ORIGINAL ARTICLE

# A composite model including visfatin, tissue polypeptide-specific antigen, hyaluronic acid, and hematological variables for the diagnosis of moderate-to-severe fibrosis in nonalcoholic fatty liver disease: a preliminary study

Alina Chwist[1], Marek Hartleb[1], Andrzej Lekstan[2], Michał Kukla[1], Krzysztof Gutkowski[1], Maciej Kajor[3]

1 Department of Gastroenterology and Hepatology, Medical University of Silesia, Katowice, Poland
2 Department of Digestive Tract Surgery, Medical University of Silesia, Katowice, Poland
3 Department of Pathomorphology, Medical University of Silesia, Katowice, Poland

**KEY WORDS**

liver fibrosis, nonalcoholic liver disease, nonalcoholic steatohepatitis, noninvasive diagnosis

**ABSTRACT**

**INTRODUCTION** Histopathological risk factors for end-stage liver failure in patients with nonalcoholic fatty liver disease (NAFLD) include nonalcoholic steatohepatitis (NASH) and advanced liver fibrosis. There is a need for noninvasive diagnostic methods for these 2 conditions.

**OBJECTIVES** The aim of this study was to investigate new laboratory variables with a predictive potential to detect advanced fibrosis (stages 2 and 3) in NAFLD.

**PATIENTS AND METHODS** The study involved 70 patients with histologically proven NAFLD of varied severity. Additional laboratory variables included zonulin, haptoglobin, visfatin, adiponectin, leptin, tissue polypeptide-specific antigen (TPSA), hyaluronic acid, and interleukin 6.

**RESULTS** Patients with NASH (NAFLD activity score of ≥5) had significantly higher HOMA-IR values and serum levels of visfatin, haptoglobin, and zonulin as compared with those without NASH on histological examination. Advanced fibrosis was found in 16 patients (22.9%) and the risk factors associated with its prevalence were age, the ratio of erythrocyte count to red blood cell distribution width, platelet count, and serum levels of visfatin and TPSA. Based on these variables, we constructed a scoring system that differentiated between NAFLD patients with and without advanced fibrosis with a sensitivity of 75% and specificity of 100% (area under the receiver operating characteristic curve, 0.93).

**CONCLUSIONS** The scoring system based on the above variables allows to predict advanced fibrosis with high sensitivity and specificity. However, its clinical utility should be verified in further studies involving a larger number of patients.

Correspondence to:
Professor Marek Hartleb, MD, PhD, Katedra i Klinika Gastroenterologii i Hepatologii, Śląski Uniwersytet Medyczny w Katowicach, ul. Medyków 14, 40-752 Katowice, Poland, phone: +48-32-789-44-01, fax: +48-32-789-44-02, e-mail: mhartleb@sum.edu.pl
Received: September 11, 2014.
Revision accepted: November 13, 2014.
Published online: November 14, 2014.
Conflict of interest: none declared.
Pol Arch Med Wewn. 2014; 124 (12): 704-712


**INTRODUCTION** Nonalcoholic fatty liver disease (NAFLD) is strongly associated with metabolic syndrome.[1] The histopathological spectrum of NAFLD ranges from isolated fatty liver through nonalcoholic steatohepatitis (NASH) to advanced fibrosis or cirrhosis with attendant complications of portal hypertension, hepatic synthetic impairment, and hepatocellular carcinoma. The determination of NASH and staging of hepatic fibrosis is the key determinant of prognosis in patients with NAFLD.[2,3]

Historically, liver biopsy has been a standard method to assess the severity of hepatic damage; however, its invasive nature and vulnerability to sample error make biopsy an inappropriate tool to screen patients for the occurrence of NAFLD or to monitor the progressive potential of this disease. The limitations of biopsy have led

to the search for alternative, noninvasive methods to detect NASH and advanced liver fibrosis. The number of serum fibrosis biomarkers has increased significantly over the past decade, and validated scores are beginning to be introduced into clinical practice to screen patients for advanced fibrosis, which influences further diagnostic and therapeutic strategies.

Most predictive models have been designed to assess patients with hepatitis C, for whom they have been validated, and few methods were specifically developed for patients with NAFLD.[4] Furthermore, some biomarker panels have been developed on the basis of routine liver function tests, and others rely on nonroutine variables directly involved in the molecular pathogenesis of fibrogenesis and fibrinolysis. These biomarkers are commonly combined with clinical risk factors for hepatic fibrosis, such as age, obesity, or diabetes.[5-12] Recently, serological parameters reflecting insulin resistance, oxidative stress, or hormonal activity of visceral adipose tissue have attracted increasing attention. Several adipokines have been implicated in the pathogenesis of NASH and NAFLD. Leptin and adiponectin have been extensively explored in patients with NAFLD,[13] but the role of recently discovered adipokines in the development of liver inflammation and fibrosis is still unclear. Visfatin is a proinflammatory and insulin-mimetic adipokine that contributes to glucose and lipid metabolism. The avaialable data on the effect of visfatin on different histopathological components of NAFLD are inconsistent.[14,15]

It has been postulated that gut microflora participates in the pathogenesis of obesity and NAFLD by damaging the intestinal mucosal barrier.[16] Moreover, some bacterial species produce small amounts of alcohol entering the portal venous system. Intercellular tight junctions are the key structures regulating paracellular trafficking of macromolecules, and zonulin is one of the physiological mediators of this process.[17] Because the serum zonulin level in obese patients is correlated with proinflammatory cytokines, it has been hypothesized that this molecule may be a link between gut microflora and the obesity-related state of microinflammation.[18]

The aim of this study was to assess the predictive power of serum levels of zonulin, haptoglobin, visfatin, hyaluronic acid, and tissue polypeptide-specific antigen (TPSA) along with readily available clinical, demographic, and laboratory variables to identify moderate-to-severe fibrosis.

## PATIENTS AND METHODS

### Study population

The study involved a total of 70 participants (30 women and 40 men) diagnosed with NAFLD and enrolled prospectively to the study between 2006 and 2013 (patients hospitalized in a tertiary referral university hospital). The mean age of the patients was 45.7 ±13.3 years (range, 20–73 years). The inclusion criteria were as follows: hyperechogenic pattern of the liver, elevation of alanine aminotransferase over 1.5 × the upper normal limit, and presence of metabolic syndrome according to the definition by the International Diabetes Association.[19] A thorough medical history included the use of drugs and alcohol consumption as well as a history of arterial hypertension, hyperlipidemia, and diabetes mellitus, defined by clinical diagnosis requiring medical therapy. A physical examination including height, weight, hip, and waist measurements was performed in each patient.

The exclusion criteria were as follows: liver cirrhosis, alcohol consumption of more than 60 g/wk in women and more than 100 g/wk in men, chronic use of drugs known to induce liver steatosis (nonsteroid anti-inflammatory drugs, amiodaron, tamoxifen, methotrexate, and corticosteroids), inflammatory bowel disease, infection with hepatitis C virus (HCV), transferrin saturation index over 50%, ceruloplasmin levels of less than 18 mg/dl, present or past coagulation disorders, clinical features of liver dysfunction or portal hypertension, and age below 18 years.

### Liver biopsy

All patients had liver biopsy performed not earlier than 2 weeks before enrollment to the study. Liver samples were obtained using Menghini needles with a diameter of 1.4 or 1.6 mm (HEPAFIX®, B. Braun Melsungen AG, Germany), in accordance with the accepted standards. The minimum length of the biopsy sample eligible for a histological assessment was 1.5 cm. An experienced pathologist blinded to the clinical data reviewed liver samples.

Fatty liver was defined as the presence of at least 5% steatosis, while steatohepatitis was diagnosed by the simultaneous presence of steatosis, inflammation, and ballooning graded according to the NAFLD activity score (NAS).[19] Depending on the result of the NAS, patients were divided into 3 groups: non-NASH (0–2 points, steatosis with or without nonspecific inflammation), indeterminate or borderline type (3–4 points), and definite NASH (≥5 points). Hepatic fibrosis was assessed from trichrome-stained sections and was staged using the Liver Fibrosis Staging System from 0 to 4.[20] Based on this system, fibrosis was categorized into 2 groups: none-to-minimal fibrosis (F0–F1) and moderate-to-severe fibrosis (F2–F3).

### Laboratory analysis

All participants underwent a routine laboratory workup, including a complete blood count, alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyltranspeptidase (γ-GTP), albumin, total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, C-reactive protein (CRP), glucose, insulin, prothrombin index, ceruloplasmin, iron parameters, and viral serology (hepatitis B surface antigen and HCV antibody).

The blood count was determined using an automated hematology analyzer, Sysmex XT-2000i (Sysmex Europe GMBX, Norderstedt, Germany). The serum levels of ALT, AST, γ-GTP, CRP, albumin, total cholesterol, HDL cholesterol, triglycerides, iron, and glucose were measured using an

autoanalyzer, Olympus AU 680 (Olympus, Dallas, Colorado, United States). Serum ferritin and insulin levels were assayed using Advia Centaur XP (Siemens, Erlangen, Germany). The prothrombin index was determined using the hemostasis testing system ACL TOP 500 (Werfen Company, Barcelona, Spain). In all 70 patients, the serum concentrations of hyaluronic acid, zonulin, and haptoglobulin were determined. In 41 participants, the serum concentrations of adiponectin, leptin, visfatin, and TPSA were assessed. Hyaluronic acid, zonulin, and haptoglobulin levels were measured by sandwich enzyme-linked immunosorbent assays (ELISA; Teco Medical, Switzerland, Immundiagnostic AG, Germany, and Zytomed, Berlin, Germany, respectively). The concentrations of adiponectin, leptin, visfatin, and interleukin 6 were determined using ELISA kits (Human Adiponectin ELISA KIT, BIOVENDOR, Laboratorni medicina a.s., Brno, Czech Republic, and PHOENIX PHARMACEUTICALS, Karlsruhe, Germany), and the TPSA concentration was measured by an immunoenzymatic assay with the use of Immulite 1000 analyzer (Siemens). All measurements were performed according to manufacturers' guidelines.

The demographic and clinical data of patients in this study were used for testing several well-known predictive models of advanced fibrosis. APRI (aspartate aminotransferase to platelet count ratio) was calculated as a ratio of AST (IU/l) to platelet count ($\times 10^3/mm^3$), which typically yields a range of results from 0.1 to 8.0. According to an original method, the BAAT score (body mass index, age, alanine aminotransferase, triglycerides) was calculated by designating 1 point to the following parameters: body mass index (BMI) >28kg/m$^2$, age >50 years, ALT >2× the upper normal limit, and triglycerides. The BARD score (body mass index, alanine aminotransferase to aspartate aminotransferase ratio, diabetes) was calculated by designating 0–2 points to the following parameters: BMI ≥28 kg/m$^2$ = 1; BMI <28 kg/m$^2$ = 0; AST/ALT ratio ≥0.8 = 2; AST/ALT ratio <0.8 = 0; and new-onset or preexisting diabetes = 1. A total of 2–4 points indicated significant fibrosis. The NAFLD fibrosis score (NFS) included age, hyperglycemia, BMI, platelet count, albumin, and AST/ALT ratio. By applying the low cut-off score (–1.455), advanced fibrosis was excluded, and the high cut-off score (0.676) indicated the presence of advanced fibrosis.

Written informed consent was obtained from each participant enrolled to the study. The study protocol was approved by the Ethics Committee of the Medical University of Silesia and conformed to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008).

**Statistical analysis** The calculations were done using the Statistica analysis package (version 10; StatSoft, Kraków, Poland). The distribution of quantitative variables was tested by the Shapiro–Wilk test. For variables with normal distribution, means and standard deviations were calculated. In the case of variables deviating from the normal distribution, the median values with interquartile ranges were calculated.

Subjects were divided into the following subgroups: none-to-minimal fibrosis (F0 and F1) and moderate-to severe fibrosis (F2 and F3). Selected variables were compared between the groups using the *t* test and Mann–Whitney test. A nonparametric analysis of variance (Kruskall–Wallis test) for continuous variables was used when more than 2 groups were compared. For comparisons of categorical variables, such as the prevalence of disease or condition, the $\chi^2$ homogeneity test was used. A *P* value of less than 0.05 was considered statistically significant.

The principal objective of the study was to predict the presence of moderate-to-severe fibrosis by a combination of demographic, clinical, and laboratory data. For this purpose, univariate and multivariate regression analyses were performed. In our regression models, predictor variables were the demographic, clinical, and laboratory parameters and the dependent variable was the presence of moderate-to-severe fibrosis found on histopathological assessment (F2 and F3). For the construction of a multivariate model, only variables demonstrating significant interactions were considered (after performing the interaction analysis). A logistic regression analysis was performed for the dependent variable (F2 and F3) with sigma limitations in a stepwise backward model with v-fold cross validation.

The predictive power of the variables differentiating none-to-minimal fibrosis from moderate-to-severe fibrosis, namely the sensitivity, specificity, positive/negative predictive values, and area under the receiver operating characteristic (AUROC) curves with 95% confidence intervals were calculated for each model.

**RESULTS** The demographic and clinical characteristics of the patients according to the severity of fibrosis are presented in **TABLE 1**. The variables independently associated with definite NASH in a multivariate analysis (NASH activity score greater than 4) are shown in **TABLE 2**. Patients with fibrosis staged F0 and F1 (none-to-minimal fibrosis; n = 54) differed significantly from patients with fibrosis staged F2 and F3 (moderate-to-severe fibrosis; n = 16) with respect to the prevalence of arterial hypertension, diabetes, coronary heart disease, and hypothyroidism (**TABLE 3**). The variables showing a significant change across the progression of liver fibrosis from F0 to F3 in the Kruskall–Wallis test included age ($P$ <0.01), serum levels of hyaluronic acid ($P$ <0.001), and AST/ALT ratio ($P$ <0.01).

The results of univariate and multivariate analyses according to the severity of fibrosis are shown in **TABLE 4**. In the multivariate analysis, the variables with an independent effect on moderate-to-severe fibrosis were age, arterial hypertension, platelet count, red blood cell (RBC) count, serum

**TABLE 1** Demographic and laboratory characteristics of patients with nonalcoholic fatty liver disease according to severity of fibrosis

| Variable | Moderate-to-severe fibrosis (F2–F3) | | | None-to-minimal fibrosis (F0–F1) | | | *P* value |
|---|---|---|---|---|---|---|---|
| | mean ±SD | median ±IQR | range | mean ±SD | median ±IQR | range | |
| age, y | 54.2 ±10.6 | 52 ±35 | 38–73 | 43.1 ±13.07 | 42 ±48 | 20–68 | 0.003 |
| weight, kg | 91.2 ±19.3 | 91.3 ±65 | 63–128 | 90.0 ±16.6 | 87 ±75.5 | 64.5–140 | 0.84 |
| height, cm | 167 ±10.7 | 165 ±30 | 153–183 | 171 ±10.5 | 172 ±41 | 152–193 | 0.21 |
| BMI, kg/m$^2$ | 32.3 ±3.89 | 31.8 ±12.6 | 26.5–39.1 | 30.7 ±4.65 | 29.9 ±25.7 | 23.7–49.5 | 0.1 |
| waist circumference, cm | 111 ±9.8 | 113 ±33 | 92–125 | 108 ±9.73 | 105 ±59 | 94–153 | 0.07 |
| hip circumference, cm | 109 ±11.5 | 110 ±44 | 85–129 | 103 ±10.3 | 102 ±51 | 87–138 | 0.02 |
| hip-to-waist ratio | 0.98 ±0.1 | 1 ±0.4 | 0.83–1.2 | 0.96 ±0.06 | 0.96 ±0.27 | 0.84–1.11 | 0.28 |
| SBP, mmHg | 134 ±10 | 130 ±30 | 120–150 | 131 ±12.4 | 130 ±60 | 100–160 | 0.45 |
| DBP, mmHg | 83.1 ±6.02 | 80 ±20 | 70–90 | 84.6 ±8.76 | 85 ±40 | 60–100 | 0.40 |
| triglycerides, mg/dl | 176 ±47.4 | 169 ±172 | 103–275 | 199 ±98.5 | 169 ±445 | 73–518 | 0.89 |
| total cholesterol, mg/dl | 208 ±47.2 | 193 ±162 | 144–306 | 229 ±47.8 | 229 ±224 | 130–354 | 0.13 |
| HDL cholesterol, mg/dl | 40.1 ±10.7 | 37 ±37 | 28–65 | 43.9 ±9.54 | 41 ±36 | 31–67 | 0.13 |
| albumin, g/dl | 4.09 ±0.75 | 4.3 ±2.6 | 2.50–5.1 | 4.36 ±0.82 | 4.5 ±4.95 | 2.05–7 | 0.11 |
| hemoglobin, g/dl | 14.6 ±1.43 | 14.5 ±5.7 | 11.9–17.6 | 15.09 ±1.15 | 15.2 ±4.3 | 12.8–17.1 | 0.13 |
| RBC count, 10$^6$/mm$^3$ | 4.61 ±0.43 | 4.59 ±1.2 | 41–5.2 | 4.98 ±0.42 | 5.01 ±1.99 | 3.71–5.7 | 0.006 |
| RBC count distribution width | 13.8 ±0.74 | 13.6 ±2 | 13.1–15.1 | 13.2 ±0.7 | 13.1 ±3.1 | 12.2–15.3 | 0.03 |
| platelet count, 10$^3$/mm$^3$ | 168 ±56.9 | 166 ±228 | 97–325 | 229 ±47.9 | 237 ±240 | 121–361 | 0.0001 |
| INR | 1.06 ±0.08 | 1.07 ±0.3 | 0.87–1.2 | 1.02 ±0.08 | 1.01 ±0.37 | 0.87–1.24 | 0.08 |
| ALT, U/l | 115 ±89.9 | 89 ±349 | 27–376 | 92.6 ±59.4 | 79 ±335 | 13–348 | 0.54 |
| AST, U/l | 89.5 ±86.9 | 60 ±351 | 35–386 | 51.5 ±26.3 | 45 ±141 | 14–155 | 0.01 |
| AST/ALT ratio | 0.84 ±0.37 | 0.71 ±1.5 | 0.44–1.9 | 0.69 ±0.66 | 0.59 ±4.92 | 0.31–5.23 | 0.01 |
| γ-GTP, U/l | 108 ±64.8 | 90 ±225 | 21–246 | 80.1 ±51.4 | 63 ±222 | 24–246 | 0.09 |
| iron, µg/dl | 117 ±45.9 | 112 ±165 | 39–204 | 107 ±35.2 | 99 ±161 | 42–203 | 0.44 |
| ferritin, ng/ml | 360 ±365 | 295 ±1446 | 29.7–1476 | 256 ±157 | 227 ±674 | 11.2–685 | 0.53 |
| iron/TIBC | 0.35 ±0.16 | 0.29 ±0.6 | 0.1–0.7 | 0.3 ±0.09 | 0.29 ±0.38 | 0.12–0.5 | 0.16 |
| CRP, mg/dl | 2.89 ±1.92 | 2.39 ±6.8 | 0.57–7.4 | 2.81 ±2.37 | 2.42 ±11.3 | 0.18–11.5 | 0.65 |
| glucose, mg/dl | 122 ±43.8 | 107 ±133 | 68–201 | 102 ±23.6 | 96 ±114 | 64–178 | 0.19 |
| insulin, mIU/ml | 17.3 ±7.87 | 17.7 ±30.8 | 5.59–36.4 | 16.2 ±14.9 | 12.7 ±95.9 | 4.16–100 | 0.1 |
| HOMA-IR | 4.57 ±2.54 | 4.17 ±7.4 | 1.78–9.2 | 2.99 ±1.34 | 2.86 ±5.55 | 1–6.55 | 0.02 |
| visfatin, ng/ml | 31.9 ±8.87 | 30.5 ±29 | 18.4–47.4 | 27.5 ±9.98 | 24.5 ±39.6 | 17.2–56.8 | 0.14 |
| interleukin 6, pg/ml | 2.15 ±1.38 | 1.23 ±3.3 | 0.94–4.3 | 1.42 ±0.66 | 1.23 ±2.68 | 0.82–3.5 | 0.33 |
| TPSA, U/l | 406 ±207 | 301 ±578 | 213–791 | 252 ±208 | 211 ±770 | 15–785 | 0.02 |
| leptin, ng/ml | 83.2 ±86.7 | 55.9 ±273 | 33.2–307 | 46.7 ±33.7 | 35.7 ±147 | 9.9–157 | 0.05 |
| adiponectin, µg/ml | 5.55 ±2.44 | 5.52 ±6.6 | 2.52–9.1 | 5.18 ±1.77 | 4.79 ±6.36 | 3.14–9.51 | 0.89 |
| hyaluronic acid, ng/ml | 78.2 ±118 | 36.2 ±486 | 14.2–501 | 24.5 ±30.7 | 15.5 ±197 | 4.72–202 | 0.0003 |
| haptoglobin, mg/dl | 134 ±18.4 | 133 ±56 | 109–165 | 131 ±19.6 | 131 ±63 | 101–164 | 0.57 |
| zonulin, ng/ml | 6.93 ±1.76 | 7.04 ±6.4 | 4.01–10.4 | 6.69 ±1.95 | 6.64 ±8.78 | 2.18–10.9 | 0.67 |
| right hepatic lobe, mm | 137 ±12.6 | 140 ±35 | 120–155 | 137 ±13.7 | 140 ±55 | 105–160 | 0.84 |

Conversion factors to SI units are as follows: for triglycerides, 0.0114; glucose, 0.05551; cholesterol and HDL cholesterol, 0.02586; iron, 0.179; ferritin, 2.247; albumin,10; and haptoglobin, 0.10.

Abbreviations: ALT – alanine transaminase, AST – aspartate transaminase, BMI – body mass index, CRP – C-reactive protein, DBP – diastolic blood pressure, HDL – high-density lipoprotein, HOMA-IR – homeostasis model assessment of insulin resistance, INR – international normalized ratio, IQR – interquartile range, RBC – red blood cell, SBP – systolic blood pressure, SD – standard deviation, TIBC – total iron binding capacity, TPSA – tissue polypeptide-specific antigen, γ-GTP – γ-glutamyltranspeptidase

concentrations of hyaluronic acid, and AST activity. After the exclusion of patients with fibrosis stage 1, additional independent variables were waist circumference, diabetes, international normalized ratio, and RBC distribution width (RDW).

In a cohort of 70 patients, the ROC curve analyses were performed for all available parameters to detect those with the best predictive value for moderate-to-severe fibrosis. In a further analysis, only variables showing the AUROC curves over 0.75 were taken into account. The variables that met this criterion were platelet count (AUROC curve, 0.83; cut-off, 137 ×10$^3$/mm$^3$), serum concentration of hyaluronic acid (AUROC curve,

**TABLE 2** Distribution of variables and multivariate analysis of the patient cohort according to nonalcoholic fatty liver disease fibrosis score

| Variable | NAFLD activity score | | | Multivariate analysis (all patients included) | |
|---|---|---|---|---|---|
| | <5 (n = 43) | ≥5 (n = 27) | *P* value | odds ratio (95% CI) n = 70 | *P* value |
| visfatin, ng/ml | 23.4 ±13.2 | 28.3 ±9.95 | 0.16 | 1.03 (0.96–1.11) | 0.38 |
| interleukin 6, pg/ml | 1.07 ±0.59 | 1.43 ±1.53 | 0.14 | 1.90 (0.8–4.51) | 0.15 |
| TPSA, U/l | 221 ±254 | 384 ±243 | 0.04 | 1.00 (1–1.008) | 0.03 |
| leptin, ng/ml | 33.4 ±32 | 43.5 ±39.6 | 1.14 | 1.01 (0.99–1.03) | 0.14 |
| adiponectin, µg/ml | 4.84 ±2.70 | 4.75 ±2 | 0.60 | 1.10 (0.75–1.61) | 0.63 |
| hyaluronic acid, ng/ml | 17.2 ±24.1 | 21.8 ±43 | 0.24 | 1.01 (0.99–1.02) | 0.15 |
| haptoglobin, mg/dl | 137 ±26 | 116 ±38 | 0.001 | 0.96 (0.93–0.98) | 0.003 |
| zonulin, ng/ml | 6.43 ±2.40 | 7.54 ±1.9 | 0.003 | 1.55 (1.13–2.12) | 0.006 |
| HOMA-IR | 2.42 ±1.74 | 4.26 ±2.8 | 0.004 | 2.01 (1.17–3.45) | 0.01 |
| insulin, mU/ml | 11.6 ±8.40 | 17.4 ±9.8 | 0.002 | 1.02 (0.98–1.07) | 0.23 |
| platelet count, $10^3/mm^3$ | 235 ±56 | 188 ±77 | 0.01 | 0.99 (0.98–0.99) | 0.03 |
| AST, U/l | 42 ±18 | 67 ±44 | 0.001 | 1.03 (1–1.06) | 0.007 |

Data are presented as means ± SD.

Abbreviations: CI – confidence interval, others – see **TABLE 1**

**TABLE 3** Prevalence of comorbidities in patients with nonalcoholic fatty liver disease according to the severity of fibrosis

| Variable | F0–F1 | F2–F3 | *P* value |
|---|---|---|---|
| sex, female/male | 21/33 (39/61) | 9 /7 (56/44) | 0.22 |
| arterial hypertension | 24 (44) | 14 (88) | 0.002 |
| hyperlipidemia | 19 (35) | 3 (19) | 0.21 |
| diabetes | 9 (17) | 8 (50) | 0.006 |
| ischemic heart disease | 2 (4) | 4 (25) | 0.007 |
| hypothyroidosis (hormonal substitution) | 3 (6) | 4 (25) | 0.02 |

Data are presented as number (percentage).

0.80; cut-off, 53.8 ng/ml), and RBC/RDW ratio (AUROC curve, 0.77; cut-off, 0.31).

In order to build our own predictive model of moderate-to-severe fibrosis, we used 6 variables with the highest AUROC curves (age, platelet count, RBC/RDW ratio, visfatin, TPSA, and hyaluronic acid) and 1 point was attributed to each variable lying beyond the cut-off value; therefore, the highest score for 1 patient was 6. Thirty-eight patients in whom all necessary variables were accessible were enrolled to construct this model. Sixteen patients had no fibrosis, 14 had minimal fibrosis (F1), and 8 had fibrosis stage F2 or F3. Obtaining 3 or more points classified the patient to the group of advanced fibrosis with a sensitivity of 75% and specificity of 100% (AUROC curve was 0.93; positive predictive value was 1.00; and negative predictive value was 0.89). Our predictive model was compared with other scoring systems of recognized effectiveness, namely, APRI, BARD, BAAT, and NFS (**FIGURE**).

**DISCUSSION** The major aim of this study was to predict the presence of moderate-to-severe fibrosis in NAFLD on the basis of a combination of demographic, clinical, and laboratory variables. The study prospectively included patients with biopsy-proven NAFLD with an extensive collection of clinical, laboratory, and histological data. Laboratory variables involved easily accessible data of known and unknown significance to predict liver injury, and additional parameters such as haptoglobin, zonulin, cytokeratin 18, interleukin 6, hyaluronic acid, adiponectin, leptin, and visfatin. All liver biopsies were assessed by a single hepatopathologist using a standardized pathologic protocol. The model predicting advanced fibrosis was validated by 2 different methods, namely, multivariate regression and ROC curve analyses.

NASH is characterized by composite histopathological abnormalities encompassing steatosis, hepatocyte injury, and inflammation. About 15% of patients with NASH develop cirrhosis.[21] Given that NASH and hepatic fibrosis are the surrogate markers for progressive liver disease and premature death from cardiovascular complications, early identification of these patients could have important prognostic significance. In our study, independent predictors of NASH were HOMA-IR, serum AST activity, platelet count, and serum levels of haptoglobin, zonulin, and TPSA. These results confirm the significance of insulin resistance and hepatocyte apoptosis for the pathogenesis of NASH. At present, cytokeratin 18 (CK18) fragments are the only recognized biomarker for NASH despite several limitations.[22] TPSA is synonymous with the CK18 molecule, which yields worse results in the identification of NASH than its caspase-3-derived fragments. The strength of our study lies in that we identified new potential biomarkers for NASH, such as haptoglobin and zonulin. These proteins are functionally interrelated because zonulin is the precursor of haptoglobin. Recently, it has been shown that mucosal permeability for endotoxins and other bacterial products is essential for the development of hepatic inflammation and fibrosis by activating toll-like receptors expressed on the surface

**TABLE 4** Univariate and multivariate analysis according to the severity of fibrosis

| Variable | Fibrosis stage | | | Univariate analysis | | Multivariate analysis without F1 | | Multivariate analysis all patients included | |
|---|---|---|---|---|---|---|---|---|---|
| | F0 (n = 32) | F0–F1 (n = 54) | F2–F3 (n = 16) | *P* value (F0 vs. F2–F3) | *P* value (F0–F1 vs. F2–F3) | odds ratio (95% CI) (n = 48) | *P* value | odds ratio (95% CI) (n = 70) | *P* value |
| age, y | 41.6 ±11.7[a] | 43.1±13.1[a] | 54.2±10.6[a] | 0.0007 | 0.003 | 1.1 (1–1.18) | 0.004 | 1.07 (1.02–113) | 0.006 |
| waist circumference, cm | 102 ±8.29[a] | 102 ±13 | 110 ±18 | 0.02 | 0.02 | 1.08 (1–1.15) | 0.03 | 1.05 (0.99–1.11) | 0.06 |
| diabetes | 6 (19) | 9 (17) | 8 (50) | 0.02 | 0.006 | 2.1 (1.07–4.03) | 0.03 | 0.65 (0.33–1.3) | 0.22 |
| arterial hypertension | 14 (44) | 24 (44) | 14 (88) | 0.003 | 0.002 | 3 (1.3–6.8) | 0.009 | 2.96 (1.34–6.5) | 0.007 |
| platelet count, $10^3/mm^3$ | 235 ±60 | 229 ±47.9[a] | 166 ±63 | 0.0003 | 0.0001 | 0.97 (0.96–0.99) | 0.003 | 0.97 (0.96–0.99) | 0.001 |
| AST, U/l | 44.5 ±29 | 45 ±27 | 60 ±52 | 0.02 | 0.01 | 1.02 (0.99–1.04) | 0.09 | 1.02 (1–1.04) | 0.04 |
| AST/ALT ratio | 0.53 ±0.29 | 0.59±0.27 | 0.71 ±0.36 | 0.008 | 0.01 | 1.24 (0.55–2.79) | 0.6 | 1.38 (0.6–3.1) | 0.43 |
| RBC distribution width | 13.1 ±0.5[a] | 13.2±0.7[a] | 13.8 ±0.74[a] | 0.01 | 0.04 | 7.77 (1.13–53.6) | 0.04 | 2.84 (0.95–8.5) | 0.06 |
| erythrocyte count, 106/mm3 | 5.00 ±0.46[a] | 4.98±0.42[a] | 4.61 ±0.43[a] | 0.01 | 0.006 | 0.16 (0.03–0.73) | 0.02 | 0.14 (0.03–0.64) | 0.01 |
| INR | 1.01 ±0.06[a] | 1.02±0.08[a] | 1.06 ±0.08[a] | 0.01 | 0.08 | 0.02 (0–0.63) | 0.03 | 0.23 (0.01–5.23) | 0.35 |
| hyaluronic acid, ng/ml | 12.8 ±18.7 | 15.5±22.5 | 36.2 ±61.4 | 0.0001 | 0.0003 | 1.06 (1.02–1.1) | 0.006 | 1.02 (1–1.04) | 0.03 |
| TPSA, U/l | 168 ±232 | 211 ±260 | 301±264 | 0.02 | 0.02 | 1 (1–1.01) | 0.07 | 1 (1–1.01) | 0.07 |

Data are presented as means ±SD (a), median ±IQR, or number (percentage).

Abbreviations: see TABLES 1 and 2

of hepatocytes and Kupffer cells.[16] Zonulin is an important component of the intestinal mucosal barrier, and all factors leading to the loss of mucosal integrity might be a source of increased release of zonulin into circulation. In a recently published study, morbidly obese patients showed the elevated serum levels of zonulin, which correlated with the number of bacterial colonies in the large bowel.[18] Increased zonulin levels were also found in patients with insulin resistance irrespective of their body mass.[23] Haptoglobin is an acute phase protein synthesized in the liver. In a Japanese study comprising 126 patients with NAFLD, fucosylated haptoglobin levels were selectively increased in patients meeting the histopathological criteria for NASH and showed a close relationship with hepatocyte ballooning.[24] Further studies investigating haptoglobin and zonulin as the predictors of NASH are needed.

Our study confirms previous data that the presence of the components of metabolic syndrome, such as diabetes, arterial hypertension, and ischemic heart disease, increase the risk of advanced hepatic fibrosis. Interestingly, moderate-to-severe fibrosis occurred more often in patients with hypothyroidism.[25] An effect of thyroid hormones on hepatic fibrosis is poorly understood and should be further explored. In our study, advanced fibrosis was not associated with hyperlipidemia. This finding may correspond with the lack of a significant effect of hypolipemic drugs on liver histopathology in NAFLD.

In this study, age, waist circumference, platelet count, AST/ALT ratio, erythrocyte count, RDW, international normalized ratio (INR), and serum levels of AST, hyaluronic acid, TPSA, and visfatin were found to be the independent predictors of moderate-to-severe fibrosis in the multivariate analysis or ROC curve analyses. These variables were even more closely related with advanced fibrosis when patients with fibrosis stage 1 were excluded from the analysis. This observation can be easily explained because the boundaries between the lack of and minimal fibrosis may be subject to many errors in both histopathological and noninvasive assessments.[16] Similarly to other studies, we found that the serum level of hyaluronic acid correlates with the progression and extension of liver fibrosis. Accordingly, hyaluronic acid is a component of several fibrosis tests based on laboratory parameters.[26] TPSA was a variable that was increased in both NASH and advanced fibrosis. Recently, it has been shown that serum CK18 levels correlate with hepatic angiogenesis, which is a necessary step for the development of fibrosis.[27] In our study, waist circumference, but not BMI, was associated with advanced fibrosis, thus showing that mesenteric fat is a more important predictor of significant fibrosis than body weight, which again emphasizes the metabolic role of visceral adipose tissue.

Visfatin is an adipokine predominantly expressed and secreted by visceral adipocytes and macrophages infiltrating the adipose tissue. This

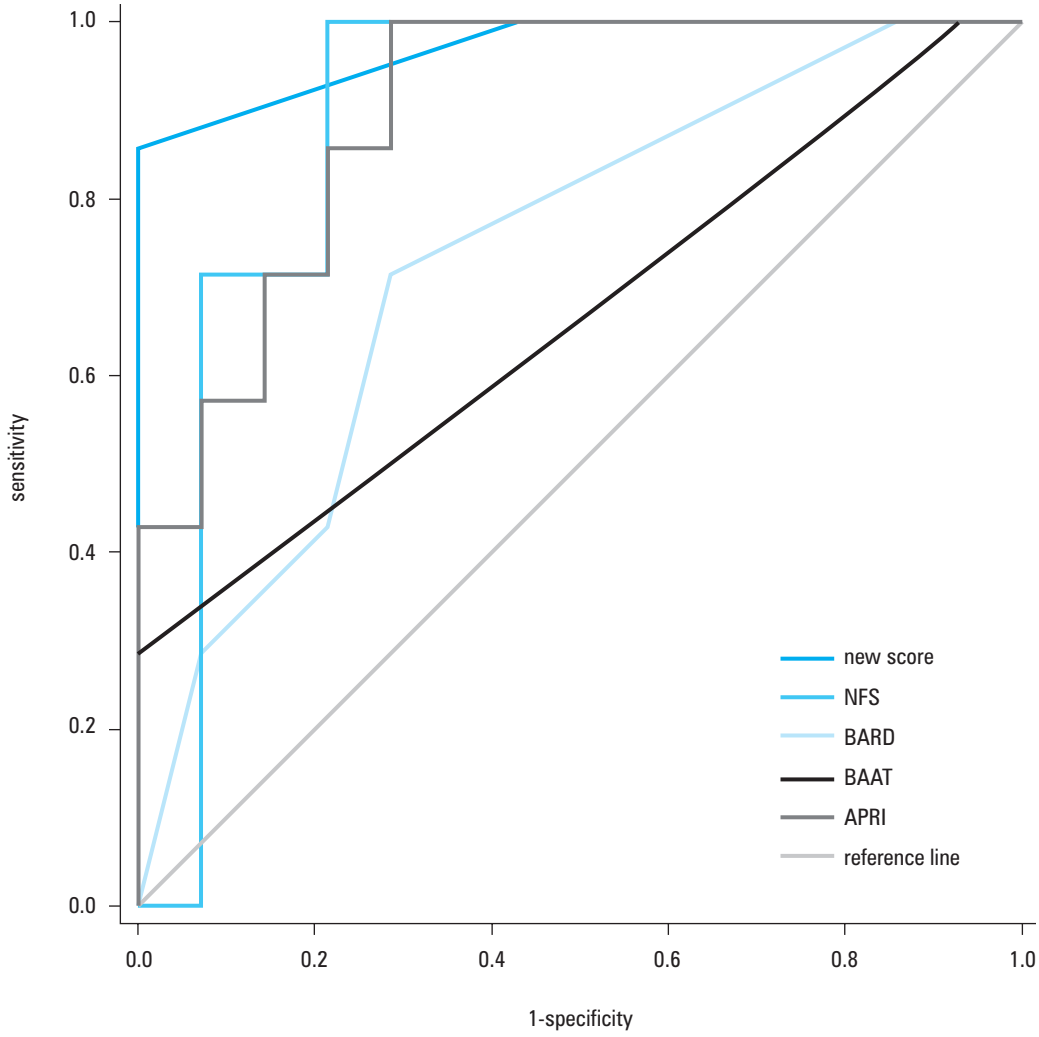


**FIGURE** Area under the receiver operating characteristic curves of various scoring systems for predicting moderate--to-severe fibrosis

Abbreviations: APRI – aspartate aminotransferase to platelet count ratio, BAAT – body mass index, age, alanine aminotransferase, triglycerides, BARD – body mass index, alanine aminotransferase to aspartate aminotransferase ratio, diabetes, NFS – nonalcoholic fatty liver disease fibrosis score

protein shows multiple functions as a regulator of energy homeostasis, insulin receptor sensitivity, innate immunity, and inflammation. Studies on circulating visfatin in NAFLD patients provided conflicting results that showed either increased or normal levels.[28-31] However, serum visfatin and its liver expression positively correlated with proinflammatory cytokines and histological inflammatory activity of hepatic portal tracts.[28-30] Moreover, in our previous study, the hepatic expression of visfatin in morbidly obese patients was positively correlated with the hepatic fibrosis stage.[15] In the current study, the levels of visfatin below 32 ng/ml presented a high negative predictive value for advanced fibrosis; therefore, such results would allow to avoid liver biopsy in numerous patients with NAFLD.

Moderate-to-severe fibrosis may be associated with clinically latent metabolic liver insufficiency and portal hypertension. Indeed, we found a small but significant increase in INR and reduction of platelet count in these patients. A decrease in erythrocyte count and abnormal RDW may also be associated with portal hypertension that predisposes to a microscopic loss of RBCs through the gastrointestinal mucosa subjected to ischemia.

There are several commonly accepted scoring systems predicting significant or advanced fibrosis in NAFLD.[4,11] The BARD score including the BMI, AST/ALT ratio, and presence of diabetes was validated on a large sample of American patients with NAFLD,[8] but was also positively re-evaluated in the Polish population with fatty liver.[32] The NFS incorporates 6 easily accessible variables and shows great accuracy for advanced fibrosis. It is the most extensively studied scoring system with external validation in 13 studies, involving more than 3000 patients.[33] The BAAT and APRI scores are older scores showing poor sensitivity but reasonable specificity for advanced fibrosis.[34,35] On the basis of the 6 variables reaching the AUROC curves over 0.75 in the prediction of significant and advanced fibrosis, we constructed our own scoring system, which performed as good or better (AUROC curve, 0.93; sensitivity,

75%; specificity, 100%) than the previously published predictive models; however, it should be stressed that those findings need to be reproduced in much larger studies.

Our study has several limitations including a cross-sectional design, the lack of external validation of our scoring system, and a small number of patients with advanced fibrosis. Therefore, the clinical utility of our scoring system needs to be confirmed in a larger validation set of patients with NAFLD.

In summary, we found several novel and easily available clinical and laboratory variables to identify patients who are at risk of progressive liver disease. Moreover, we developed a noninvasive, easy-to-use scoring system to accurately predict moderate-to-severe fibrosis in NAFLD. Such a scoring system would be clinically valuable because patients with advanced fibrosis require closer monitoring or inclusion in clinical trials testing novel therapies.

**Acknowledgements** A financial support for the project was provided by the Medical University of Silesia and was granted to MH (no. KNW-1-192/09).

**Contribution statement** AC contributed to the design of the research and drafted the manuscript; MH participated in the study design, helped to prepare the manuscript, made the final language edition, and coordinated funding for the project; AL analyzed the data; MK helped with laboratory work; KG helped with the preparation of the manuscript; MK was responsible for the histopathological part of this study. All authors edited and approved the final version of the manuscript.

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ARTYKUŁ ORYGINALNY

# Wieloczynnikowy model rozpoznawania średniego i zaawansowanego włóknienia wątroby w niealkoholowej chorobie stłuszczeniowej oparty na stężeniach wisfatyny, tkankowego swoistego antygenu polipeptydowego, kwasu hialurunowego i wskaźnikach hematologicznych – badanie wstępne

Alina Chwist[1], Marek Hartleb[1], Andrzej Lekstan[2], Michał Kukla[1], Krzysztof Gutkowski[1], Maciej Kajor[3]

1 Klinika Gastroenterologii i Hepatologii, Śląski Uniwersytet Medyczny, Katowice
2 Klinika Chirurgii Przewodu Pokarmowego, Śląski Uniwersytet Medyczny, Katowice
3 Katedra i Zakład Patomorfologii, Śląski Uniwersytet Medyczny, Katowice

**SŁOWA KLUCZOWE**

nialkoholowa choroba stłuszczeniowa wątroby, nialkoholowe stłuszczeniowe zapalenie wątroby, nieinwazyjna diagnostyka, włóknienie wątroby

Adres do korespondencji:
prof. dr hab. med. Marek Hartleb, Katedra i Klinika Gastroenterologii i Hepatologii, Śląski Uniwersytet Medyczny, ul. Medyków 14, 40-752 Katowice, tel.: 32-789-44-01, fax: 32-789-44-02, e-mail: mhartleb@sum.edu.pl
Praca wpłynęła: 11.09.2014.
Przyjęta do druku: 13.11.2014.
Publikacja *online*: 14.11.2014.
Nie zgłoszono sprzeczności interesów.
Pol Arch Med Wewn. 2014; 124 (12): 704-712


**STRESZCZENIE**

**WPROWADZENIE** Histopatologicznymi czynnikami ryzyka schyłkowej niewydolności wątroby u pacjentów z niealkoholową chorobą stłuszczeniową wątroby (*nonalcoholic fatty liver disease* – NAFLD) są niealkoholowe stłuszczeniowe zapalenie wątroby (*nonalcoholic steatohepatitis* – NASH) oraz zaawansowane włóknienie wątroby. Istnieje zapotrzebowanie na nieinwazyjne metody diagnostyczne tych dwóch stanów chorobowych.

**CELE** Celem pracy było poszukiwanie nowych parametrów laboratoryjnych posiadających potencjał predykcji obecności w NAFLD zaawansowanego włóknienia wątroby (stopień 2 i 3).

**PACJENCI I METODY** Do badania włączono 70 pacjentów, u których rozpoznano na podstawie badania histopatologicznego NAFLD o różnym stopniu zaawansowania zmian. Dodatkowe parametry laboratoryjne obejmowały: zonulinę, haptoglobinę, wisfatynę, adiponektynę, leptynę, antygen specyficznego polipetydu tkankowego (*tissue polypeptide-specific antigen* – TPSA), kwas hialuronowy i interleukinę 6.

**WYNIKI** Ppacjenci z NASH (≥5 punktów w skali aktywności NAFLD) mieli znamiennie większe wartości HOMA-IR oraz stężenia wisfatyny, haptoglobiny i zonuliny w surowicy w badaniu histologicznym w porównaniu do pacjentów bez NASH. Zaawansowane włóknienie stwierdzono u 16 (22,9%) pacjentów, a czynnikami ryzyka związanymi z tym stanem były: wiek, stosunek liczby erytocytów do rozpiętości rozkładu objętości erytrocytów (*red blood cell distribution width* – RDW), liczba płytek oraz stężenia wisfatyny i TPS w surowicy. Na podstawie tych wskaźników opracowano system punktowy odróżniający pacjentów z NAFLD posiadających i nieposiadających zaawansowanego włóknienia wątroby z 75% czułością i 100% swoistością (pole pod krzywą ROC 0,93).

**WNIOSKI** System punktowy oparty na tych wskaźnikach pozwala przewidzieć zaawansowane włóknienie wątroby z dużą czułością i swoistością. Jego przydatność kliniczna powinna być jednak zweryfikowana w kolejnych badaniach przeprowadzonych na większej liczbie pacjentów.