPa) | 22 | 16 | |
| >9.8 (kPa) | 24 | 23 | |
| Liver stiffness value, kPa | 10.12 ± 10.38 | 7.26 ± 6.42 | 0.013 |

7.26 ± 6.42 kPa, $P = 0.013$). The proportion of patients with liver significant fibrosis and advanced fibrosis in the smoking group was significantly higher than that in the nonsmoking group ($P = 0.046$).

*3.3. Risk Factors Associated with Fibrosis in the Patients with NAFLD.* The univariate and multivariate analyses were conducted to explore the risk factors associated with fibrosis among the patients with NAFLD. The results are shown in Table 3. The univariate analysis showed that age, weight, high AST level, low PLT level, and smoking were the risk factors associated with liver fibrosis in the smokers with NAFLD. Conversely, the multivariate analysis showed that age (OR = 1.029, $P = 0.021$), high AST level (OR = 1.0121, $P = 0.025$), and smoking (OR = 1.294, $P = 0.015$) were the independent risk factors associated with liver fibrosis in the patients with NAFLD.

*3.4. Clinical Characteristics of the Patients with NAFLD with and without Liver Fibrosis.* To analyze the related factors for liver fibrosis in the smokers with NAFLD further, we subdivided the smokers into the fibrosis and nonfibrosis subgroups. The clinical characteristics of these two subgroups are shown in Table 4. The average age ($P = 0.032$) and AST level ($P = 0.001$) in the fibrosis group were significantly higher than those in the nonfibrosis group, while the PLT level was lower in the fibrosis group than in the nonfibrosis group ($P = 0.036$). In addition, the proportion of

Table 3: Risk factors associated with fibrosis in NAFLD patients.

| Variables | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| | OR | 95% CI | $P$ | OR | 95% CI | $P$ |
| Sex | 1.622 | 0.366–3.322 | 0.632 | | | |
| Age | 1.022 | 1.006–1.097 | 0.019 | 1.029 | 1.004–1.055 | 0.021 |
| Height | 0.936 | 0.875–1.001 | 0.055 | | | |
| Weight | 1.066 | 1.028–1.107 | 0.001 | | | |
| SBP | 1.014 | 0.958–1.073 | 0.632 | | | |
| DBP | 0.922 | 0.842–1.009 | 0.076 | | | |
| ALT | 0.998 | 0.994–1.002 | 0.339 | | | |
| AST | 1.009 | 1.001–1.017 | 0.020 | 1.012 | 1.002–1.061 | 0.025 |
| PLT | 0.991 | 0.985–0.996 | 0.001 | | | |
| Smoking index | 1.305 | 1.152–2.611 | 0.011 | 1.294 | 1.087–2.087 | 0.015 |
| T2DM | 1.022 | 0.998–1.525 | 0.062 | | | |

Table 4: Characteristics of NAFLD patients with smoking with or without liver fibrosis.

| Variables | Nonalcoholic fatty liver disease with smoking | | $P$ |
|---|---|---|---|
| | Fibrosis | Nonfibrosis | |
| Sample size | 46 | 52 | |
| Sex (F/M) | 1/45 | 2/50 | 0.632 |
| Age (years) | 48.78 ± 11.65 | 43.09 ± 13.92 | 0.032 |
| Height (cm) | 168.17 ± 6.51 | 168.52 ± 6.49 | 0.794 |
| Weight (kg) | 67.37 ± 13.57 | 65.79 ± 11.56 | 0.535 |
| SBP (mm/Hg) | 125.71 ± 14.25 | 128.17 ± 10.82 | 0.337 |
| DBP (mm/Hg) | 80.76 ± 8.92 | 83.21 ± 6.64 | 0.124 |
| ALT | 87.91 ± 82.58 | 72.12 ± 81.30 | 0.344 |
| AST | 83.78 ± 38.15 | 53.74 ± 46.76 | 0.001 |
| PLT | 201.55 ± 70.21 | 228.99 ± 55.43 | 0.036 |
| T2DM (Y/N) | 14/32 | 7/45 | 0.041 |
| Smoking index | 583.26 ± 480.72 | 388.63 ± 458.52 | 0.043 |

patients with diabetes mellitus in the fibrosis group was significantly higher than that in the nonfibrosis group ($P = 0.041$); the smoking index was significantly higher in the fibrosis group than in the nonfibrosis group ($P = 0.043$).

*3.5. Risk Factors Associated with Fibrosis in Smokers with NAFLD.* We further analyzed the factors associated with liver fibrosis in the smokers with NAFLD, and the results are shown in Table 5. The univariate analysis showed that age, high AST level, low PLT level, smoking index, and diabetes mellitus were the risk factors for fibrosis among these patients. Conversely, the multivariate analysis indicated that high AST level (OR = 1.040, $P = 0.029$), smoking index (OR = 1.220, $P = 0.019$), and diabetes mellitus (OR = 1.054, $P = 0.032$) were the independent risk factors for liver fibrosis among them.

## 4. Discussion

In this study, we confirmed that smoking is closely associated with NAFLD. Moreover, we further confirmed that it is closely related to liver fibrosis in NAFLD. The LS value of the smokers with NAFLD was significantly higher than that of the nonsmokers with NAFLD. Older age, high AST level,

Table 5: Risk factors for fibrosis in NAFLD patients with smoking.

| Variables | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| | OR | 95% CI | *P* | OR | 95% CI | *P* |
| Sex | 1.022 | 0.395–1.895 | 0.614 | | | |
| Age | 1.049 | 1.004–1.095 | 0.029 | | | |
| Height | 0.956 | 0.867–1.054 | 0.983 | | | |
| Weight | 1.069 | 1.007–1.136 | 0.176 | | | |
| SBP | 1.036 | 0.943–1.138 | 0.113 | | | |
| DBP | 0.870 | 0.747–1.014 | 0.076 | | | |
| ALT | 0.997 | 0.991–1.004 | 0.310 | | | |
| AST | 1.024 | 1.008–1.040 | 0.004 | 1.040 | 1.004–1.078 | 0.029 |
| PLT | 0.991 | 0.982–1.000 | 0.010 | | | |
| Smoking index | 1.666 | 1.187–2.338 | 0.014 | 1.220 | 1.040–1.878 | 0.019 |
| T2DM | 1.199 | 1.036–3.991 | 0.011 | 1.054 | 1.067–3.050 | 0.032 |

and smoking were found to be the independent risk factors for liver fibrosis in the patients with NAFLD. Conversely, high AST level, smoking index, and diabetes mellitus were determined to be the independent risk factors for liver fibrosis in the smokers with NAFLD. These results imply that smoking is not only associated with liver fibrosis in NAFLD but also increases the risk for liver fibrosis as the smoking index increases.

The pathogenesis of NAFLD is not fully understood [1]. A widely accepted conclusion is that NAFLD is a genetic-environment-metabolism-related disease [1, 2]. Consumption of food high in calorie and fructose, refined carbohydrates, and sugar-sweetened beverages has been associated with NAFLD [1]. Recently, several genetic modifiers of NAFLD have been identified [25–28]. Among them, the best-characterized genetic association was found with PNPLA3, which was initially identified from genome-wide association studies and confirmed in multiple cohorts [29–32]. Liver biopsy is the gold standard for the diagnosis of NAFLD [1]. However, it cannot be routinely used because of its invasiveness. Noninvasive techniques, such as the use of FibroScan and ultrasonography, are beginning to be used for the diagnosis of NAFLD [20, 22]. Their accuracy has been confirmed in many studies [2, 16, 20–22].

The toxic and harmful substances produced by smoking can damage the antioxidant system, including cytochrome P450 and inflammatory cytokines [33]. Although the effects of smoking on CHB infection and cirrhosis have been reported [17, 34, 35], there is limited information on the relationship between smoking and NAFLD. Hamabe et al. [19] reported that smoking is an independent risk factor for NAFLD. Suzuki et al. [18] reported that it is associated with high levels of ALT in patients with NAFLD. Herein, we found that smoking is an independent risk factor for liver fibrosis in NAFLD. For patients with NAFLD, timely smoking cessation education should be provided, and a liver fibrosis test is also necessary. However, we found a connection between smoking index and liver fibrosis among NAFLD patients. However, in this population, the smoking index and liver fibrosis grade did not show with dose response in our study. We further found that diabetes mellitus and the smoking index were the independent risk factors for fibrosis in the smokers with NAFLD. The relationship between smoking and diabetes mellitus has been well established. If smokers with NAFLD are diagnosed with diabetes mellitus, the possibility of liver fibrosis development may increase. In our study, we found that diabetes mellitus is an independent factor for liver fibrosis among NAFLD patients with smoking. Although we did not find that DM is an independent factor for liver fibrosis among all NAFLD patients, the reason may due to the relatively small patients enrolled in our study with only 12 patients diagnosed with diabetes mellitus in NAFLD without smoking. In addition, since smoking is also an independent risk factor for DM, this may impair the association of DM and liver fibrosis among all NAFLD patients.

This study has some limitations. First, the sample size of the study is relatively small. Second, the study data were collected from a single center. Given the cross-sectional nature of this study, prospective studies should be conducted to corroborate the conclusions. Multicenter clinical studies are also warranted to confirm our results for screening and early diagnosis of liver fibrosis in patients with NAFLD.

In conclusion, smoking is closely related to liver fibrosis in NAFLD. Older age, high AST level, and smoking are the independent risk factors for liver fibrosis in NAFLD. Conversely, high AST level, smoking index, and diabetes mellitus are the independent risk factors for liver fibrosis in smokers with NAFLD. For patients with NAFLD, priority screening and timely intervention should be provided if they are found to have risk factors for liver fibrosis.

## Data Availability

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

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

## Authors' Contributions

Hongjie Ou and Yaojie Fu contributed equally to this work.

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