# The Relationship of Serum Hemojuvelin and Hepcidin Levels with Iron Overload in Nonalcoholic Fatty Liver Disease

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Received: 14.04.2015 Accepted: 08.07.2015

# ABSTRACT

**Background & Aims**: Mild iron overload is frequently reported in patients with nonalcoholic fatty liver disease (NAFLD). Hepcidin is the master iron-regulatory peptide and hemojuvelin (HJV) is the key regulator of iron-dependent secretion of hepcidin. The aims of this study were to evaluate serum HJV and hepcidin levels in patients with biopsy-proven NAFLD with and without hepatic iron overload, and to identify potential associations of HJV and hepcidin with the clinical characteristics of the patients enrolled.

**Methods**: Serum levels of HJV and hepcidin were measured in 66 NAFLD patients with (n=12) and without (n=54) iron overload, and controls (n=35) by enzyme-linked immunosorbent assay. Hemojuvelin and hepcidin levels were assessed in relation to clinical characteristics and liver histologic evaluation of the participants. **Results**: Significantly lower serum HJV (281.1 [239.2-353.6] vs. 584.8 [440.3-661] ng/ml, p<0.001) and similar serum hepcidin levels  $(60.5\pm31.1 \text{ vs. } 55.8\pm11.9 \text{ ng/ml}, p=0.285)$  were found in NAFLD patients when compared to controls. İron-overloaded NAFLD patients had significantly lower HJV (249.9 [187.6-296.3] vs. 292.9 [243-435] ng/ml, p=0.032) and significantly higher hepcidin (78.4±35.5 vs. 56.5±28.9ng/ml, p=0.027) levels than NAFLD patients without iron overload. Fibrosis stage was significantly higher in iron overloaded NAFLD group (p<0.001). Ferritin levels correlated significantly both with HOMA-IR (r=0.368, p=0.002) and fibrosis stage (r=0.571, p<0.001).

**Conclusions:** Our findings suggest that HJV levels are low in NAFLD and even lower in iron overloaded NAFLD, while hepcidin levels are higher in NAFLD with iron overload. The gradually decreased HJV and increased hepcidin concentrations in our patients most likely reflect the physiological response to iron accumulation in the liver.

Key words: hemojuvelin - hepcidin - iron - nonalcoholic fatty liver disease.

Abbrevations: ALT: alanine aminotransferase; AST: aspartate aminotransferase; BMI: Body-mass index; BMP-HJV: bone morphogenetic protein-hemojuvelin; FP-1:ferroportin-1; GGT: gamma-glutamyl transpeptidase; HDL-C: high density lipoprotein cholesterol; HIC: hepatic iron concentration; HJV: hemojuvelin; HOMA-IR: homeostasis model of insulin resistance; hs-CRP: high-sensitivity c-reactive protein; LDL-C: low density lipoprotein cholesterol; mHJV: membrane-bound hemojuvelin; mRNA: messenger ribonucleic acid; NAFLD: nonalcoholic fatty liver disease; NAFLD-Fe: NAFLD without iron overload; NAFLD+Fe: NAFLD with iron overloaded; NASH: nonalcoholic steatohepatitis; HJV: soluble hemojuvelin; Tf: transferrin.

## **INTRODUCTION**

With the increasing prevalence of obesity and insulin resistance in the Western world, nonalcoholic fatty liver disease (NAFLD) has become a major cause of chronic liver disease. Based on studies using different diagnostic methods, the current estimates of NAFLD worldwide prevalence vary from 6.3% to 33.0% (average 20%) [1]. Increased liver iron deposits have been reported in patients with NAFLD. Approximately 30% of NAFLD patients have elevated ferritin and a specific form of iron overload has been described and named "insulinresistance associated" or "dysmetabolic" hepatic iron overload syndrome [2, 3].

The liver represents the major site of iron deposition in the body and has a central role in iron homeostasis through the regulation of hepcidin production. Hepcidin is a master ironregulatory peptide, which is secreted mainly by hepatocytes in response to iron perturbations and inflammation and hypoxia. Hepcidin exerts its regulatory functions on iron homeostasis via binding to ferroportin and blocking cellular iron export [4, 5]. Although several studies have been held regarding the messenger RNA (mRNA), serum and urine levels of hepcidin, the role played by hepcidin in the pathogenesis of NAFLD still remains controversial.

While Aigner et al. [6] showed that the mRNA expression of hepcidin was increased in NAFLD patients with iron overload, Barisani et al. [7] demonstrated that mRNA and urinary hepcidin levels normalized for the amount of iron overload showed a significantly lower ratio in patients with dysmetabolic hepatic iron overload. Vuppalanchi et al. [8] reported that the presence of NAFLD was not associated with higher serum levels of hepcidin whereas Demircioglu et al. [9] concluded that hepcidin levels were significantly higher in obese children with NAFLD than in those without NAFLD.

Recently, it has been shown that hepcidin expression is regulated through the bone morphogenetic proteinhemojuvelin (BMP-HJV) signaling pathway. Hemojuvelin is a member of the repulsive guidance molecule family and appeared lately to be a key regulator of iron-dependent secretion of hepcidin. This iron-regulatory protein is mainly expressed in the liver, skeletal muscle, and heart and its production follows a specific process. Hemojuvelin is a glycosyl-phosphatidylinositol-anchored membrane protein (mHJV) that, in conditions of iron deficiency or hypoxia, is cleaved by furin and produces soluble extracellular domain fragments (sHJV) which are measurable in the serum. The furin mRNA level is negatively regulated by iron concentrations [10, 11]. The sHJV produced by furin cleavage downregulates hepcidin expression by competing with hepatocyte membranebound mHJV for BMP binding. When not inhibited by sHJV, this binding starts a cascade ending with an active protein complex that translocates into the nucleus and regulates hepcidin expression positively [12, 13].

In the present study we aimed to evaluate serum HJV and hepcidin levels in patients with biopsy-proven NAFLD with and without hepatic iron overload, and identify any potential correlations of HJV, hepcidin or other routinely used markers of iron metabolism with various epidemiological, laboratory and histological characteristics of the NAFLD patients.

#### MATERIAL AND METHODS

In this observational case-control study, 66 patients with NAFLD and 35 healthy control subjects were enrolled. The study protocol was approved by the local Ethics Committee and all subjects gave their written informed consent to participate in the study. Patients with NAFLD were seen consecutively at the outpatient clinics of Sisli Hamidiye Etfal Education and Research Hospital between January 2013 and August 2014. All the patients had elevated alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels for at least 6 months, and steatosis at ultrasonography (US). They had no history of hepatotoxic drugs, hormone replacement therapy or herbal products, consumption, were not drinking alcohol more than 20 g/day, had no viral hepatitis or autoimmune hepatitis, hemochromatosis, Wilson's disease, alpha-1 antitrypsin deficiency, biliary disease, anemia, ischemic cardiac or cerebrovascular disease, impaired renal function or malignancies. Concomitant inflammatory diseases potentially capable of causing hyperferritinemia were ruled out on the basis of clinical signs or abnormal blood test results (erythrocyte sedimentation rate, serum rheumatoid factor, and C reactive protein). The control group had no illness, no inflammatory disease, no usage of alcohol, drug or herbal substances, no history of previous liver diseases, were negative for viral hepatitis serology tests and had normal liver at US. All patients and controls were of Turkish descent.

All subjects underwent physical examination, anthropometric measurements, and biochemical screening. The weight and height of the participants were measured with a calibrated scale and body-mass index (BMI) was computed as body weight/(height)<sup>2</sup>. The estimate of insulin resistance was calculated using the homeostasis model of insulin resistance (HOMA-IR) index: insulin resistance = fasting plasma insulin (in microunits per milliliter) x fasting plasma glucose (in millimoles per liter) / 22.5.

Routine blood chemistry analyses were performed at the central laboratory of our center. For HJV and hepcidin analyses, all blood samples were collected from an antecubital vein, between 08.00 and 09.00 a.m. after an overnight fasting. Blood was drained into a tube containing ethylene diaminetetraacetate and samples were centrifuged for 15 minutes at 1000g. Then the plasma was removed immediately and stored frozen at -80°C until analyzed.

Serum hepcidin concentrations were measured by a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Catalog No: EIA-4705, DRG Diagnostics, Marburg, Germany), according to the manufacturer's instructions. Intra-assay and inter-assay coefficients of variation were <10% and <12%, respectively. Serum HJV concentrations were measured using Human HJV ELISA kit (Catalog No: CK-E90162, EASTBIOPHARM, Hangzhou, China), according to the manufacturer's instructions. The assay is based on the method of the quantitative sandwich enzyme immunoassay. Intra-assay and inter-assay coefficients of variation were <10% and <12%, respectively.

Liver biopsies were performed under US guidance by using a 16-gauge Hepafix needle. All biopsy specimens were placed in formalin solution for fixation and embedded in paraffin blocks. Serial sections (sectioned at 4-mm intervals) were stained with hematoxylin-eosin, Mallory Trichrome for morphologic evaluation, and Perls' stain was used to determine the liver iron load. An experienced hepatopathologist blinded to the subjects' details evaluated biopsy specimens based on the presence of steatosis, inflammation and ballooning. According to the decision of the hepatopathologist, in terms of the presence or absence of nonalcoholic steatohepatitis (NASH), we divided our NAFLD study group into two subgroups as NASH and non-NASH. The same experienced hepatopathologist also scored each liver biopsy specimen using the National Institute of Diabetes and Digestive and Kidney Diseases Nonalcoholic Steatohepatitis (NIDDK NASH) Clinical Research Network scoring system [14]. Steatosis was scored from 0 to 3 with a four-grade scoring system from S0 to S3: S0, no steatosis or < 5%; S1, 5–33%; S2, 33–66%; and S3, > 66%. Lobular inflammation was graded as follows: stage 0, no foci; stage 1, <2 foci per 200x field; stage 2, 2–4 foci per 200x field; stage 3, >4 foci per 200x field. Ballooning degeneration was scored as follows: score 0, no ballooning cells; score 1, few balloon cells and score 2, many balloon cells or prominent ballooning. Fibrosis was staged as follows: stage 0, no fibrosis; stage 1, perisinusoidal or periportal fibrosis; stage 2, perisinusoidal and portal/periportal fibrosis; stage 3, bridging fibrosis; and stage 4, cirrhosis.

Siderosis was assessed semiquantitatively on histopathological examination of Perls' stained liver biopsy samples. A score of 0 to 4 was used: 0, granules absent or barely discernible at a magnification of 400x; 1, barely discernible granules at a magnification of 200x but easily confirmed at 400x; 2, discrete granules resolved at 100x magnification; 3, discrete granules resolved at 25x; and 4, massive granules visible even at 10x magnification [15].

Patients were considered to have NAFLD with iron overload when both biochemical signs of iron overload: elevated serum ferritin concentration and hepatic iron deposition (positive Perls' stain > grade 1) were detected. Accordingly, patients with a histological and clinical diagnosis of NAFLD and the absence of stainable iron and normal serum iron variables were identified as having NAFLD without iron overload.

#### Statistical analysis

All analyses were performed using SPSS version 21.0 for Windows (IBM Inc, Chicago, Illinois, USA). The variables were investigated using visual (histograms, probability plots) and analytical methods (Kolmogorov-Smirnov/Shapiro-Wilk tests) to determine whether or not they were normally distributed. Ordinal variables and continuous variables that do not have normal distribution were compared by the Mann-Whitney U test. The Student t-test was used to evaluate differences between the two study groups in normally distributed continuous variables such as age, BMI, transferrin saturation and hepcidin level. The Kruskal-Wallis test was used to compare NAFLD with iron overload, NAFLD without iron overload and control groups. A value of p<0.017, calculated by Bonferroni correction, was considered statistically significant in the posthoc comparisons. Correlations among the study variables were analyzed by the Pearson and Spearman's tests depending on the normality of variables. A p-value <0.05 was considered to show a statistically significant result. The capacity of serum HJV values in predicting the presence of iron overloaded NAFLD were analyzed using ROC (receiver operating characteristics) curve analysis. The optimal cut-off value was determined as the point on the ROC curve that is closest to the upper left corner. Sensitivity, specificity, positive and negative predictive values were calculated for this cut-off value.

# RESULTS

The main clinical and biochemical characteristics of the NAFLD patients and control subjects are shown in Table I. Age, gender distribution, hemoglobin, and transferrin saturation levels were similar in NAFLD and control groups. In the NAFLD group BMI, HOMA-IR, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), total and low density lipoprotein (LDL) cholesterol, triglyceride, high sensitive C reactive protein (hs-CRP) and ferritin levels were significantly

Table I. The main clinical and biochemical characteristics of the NAFLD patients and controls

	NAFLD patients (n=66)	Controls (n=35)	P value
Age	44.4±11.2	43.0±9.1	0.507
Sex (male/female)	23/43	15/20	0.429
BMI kg/m²	32.4±4.5	22.3±2.8	< 0.001
ALT, U/L	75.5 (52.5-102)	170 (10-28)	< 0.001
AST, U/L	53.5 (40-69.5)	18.0 (15-21)	< 0.001
GGT, U/L	41.5 (26-65.2)	10.0 (7-20)	< 0.001
Total cholesterol, mg/dL	202.5 (190-234)	172.0 (152-200)	< 0.001
LDL-C, mg/dL	159.9 (147.3-188.5)	130.8 (116.2-164.2)	< 0.001
HDL-C, mg/dL	47.0 (40.7-53)	62.5 (54.8-68)	< 0.001
TG, mg/dL	167.5 (130-205)	142.0 (100-151.8)	< 0.001
HOMA-IR	4.09 (2.7-6)	1.89 (1.23-2.84)	< 0.001
hs-CRP, mg/L	4.60 (2.2-7)	1.25 (1.1-1.8)	< 0.001
Hemoglobin, g/dL	13.9 (12.8-14.8)	13.6 (13.2-15.1)	0.858
Tf saturation, %	29.7±12.3	27.6±16.1	0.512
Ferritin, ng/mL	140.1 (92.5-229.5)	98.0 (28.8-134.8)	< 0.001
HJV, ng/ml	281.1 (239.2-353.6)	584.8 (440.3-661)	< 0.001
Hepcidin, ng/ml	60.5±31.1	55.8±11.9	0.285

Values are presented using means  $\pm$  standard deviations for normally distributed and medians and first and third quartiles in the brackets for the non-normally distributed variables. BMI: body mass index; ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: gamma-glutamyl transpeptidase; LDL-C: low density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol; TG: triglyceride; HOMA-IR: homeostasis model assessment of insulin resistance; hs-CRP: high-sensitivity c-reactive protein; Tf saturation: transferrin saturation; HJV: hemojuvelin. higher, and high density lipoprotein (HDL) cholesterol levels were significantly lower than in the control group. While serum hepcidin levels were similar (p=0.285), significantly lower serum HJV levels (281.1 [239.2-353.6] vs. 584.8 [440.3-661] ng/ml, p<0.001) were found in NAFLD patients compared to the control group.

When evaluated according to serum ferritin concentrations and hepatic iron deposition as detected by positive Perls' stain (> grade 1), 12 of the NAFLD patients were classified as iron overloaded (NAFLD+Fe) and 54 of them were without any sign of iron overload (NAFLD-Fe). When investigating the NAFLD patients, we found that patients with iron overload were older and had a higher HOMA-IR than patients without iron overload. No differences in BMI, serum transaminase levels, lipid profile, hemoglobin and hs-CRP concentrations were observed. Besides having significantly higher serum ferritin concentrations and transferrin saturations than NAFLD patients without iron overload, iron-overloaded NAFLD patients also had significantly lower HJV levels (p=0.032) and significantly higher hepcidin levels (p=0.027) (Table II, Fig. 1a,b).

In order to detect the differentiating power of HJV levels of NAFLD+Fe patients from NAFLD-Fe patients, we synthesized ROC analysis and found AUC as 0.699 (Fig. 2). The optimal cut-off value investigated by ROC analysis for the diagnosis of iron overloaded NAFLD was 268.3 ng/ml, with a sensitivity of 75.0%, specificity of 66.7%, positive predictive value of 75.0%, and a negative predictive value of 72.7%.

The NAFLD patients were also divided into two groups according to the morphopathologic evaluation of the liver:

52 patients were grouped as NASH and the remaining 14 as non-NASH. When NASH and non-NASH patients were compared according to iron metabolism biomarkers, serum ferritin concentrations (136.7 [80-224.2] vs. 145.6 [128.9-317.5] ng/ml, p=0.233), transferrin saturation ( $30.4\pm11.7$  vs. 29.5±12.5, p=0.812), hepcidin levels ( $60.2\pm29.9$  vs.  $61.5\pm36.7$  ng/ml, p=0.897) and HJV levels (290.6 [243.2-351.2] vs. 251.8 [211-932.7] ng/ml, p=0.678) were found to be similar (Fig. 3a,b).

Nine (75%) of iron-overloaded NAFLD patients and 43 (79.6%) of NAFLD patients without iron overload had NASH and there was no statistically significant difference in terms of presence of NASH between the two groups (p=0.708). Moreover, when evaluated histologically, iron-overloaded NAFLD patients and NAFLD patients without liver iron accumulation were similar with regard to the grade of steatosis, lobular inflammation and ballooning. Although stage of fibrosis was significantly higher in the NAFLD+Fe group than in the NAFLD-Fe group (p < 0.001), there was no correlation with the grade of Perls' stain and stage of fibrosis.

When association of iron metabolism biomarkers with the clinical, biochemical and histological characteristics of NAFLD patients was assessed, serum hepcidin levels showed a positive but weak correlation with ferritin concentrations (r=0.266, p=0.031). There was no significant correlation between HJV levels and any of the determinants studied. Ferritin levels showed significant correlations with both HOMA-IR (r=0.368, p=0.002) and stage of fibrosis (r=0.571, p<0.001). Also HOMA-IR levels and stage of fibrosis were correlated with each other (r=0.458, p<0.001).

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	NAFLD+Fe group (n=12)	NAFLD-Fe group (n=54)	Controls (n=35)	P value	
Age	50.8±8.2*†	43±11.3	43.0±9.1	0.040	
Sex (males/females)	6/6	17/37	15/20	0.357	
BMI kg/m²	32±5.3†	32.4±5.4‡	22.3±2.8	< 0.001	
ALT, U/L	96.0 (71.5-116.5)†	68.5 (49.8-98)‡	170 (10-28)	< 0.001	
AST, U/L	58.0 (42-90.5)†	52.0 (40-66.5)‡	18.0 (15-21)	< 0.001	
GGT, U/L	45.5 (32.5-55.8)†	40.0 (25.8-66.3)‡	10.0 (7-20)	< 0.001	
Total cholesterol, mg/dL	202.5 (196.5-212.5)†	203.0 (190-234)‡	172.0 (152-200)	< 0.001	
LDL-C, mg/dL	161.4 (145.5-176.5)†	159.9 (148.4-192.6)‡	130.8 (116.2-164.2)	< 0.001	
HDL-C, mg/dL	42.5 (39-53.5)†	47.0 (42-53)‡	62.5 (54.8-68)	< 0.001	
TG, mg/dL	151.0 (125.5-224.5)†	108.0 (134-204)‡	142.0 (100-151.8)	< 0.001	
HOMA-IR	6.33 (4.98-6.92)*†	3.53 (2.58-5.28)‡	1.89 (1.23-2.84)	< 0.001	
hs-CRP, mg/L	2.7 (1.4-8.5)	4.9 (2.5-7.1)‡	1.25 (1.1-1.8)	< 0.001	
Hemoglobin, g/dL	14.3 (12.8-15.1)†	13.9 (12.8-14.7)	13.6 (13.2-15.1)	0.785	
Tf saturation, %	38.6±13.4*†	27.7±11.2	27.6±16.1	0.028	
Ferritin, ng/mL	432.8 (385.2-478.7)*†	129.8 (78.7-169.7)‡	98.0 (28.8-134.8)	< 0.001	
HJV, ng/ml	249.9 (187.6-296.3)*†	292.9 (243-435)‡	584.8 (440.3-661)	< 0.001	
Hepcidin, ng/ml	78.4±35.5*†	56.5±28.9	55.8±11.9	0.022	

**Table II**. The main clinical and biochemical characteristics of the NAFLD+Fe, NAFLD-Fe and control groups

\* NAFLD+Fe vs NAFLD-Fe p<0.05; † NAFLD+Fe vs Control p<0.05; ‡ NAFLD-Fe vs Control p<0.05. The column on the right represents p values obtained by the comparison of all three groups by Kruskal-Wallis test. A value of p <0.017, calculated by Bonferroni correction, was considered statistically significant in the post-hoc comparisons. Values are presented using means  $\pm$  standard deviations for normally distributed and medians and first and third quartiles in the brackets for the non-normally distributed variables. For abbreviations se Table I.



**Fig. 1.** a) Serum hemojuvelin concentrations in NALFD+Fe , NAFLD-Fe and control groups; b) Serum hepcidin concentrations in NALFD+Fe , NAFLD-Fe and control groups.



**Fig. 2.** ROC curve to differentiate NAFLD+Fe patients from NAFLD-Fe patients according to serum hemojuvelin levels (AUC=0.699).

# DISCUSSION

We investigated serum HJV and hepcidin levels in biopsyproven NAFLD in patients with and without hepatic iron



**Fig. 3.** a) Serum hemojuvelin concentrations in NASH, non-NASH and control groups; b) Serum hepcidin concentrations in NASH, non-NASH and control groups.

overload, and aimed to identify any potential correlation of HJV, hepcidin and other routinely used markers of iron metabolism with various epidemiological, laboratory and histological characteristics of the study participants.

The analysis of iron-regulatory molecules in the serum revealed low levels of the iron-sensing molecule HJV in both NAFLD groups. Moreover, we observed that the serum levels of the master iron-regulatory peptide hepcidin are increased in NAFLD patients with iron overload. Thus, the increased hepcidin concentrations in our patients most likely reflect the physiologic response to liver iron accumulation, because hepcidin concentrations in NAFLD patients without iron accumulation were similar to hepcidin concentrations in controls. We also found a positive although weak correlation between ferritin and hepcidin levels, thus the increased hepcidin formation especially in NAFLD patients with iron overload might be responsible for the increase in ferritin levels. Besides the increased serum hepcidin levels, serum HJV concentrations were significantly lower in NAFLD patients than in controls and even lower in NAFLD patients with than in those without iron overload. So far, HJV has been identified as a key regulator of hepcidin formation in response to iron

Boga et al

accumulation, with a particular role in the sensing of dietary iron overload independently from inflammation [16]. When the conflicting results of the studies investigating serum hepcidin levels in NAFLD with and without iron overload are remembered and the progressive iron accumulation in hepatic HJV down-regulated cases such as juvenile hemochromatosis [17] are kept in mind, the pathophysiological significance of the markedly decreased HJV levels as a promising serum iron biomarker for the future studies in assessing iron overload is better understood.

In the study of Aigner et al. [6] from Austria, analysis of iron-regulatory molecules in liver tissue revealed compatible results with the analysis of the serum levels of same molecules in our study. Besides finding a decrease in the hepatic expression of the iron-export protein ferroportin-1 (FP-1), they reported that HJV mRNA expression in the liver was significantly lower in NAFLD patients, with a more prominent reduction in ironoverloaded NAFLD subjects, and hepatic hepcidin mRNA expression was significantly higher in NAFLD patients with iron overload than in controls and NAFLD subjects without iron overload. Thus, they concluded that iron accumulation in NAFLD may result from decreased iron mobilization from hepatocytes due to low expression of FP-1 and HJV. It was previously reported that serum levels of hepcidin correlate with hepatic hepcidin mRNA expression [18, 19]. This data makes the results of the present study coherent with the study of Aigner et al. [6]. In their study they also reported improvement in liver function tests and reduction in inflammatory cytokines in response to treatment by phlebotomy. This treatment option may also give rise to a practical use of serum HJV levels in the future both for detecting and following up iron overload in NAFLD patients although studies with larger scales will obviously be needed to confirm our and their results and to see the efficiency of utilizing serum HJV levels.

In the general population, the association of elevated serum ferritin concentrations and insulin resistance was demonstrated [20]. Several studies have also demonstrated that serum ferritin is a good indicator of insulin resistance in hepatitis C patients [21, 22]. After the groundbreaking study of Mendler et al. [2], insulin resistance associated or dysmetabolic hepatic iron overload syndrome was accepted as a specific syndrome demonstrating the histologic evidence of NAFLD. In the present study, besides finding higher ferritin levels in NAFLD patients, we also found that ferritin levels correlated well with insulin resistance, concordant with the literature data.

It is known that insulin itself may have profibrogenic properties. Paradis et al. [23] reported that incubation of hepatic stellate cells with glucose or insulin leads to overexpression of connective tissue growth factor, which is involved in liver fibrosis. In the present study we found that insulin resistance was associated with the grade of fibrosis. This finding supports the study of Paradis et al. that suggests hyperglycemia and insulin as key-factors in the progression of fibrosis in patients with NAFLD.

Conflicting data have recently been reported on the role of iron in the liver damage of NAFLD patients. George et al. [24] and Bonkovsky et al. [25] showed that patients with NASH and iron overload had a more severe liver disease and more intense fibrosis, whereas Younossi et al. [26] and Angulo et al. [27] did not observe any relation between iron and clinical or pathological outcomes in patients with NAFLD. The limited number of NAFLD patients with positive Perls' stain prevented us to make inferences regarding the association of liver damage and hepatic iron. But when ferritin levels were assessed, we found that serum ferritin concentrations showed a positive correlation with the stage of hepatic fibrosis. In their study of 263 NAFLD patients, Bugianesi et al. [28] concluded that the increased level of serum ferritin was the most relevant independent predictor of severe fibrosis, a result concordant with our finding.

Several caveats are inherent in this study. First, the relatively small sample size limits the generalizability of our conclusions. Second, our sample included subjects of Turkish ethnicity, so that results cannot be extrapolated to populations with different ethnic backgrounds. Thirdly, we were unable to study hepatic HJV and hepcidin expression in liver biopsies. Such data would generally provide more information about the source of decreased HJV and increased circulating hepcidin and the regulation of hepcidin expression in NAFLD. The routine study of hepatic HJV and hepcidin expressions are not suitable in clinical practice due to its invasive nature. For these reasons, the measurements of serum HJV and hepcidin levels may be feasible for providing a noninvasive assessment of body iron status and useful in predicting hepatic iron overload in everyday practice. Finally, due to financial constraints we were unable to measure the hepatic iron concentration (HIC) of the patients. But it must be kept in mind that as the determination of HIC provides a measure of iron in dry liver tissue specimens, it can be influenced by the fat content of hepatocytes and by the way samples are preserved. Actual results in the studies regarding the amount of intrahepatic iron contents are conflicting, in part due to the different techniques used to determine HIC. When hepatic iron stores were assessed by both HIC and Perls' stain, the results were poorly correlated [24, 26] and the association between fibrosis and hepatic iron store was stronger for Perls' stain [29].

One can speculate that the absence of gene analysis for mutations of the hemochromatosis gene, HFE, may be a limitation of this study. But although an increased prevalence of HFE mutations has been described in patients with NAFLD, subsequent studies have failed to confirm a significant increase in their hepatic iron burden and no association was found among hepatic iron, HFE mutations, and severity of liver damage [26, 29]. Also, a large study of 4,633 individuals, performed in order to detect hemochromatosis gene mutations in Turkish population, demonstrated that there was no Cys282Tyr gene mutation, 0.23% had heterozygotic H63D mutation, and only one case had homozygotic H63D mutation [30]. Similarly, in another recent study of 3,060 individuals, mutational analysis also revealed no homozygotic C282Y or compound heterozygosity, while two subjects had heterozygotic H63D mutation [31]. In these studies it was seen that the prevalence of hemochromatosis in the Turkish population was much lower than that in populations of Nordic or Celtic ancestry and the C282Y mutation was nonexistent. Therefore, the absence of gene analysis might not be an important limitation of the present study.

## CONCLUSIONS

To the best of our knowledge, this is the first published study measuring serum HJV levels in NAFLD patients. Analysis of iron-regulatory molecules in the serum revealed significantly lower serum HJV levels in NAFLD patients than in control subjects and even lower in iron-overloaded NAFLD patients than in NAFLD patients without iron overload. Moreover, we observed that serum hepcidin levels were increased in NAFLD patients with iron overload. It is also noteworthy that ferritin levels, insulin resistance and fibrosis stage showed significant correlations with each other.

The findings of this study suggest that the gradually decreased HJV and increased hepcidin concentrations in our patients most likely reflect the physiological response to liver iron accumulation. It can be concluded that these biochemical markers of iron metabolism in fatty liver disease may provide important contributions to the early diagnosis and treatment options in NAFLD patients with iron overload. It would be important to confirm these findings in a larger patient population and further studies should be conducted in order to elucidate the importance of these findings.

**Conflicts of interest.** There are no financial or other relations that could lead to a conflict to interest.

Authors' contribution: S.H. designed the study, collected the data and drafted the manuscript, revised and formed its final version. H.A. and C.A. were involved in drafting the manuscript, revised it critically for important intellectual content. A.R.K. participated in the design of the study and performed the statistical analysis. M.B. participated in the design of the study and helped in drafting the manuscript. M.B.Y.O. helped in designing the study, evaluated the liver biopsy specimens. S.T.N. helped in designing the study, measured the enzyme-linked immunosorbent assays, helped in statistical analysis. All authors gave final approval of the version to be published.

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