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Role of oxidative stress and insulin resistance in disease severity of non-alcoholic fatty liver disease

LIVER

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Original Article

ABSTRACT

Background/Aims: Oxidative stress and insulin resistance (IR) are major contributors in the pathogenesis of non-alcoholic fatty liver disease (NAFLD). The purpose of this study was to find the relation between oxidative stress parameters and histopathological findings in NAFLD patients with and without insulin resistance (IR).

Materials and Methods: Thirty-two patients with no alcohol intake and biopsy-proven diagnosis of NAFLD were studied (M/F: 17/15; mean age 46.5±11.4 years). Twenty-one NAFLD patients with IR were compared with 11 patients without IR. The fasting insulin level was measured, and the insulin resistance index was calculated using the homeostasis model assessment (HOMA) method. Malondialdehyde (MDA) and superoxide dismutase (SOD) activities were measured in tissue and serum specimens. Glutathione (GH) was measured in tissue homogenates. Nitric oxide (NO), vitamin E and C levels were measured in serum.

Results: Patients with IR had significantly higher tissue MDA levels (p=0.001) and significantly decreased tissue SOD and GH levels (p=0.001 and 0.002, respectively) than those without IR. The steatosis grade, necroinflammatory grade and stage were significantly higher in patients with IR (p=0.035, 0.003 and 0.001, respectively). HOMA IR significantly correlated with the necroinflammatory grade, stage, tissue MDA, SOD and GH (p=0.013, 0.001, 0.008, 0.001 and 0.001, respectively). Serum MDA (β=1.88, p=0.002), serum SOD (β=0.57, p=0.006), tissue MDA (β=0.22, p=0.006), tissue SOD (β=1.48, p=0.071) and stage (β=2.81, p=0.003) were independently associated with increased HOMA IR. Increased MDA [OR: 1.51; 95% CI: (1.03–2.22); p=0.034] was a risk factor for non-alcoholic steatohepatitis (NASH), and increased SOD activity had a preventive effect against NASH [OR: 0.008; 95% CI: (0.001–0.98); p=0.04].

Conclusion: This study shows that insulin resistance in NAFLD correlates with enhanced oxidative stress. Histopathological disease severity significantly correlated with oxidative stress parameters. These data show that NAFLD patients with IR may have increased risk for disease progression.

Keywords: Oxidative stress, insulin resistance, non-alcoholic fatty liver disease

INTRODUCTION

The pathogenic mechanism underlying non-alcoholic fatty liver disease (NAFLD) and progression from non-alcoholic fatty liver (NAFL) to non-alcoholic steatohepatitis (NASH) is not entirely understood. Oxidative stress (OS) and insulin resistance (IR) are major contributors in the pathogenesis of NAFLD. IR leads to an increase in lipolysis in peripheral fat tissue and subsequent elevated serum concentration of free fatty acids (FFAs), which causes triglyceride accumulation in the liver (1). It is suggested that increased accumulation of liver triglycerides causes increased OS in the hepatocytes of animals and humans (2).

Oxidative stress caused by reactive oxygen species (ROS) is however known to be one of the major factors in disease progression. Many experimental models (3) and human studies (4-6) have shown a strong relation between the severity of NASH and the degree of OS.

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To make a comparison between NAFLD progression and the severity of OS, we compared the histopathological changes and OS parameters in our NAFLD patients with and without IR.

MATERIALS AND METHODS

Patients and inclusion criteria

Thirty-two non-drinking patients (17 men and 15 women; mean age 46.5±11.4 years) with biopsy-proven NAFLD were enrolled into the prospective study in our university hospital clinic. Informed consent was obtained from each patient. Twenty-one NAFLD patients with IR were compared with 11 patients without IR.

Patients were referred for the assessment of abnormal liver function tests or hepatic steatosis, and hepatomegaly was detected under ultrasonography (USG). Most patients were referred from the internal medicine clinic and endocrinology department of our hospital. Patients with alcohol intake (all patients were teetotallers) or with a history of recent potentially hepatotoxic drug intake were excluded. Diagnosis was based on histology with exclusion of other aetiologies such as chronic viral hepatitis, primary biliary cirrhosis, metabolic liver diseases, autoimmune liver disease, α1-antitrypsin deficiency, Wilson's disease, hemochromatosis and sclerosing cholangitis.

The fasting insulin level was measured, and the insulin resistance index was calculated using the homeostasis model assessment method: HOMA IR (%): [glucose (mg/dL)/18]×[fasting insulin (mU/mL)/22.5]. The American Diabetes Association criteria for diabetes were used. Patients with fasting serum glucose levels of more than 126 mg/dL or a 2-h glucose level of more than 200 mg/dL during an oral glucose tolerance test were considered to have diabetes mellitus.

After exclusion of the secondary NAFLD conditions, patients with elevated ALT, steatosis or hepatomegaly in USG underwent liver biopsy. A percutaneous transcostal liver biopsy was performed under ultrasonic guidance. Biopsy materials that contained more than five portal areas were evaluated. According to HOMA IR, patients with biopsy-proven NAFLD diagnosis were then divided into two groups: the IR group (n=21) and non-IR group (n=11).

Histological analysis

The histological findings were interpreted according to the classification proposed by Brunt et al. (7). Liver histological sections were fixed with 10% formaldehyde, and tissue sections cut from paraffin-embedded blocks were stained using hematoxylin and eosin. To determine the fibrotic tissue component, Masson trichrome and Gomory reticulum staining was performed. The same pathologist, who was blinded to the clinical and biochemical data, reviewed all liver biopsy specimens. The severity of steatosis was graded from 1 to 3 according to the percentage of cells with fatty droplets (1: 10–33%; 2: 33–66%; 3: >66%). Necroinflammatory activity was graded using a 4-point scale (0: absent; 1: mild; 2: moderate and 3: severe). As previously reported by Brunt et al. (7), NASH was defined by the presence of hepatic steatosis, cytologic ballooning, scattered (mainly acinar or portal) inflammation with or without Mallory bodies and/or fibrosis. The stage of fibrosis was measured using a 4-point scale: stage 1, zone 3 perisinusoidal/pericellular fibrosis; stage 2, zone 3 perisinusoidal/pericellular fibrosis with periportal fibrosis; stage 3, zone 3 perisinusoidal/pericellular fibrosis and portal fibrosis with bridging fibrosis and stage 4, cirrhosis.

Quantitation of OS parameters

Serum samples and liver biopsy samples were stored at –80°C until analysis. Malondialdehyde (MDA) and superoxide dismutase (SOD) activities were measured in tissue and serum specimens. Glutathione (GH) was measured in tissue homogenates. Nitric oxide (NO), vitamin E and C levels were measured in serum. MDA was measured in liver homogenates and serum using a thiobarbituric acid reactivity assay (8). Serum and tissue SOD activities were determined through the inhibition of the nitroblue tetrazolium reduction with xanthine/xanthine oxidase, as previously described by Sun et al. (9). Tissue GH concentration was measured using the method described by Beutler et al. (10). Metaphosphoric acid was used for protein precipitation, and 5,5´dithiobis-2-nitrobenzoic acid was used for colour development. Human TNFR1 twin enzyme-linked immunosorbent assay (ELISA) (HyCult Biotechnology) was used for the measurement of serum tumour necrosis factor (TNF) receptor (TNFsRp55) levels. NO levels were determined using the Griess reaction. A commercially available ELISA kit (R&D systems, Quantikine; Wiesbaden – Nordenstadt, Germany) was used for this propose. Plasma vitamin C was spectrophotometrically assayed using the 2,4-dinitrophenylhydrazine method of Omaye et al. (11). Plasma vitamin E concentration was determined according to the methods of Maritim et al. (12).

Statistical analysis

We compared the data between the two groups using the unpaired Student's t-test and k^2test. Correlation analysis was performed using Pearson's and Spearman's correlation. Risk factors for histopathological severity were identified using multiple regression analyses. Area under the receiver operating characteristic (ROC) curve was used to identify the ability of studied parameters to predict advanced fibrosis. Post-hoc power analysis was used to provide a power calculation of the study. P-values of <0.05 were considered statistically significant. All statistical analyses were performed using SPSS® statistical software (version 15.0, IBM Corp.; New York, USA).

RESULTS

A total of 32 patients (17 men and 15 women) with NAFLD were identified through liver biopsies. The mean age of the whole cohort was 46.5±11.4 years. A total of 21 patients with NAFLD and insulin resistance were compared with 11 NAFLD

patients without IR. The groups, which were allocated according to their insulin resistance, were similar in age and gender. The body mass index (BMI) was 30.4±5.08 in the IR group and 29.51±3.12 in the non-IR group. Five (45%) of 11 patients without IR and 10 (47%) of 21 patients with IR had BMI>30. There were no significant differences between the groups. Table 1 presents the characteristics of the examined groups.

Post-hoc power analysis was used to provide a power calculation of the study. Study power calculated by post-hoc power analysis was 72% with a 17% difference and 12% standard deviation between the groups, consisting of 21 patients in one group and 11 in the other at an α-level of 0.05.

Clinicohistological correlations

The steatosis grade, necroinflammatory grade and stage were significantly higher in patients with IR (p=0.035, p=0.003 and p=0.001, respectively) (Figure 1).

HOMA IR significantly correlated with the necroinflammatory grade and stage (p=0.013 and 0.001, respectively). The ROC curves used to establish the discriminative power of the HOMA IR index for necroinflammatory and fibrosis severity and for differentiating NASH from simple fatty liver showed statistically significant values (AUROC=0.069, 0.58 and 0.57, respectively) (Figure 2-4).

OS parameters

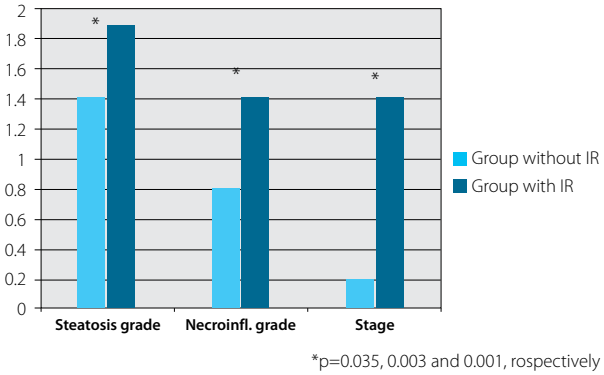
Patients with IR had significantly higher tissue MDA levels (p=0.001) and significantly decreased tissue SOD and GH levels (p=0.001 and 0.002, respectively) than those without IR (Table 2).

Table 1. Biochemical and demographic parameters of NAFLD patients with and without insulin resistance

Parameters	Group without IR (n=11)	Group with IR (n=21)	p
Age (years)	47.11±6.21	45.96±11.76	NS
BMI (kg/m2)	30.4±5.08	29.51±3.12	NS
BMI<25	n=2	n=4	
BMI: 26–29	n=4	n=7	
BMI: 30–34	n=4	n=8	
BMI≥35	n=1	n=2	
ALT (U/l) (NR: 5–37)	62.12±8.21	88.12±30.23	0.04
AST (U/l) (NR: 5–37)	49.45±11.12	62.20±25.14	0.045
γ GT (U/l) (NR: 7–49)	60.08±28.17	65.96±48.16	NS
ALP (U/l)(NR: 38–155)	140.95±93.07	142.04±82.55	NS
Cholesterol (mg/dL)	219.1±66.70	232.6±42.45	NS
Triglyceride (mg/dL)	183.12±86.15	192.42±74.86	NS

Data are presented as mean±standard deviation.
NAFLD: non-alcoholic fatty liver disease; NS: statistically not significant; BMI: Body Mass Index; γ-GT: g-glutamyl transpeptidase; ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; IR: insulin resistance; NR: normal range

HOMA IR significantly correlated with tissue MDA, SOD and GH (p=0.008, 0.001 and 0.001, respectively). Serum MDA (β=1.88, p=0.002), serum SOD (β=0.57, p=0.006), tissue MDA (β=0.22, p=0.006), tissue SOD (β=1.48, p=0.071) and stage (β=2.81, p=0.003) were independently associated with increased HOMA IR. Increased MDA [OR: 1.51; 95% CI: (1.03–2.22); p=0.034] was a risk factor for NASH, and increased SOD activity had a pre-



*p=0.035, 0.003 and 0.001, rospectively

Figure 1. Histopathological correlations between patients with non-alcoholic fatty liver disease (NAFLD) with and without insulin resistance (IR)

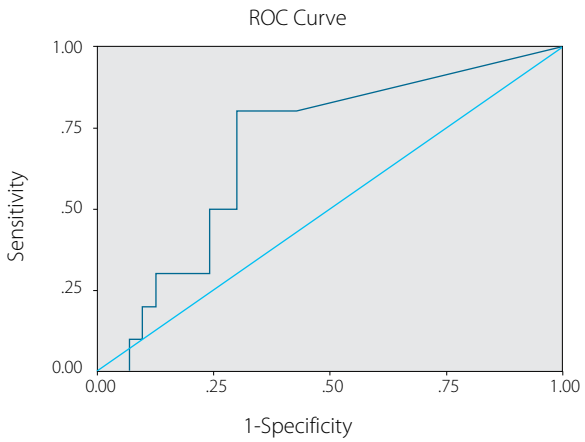


Figure 2. Discriminative power of IR index for necroinflammatory grade, AUROC: 0.69

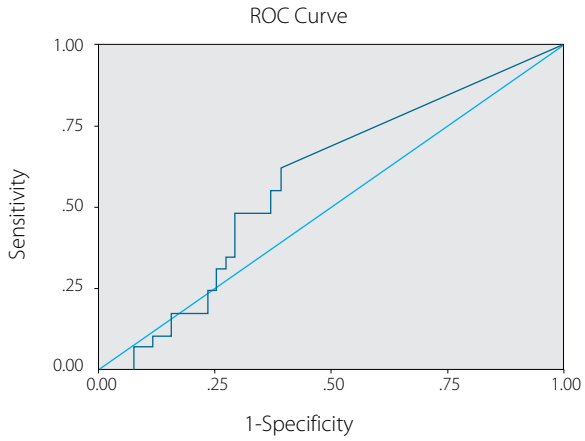


Figure 3. Discriminative power of insulin resistance (IR) index for stage, AUROC: 0.58

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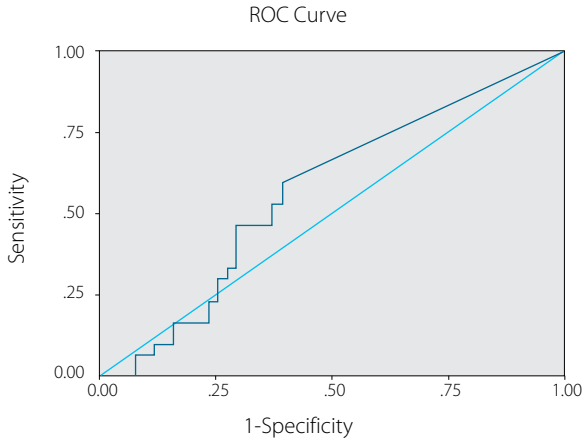

Figure 4. Discriminative power of insulin resistance (IR) index for NASH, AUROC: 0.57

Table 2. Oxidative stress parameters in non-alcoholic fatty liver disease groups with and without IR

Parameters	Group without IR (n=11)	Group with IR (n=21)	p
Serum MDA mmol/L	3.94±0.84	4.07±0.66	NS
Tissue MDA mmol/g wet tissue	40.29±8.44	51.4±8.17	0.001
Serum SOD U/mL	25.04±1.11	24.83±1.32	NS
Tissue SOD U/mg protein	2.47±0.92	1.0±0.52	0.001
Tissue GH mmol/g wet tissue	2.47±0.92	1.52±0.62	0.002
Serum vitamin E (mg%)	0.86±0.14	0.84±0.13	NS
Serum vitamin C (mg%)	1.14±0.13	1.07±0.13	NS
Serum NO mmol/L	43.09±8.03	47.71±9.48	NS

Data are presented as mean±standard deviation.
NS: statistically not significant (p<0.05); IR: insulin resistance; MDA: malondialdehyde; SOD: superoxide dismutase; GH: glutathione; NO: nitric oxide

ventive effect against NASH [OR: 0.008; 95% CI: (0.001–0.98); p=0.04). Patients with NASH had significantly higher tissue MDA levels (p=0.035) and significantly decreased tissue SOD and GH levels (p=0.004 and 0.003, respectively) than those with NAFL. Patients with NAFL had significantly higher serum vitamin E and C levels than those with NASH (p=0.03 and 0.038, respectively) (Table 3).

DISCUSSION

The main pathogenic features in NAFLD are insulin resistance and OS (12). It was suggested that IR suppresses glycogenesis, promotes gluconeogenesis and glycogenolysis and increases the release of FFA from adipose tissue (13). The increased fatty acid influx to the liver and the subsequent induction of the lipotoxicity is the main course for increased ROS production and disease progression from simple steatosis to NASH. Although simple steatosis generally has a benign clinical course, NASH, which can be present in one-third of NAFLD cases, is a progressive disease that can advance to liver cirrhosis and hepatocellular carcinoma (HCC) (14,15).

Table 3. Oxidative stress parameters in non-alcoholic fatty liver disease groups with NAFL and NASH

Parameters	Group without NAFL (n=27)	Group with NASH (n=49)	p
Serum MDA mmol/L	4.8±0.71	5.11±1.21	NS
Tissue MDA mmol/g wet tissue	39.29±11.35	48.4±9.12	0.035
Serum SOD U/mL	27.12±3.11	25.14±4.32	NS
Tissue SOD U/mg protein	3.07±1.64	2.04±0.42	0.004
Tissue GH mmol/g wet tissue	3.17±1.92	2.02±0.98	0.003
Serum vitamin E (mg%)	1.66±0.24	0.78±0.26	0.03
Serum vitamin C (mg%)	2.31±0.72	1.27±0.26	0.038
Serum NO mmol/L	45.12±7.81	50.71±6.41	NS

Data are presented as mean±standard deviation.
NS: statistically not significant (p<0.05); NAFL: non-alcoholic fatty liver; NASH: non-alcoholic steatohepatitis; MDA: malondialdehyde; SOD: superoxide dismutase; GH: glutathione; NO: nitric oxide

The pathogenesis of NAFLD is closely related to the presence of insulin resistance. In the study of Leach et al. (16), plasma fasting glucose, serum insulin and HOMA-IR were all significantly higher in patients with NASH than in the control group. Diez-Rodriquez (17) found that HOMA-IR was statistically related to the necroinflammatory grade. Using ultrasound to detect fatty liver in obese children and adolescents, different studies have reported that patients with NAFLD had more prevalent IR and QUICKI and the severity of fatty liver was positively related to IR by HOMA. Therefore, it has been suggested that markers of insulin sensitivity could be useful screening parameters for NAFLD in obese children (18-20). In our study, HOMA IR significantly correlated with the necroinflammatory grade and stage. The HOMA IR index showed statistically significant values for differentiating NASH from simple fatty liver. The ROC curves used to establish the discriminative power of the HOMA IR index for necroinflammatory and fibrosis severity and for differentiating NASH from simple fatty liver showed statistically significant values. The steatosis grade, necroinflammatory grade and stage were significantly higher in patients with IR.

Oxidative stress is the major promoter of necroinflammation in NASH through lipid peroxidation. However, only few studies have investigated markers of systemic OS in patients with NAFLD. Sanyal et al. (4) demonstrated that a marker concerning OS was elevated in liver biopsies from patients with NAFLD and was even higher in NASH than in steatosis alone. In another study, OS parameters in the liver tissue of patients with NAFLD significantly correlated with the inflammation grade, and there was a significant negative relation between the steatosis degree and antioxidant levels in tissue (21).

Oxidative stress is described as a disturbance in the balance between pro-oxidants and antioxidants. OS can result from increased ROS or reactive nitrogen species (RNS) production or from decreased antioxidant levels.

Superoxide dismutase is an antioxidant enzyme that changes superoxide anion radicals into hydrogen peroxide and molecular oxygen (22). With regard to lipid metabolism in the liver, it was shown that SOD1-knockout mice presented with increased lipid peroxidation and hepatic TG accumulation because of abnormal lipid metabolism in mouse liver (23). Furthermore, SOD-knockout mice hepatocytes included abnormally enlarged mitochondria (24). Kondo et al. (22) reported that high levels of OS caused by defects in the antioxidant system as result of concomitant deficiency of SOD in SOD-knockout mice resulted in hepatic lipid accumulation via altered lipid metabolism.

Depletion of GH, a major cellular antioxidant, has been reported in NAFLD (25). Leach et al. (16) recently demonstrated that NASH was an independent predictor of decreased GH levels and patients with NASH had significantly lower levels of antioxidants.

In our study, increased SOD activity had a protective effect against NASH. Patients with NASH had significantly decreased tissue SOD and GH levels than those with NAFL. Our data suggest that high levels of OS caused by defects in the antioxidant system resulted in histopathological disease severity.

The activity of antioxidant enzymes is increased in patients with NASH (26). However, the total antioxidant capacity in NASH patients is apparently insufficient to compensate for OS (27). Furthermore, the intake of dietary antioxidants such as vitamin C and E is significantly lower in patients with NASH than in healthy controls (28).

Malonildialdehyde (MDA), a product of polyunsaturated fatty acids and ROS, is widely used as a marker of lipid oxidation. Hepatic oxidative injury was associated with the development of antibodies toward protein-adducted MDA in a significant proportion of children with NAFLD (63%) and in ~40% of adult patients with NAFLD (25). In a previous study, plasma NO and plasma MDA levels were significantly higher, and plasma vitamin E and C levels were significantly lower in patients with NASH than in those with NAFL (29).

Our patients with NAFL had significantly higher serum vitamin E and C levels than those with NASH. We demonstrated that OS was associated with increased risk of NASH and antioxidative agents showed a preventive effect against NASH. In our study, increased MDA was a risk factor for NASH and increased SOD activity had a preventive effect against NASH. Patients with NASH had significantly higher tissue MDA levels and significantly decreased tissue SOD and GH levels than those with NAFL. With regard to lipid metabolism in the liver, we showed that a decrease in antioxidative agents exhibited increased lipid peroxidation and subsequent inflammation in the liver. This result suggests that superoxide anion radicals are involved in abnormal lipid metabolism in the liver. Further studies will be required to clarify the molecular mechanism involved in the alteration of lipid homeostasis and metabolism of ROS.

Insulin resistance is involved in liver steatosis and in the development of OS by decreasing mitochondrial beta oxidation, which causes activation of other oxidation pathways, thereby contributing to high ROS levels (30-32). Persistent OS can diminish insulin action through the activation of serine–threonine kinase cascades, which in turn phosphorylate several targets, including insulin receptor and insulin receptor substrate (IRS) proteins, with consequent decrease of insulin-stimulated tyrosine phosphorylation (33). As suggested by Videla et al. (34), the onset of IR could further increase ROS generation through CYP2E1 induction, mitochondrial dysfunction and pro-inflammation, all of which lead to the progression from steatosis to steatohepatitis. This observation was also supported by another study that showed an exacerbation of OS in patients with both NAFLD and T2DM (35).

Our patients with IR had significantly higher tissue MDA levels and significantly decreased tissue SOD and GH levels than those without IR. Serum and tissue MDA and SOD levels and tissue GH level and stage were independently associated with increased HOMA IR. Further studies will be required to clarify the molecular mechanism involved in the alteration of carbohydrate metabolism and insulin resistance and ROS metabolism caused by the depletion of antioxidants in NAFLD. However, our data suggest that high levels of OS caused by defects in the antioxidant system resulted in histopathological disease severity via altered carbohydrate metabolism and insulin resistance, and these observations together support that OS and insulin resistance might be possible causes for disease progression in NAFLD.

Although several clinical trials have shown that antioxidative therapy with vitamin E can effectively control hepatitis pathology in the short term, the long-term effects remain obscure and have often proved to be ineffective in many other diseases. In our study, there was no statistically significant difference in vitamin E and C levels between patients with and without IR. However, our patients with NAFL had significantly higher serum vitamin E and C levels than those with NASH.

The limitation of this study is the small number of NAFLD patients without IR. Therefore, we calculated the power of the study. Using post-hoc power analysis, the calculated study power was 0.72. Even though our study power was at a statistically significant level, further investigations should be performed with larger patient groups.

This study demonstrates that insulin resistance in NAFLD is associated with enhanced OS. Histopathological disease severity significantly correlated with OS parameters. These data indicate that NAFLD patients with IR may have increased risk for disease progression. Therapeutic interventions should be aimed to improve hepatic insulin sensitivity in NAFLD.

Ethics Committee Approval: N/A.

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - K.E.; Design - K.E., C.B.; Supervision - K.E.; Materials - K.E., C.B., A.K., H.I., T.M., D.A., S.A., G.I., S.H.; Data Collection and/or Processing - K.E., C.B., A.K., H.I., T.M., D.A., S.A., G.I., S.H.; Analysis and/or Interpretation - K.E., C.B., A.K., H.I., T.M., D.A., S.A., G.I., S.H.; Literature Review - K.E., C.B., A.K., H.I., T.M., D.A., S.A., G.I., S.H.; Writer - K.E.; Critical Review - K.E., C.B., A.K., H.I., T.M., D.A., S.A., G.I., S.H.; Other - K.E., C.B., A.K., H.I., T.M., D.A., S.A., G.I., S.H.

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