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The relationship of serum bilirubin level with histopathological parameters in patients with nonalcoholic fatty liver disease

Ali Kirik[1], Cemal Nuri Ercin[2], Hasan Gurel[3], Ali Fuat Cicek[4], Ali Cem Yekdes[1], Sultan Turel[5], Serkan Tapan[6], Teoman Dogru[7]

[1]Department of Internal Medicine, Balikesir University, School of Medicine, Balikesir, Turkey
[2]Department of Gastroenterology, University of Health Sciences, Gulhane School of Medicine, Ankara, Turkey
[3]Department of Gastroenterology, University of Health Sciences, Education and Research Hospital, Samsun, Turkey
[4]Department of Pathology, University of Health Sciences, Gulhane School of Medicine, Ankara, Turkey
[5]Department of Internal Medicine, Health Ministery Education and Research Hospital, Nigde, Turkey
[6]Section of Biochemistry, ENA Laboratory, Ankara, Turkey
[7]Department of Gastroenterology, Balikesir University, School of Medicine, Balikesir, Turkey

ABSTRACT

Objectives: Non-alcoholic fatty liver disease (NAFLD) is among the most common causes of chronic liver disease and is known as a part of metabolic syndrome (MetS), and the role of bilirubin in the pathogenesis of NAFLD is unclear. This study aimed to evaluate the relationship between bilirubin levels and histopathological findings in patients with NAFLD having no confounding factors such as morbid obesity, diabetes mellitus (DM), and hypertension.

Methods: A retrospective analysis of clinical and laboratory data of patients with biopsy-proven NAFLD was performed. The relationship between the bilirubin levels and histopathologic findings was evaluated.

Results: The subjects in the nonalcoholic steatohepatitis (NASH) group had greater AST ($p < 0.001$) and ALT ($p < 0.001$) levels than the non-NASH group. We found no difference between NASH and non-NASH groups regarding bilirubin levels. The levels of AST (p = 0.001), ALT ($p = 0.011$), insulin ($p = 0.029$), and HOMA-IR index ($p = 0.027$) were higher in fibrosis group comparing non-fibrosis group. However, bilirubin levels were not different comparing the fibrosis and non-fibrosis group. We couldn't find any relation between bilirubin levels and other parameters in correlation analysis.

Conclusion: We couldn't find any relation between the bilirubin levels and histopathological findings of the patient with NAFLD having no confounding factors such as morbid obesity, DM, and hypertension. The difference, shown in the other studies, may be the effect of other diseases related to MetS.

Keywords: NAFLD, NASH, fibrosis, bilirubin

Non-alcoholic fatty liver disease (NAFLD), a component of the metabolic syndrome (MetS), is the most common chronic liver disease worldwide. NAFLD is a disease characterized by lipid accumulation in the liver, often without secondary causes such as alcohol and steatogenic medication.[1]

The pathogenic mechanisms responsible for the

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Address for correspondence: *Ali Kirik, Asst. Prof., Department of Internal Medicine, Balikesir University, School of Medicine, Cagis, Balikesir, 10145, Turkey*
E-mail: alikirik87@hotmail.com

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development of NAFLD are complex. The key points are mainly dysregulation of fatty liver accumulation (FLA), development of hepatic insulin resistance (IR), and hyperinsulinemia. Oxidative stress is one of the leading causes of chronic inflammation and fibrosis in the liver, especially hepatocyte necrosis. In addition, increased FLA levels predispose to oxidative stress that leads to hepatic inflammation and fibrosis.[1,2] Also, it was shown that cardiovascular disease (CVD) is a more common cause of death among NAFLD patients comparing liver disease.[3,4] Also, elevated biochemical markers of atherosclerosis and systemic inflammation are often found in patients with NAFLD.5 It is interesting that CVD can be diagnosed without traditional risk factors in patients with NAFLD.[6] So, it is accepted that every patient with NAFLD should be screened for CVD and vice versa.[7]

Bilirubin is the final product molecule resulting from the breakdown of heme molecule. When the level is in the physiologic range, bilirubin has cytoprotective and beneficial metabolic effects, but it is potentially toxic if high.[8] It is reported that serum bilirubin significantly contributes to total antioxidant capacity.[9] The antioxidant effect of bilirubin molecule, which has lipid peroxidation inhibitory property, can be as strong as antioxidant vitamin E.[10] Both bilirubin and the enzymes involving bilirubin metabolism have some effects. It was shown that heme oxygenase, responsible for the degradation of heme to biliverdin, stimulated insulin products, and reduced insulin resistance.[11] In clinical studies, it was found that higher levels of bilirubin were inversely associated with IR and MetS.[12] Also, bilirubin is suggested to have a protective effect in atherogenesis, coronary artery disease (CAD), and peripheral arterial disease (PAD).[13,14]

In light of these data, the current literature's relationship between serum bilirubin level and histopathological findings such as steatohepatitis and fibrosis in patients with NAFLD remains unclear. In the study presented here, it was aimed to examine the relationship between serum bilirubin levels with steatohepatitis and fibrosis in biopsy proven NAFLD patients.

METHODS

Study design and population

In this study, biopsy-proven NAFLD patients followed in the gastroenterology clinic of a tertiary university hospital were examined retrospectively. Demographic, clinical, laboratory and biopsy data of the patients were enrolled. The study was approved by the local ethics committee of Balikesir University Medical School (date: 14.10.2020; no: 2020/179) and was complied with according to the Helsinki Declaration. In this study, which included patients diagnosed with NAFLD through liver biopsy, the main exclusion criteria were chronic alcohol use, hemochromatosis, wilson's disease, presence of viral hepatitis, type 2 DM, morbid obesity, and any other major diseases.

Clinical examination and laboratory analyses

The BMI of the patients, whose waist circumference (WC) was measured from the midpoint between the lowest rib margin and the iliac crest was evaluated using the formula obtained by dividing the weight by the square of the height. Hemoglobin (Hb), white blood cell (WBC), and platelet (PLT) levels were determined by an ABX Pentra 120 automatic hematology analyzer used for whole blood counts. Fasting plasma glucose (FPG), liver enzymes [alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT)] and cholesterol profile [triglyceride (TG), total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-C)] levels were measured by enzymatic colorimetric methods. The Friedewald Formula calculated low-density lipoprotein cholesterol (LDL-C).[15] The Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) formula determined the IR level.[16]

Liver histology

Liver biopsy materials of the patients were stained with Hematoxylin and eosin (H&E) dye and examined by a single specialist pathologist. On the other hand, Masson's trichrome stain was used to examine the fibrosis levels. Finally, histopathological findings were evaluated according to Kleiner *et al*.17, and fibrosis staging were evaluated according to Brunt *et al*.[18] NAFLD activity score (NAS) was evaluated by adding the scores obtained from steatosis, lobular inflammation and hepatocellular ballooning, and NASH was defined as a NAS $\geq$ 5. Fibrosis staging was graded on a scale from 0 to 4.

Statistically analysis

Statistical analyses were performed by SPSS 22 (Statistical Package for the Social Sciences, version

22). Kolmogorov-Smirnov and Shapiro-Wilk tests were used to assess the assumption of normality. Parametric values were reported as the mean ± standard deviation, and non-parametric values were reported as the median (25.-75. percentiles). Mann-Whitney U-test and independent samples t-test evaluated differences between groups as appropriate. For possible relationships, Spearman and Pearson correlation analyses were performed between two parametric values. $P < 0.05$ was accepted as statistically significant.

RESULTS

Descriptive and comparative statistics of NASH and Non-NASH groups are summarized in Table 1. The distribution of age, BMI, and WC were not different between the NASH and non-NASH groups. Among the biochemical parameters, the subjects in the NASH group had greater AST ($p < 0.001$) and ALT ($p < 0.001$) levels than the non-NASH group. The distribution of age, BMI, and WC did not differ between the fibrosis and non-fibrosis groups. The levels of AST ($p = 0.001$), ALT ($p = 0.011$), insulin ($p = 0.029$), and HOMA-IR index ($p = 0.027$) were higher in the fibrosis group comparing the non-fibrosis group (Table 2).

We found no difference between NASH [direct bilirubin = 0.15 (0.10-0.22), indirect bilirubin = 0.63 (0.42-0.90)] and non-NASH groups [direct bilirubin = 0.15 (0.11-0.20), indirect bilirubin = 0.58 (0.40-0.77)] regarding bilirubin levels (respectively $p = 0.888$, $p = 0.286$). Additionally, there was no difference between with fibrosis group [direct bilirubin = 0.15 (0.11-0.20), indirect bilirubin = 0.17 (0.11-0.20)] and

Table 1. Descriptive and comparative statistics of NASH and Non-NASH groups

Variables	Values		*p*
	NASH n =53	Non-NASH n =76	
Age (years)	31.13 (± 5.86)	32.83 (± 6.20)	0.120***
BMI (kg/m²)	28.20(26.07-29.77)*	28.40 (26.50-31)*	0.450**
WC (cm)	100 (96-105)*	98.50 (96-104)*	0.745**
FPG (mg/dL)	94.06 (± 11.07)	92.73 (± 11.09)	0.507***
TC (mg/dL)	201.92 (± 44.24)	205.45 (± 45.29)	0.664***
LDL-C (mg/dL)	119.80 (± 33.67)	128.58 (± 35.60)	0.170***
TG (mg/dL)	160.50 (122-265.25)*	166 (108-251)*	0.600**
HDL-C (mg/dL)	38 (35-44.75)*	41 (36.75-46)*	0.156**
ALT (U/L)	121(95-166.50)*	91 (64.50-115.25)*	**<0.001****
AST (U/L)	56 (41.50-65.50)*	44.50 (35-53.75)*	**<0.001****
GGT (U/L)	60 (46.25-81.50)*	58 (44-87.75)*	0.814**
Direct bilirubin (mg/dL)	0.15 (0.10-0.22)*	0.15 (0.11-0.20)*	0.888**
Indirectbilirubin(mg/dL)	0.63 (0.42-0.90)*	0.58 (0.40-0.77)*	0.286**
UricAcid (mg/dL)	6.67 (5.73-7.23)*	6.58 (5.70-7.07)*	0.390**
Hb (g/dL)	16.12 (± 0.95)	15.63 (± 0.97)	**0.008*****
WBC (x 10³)	7100 (6100-8600)*	7150 (6200-8675)*	0.968**
Plt (x 10³)	225829.79 (± 41730.14)	244147.06 (± 54433.10)	0.054***
Insulin (µU/mL)	14.45 (10.37-20.21)*	12.48 (9.56-20.16)*	0.361**
HOMA-IR	3.39 (2.34-4.79)*	2.78(2.10-4.95)*	0.411**
Hs-CRP (pg/mL)	2.02 (1.25-3.12)*	2.03 (1.16-3.50)*	0.810**

NASH: nonalcoholic steatohepatitis; BMI: Body mass index; WC: waist circumference; FPG: fasting plasma glucose; TC: total cholesterol; LDL-C: low-density lipoprotein cholesterol; TG: triglicerides; HDL-C: high-density lipoprotein cholesterol; ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: gamma-glutamyl transferase; Hb: Hemoglobin; WBC: White blood cell; Plt: platelet; HOMA-IR: homeostasis model assessment for insulin resistance; Hs-CRP: high-sensitivity C-reactive protein

*median(25.-75. Percentiles)

** Mann Whitney U test

*** Independentsample t- test

Table 2. Descriptive and comparative statistics of fibrosis and non fibrosis groups

Variables	Values		*p*
	With Fibrosis n = 84	Without Fibrosis n = 45	
Age (years)	32.44 (± 6.61)	31.56(± 5.01)	0.396***
BMI (kg/m²)	28.40 (26.10-30.50)*	28.20 (26.42-29.85)*	0.723**
WC (cm)	100 (96-105)*	98 (97-102)*	0.542**
FPG (mg/dL)	93.83 (± 10.71)	92.23 (± 11.74)	0.437***
TC (mg/dL)	203.34(± 47.73)	205.27 (± 38.90)	0.818***
LDL-C (mg/dL)	123.95 (± 38.48)	126.82 (± 27.73)	0.633***
TG (mg/dL)	167 (115-261)*	155.50 (110-253.75)*	0.820**
HDL-C (mg/dL)	40.50 (36-47)*	40 (35-43.75)*	0.276**
ALT (U/L)	111 (85.50-148.75)*	89 (69.50-114)*	**0.011****
AST (U/L)	51.50 (40-64.75)*	41 (35-51.50)*	**0.001****
GGT (U/L)	58 (44-77)*	59 (45-90)*	0.797**
Direct bilirubin (mg/dL)	0.15 (0.11-0.20)*	0.17 (0.11-0.20)*	0.527**
Indirectbilirubin (mg/dL)	0.60(0.40-0.80)*	0.60 (0.40-0.82)*	0.976**
UricAcid (mg/dL)	6.67 (6.06-7.18)*	6.38 (5.58-7.13)*	0.174**
Hb (g/dL)	15.87 (± 1.06)	15.73 (± 0.82)	0.461***
WBC (x 10³)	7100 (6250-8525)*	7200 (6050-8850)*	0.799**
Plt (x 10³)	241256.41 (± 50103.38)	226972.97 (± 49869.54)	0.155***
Insulin (µU/mL)	15.25 (10.27-23.91)*	11.41 (9.31-16.13)*	**0.029****
HOMA-IR	3.63 (2.34-5.43)*	2.48 (2.09-4.06)*	**0.027****
Hs-CRP (pg/mL)	2.11 (1.30-3.45)*	1.75 (1.11-2.89)*	0.257**

BMI: Body mass index; WC: waist circumference; FPG: fasting plasma glucose; TC: total cholesterol; LDL-C: low-density lipoprotein cholesterol; TG: triglicerides; HDL-C: high-density lipoprotein cholesterol; ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: gamma-glutamyl transferase; Hb: Hemoglobin; WBC: White blood cell; Plt: platelet; HOMA-IR: homeostasis model assessment for insulin resistance; Hs-CRP: high-sensitivity C-reactive protein
*median(25.-75. Percentiles)
** Mann Whitney U test
*** Independentsample t- test

without fibrosis groups [direct bilirubin = 0.60 (0.40-0.80), indirect bilirubin = 0.60 (0.40-0.82)] regarding bilirubin levels (respectively $p = 0.527$, $p = 0.976$).

DISCUSSION

In this study, we investigated the bilirubin levels among subjects with biopsy-proven NAFLD grouped as either with or without NASH and with or without fibrosis who have no confounding factors such as hypertension, DM, and obesity. We couldn't find any difference in comparing the groups. Including only the subjects free from any confounding factor is an important feature of the present investigation.

Bilirubin exerts an antioxidant effect by inhibiting the activity of NAD(P)H oxidase, which paves the way for the formation of superoxide radicals.[19] However, bilirubin has anti-inflammatory effects, which inhibit the formation of fibrogens via heme oxygenase-1.[20] At the same time, it is shown that higher bilirubin levels are inversely associated with insulin level, IR, and DM, the factors defined in the pathogenesis of NAFLD. From a cardiovascular perspective, it is stated that elevated bilirubin levels are also related to the reduced risk of CVD, including CAD, stroke, and PAD.[12]

A cross-sectional study of 17,348 participants compared the subjects with and without ultrasonography diagnosed NAFLD. The bilirubin levels were significantly low in the NAFLD group. It is emphasized that NAFLD, which is observed to be associated with serum bilirubin level, decreases with the increase in bilirubin level.[21] Chang Y *et al.* conducted a prospective cohort study among middle-aged Korean workers with no evidence of liver disease and no major risk factors for liver disease. The patients evaluated within the scope of the study were

followed up for a period of seven years and checked with USG at certain periods. Interestingly, it has been observed that higher direct bilirubin levels result in a reduced risk of developing NAFLD, even adjusting for various metabolic parameters, including obesity, IR, DM, history of CVD, and malignancy. They stated the effect of oxidative stress and IR in the pathogenesis of NAFLD and the association between low serum bilirubin levels and increased HOMA-IR and insulin levels. Interestingly, they found the persistence of the association between increased direct bilirubin and lower incidence of NAFLD after adjusting for a variety of measures of IR and after restricting the analysis to participants with no evidence of IR. In conclusion, they reported that the protective role of direct bilirubin in these patients is independent of IR.[22] In parallel with the previous study, Tian J *et al*. found a relationship between low direct bilirubin levels. They reduced NAFLD risk among the middle-aged and elderly Chinese population. They explained this as the anti-inflammatory effect of bilirubin, especially direct bilirubin, inhibiting oxidative stress and IR and altering glucose metabolism. The important part of these results was that this association was independent of classical risk factors, including liver enzymes, DM, MetS features, CAD, and other classical metabolic risk factors.[23]

Two studies evaluate the relationship between bilirubin levels and histopathologic findings in patients with NAFLD. In a retrospective study including the patients' biopsy-proven NAFLD, they investigated the relationship between the unconjugated bilirubin (UCB) levels and histopathological findings. They found that unconjugated hyperbilirubinemia was inversely associated with NASH. They speculated that these findings are because of the inhibition of the pathogenesis of NASH via the potent antioxidant, anti-inflammatory and anti-fibrogenic effect of UCB.[24] Another study, including two hundred and eighty-five patients with biopsy-confirmed NAFLD, investigated the relationship between steatosis, inflammation, and fibrosis with UCB levels. They found that UCB levels were decreased in patients with NASH and advanced inflammation and fibrosis. Also, they demonstrated by logistic regression analysis that low UCB levels are independently associated with advanced liver inflammation and fibrosis. These results were interpreted as a lack of the effect of UCB, the main endogenous lipid antioxidant, leading to the progression of liver injury in patients with NASH.[25] Conversely, we couldn't find any relation between bilirubin levels with histological findings in our study participants. In these studies, some of the participants in NAFLD groups were diabetic, obese, or hypertensive. Still, in our study, none of the patients had confounding factors for NAFLD, such as morbid obesity, DM, and hypertension. We think that the real relationship between the bilirubin and histological findings in NAFLD can be evaluated in this unique group. The low bilirubin levels in patients with NAFLD found in these studies can be the effect of the other confounding factors seen in MetS.

Limitations

This study has several limitations. Since our study is cross-sectional, it does not reflect all NAFLD patients. Secondly, all participants were men, and it remains to be determined if these results were similar even in women. Finally, HOMA-IR index has been used for the evaluation of insulin resistance and is not the gold standard method.

CONCLUSION

In light of these data, it was found that serum bilirubin level was not different in either the steatohepatitis group or the fibrosis group compared to the control group in biopsy-proven NAFLD patients. It is thought that conducting our study in a patient group without confounding factors such as morbid obesity, DM, and hypertension will contribute positively to the literature. On the other hand, randomized controlled studies are needed to understand the role of bilirubin in the development and progression of NAFLD.

Conflict of Interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical Approval

The protocol of the study was approved by the Medical Ethics Committee of Balikesir University, School of Medicine Balıkesir, Turkey. (Decision number: 2020/179, date: 14.10.2020).

Authors' Contribution

Study Conception: CNE,AK, TD; Study Design: CNE,AK, TD; Supervision; CNE, TD; Materials: ST, AÇ; Data Collection and/or Processing: ST, AÇ; Analysis and/or Data Interpretation: ACY, ST; Literature Review: CNE,AK, TD; Critical Review: CNE, TD; Manuscript preparing: CNE, AK, TD.

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