Myeloperoxidase and calprotectin; Any role as non-invasive markers for the prediction of ınflammation and fibrosis in non-alcoholic steatohepatitis?

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ABSTRACT

Background/Aims: Specific serum markers reflecting hepatic inflammation and fibrosis are required to tailor the treatment strategies in non-alcoholic steatohepatitis (NASH). We aimed to investigate the roles of myeloperoxidase (MPO) and calprotectin in predicting the hepatic inflammation status and disease severity in NASH.

Materials and Methods: A total of 48 patients with biopsy-proven NASH and 25 healthy volunteers with normal weight were prospectively enrolled. Serum MPO and calprotectin levels were compared between the NASH and control groups. Hepatic MPO and calprotectin expressions were compared in terms of histologic non-alcoholic fatty liver disease activity scores (NAS) (low NAS [≤4] vs. high NAS [>5]) and fibrosis stage (insignificant [F0–1]/significant [F2–4]).

Results: Serum MPO and calprotectin levels were not significantly different between the NASH and control groups. In the subgroup analysis, hepatic MPO expression was significantly increased in patients with NASH with significant fibrosis than in those with insignificant fibrosis (F2–4: 7.04±3.61 vs. F0–1: 4.83±2.42, p=0.01). We found no difference between the groups with low and high NAS with regard to serum MPO and calprotectin levels and hepatic MPO and calprotectin expressions.

Conclusion: This study demonstrated that hepatic MPO expression can reflect advanced fibrosis in NASH. However, when serum MPO and calprotectin levels were evaluated as potential serum markers, both did not associate with hepatic inflammation status and fibrosis stage in NASH. Therefore, our study results preclude their use as serum markers for hepatic inflammation in NASH.

Keywords: Non-alcoholic fatty liver disease, inflammation, fibrosis, oxidative stress, enzyme-linked immunosorbent assay

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has a spectrum ranging from simple hepatic steatosis to steatosis with inflammation, namely non-alcoholic steatohepatitis (NASH). NASH itself may exhibit a disease severity profile from mild fibrosis to cirrhosis and hepatocellular carcinoma. The inflammatory process leads to the progression of simple steatosis toward NASH, which may in turn result in liver cirrhosis (1, 2). Thus, the research for non-invasive surrogate markers predicting the hepatic inflammatory and fibrotic states in NAFLD is the ultimate target of preventive medicine.

Innate immunity is involved in the inflammatory process of NASH (3). Neutrophils are one of the most important components of the innate immunity because of their phagocytic and antimicrobial properties. Recent studies have highlighted the presence of neutrophils in the liver inflammation caused by NASH (4). Neutrophils exhibit their activity through granules containing enzymes, including myeloperoxidase (MPO) and calprotectin, or through proteins stored in their cytoplasm (5, 6). In addition to inflammation, oxidative stress, in which MPO plays a role, has been shown to be a significant contributor to the progression of NAFLD (7, 8).

MPO is the predominant protein secreted by neutrophils as a result of phagocyte activation and to lesser extent by monocytes, macrophages, and Kupffer cells (9, 10). MPO is a member of the peroxidase enzyme family, just like lactoperoxidase, eosinophil peroxidase, and thyroid peroxidase. In addition to the peroxidation activity, MPO catalyzes the conversion of hydrogen peroxide and chloride ion to hypochlorous acid, which exhibits potent an-

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timicrobial and cytotoxic activity as an effective oxidant. The reactive bioproducts of the oxidative stress that results from the chlorination activity of MPO play role in the pathogenesis of NASH and other diseases, including atherosclerosis and neurodegenerative diseases, such as Alzheimer's disease or multiple sclerosis (11-13). It has also been demonstrated that increased serum levels of MPO are associated with the severity of coronary artery disease (14), but the data evaluating the disease severity of NASH and serum MPO levels are scarce.

Calprotectin is another protein found abundantly in neutrophils. The soluble form of calprotectin has both bacteriostatic and cytokine-like effects. It is secreted from the neutrophils activated by bacterial infections or inflammation; thus, its level rises in serum in both the situations. In previous studies, serum concentrations of calprotectin have been shown to be increased in rheumatoid arthritis and multiple sclerosis, whereas inflammatory bowel disease and colorectal cancer were associated with an increase in the stool concentrations of calprotectin. In most of these diseases, the correlation between serum calprotectin level and disease activity has been proven (5, 15-18). However, the data evaluating the association between serum calprotectin levels and NASH disease activity do not exist.

Given that the inflammatory process and oxidative stress play important roles in the pathogenesis of NASH, we aimed to study the roles of MPO and calprotectin, the markers of inflammation, in NASH. Serum MPO and calprotectin levels were investigated with regard to hepatic inflammation status and fibrosis in patients with biopsy-proven NASH, and we compared the results with those of healthy volunteers. We also aimed to examine the association of hepatic MPO and calprotectin expressions with NASH disease activity and fibrosis.

MAIN POINTS

- *Myeloperoxidase (MPO) and calprotectin are both well-established inflammatory markers and demonstrated to be increased in many inflammatory disease, but their role in non-alcoholic steatohepatitis (NASH) is uncertain.*
- *We investigated the potential association of hepatic and serum MPO and calprotectin with fibrosis and inflammatory component of NASH.*
- *We demonstrated that increased hepatic MPO expression can reflect significant fibrosis in patients with NASH.*
- *Serum levels of myeloperoxidase and calprotectin are not useful in reflecting either inflammatory or fibrosis component of NASH.*

MATERIALS AND METHODS

Patient Selection

In this prospective case-control study, 48 patients with biopsy-proven NASH who were older than 18 years were included as the study group. A total of 25 healthy volunteers, matched for age and sex with the study group, were included as the control group. The control group was selected from the voluntary individuals who were admitted to our gastroenterology outpatient clinic and were deemed normal after through ultrasound and laboratory investigations showing no steatosis in their liver for complaints, such as dyspepsia and bloating, and without any sign of liver disease, significant alcohol intake, or medications leading to NAFLD. Patients with active infection; rheumatologic disease; chronic renal failure (creatinine>1.4 mg/dL); congestive heart failure; coronary artery disease; malignancy; pregnancy; daily alcohol consumption exceeding 30 g for men and 20 g for women; liver disease owing to causes other than NAFLD, such as viral hepatitis, hemochromatosis, Wilson's disease, autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, biliary obstruction, and alpha-1 antitrypsin deficiency; and drug use that may lead to hepatosteatosis, such as estrogen, amiodarone, steroids, tamoxifen, methotrexate, and valproate, were excluded.

All the patients underwent physical examination and anthropometric measurements. Body-mass index (BMI) was calculated from the height and weight measurements. Metabolic syndrome (MetS) was diagnosed according to adult treatment panel (ATP) III criteria (19). Blood pressure measurement was performed in a quiet room with a sphygmomanometer after the patients rested for more than 10 minutes.

Determination of Serum MPO and Calprotectin Levels

Blood samples were taken from the antecubital veins in all patients between 8-9 a.m. with at least 8 hours of fasting and immediately centrifuged at 2,500 g for 10 minutes. Aliquots of the serums sample were kept frozen at −80°C and dissolved only once before the analysis. All the serum samples were evaluated irrespective to the clinical information.

Serum levels of calprotectin were studied using the commercial kit with enzyme-linked immunosorbent assay (ELISA) (Calprotectin, Immunodiagnostic AG, Germany). According to the information given by the manufacturer, the coefficient of variability is reported as 6.16%-4.48%-4.90% for 878.58-1,750.10-877.13 ng/mL concen-

trations in study and 7.49–10.32–12.86% for 768.62-813.97-1,584.67 ng/mL concentrations between the studies. The serum samples were assayed at 1:50 dilution.

Serum levels of human MPO were studied using the commercial kit with ELISA (Human Myeloperoxidase, Boster Immunoleader, USA). The serum human MPO measurement range was determined to be 312-20,000 pg/mL, and the analytical sensitivity was <10 pg/mL according to the information provided by the manufacturer. The serum samples were assayed at 1:10 dilution.

Liver Histology and Immunohistochemical Staining

Biopsy specimens were fixed with 10% formaldehyde and embedded in paraffin blocks. Tissue sections (4-micron in thickness) were stained with hematoxylin-eosin and Masson's trichrome and examined under the light microscope. An experienced pathologist, blinded to the clinical information, assessed the biopsy specimens according to the NASH clinical study network scoring system. The histologic NAFLD activity score (NAS) is calculated by the summation of steatosis (0-3), lobular inflammation (0-3), and ballooning (0-2). Scores between 0-4 and >4 are accepted as low NAS and high NAS, respectively (20). Fibrosis stages were stratified as insignificant fibrosis (F0-1) and significant (F2-4) fibrosis.

Streptavidin-biotin-peroxidase immunohistochemical staining was performed to show the expression of calgranulin A/B and MPO in formalin-fixed paraffin-embedded tissues, and 3-µm thick sections that were obtained from the tissue blocks were stained according to the manufacturer's instructions. For antigen retrieval, ethylene diamine tetra acetate solution (pH 8) in microwave with 400 W power and citrate buffer solution (pH 6) for calgranulin A/B and MPO were used. To prevent non-specific staining, protein blockage (SensiTek HRP Anti-Polyvalent Lab Pack, ScyTek Laboratories, US) was performed. The tissue sections were incubated in 1/400 dilution for calgranulin A antibody (C-10, Santa Cruz Biotechnology) and 1/200 dilution for both calgranulin B (B-5, Santa Cruz Biotechnology) and MPO antibody (B-5, Santa Cruz Biotechnology) at room temperature for 60 minutes. Biotinylated secondary antibody (SensiTek HRP Anti-Polyvalent Lab Pack, ScyTek Laboratories, US) was applied for 20 minutes. After washing with phosphate-buffered saline, the tissue sections were incubated in streptavidin-peroxidase (SensiTek HRP Anti-Polyvalent Lab Pack, ScyTek Laboratories, US) for 20 minutes. Visualization was performed by 3,3'-diaminobenzidine chromogen, and Mayer's hematoxylin was used as a counter-stain.

Statistical Analysis

The data on continuous variables were presented as mean±standard deviation and categorical variables as absolute numbers and percentages (n [%]). For the comparison of quantitative variables, the Student's t-test was used for independent groups with normal distribution and the Mann–Whitney U test for groups without normal distribution. The Kruskal-Wallis test was used for the comparison of 3 groups without normal distribution. Pearson chi-squared, Fisher-Freeman-Halton, and Yates continuity correction tests (Yates corrected chi-squared test) were used for the comparison of qualitative data. Correlation between 2 independent parameters was analyzed using the Spearman's correlation analysis. All statistical analyses were conducted using the Statistical Package for the Social Sciences (SPSS) software version 20.0 (IBM Corp.; Armonk, NY, USA). A p value of <0.05 was considered statistically significant.

Ethics Approval

The study protocol was approved by the local ethics committee (70737436-050.06.04-1300082275), and informed consent was obtained from all the individuals who participated in the study.

RESULTS

Of the 48 patients with NASH, 29 (60.4%) were men and the mean age was 42.5 (18–63) years. Age and sex distributions of the study and control groups were similar. BMI, presence of MetS, and serum levels of liver aminotransferases were significantly higher in patients with NASH than in the control group. Approximately half of the patients with NASH were obese, and the remaining patients were overweight, except for 3 patients. MetS was observed in the 54.3% (n=25) of patients with NASH. The liver biopsy results of 48 patients with NASH revealed high NAS in 30 (62.5%) and low NAS in 18 (37.5%) patients. Baseline demographics and general characteristics of patients are summarized in Table 1.

Serum calprotectin and MPO levels were not statistically different between the NASH and control groups (Calprotectin [NASH], 968.58±673.61 ng/mL; [control] 1,524.37±835.12 ng/mL; p=0.074 and MPO [NASH], 14.52±7.58 U/mL; [control], 16.25±7.14 U/mL, p=0.259). The subgroup analysis showed that the serum calprotectin levels were slightly increased in patients with NASH with high NAS compared with those of patients with low NAS, but the difference was not statistically significant (1,072.83±688.44 ng/mL vs. 773.125±618.46 ng/mL, respectively; p=0.142). In addition, the serum

MPO levels did not differ among the high and low NAS groups (14.14±5.30 U/mL vs. 15.15±10.49 U/mL, respectively; p=0.523). Similarly, serum calprotectin and MPO levels were similar in patients with NASH with insignificant and significant fibrosis (Calprotectin: [F0–1], 1,029.06±749.39 ng/mL vs. [F2–4], 830.35±449.38 ng/mL; p=0.363 and MPO: [F0–1], 15±8 U/mL vs. [F2–4], 13.46±6.66 U/mL; p=0.520]. When we compared the serum calprotectin and MPO levels in patients with NASH with and without MetS, we did not find any significant difference between the 2 groups (Calprotectin: MetS [+], 812.08±442.85 ng/mL vs. MetS [–], 1,233.09±1,095.05 ng/mL, p=0.09 and MPO: MetS [+], 14.47±7.65 U/mL vs. MetS (–), 14.44±7.96 U/mL, p=0.987). Comparison of the serum calprotectin and MPO levels in patients with NASH with regard to NAS score and fibrosis stage is presented in Table 2.

Hepatic calprotectin and MPO expression were not different between the low and high NAS groups (17.69±4.64 ng/mL and 5.46±2.92 ng/mL vs. 17.05±4.61 ng/mL and 5.56±3.08 ng/mL, p=0.642 and p=0.910, respectively). Hepatic MPO expression was increased in the significant fibrosis group (F0–1: 4.83±2.42 ng/mL vs. F2–4: 7.04±3.61 ng/mL, p=0.01), but calprotectin expression was similar among the different degrees of fibrosis (F0–1: 16.96±4.58 ng/mL vs. F2–4: 18±4.66 ng/mL, p=0.472). Histologic images of MPO- and calpro-

Table 1. General characteristics and demographics of whole cohort and subgroups.

	Control group (n=25)	Study group (n=48)	p	Low NAS (n=18)	High NAS (n=30)	p
Age (years)	39.56±9.50	42.58±11.27	0.190	42.17±10.90	42.83±11.66	0.786
BMI (kg/m^2)	24,48±2,29	30,50±4,69	0.009	29,83±3,98	30.90±5.09	0.396
Waist circumference(cm)	100.55±12.91	105.15±11.44	0.087	103.22±9.00	106.30±12.68	0.188
Gender;						
Male	16 (64%)	29 (60.4%)	0.485	11 (61.1%)	18 (60%)	0.592
Female	9 (36%)	19 (39.6%)		7 (38.9%)	12 (40%)	
BMI;						
Normal	13 (52%)	3 (6.3%)	0.001*	1 (5.6%)	2 (6.7%)	0.575
Overweight	12 (48%)	22 (45.8%)		10 (55.6%)	12(40%)	
Obese	0	23 (47.9%)		7 (38.9%)	16 (53.3%)	
Systolic blood pressure	110.46±12.38	121.40±15.09	0.003*	120.05±18.14	122.24±13.11	0.634
Diastolic blood pressure	70.58±8.85	78.55±9.97	0.002*	77.77±9.77	79.03±10.23	0.679
AST	17.61±4.65	50.06±20.14	0.001*	43.16±18.89	54.20±20.02	0.063
ALT	20.94±8,95	93.92±43.61	0.001*	78.77±43.49	103.00±41.77	0.066
HDL	52.33±12.29	46.20±12.34	0.081	48.17±11.16	44.96±13.07	0.407
LDL	114.67±29.01	122.91±43.97	0.468	132.64±53.58	116.77±36.45	0.248
Triglyceride	111,56±40,95	173,63±89,25	0.001*	157.82±90.18	183.96±88.86	0.357
MetS	0 (0%)	25 (54.3 %)	0.001*	8 (47.1 %)	17 (58.6 %)	0.447

BMI: body mass index; AST: aspartate aminotransferase, ALT: alanine aminotransaminase; HDL: high density lipoprotein; LDL: low-density lipoprotein, MetS: Metabolic syndrome. *p<0.05

Table 2. Calprotectin and MPO levels in serum and liver biopsies of the study and control groups.

	Control group (n=25)	Study group (n=48)	p	Low NAS (n=18)	High NAS (n=30)	p
Serum calprotectin	968.58±673.61	1524.37±835.12	0.074	773.125±618.46	1072.83±688.44	0.142
Serum MPO	14.52±7.58 (12.9)	16.25±7.14 (13.4)	0.259	15.15±10.49 (12.8)	14.14±5.30 (13.6)	0.523
Calprotectin-stained cell count	17.29±4.58	-	-	17.69±4.64	17.05±4.61	0.642
MPO-stained cell count	5.52±2.99	-	-	5.46±2.92	5.56±3.08	0.910

tectin-expressing cells in the liver biopsy of patients with NASH are shown in Figure 1. When each histological parameter was considered individually, we did not observe any statistical difference among patients having higher grades of steatosis (grades 2 and 3), lobular inflammation (grades 2 and 3), or ballooning (grade 2) and lower grades of steatosis (grade 1), lobular inflammation (grades 0 and 1), or ballooning (grades 0 and 1) in terms of calprotectin or MPO expression in the liver biopsies. Association of hepatic MPO and calprotectin expressions with histological findings in patients with NASH are exhibited in Table 3.

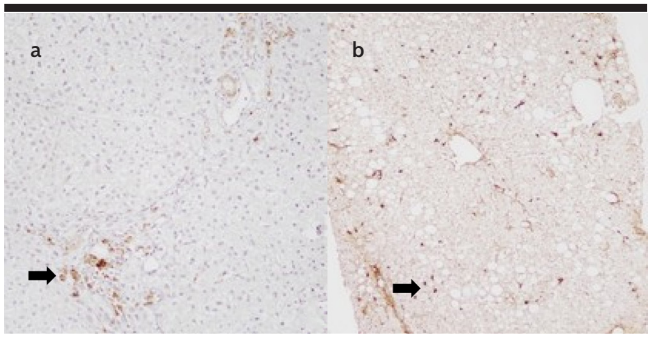

Figure 1. a, b. a) Myeloperoxidase-expressing cells in the liver biopsy of patients with non-alcoholic steatohepatitis, hematoxylin and eosin staining, 400x. b) Calprotectin-expressing cells in the liver biopsy of patients with non-alcoholic steatohepatitis, hematoxylin and eosin staining, 400x.

Table 3. Analysis of MPO and calprotectin stained cell counts in liver biopsies in comparison to steatosis, lobular inflammation and ballooning.

	MPO-stained cell count	Calprotectin-stained cell count
Steatosis		
1 (n=12)	5.57±2.63 (5.3)	19.27±3.93 (19.3)
2+3 (n=36)	5.51±3.14 (4.6)	16.63±4.64
(15.4)		
	p=0.65	p=0.072
Lobular inflammation		
0+1 (n=24)	4.83±2.70(4.4)	17.38±3.94 (17.3)
2+3 (n=24)	6.22±3.17 (5.4)	17.20±5.24 (15.9)
	p=0.110	p=0.892
Ballooning		
0+1 (n=29)	5.48±2.64 (5.3)	17.46±4.72 (16.3)
2 (n=19)	5.60±3.55 (4.3)	17.03±4.48 (17.0)
	p=0.890	p=0.749

*p values are denoted in the boxes for comparison of each figures

DISCUSSION

The results of this study showed that serum MPO or calprotectin levels did not reflect the histological inflammation status in NASH as they did not differ between patients with NASH and control group. In the subgroup analysis of patients with NASH with high and low NAS, we did not find any significant difference in the serum MPO and calprotectin levels. When we investigated the liver biopsies of patients with NASH for hepatic MPO or calprotectin expressions, we did not observe any difference in patients with NASH with low and high NAS. Hepatic MPO expression was found to be associated with significant fibrosis; however, there was no significant difference in the serum MPO levels of the control and NASH groups. The most likely explanation for this disparity between the serum and hepatic MPO levels in patients with NASH could be that MPO and calprotectin are mainly secreted from the activated neutrophils in the inflamed liver and only lower levels are released into the serum. Because of the crucial role played by the inflammatory process and oxidative stress in the pathogenesis of NASH, our hypothesis was to find the increased serum MPO and calprotectin levels in the NASH group compared with the control group. The results of this study showed that these inflammatory biomarkers were not reliable for predicting the varying degrees of hepatic inflammation, neither from the serum nor from the liver tissues of patients with NASH, although our results point to an important link between the hepatic MPO expression and fibrosis development.

The association of NAFLD and MPO in the serum and hepatocytes was studied in a population of patients with morbid obesity who underwent bariatric surgery, which revealed that the serum MPO levels of patients who were morbidly obese and with NASH were significantly higher than those of patients who were morbidly obese but with simple steatosis (13). The same study demonstrated increased MPO-expressing cell counts in the liver biopsies of patients with NASH than those of patients with simple steatosis. The authors explained the findings by increased chemokines in the liver, which were secreted in response to the oxidation products generated by MPO. In another study, plasma MPO and calprotectin levels assessing neutrophil activation were found to be significantly higher in patients with morbid obesity than in controls with normal weight; it was suggested that morbid obesity consistently led to chronic inflammation through activation of neutrophils and thus by the activation of the innate immune system (21). This study has shown no difference between the serum MPO levels of the NASH and healthy groups

and hepatic MPO expression in the liver biopsies of patients with low and high NAS. There are a few reasons for the differences between the results of those 2 studies published by the same group and our study. The first study had no healthy control group. Furthermore, our cohort did not contain patients with solely simple steatosis. The second study compared patients with morbid obesity having a BMI of approximately 46 kg/m^2 with healthy controls with normal weight, whereas the average BMI of patients with NASH in our study was only 30.5 kg/m^2. Our study demonstrated an insignificant difference between the healthy controls and patients with NASH with regard to serum MPO and calprotectin levels.

Our initial hypothesis was to find increased MPO and calprotectin levels in the serum of patients with NASH compared with the control group. However, we found no difference despite prominent hepatic inflammation and MPO- and calprotectin-expressing cell counts in the liver biopsies. We could not compare the hepatic MPO and calprotectin expression of the control group with those of patients with NASH because we did not obtain biopsies from the controls; however, apparently, the hepatic inflammation involving infiltration by neutrophils and other MPO/calprotectin-expressing cells did not translate into increased serum MPO/calprotectin levels in patients with NASH than in the controls. Cottam DR et al. (22) revealed impaired neutrophil activation and migration with reduced CD62L expression in patients with morbid obesity than in individuals with normal weight. This evidence of immune dysfunction can explain the statistically insignificant calprotectin levels in patients with NASH than in the controls in this study. Although calprotectin has been shown to have an impact on the development, diagnosis, and progression of NAFLD in rat models (23), there is no evidence showing the association of calprotectin with NAFLD in humans in the literature. Our study revealed that serum calprotectin levels and hepatic calprotectin expression were not associated with NASH or disease activity.

Our study had some limitations. The main limitation was the case-control design of the study; therefore, the role of MPO and calprotectin in the pathogenesis of NASH was not investigated by examining the causal associations in specific study groups, such as patients with morbid obesity, established diabetes, high plasma lipids, or high alanine aminotransferase values. Instead, we enrolled consecutive patients with NASH from our outpatient clinic. Moreover, the small number of participants prevented the generalization of the results. The individuals in the control group in our study had normal liver functions and no medication or significant alcohol intake; however, we did not check the general inflammation or infection status in detail other than through physical examination, laboratory tests, or hepatic ultrasound imaging. Nevertheless, this is the first study investigating the role of serum MPO/calprotectin levels and hepatic MPO/calprotectin expression from the liver biopsies in patients with NASH. The prospective design with the enrolment of patients with biopsy-proven NASH is another remarkable aspect of our study.

In conclusion, this study demonstrated that hepatic MPO expression could be a potential marker of fibrosis in NASH. However, the serum levels of MPO and calprotectin, which are inflammation markers and mainly reflect the neutrophil function, are neither associated with NASH inflammation status nor fibrosis. Further studies with larger populations of patients with NASH are required to understand the role of intrahepatic MPO and to decide whether it can be a potential target for NASH treatment.

Ethics Committee Approval: The study protocol was approved by the local ethics committee (Approval number: 70737436-050.06.04-1300082275, Approval date: 16.05.2013).

Informed Consent: Informed consent was obtained from all the individuals who participated in the study.

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Conflict of Interest: The authors have no conflict of interest to declare.

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