# Apoptosis and Disease Severity is Associated with Insulin Resistance in Non-alcoholic Fatty Liver Disease

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## Abstract

***Background & Aims :*** **Non-alcoholic fatty liver disease (NAFLD) is associated with insulin resistance (IR). We evaluated whether IR contributes to hepatocyte apoptosis, inflammation, and fibrosis in NAFLD.**

***Methods :*** **Forty-four teetotaller patients with biopsy-proven diagnosis of NAFLD were enrolled. Twenty-eight NAFLD patients with IR were compared with 16 subjects without IR. For apoptotic activity caspase 3 and 8, transcription nuclear factor kB (NF-kB), and anti-apoptotic Bcl-2 protein were determined through immunohistochemical methods.**

***Results :*** **HOMA-IR index was significantly correlated with the stage and caspase 3- and 8 levels (p= 0.001, 0.02, and 0.01, respectively). HOMA-IR index was independently associated with the severity of fibrosis ($\beta$ = 5.9, p = 0.001), caspase-3 ($\beta$ = 0.16, p = 0.001), and caspase-8 ($\beta$ =0.032, p = 0.018) levels. TNF-sRp55 level was positively correlated with HOMA-IR index (p = 0.024). Patients with IR had significantly higher necroinflammatory grade, stage, caspase-3, and caspase-8 levels than those without IR (p = 0.022, 0.007, 0.031, and p = 0.011, respectively). HOMA-IR index had statistically significant values for distinguishing of severe necroinflammatory grade, stage and for differentiating NASH from simple fatty liver (AUC = 0.78, 0.76, and 0.82, respectively).**

***Conclusion :*** **This study demonstrates that IR in NAFLD is associated with enhanced hepatocyte apoptosis and histopathologic disease severity. These data indicate that NAFLD patients with IR may have increased risk for disease progression.** (Acta gastroenterol. belg., **2017**, 80, **271-277**).

**Key words :** Apoptosis, insulin resistance, and fatty liver disease

## Introduction

Non-alcoholic fatty liver disease (NAFLD) is a prevalent liver disorder in Western countries, which can be seen in a wide range of clinical and histopathologic presentations such as simple hepatic steatosis, non-alcoholic steatohepatitis, liver cirrhosis, and hepatocellular cancer (1). Simple liver steatosis in NAFLD is generally asymptomatic and has a benign clinical course. Non-alcoholic steatohepatitis (NASH), on the other hand, is characterized by liver inflammation and fibrosis, and can progress to liver cirrhosis. The complex mechanisms that lead to the development of steatosis and its progression to NASH are still not clear (2). Although some pathogenetic hypothesis have been postulated. Day et al(3) first proposed the current concept of the "two-hit hypothesis in NAFLD". The first hit is primarily IR, followed by increased dietary intake and enhanced hepatic lipogenesis, peripheral lipolysis and subsequently accumulation of free fatty acids (FFAs) and triglycerides (TGs) in hepatocytes. The second hit is a combination of oxidative stress, lipid peroxidation, mitochondrial dysfunction and the release of reactive oxygen species (ROS) which trigger hepatic apoptosis and progressive liver injury (3). The frequent co-occurrence of NAFLD with obesity, type 2 diabetes mellitus (DM), dyslipidemia, and metabolic syndrome suggests that insulin resistance (IR) may play an important role in the pathogenesis of this disease(4). IR increases peripheral lipolysis in adipose tissue that leads to increase in the delivery of free fatty acids (FFAs) to the liver and de novo lipogenesis (5,6). In addition, lipid overload leads to dysregulated insulin secretion and changes in the expression of peroxisome proliferator-activated receptor(PPAR)-$\alpha$, glucokinase, the glucose transporter-2, pre-pro-insulin and pancreatic duodenal homeobox-1, which can lead to IR as a result of FFA induced B-cell apoptosis (7). Furthermore, increased hepatocyte apoptosis is thought to play a role in the progression of liver disease in subjects with NAFLD (8). Nevertheless, the exact pathophysiologic mechanisms that underlie this condition have not been completely understood. In previous studies on mice, insulin exerts antiapoptotic functions in many cell types and has been linked to insulin-mediated activation (9-11). Number of clinical studies on patients with NAFLD showed that impairment of insulin signaling in liver was closely associated with increased hepatocyte apoptosis (12,13). Therefore, we aimed to elucidate whether IR may contribute to hepatocyte apoptosis and the subsequent induction of inflammation and fibrosis in NAFLD.

## Patients and Methods

### *Patients*

Fifty-four subjects with biopsy-proven NAFLD were enrolled in to the study in our university hospital clinic. Patients were referred for the assessment of elevated cytonecrotic or cholestatic liver enzymes or hepatic

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steatosis detected under ultrasonography (US) from the internal medicine outpatient clinic or endocrinology departments of our hospital. The exclusion criteria were as follows : history of alcohol consumption (>30g/day), HBsAg and/or anti-HCV positivity, autoimmune hepatitis, Wilson's disease, hemochromatosis, or other chronic liver diseases and use of steatogenic drugs (e.g. corticosteroid, methotrexate). Informed consent was obtained from each participating subject.

*Pathologic examination*

The same pathologist (NK), who was blinded to the clinical and biochemical data, reviewed all liver biopsy specimens. Necroinflammatory activity and fibrosis were defined using the scoring system described by Brunt et al. (14). 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 (14).

*Laboratory evaluations*

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), fasting insulin, fasting glucose and the lipid profile high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceride and total cholesterol were analyzed using standard laboratory methods. The insulin resistance (IR) index was calculated using the homeostasis model assessment (HOMA) method (IR% = glucose mg/dL/18 x fasting insulin mIU/mL/22.5).

*Apoptosis parameters*

Caspase 3 and 8, transcription nuclear factor kB (NF-kB), antiapoptotic B-cell lymphoma 2 protein (Bcl-2 protein) were studied using immunohistochemical methods in biopsy specimens. Tumor necrosis factor receptor (TNF-sRp55) level was measured in serum using a commercially- available human sTNFR-1 twin enzyme-linked immunosorbent assay (ELISA) kit with a sensitivity of 25 pg/mL and without cross-reactivity between the two assays (HyCult Biotechnology ; Uden, The Netherlands) at room temperature. Formalin-fixed, paraffin-embedded liver biopsy samples were cut into 5-μm-thick slices. The sections were soaked in 3% hydrogen peroxide and then incubated using the ultra-V block procedure. Antigen retrieval was performed after this process. Immunohistochemistry was performed using polyclonal rabbit antibodies. the peroxyblocks underwent the ultra V block procedure after microwave incubation : primary antibodies caspase 3 Ab-4 rabbit polyclonal RB-1197-P1 (1/100 dilution Neomarkers), caspase 8 Ab-4 rabbit polyclonal RB-1200-P1 (1/100 dilution Neomarkers), NF-kB P50 Ab-2 rabbit polyclonal RB- 1648-R7 (ready to use Neomarkers), Bcl-2 alpha Ab-1(100/D5) MS-123-R7 (ready to use Neomarkers) were applied. The sections were incubated with biotinylated secondary antibody (goat anti-rabbit IgG-HRP Sc-2030, Lot number : 01504, Santa Cruz Biotechnology), streptavidin–peroxydase conjugate and substrate-chromogen solution. Nuclear and counterstaining was achieved with hematoxylin and eosin. Staining intensity was defined as a percentage and scaled using a 3-point scoring system (0, no staining ; 1, positive staining <30% of cells per high-power field ; 2, positive staining in 30-70% of cells, and 3, positive staining >70% of cells per high-power field).

*Statistical analysis*

Categorical data are presented as numbers and percentages. Continuous data are presented as means and standard deviations or median values according to the distribution of the data. The normality of the distribution of the data was assessed using the Shapiro-Wilk test. Chi-square, Student's t-test, and Mann-Whitney U tests were used to compare the groups. Pearson or Spearman correlation analyses were utilized for correlation analyses. Multivariate linear regression analysis and receiver operator characteristic (ROC) curve analyses were also used. All statistical analyses were performed using SPSS statistical software version 21.0 (Chicago, Illinois, USA). P value <0.05 was considered statistically significant.

## Results

Forty-four patients with no alcohol intake and biopsy-proven NAFLD were studied. The demographic and baseline characteristics of the patients are shown in table I. Twenty-eight NAFLD patients with IR (IR group) were compared with 16 NAFLD subjects without IR (non-IR group). The mean age and sex distribution of the groups were similar. The mean body mass index (BMI) of the IR group was significantly higher than the group without IR. Total cholesterol, ALT, and C-reactive protein (CRP) levels of the patients with IR group were significantly higher than those without IR, other liver enzymes and the rest of the lipid profile were similar in both groups.

Among 44 patients with NAFLD, 27 (61.4%) had NASH. The NASH group had a significantly higher rate of IR compared with those with only NAFLD (81.5% vs. 35.3%, p = 0.002).

Among patients with NAFLD, 11 (25%) had advanced fibrosis (fibrosis grade ≥2). Patients with advanced fibrosis had tended to have higher caspase 8 (54.1±25.3 vs. 36.2±27.9, p = 0.069) and higher caspase 3 levels (61.4±23.7 vs. 44.2±26.9, p b= 0.068) compared with those with no or mild fibrosis. However, NFKB, Bcl-2, and TNF-sRp55 levels were similar between these groups (p>0.4 for all comparisons).

HOMA-IR index was significantly correlated with stage, and caspase-3 and -8 levels (r = 0.9, 0.39, 0.37 and p = 0.001, 0.02, and 0.01 respectively). Linear regression analyses revealed that HOMA-IR was

Table 1. — **Baseline demographic and laboratory features of patients with or without IR**

| Variables | Group with IR NAFLD patients | Group without IR NAFLD patients | p |
|---|---|---|---|
| Sex (F/M) | 10/6 | 13/15 | 0.7 |
| Age | 48.5±10 | 47±9 | 0.1 |
| BMI | 30.9±3.6 | 26.6±1.1 | **0.001** |
| AST | 59±7 | 38±5 | **0.05** |
| ALT | 70±6 | 47±4 | **0.01** |
| ALP | 165±12 | 170±23 | 0.8 |
| GGT | 98±10 | 83±17 | 0.2 |
| Total Cholesterol | 227±35 | 196±40 | 0.4 |
| Triglycerides | 203±20 | 146±17 | 0.1 |
| LDL | 136±35 | 122±40 | 0,3 |
| HDL | 42±12 | 44±7 | **0.006** |
| CRP | 6.4±0.9 | 3.2±0.3 | **0.01** |

independently associated with the severity of fibrosis (β = 5.9, p = 0.001), caspase-3 (β = 0.16, p = 0.001), and caspase-8 (β = 0.032, p = 0.018) levels. Patients with IR had significantly higher necroinflammatory grade, stage, caspase-3 and caspase-8 levels than those without IR (p = 0.022, 0.007, 0.031, and 0.011, respectively). (II and Figure 1,2,3).

The ROC curves used to establish the discriminative power of IR index for necroinflammatory grade and fibrosis severity and for differentiating NASH from simple fatty liver showed statistically significant values. The serum HOMA-IR cut-off value for the prediction of necroinflammatory grade was 4.6, at this threshold the sensitivity and specificity were 77% and 78%, respectively (AUC 0.78 ; 95% CI :[0.63-0.92] ; p<0.004). The cut-off value for the prediction of fibrosis severity was 8.6, at this threshold the sensitivity and specificity were 73% and 79%, respectively (AUC 0.76 ; 95% CI :[0.56-0.94] ;

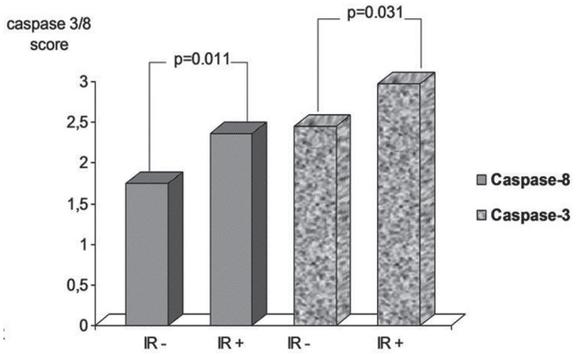


Fig. 1. — Caspase 3/8 score between groups with IR and without IR

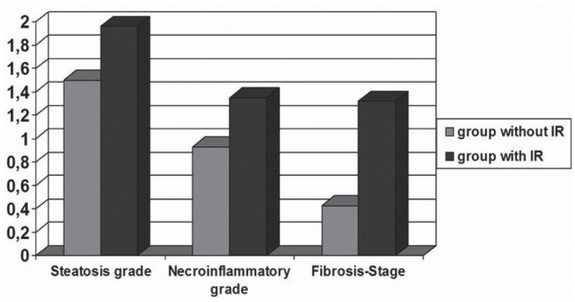


Fig. 2. — Histopathological correlation between NAFLD patients with or without IR

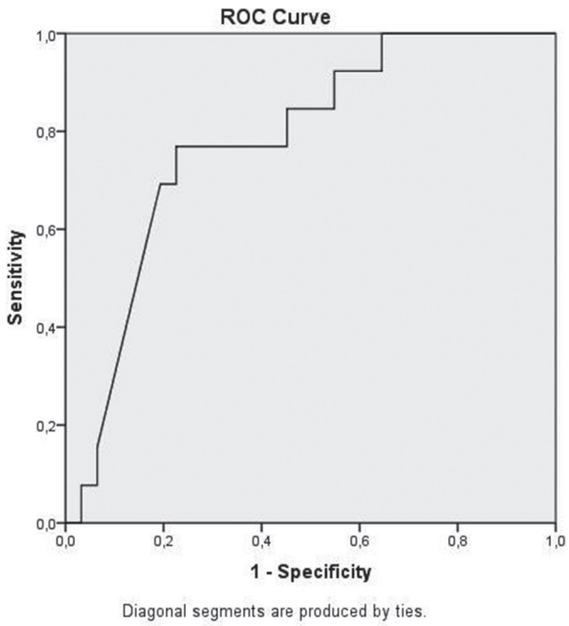


Fig. 3. — Discriminative power of IR index for necroinflammatory grade. (AUC 0.78 p<0.004)

Table 2. — **Immunohistochemical variables in NAFLD groups with and without IR**

| Parameters | Group without IR (16) | Group with IR (28) | p |
|---|---|---|---|
| Caspase 3 | 37.18±23.16 | 55.37±27.10 | **0.031** |
| Caspase 8 | 26.66±20.41 | 49.23±28.86 | **0.011** |
| NF-kB | 37.66±26.17 | 45.17±26.89 | NS |
| Bcl-2 | 13±2.73 | 14.72±10.63 | NS |
| TNF-sRp55 | 2637.71±203.06 | 2931.91±323.49 | **0.046** |

Data are presented as mean ± standard deviation ; NS=Statistically not signicant(p<0.05)

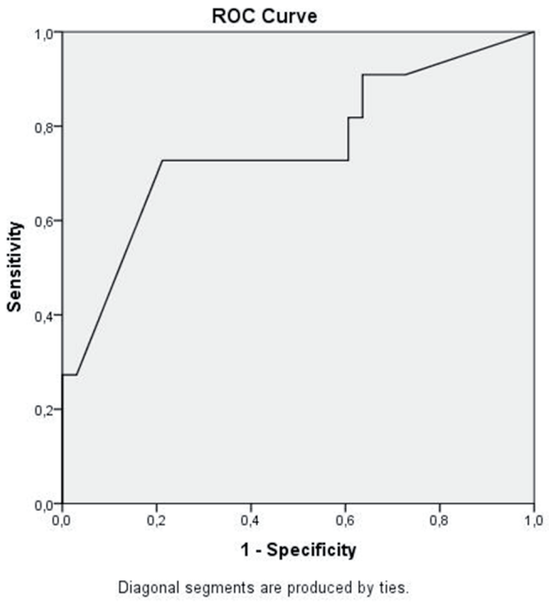


Fig. 4. — Discriminative power of IR index for fibrosis stage (AUC 0.76, p<0.01).

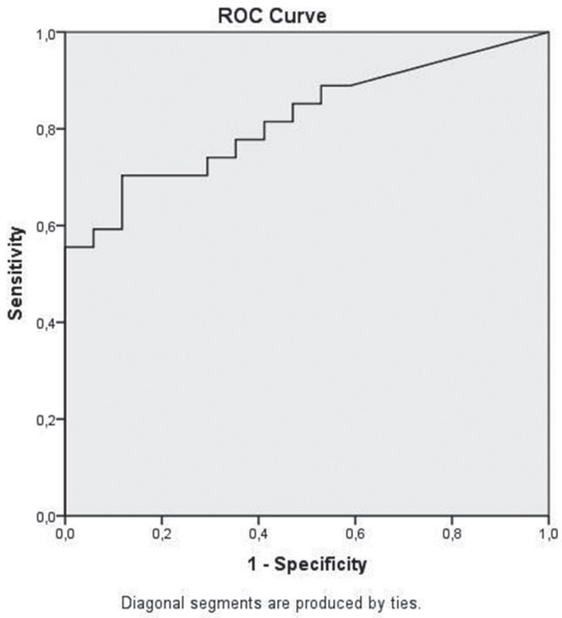


Fig. 5. — Discriminative power of IR index for NASH (AUC 0.82, p<0.001)

p<0.01). The cut-off value for the prediction of NASH was 3.9, at this threshold the sensitivity and specificity were 70% and 89% (AUC 0.82, 95% CI :[0.69-0.95] ; p<0.001) (Figures 4 and 5). Patients with IR had significantly higher TNFsRp55 levels than those without IR (2637.71±203.06 vs. 2931.91±323.49, p = 0.046). The increase in plasma TNF-sRp55 levels correlated with HOMA-IR index (r = 0.51, p = 0.024).

## Discussion

Non-alcoholic fatty liver disease is still the most frequent cause of liver diseases (15). NAFLD is associated with lipid deposition in the liver, and might cover a wide range of conditions from simple steatosis with no cellular damage or inflammation, to NASH, which is associated with cellular damage and varying levels of fibrosis (16). Hyperinsulinemia (HI) is a common problem in patients with NASH, and its role in the pathogenesis of steatohepatitis is still not yet exactly known. However, the level of HI in patients with NAFLD is known to be correlated with the level of liver steatosis (4). Furthermore, the association of NAFLD and IR was found related with hepatocyte apoptosis in these patients (17-19).

n our study, we evaluated whether IR may contribute to hepatocyte apoptosis, inflammation, and fibrosis in NAFLD. HOMA-IR index was significantly associated with fibrosis stage and caspase-3 and -8 levels. Patients with IR had significantly higher necroinflammatory grade, fibrosis stage, and caspase-3, and -8 than those without IR.

A protease group called caspases is a product of cellular damage, which is responsible for cell fractionation during apoptosis (20). Caspase-3 and -8 play key roles in apoptosis pathways (21). As a consequence, novel strategies based on caspase activity have recently gained attention in the development of diagnosis and treatment strategy in patients with NASH. Preclinical mice studies with NASH models showed protective effects of pancaspase inhibitors on both inflammation and fibrosis parameters (22-24). Other studies also showed the roles of apoptosis on liver cell damage, tissue inflammation, fibrosis, and progression of cirrhosis (25). Significant advances were achieved in recent years about the pathogenesis and progression of NAFLD. Several studies showed that hepatocyte apoptosis had a role in the progression of NAFLD to nonalcoholic steatohepatitis (NASH), liver fibrosis, cirrhosis, and even hepatocellular cancer (26, 27). Giovanni et al. (28) showed high levels of apoptotic cells in NAFLD patients in their study. They also reported that there was a significant correlation

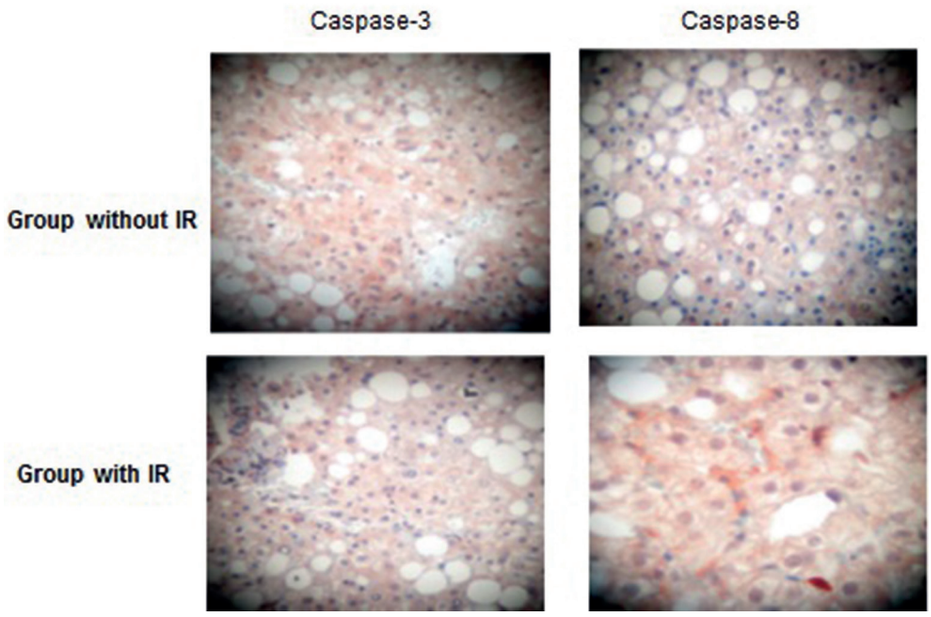


Fig. 6. — Immunostaining scores for caspase 3 and 8 in patients with IR and without IR(Activated caspase 3 and 8 were strongly expressed in hepatocytes of patients with IR)

between apoptotic cell ratios and lobular inflammation. In a similar study, Feldstein et al reported that levels of hepatocyte apoptosis, liver damage, and fibrosis were related with each other(29). Finally, Ferreira et al. showed the effects of association between caspase-3 activity and apoptosis on the degree of liver damage in patients with NAFLD, and also that apoptosis was closely related to IR (2). We also found that patients with NASH and IR had high levels of caspase 3 ($p = 0.031$), which indicated enhanced apoptotic activity. Caspase-8 has an important role in the activation of other caspases in the apoptosis cascade and in the execution of caspase pathways (30). Experimental studies showed that caspase-8 expression was positively correlated with hepatic steatosis degree and hepatic apoptosis rate in NAFLD (31). We also found that patients with IR had significantly higher caspase-8 expression than those without IR. This finding supports the association of apoptosis and IR in patients with NAFLD.

Bcl-2 protein, which plays a role in the regulation of mitochondrial-apoptosis system, is a member of regulatory proteins responsible for regulation of the complex apoptosis pathway (32). Bcl-2 is mainly located at the outer part of mitochondrial membrane and has anti-apoptotic activity (33). In line with this, inhibition of Bcl-2 resulted in progression of apoptosis, and dominance of Bcl-2 in the environment was associated with cellular survival (12). Bassat et al.(12,34) observed that there was a negative correlation between Bcl-2 levels and lobular inflammation in patients with NAFLD. Similar results were also reported in the study by Giovanni et al.(28). However, we found no significant correlation between bcl-2 levels and IR and histopathologic findings.

Another indirect indicator for apoptotic activity is NF-kB. NF-kB is a transcription factor that can be found in every cell and has an important role in the regulation of inducible gene expression in immune and inflammatory events (35,36). Previous experimental studies about the role of NF-kB on liver damage reported conflicting results. However, significant acute liver damage and liver regeneration were found in mice with NF-kB1 deletion (37,38). Moreover, in another experimental study, NF-kB1 down-regulation resulted in development and progression of fibrosis in mice liver with NASH (39). On the other hand, studies in rodent models of NAFL/NASH showed that increased NF-ϰB activity was also associated with the development of steatosis, hepatic insulin resistance, and inflammation (40,41).A similar result was reported in study by Ribeiro et al. They showed that NF-kB expression was associated with the degree of inflammation and fibrosis in patients with NASH(13). However, in our study, NF-ϰB1 levels were not associated with IR and histopathologic findings.

Tumor necrosis factor α plays a central role in proinflammatory and apoptotic processes against endotoxins. This cytokine exerts its effects by binding to cellular surface receptors, namely TNF-sRp55 and TNF-sRp75. Adipose tissue is an important resource of TNF-α (42). Some studies in rodent models of hepatosteatosis showed liver inflammation and significant focal hepatocyte necrosis in response to endotoxemia (43).Soluble TNF-α receptors p55 is a proteolytic extracellular

degradation product of TNF-α membrane receptor associated lysis (44). Several previous studies reported an association between TNF-sRp55 levels and the degree of hepatocellular damage (45,46). Ruiz et al. reported that TNF-sRp55 levels were significantly increased in alcoholic cirrhosis patients compared with controls, and that TNF-sRp55 levels were associated with the severity of fibrosis(45). In another study by Crespo et al., it was reported that TNF-sRp55 levels were high in liver tissues with NASH, and TNF-sRp55 expression was related to the degree of inflammation and liver tissue damage in patients with NASH (47). We also found that patients with IR and severe histopathologic findings had significantly higher TNF-sRp55 levels than those without IR, which was associated with steatosis grade, necroinflammatory grade, and fibrosis stage. These data suggest that liver injury may be mediated on secreted TNF-α signaling through the TNF-sRp55 receptor.

The increase release of FFAs from adipose tissue caused by IR leads to the transcription of TNF-α. The increase delivery of TNF-α to the liver increases the generation of reactive oxygen species. This oxidative stress and TNF-α induction sensitize hepatocytes to apoptotic cell death. The initiator caspase-8 is activated by TNF-α, and caspase-3 release is generated by oxidative stress. In our study, patients with IR had significantly higher TNF-sRp55, caspase-3 and -8 levels and severe histopathological changes in comparison to those without IR.

In conclusion, this study demonstrates that IR is associated with enhanced hepatocyte apoptosis in NAFLD. Histopathologic disease severity was significantly correlated with hepatocyte apoptosis. These data indicate that NAFLD patients with IR may have increased risk for disease progression. Further studies are needed to investigate the effect of therapeutic interventions aimed at improving hepatic insulin sensitivity and subsequently attenuating liver injury and histopathologic progression in NAFLD.

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